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Design and synthesis of novel bioactive peptides  
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*Alla mia famiglia,  
che porto sempre nel cuore*



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## Abstract

Nowadays there's a growing interest in biologically active peptides for the development of new therapeutics; however in some cases, they could not directly use as drugs, due to their inherent limitations, such as rapid metabolism and low oral activity. As a result, peptides are modified into peptidomimetics with specific characteristics, in a rational design.

The present PhD project is focused on the synthesis of several peptides and peptidomimetics, structurally different and presenting individual features, properties, targets and pharmaceutical applications. In particular, two are the research studies we've developed during the three years, these are the design of novel Carnosine-like derivatives and of new Farnesyl Transferase Inhibitors (FTIs).

Concerning the first topic, we investigated how Carnosine ( $\beta$ -alanyl-L-histidine) structural changes influence its role as scavenger of HNE (4-hydroxy-*trans*-2,3-nonenal) and other toxic aldehydes.

For this reason we modified the carnosine structure firstly replacing the *Hystidinil*-portion with different aromatic system, secondly substituting the  $\beta$ -*alanyl* portion with ten different amino acids, chosen in order to cover exhaustively the available chemical space. Finally we rigidified the whole structure, inserting a 2-oxazolidinone; the entire compound underwent biological evaluation, testing their ability to quench HNE.

As a result, some of the twenty dipeptides showed impressing scavenging activities and great selectivity towards toxic aldehydes, suggesting us that they can represent truly promising candidates for the design of improved carnosine derivatives.

Regarding the second subject, we designed, synthesized and tested several peptidomimetics of the CAAX box, where CAAX is the sequence Cysteine-Valine-Isoleucine-Methionine, able to block the farnesylation of RAS proteins and therefore cell proliferation.

The design started from a nanomolar range FTI, previously synthesized by our group, where the central dipeptide (AA) is replaced with a 4-amino-2-o-tolylbenzoyl spacer and the Cysteine (C) with the residue 2-amino-4-thiazolylacetyl. The synthesis of the novel FTIs followed two separate approaches; at first we kept the aromatic spacer and modified the N-terminal residue with other heterocycles; the unimproved antiproliferative activity suggested us to apply other kind of modification. Therefore we replaced the o-tolyl with six heteroaromatic residues, in addition the synthesized compounds presented, as N-terminal residue, the 2-amino-4-thiazolylacetyl itself or the 1,4-benzodioxan-2-ylmethyl or the 1,4-benzodioxan-2-ylformyl. In all the three series of compounds, the 2-thienyl, 1-naphtyl and the 3-furanyl derivatives showed the highest FTase inhibition, at low micromolar level.

Taken together, our biological activities provide interesting results, confirming that peptides and peptidomimetics should be employed as therapeutics.



## Chapter 1: Carnosine

Carnosine (*L*-Car;  $\beta$ -alanyl-*L*-histidine) is an endogenous dipeptide widely and abundantly distributed in the muscle and nervous tissues of several animal species. It was discovered more than a century ago by the Russian scientists Gulewitsch and Amiradzibi: it was extracted for the first time from Liebig's in 1900, becoming the first ever peptide isolated from animal tissue<sup>1</sup>.

Carnosine is the best known representative of a series of imidazole dipeptides such as homocarnosine ( $\gamma$ -aminobutyryl-*L*-histidine) (Figure 2), Anserine ( $\beta$ -alanyl-*N*- $\pi$ -methyl-*L*-histidine) and Balenine ( $\beta$ -alanyl-*N*- $\tau$ -methyl-*L*-histidine), which have long been reported to be present at high concentrations in skeletal muscle and in the central nervous system of vertebrates (Figure 1).

Specifically, the  $\beta$  alanyl containing peptides are mainly found in skeletal muscle, whereas the  $\gamma$ -aminobutyryl containing peptides are typical of the central nervous system, probably due to the availability of its precursor  $\gamma$ -aminobutyric acid in this tissue.

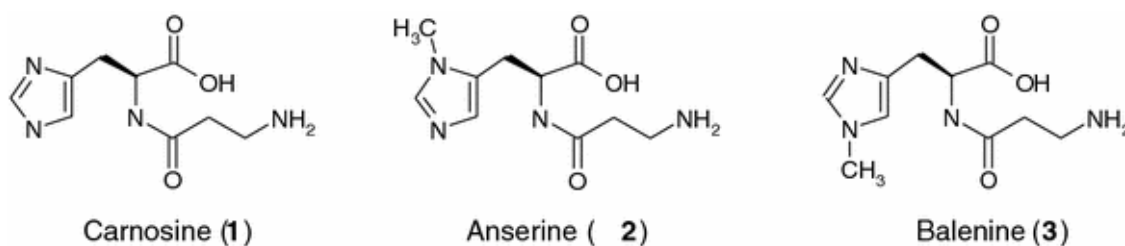


Figure 1: Naturally occurring histidine-containing dipeptides

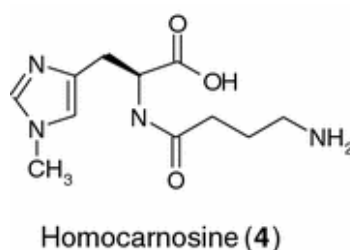


Figure 2: Homocarnosine

<sup>1</sup> Gulewitsch, W., and Amiradzibi, S. , 1900, *Ber. Deutsch. Chem. Ges.*, 33, 1902-1903;

The concentration of Carnosine in skeletal muscle of vertebrates varies according to the species, from 0.6 mM in mice up to 10 and 30 mM in humans and horses, respectively<sup>2</sup>.

However, the dipeptide is also present at high concentrations (1–2 mM) in the olfactory bulb of the mammalian brain and has been detected in muscles of some invertebrates such as crabs, shrimp, and oysters<sup>3</sup>.

Homocarnosine is detected at concentration of 0.3–1.5 mM in different regions of the human brain<sup>4</sup>.

Anserine is abundant in rabbit leg muscle (17mM) and chicken pectoral muscle (43 mM), whereas balenine is mainly found in muscles of marine-mammals such as whales and dolphins (up to 45mM).

Carnosine homeostasis is regulated by (1) the rate and magnitude of  $\beta$ -alanine uptake within muscle fibres, (2) serum biosynthesis and, in the absence of sufficient  $\beta$ -alanine within the diet, (3) hepatic synthesis of the amino acid and its transport to skeletal muscle.

Specific enzymes are involved in Carnosine synthesis and degradation; in particular Carnosine is known to be synthesized from  $\beta$ -alanine and L-histidine by an ATP-dependent synthase (EC 6.3.2.11), which has been purified from different sources<sup>5</sup> and is shown to also catalyze the synthesis of homocarnosine (Figure 2).

The synthesis, here reported, consists of two different steps:

1.  $\beta$ -Alanine + ATP + Enzyme +  $Mg^{2+} \rightleftharpoons$  Enzyme- $\beta$ -alanyl adenylate + pyrophosphate
2. Enzyme- $\beta$ -alanyl adenylate + L-histidine  $\rightleftharpoons$  AMP + Enzyme + L-Carnosine

Reaction 1 is a  $Mg^{2+}$ -dependent  $\alpha$ -amino acid activation, bringing to the intermediate  $\beta$ -alanyl adenylate; some support for the involvement of pyrophosphate was the strong inhibition produced by this compound on  $\beta$ -alanyl peptide synthesis with crude enzyme.

<sup>2</sup> Crush, K. G., **1970**, *Comp. Biochem. Physiol.*, 34, 3–30;

<sup>3</sup> Cameron, J. N., **1989**, *J. Exp. Biol.*, 143, 543–548; Boldyrev, A. A. , **2007**, *Carnosine and Oxidative Stress in Cells and Tissues*, Nova Scientific Publisher, New York;

<sup>4</sup> Kish, S. J., Perry, T. L., and Hansen, S., **1979**, *J. Neurochem.*, 32, 1629 –1636,

<sup>5</sup> Kalyankar, G. D., and Meister, A., 1959, *J. Biol. Chem.*, 234, 3210–3218; Stenesh, J. J., and Winnick, T., **1960**, *Biochem. J.*, 77, 575–581; Skaper, S. D., Das, S., and Marshall, F. D., **1973**, *J. Neurochem.*, 21, 1429–1445; Horinishi, H., Grillo, M., and Margolis, F. L., **1978**, *J. Neurochem.*, 31,909–919; Drozak J. Et all, **2010**, *J Biol Chem*, 285, 9346-9356;

An excess of adenosine 5'-phosphate (AMP) also depressed Carnosine formation, moreover ADP exerted an even stronger effect. (Figure 3).

Reaction 2, differently from Reaction1, does not require added magnesium ions.

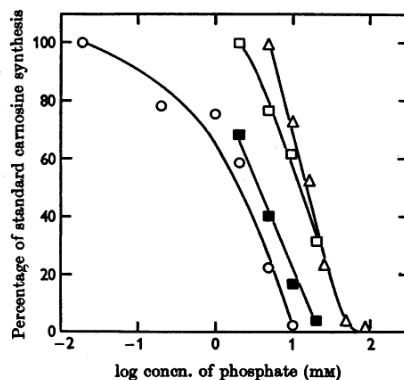


Figure 3:

Effect of phosphate compounds on Carnosine synthase activity. The values are in terms of standard assay conditions, with 2 mM-ATP present. (○: Pyrophosphate; ▲: orthophosphate; □: AMP; ■: ADP).

Carnosine degradation is performed by two proteins, named carnosinases, all belonging to the large family of metalloproteases, identified and characterized a few years ago<sup>6</sup>.

Two novel cDNAs code for two proteins of 56.8 and 52.7 kDa, named CN1 and CN2 respectively: whereas human CN1 mRNA and protein are brain-specific, CN2 codes for a ubiquitous protein.

CN1, a pure human carnosinase, was identified as homodimeric dipeptidase (EC 3.4.13.20) with a narrow substrate specificity for Xaa-His dipeptides, including those with Xaa =  $\beta$ -Ala (Carnosine,  $K_m$  1.2 mM), N-methyl  $\beta$ -Ala, Ala, Gly, and  $\gamma$ -aminobutyric acid (homocarnosine,  $K_m$  200  $\mu$ M), having an isoelectric point of pH 4.5 and maximal activity at pH 8.5. Interestingly, deficiency of this enzyme leads to hypercarnosinemia and hypercarnosinuria and was associated with neurological symptoms<sup>7</sup>.

CN2, the cytosolic nonspecific protein, (EC 3.4.13.18) is a dipeptidase not limited to Xaa-His dipeptides, it requires  $Mn^{2+}$  for full activity, and it is sensitive to inhibition by bestatin ( $IC_{50}$  7 nM). This enzyme does not degrade homocarnosine and hydrolyzes Carnosine only at alkaline pH with an optimum at pH 9.5.

<sup>6</sup> Teufel, M. et al, **2003**, *J. Biol. Chem.*, 278, 6521–6531;

<sup>7</sup> Murphey, W. H., Lindmark, D. G., Patchen, L. I., Housler, M. E., Harrod, E. K., and Mosovich, L., **1973**, *Pediatr. Res.* 7, 601–606; Willi, S. M., Zhang, Y., Hill, J. B., Phelan, M. C., Michaelis, R. C., and Holden, K. R., **1997**, *Pediatr. Res.* 41, 210–213

## Carnosine roles

In the latest years, thanks to a large number of works and studies, several roles of *L*-Carnosine have been postulated; it is now well known that it is related to:

- ✓ Intracellular buffering;
- ✓ Ergogenic aid;
- ✓ Cell senescence;
- ✓ Neurotransmission;
- ✓ Glycation;

### ***Carnosine as intracellular buffer***

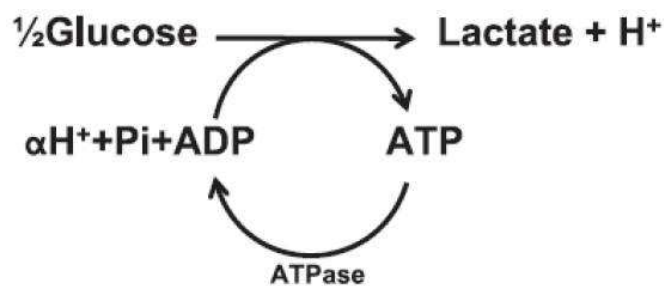
Intracellular buffering is provided mainly by bicarbonate, phosphates and proteins, nevertheless a part may also be played by free histidine and small peptides containing histidine.

Although the free histidine content in the majority of animal tissues is low, a variety have been found to possess substantial amounts of histidine-containing dipeptides, most commonly  $\beta$ -alanyl-*L*-histidine, actually *L*-Carnosine, either without substitutions or methylated at the 1-position, as in the case of Anserine, or at the 3-position, for instance Ophidine or Balenine. At 25°C the pK of Carnosine is 6.83 whereas that of anserine is 7.04, so the combination of appropriate pK and high concentration leads to significant intracellular buffer action.

***Carnosine in ergogenic aid***

As people exercise to voluntary failure, multiple mechanisms (psychological, neurological, metabolic, etc.) may undermine performance.

Since Carnosine is an intramuscular compound, it is important to examine how conditions within cells, as exercise, progress to the point of fatigue. In the quest for sufficient ATP, perhaps the greatest exercise-induced intracellular change is the rapid accrual of lactate and H<sup>+</sup> that typifies metabolic acidosis (Figure 4).



**Figure 4:**

**Anaerobic glycolysis and ATP hydrolysis schematic; Carnosine buffers H<sup>+</sup> increases**

Lactate and acid metabolites accrue commensurate to the degree by which intracellular aerobic capacity is exceeded, such as when anaerobic glycolysis serves as the primary ATP resynthesis pathway.

At maximal exercise rates, such bouts last from 15 to 240 s, intramuscular glycolytic rates may rise 1000-fold as persons go from rest to supramaximal exercise. With supramaximal exercise, intracellular ATP declines up to 40% and causes a near complete depletion of phosphocreatine as well as elevations in lactate and H<sup>+</sup>. Intramuscular pH, from resting values of ~7.1, may decline from supramaximal activity to less than 6.5, which represents a four-fold rise in H<sup>+</sup> and an intracellular increase of up to 54 mmol·kg<sup>-1</sup>.

When such increases are added to other reactions that raise the total proton load intracellular H<sup>+</sup> levels exceed 100 mmol·kg<sup>-1</sup>. Concomitant lactate increases also occur with higher glycolytic rates; intramuscular and plasma levels for the metabolite rise by up to 40 and 25 mmol·L<sup>-1</sup>.

A strong linear relationship exists between muscle pH decrements and intracellular lactate and pyruvate values. Thus examinations of blood lactate changes over time, which quantify the rate of the metabolite's entry into and removal from the

vasculature, foretell concurrent intracellular  $H^+$  changes in response to, and recovery from, exercise.

Upon cessation of exercise, force output and intracellular pH restoration is rapid, yet the former recovers at a faster rate. For each measure, recovery is slower than their rate of loss incurred from supramaximal exercise.

Adverse intracellular changes from  $H^+$  accrual include phosphofructokinase inhibition, impaired phosphocreatine resynthesis, slower cross bridge transitions from low- to high-force states, losses in maximal shortening velocity, lowered glycolytic rates, competitive inhibition with  $Ca^{2+}$  at the troponin C subunit, and delayed  $Ca^{2+}$  reuptake by the sarcoplasmic reticulum. Such changes compromise exercise and recovery rates, which is a concern if successive bouts of supramaximal activity are to occur. If contractions proceed at high force outputs, which are common to supramaximal exercise, high  $H^+$  levels also impair intramuscular  $Ca^{2+}$  release and compromise  $Ca^{2+}$ -ATPase activity.

To deal with such conditions, cells utilize multiple mechanisms to remove lactate and  $H^+$ , as well as buffers to mitigate the effects of metabolic acidosis.

Lactate and  $H^+$  efflux is facilitated by monocarboxylate transporters such as MCT1 and MCT4.

Unlike Carnosine, which appears to have no ceiling with respect to its intramuscular concentration, other intracellular buffers such as proteins, bicarbonates, citrates and NAD<sup>+</sup>-dependent redox reactions, are under tight homeostatic control.

In untrained persons, Carnosine typically adds a mere 7%–10% to their intracellular buffering capacity and neutralizes 2.4–10.1 mmol  $H^+$ ·kg<sup>-1</sup> dry mass as intramuscular pH declines. Nevertheless, during exercise-induced metabolic acidosis, Carnosine is particularly effective as an  $H^+$  acceptor, due to its imidazole ring and a concomitant shift in its pKa to 6.83, which is rare for intracellular buffers and vital to maintain pH.

Unlike most buffers, Carnosine is potent over a broad pH range in type I and II muscle fibres from the rapid and substantial accrual of intracellular  $H^+$  and lactate. Carnosine levels range from 4 to 7 and 9 to 13 mM within type I and II fibres respectively.

Since buffer capacity ( $\beta$ ) is a quantitative index of the resistance of a buffer solution to change pH upon added  $H^+$ , its formula is as follows:

$$\beta = (\Delta n) / (\Delta pH)$$

where  $\Delta n$  represents the incremental additions of proton equivalents to a buffered system and  $\Delta pH$  is the observed pH changes.

This equation is commonly known as the buffer derivative, in which pH (*i.e.*,  $-\log_{10}[H^+]$ ) is expressed as  $[H^+]$  and where the  $\beta$  in the presence of a neutralizing agent with an acid dissociation constant represented by  $Ka$ , is defined as:

$$\beta = 2.303 \left( \frac{Kw}{[H^+]} + [H^+] + \frac{C_{buf}Ka[H^+]}{(Ka + [H^+])^2} \right)$$

It is important to note, for near neutral pH's, the first ( $Kw/[H^+]$ ) and second ( $[H^+]$ ) terms do not contribute significantly to Carnosine's prowess as a buffer.

$\beta$  can be calculated at pH = 7.1 for a lower limit of  $4 \times 10^{-3}$  M Carnosine in type I fibres to have a buffer capacity of  $9.1 \times 10^{-4}$ .

At pH = 7.1 for Carnosine measured at the higher range found in type II fibres, *i.e.*, at  $13 \times 10^{-3}$  M, the buffer capacity rises to  $2.95 \times 10^{-3}$ , approximately a 3.2-fold increase.

At a pH of 6.5, the 4 mM Carnosine only slightly changes to  $8.7 \times 10^{-4}$ , and for the 13 mM Carnosine to  $2.8 \times 10^{-3}$ . Thus as pH declines from 7.1 to 6.5 in exercising muscle, Carnosine's buffer capacity is well preserved in type I and II fibres.

Carnosine's ability to absorb protons is well maintained over a substantial pH range and makes it a unique and real valuable molecule.

***Carnosine and cell senescence***

Carnosine has been implicated as an anti-ageing agent for different reasons<sup>8</sup>:

- (i) it can delay senescence in cultured human fibroblasts,
- (ii) it can reverse the senescent phenotype in cultured human cells and preserve a juvenile phenotype in cultured rodent cells;
- (iii) it is present in long-lived mammalian tissues at surprisingly high concentrations (up to 20 mM in human muscle) which may decline with age;
- (iv) tissue levels in mammals appear to correlate directly with the maximum life-span of the species.

Many proposals have been made regarding the cellular action of Carnosine including a role as an anti-oxidant and oxygen free radical scavenger, a physiological buffer, a histidine source and an immunostimulant.

The anti-oxidant and oxygen free radical-scavenging activities of Carnosine have been demonstrated in many studies<sup>9</sup>. However, other anti-oxidants do not have the anti-ageing actions on cultured human fibroblasts described by McFarland and Holliday suggesting that additional properties of the dipeptide are responsible for, or contribute to, these effects. More recent evidences show that Carnosine can react with low-molecular-weight aldehydes and ketones indicating that it might also act as a naturally occurring anti-glycating agent<sup>10</sup>.

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<sup>8</sup> (a) Hipkiss A. R., **1998**, *Int. J. Biochem. Cell Biol.*, 30, 863–868; (b) McFarland G. A. and Holliday R., **1994**, *Exp. Cell Res.*, 212, 167–175; (c) McFarland G. A. and Holliday R., **1999**, *Exp. Gerontol.*, 34, 35–45; (d) Kantha S. S. et al., **1996**, *Biochem. Biophys. Res. Commun.*, 223, 278–282; (e) Johnson P., Fedyna J. S., Schindzielorz A., Smith C. M. and Kasvinshy P. J., **1982**, *Biochem. Biophys. Res. Commun.*, 109, 769–775; (f) Stuenkel H. J. and Kunze K., **1999**, *Arch. Gerontol. Geriatrics*, 29, 107–113; (g) Hipkiss A. R., Holliday R., McFarland G. and Michaelis J., **1993**, *Lifespan*, 4, 1–3; (h) Munch G., Thome J., Foley P., Schinzel R. and Riederer P., **1997**, *Brain Res. Rev.*, 23, 134–143; (i) Quinn P. R., Boldyrev A. A. and Formazyuk V. E., **1992**, *Mol. Aspects Med.* 13: 379–444;

<sup>9</sup> (j) Kohen R., Yamamoto Y., Cundy K. C and Ames B. N., **1988**, *Proc. Natl. Acad. Sci. USA*, 95, 2175–2179; (k) Boldyrev A. A., Formazyuk V. E. and Sergienko V. I., **1994**, *Sov. Sci. Rev. D. Physicochem. Biol.*, 13, 1–60; (l) Aruoma O. I., Laughton M. J. and Halliwell B., **1989**, *Biochem. J.*, 264, 863–869;

<sup>10</sup> (m) Hipkiss A. R., Michaelis J., Syrris P., Kumar S. and Lam Y., **1994**, *Biochem. Soc. Trans.*, 22, 399S; (n) Hipkiss A. R., Michaelis J. and Syrris P., **1995**, *FEBS Lett.*, 371, 81–85; (o) Hipkiss A. R., Michaelis J., Syrris P. and Dreimanis M., **1995**, *Hum. Biol.*, 1, 59–70;



***Carnosine as neurotransmitter***<sup>11</sup>

The detection of high levels of Carnosine in the olfactory system of several mammalian species, including humans, and its specific localization in the olfactory receptor neurons, led to the hypothesis that the dipeptide could exert a putative role in neurotransmission within this sensory system.

The olfactory receptor neurons (primary olfactory neurons) are typical bipolar neurons located among non-neuronal supporting cells in the olfactory neuroepithelium lining the nasal cavity. These receptor cells send their axon to the glomerular layer of the olfactory bulb, where they make synapses with the dendrites of mitral, tufted and periglomerular cells.

Immunocytochemical studies carried out at both the light and electron microscopical level clearly showed that Carnosine is contained within these axons and in their synaptic terminals.

At present, the aminoacid glutamate is the main excitatory neurotransmitter candidate in several sensory systems, including the primary olfactory pathway. By using post-embedding immunogold techniques, the coexistence of Carnosine and glutamate has been demonstrated in the synaptic terminals of the mouse olfactory neurons, as well as in hair cells of the inner ear and photoreceptors of frogs.

These data, along with the existence of Carnosine synthetase and Carnosinase activities in the olfactory bulb and mucosa (higher than in other brain regions or tissues) and of binding sites for Carnosine in the olfactory bulb, support the hypothesis for an unknown role of the dipeptide Carnosine in neuromodulation in glutamatergic sensory neurons.

Nevertheless, biochemical studies indicated that Carnosine is localized in the cytosol of the olfactory bulb and epithelium, not associated with synaptic vesicles; indeed, morphological studies carried out using high resolution techniques were not able to clearly show a high concentration of the dipeptide in synaptic vesicles. A calcium-dependent depolarization stimulated release of Carnosine has been shown in olfactory bulb synaptosomes and inward current responses have been induced in olfactory bulb cultured slices after application of Carnosine; however, it is known that

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<sup>11</sup> Bonfanti L., Peretto P., De Machis S., Fasolo A., **1999**, *Progress in Neurobiology*; 59, 333-353;

Carnosine leaks out of tissue during preparation of synaptosomes or tissue slices and can also be released as a slow spontaneous process which is independent of depolarization.

Definitive physiological evidence for a role of this dipeptide in neurotransmission is at present unavailable. Indeed, in vivo studies have provided conflicting results.

Direct injection of the dipeptide into the olfactory bulb glomerular layer of rabbits revealed an increase in frequency of evoked potentials within the first minute after injection<sup>12</sup>, but Carnosine applied by microiontophoresis in the rat olfactory bulb was mainly with-out effect on mitral cells.

Similarly, Nicoll et al<sup>13</sup> found no effect using in vitro preparations of the turtle and frog olfactory bulb. Finally, a glutamate receptor-mediated release of Carnosine and  $\beta$ -alanine, dependent on elevated intracellular  $Ca^{2+}$ , has been recently shown to occur in oligodendrocytes.

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<sup>12</sup> Gonzalez-Estrada, M.T., Freeman, W.J., **1980**, *Brain Res.*, 202, 373-386;

<sup>13</sup> Nicoll, R.A., Alger, B.E., Jahr, C.E., **1980**, *Proc. R. Soc. Lond. (B)*, 210, 133-149;

### ***Carnosine as glycating agent***

Reactive carbonyl species (RCS) are electrophilic molecules which react with nucleophilic groups in proteins, yielding oxidative-based non-enzymatic protein adducts. These covalent adducts can be subdivided into two major classes, depending on the source of the reactive species.

Advanced glycation end products (AGEs) are generated by sugars or sugar derivatives including dicarbonyl derivatives such as glyoxal (GO), methylglyoxal (MGO), malondialdehyde (MDA) and desoxyglucosone, while lipid-oxidation end products (ALEs) are formed from the oxidative reactions of lipids comprising  $\alpha,\beta$ -unsaturated carbonyls such as 4-hydroxy-2-nonenal (HNE), 4-oxo-2-nonenal (ONE) and acrolein (ACR).

AGEs and ALEs are involved in oxidative cellular damage through different mechanisms including protein dysfunction, protein oligomerization and fibrillogenesis, altered signal transduction, immune response and activation of the receptor for AGEs (RAGE) which is a type I transmembrane glycoprotein of the immunoglobulin superfamily of cell surface receptors.

AGEs and ALEs have been widely accepted as biomarkers for oxidative-based diseases. Moreover, considering that AGEs and ALEs are involved in the pathogenesis of several diseases, including diabetes and arteriosclerosis, they are now also considered as promising targets for therapeutic intervention. This is promoting the design of carbonyl scavengers able to trap RCSs converting them into nontoxic and easily excretable derivatives so inhibiting protein carbonylation and all downstream pathways.

Although clinical investigations on humans are still very limited, several *in vitro* and *in vivo* animal studies demonstrated that Carnosine is able to detoxify RCS, inhibiting AGEs and ALEs formation and restraining oxidative-based diseases. The mechanism by which Carnosine prevents AGEs and ALEs formation is still under investigation and the involvement of multiple molecular mechanisms should be considered, given that the formation of AGEs and ALEs can involve different reactions and several catalysts including transition metals.

Nevertheless, there is enough evidence to indicate that Carnosine acts by a direct quenching mechanism<sup>14</sup>; specifically, Carnosine reacts with  $\alpha,\beta$ -unsaturated aldehydes through a multi-step mechanism involving the initial formation of a reversible unsaturated imino intermediate followed by the key intramolecular Michael addition between the histidine imidazole ring and the acceptor  $\beta$ -carbon atom (Figure 5).

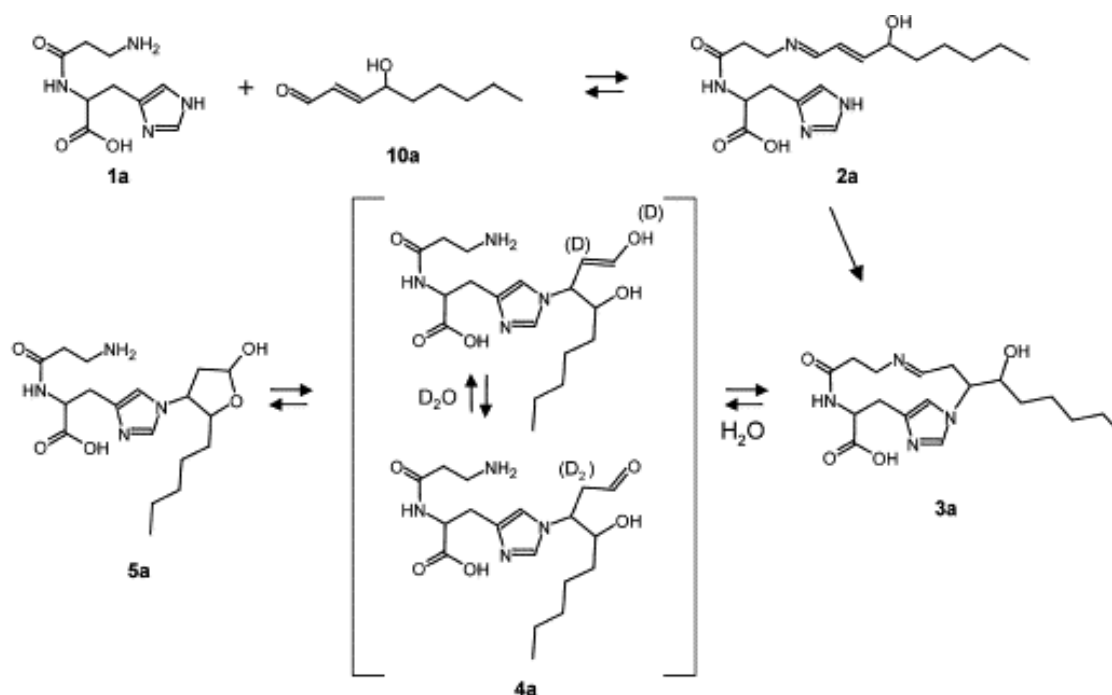


Figure 5: Proposed reaction mechanism of carnosine with 4-hydroxy-trans-2,3-nonenal (HNE)

The imino intermediate acts as an intramolecular catalyst promoting a stable approach between the two reacting centers, this is confirmed also from the fact that a mixture of the two separate amino acids ( $\beta$ Ala + L-His) does not possess a significant quenching activity.

Similarly, the reversible nature of the imino intermediate can explain why Carnosine selectively quenches reactive  $\alpha,\beta$ -unsaturated carbonyls without stably trapping physiological carbonyl compounds.

<sup>14</sup> Aldini G, Carini M, Beretta G, Bradamante S, Maffei Facino R, **2002**, *Biochem Biophys Res Comm*, 298, 699-706;

## Carnosine pharmacokinetics

In humans, diet represents the main source of Carnosine and histidine derivatives (HD), which are present in a significant amount in red and white meats. Following ingestion, dietary Carnosine is taken up by intestinal cells and readily absorbed intact.

The intestinal absorption is connected with H<sup>+</sup>/peptide cotransporter 1 (PEPT1) and human peptide/histidine transporter 1 (hPHT1).

The high-affinity type H<sup>+</sup>/peptide cotransporter 2 (PEPT2) may be involved in the transport of CAR in the rat cerebellum, choroid plexus epithelial cells, astrocytes, cardiomyocytes and kidney.

Moreover, PHT1, which may also mediate CAR transport, is observed throughout the whole brain; PEPT2 has been implicated in modulation of the disposition of exogenous CAR, but not in the homeostatic control of endogenous CAR levels in skeletal muscle<sup>15</sup>.

The enzymatic hydrolysis of the peptide bond represents the main metabolic fate of Carnosine, and it mainly occurs in the plasma by, as shown before, a specific serum hydrolase that cleaves the β-alanine–L-Histidine peptidic bond.

The two hydrolyzed amino acids are then delivered to tissues and then again synthesized to Carnosine in those tissues characterized by the presence of Carnosine synthetase, such as skeletal muscle, heart and in some regions of the CNS<sup>16</sup>.

The ADME (Adsorption, Distribution, Metabolism, and Excretion) profile of Carnosine in humans is not fully elucidated, mainly due to the lack of specific and sensitive methods for their measurement in biological matrices as well as the scarcity of ADME studies in humans.

Gardner<sup>17</sup> first described the urinary and plasma profile of Carnosine and β-alanine in healthy volunteers, after ingesting 4 g of Carnosine, and using an ion-exchange amino acid analyzer with ninhydrin detection for β-alanine and Carnosine quantitation. The

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<sup>15</sup> Bhardwai RK, Herrera-Ruiz D, Eltoukhy N, Saad M, Knipp GT, **2006**, *Eur J Pharm Sci*, 27, 533–542; Geissler S, Zwarg M, Knutter I, Markwardt F, Brandsch M, 2010, *FEBS J*, 277, 790–795;

<sup>16</sup> Aldini G, Facino RM, Beretta G, Carini M., **2005**, *Biofactors*, 24, 77–87;

<sup>17</sup> Gardner ML, Illingworth KM, Kelleher J, Wood D, **1991**, *J Physiol*, 439, 411–422;

amount of Carnosine recovered in urine was found to range from 1.2 to 14% of the ingested dose, while only small amounts of Carnosine were detected in the blood.

In addition, it has also been performed the HPLC of the plasma, studying Carnosine levels in healthy volunteers, following consumption of 200 g of ground beef (equivalent to a Carnosine dose of almost 250 mg).

Carnosine was detected in plasma 15 min after beef consumption, and maximal concentration was recorded 2.5 h after consumption (C<sub>max</sub> 150 μM).

More recently, the profile of histidine dipeptides in plasma and urine after ingesting beef, chicken, chicken broth or pure peptides in humans has been reported.

A significant excretion of HD, and in particular of Anserine, was found in urine, despite the low and in many cases undetectable content of HD peptides in the serum<sup>18</sup>.

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<sup>18</sup> Park YJ, Volpe SL, Decker EA , **2005**, *J Agric Food Chem*, 53, 4736–4739; Yeum KJ, Orioli M, Regazzoni L, Carini M, Rasmussen H, Russell RM, Aldini G , **2010**, *Amino Acids*, 38, 847–858;

## Carnosine: a novel therapeutic approach<sup>19</sup>

Many claims have been made about the therapeutic actions of Carnosine. These also include antihypertensive effects, as well as immunomodulating actions, wound healing and acting as an anti-inflammatory agent.

It is also used in the treatment of acute spinal cord injury<sup>20</sup>; positive effects of Carnosine were also demonstrated on survival and learning ability of animals under ischemic injury<sup>21</sup>.

In the context of neurodegenerative disorders, Carnosine has been suggested as an inhibitor of Amyloid $\beta$  toxicity in vitro. Moreover, it has been reported that Carnosine has a strong effect in restoring mitochondrial functioning and in counteracting amyloid pathology in triple-transgenic Alzheimer's disease model mice.

Very recently, a proteomic approach revealed that L-Carnosine affects tumor cell growth by causing an interference with protein folding/processing and HIF-1 $\alpha$  signaling in glioblastomas.

The metal binding ability of Carnosine especially for copper (II) and zinc (II) ions has extensively been studied. The copper- and zinc-mediated neurotoxicity involved in several pathologies, such as amyotrophic lateral sclerosis, Alzheimer's and Parkinson's diseases might be reduced or prevented by endogenous metal-chelating agents, such as Carnosine itself.

Recently, it has been also proven that polaprezinc, the zinc (II)-Carnosine complex, is effective for the recovery of ulcers and other lesions in the alimentary tract<sup>22</sup>.

The peptide nature of Carnosine imposes limitations in its therapeutical uses, mainly associated with the breakdown caused by the carnosinases.

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<sup>19</sup> Bellia F., Vecchio G., Rizzarelli E., **2012**, *Amino Acids*, 43, 153-163

<sup>20</sup> Di Paola R, Impellizzeri D, Salinaro AT, Mazzon E, Bellia F, Cavallaro M, Cornelius C, Vecchio G, Calabrese V, Rizzarelli E, Cuzzocrea S, **2011**, *Biochem Pharmacol.*, doi:10.1016/j.bcp.2011.07.074;

<sup>21</sup> Dobrota D, Fedorova T, Stvolinsky S, Babusikova E, Likavcanova K, Drgova A, Strapkova A, Boldyrev A, **2005**, *Neurochem Res*, 30, 1283-1288; Gallant S, Kukley M, Stvolinsky S, Bulygina E, Boldyrev A, **2000**, *Tohoku J Exp Med*, 191, 85-99;

<sup>22</sup> Katayama S, Nishizawa K, Hirano M, Yamamura S, Momose Y, **2000**, *J Pharm Pharm Sci*, 3, 114-117; Matsukura T, Tanaka H., **2000**, *Biochemistry (Mosc)*, 65, 817-823;

Several attempts have been made to overcome this limitation, essentially through the derivatization of Carnosine or the synthesis of its structural analogues<sup>23</sup>, but only N-acetyl Carnosine has been used as prodrug for some pharmaceutical applications.

The conjugation of Carnosine with several types of organic molecules has the main purpose of reducing the carnosinase action on the peptide moiety, improving the multifunctional activity, because of the potential beneficial role of the conjugating moiety and the synergism with the peptide properties as well.

Finally, the functionalized group could aim at the delivery to a specific target.

Another interesting consideration is that the enantiomer *D*-Carnosine ( $\beta$ -alanyl-*D*-histidine, *D*-Car) has been reported as a very promising dipeptide, because it surprisingly maintains the same activity of *L*-enantiomer.

In addition, *D*-Car is not hydrolyzed by Carnosinase, therefore an acceptable concentration is maintained in the serum<sup>24</sup>.

*D*-Carnosine has a reasonable beneficial effect; however *D*-Car is less bioavailable than *L*-Car, because it is not recognized by hPepT1, a transporter responsible for the uptake of a broad array of small peptides in the colon.

Nevertheless, increasing attention has been paid also on the functionalization of *D*-Carnosine and several compounds have been produced with the aim of increasing itself bioavailability.

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<sup>23</sup> Guiotto A, Calderan A, Ruzza P, Borin G, **2005**, *Curr Med Chem*,12, 2293–2315;

<sup>24</sup> Vistoli G, Orioli M, Pedretti A, Regazzoni L, Canevotti R, Negrisoni, G, Carini M, Aldini G, **2009**, *ChemMedChem*, 4, 967–975;



## Carnosine derivatives as new therapeutics: state of art

The chemical modification of Carnosine is a very promising approach to realize therapeutic Carnosinase resistant molecules based on *L*-Carnosine, therefore a large number of Carnosine-like derivatives have been synthesized.

Due to the way the structure has been modified, the possible derivatives could be classified in:

- A. Histidyl-containing Carnosine analogues bearing hydrazide or 1,2-diol moieties;
- B. Carnosine- derivatives, modified on the amino group;
- C. Carnosine-derivatives, modified at the carboxylic group;
- D. Double-functionalized Carnosine-derivatives;
- E. Ethylenic chain-modified Carnosine-derivatives;
- F. *D*-Carnosine derivatives.

### ***Histidyl-containing Carnosine analogues bearing hydrazide or 1,2-diol moieties***<sup>25</sup>

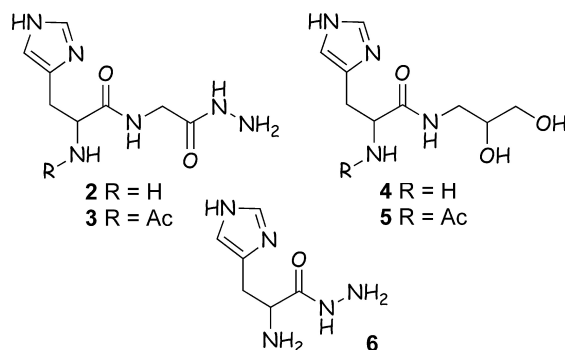
After the elucidation of the mechanism of Carnosine- $\alpha,\beta$ -unsaturated aldehyde adduct, explained before, and the consideration that the formation of the Schiff base is the rate-determining step, Guiotto and co-workers decided to substitute the primary amine of  $\beta$ -alanine with different nucleophiles to produce carnosine analogues able to form more stable adducts with aldehydes.

They focused on 1,2-diols, which react with carbonyls yielding cyclic acetals, and hydrazides, that are among the strongest aldehyde-sequestering moieties.

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<sup>25</sup> Guiotto A, Calderan A, Ruzza P, Borin G, **2005**, *Curr Med Chem*, 12, 2293–2315;

In addition, they placed the histidine residue at the C-terminus instead of the N-terminus, in the attempt to avoid recognition by the specific enzyme Carnosinase (Figure 6).



**Figure 6:**

(2) L-histidylglycyl hydrazide, (3) N-acetyl-L-histidylglycyl hydrazide, (4) 2-amino-N-(2,3-dihydroxypropyl)-3-(1H-imidazol-4-yl)propionamide, (5) 2-acetylamino-N-(2,3-dihydroxypropyl)-3-(1H-imidazol-4-yl)propionamide, (6) L-histidyl hydrazide

In preliminary tests, performed using *trans*-2-nonenal as model (scavenger/aldehyde ratio 1:20), all the synthesized compounds formed stable adducts with the  $\alpha,\beta$ -unsaturated aldehyde.

In particular, the hydrazides **2** (H-HisGly-NHNH<sub>2</sub>) and **6** (H-His-NHNH<sub>2</sub>) reacted almost completely, proving to be more efficient than Carnosine.

Compounds **3** and **5** (**2** and **4** N-acetyl derivatives) were far less reactive, suggesting a possible role as prodrugs.

The 1,2-diol derivative **4** showed a slower reactivity, but eventually matched carnosine after 4 h.

The efficacy of compound **6** was evidenced in two sets of experiments, showing that it is virtually nontoxic at concentrations as high as 1 mM, but has a strong cytoprotective activity toward SH-SY5Y neuroblastoma cells and rat hippocampal neurons treated with HNE.

### Carnosine-like derivatives, modified on the amino group

A wide number of Carnosine derivatives have been synthesized modifying the amino group of the dipeptide.

As seen before, the presence of the primary amino group should be mandatory for the quenching towards RCS species. However, they could be recognized from hPepT1 because the amino group has been tested not to be essential for the recognition of the transporter.

These derivatives are reported here below (Figure 7).

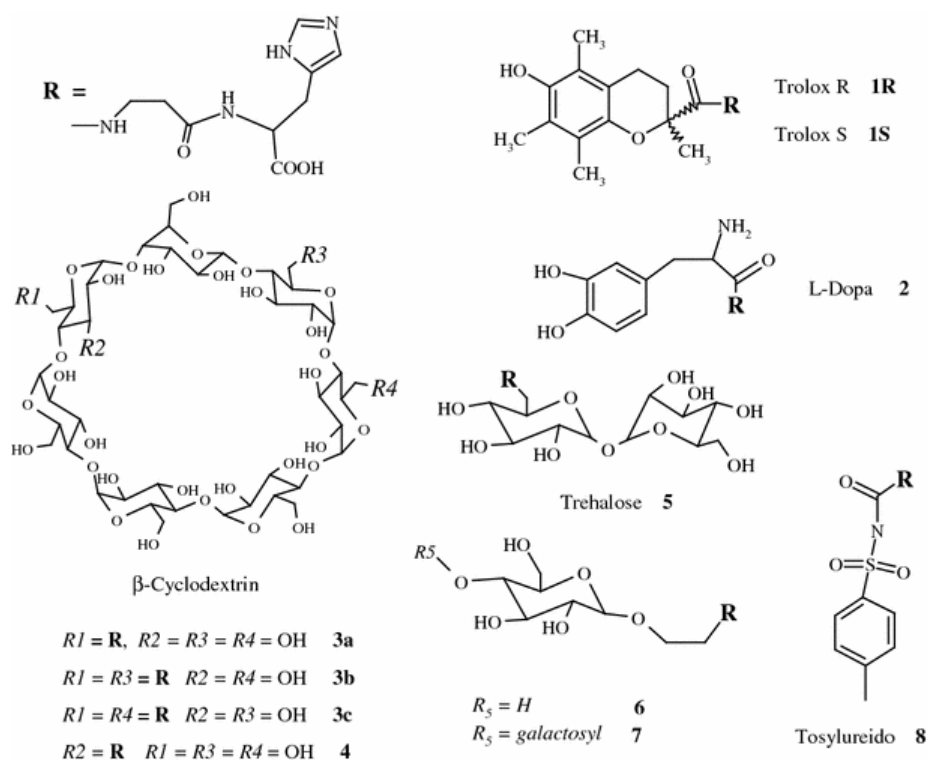


Figure 7: L-Carnosine derivatives at the amino group

The amino group of carnosine has been modified, introducing:

- ✓ Trolox, a well-known antioxidant compound (**1R**; **1S**),
- ✓ L-Dopa (**2**),
- ✓ Cyclodextrins (**3a**, **3b**, **3c**, **4**),
- ✓ Trehalose, lactose and glucose (**5**, **6**, **7**);
- ✓ 4-Toluensulfonylureido (**8**).

The aim that has moved the synthesis of the majority of these derivatives was the obtainment of Carnosinase-resistant compounds with antioxidant activity, considering that the antioxidant activity is generally due to carnosine moiety.

The Carnosine derivatives with *R* and *S*-trolox, the water-soluble analog of alpha-tocopherol acylated derivatives [(*S,S*)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid)], have been designed to exploit the cooperative effect of the beneficial activities showed by the constituents<sup>26</sup>.

Several assays were performed in order to study the antioxidant activity of trolox conjugates: red blood cell hemolysis, DPPH, and lipoprotein oxidation. As a matter of fact, the conjugates generally show an average activity between those of trolox and carnosine. A higher quenching activity against the DPPH radical has been reported for the conjugates with respect to that of the constituents. In this assay, the enantioselective activity has been shown and the conjugate with *R*-trolox (**1R**) is more active than the epimer with *S*-trolox (**1S**).

No hydrolysis by carnosinase in the human serum has been observed for these conjugates. The antioxidant effects of trolox conjugates have also been tested on the lifespan of the fruit fly, *Drosophila melanogaster*. The findings obtained in that study show that **1S** is more active than carnosine. On the contrary, **1R** is less active than the dipeptide. These data suggest the presence of additional cellular targets in comparison with simple neuronal cells to be acted upon by exposing *D. Melanogaster* to these compounds.

*L*-Car has also been modified with *L*-Dopa (L-3,4-dihydroxyphenylalanine) with the aim of reducing the reactive oxygen species generated by the *L*-Dopa treatment in Parkinson's disease<sup>27</sup>.

The conjugate **2**, designed as a potential prodrug for Parkinson's disease, has not shown significant antioxidant activity in vivo.

The third class of Carnosine derivatives are the glycoconjugates.

Carnosine has been functionalized with  $\beta$ -cyclodextrin, in different positions of the sugar<sup>28</sup>. The pharmaceutical use of cyclodextrins is commonly used for their ability to

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<sup>26</sup> Stvolinsky SL, Bulygina ER, Fedorova TN, Meguro K, Sato T, Tyulina OV, Abe H, Boldyrev AA, **2010**, *Cell Mol Neurobiol*,30, 395–404;

<sup>27</sup> Sozio P, Iannitelli A, Cerasa LS, Cacciatore I, Cornacchia C, Giorgioni G, Ricciutelli M, Nasuti C, Cantalamessa F, Di Stefano A, **2008**, *Arch Pharm (Weinheim)*,341, 412–417;

include and stabilize drugs; for this reason, several inclusion complexes are commercially available. This specific conjugation stabilizes the dipeptide to the carnosinase hydrolysis and confers a higher antioxidant activity than that of the natural dipeptide, as it has been found by the pulse radiolysis method.

Other glycoconjugates with monosaccharides and disaccharides have been synthesized; in particular the trehalose derivative (**5**) has been accomplished to combine the properties of trehalose and Carnosine<sup>29</sup>. Trehalose is a sugar distributed in many living systems and used in cosmetics with protective and moisturising functions. Its ability to protect proteins against the denaturation process and conformational changes has been focused on and related to potential application in the treatment of Huntington's disease. The carnosine trehalose conjugate has been tested in the LDL assay.

As in the case of the cyclodextrin moiety, the trehalose increased the antioxidant properties and protected carnosine from the degradation by carnosinase.

With the aim of selectively addressing Carnosine and its antioxidant function, glucose and lactose derivatives have been obtained (**6**, **7**). An important physiological aim of the conjugation is to enhance the bioavailability of the dipeptide, by facilitating the site-specific transport to different tissues.

In addition, the consideration that the animal lectins and galectins are important mediators in inflammatory diseases and that lectins play a key role in recognition processes, has prompted efforts in the syntheses of the glycoconjugates.

As a result, these kinds of derivatives are stable to Carnosinases<sup>30</sup>.

The conjugation of 4-Toluensulfonylureido to Carnosine has been performed, the resulting compound was tested as a target moiety for the delivery to tumor cells<sup>31</sup>. Compounds containing this aromatic moiety have been shown to act as anticancer agents for their ability to inhibit the carbonic anhydrase in tumor cells.

4-Toluensulfonylureido carnosine (**8**) has been shown to be stable to serum carnosinase and to have good affinity for the hPetT1 transporter. However, its

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<sup>28</sup> La Mendola D, Rizzarelli E, Vecchio G, **2002**, *EP1176154*; Bellia F, Amorini AM, La Mendola D, Vecchio G, Tavazzi B, Giardina B, Di Pietro V, Lazzarino G, Rizzarelli E, **2008**, *Eur J Med Chem*, 43, 373–380; Mineo P, Vitalini D, La Mendola D, Rizzarelli E, Scamporrino E, Vecchio G, **2004**, *J Inorg Biochem*, 98, 254–265;

<sup>29</sup> Rizzarelli E, Vecchio G, Lazzarino A, Amorini AM, Bellia F, **2007**, *EP1860116*;

<sup>30</sup> Lanza V, Bellia F, D'Agata R, Grasso G, Rizzarelli E, Vecchio G, **2011**, *J Inorg Biochem*, 105, 181–188;

<sup>31</sup> Nielsen CU, Supuran CT, Scozzafava A, Frokjaer S, Steffansen B, Brodin B, **2002**, *Pharm Res*, 19, 1337–1344;

transepithelial transport was very low thus excluding such an application for carnosine tosylate.

Nanoparticles (NPs) are a new class of carriers in nanomedicine, with special applications in the case of cancer. Recently, the interest for NPs has been increasing and also NPs based on carnosine have been obtained.

Carnosine has been functionalized with *L*-lipoic acid to synthesize gold NPs<sup>32</sup>. These NPs have been synthesized as activators of carbonic anhydrase. The role of carnosine could be related to the presence of histidine, being that the activity of carnosine nanoparticles is very similar to that of histidine nanoparticles.

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<sup>32</sup> Saada MC, Montero JL, Vullo D, Scozzafava A, Winum JY, Supuran CT, **2011**, *J Med Chem*, 54, 1170–1177;

### Carnosine-like derivatives, modified at the carboxylic group

The majority of this class of derivatives are amides of Carnosine<sup>33</sup> (Figure 8).

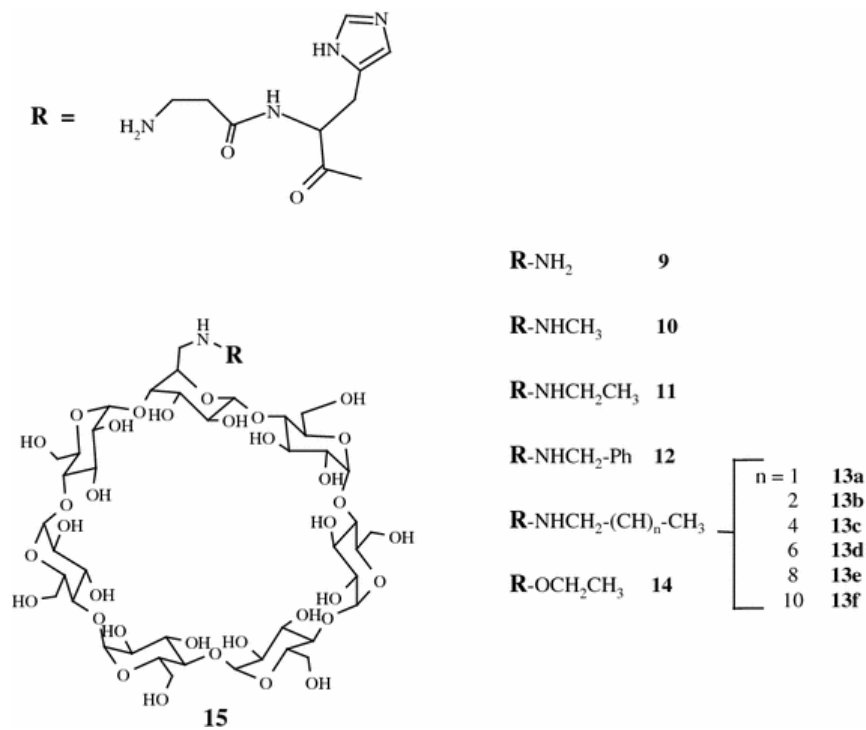


Figure 8: L-Carnosine derivatives at the carboxylic group

The amide functionalization seems to be a promising strategy because it deeply modifies the resistance of the Carnosine versus the human serum Carnosinase, it has been proved that the carboxylic group is important in the recognition done by the carnosinase enzymes; therefore the conversion of carboxyl group into amide makes the derivatives very stable to the carnosinase action.

The most important Carnosine derivatives are:

- ✓ The simple amido-Carnosine (**9**);
- ✓ Other different amides (**10-14**);
- ✓ The amide obtained through conjugation with amino- $\beta$ -cyclodextrin (**15**).

It is interesting that the modification of the carboxyl group maintains the HNE (4-hydroxy-trans-2-nonenal) quenching activity of the carnosine moiety, though the derivatives show a lower activity than that of the dipeptide.

<sup>33</sup> Lanza V, Bellia F, D'Agata R, Grasso G, Rizzarelli E, Vecchio G, **2011**, *J Inorg Biochem*, 105, 181–188;

The activity has also been studied in cell cultures. The amido derivative **13a**, which is moderately more hydrophobic with respect to **9**, has been able to protect primary hippocampus neurons against HNE-induced death, showing a very significant increase in comparison to *L*-Carnosine.

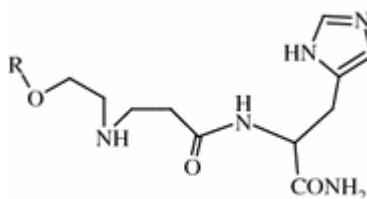
Derivative **13a** is also able to cross the blood brain barrier (BBB) and to concentrate in the rat brain after intravenous administration. These results render **13a** a very promising neuroprotective agent.



***Double-functionalized Carnosine-like derivatives***

In addition to the Carnosine-like derivatives, modified at the aminic or carboxylic function, also compounds double functionalized have been prepared.

In particular, the amide of Carnosine has also been glycoconjugated at amino group in order to study the metal complexing ability (Figure 9).



R = glucosyl    **16**

R = lactosyl    **17**

**Figure 9: Double-functionalized L-Carnosine derivatives**

As expected, these novel molecules revealed to be very promising systems that are able to increase Carnosine bioavailability.

They are also potentially able to act as chelating agents in the development of clinical approaches for the regulation of copper (II) homeostasis in the field of medicinal inorganic chemistry.

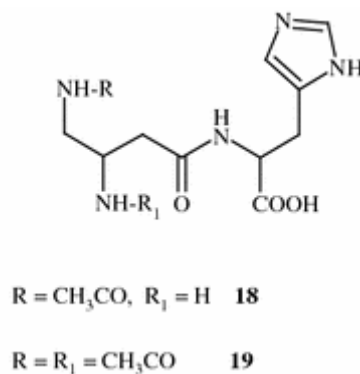
The presence of the sugar renders these derivatives capable of recognizing important biological systems such as the lectins. This feature should localize copper (II) chelating activity to the target tissue or even to the target cell compartment of interest.

**Ethylenic chain-modified Carnosine-like derivatives<sup>34</sup>**

In order to investigate in more detail the effect of N-acetylation on the biochemical properties of Carnosine, derivatives whose side chain has been introduced on the ethylenic chain of beta alanine have also been synthesised.

For this purpose, it seemed interesting to replace the residue of  $\beta$ -alanine with L(+)-2,3-diaminopropionic acid. This chemical modification leads to a combination of a close structural similarity to the native model with the possibility to bear two N-acetylamino groups or to maintain a free extra amino group.

Hence, the two novel synthesised Carnosine analogues, containing 2,3-diaminopropionic acid with a different degree of N-acetylation, are reported in Figure 10.



**Figure 10: Carnosine derivatives modified on the ethylenic chain**

The compounds have been tested either as substrates or inhibitors of human serum carnosinase: the enzyme resistance was higher in the case of **19**, while compound **18** showed only partial carnosinase resistance.

In addition, interestingly enough, derivative **19** revealed to be also able to inhibit carnosinase.

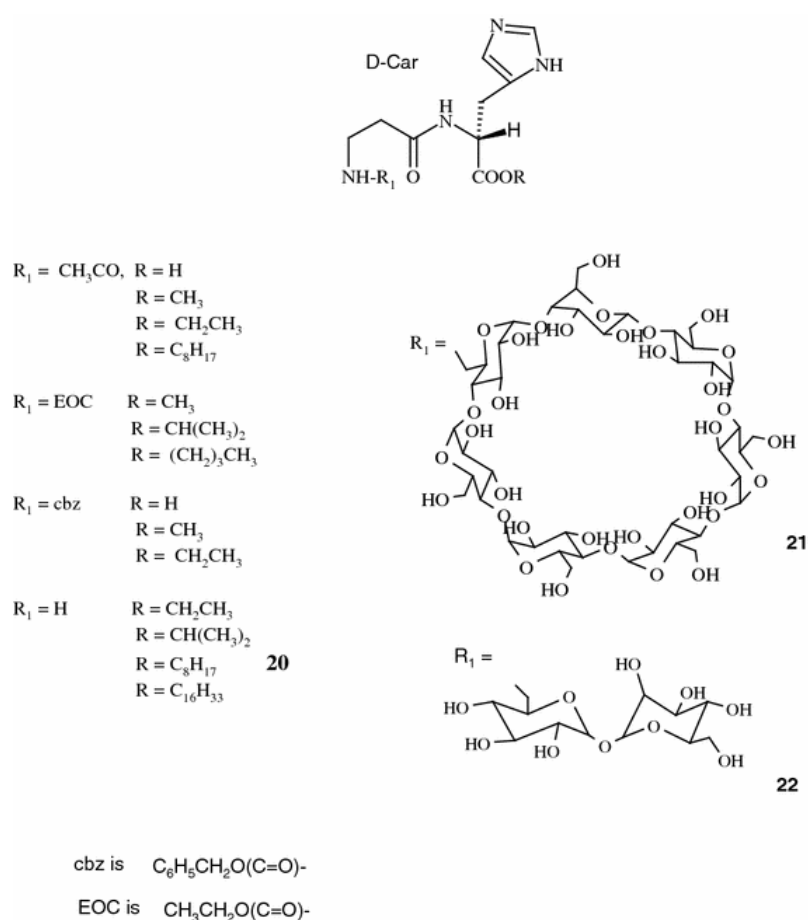
The antioxidant and free radical scavenger properties of these compounds have been also investigated by their ability to inhibit reactions induced by hydroxyl radicals and by peroxyxynitrite.

<sup>34</sup> Cacciatore I, Cocco A, Costa M, Fontana M, Lucente G, Pecci L, Pinnen F, **2005**, *Amino Acids*, 28, 77–83;

**D-Carnosine derivatives**

As explained before in this thesis, the design and synthesis of *D*-Carnosine derivatives prodrugs have been inspired by the promising RCS scavenger ability of *D*-Carnosine itself.

The prodrugs of *D*-Car have been designed mainly based on computed lipophilicity<sup>35</sup>. Derivatives with both amine and carboxyl groups were synthesized (Figure 11) in order to study the hydrolysis mechanism in rat plasma.



**Figure 11: D-Carnosine derivatives**

The most stable derivatives have been excluded from an in vivo investigation. The octyl ester of *D*-Car (**20**) has been selected to undergo an extensive evaluation in the Zucker rat.

<sup>35</sup> Orioli M, Vistoli G, Regazzoni L, Pedretti A, Lapolla A, Rossoni G, Canevotti R, Gamberoni L, Previtali M, Carini M, Aldini G, **2011**, *ChemMedChem*, 6, 1269–1282;

Several protective actions have been observed:

- ✓ the reduction of markers of carbonyl stress, such as advanced glycoxidation product (AGE);
- ✓ the reduction of hyperlipidemie;
- ✓ the prevention of renal and vascular injuries.

These derivatives have also been synthesized in order to compare the stereochemical differences of the *L*- and *D*-Car conjugates.

Cyclodextrin (**21**) and trehalose (**22**) conjugates of *D*-Carnosine have been structurally investigated and their ability to complex metal ions has been correlated to the chirality of the histidine ring<sup>36</sup>.

These systems revealed to be very interesting examples in the field of stereochemistry, but their potentialities as therapeutic agents have not been investigated yet.

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<sup>36</sup> Grasso GI, Arena G, Bellia F, Maccarrone G, Parrinello M, Pietropaolo A, Vecchio G, Rizzarelli E, **2011 (a)**, *Chemistry*, 17, 9448–9455; Grasso GI, Bellia F, Arena G, Vecchio G, Rizzarelli E, **2011 (b)**, *Inorg Chem*, 50, 4917–4924;

## Aim of the work

Considering all the possible therapeutic potentialities of Carnosine, in the present work several derivatives have been designed in order to:

- ✓ increase quenching activity towards cytotoxic RCS,
- ✓ maintain a selectivity versus cytotoxic species, avoiding an activity towards endogenous ones;
- ✓ ensure a satisfactory oral bioavailability by preventing carnosinase-catalyzed hydrolysis while preserving active absorption by hPepT1.

A critical analysis of all reported carnosine analogues, combined with computational studies involving serum carnosinase and peptide transporters<sup>37</sup>, revealed that the carboxyl terminus and the  $\beta$ -alanine carbon skeleton can be largely modified to improve pharmacokinetic profile without detrimentally affecting quenching activity.

As seen before, C-terminus capping is a well-known strategy to enhance plasma stability by hampering peptide recognition by all hydrolases, thus suggesting that such a modification should prevent the hydrolytic effects of both specific carnosinases and nonspecific peptidases.

On the contrary, the primary amino group should be basic enough to allow the Schiff-base formation with  $\alpha,\beta$ -unsaturated aldehydes; also the imidazole ring is mandatory for quenching activity and could be modified only with other moieties able to form the Michael adduct, even if causing changes in efficiency and/or selectivity.

Starting from these simple SARs, the Carnosine structure has been modified, following three different approaches:

1. replacing the Hystidinil-portion with a different nucleophilic aromatic system, specifically the furan, the thiophene and the p-methoxy-aniline (Figure 12);

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<sup>37</sup> Vistoli G, Pedretti A, Cattaneo M, Aldini G, Testa B, **2006**, *J Med Chem*, 49, 3269-3277; Pedretti A, De Luca L, Marconi C, Negrisoni G, Aldini G, Vistoli G., **2008**, *ChemMedChem*, 3, 1913-1921; Pedretti A, De Luca L, Marconi C, Regazzoni L, Aldini G, Vistoli G., **2011**, *Bioorg Med Chem.*, 19, 4544-4551;

- substituting the  $\beta$ -alanyl portion with ten different aminoacids, chosen in order to cover exhaustively the available chemical space; the synthesized diastereoisomeric pairs of Histidine-containing dipeptides are shown in Figure 14;
- rigidifying the whole structure, with the insertion of a 2-oxazolidinone that includes the amino- and the C-terminal-portion of the Histidine (Figure 15)

The first approach wanted to evaluate the substitution of the Histidine ring with other moieties, nucleophilic enough to form the Michael adduct.

Following this idea, several aromatic rings were chosen, in order to have a balance between reactivity and nucleophilicity of the ring itself (Figure 13).

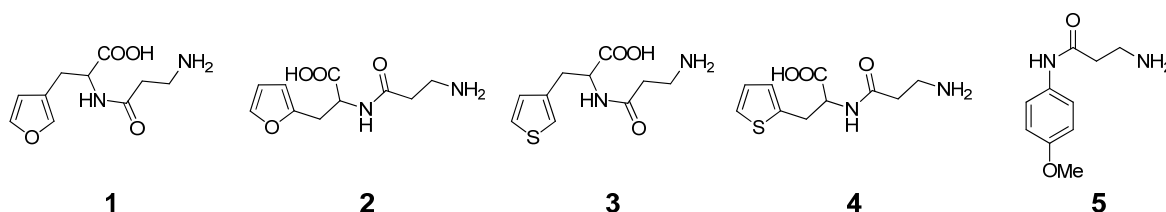


Figure 12

The nucleophilicity ( $\omega$ ) is expressed, as we can see from the Figure 13 below, from a mathematic ratio.

This nucleophilicity scale was derived by HOMO/LUMO energies ( $\mu$  and  $\eta$ ) of the two interacting partners (A and B) as here reported for imidazole ring with acrolein.

Such calculations suggest that furan and thiophene could have a conformational profile and reactivity rather similar to those of imidazole; therefore they have been chosen as substituting moieties.

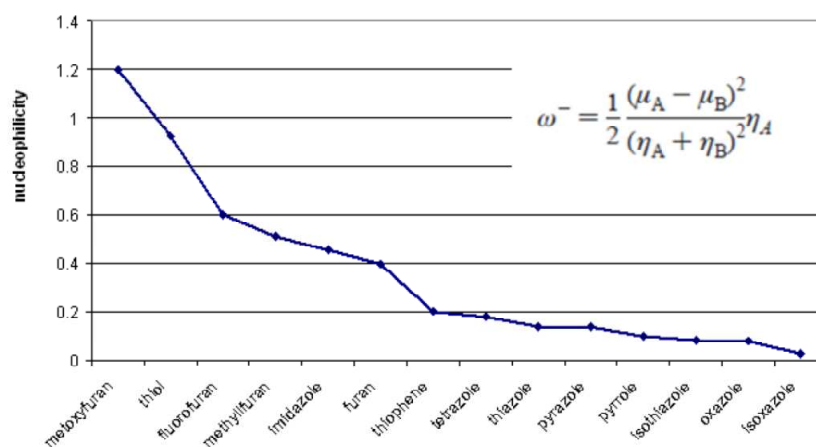


Figure 13: Nucleophilic scale for different aromatic rings

In the second approach (Figure 14), since the Carnosine carboxyl group is not required for carbonyl scavenging and its modifications can improve the resulting pharmacokinetic profile, as it has been explained above, the dipeptides were prepared with the C-terminus capped by a methyl ester so as to study dipeptides which should be still recognized by peptide transporters and resistant to proteolysis. Moreover, the study considered diastereoisomeric pairs of dipeptides produced by alternating the absolute configuration of the Histidine residue with a view to revealing the effect of configuration on quenching activity.

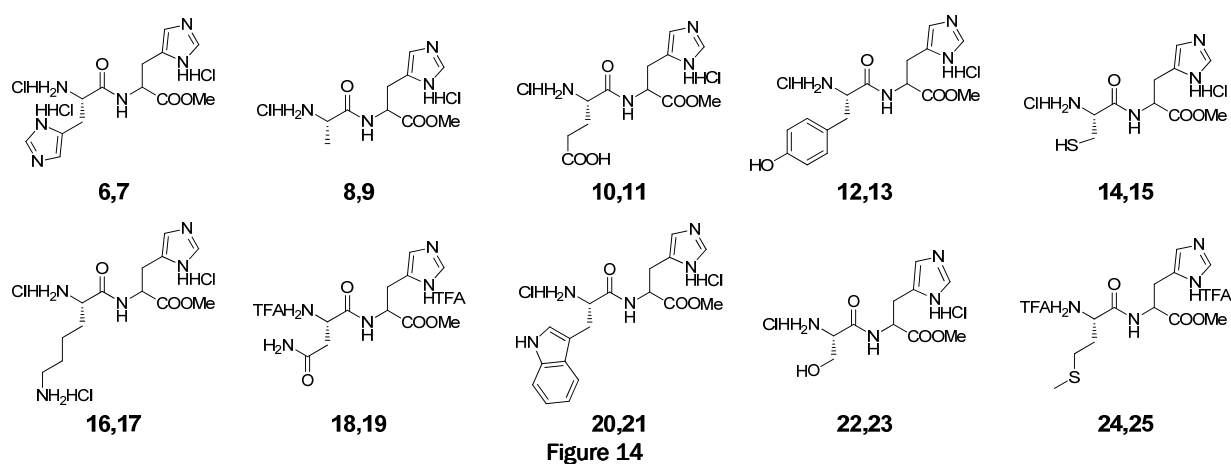


Figure 14

Indeed, while the two Carnosine enantiomers have almost the same quenching activity, a result easily explainable since two enantiomers must possess the same chemical reactivity, the introduction of a second chiral center should allow diastereoisomeric differences useful for a better understanding of the precise scavenging mechanism.

The third approach (Figure 15) was performed in order to follow a new research line, not yet explored that is the total rigidifying of the structure. The synthesised molecule includes all the mandatory features explained before.

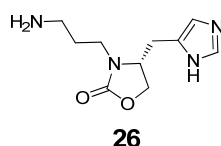
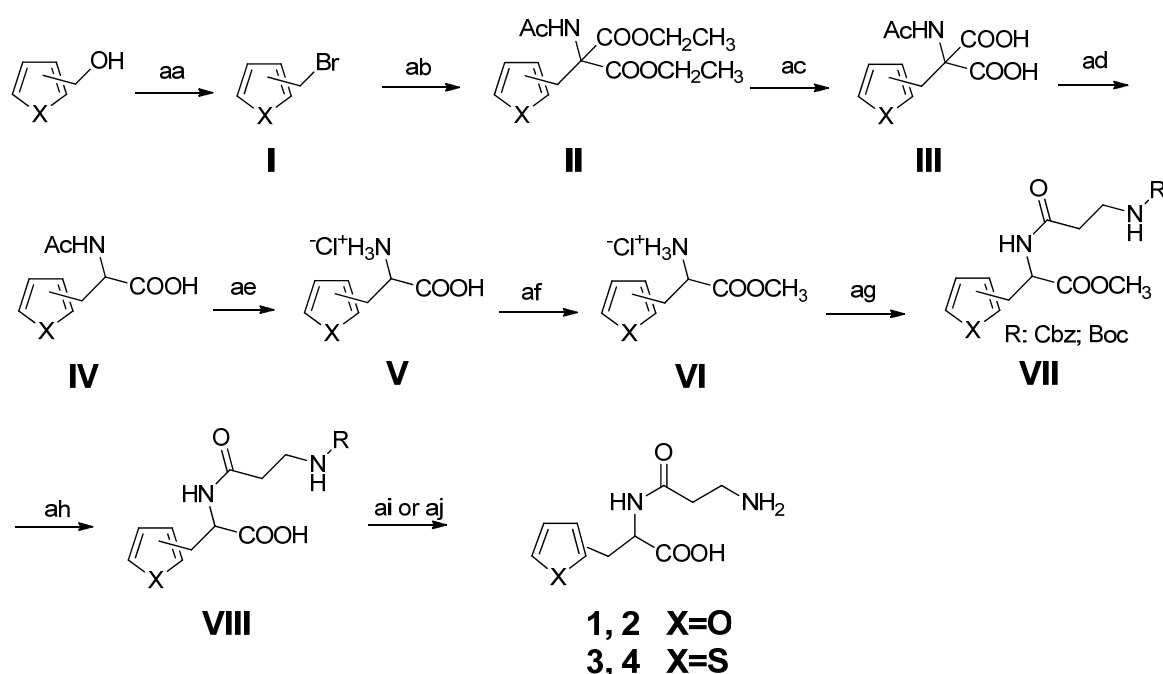


Figure 15

## Synthetic scheme

The synthetic pathway has been peculiar for each of the three different classes of compounds.

The Carnosine-like derivatives reported in Figure 16 were prepared starting from the 2- or 3- furfuryl alcohol and 2- or 3-thiophenemethanol which are commercially available.



Reagents and conditions: (aa)  $\text{PBr}_3$ , THF,  $-5^\circ\text{C}$ ; (ab)  $t\text{-BuOK}$ , diethyl acetamidomalonate, THF,  $\text{Et}_2\text{O}$ , reflux; (ac)  $\text{NaOH}$  4M, dioxane, reflux; (ad)  $\text{CH}_3\text{COOH}$ , dioxane, reflux; (ae)  $\text{NaOH}$  30%, reflux;  $\text{HCl}$  conc, methanol; (af)  $\text{SOCl}_2$ , trimethylorthoformate, MeOH,  $50^\circ\text{C}$ ; (ag) R- $\beta$ -Alanine, HOBt, EDAC, DIPEA, DCM, RT; (ah)  $\text{NaOH}$  4M, MeOH, RT; (ai)  $\text{H}_2$  Pd/C 5%, MeOH, RT; (aj) TFA, anisole.

Figure 16

The alcoholic derivative underwent firstly bromination (aa), using  $\text{PBr}_3$  in THF, with good yields and purity of the products, and then nucleophilic substitution (ab) from the diethyl acetamidomalonate, operating in THF using potassium *tert*-butoxide as base.



The ethylic esters hydrolysis (ac) is achieved in basic conditions, accomplishing the bi-carboxylic derivative, which, after treatment with acetic acid (ad), let the obtainment of compound **IV**.

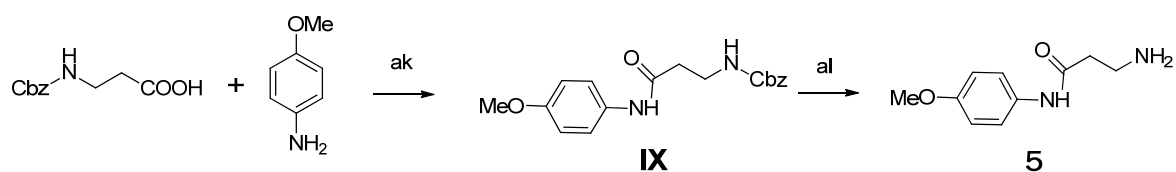
The basic hydrolysis of the acetamidic function (ae) and the esterification of the free carboxylic acid, achieved in Methanol using thionyl chloride (af), yielded compound **VI**.

**VI** was coupled, using HOBt and EDAC as coupling reagents (ag), with  $\beta$ -alanine, differently protected for the furan- and thiophene- derivatives. In the first case, the aminic function is capped with benzyl chloroformate, and, after basic hydrolysis of the esteric function (ah), the Cbz- is easily removed by hydrogenolysis.

On the other hand, the 2-thiophene-derivative was initially protected, one more time, with benzyl chloroformate; nevertheless in this case, after basic hydrolysis of the esteric function (ah), the Cbz removal wasn't possible by hydrogenolysis, due to the poisoning action of the sulphur on the Pd/C. Therefore the final compound was accomplished treating the compound with Trifluoroacetic acid, in presence of anisole as scavenger.

In order to avoid such difficulties, in the synthetic pathway of the 3-thiophene compound, the  $\beta$ -alanine was protected with di-*tert*-butyl-dicarbonate, whose removal was easily achieved in acidic conditions.

The synthesis of compound **5** is here reported (Figure 17).



Reagents and conditions: (ak) Cbz- $\beta$ -Alanine, HOBt, EDAC, DIPEA, DCM, RT; (al) H<sub>2</sub> Pd/C 5%, MeOH, RT.

**Figure 17**

It started from the commercially available *p*-methoxy-aniline, that is coupled with Cbz- $\beta$ -alanine in dichloromethane, using HOBt and EDAC as coupling reagents (ak). The hydrogenolysis (al) accomplished the final product in good yield.

Concerning the second approach<sup>38</sup>, the dipeptides were prepared by maintaining the N-terminal residue in its natural *L* configuration and alternating the configuration of the Histidine residue, in order to obtain the desired diastereoisomeric pairs of general formula NH<sub>2</sub>-*L*-X-(*L* or *D*)-His-OMe.

All dipeptides were synthesized as methyl esters to facilitate their physicochemical profiling, characterized by <sup>1</sup>H NMR spectroscopy and analyzed by reverse-phase HPLC under conditions effective in diastereomer separation, in order to prove their >98% purity and, what is more, the absence of any epimer.

The synthesis of each dipeptide started by preparing the specific *L*-amino acid, when non commercially available: the amino group is protected using benzyl chloroformate in basic conditions, the eventual functional groups on the lateral chain are suitably protected.

The *L*- and *D*- Histidine were prepared as methyl ester dihydrochloride (**X**), treating the amino acid with thionyl chloride and trimethyl orthoformate in methanol, and then coupled with each *L* protected amino acid in DMF, using HOBt and EDAC as coupling reagents, yielding **XI** (Figure 18).

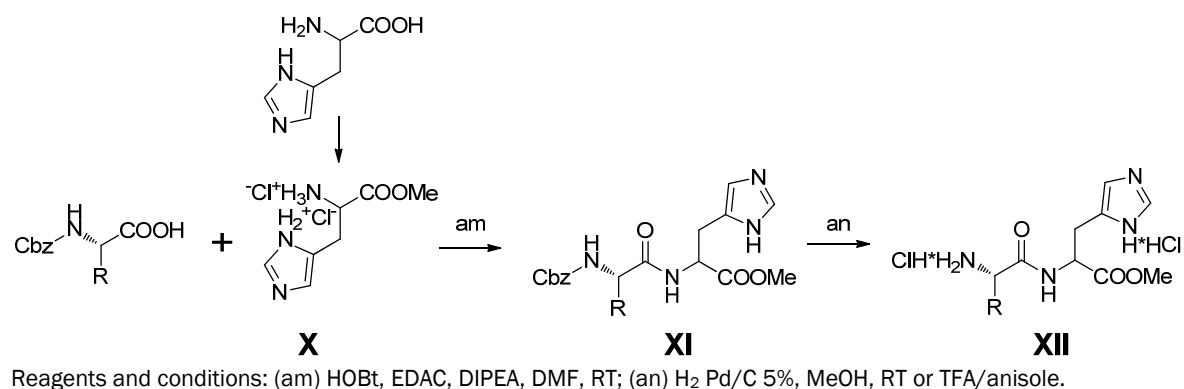


Figure 18

The resultant crude full-protected dipeptide was purified by column chromatography.

The purification conditions of each single dipeptide and the respective yields are listed in Table 1.

<sup>38</sup> Vistoli G, Straniero V, Pedretti A, Fumagalli L, Bolchi C, Pallavicini M, Valoti E, Testa B, **2012**, *Chirality*, 24, 566-576;

Dipeptide	Yield (%)	Eluent
<i>Z-L-Hys-L-Hys-OMe</i>	47	AcOEt/MeOH (95:5)
<i>Z-L-Hys-D-Hys-OMe</i>	50	AcOEt/MeOH (95:5)
<i>Z-L-Val-L-Hys-OMe</i>	60	DCM/MeOH (90:10)
<i>Z-L-Val-D-Hys-OMe</i>	46	DCM/MeOH (90:10)
<i>Z-L-Glu(<math>\gamma</math>Bn)-L-Hys-OMe</i>	49	AcOEt/MeOH (95:5)
<i>Z-L-Glu(<math>\gamma</math>Bn)-D-Hys-OMe</i>	66	AcOEt/MeOH (95:5)
<i>Z-L-Tyr(Bn)-L-Hys-OMe</i>	51	DCM/MeOH (95:5)
<i>Z-L-Tyr(Bn)-D-Hys-OMe</i>	49	DCM/MeOH (95:5)
<i>Z-L-LysZ-L-Hys-OMe</i>	55	DCM/MeOH (90:10)
<i>Z-L-LysZ-D-Hys-OMe</i>	50	DCM/MeOH (90:10)
<i>Z-L-Asn-L-Hys-OMe</i>	36	DCM/MeOH (96:4)
<i>Z-L-Asn-D-Hys-OMe</i>	38	DCM/MeOH (96:4)
<i>Z-L-Trp-L-Hys-OMe</i>	92	AcOEt/MeOH (95:5)
<i>Z-L-Trp-D-Hys-OMe</i>	86	AcOEt/MeOH (95:5)
<i>Z-L-Ser(tBu)-L-Hys-OMe</i>	73	AcOEt/MeOH (95:5)
<i>Z-L-Ser(tBu)-D-Hys-OMe</i>	80	AcOEt/MeOH (95:5)
<i>Z-L-Met-L-Hys-OMe</i>	50	DCM/MeOH (90:10)
<i>Z-L-Met-D-Hys-OMe</i>	63	DCM/MeOH (90:10)
<i>Z-L-Dmt-L-Hys-OMe</i>	51	AcOEt
<i>Z-L-Dmt-D-Hys-OMe</i>	58	AcOEt

**Table 1: Yields and elution conditions of the full protected dipeptides**

Each purified dipeptide was analyzed by HPLC at 1 ml/min flow rate (0.5 ml/min for the dipeptides containing Asparagine or Glutamic acid) with the elution gradient stated in Table 2.

Time	Water (%)	ACN (%)	1% aqueous TFA (%)
$t_0$	90	0	10
$t_3 = 25'$	75	15	10
$t_4 = 35'$	10	80	10
$t_5 = 40'$	90	0	10

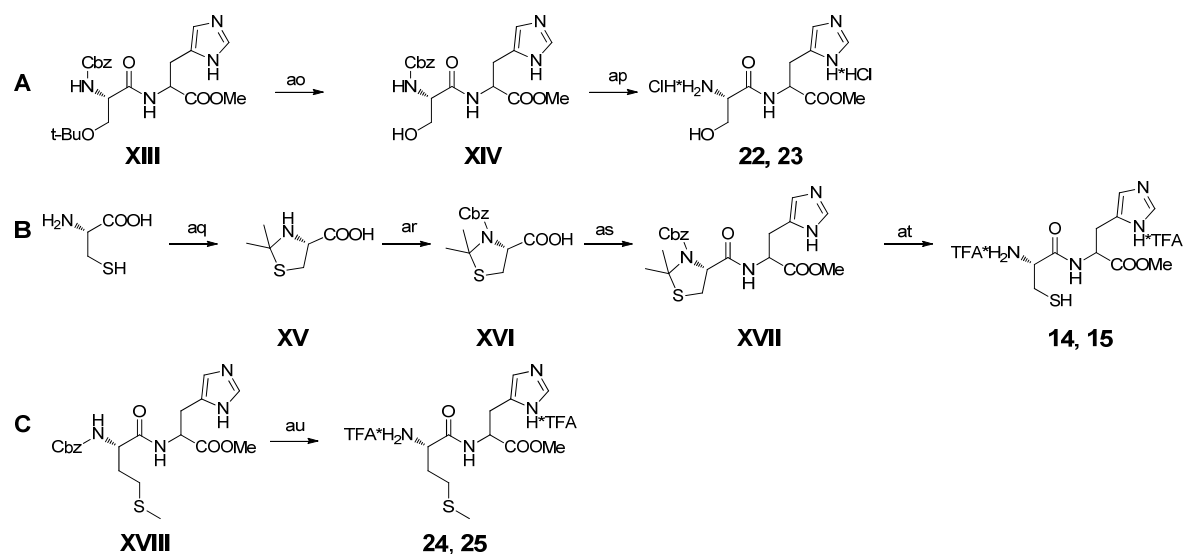
ACN, acetonitrile; TFA, trifluoroacetic acid.

**Table 2: Elution gradient for the analysis of full-protected dipeptides by HPLC**

Under such conditions, proved to be effective in separating all the diastereoisomeric pairs of the dipeptides epimers at the histidine stereocentre, each purified dipeptide was found exempt from its diastereoisomers.

The fully protected dipeptides, with the exception of those containing serine, cysteine, and methionine, were deprotected by hydrogenolysis in a mixture of dry methanol/ 1.25M methanolic hydrogen chloride, in the presence of 10% Pd/C.

In the case of Serine (Figure 19-A), the Serine suitably protected was coupled with both the methyl ester of the Histidine dihydrochlorides, yielding the dipeptide **XIII** according to the general coupling procedure. The side chain protection was removed stirring the dipeptide in a mixture 7:3 DCM/ TFA, in the presence of anisole (ao). The crude products **XIV** underwent hydrogenolysis (ap), giving the final dipeptide, as dihydrochloride (**XV**).



Reagents and conditions: (ao) TFA/DCM= 7/3, anisole; (ap) MeOH\*HCl 1,25M, MeOH; (aq) Acetone; (ar) Benzyl chloroformate, DIPEA, ACN; (as) HOBt, EDAC, DIPEA, DMF; (at) TFA, anisole; (au) TFA, anisole.

Figure 19

The synthesis of Cysteine containing dipeptides (Figure 19-B) started from the formation of the dimethyl thiazolidine (aq; **XV**), that is sequentially protected on the aminic function (ar) with benzyl chloroformate, in Acetonitrile and DIPEA (**XVI**). The isolation of the protected compound was followed by the coupling with both the Histidine dihydrochlorides, following the general method (as). The full protected dipeptides (**XVII**) were stirred in TFA, in the presence of anisole, yielding the final products (at).

The full protected Methionine containing dipeptides (**XVIII**) were obtained with the general coupling procedure (Figure 19-C); the consecutive treatment in TFA in the presence of anisole (au) accomplished the final compounds, in quantitative yield.

Each final dipeptide, methyl esterified at the C-terminal amino acid histidine, was analyzed by HPLC at 1 ml/min flow rate (0.5 ml/min for the dipeptides containing asparagine or glutamic acid) with the elution gradient stated in Table 3.

Time	Water (%)	ACN (%)	1% aqueous TFA (%)
$t_0$	90	0	10
$t_1 = 5'$	89	1	10
$t_2 = 10'$	85	5	10
$t_3 = 25'$	75	15	10
$t_4 = 35'$	10	80	10
$t_5 = 40'$	90	0	10

Table 3: Elution gradient for the analysis and the preparative purification of deprotected dipeptides, by HPLC

When purity was found lower than 98% (UV detector 220 nm), the dipeptide was purified and isolated as di-trifluoroacetate by reverse-phase HPLC under the same conditions reported previously for the analyses, but using a preparative C-18 column.

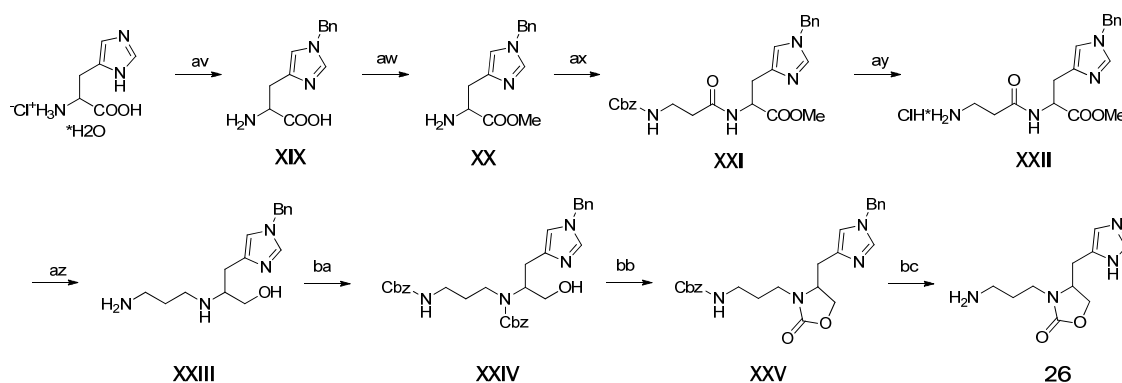
The third approach has been the rigidifying of the whole molecule (Figure 20), obtained with the insertion of a 2-oxazolidinone, that includes the amino- and the C-terminal-portion of the Histidine; the synthesis started from the Histidine monohydrate monochloride, which reacted with benzylchloride, in liquid  $\text{NH}_3$  and Na (av).

The achieved solid (XIX) was then treated in Methanol with Methanolic hydrogen chloride (aw), giving the corresponding methyl ester. The Histidine suitably protected was sequentially coupled with the  $\beta$ -alanine (ax), having the amine function protected as benzyl carbamate, under the usual coupling condition.

The hydrogenolysis (ay) and the consecutive reduction (az) of both the amide and ester functions, achieved heating the dipeptide in THF in the presence of Lithium aluminum hydride, yielded a very polar compound (XXIII), having free primary amines.

Therefore, the two aminic functions were transformed in the corresponding benzyl carbamates, using benzyl chloroformate and DIPEA in THF (ba).

The formation of the oxazolidinone (XXV) was simply obtained treating the resulted compound with sodium hydride in dry THF; the consecutive hydrogenolysis allowed the desired compound.



Reagents and conditions: (av) Na, BnCl,  $\text{NH}_3$ ; (aw) MeOH·HCl 1,25M, MeOH; (ax) Cbz- $\beta$ -alanine, HOBt, EDAC, DIPEA, DMF; (ay)  $\text{H}_2$ , Pd/C 10%, MeOH·HCl, MeOH; (az)  $\text{LiAlH}_4$ , heating, THF; (ba) benzyl chloroformate, DIPEA, THF; (bb) NaH, THF; (bc)  $\text{H}_2$ , Pd/C 10%, MeOH·HCl, MeOH.

Figure 20

This compound was analyzed by reverse phase HPLC, at 0.5 ml/min flow rate with the elution gradient stated in Table 3, and further purified by reverse phase preparative column, using the same conditions reported previously for the analyses but using a preparative C-18 column.

## Physicochemical, computational and biological evaluation

All the novel Carnosine-like derivatives were biologically evaluated; in addition the set of diastereoisomeric dipeptides underwent physicochemical profiling, computational studies and biological evaluation.

### *Physicochemical data*

The physicochemical properties of the dipeptides were used to investigate configurational effects on ionization and lipophilicity, as well as to develop correlative models able to predict the diastereoisomeric differences.

The amino acids, as explained before, were chosen to cover exhaustively the available chemical space, even though the zwitterionic character of the glutamate-containing dipeptides impeded their accurate lipophilicity evaluation, and the dipeptides were synthesized as methyl esters. This choice facilitated their physicochemical profiling; indeed, esterification (1) reduces polarity, (2) simplifies the ionization scheme, and (3) abolishes the zwitterionic behavior that would prevent experimental log P determination.

The attention was focused on the accurate measurement of log P<sup>N</sup><sup>39</sup> only because the log P values for most ionized states were somewhat out of the range of the apparatus (i.e., log P<sup>i</sup> < -1).

The physicochemical results are compiled in Table 4 that includes ionization constants (namely pK<sub>1</sub> for the N-terminus, pK<sub>2</sub> for the Histidine imidazole ring, and eventually, pK<sub>3</sub> when the second side chain possessed an ionizable group), lipophilic data (i.e., log P<sup>N</sup> and logD<sup>7.4</sup>) plus the corresponding diastereoisomeric differences and some overall averages.

Physicochemical data for some well-known natural histidine-containing dipeptides (i.e., carnosine, β-Ala-His and homocarnosine, γ-aminobutyryl-His) were also included for easy comparison.

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<sup>39</sup> Log P<sup>N</sup>: normalized partition coefficient; logD<sup>7.4</sup>: distribution coefficient at pH=7.4 (the physiological pH of blood serum).

Dipeptide	pK <sub>1</sub>	pK <sub>2</sub>	pK <sub>3</sub>	ΔpK <sub>mean</sub>	log P <sup>N</sup>	log D <sup>7.4</sup>	Rotors
Carnosine*	2.76	6.72	9.32	—	— <sup>a</sup>	— <sup>a</sup>	5
Homocarnosine*	2.75	6.79	9.88	—	— <sup>a</sup>	— <sup>a</sup>	6
<i>L</i> -Asn- <i>D</i> -His-OMe	7.28	6.06	—	—	0.65	0.41	8
<i>L</i> -Asn- <i>L</i> -His-OMe	7.04	5.98	—	—	0.20	-0.14	8
Δ(Asn-His-OMe)	0.24	0.08	—	0.16	0.45	0.55	—
<i>L</i> -Cys- <i>D</i> -His-OMe	6.98	5.94	9.19	—	2.46	0.90	7
<i>L</i> -Cys- <i>L</i> -His-OMe	6.96	5.94	9.10	—	1.78	0.47	7
Δ(Cys-His-OMe)	0.02	0.00	0.09	0.04	0.68	0.43	—
<i>L</i> -Glu- <i>D</i> -His-OMe	7.97	6.30	3.71	—	— <sup>a</sup>	— <sup>a</sup>	8
<i>L</i> -Glu- <i>L</i> -His-OMe	7.99	6.44	3.79	—	— <sup>a</sup>	— <sup>a</sup>	8
Δ(Glu-His-OMe)	0.02	0.14	0.08	0.08	— <sup>a</sup>	— <sup>a</sup>	—
<i>L</i> -His- <i>D</i> -His-OMe	7.40	6.35	4.92	—	-0.77	-1.08	8
<i>L</i> -His- <i>L</i> -His-OMe	7.48	6.42	5.15	—	0.10	-0.05	8
Δ(His-His-OMe)	0.08	0.07	0.23	0.12	0.87	1.03	—
<i>L</i> -Lys- <i>D</i> -His-OMe	7.65	6.03	10.30	—	-0.79	-1.01	10
<i>L</i> -Lys- <i>L</i> -His-OMe	7.63	6.66	10.78	—	-0.47	-0.86	10
Δ(Lys-His-OMe)	0.02	0.63	0.48	0.38	0.32	0.15	—
<i>L</i> -Met- <i>D</i> -His-OMe	7.52	6.19	—	—	2.96	2.69	8
<i>L</i> -Met- <i>L</i> -His-OMe	7.30	6.10	—	—	1.94	1.73	8
Δ(Met-His-OMe)	0.22	0.09	—	0.16	1.02	0.96	—
<i>L</i> -Ser- <i>D</i> -His-OMe	7.32	6.26	—	—	0.36	0.20	7
<i>L</i> -Ser- <i>L</i> -His-OMe	7.23	6.24	—	—	0.47	0.32	7
Δ(Ser-His-OMe)	0.09	0.02	—	0.06	0.11	0.12	—
<i>L</i> -Trp- <i>D</i> -His-OMe	7.57	6.34	—	—	0.71	0.44	8
<i>L</i> -Trp- <i>L</i> -His-OMe	7.54	6.43	—	—	1.17	0.89	8
Δ(Trp-His-OMe)	0.03	0.09	—	0.06	0.46	0.45	7
<i>L</i> -Tyr- <i>D</i> -His-OMe	7.57	6.41	9.86	—	0.02	-0.13	8
<i>L</i> -Tyr- <i>L</i> -His-OMe	7.52	6.41	9.69	—	0.15	0.01	8
Δ(Tyr-His-OMe)	0.05	0.00	0.17	0.07	0.13	0.14	—
<i>L</i> -Val- <i>D</i> -His-OMe	8.09	6.30	—	—	-0.13	-0.56	—
<i>L</i> -Val- <i>L</i> -His-OMe	7.72	6.30	—	—	0.44	0.24	7
Δ(Val-His-OMe)	0.37	0.00	—	0.18	0.57	0.80	7
Means	7.52	6.27	7.65	—	0.66	0.15	—
( <i>L</i> , <i>L</i> ) Means	7.48	6.31	7.69	—	0.74	0.28	—
( <i>L</i> , <i>D</i> ) Means	7.57	6.23	7.62	—	0.59	0.02	—
ΔMean	0.12	0.06	0.14	0.11	0.61	0.65	—
Standard deviation	0.40	0.35	2.88	—	1.06	0.93	0.86

The number of rotatable bonds is also reported as an index of dipeptide flexibility.

<sup>a</sup>Because of their zwitterionic nature, lipophilic data for carnosine, homocarnosine and glutamate-containing dipeptides cannot be accurately determined.

**Table 4: Determined physicochemical properties, plus the corresponding diastereoisomeric differences and some general mean values.**

## pK<sub>a</sub> values

Comparing the pK<sub>1</sub> values of the dipeptides with those of the corresponding free amino acids confirms that the amino group is markedly less basic when belonging to a peptide.

Indeed, although the pK<sub>1</sub> values in free amino acids are almost always greater than 9, they rarely exceed 8 in the analyzed dipeptides because of the electron-withdrawing effect of the peptide bond. This electronic effect is particularly evident when considering the pK<sub>1</sub> values of the included natural histidine-containing dipeptides. Indeed, the shifting of the amino group from the α to the β position (as seen in Carnosine) induces a pK<sub>1</sub> increase of more than one logarithmic unit, whereas further shifting to the γ position (as seen in homocarnosine) has a more modest effect on pK<sub>1</sub>, even though it should be noted that the electron-withdrawing effect of the peptide

bond is still significant also in a  $\gamma$  position as evidenced by a comparison with the clearly greater basicity of alkylamines (e.g., butylamine  $pK = 10.77$ ).

Moreover, the obtained  $pK_1$  values are in line with those reported for unprotected dipeptides, thus indicating that the esterification of the C-terminus does not induce pronounced effects on the  $pK_1$  values, but it may influence differences between diastereoisomers.

The  $pK_2$  values of the imidazole ring remain in a narrow range somewhat above that of free Histidine ( $pK_2 = 5.97$ ) and are rather similar to those of the reported endogenous dipeptides, thus being scarcely affected by the vicinal residue.

The third ionization constant, when present, shows marked differences compared with the corresponding values for the free residues probably because of a mutual influence between adjacent ionizable side chains.

For example, the low basicity of the second imidazole ring in the  $NH_2$ -L-His- (*L* or *D*)-His-OMe dipeptides indicates that the influence between contacting basic moieties destabilizes the electrical state with both protonated imidazole rings.

Also,  $NH_2$ -L-Cys- (*L* or *D*)-His-OMe dipeptides show higher  $pK_3$  values compared with that of the sulfhydryl group in free cysteine ( $pK = 8.33$ ), whereas Lysine-containing and Tyrosine-containing dipeptides show  $pK_3$  values similar to those of the corresponding free amino acids.

When considering the mean difference in the  $pK$  values of each diastereoisomeric pair ( $\Delta pK_{\text{mean}}$ ), there are three different behaviors that depend on conformational flexibility and intramolecular interactions.

They can be schematized as follows:

1. When the dipeptide is characterized by significant intramolecular interactions (mainly, but not exclusively, between side chains), it shows low diastereoisomeric differences ( $\Delta pK_{\text{mean}} < 0.1$ ) because the configurational effects are restrained by conformational rigidity;
2. When the dipeptide does not show relevant intramolecular interactions (apart from some weak hydrophobic contacts), it exhibits intermediate diastereoisomeric differences ( $0.1 < \Delta pK_{\text{mean}} < 0.2$ ), thus indicating that the configurational effects are paralleled by a good conformational flexibility;
3. When the dipeptide conformation is affected by relevant intramolecular repulsions (as in the case of  $NH_2$ -L-Lys- (*L* or *D*)-His-OMe dipeptides between



the ammonium heads), it exhibits large diastereoisomeric differences ( $\Delta pK_{\text{mean}} > 0.2$ ), thus indicating that the configurational effects are amplified by an extreme conformational mobility.

### ***Lipophilicity***

Comparing the present lipophilicity data (Table 4) with literature values confirms the remarkable polarity of these dipeptides, and, despite the impossibility of a direct comparison, suggests that hydrophilicity is only slightly reduced by methyl esterification.

Specifically, most  $\log P^N$  values are found in the range  $0 < \log P^N < 1$ , and only the Cysteine-containing and Methionine-containing dipeptides show markedly higher  $\log P^N$  values.

The extrapolated  $\log D^{7.4}$  values are well correlated with the  $\log P^N$  values ( $r^2 = 0.93$ ) and were found to be slightly lower than the latter. This may indicate that the neutral forms play a relevant role at pH 7.4, a result explainable by the weak basicity of the amino groups.

The diastereoisomeric differences in lipophilicity values ( $\Delta \log P^N$  and  $\Delta \log D^{7.4}$ ) are well understandable considering the discussed conformational properties. The data reported in the table reveal that homochiral isomers are, on average, more lipophilic than the heterochiral isomers as confirmed by both  $\Delta \log P^N$  and  $\Delta \log D^{7.4}$  values. This can be explained by the greater side chain accessibility of the heterochiral isomers that results in more polar derivatives and suggests that the hydrophilicity increase due to a greater imidazole exposition is rarely counterbalanced by the second side chain. Only the significant apolarity of the Cysteine and Methionine side chains can overcome the polarity of the imidazole ring, and indeed, sulfur-containing dipeptides show an opposite trend, their (*L, D*)-isomers being more lipophilic than the (*L, L*)-isomers.

Conversely, the accessibility of the dipeptide termini shows a reduced influence as demonstrated by the higher lipophilicity of the (*L, L*)-isomers despite the greater accessibility of both termini. This may imply that the hydrophilic contributions of the termini, and in particular that of the ionizable N-termini, are so crucial to be almost independent on conformational and configurational effects.

Collectively, lipophilicity data show significant diastereoisomeric differences that appear more pronounced than those observed for the ionization constants and also more homogeneously distributed.

However, one can also recognize here a constraining effect of the intramolecular interactions as seen, for example, when comparing  $\Delta \log P^N$  for  $\text{NH}_2\text{-L-Met-(L or D)-His-OMe}$  with that of  $\text{NH}_2\text{-L-Ser-(L or D)-His-OMe}$  (1.02 vs. 0.11).

Altogether, the lipophilicity data confirm that peptide isomerisation can have a remarkable impact on lipophilicity profile, essentially because of a greater exposition of the side chains in the heterochiral peptides. Clearly, the resulting effect depends on the hydrophilicity of the side chains. In our Histidine-containing dipeptides, isomerization almost always induced a log P decrease ascribable to the polarity of the imidazole moiety, whereas the heterochiral diastereoisomers of apolar peptides are expected to be more lipophilic (and thus more prone to amyloid deposition) as confirmed here for the sulfur-containing dipeptides.

### **MD simulations**

The property space of the dipeptides was investigated by calculating the following properties for each conformer stored during the MD simulations in vacuo, in water, and in chloroform:

1. Its radius of gyration, a well-known descriptor encoding molecular shape and size<sup>40</sup>, and whose variations can be used to estimate molecular flexibility<sup>41</sup>;
2. Its conformer-dependent log  $P^{\text{MLP}}$  value (also known as virtual log P, as computed here by the Molecular Lipophilicity Potential (MLP) approach<sup>42</sup>;
3. Its polar surface area (PSA), which parameterizes their H-bonding capacity<sup>43</sup>.

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<sup>40</sup> Vree C, Mayr SG., **2010**, *New. J.Phys.*;12, 023001;

<sup>41</sup> Vistoli G, Pedretti A, Testa B., **2011**, *Future Med Chem.*, 3, 995–1010;

<sup>42</sup> Gaillard P, Carrupt PA, Testa B, Boudon A., **1994**, *J Comput Aided Mol Des*, 8, 83–96;

<sup>43</sup> Clark DE., **2011**, *Future Med Chem.*, 3, 469–84;

For each property, the resulting space is described by the mean plus the range (namely, the difference between maximum and minimum values) of the computed values, as well as the sensitivity computed as the ratio between property range and number of rotatable bonds.

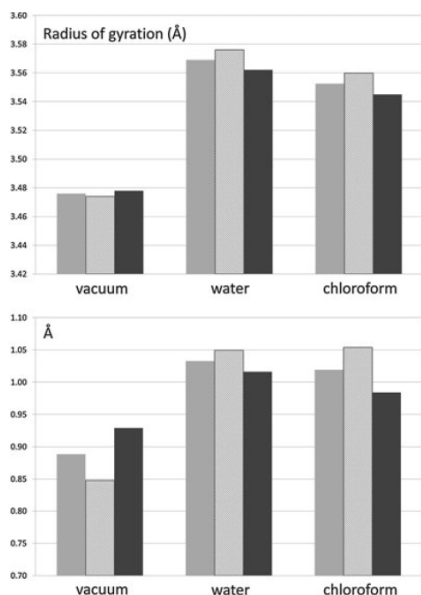


Figure 21: Comparison of the overall means (upper plot) and ranges (lower plot) for the radius of gyration as calculated by molecular dynamics simulations in vacuo, in water, and in chloroform and computed for all dipeptides (light gray bars), homochiral peptides (dashed bars), and heterochiral peptides (dark gray bars).

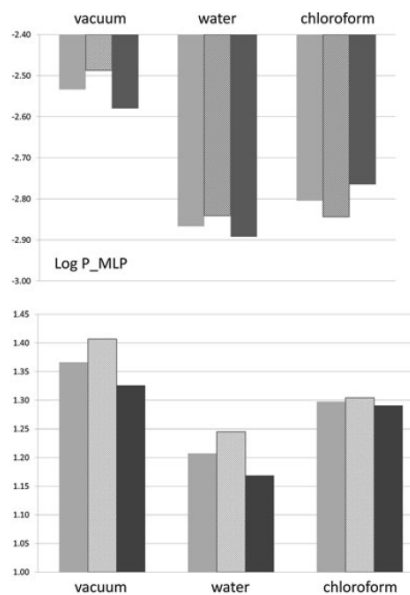


Figure 22: Comparison of the overall means (upper plot) and ranges (lower plot) for the log  $P_{MLP}$  values as calculated by molecular dynamics simulations in vacuo, in water, and in chloroform and computed for all dipeptides (light gray bars), homochiral peptides (dashed bars), and heterochiral peptides (dark gray bars).

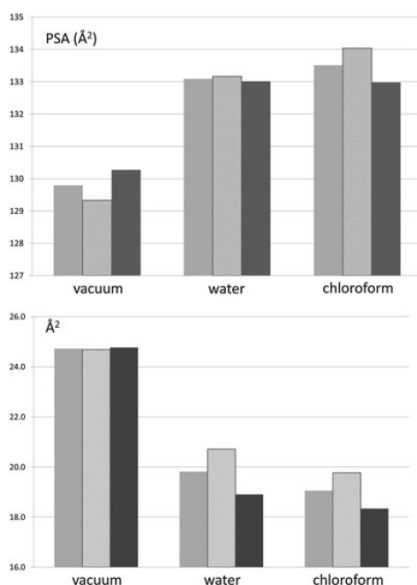


Figure 23: Comparison of the overall means (upper plot) and ranges (lower plot) for the polar surface area (PSA) as calculated by molecular dynamics simulations in vacuo, in water, and in chloroform and computed for all dipeptides (light gray bars), homochiral peptides (dashed bars), and heterochiral peptides (dark gray bars).

Figure 21, Figure 22 and Figure 23 compare the overall averages and ranges as obtained in the simulated media for the radius of gyration (Fig. 21), lipophilicity (Fig. 22), and the PSA (Fig. 23).

With regard to the radius of gyration, Figure 21 shows that all simulated dipeptides assumed more folded conformations in vacuo because, essentially, of intramolecular polar interactions.

In contrast, the monitored conformations appear largely more extended in water and chloroform probably because of strong solute–solvent interactions. Conceivably, the effect is more pronounced in water where the extended geometries are stabilized by strong polar contacts, whereas the effect in chloroform is mainly due to a greater molecular friction experienced by extended geometries.

Conformational variability, as encoded by the ranges of values, is greater in solvents than in vacuum, a result that depends on the abundance of extended geometries in the investigated solvents. The diastereoisomeric differences are quite marginal and without well-defined trends for both means and ranges, thus suggesting that the conformation profiles of all isomers are similarly affected by the simulated media.

The  $\log^{PMLP}$  means (Fig. 22) are in line with the polarity of the simulated environments. Indeed, a vacuum represents the most apolar medium, water as the most polar one, where as chloroform properties are intermediate. Figure 22 confirms that molecules tend to adapt their physicochemical properties to those of the environment without restraining their conformational spaces (as evidenced by the ranges of radius of gyration). The global  $\log^{PMLP}$  means are in encouraging agreement with the experimental data because the heterochiral dipeptides appear more hydrophilic than the homochiral ones in a vacuum and in water because of a greater accessibility of their side chains.

The  $\log^{PMLP}$  means show an opposite trend in chloroform probably because the exposed side chains tend to collapse in an apolar solvent.

Unlike the trends shown by the radius of gyration, the overall  $\log^{PMLP}$  ranges clearly decrease when the dipeptides are simulated in a solvent. This implies that the property adaptability is achieved by narrowing the property space while expanding the corresponding conformational space because the medium is able to select in each conformational cluster those conformers whose polarity most resembles its own. This confirms that conformational space and property spaces are only partly related and

that each cluster of conformers spans most of the property space. Albeit with minimal differences, the computed ranges suggest that the  $\log^{PMLP}$  variability is greater in the homochiral peptides presumably because of a variable exposition of their termini. The range differences vanish in chloroform probably because here, the polar termini tend to minimize their exposure regardless of configuration.

The overall averages and ranges for PSA (Fig. 23) are in line with those of  $\log^{PMLP}$ , although the reported differences are far less pronounced allegedly because of the homogeneity in PSA values. Specifically, it is confirmed the adaptability of the dipeptides simulated in solvents of different polarity and the constraining effects that the solvents exert on property variability as seen in PSA ranges. Similar to what was observed for the radius of gyration, the diastereoisomeric differences are marginal and without defined trends.

The investigated property spaces suggest that genuine physicochemical properties (namely,  $\log^{PMLP}$ ) are more sensible to both environmental and configurational effects compared with geometrical (radius of gyration) or mixed (PSA) properties.

### ***Quenching ability***

Each Carnosine-like derivative has been biologically evaluated, studying the quenching activities toward 4-hydroxy-2-nonenal (HNE), chosen as representative of  $\alpha,\beta$ -unsaturated aldehydes (Table 5 and Table 6).

Compound	% HNE quenching
Carnosine	48.20
<b>1</b>	2.9
<b>2</b>	2.8
<b>3</b>	5.82
<b>4</b>	14.49
<b>5</b>	5.8
<b>26</b>	12.15

**Table 5: HNE quenching activity of the synthesised Carnosine-like derivatives**

Dipeptide	% HNE		% PYR		HNE/PYR
	Quenching	Std dev	Quenching	Std dev	
<b>Carnosine</b>	48.20	—	5.57	—	8.65
<b>6</b>	8.86	2.13	7.70	0.78	1.15
<b>7</b>	6.00	1.75	1.82	1.14	3.30
$\Delta$ (His-His-OMe)	-2.86	$p > 0.05$	-5.88	$p < 0.05$	+2.15
<b>8</b>	16.92	1.27	3.62	4.27	4.67
<b>9</b>	4.97	2.46	0.87	0.42	5.71
$\Delta$ (Val-His-OMe)	-11.95	$p < 0.05$	-2.75	$p > 0.05$	+1.04
<b>10</b>	13.12	2.15	0.73	1.19	17.97
<b>11</b>	12.21	4.37	0.76	0.78	16.07
$\Delta$ (Glu-His-OMe)	-0.91	$p > 0.05$	+0.03	$p > 0.05$	-1.91
<b>12</b>	27.44	1.08	1.29	1.08	21.27
<b>13</b>	12.53	0.78	4.02	0.29	3.12
$\Delta$ (Tyr-His-OMe)	-14.91	$p < 0.05$	+2.73	$p < 0.05$	-18.15
<b>14</b>	100.00	—	40.78	0.73	2.45
<b>15</b>	100.00	—	25.26	1.56	3.96
$\Delta$ (Cys-His-OMe)	0.00	ND	-15.52	$p < 0.05$	+1.51
<b>16</b>	10.20	0.98	0.35	0.11	29.14
<b>17</b>	31.27	1.06	1.87	0.54	16.72
$\Delta$ (Lys-His-OMe)	+21.07	$p < 0.05$	+1.52	$p < 0.05$	-12.42
<b>18</b>	9.50	3.27	1.14	0.64	8.33
<b>19</b>	9.59	3.08	1.00	0.44	9.59
$\Delta$ (Asn-His-OMe)	+0.09	$p > 0.05$	-0.14	$p > 0.05$	+1.26
<b>20</b>	18.83	3.75	5.81	0.55	3.24
<b>21</b>	11.71	2.51	4.20	0.23	2.79
$\Delta$ (Trp-His-OMe)	-7.12	$p < 0.05$	-1.61	$p < 0.05$	-0.45
<b>22</b>	5.91	0.37	2.08	1.55	2.84
<b>23</b>	5.18	0.14	0.10	0.65	51.80
$\Delta$ (Ser-His-OMe)	-0.73	$p > 0.05$	-1.98	$p > 0.05$	+48.96
<b>24</b>	1.81	1.49	3.11	0.29	0.58
<b>25</b>	0.17	0.80	2.61	0.17	0.07
$\Delta$ (Met-His-OMe)	-1.64	$p > 0.05$	-0.50	$p < 0.05$	-0.52
Means	20.31 $\pm$ 28.31		5.46 $\pm$ 9.94		10.24 $\pm$ 12.64
(L, D) Means	21.26 $\pm$ 28.59		6.66 $\pm$ 12.22		9.17 $\pm$ 10.01
(L, L) Means	19.36 $\pm$ 29.53		4.25 $\pm$ 7.51		11.31 $\pm$ 15.32
$\Delta$ Mean	-1.90 $\pm$ 9.64		-2.41 $\pm$ 5.18		+2.15 $\pm$ 17.79

Table 6: HNE/ PYR quenching activity of the dipeptides

In addition, the dipeptides, which revealed to be promising candidates, were also tested towards pyridoxal (PYR), chosen as representative of endogenous carbonyl compounds.

These quenching activities are expressed as percentages of quenched carbonyl compound after a fixed incubation time of 180 min. Table 6 reports also the corresponding standard deviations as well as the dipeptide selectivity calculated as the ratio between the quenching activities toward HNE and PYR.

### ***HNE Quenching***

The data referring to the first five Carnosine-like derivatives and the rigidified one are not comparable to the one of the Carnosine itself; on the other hand some of the twenty dipeptides possess an impressive scavenging activity since they completely quenched HNE during the incubation time, regardless of their capping and configuration.

These data suggest that the Cysteine-containing dipeptides do not follow the multistep mechanism involving the amino and imidazolyl moieties as seen for Carnosine, while their extreme reactivity seems to be due almost exclusively to the marked nucleophilicity of the thiol group. Such a mechanism is indirectly confirmed by the virtually undetectable activity of the Methionine-containing dipeptides and suggests that these very reactive dipeptides cannot be considered promising scaffolds in the design of improved carbonyl quenchers because the remarkable thiol nucleophilicity renders them unsafely reactive molecules acting by nonspecific mechanisms.

In addition, thiol-containing drugs could potentially toxic compounds since they can react with Cysteine thiol functions yielding protein-drug mixed disulfides and these adducts can impair the physiological functions of the modified proteins.

What's more, thiol-containing drugs can behave as haptens when the mixed disulfides become antigenic determinants and initiate an immune response which can culminate in hypersensitivity and, consequently, idiosyncratic reactions (IDRs) depending on the body's capacity to detoxify these adducts.

Nevertheless, some dipeptides show remarkable quenching activities which deserve specific considerations.

The significant activity of the Lysine containing peptides can be explained considering that they possess two primary amines, favouring the formation of the imino intermediate regardless of which amino group is the more reactive one.

In general, dipeptides containing aromatic residues show marked quenching activities probably because the aromatic rings facilitate interactions between the imidazole ring and the acceptor  $\beta$ -carbon atom through  $\pi$ - $\pi$  stacking with a beneficial effect already seen for the  $\beta$ -aryl amino acids<sup>44</sup>.

Meanwhile, the dipeptides presenting the double Histidine, show modest activities suggesting interference between imidazole rings, causing a prevalently negative effect on the whole scavenging mechanism.

Finally, the weak quenching activity of the dipeptides containing hydroxyl functions (specifically Serine and Tyrosine containing peptides) suggests the possibility to yield also hemiacetal intermediates that do not enhance the scavenging activity.

Considering the totality of the dipeptides, the pairs of diastereoisomers show only marginal quenching differences since the heterochiral isomers ( $19.69 \pm 28.07$ ) are barely more active than their homochiral diastereoisomers ( $18.58 \pm 28.52$ ), and this result may be explained considering the heterogeneity of the examined dipeptides which prevents general trends to be uncovered.

Nevertheless, several pairs of diastereoisomers exhibit statistically significant differences, which also reflect the dissimilar physicochemical features.

Table 6 underlines that 11 diastereoisomeric pairs out of 20 show statistically significant differences (i.e.  $p < 0.05$ ) and for 7 pairs out of 11 the heterochiral dipeptide is more active than the homochiral one thus substantiating the trend observed in the overall means. This result can be explained by the conformational differences between diastereoisomers, evidenced in previously, after the physicochemical evaluation.

Consequently, the greater activity of heterochiral dipeptides is easily interpretable in terms of increased accessibility of the Histidine side chain that renders the imidazole ring more prone to the Michael addition, while the intramolecular

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<sup>44</sup> Vistoli G, Orioli M, Pedretti A, Regazzoni L, Canevotti R, Negrisoni G, Carini M, Aldini G., **2009**, *ChemMedChem.*, 4, 967-75;



interactions which characterize the homochiral isomers shield the imidazole ring preventing its approach to the unsaturated imine.

For both Lysine-containing pairs of diastereoisomers, the homochiral isomers are conversely more active than the corresponding heterochiral ones. This finding may suggest that here the imino intermediate is mostly produced by the Lysine's  $\epsilon$ -amino group (rather than by the N-terminus) which is stably closer to the imidazolyl ring in the homochiral dipeptides thus favouring the key Michael addition.

A closer analysis confirms that aromatic and positively charged side-chains have generally a significantly beneficial effect; aromatic residues promote contacts between the imidazolyl ring and the  $\beta$ -carbon atom as discussed above, while positively charged residues may promote the initial imino formation.

On the contrary, negatively charged and H-bonding side-chains show an overall negative effect on the quenching activity: the former can stabilize ion-pairs with primary amine thus hampering the imino formation, while H-bonding residues may engage imidazole ring and imino function in intramolecular H-bonds which shield their nucleophilicity or preclude their contacts.

Generally, hydrophobic residues have a modest effect on quenching activity and at least they can hamper the necessary approach of the reactive groups by steric hindrance.

### ***PYR quenching and peptide selectivity***

Considering the promising HNE quenching activity of some of the dipeptides, it has been also tested their scavenging ability toward an endogenous carbonyl compound (the Pyridoxal), in order to study their selectivity.

The data shown in Table 6 reveal that all the considered derivatives possess very modest quenching activities toward pyridoxal.

Specifically, Cysteine-containing dipeptides have the greatest quenching activity; this could be explained by the marked nucleophilicity of their thiol group.

In addition, the detectable activity of some hydroxyl-containing dipeptides could be due to their capacity to form hemiacetal adducts.

The very poor activity toward PYR of the examined dipeptides confirms that they are unable to yield stable imino derivatives with physiological carbonyls. On average, all peptides show the same poor activity regardless of their capping and absolute configuration, which indeed have a limited impact on the reactivity of the amino group.

Concerning the HNE/PYR selectivity, all tested dipeptides show a marked selectivity for HNE given their very weak quenching activity toward pyridoxal. More importantly, such a remarkable selectivity emphasizes that the HNE quenching of these dipeptides cannot be restricted to the formation of a mere imino function but may also involve a crucial Michael addition.

In conclusion, the present PhD thesis suggests that some dipeptides show a quenching activity only slightly weaker than that of Carnosine; in addition this scavenging activity is endowed by a markedly greater selectivity, therefore they can represent truly promising candidates for the design of improved Carnosine derivatives.

## Chapter 2: Ras proteins<sup>45</sup>

Cell growth, differentiation and survival are regulated by a complex combination of extracellular signals and intracellular signaling cascades.

The Ras proteins, a family of monomeric G molecules (H-ras, N-ras and K-ras) act as “molecular switches”, as linker from extracellular signals to intracellular ones, through membrane receptors.

### **Ras family**

H-, N- and K-ras are members of a highly homologous group of approximately 21 kDa monomeric, membrane-localized GTPases whose structure and function has been extensively studied<sup>46</sup>. The p21 Ras proteins are formed of H-, N- and K-ras, with K-ras existing in two alternatively spliced forms, 4A and 4B, resulting in differing C-terminal residues which are important for post-translational modification.

Every single Ras protein share approximately 85% sequence homology, there's also a larger Ras superfamily, which includes other proteins such as R-ras, M-ras, TC21, Rap1A, Rap1B, RalA and RalB, sharing 40–50% sequence homology<sup>47</sup>.

In its totality, the Ras superfamily includes over 150 small GTPases.

The Ras proteins length is of 188 amino acids for H-ras, N-ras and K-ras 4A, while K-Ras4B is an amino acid longer.

Amino acids 1–165 are highly conserved between the four Ras proteins while the carboxy terminal 25 amino acids could have a considerable variation. In the conserved domain there are several motifs important for protein function including GTP binding, effector binding and Switch I and Switch II loops responsible for Guanine nucleotide exchange factor (GEF) and GTPase activating protein (GAP) interactions (Figure 24).

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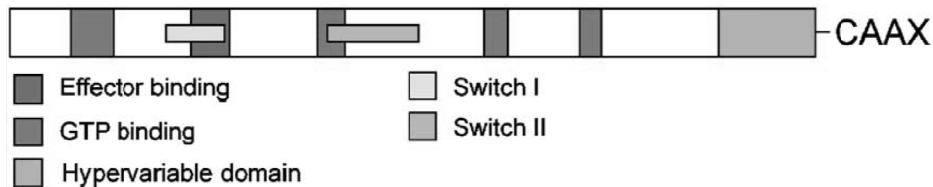
<sup>45</sup> Friday B.B., Adjei A.A., **2005**, *Bioch Biophys Acta*, 1756, 127-144;

<sup>46</sup> C.A. Ellis, G. Clark, **2000**, *Cell. Signal.*, 12, 425–434; A.A. Adjei, **2001**, *Curr. Pharm. Des.*, 7, 1581–1594; A.A. Adjei, **2001**, *J. Natl. Cancer. Inst.*, 93, 1062–1074; M. Barbacid, **1987**, *Annu. Rev. Biochem.*, 56, 779–827; D.R. Lowy, B.M. Willumsen, **1993**, *Annu. Rev. Biochem.*, 62, 851–891;

<sup>47</sup> A. Ehrhardt, G.R.A. Ehrhardt, X. Guo, J.W. Schrader, 2002, *Exp. Hematol.*, 30,x 1089–1106;

The variable region within the carboxy-terminus contains sequences important for post-translational modification, including the CAAX box that is responsible for targeting lipid modification.

K-ras 4A and K-ras 4B differ in this region, which results in differential post-translational modification.

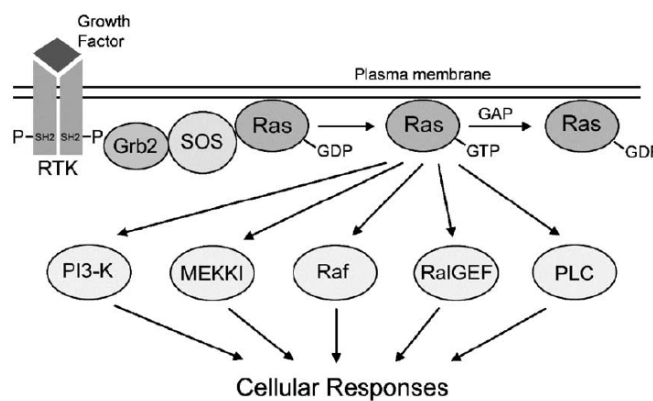


**Figure 24: Schematic depiction of K-ras structure.**

### ***Ras proteins activation***

The crystal structure of Ras reveals that the proteins exist in two different forms, a GDP bound “off” state and a GTP bound “on” state, which cycle from one to the other in response to activation of various receptors.

The activation of the Ras pathway is complex because of the wide range of stimuli that can initiate Ras signalling (Figure 26).



**Figure 25: Ras activation and signaling cascade**

Ras activation begins with stimulation of a vast array of upstream receptors including receptor tyrosine kinases, integrins, serpentine receptors, heterotrimeric G-proteins and cytokine receptors.

The best described pathway is stimulation of Ras via a receptor tyrosine kinase such as EGF receptor; the binding of a ligand to EGF receptor causes oligomerization

of the receptor. This process results in juxtaposition of the cytoplasmic, catalytic domains in a manner that allows activation of the kinase activity and transphosphorylation.

Adaptor proteins such as Grb2 are now able to recognize Sequence Homology 2 (SH2) domains which, in turn, recruit Guanine nucleotide Exchange Factors (GEFs) like SOS-1 or CDC25 to the cell membrane.

The GEF is now capable of interacting with Ras proteins at the cell membrane to promote a conformational change and the exchange of GDP for GTP. Subcellular localization of GEFs is thought to be a key event in Ras activation. Indeed, when SOS-1 or CDC25 are constitutively targeted to the cell membrane, they have enhanced ability to transform NIH 3T3 cells. In addition, the interesting consideration that the pathway is bidirectional, whereby RAS regulates SOS activity, suggests that regulation of this key component of Ras signaling is a very complex mechanism.

Hydrolysis of the GTP to GDP terminates Ras activation; nevertheless Ras proteins have intrinsically low GTPase activity.

The GTPase activity is stimulated by GAPs such as NF1-GAP/neurofibromin and p120-GAP leading to inactivation, and consequently preventing prolonged Ras stimulated signaling. The importance of GAPs in regulating Ras is highlighted by the fact that oncogenic ras mutations almost uniformly abolish the interaction of GAPs and Ras, as described above. In addition, NF1-GAP is a tumor suppressor gene and dysfunction of this gene results in neurofibromatosis type 1, a clinical disorder that places patients at high risk for optic gliomas, astrocytomas, nerve sheath tumors, rhabdomyosarcomas and pheochromocytomas. Ras signaling is normally transient, both because of the intrinsic GTPase activity and the action of GAPs.

However, prolonged or constitutive Ras signaling as a result of ras mutations, NF-1 dysfunction or Ras overexpression is a key event in Ras induced oncogenesis.

### ***Ras proteins effectors***

Activated Ras include a large number of effectors, which serve to regulate myriad cell functions, including growth, survival, differentiation and angiogenesis.

These pathways become more complex as the number of effectors increases and the complexity of regulation by cross-talk between pathways is better understood.

The best characterized Ras effector is the Raf family of Serine/Threonine kinases, including Raf-1, A-Raf and B-Raf.

Raf kinase is a key component of the mitogen-activated protein kinase (MAPK) pathway comprising Raf/MEK/ERK.

Raf is recruited, following Ras activation, to the cell membrane through binding to the switch I domain of Ras and also by lipid binding. Full activation of Raf requires multiple cofactors and phosphorylation steps, and is therefore a complex event not fully understood.

K-ras 4B is the best Ras isoform which most efficiently activates Raf-1, although K-ras 4A, H-ras and N-ras are also capable of activate the pathway. Raf plays a critical role in Ras signaling and oncogenesis, this is due of several reasons.

First, dominant-negative forms of Raf are capable of suppressing H-ras induced cell transformation. Second, constitutively active forms of Raf possess transforming activity comparable to Ras. Third, activating BRAF mutations are found at high frequency in many human cancers, including malignant melanoma (66%) and colon cancer (12%).

However, Raf is not the only effector of Ras in oncogenesis; Raf activation stimulates a signaling cascade by phosphorylation of MEK1 and MEK2, which successively phosphorylate and activate ERK1 and ERK2. Activation of ERK is critical for a large number of Ras-induced cellular responses. ERK1 and ERK2 phosphorylate and activate a variety of transcription factors and kinases, including Elk-1, c-Ets1, c-Ets2, p90RSK1, MNK1, MNK2, as well as other proteins such as the anti-proliferative protein Tob. Many of these Erk targets have been implicated in Ras induced cell transformation.

Phosphoinositide 3V-kinases (PI3-K) generate mitogenic phosphoinositol lipids by phosphorylating various phosphatidylinositol substrates. Of the three known classes of PI3- Ks, each of the three related p110 kinase subunits of class 1 are able to bind Ras through a Ras binding domain similar to those found in Raf and Ral-GDS.

GTP bound Ras stimulates PI3-K activity; in NIH 3T3 cells, both Raf and PI3-K are necessary for cell transformation.

The importance of PI3-K in Ras mediated tumorigenesis likely occurs through several downstream targets of PI3-K. Akt/PKB is a downstream target of PI3-K that plays a significant role in regulation of apoptosis through proapoptotic proteins such as Bad and caspase 9.

Constitutive Akt/PKB activity was found in 16/17 NSCLC cell lines tested, and the activity was PI3-K dependent as treatment with PI3-K inhibitors decreased Akt/PKB activation and induced apoptosis. An alternative target of PI3-K is Rac, a G protein that can be activated by phosphatidyl 3,4,5-triphosphate, a PI3-K product. Rac is important in regulating cytoskeletal rearrangement, which along with membrane ruffling is induced by Ras mediated transformation. Ras-controlled membrane remodelling through Rac is dependent on PI3-K activity, therefore, PI3-K is an important mediator of Ras induced cell transformation.

The GAPs p120 and NF-1/neurofibromin function as Ras effectors in addition to their roles in the negative regulation of Ras through stimulation of GTP hydrolysis. Constitutively active Ras induces the association of p120 and syndecan-2, a complex that can provide docking sites for the oncogene src.

MEKK1 is a Serine/Threonine kinase activated in response to multiple signals including growth factors and cytokines to promote cell survival and apoptosis through a number of mediators such as JNK, SAPK, 14-3-3 and NFkB. MEKK1 may also regulate both Raf and ERK, providing for cross talk between multiple signalling pathways. Indeed, MEKK1 appears to induce apoptosis by dysregulation of a number of pathways including ERK, JNK and p38.

RalGEFs regulate the function of the G proteins RalA and RalB in the same manner that SOS and cdc25 regulate Ras activity, by stimulating the exchange of GDP for GTP.

The RalGEF family contains at least three members with Ras binding domains, RalGDS, Rgl and Rgl2/Rlf.

A dominant negative Ral protein is capable of blocking Ras induced transformation of mouse fibroblasts; this function is at least in part mediated by RalGDS, as RalGDS knock out mice demonstrate resistance to the development of skin tumors in a model dependent on Ras activation.

### Ras proteins post-translational modification

Ras proteins are involved in a multi-stage post-translational modification<sup>48</sup>; their membrane localization is essential for function, but they are synthesised in the cytoplasm, as small and hydrophilic compounds.

Therefore they must undergo a four stage post-translational lipid modification, occurring at the carboxyterminal tetrapeptide, or 'CAAX box' (where "C" is Cysteine, "A" is any aliphatic residue, and "X" is any other residue), (Figure 26):

1. Ras proteins prenylation, resulting in the addition of a farnesyl group to the carboxy terminus; the process is catalyzed by an isoprenyltransferase enzyme;
2. Proteolytic cleavage of the terminal AAX motif by CAAX proteases Rce1 or Afc1;
3. Carboxymethylation at the terminus, via S-adenosyl methionine (SAM) and a specific transferase;
4. Palmitoylation of the SH group of cysteine residues, close to the carboxy terminus, operated by a prenyl protein-specific palmitoyltransferase (PPPTase);

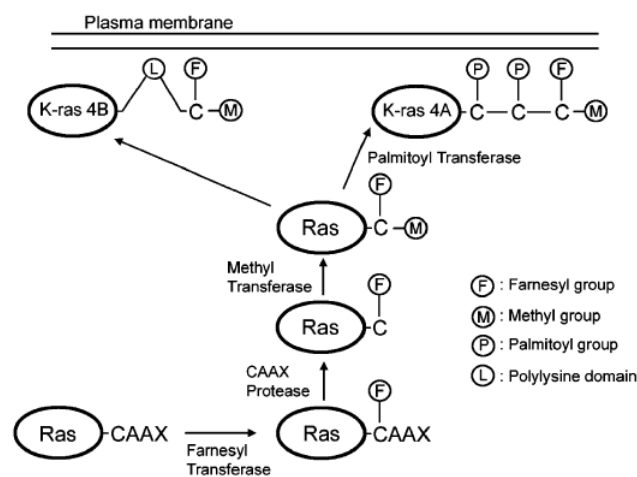


Figure 26: Ras post-translational modification

<sup>48</sup> I.M. Ghobrial, A.A. Adjei, **2002**, *Hematol. Oncol. Clin. N. Am.*, 16, 1065–1088; A.A. Adjei, **2001**, *Cancer Chemother. Biol. Response Modif.*, 19, 149–164; K.N. Cho, K.I. Lee, **2002**, *Arch. Pharm. Res.*, 25, 759–769;



**Ras proteins and oncogenesis**

The importance of Ras signaling in cell growth and survival is evidenced by the importance of Ras in oncogenesis. Specifically, K-ras was identified as a transforming protein in human tumors that was analogous to the transforming protein of the Kirsten murine sarcoma virus.

Several K-ras point mutations have been identified that result in constitutive activation. These mutations are found at high frequency (from 10% to 90%) in almost all the human tumors (Figure 27).

Tumor type	Frequency
Pancreatic	72–90%
Colorectal	32–57%
Lung	15–50%
Endometrium	5–50%
Gallbladder	14–38%
Multiple myeloma	16–33%
Testicular	9–12%

**Figure 27: Prevalence of k-ras mutations in selected human tumors**

Pancreatic tumors appear to have a particular propensity for K-ras mutations, with identifiable mutations in approximately 72–90% of all pancreatic tumors.

Alternatively, some tumors have a relatively high prevalence of mutations in other *ras* genes, but only rare K-ras mutations; for instance N-ras mutations are common in both primary melanomas and melanoma cell lines, while K-ras mutations are rare.

The differential spectrum of *ras* mutations among the various tumor types likely reflects their expression levels and cell-specific roles in survival and growth.

There are differences between the Ras proteins in their post-translational modifications, GEFs and effectors.

In the absence of activating mutations, K-ras may still play a role in oncogenesis via Ras gene amplification, overexpression or upstream activation of the pathway. Each of these potential cellular alterations will produce increased activation of Ras effectors, thereby promoting development of tumors.

In esophageal adenocarcinomas, 40% of the tumors were found to have amplification of the K-ras gene; in addition, morphologic transformation of 10T1/2 cell lines is associated with overexpression of wild-type K-ras.

Although *ras* mutations are rare in breast cancer, Ras is highly active in approximately 50% of tumors compared to benign tissue, and this is associated with expression of epidermal growth factor and HER-2 receptors.

In low-grade ovarian serous carcinomas, active MAPK was present in 41% of the tumors tested that did not have either K-ras or BRAF activating mutations. The importance of Ras activation in the absence of activating mutations is also suggested by the success of receptor tyrosine kinase targeted therapies, such as anti Her-2 monoclonal antibody in breast cancer and anti-EGFR monoclonal antibodies in colon cancer.

### ***Ras as pharmaceutical target***

Because of its crucial role in oncogenesis, various strategies have been developed to target K-ras for the treatment of human cancers.

These strategies have ranged from inhibiting protein expression via anti-sense oligonucleotides to blocking post-translational modification, to inhibiting downstream effectors.

The best results were obtained interfering with Ras post-translational modification, and therefore with Ras-driven cell transformation. In particular, prenylation, that is the first reaction of the multiple post-translational modifications, is the only one crucial for Ras biological activity; the others are dispensable<sup>49</sup>.

Thus, interference with Ras prenylation (catalyzed by specific isoprenyltransferase enzymes) offered an interesting strategy in the interference with Ras-driven cell transformation.

Prenylated proteins can be grouped into two major classes: the one containing the CAAX motif and the so-called CC- or CXC-containing proteins.

The former class contains a diverse group of proteins, whereas the latter is almost exclusively composed of members of the Rab family; they are small GTP binding proteins that participate in intracellular membrane trafficking.

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<sup>49</sup> Kato K, Cox AD, Hisaka MM, Graham SM, Buss JE, Der CJ, **1992**, *Proc Natl Acad Sci USA*, 89, 6403–6407;

Three known enzymes catalyze isoprenoid addition to proteins; these are termed protein farnesyltransferase (FTase), protein geranylgeranyltransferase type I (GGTase-I), and protein geranylgeranyltransferase type II (GGTase-II).

FTase and GGTase-I are closely related and transfer a farnesyl group or a geranylgeranyl group from farnesyl diphosphate (FPP) and geranylgeranyldiphosphate (GGPP), respectively, to the cysteine residue of CAAX-containing proteins.

FTase catalyzes the transfer of a 15-carbon isoprenyl group on farnesyl diphosphate (FPP) to its protein substrates via the formation of a covalent thioether bond. GGTase-I similarly transfers a 20-carbon isoprenyl group to its target proteins.

The carboxyl-terminal residue of the CAAX motif (i.e. the "X") in general determines which isoprenoid will be added to a protein<sup>50</sup>. When "X" is Serine, Methionine, or Glutamine, proteins are recognized by FTase whereas a Leucine at this position results in modification by GGTase-I.

However, these preferences are not absolute and there is no exact rule to predict the CAAX specificity for prenyltransferases. All Ras proteins are preferentially farnesylated by FTase, but if FTase activity is inhibited K-Ras and N-Ras can be geranylgeranylated by GGTase.

These biochemical data suggest that a combination of FTase and GGTase inhibitors may be needed to inhibit the prenylation of K-Ras or N-Ras proteins. However, FTase inhibitors are sufficient to achieve growth suppression in cancer cells and co-application of GGTase inhibitors does not increase this effect.

In addition, there was also the proof that mutated Ras proteins remain active when geranylgeranylated, but that normal H-Ras protein exerts growth-suppressive effects when geranylgeranylated.

On the other hand, GGTase-II transfers geranylgeranyl groups from GGPP to both Cysteine residues of CC- or CXC- containing proteins in a process mechanistically distinct from that of the CAAX proteins<sup>51</sup>.

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<sup>50</sup> Casey PJ, Thissen JA, Moomaw JF., **1991**,. *Proc. Natl. Acad. Sci. USA*, 88, 8631-8635; Yokoyama K, Goodwin GW, Ghomashchi F, Glomset JA, Gelb MH., **1991**, *Proc. Nutri. Acad. Sci. USA*, 88, 5302-5306;

<sup>51</sup> Seabra MC, Goldstein JL, Sudhof TC, Brown MS., **1992**, *J. Biol. Chem*, 267, 14497-14503; Horiuchi H, Kawata M, Katayama M, Yoshida Y, Musha T, et al. **1991**,. *J. Biol. Chem*,. 266, 16981-16984; Farnsworth CC, Seabra MC, Ericsson LH, Gelb MH, Glomset JA. **1994**,. *Proc. Natl. Acad. Sci. USA*, 91, 11963-11967;

## FTase<sup>52</sup>

Mammalian protein Farnesyltransferase (FTase) was first identified and purified to homogeneity from rat brain cytosol.

FTase is a heterodimer consisting of 48 kDa ( $\alpha_{F/GGI}$ )- and 45 kDa ( $\beta_F$ )- subunit polypeptides; the nomenclature  $\alpha_{F/GGI}$  is chosen because the  $\alpha$  subunit is also a component of GGTase-I. Substrates for FTase in mammalian cells include all known Ras proteins, nuclear lamins A and B, the  $\gamma$  subunit of the retinal trimeric G protein transducin, rhodopsin kinase, and a peroxisomal protein of unknown function termed PxF.

The mammalian  $\alpha_{F/GGI}$  subunit is formed of 377 amino acid and has a calculated molecular weight is 44 kDa. A string of nine consecutive Proline residues near the amino terminus is responsible for the apparent molecular weight of 48 kDa observed on SDS-PAGE.

The mammalian  $\beta_F$  subunit consists of 437 residues with a calculated molecular weight of 48.6 kDa. The  $\alpha_{F/GGI}$  and  $\beta_F$  subunits of mammalian FTase show about 30% and 37% identity with the proteins encoded by the *S. Cerevisiae* genes RAM2 and RAM1 (also known as *DPR1*), respectively.

The two yeast genes were originally identified in a genetic screen of suppressors of RAS2<sup>val19</sup>, a mutationally activated RAS allele; RAM1 was also identified based on its involvement in **a**-factor (a *S. Cerevisiae* carboxymethylated pheromone) processing and as a suppressor of G protein function.

Mutations in either RAM1 or RAM2 abolish FTase activity, and coexpression of the two genes in *E. coli* produces FTase activity that can farnesylate **a**-factor peptide and Ras protein substrates.

A compelling property of FTase is that it can recognize short peptides containing appropriate CAAX motifs as substrates, where “C” is cysteine, “A” is any aliphatic

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<sup>52</sup> Zhang F.L., Casey P.J., **1996**, *Annu Rev. Biochem.*, 65, 241-269;

residue, and “X” is any other residue. Specificity in recognition of CA<sub>1</sub>A<sub>2</sub>X sequences by FTase indicates that the A<sub>1</sub> position has a relaxed amino acid specificity, whereas variability at A<sub>2</sub> and X are more restricted.

Basic and aromatic side chains are tolerated at A<sub>1</sub> but not at A<sub>2</sub>, whereas acidic residues are not well tolerated at either position. Moreover, substitution at the A<sub>2</sub> position by an aromatic residue in the context of a tetrapeptide creates a molecule which is not a substrate for FTase but rather is a competitive inhibitor<sup>53</sup>.

FTase is a Zinc metalloenzyme. Dialysis versus chelating reagents, such as EDTA, is able to completely inactivate the enzyme. Neither Zn<sup>2+</sup> nor Mg<sup>2+</sup> alone restore the activity of metal-depleted FTase, on the other hand addition of both Zn<sup>2+</sup> and Mg<sup>2+</sup> fully restores it.

The dependence on millimolar levels of Mg<sup>2+</sup> for full activity indicates that it is probably not an integral component of FTase; measurement of the zinc content of recombinant FTase has confirmed that FTase contains one mole of zinc per mole of enzyme.

The zinc isn't required for FPP binding, but it is mandatory for protein substrate binding. Whether the zinc plays a structural role or whether it is directly involved in catalysis is not yet known.

One possibility for a catalytic role of the zinc is that the metal could activate the sulfhydryl of the substrate protein cysteine residue and make it more nucleophilic. Evidence for such a “metalloactivation of cysteine” mechanism has been found in a DNA repair enzyme termed Ada, which catalyzes a reaction chemically similar to that of FTase.

Metal substitution studies combined with spectroscopic analysis could provide evidence for such a mechanism.

FTase can bind its two substrates, peptide and FPP respectively, in an independent manner.

Binding of peptide substrate has been closely examined by NMR using a transferred NOE approach, revealing that the CAAX sequence of a peptide substrate adopts a Type I β-turn conformation when bound to the enzyme. A similar study of

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<sup>53</sup> Goldstein JL, Brown MS, Stradley SJ, Reiss Y, Gierasch LM., **1991**. *J. Biol. Chem.*, 266, 15575-15578; Brown MS, Goldstein JL, Paris KJ, Burnier JP, Marsters JC., **1992**, *Proc. Natl. Acad. Sci. USA*, 89, 8313-8316; Reiss Y, Stradley SJ, Gierasch LM, Brown MS, Goldstein JL., **1991**. *Proc. Natl. Acad. Sci. USA*, 88, 732-36;

binding of a peptidomimetic inhibitor of FTase termed **L-739,787** revealed a slightly different conformation most closely approximating a Type III  $\beta$ -turn.

Binding of FPP by FTase is of such high affinity that the complex can be isolated by gel filtration. No covalent adduct is involved, however, because FPP can be released intact by denaturing the enzyme. Although kinetic analysis indicates that FTase has distinct binding sites for its two substrates, direct evidence for the formation of a ternary complex has not been obtained.

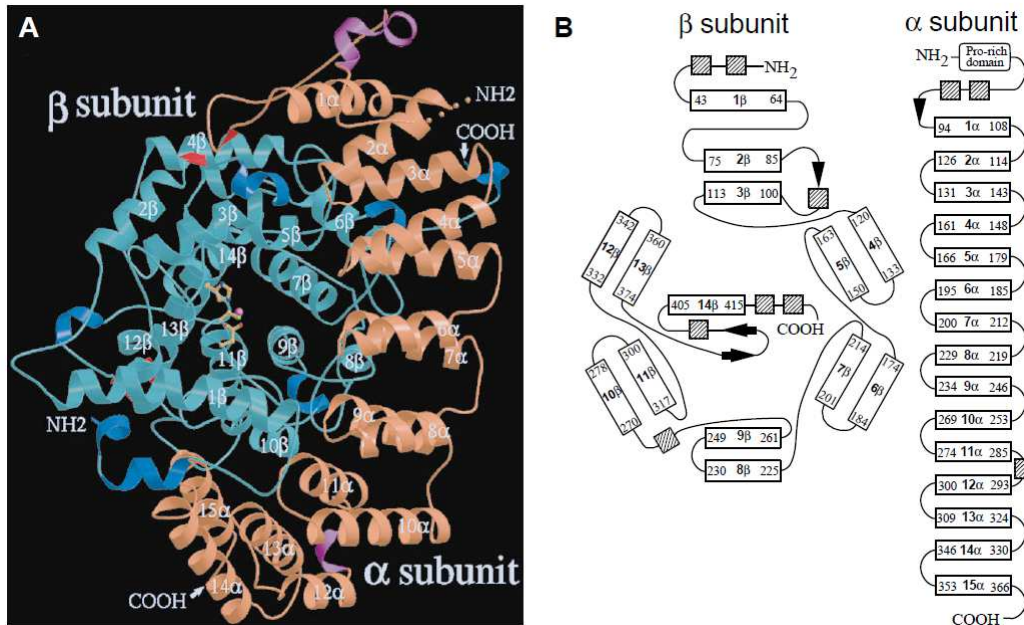
The binding sites for both substrates seem to lie on the  $\beta$  subunit of the enzyme, and the binding site for the peptide substrate may be near the interface of the two subunits.

The publication of the first X-ray crystallographic structure of an FTase enzyme in 1997<sup>54</sup> (Figure 28) was a major breakthrough in FTase research; five years later it was published the first X-ray structures for the FTase ternary complex (FTase–FPP–Peptide) and product complex, added to the structure of the FTase binary complex (FTase–FPP).

The publication of such a complex made available a complete series of structures representing the major steps along the reaction coordinate of this enzyme.

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<sup>54</sup> Hee-Won Park *et al.*, **1997**, *Science*, 275, 1800;



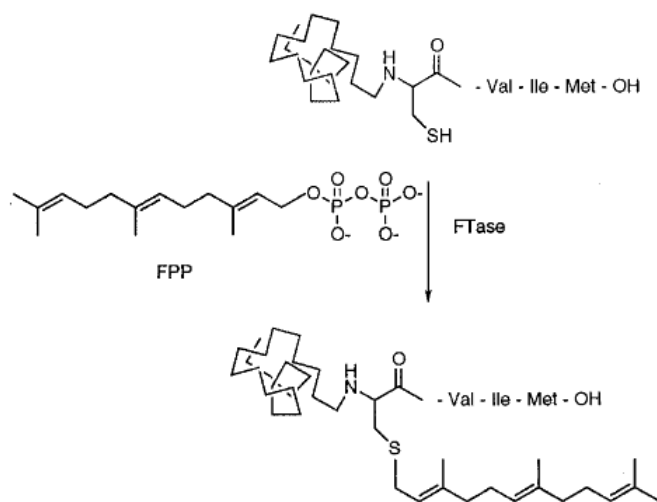
**Figure 28: Structure of the FTase heterodimer.**

**(A) Overall view of the structure.** In the  $\alpha$ -subunit, the  $\alpha$ -helices are orange,  $3_{10}$  helices are magenta, and the  $\beta$ -strand is red. In the  $\beta$ -subunit, the  $\alpha$ -helices are cyan,  $3_{10}$  helices are blue, and the  $\beta$  strands are red. The zinc ion is a magenta sphere and its three amino acid ligands are yellow. The secondary structures of the  $\alpha$  subunit include 15  $\alpha$ -helices, 3 short  $3_{10}$  helices, and a short  $\beta$ -strand. The  $\beta$  subunit contains 14  $\alpha$ -helices, 7 short  $3_{10}$  helices, and 3 short  $\beta$ -strands.

**(B) Topology diagram.** The open boxes represent  $\alpha$ -helices, the striped boxes  $3_{10}$  helices, and the arrows  $\beta$ -strands. In both subunits, the residue numbers for  $\alpha$ -helices are shown. Each of the 15 helices ( $1\alpha$  to  $15\alpha$ ) of the  $\alpha$ -subunit are 8 to 17 residues in length, and the connecting loops are 4 to 8 residues. Helices  $1\beta$  to  $14\beta$  of the  $\beta$ -subunit contain between 9 and 22 residues. In the  $\alpha$ -subunit, the three  $3_{10}$  helices are four to five residues in length ( $65\alpha$ – $69\alpha$ ,  $70\alpha$ – $73\alpha$ , and  $288\alpha$ – $291\alpha$ ). A three-residue  $\beta$ -strand ( $89\alpha$ – $91\alpha$ ) in the  $\alpha$ -subunit interacts with a  $\beta$ -strand ( $87\beta$ – $89\beta$ ) in the  $\beta$ -subunit at the subunit interface. In the  $\beta$ -subunit, each of seven  $3_{10}$  helices consists of less than seven residues ( $23\beta$ – $27\beta$ ,  $28\beta$ – $34\beta$ ,  $91\beta$ – $96\beta$ ,  $264\beta$ – $268\beta$ ,  $389\beta$ – $393\beta$ ,  $423\beta$ – $427\beta$ , and  $433\beta$ – $437\beta$ ) and the three  $\beta$ -strands are less than four residues in length ( $87\beta$ – $89\beta$ ,  $375\beta$ – $378\beta$ , and  $381\beta$ – $384\beta$ ). The NH<sub>2</sub>-terminal proline-rich domain (residues 1 to 54) is disordered in the crystal.

## FTase inhibitors

Starting from the prenylation mechanism and from its substrate (Figure 29), several different approaches were performed during the years, looking for active inhibitors of FTase.



**Figure 29: Ras proteins prenylation**

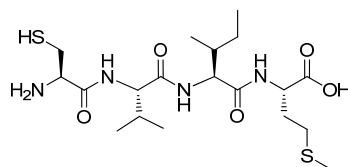
The main classes of Ftase inhibitors (FTIs) are:

1. Peptidomimetics, analogues of the tetrapeptide CAAX;
2. FPP analogues, able to interact with the binding site;
3. Bisubstrate analogues, incorporating in their structure both a FPP moiety and a CAAX peptidomimetics;
4. Natural inhibitors;



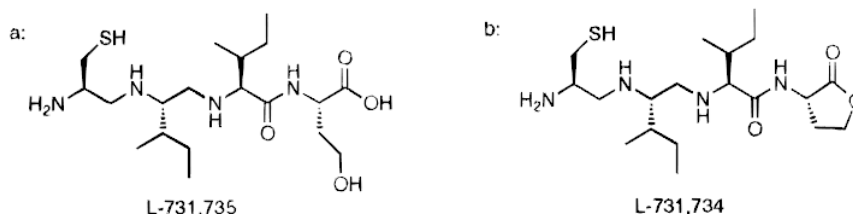
**Peptidomimetic inhibitors**

Initial reports of farnesyltransferase inhibitors were based on the CAAX motif that is the sequence Cysteine-Valine-Isoleucine-Methionine: **CVIM**.

**CVIM**

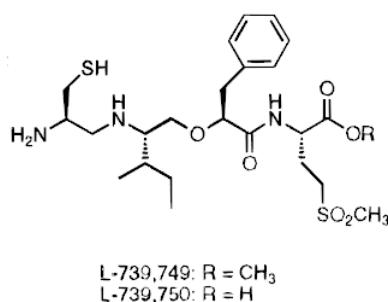
The tetrapeptide itself was found to be a competitive inhibitor with Ras p21; consecutive modifications of the tetrapeptide at the “Ile” position, with its substitution with the aromatic amino acid Phenylalanine, led to a potent Ras FTase inhibitor with an  $IC_{50}$  of 25 nM against bovine FTase.

Further modifications have also been reported, such as the introduction of reduced bond isosteres and homoserine for Methionine, leading to **L-731,735**, which has an  $IC_{50}$  of 18 nM against bovine FTase. The corresponding homoserine lactone **L-731,734** had an  $IC_{50}$  of 280 nM and was active in cells (Figure 30).

**Figure 30: Peptidomimetic inhibitors-(part I)**

The above-mentioned compound inhibited Ras processing in v-Ras-transformed cells and growth in soft agar and reduced Ras farnesylation by 50% in NIH 3T3 fibroblasts in culture at 50  $\mu$ M.

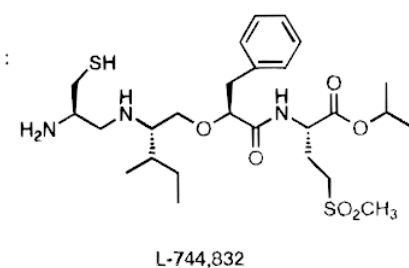
Both pseudopeptides were found to be selective for FTase ( $IC_{50}$  for GGTase-I > 10  $\mu$ M). Indeed most groups have made an attempt to increase selectivity for FTase over GGTase-I due to a perceived increased incidence of potential toxicity if both enzymes are inhibited. It is of course possible that greater in vivo efficacy will be demonstrated with ‘less selective agents’, especially since K-Ras is known to be geranylgeranylated.



**Figure 31: Peptidomimetic inhibitors-(part II)**

**L-739,749**, the methyl ester analogue of **L-739,750** (Figure 31), is also a pseudopeptide based on the CAAX motif. It is a potent selective inhibitor of bovine FTase, with an IC<sub>50</sub> of 240 nM. In cells, it was found to inhibit processed Ras by 50% at a concentration between 0.1 and 1 μM; however, it was found to also affect the regulation of actin stress fiber formation.

Another CAAX mimetic, **L-744,832** (Figure 32), was found to be active in an in vivo model. In MMTV-v-Ras mice bearing palpable tumors, daily administration of **L-744,832** caused complete tumor regression after 2 weeks of treatment (40 mg/kg). These in vivo results, and particularly tumor regression, with various CAAX mimetics are truly significant demonstrating the efficacy of FTase inhibitors as antitumor agents. It will be important to determine the range of tumor types that these compounds are active against for clinical development.



**Figure 32: Peptidomimetic inhibitors-(part III)**

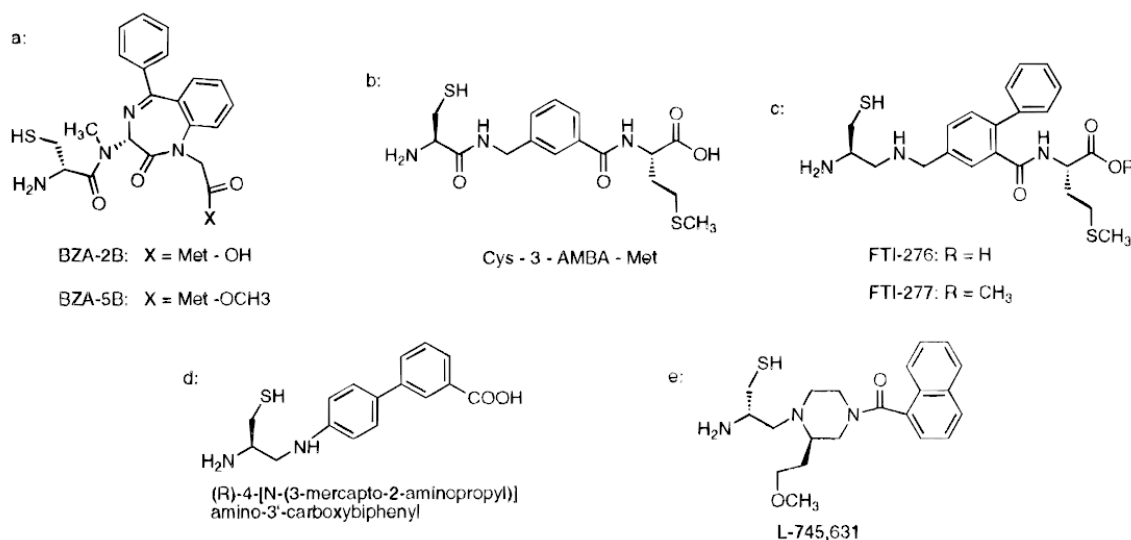
Systematic modifications of the CVFM tetrapeptide by replacement of the amino-terminal amide bonds led to the inhibitor **B581** (Figure 33), which has an IC<sub>50</sub> of 0.021 μM against bovine FTase. It was shown to inhibit Ras processing in cells with an IC<sub>50</sub> of 50 μM. It was reported to be 40-fold selective toward FTase versus GGTase-I in vitro and in NIH 3T3 cells expressing oncogenic (Leu61) H-Ras-CVLS (for FTase) and oncogenic (Leu61) H-ras-CVLL (for GGTase-I).



FTase, and in a clonogenic assay (H-Ras-transformed NIH 3T3 cells) this compound had an  $IC_{50}$  of 3.8  $\mu$ M.

Replacement of the two aliphatic amino acids by a benzodiazepine mimicking a peptide turn, **BZA-2B** (Figure 35-a), gave a potent inhibitor of FTase with an  $IC_{50}$  of 0.85 nM against recombinant rat FTase. The corresponding methyl ester, **BZA-5B** (Figure 35-a), was found to be active in cells. At micromolar concentrations, it was able to restore normal growth to Ras-transformed cells. **BZA-5B** interrupts the MAP kinase activation pathway in H-Ras-transformed cells. Recently, it has been reported that **BZA-5B** blocks farnesylation of the lamin proteins with an  $IC_{50}$  comparable to that obtained for Ras p21. However, it did not interfere with a variety of cellular functions expected to be farnesylation dependent such as cell growth and viability.

Although the above results show no interference with cellular function, the blockade of farnesylation of other proteins should still be of concern. As more potent agents are developed and tested in various in vivo models, it will become of utmost importance to monitor closely if indeed proteins such as lamin-B and transducin are post-translationally modified in the presence of Ras FTase inhibitors as well as and their effects on the viability of the cells.



**Figure 35: Peptidomimetic inhibitors-part VI**

The two aliphatic residues have also been replaced by a hydrophobic spacer, 3-(aminomethyl)benzoic acid (**3-AMBA**) (Figure 35-b). Cys-3-AMBA-Met inhibits p21 Ras FTase from human colon carcinoma (COLO-205) and Burkett's lymphoma (Daudi) with  $IC_{50}$  values of 60 and 120 nM, respectively.

Substitution of the two aliphatic residues was also carried out with 4-AMBA and 3- and 4-aminobenzoic acid (3- and 4-ABA). Cys- 4-AMBA-Met was 17-fold less potent than the corresponding 3-AMBA analogue, and Cys-3-ABA-Met also showed reduced activity. However, Cys-4-ABA-Met had an IC<sub>50</sub> of 50 nM. Introduction of functionality such as N,S-di-Cbz-Cys-3-AMBA-Met-OCH<sub>3</sub> led to compounds which could penetrate NIH 3T3 cells and disrupt p21 Ras plasma membrane association.

Substitution at the 2-position of the 4-aminobenzoic acid moiety with a phenyl group led to a potent inhibitor (**FTI-276**) of Ras FTase with an IC<sub>50</sub> of 0.5 nM, against human FTase (Figure 35-c), and it was 100-fold selective, with an IC<sub>50</sub> of 50 nM against human GGTase-I. The corresponding methyl ester (**FTI-277**; Figure 35-c) has an IC<sub>50</sub> of 50 nM against human FTase but inhibits H-Ras processing in whole cells with an IC<sub>50</sub> of 100 nM and induces accumulation of cytoplasmic nonfarnesylated H-Ras that was able to bind Raf and form a Ras/Raf complex where Raf was not activated. **FTI-277** did not inhibit processing of Rap by GGTase-I at concentrations as high as 10 μM.<sup>136</sup> The inhibitor **FTI-276** blocked the growth in nude mice of a human lung carcinoma expressing oncogenic K-Ras (50 mg/kg, dosed ip for 36 days). **FTI-276** also inhibits oncogenic signaling and tumor growth of NIH 3T3 cells transformed with the Ras oncogene at 20 μM.

Further design led to the replacement of the AAX tripeptide with biphenyl derivatives. The analogue (R)-4-[N-(3-mercapto-2-aminopropyl)amino]-3'-carboxybiphenyl (Figure 35-d) was found to inhibit rat brain FTase with an IC<sub>50</sub> of 50 nM, while it inhibited GGTase-I with an IC<sub>50</sub> of 100 μM. It disrupted H-Ras processing at a concentration of 50 μM (human H-Ras oncogenetransformed Balb/c 3T3 cells).

More recently, a piperazine analogue, **L-745,631**, was reported to suppress tumor growth in nude mice (Figure 35-e). It was found to be potent against bovine FTase (IC<sub>50</sub> = 5 nM) and selective (IC<sub>50</sub> against GGTase-I = 10 μM). It was shown to inhibit Ras processing with an IC<sub>50</sub> of 0.5 μM and to be noncytotoxic to NIH 3T3 cells at concentrations up to 100 μM. In H-Rastransfected NIH 3T3 cells, it suppresses tumor growth by 75% at a dose of 40 mg/kg (sc administration).

The bioactive conformation of the CAAX-based FTase inhibitors has been studied by NMR spectroscopy. One study suggested that the tetrapeptide of the sequence KTKCVFM adopted a type I β-turn conformation in the bound state.

This conclusion was obtained by studying the heptapeptide by NMR spectroscopy in the presence of the FTase enzyme analyzing the transferred nuclear Overhauser effects (NOEs). However activity of the inhibitors where the two aliphatic amino acids have been replaced by 3- or 4-aminobenzoic acid or 3- or 4-(aminomethyl)benzoic acid would argue against a  $\alpha$ -turn since, for these tetrapeptide mimetics, the flexibility of the aminobenzoic acid spacer is different than the two amino acids, Val-Phe. In particular, for the 4-aminobenzoic acid spacer, it is not possible for the molecule to adopt a  $\beta$ -turn conformation, and yet it is a potent FTase inhibitor (50 nM). These two different sets of results suggest that the conformation of the terminal CAAX motif needs to be investigated further.

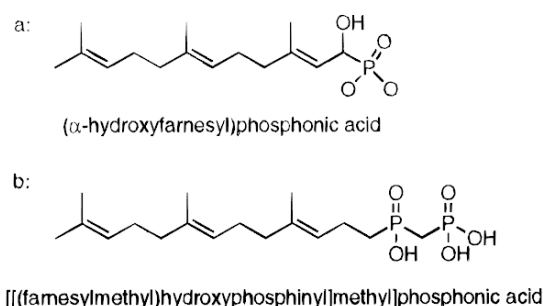
The disclosure of the structure of the FTase enzyme will hopefully further our understanding of the binding of specific classes of inhibitors and provide insight into the bioactive conformation of the CAAX peptidomimetics. Meanwhile, to investigate the binding sites of the substrates, there have been reports using radiolabeled FPP analogues suggesting that the  $\beta$ -subunit contributes significantly to the recognition and binding of the isoprenoid substrate. However, there has been no work with elucidating the site for the protein substrate, and at this point interaction with the R-subunit cannot be precluded.

## FPP analogues

Since the FTase catalyzed Ras farnesylation involves two substrates, FPP and Ras, there has been interest in the design of molecules that can mimic the FPP substrate as a strategy for blocking FTase activity.

Unlike the peptidomimetic strategy, FPP analogues have an inherent disadvantage in functioning as FTase inhibitors. The problem comes from inhibitor specificity for FTase over other FPP utilizing enzymes such as squalene synthase. As FPP is an important intermediate in cholesterol biosynthesis, nonspecific binding by FPP analogues will cause side effects

The design of FPP analogues started by substituting the hydrolyzable diphosphate fragment in FPP: in the first derivative, the  $\alpha$ -hydroxyfarnesylphosphonic acid (Figure 36-a), a monophosphonate replaced the pyrophosphate group in FPP. In the second analogue, farnesylmethylhydroxyphosphinyl methyl phosphonic acid (Figure 36-b), the diphosphate oxygen atom is replaced with a methylene group.



**Figure 36: FPP analogues (part I)**

Both the derivatives have no hydrolyzable bonds and have been shown to be competitive to FPP and non competitive to Ras protein in inhibiting FTase with inhibition constants of 5.2 and 8.30 nM, respectively<sup>55</sup>.

The first compound was also proved to inhibit squalene synthase in vitro with an IC<sub>50</sub> of 630 nM. The inhibition of H-Ras processing in whole cells with this compound was observed at a concentration of 1  $\mu$ M.

<sup>55</sup> Pompliano, D. L.; Rands, E.; Schaber, M. D.; Mosser, S. D.; Anthony, N. J.; Gibbs, J. B., **1992**, *Biochemistry*, 31, 3800-3807; Gibbs, J. B.; Pompliano, D. L.; Mosser, S. D.; Rands, E.; Lingham, R. B.; Singh, S. B.; Scolnick, E. M.; Kohl, N. E.; Oliff, A., **1993**, *J. Biol. Chem.*, 268, 7617-7620;

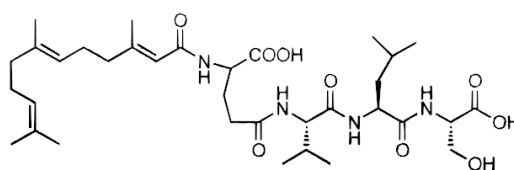




**Bisubstrate analogues**

Bisubstrate derivatives of Ras FTase incorporate the structural motifs of both FPP and the CAAX tetrapeptide<sup>57</sup>.

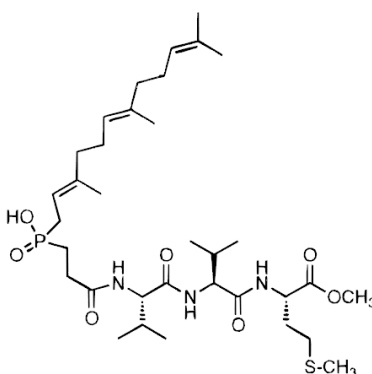
A few examples of such compounds are here reported; in the first case the substitution of thiol moiety of the CAAX motif with a carboxylic acid, together with the binding of the farnesyl chain on this carboxylic acid through a secondary amine, yields a sub-micromolar range inhibitor (Figure 38), having a  $IC_{50}$  of 0.033  $\mu$ M.



**Figure 38: Bisubstrate analogue**

The selectivity of this inhibitor versus FTase or GGTase-I is unknown, and it would be of interest to determine whether the replacement of the thiol group by a carboxylic acid group would favor one over the other and whether these compounds are competitive for FPP or the peptide or both.

A second bisubstrate analogue is the phosphinate inhibitor **BMS-186511** (Figure 39); it was shown to inhibit Ras processing in H-Ras-transformed NIH 3T3 cells at concentrations as low as 0.1  $\mu$ M and it showed a higher affinity for FTase versus GGTase-I (>2000-fold).



**Figure 39: BMS-186511**

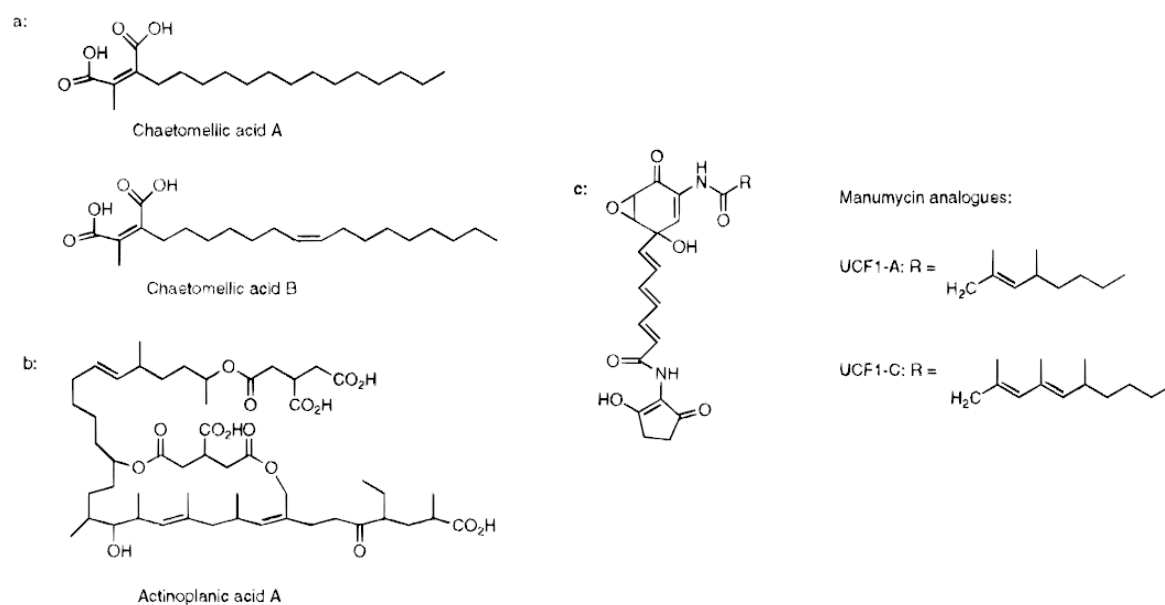
<sup>57</sup> Bhide, R. S. et al, **1994**, *BioOrg. Med. Chem. Lett.*, 4, 2107-2112; Manne, V. et al, **1995**, *Oncogene*, 10, 1763-1779;

Similar results were obtained with K-Ras-transformed NIH 3T3 cells. **BMS-186511** inhibited [ $^3\text{H}$ ]mevalonate incorporation into p21 Ras proteins in a concentration-dependent manner. At 100  $\mu\text{M}$ , the Ras farnesylation was almost completely inhibited in H-Ras- and K-Ras- transformed NIH 3T3 cells. This inhibitor was also shown to be a selective Ras FTase inhibitor (>2000-fold) and had little effect on normal cells.

### Natural inhibitors

Several natural product inhibitors of FTase have been reported, but in general their potency is not so high, compared to the one of synthetic FTIs<sup>58</sup>.

Some are competitive with farnesyl pyrophosphate (FPP), including chaetomelic acids (Figure 40-a), actinoplanic acid A (Figure 40-b) and manumycin analogues (Figure 40-c).



**Figure 40: Chaetomelic acids (a), actonoplanic acid A (b) and manumycin analogues (c) .**

<sup>58</sup> Singh, S. B. et al, **1993**, *Tetrahedron*, 49, 5917-5926; Singh, S. B., **1993**, *Tetrahedron Lett.*, 34, 6521-6524; Lingham, R. B. et al, **1993**, *Appl. Microbiol. Biotechnol.*, 40, 370-374; Singh, S. B.; Liesch, J. M.; Lingham, R. B.; Goetz, M. A.; Gibbs, J. B., **1994**, *J. Am. Chem. Soc.*, 116, 11606-11607; Hara, M.; Akasaka, K.; Akinaga, S.; Okabe, M.; Nakano, H.; Gomez, R.; Wood, D.; Uh, M.; Tamanoi, F., **1993**, *Proc. Natl. Acad. Sci. U.S.A.*, 90, 2281-2285; Tamanoi, F., **1993**, *Trends Biochem. Sci.*, 18, 349-353; Shiomi, K. Et al, **1993**, *J. Antibiot.*, 46, 229-234; Singh, S. B. et al, **1994**, *Tetrahedron Lett.*, 35, 4963-4966; Singh, S. B. et al, **1994**, *J. Org. Chem.*, 59, 6269-6302; Van der Pyl, D.; Inokoshi, J.; Shiomi, K.; Yang, H.; Takeshima, H.; Omura, S., **1992**, *J. Antibiot.*, 45, 1802-1805; Liu, W. C. et al, **1992**, *J. Antibiot.*, 45, 454-457; Singh, S. B. et al, **1995**, *Tetrahedron Lett.*, 36, 4935-4938;

Other inhibitors, such as pepticcinnamins, are competitive with the Ras peptide (Figure 41).

The remaining inhibitors do not refer neither to FPP nor to the CAAX tetrapeptide, and their mechanism of inhibition is unknown.

Examples of such inhibitors are fusidienol (Figure 42-a), preussomerins (Figure 42-b), gliotoxin (Figure 42-c), 10'-desmethoxystreptonigrin (Figure 42-d) and cylindrol A (Figure 42-e).

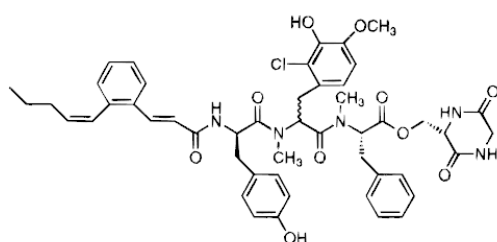


Figure 41: Pepticcinnamins

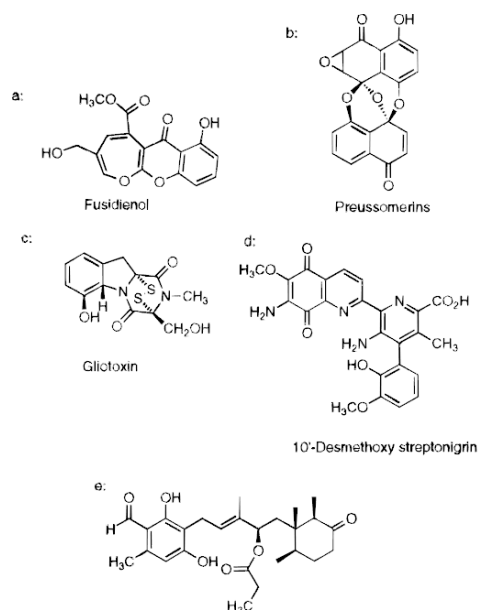


Figure 42: Non competitive natural inhibitors

The potency of this class of FTIs varies from submicromolar to micromolar (Table 7: Activity of natural FTIs). One of the manumycin analogues, UFC1-C (Figure 40-c), was shown to inhibit the growth of K-Ras-transformed fibrosarcoma at a dose of 6.3 mg/kg, administered intraperitoneally for 5 days.

natural product	IC <sub>50</sub> (μM)
chaetomelic acid-A	0.06 <sup>a</sup>
chaetomelic acid-B	0.19 <sup>a</sup>
actinoplanic acid	0.23 <sup>a</sup>
manumycin: UFC1-C	5.0 <sup>b</sup>
pepticcinnamin	0.1 <sup>a</sup>
fusidienol 1.0 <sup>c</sup>	1.0 <sup>c</sup>
preussomerin	1.2 <sup>c</sup>
gliotoxin	1.1 <sup>d</sup>
10'-desmethoxystreptonigrin	21 <sup>d</sup>
cylindrol A	2.2 <sup>c</sup>

Activity against <sup>a</sup>human FTase, <sup>b</sup>yeast FTase, <sup>c</sup>bovine FTase, or <sup>d</sup>no species of FTase.

Table 7: Activity of natural FTIs

## Aim of the work

The tetrapeptide CAAX, proven to be a potent FTase inhibitor in vitro, showed devoid of activity in vivo, therefore it inspired the design of CAAX mimetics, more metabolically stable and with enhanced cell permeability.

Among these, my research group in the latest years focused its attention in the modification of the tetrapeptide structure.

The first studies brought to mimetics having a biphenyl core in lieu of the dipeptide “AA” and, in particular, to compounds in which the biphenyl substructure, 2'-methyl or 2'-methoxy substituted, bears a 3-pyridodioxanylmethoxy residue replacing Cysteine (Figure 43).

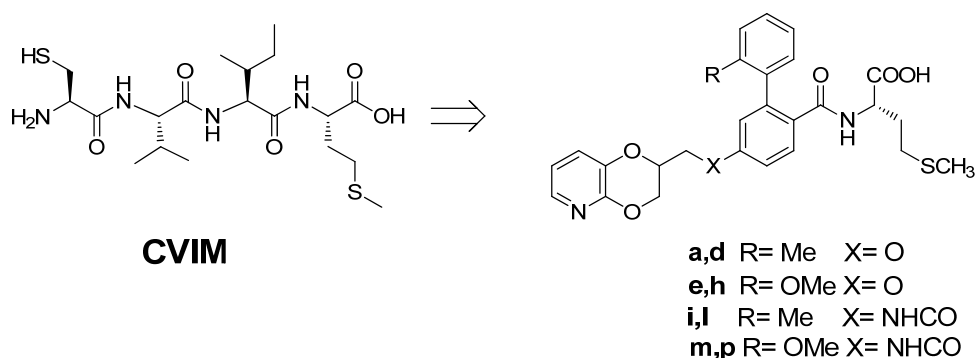


Figure 43

It has been also demonstrated that pyridodioxane nucleus, namely 2,3-dihydro-1,4-dioxino[2,3-b]pyridine, linked to the 2'-methyl or 2'-methoxy substituted biphenyl by a methyleneoxy or a methyleneaminocarbonyl bridge, can replace the 3-pyridyloxymethyl residue maintaining, if the linker is a methyleneoxy chain, a high FTase inhibition and producing micromolar antiproliferative activity on human aorta myocytes due to Ras farnesylation inhibition<sup>59</sup>.

Consistently with these results, docking of the whole series of pyridodioxane derivatives in the crystal structure of human FTase enlightens a better interaction of the enzyme with the pyridodioxanemethyl ethers than with the pyridodioxanemethyl amides and, in particular, the best interaction with the pyridodioxanemethyl ethers 2'-

<sup>59</sup> Bolchi C, Pallavicini M, Rusconi C, Diomede I, Ferri N, Corsini A, Fumagalli L, Pedretti A, Vistoli G, Valoti E, **2007**, *Bioorg. Med. Chem. Lett.*, *17*, 6192-6196;



In particular, 2-amino-4-thiazolyl is basic as pyridine and has pronounced complexation properties. Indeed, the CAAX mimetic, in which AA is replaced with 4-amino-2-phenylbenzoic acid and C with 2-amino-4-thiazolylacetic acid (Figure 45) inhibits FTase activity in the low nanomolar range and shows antiproliferative effect on rat aortic smooth muscle cells (SMC) interfering with Ras farnesylation.

These results indicate that 2-amino-4-thiazole can be regarded as an alternative to heterocycles, such as pyridine and imidazole, normally used in FTase inhibitors designed as non-thiol CAAX mimetics.

Considering these previous results, the present PhD project started from compound **A**, which exerts a nanomolar range FTase inhibition, substituting the 2-amino-4-thiazolylacetyl residue with other heterocycles.

Specifically, the chosen moieties have been the 2-mercapto-4-imidazolylacetyl-, 2-mercapto-4-imidazolylpropionyl-, 2-mercapto-4-thiazolylacetyl-, 2-mercapto-4-thiazolylpropionyl-, 2-methylthio-4thiazolylacetyl- and 2-methylthio-4thiazolylpropionyl-ones; the resulting derivatives, isopropylesters (**27**, **29**, **31**, **33**, **35** and **37**) and corresponding acids (**28**, **30**, **32**, **34**, **36** and **38**), are reported in Figure 46<sup>61</sup>.

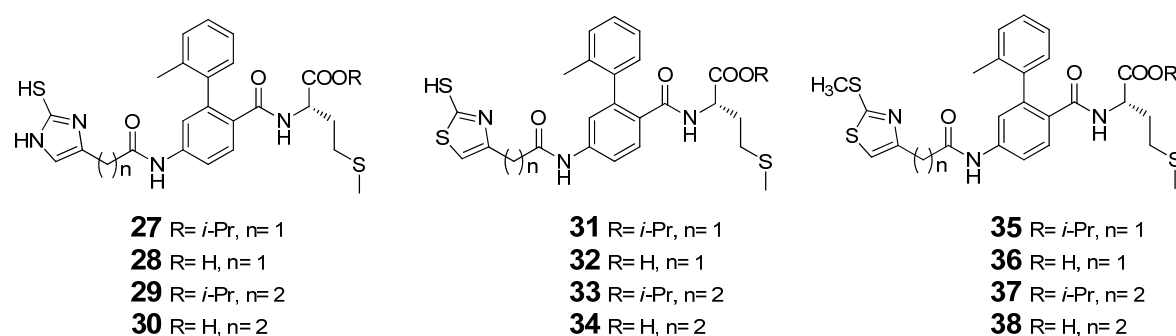


Figure 46

The antiproliferative activity of the isopropyl compounds, prepared as prodrugs, and of the relative free carboxylic acids was evaluated using two specific tests: respectively a cellular and an enzymatic assay. All the resulting data confirmed their antiproliferative activity, even if at micromolar range, suggesting us to design further FTIs, differently modified.

<sup>61</sup> Bolchi C, Pallavicini M, Bernini S K, Chiodini G, Corsini A, Ferri N, Fumagalli L, Straniero V, Valoti E; **2011**, *Bioorg Med Chem Lett*, 21, 5408-5412;

On these bases, starting from both compounds **A** and **B** (Figure 44 and Figure 45) and from an interesting work by Qian and others<sup>62</sup>, there were investigated different aromatic substitutions.

Therefore novel FTIs were designed; specifically the first modification occurred to the aromatic spacer, which mimics AA of the CAAX tetrapeptide, replacing the *o*-tolyl residue with other six heteroaromatic ones, specifically the 2-thienyl-, 3-thienyl-, 3-furanyl-, 3-furanyl-, 4-isoquinolyn- and 1-naphtyl-.

The accomplished compounds are shown in Figure 47 and Figure 48.

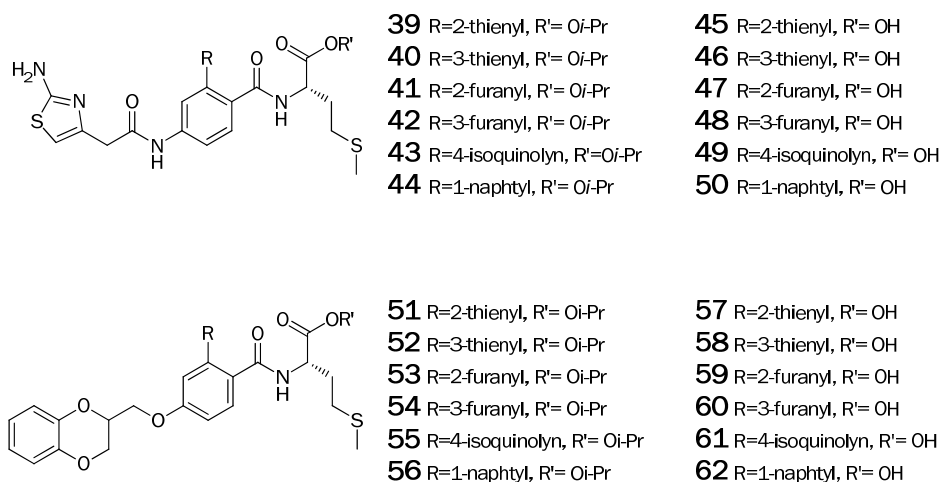


Figure 47-Figure 48

Furthermore, starting from the structure of compound **A** in Figure 44, it has been chosen to invariably connect the aromatic spacer to the terminal heterocyclic system by an amide function.

The synthesis involved all the derivatives bringing the six heteroaromatic residue together with the one having the *o*-tolyl one, so far undesigned, in order to study how such new modifications could influence the inhibitory activity.

Figure 49 reports all the yielded derivatives **63-76**.

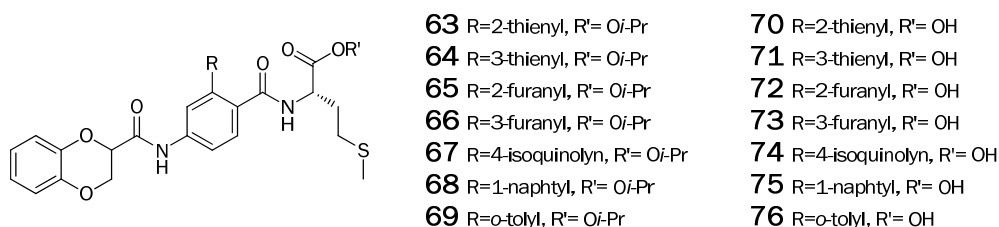


Figure 49

<sup>62</sup> Qian Y, Marugan J J, Fossum R D, Vogt A, Sebti S M, Hamilton A D, **1999**, *Bioorg. Med. Chem. Lett.*, 7, 3011-3024;

All the novel FTIs were tested with the two different assays, mentioned before: in particular the biological activity of the isopropyl esters were examined measuring the proliferation inhibition of the human aortic smooth muscle cells, as explained before. On the other hand, the FTase inhibition of the free acids was evaluated using recombinant rat FTase in a FTase fluorescent assay.



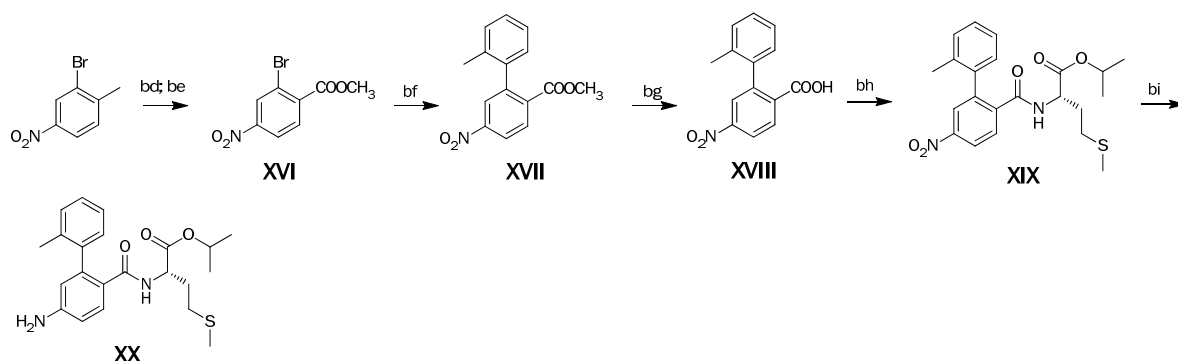
## Synthetic scheme

The synthetic pathway has been specific for each single class of novel FTIs.

The synthesis of the FTase inhibitors **27-32**, modified on the N-terminal residue and reported in Figure 46, is shown in Figure 50, 51 and 52; it started from the commercially available 2-bromo-4-nitro toluene that underwent oxidation in pyridine with potassium permanganate (bd)<sup>63</sup>.

The further conversion into methyl ester, achieved in Methanol using H<sub>2</sub>SO<sub>4</sub> as catalyst (be), afforded **XVI**, which was coupled with o-tolyl boronic acid using Suzuki conditions (bf) to give the biphenyl derivative **XVII**.

Successive reduction of nitro to amino group (bg) accomplished **XVIII**, which was condensed with the isopropyl ester of L-Methionine (bh); the resultant N-nitrobenzoylated methionine ester **XIX** underwent hydrogenolysis, yielding the corresponding aminobenzoylamide **XX**.

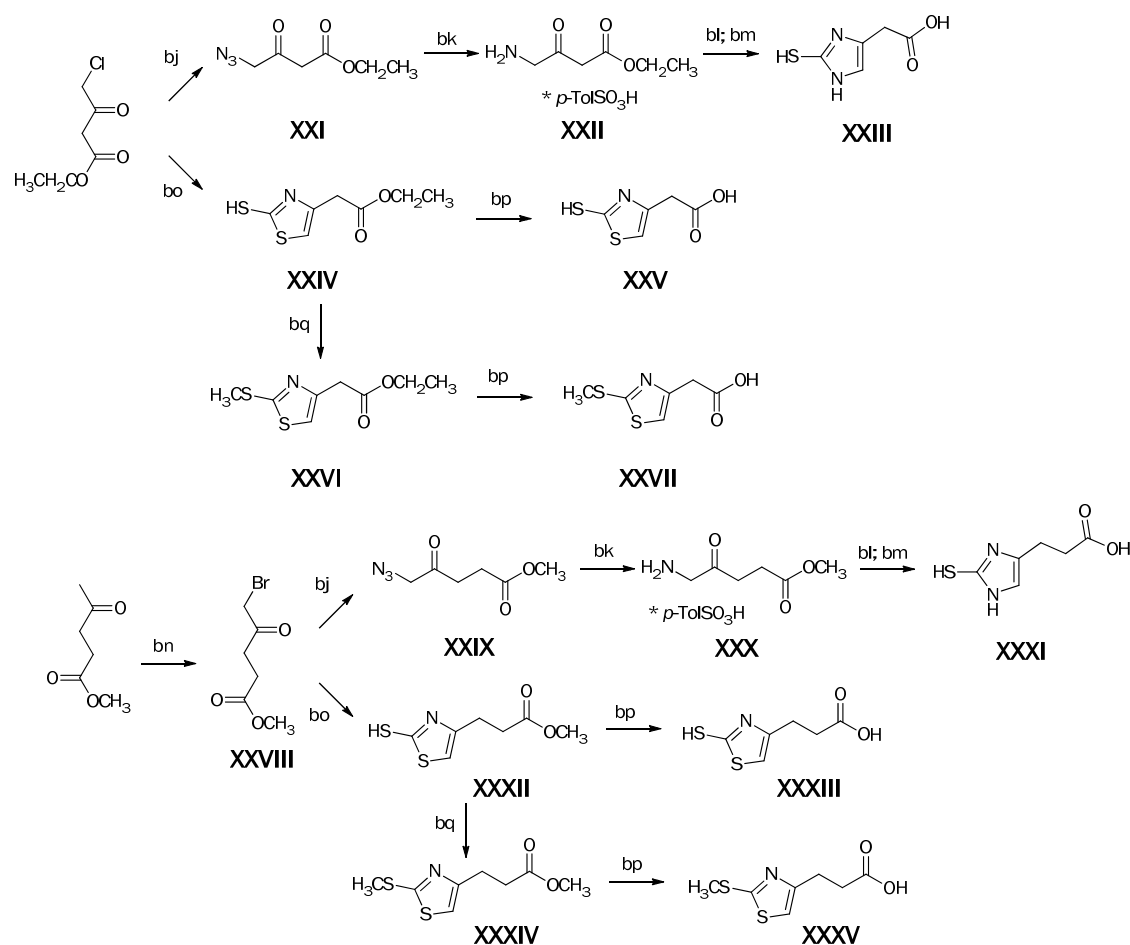


Reagents and conditions: (bd) KMnO<sub>4</sub>, Pyridine, water, 96h, reflux; (be) H<sub>2</sub>SO<sub>4</sub>, MeOH, trimethylorthoformate, 18h, reflux; (bf) tolyl boronic acid, K<sub>2</sub>CO<sub>3</sub>, Pd(PPh<sub>3</sub>)<sub>4</sub>, Toluene/MeOH/Water, 18h, 80 °C; (bg) NaOH, MeOH, 18h, RT; (bh) H-L-Met-Oi-Pr, HOBt, EDAC·HCl, DIPEA, DMF, 18h, RT; (bi) SnCl<sub>2</sub>, AcOEt, 3h, reflux;

Figure 50

This compound (**XX**) is the starting point for the condensation with all the heteroaryl acetic and propionic acids, that had to be prepared from ethyl 4-chloroacetoacetate and methyl 5-bromolevulinatate (**XXVIII**). **XXVIII** is simply yielded through bromination of the levulinic acid (bn), as shown in Figure 51.

<sup>63</sup> Bolchi C, Pallavicini M, Bernini S K, Chiodini G, Corsini A, Ferri N, Fumagalli L, Straniero V, Valoti E; **2011**, *Bioorg Med Chem Lett*, 21, 5408-5412;



Reagents and conditions: (bj)  $\text{NaN}_3$ , water, THF, 18h, RT; (bk)  $\text{H}_2$ , Pd/C 10%, TsOH, EtOH, 4h, RT; (bl) KNCS,  $\text{H}_2\text{O}$ , 2h, reflux; (bm) NaOH, MeOH, 1h, reflux; (bn)  $\text{Br}_2$ , MeOH, 60h, RT; (bo) ammonium dithiocarbamate,  $\text{H}_2\text{O}$ , 15h, reflux; (bp) NaOH, 55 °C, 1h; (bq)  $\text{CH}_3\text{I}$ , NaH, DME, 15h, RT.

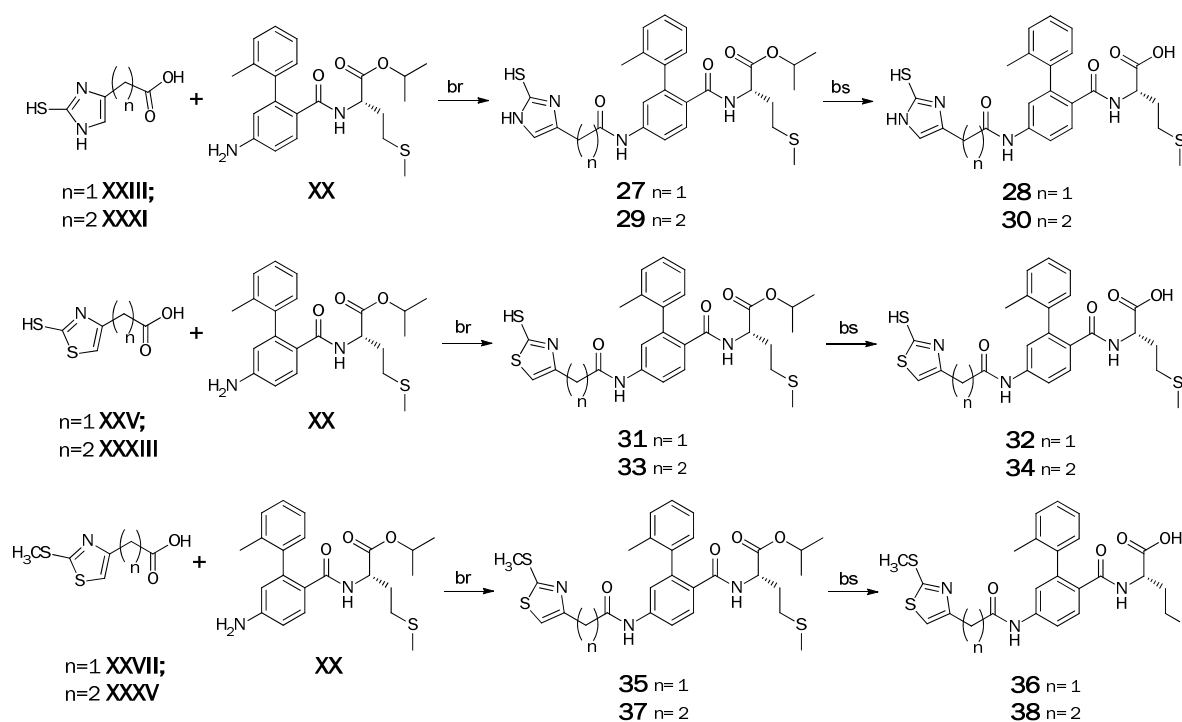
Figure 51

The heteroaryl acids were accomplished as shown in Figure 51, specifically the mercaptoimidazolyl acetic acid (XXIII) and its propionic homologue (XXXI) were obtained by conversion (bj) of ethyl 4-chloroacetoacetate and of methyl 5-bromolevulinate into the corresponding azides (XXI and XXIX), (bk) reduction to 4-aminoacetoacetate (XXII) and 5-aminolevulinate (XXX), isolated as tosylic acid salts, (bl) cyclization with potassium thiocyanate giving the mercaptoimidazole ring and the successive (bm) ester hydrolysis.

Cyclization of ethyl 4-chloroacetoacetate and methyl 5-bromolevulinate with ammonium dithiocarbamate afforded ethyl (2-mercapto-4-thiazolyl)acetate (XXIV) and methyl 3-(2-mercapto-4-thiazolyl) propionate (XXXII), respectively, which were successively converted into acids XXV and XXXIII by basic hydrolysis (bp).

The methylthio analogues, **XXVII** and **XXXV**, were obtained in the same manner, after methylation of compounds **XXIV** and **XXXII** with methyl iodide in a suspension of NaH in DME.

The condensation of each single heteroaryl acetic and propionic acid with the amino benzoylamide **XX** is achieved in DMF using HOBt and EDAC as coupling reagents; the following hydrolysis in basic conditions yielded the final products **28**, **30**, **32**, **34**, **36** and **38**, as shown in Figure 52.



Reagents and conditions: (br) HOBt, EDAC·HCl, DIPEA, DMF, 18h, RT; (bs) NaOH, MeOH, 2-3 h, RT.

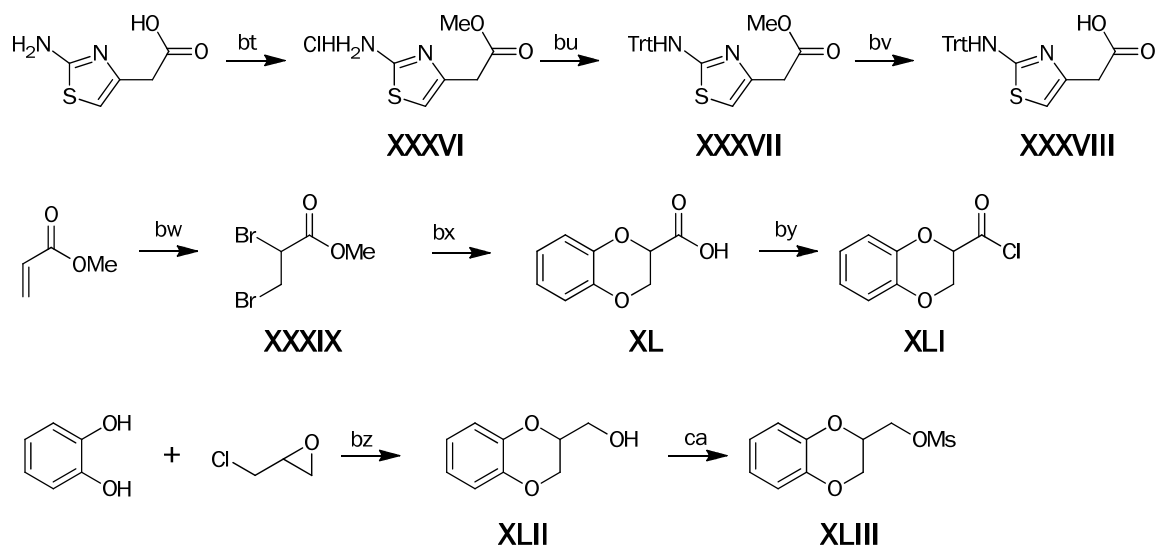
Figure 52

Concerning the second approach, the syntheses of the two different series of amidic compound (Figure 47 e 49), and of the ethereal series (Figure 48) require three different scaffold, whose preparation is shown in Figure 53.

The synthesis of the scaffold **XXXVIII** started from the 2-amino-4-thiazolyl-acetic-acid, that is firstly esterified in Methanol using thionyl chloride (bs), then tritylated at the amino function (bt) and finally desesterified in basic conditions (bu).

The compound **XLI** is prepared from the methylacrilate, through bromination (bv), condensation with the cathecol, using potassium carbonate in Isopropanol (bw), and hydrolysis of the methylester (bx). The treatment with pure thionyl chloride accomplished the 2-benzodioxanyl-acyl chloride (by).

The last scaffold (**XLIII**), that is the 2-mesiloxymethylbenzodioxane, is synthesised starting from the condensation of the epichlorohydrin on the cathecol (**bz**), followed by mesylation in DCM (**ca**), in the presence of TEA as base. These three scaffolds are stable and they are used for the synthesis of each final product.



Reagents and conditions: (bt)  $\text{SOCl}_2$ , MeOH, 12h, RT; (bu) TrtCl, TEA, DCM, 15h, RT; (bv) 2.5N NaOH, MeOH, 3h, reflux; (bw)  $\text{Br}_2$ , DCM, 30 min, RT; (bx) (1)  $\text{K}_2\text{CO}_3$ , i-PrOH, 18h, reflux; (2) NaOH, MeOH, 2h, reflux; (by)  $\text{SOCl}_2$ , Toluene, 3h, reflux; (bz)  $\text{K}_2\text{CO}_3$ , MeOH, 8h, reflux; (ca) MsCl, TEA, DCM, 1h, RT.

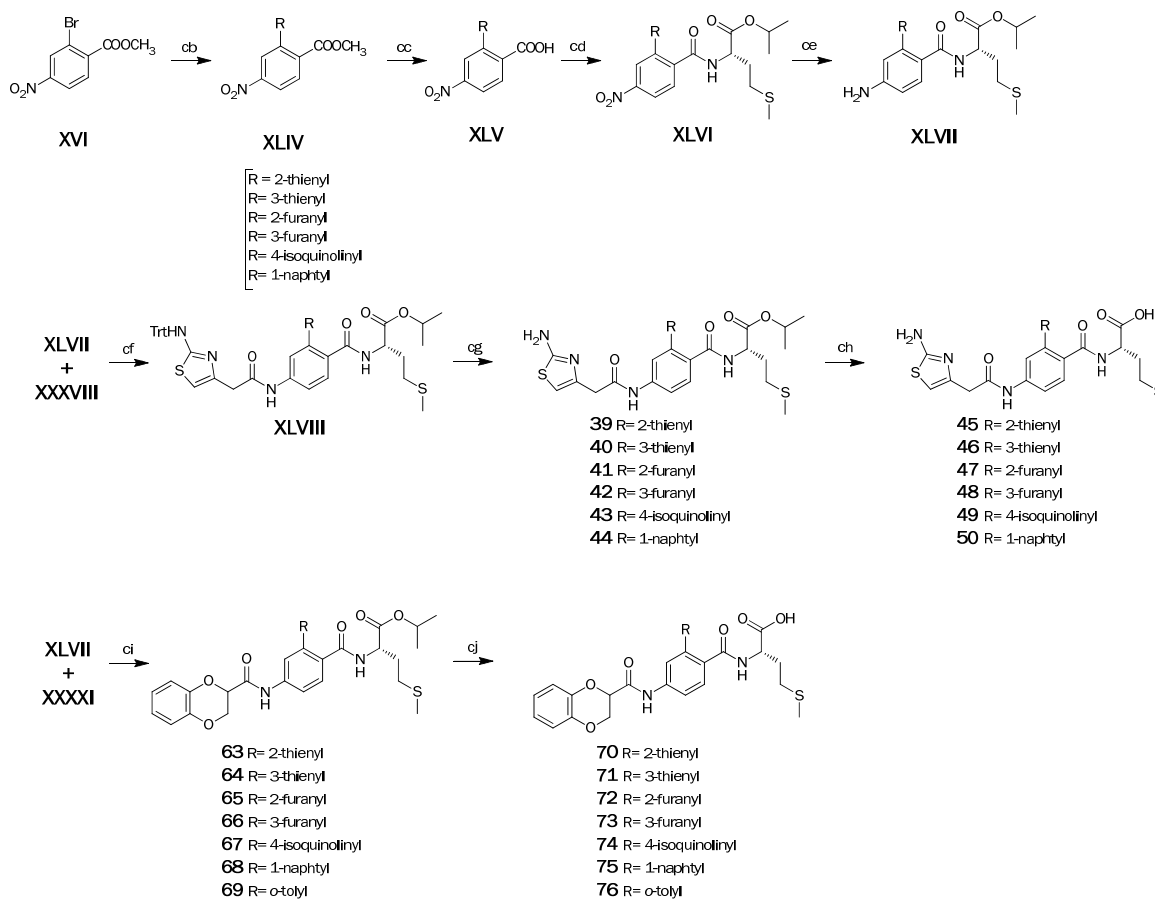
Figure 53

The synthetic pathway for the two aminic series of novel FTIs is shown in Figure 54 and started from compound **XVI**, whose preparation has been explained before (Figure 50).

**XVI** was then coupled with the six different boronic acids (**cb**). The Suzuki coupling, achieved using different boronic acids, in individual conditions (solvents and temperatures), was followed by hydrolysis (**cc**) and condensation with the isopropyl ester of *L*-methionine (**cd**); the consecutive reduction of the nitro group (**ce**), achieved in Ethyl Acetate with Stannous chloride, led to the aminic compound **XLVII**, which reacted either with acidic scaffold **XXXVIII** or scaffold **XLI**.

The condensation with the 2-trytilamino-4-tiazolyl-acetic acid is achieved in DMF using HOBt and EDAC as coupling reagents (**cf**). The sequential acidic removal of the protective trityl group (**cg**) and the hydrolysis of the esteric function (**ch**) gave the final products **45-50**.

Meanwhile the condensation with the 2-mesiloxymethyl-benzodioxane is accomplished in DCM with DIPEA (ci), and followed by hydrolysis in basic condition (cj), obtaining the second series of FTase inhibitors, as free carboxylic acid (**70-76**).

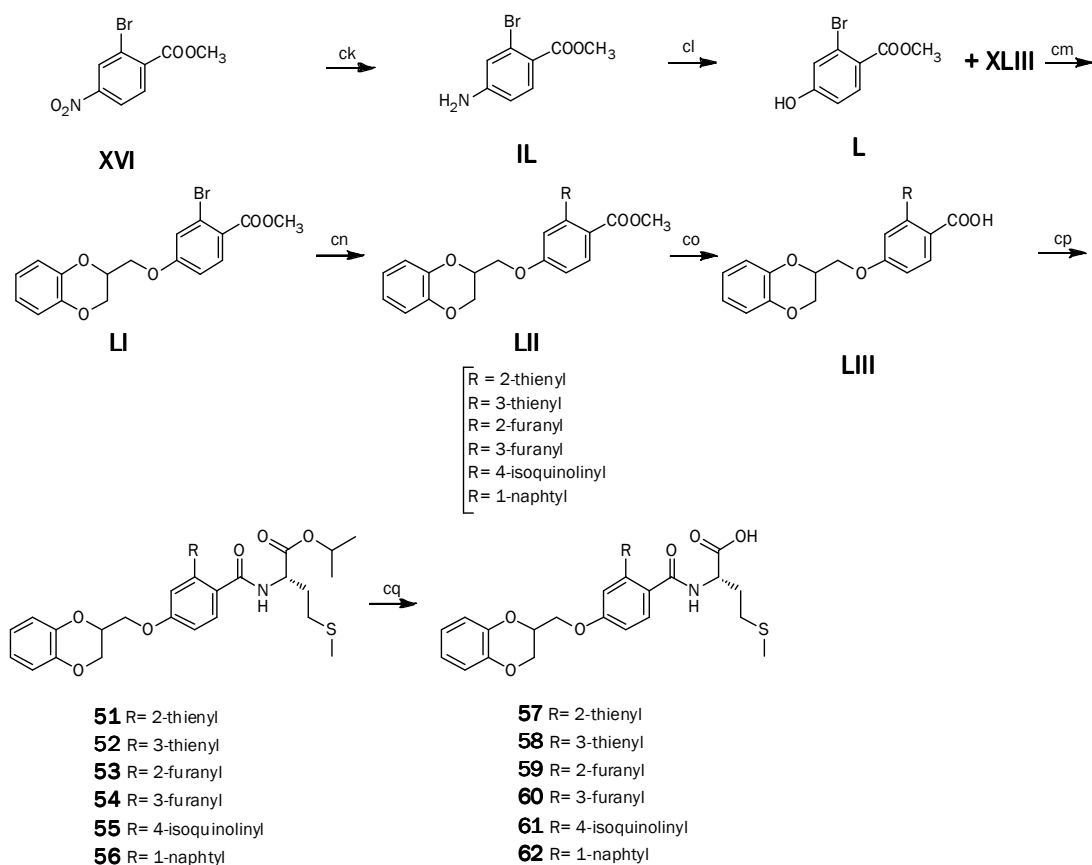


Reagents and conditions: (cb)  $\text{RB(OH)}_2$ ,  $\text{K}_2\text{CO}_3$ ,  $\text{Pd(PPh}_3)_4$ , DME or DMF or THf or Toluene/MeOH/Water, 18h, RT-100°C; (cc) NaOH, MeOH, 18h, RT; (cd) H-L-Met-Oi-Pr, HOBt, EDAC\*HCl, DIPEA, DCM or DMF, 18h, RT; (ce)  $\text{SnCl}_2$ , AcOEt, 3h, reflux; (cf) HOBt, EDAC\*HCl, DIPEA, DMF, 18h, RT; (cg) HCOOH, DCM, 18h, RT, (ch) NaOH, MeOH, 18h, RT, (ci) DIPEA, DCM, 3h, RT, (cj) NaOH, MeOH, 18h, RT.

Figure 54

As shown in Figure 55, the starting point for the synthesis of the ethereal FTIs is compound **L**, achieved, starting from **XVI**, through reduction (ck) and consecutive diazotation and replacement by hydroxyl group (cl), yielding the relative phenol.

The hydroxyl group is sequentially condensed with scaffold **XLII** in DMF and potassium carbonate (cm), the subsequent Suzuki coupling (cn) and hydrolysis (co) let to obtain the free carboxylic acid, able to be condensed with the isopropyl ester of the L-methionine (cp) and then hydrolyzed in basic conditions (cq), giving the final products **57-62**.



Reagents and conditions: (ck)  $\text{SnCl}_2$ ,  $\text{AcOEt}$ , 3h, reflux; (cl)  $\text{NaNO}_2$ ,  $\text{H}_2\text{SO}_4$  20%, 18h, reflux; (cm)  $\text{K}_2\text{CO}_3$ , DMF, 18h,  $60^\circ\text{C}$ ; (cn)  $\text{RB(OH)}_2$ ,  $\text{K}_2\text{CO}_3$ ,  $\text{Pd(PPh}_3)_4$ , DME or DMF or THF or Toluene/MeOH/Water, 18h, RT- $100^\circ\text{C}$ ; (co)  $\text{NaOH}$ , MeOH, 18h, RT; (cp) H-L-Met-Oi-Pr, HOBT, EDAC\*HCl, DIPEA, DMF, 18h, RT; (cq)  $\text{NaOH}$ , MeOH, 18h, RT.

**Figure 55**

## Biological evaluation

Two different assays have been performed in order to study the inhibition ability of the entire novel FTis. Particularly, at first FTase inhibition of the free acids was evaluated using recombinant rat FTase in a FTase fluorescent assay, then the biological activity of the isopropyl esters were examined measuring the proliferation inhibition of the human aortic smooth muscle cells (SMC).

### *In vitro FTase assay*

The effect on protein farnesylation was tested using a FTase fluorescent assay and **FTI-276** (Figure 35-c) and **B** (Figure 45) as reference compounds

**FTI-276** and **B**, as explained before, were proved to inhibit FTase with an  $IC_{50}$  of, respectively, 0.5 nM and of 49 nM.

In this test, the substrates used were Dansyl-Gly-Cys-Val-Leu-Ser-OH peptide (Calbiochem) and farnesyl-pyrophosphate (FPP) (SIGMA).

Each reaction mixture contained Dansyl peptide (final concentration 3  $\mu$ M), FPP (final concentration 10  $\mu$ M) and n-docecyl- $\beta$ -D-maltoside in 25  $\mu$ L of Assay buffer (MTT 5.8 mM,  $MgCl_2$  12 mM,  $ZnCl_2$  12 mM, Tris pH 7.5 52 mM) aliquoted in a 384 wells plate.

The reaction was started by adding of rat recombinant FTase (0.25  $\mu$ g per reaction) and conducted in the presence or absence of tested compounds (20  $\mu$ M).

The fluorescent signal was recorder by using the Fluoroskan Ascent Microplate Fluorometer (Labsystem) every 15 s for 5 min.

The inhibitory activity (% vs control) and the  $IC_{50}$  values were calculated from the fluorescent signal at the steady state, and the respective values are listed in Table 8.

Compound	FTase Assay (inhibition at 20 $\mu$ M)	IC <sub>50</sub>
FTI-276	-	IC <sub>50</sub> = 0.005 $\mu$ M
B	-	IC <sub>50</sub> = 0.049 $\mu$ M
28	-	IC <sub>50</sub> = 0.12 $\mu$ M
30	-	n.a.
32	-	IC <sub>50</sub> = 0.54 $\mu$ M
34	-	IC <sub>50</sub> = 0.53 $\mu$ M
36	-	n.a.
38	-	IC <sub>50</sub> = 0.41 $\mu$ M
45	53.8%	n.d.
46	20.9%	n.d.
47	15.4%	n.d.
48	98.7%	IC <sub>50</sub> = 2.8 $\mu$ M
49	0%	n.d.
50	90.4%	n.d.
57	90.0%	n.d.
58	96.6%	IC <sub>50</sub> = 5.0 $\mu$ M
59	51.5%	n.d.
60	48.7%	n.d.
61	14.5%	n.d.
62	41.7%	n.d.
70	6.2%	n.d.
71	0%	n.d.
72	4.8%	n.d.
73	72.9%	n.d.
74	0%	n.d.
75	67.7%	n.d.
76	73.9%	n.d.

**Table 8:** Inhibition of FTase activity (n.d.: not determined)

### *Cell proliferation assay*

In order to study whether the compounds ability to interfere with FTase activity in vitro was maintained also in cultured cells, the Ras prenylation was investigated, by SDS-PAGE from a total cell lysates of rat SMCs.

Cells were incubated for 72 h, at 37 °C, in the presence and in the absence of our compounds, at three different concentrations: 100, 250 and 500  $\mu$ M; at the end of this incubation period the cell number was determined and the value was subtracted with the starting number of cells.



The IC<sub>50</sub> values were then calculated by nonlinear regression curve and are summarized in Table 9.

Compound	Cell proliferation Assay (MTT) (IC <sub>50</sub> )
B	28.5 μM
27	226 μM
29	n.a.
31	21 μM
33	25 μM
35	n.a.
37	n.a.
39	200 μM
40	123 μM
41	110 μM
42	259 μM
43	252 μM
44	81 μM
51	81 μM
52	n.a.
53	98.9 μM
54	61.5 μM
55	n.a.
56	155 μM
63	n.a.
64	333 μM
65	n.a.
66	478 μM
67	n.a.
68	38 μM
69	n.a.

Table 9: Inhibition of rat SMC proliferation (n.a.: not active)

### *Discussion and conclusion*

The entire compounds, modified on the N-terminal residue and reported in Figure 46 (**27-38**), were biologically evaluated; the results suggest that compounds **31** and **33** show an improved antiproliferative activity, compared to the one of compound **B**.

In contrast, their compelling inhibitory capacity is not confirmed by the FTase inhibition data of the corresponding free carboxylic acids (**32** and **34**); this should be related to the toxic events that could affect the molecule itself.

In addition, the introduction of SH substituent, instead of NH<sub>2</sub>, into the 2-position of thiazole, couldn't improve the activity due to the weak basicity of the 2-aminothiazole opposed to the weak acidity of the 2-mercaptothiazol.

Such a difference cannot be considered uninfluential, especially in molecules with a carboxylic head, and it is not accidental that the fragment mimicking Cysteine in the known CAAX mimetics preferably has weakly basic or neutral character rather than acidic.

Moreover, the tautomeric thione-thiol equilibrium might further justify the remarkable decrease of activity. Obviously, this does not mean that SH methylation is sufficient to restore the FTase activity, as demonstrated by compounds **36** and **38**, which are also modestly or totally inactive.

Considering the three series of FTIs modified on the aromatic spacer, the interesting results coming from the substitution of the *o*-tolyl with a naphthalenic system (**50** and **75**) indicate that, indeed, a bicyclic system could fit the enzymatic cavity.

On the other hand, such a hindrance, in addition to the introduction of a nitrogen atom, causes a total decrease in the inhibitory activity (**49**, **61** and **74**)

Further investigations should be performed on these molecules, in order to completely define how the isoquinoline ring could influence the FTase activity.

Comparing the enzymatic inhibitions of the two amidic series of all the derivatives (**45-50** versus **70-76**), there is clear evidence that the preservation of the 2-amino-4-thiazolylmethyl chain causes a better FTase inhibition, confirming the activity of the precursor **B**.

The FTase inhibition activity of the thienyl derivatives (**45-46**, **57-58** and **70-71**), differently substituted at the N-terminal, reveals that the introduction of the thiophene, a classical example of benzene isosteric substitution, results in a beneficial effect. Specifically, for all these compounds there is a regular trend: the enzymatic

inhibition is maximal for the ethereal derivatives, intermediate for the 2-amino-4-thiazolylmethyl- amidic series and minimal for the benzodioxanyl- amidic class.

A different outcome results from the furanyl FTIs; the 2-furanyl compounds, even less active than the corresponding thionyl derivatives, have the same trend explained before (FTase inhibition scale: **72** < **47** < **59**).

On the contrary, the most potent of the 3-furanyl FTIs is **48**, belonging to the 2-amino-4-thiazolylmethyl- amidic class. This could be due to a different arrangement of the furanyl ring in the FTase cavity, causing a specific interaction of the molecule with the enzyme; this hypothesis should be confirmed by further computational studies.

It's interesting to notice that there isn't a fixed correlation between FTase inhibition and antiproliferative activity of the corresponding isopropyl ester; this could be due, as explained before, to differences in bioavailability, cell penetration and cellular toxicity

In conclusion, it's now pending how the aromatic spacer specifically interacts with the catalytic site of FTase; in particular it's important to understand if there are any HBD points, able to stabilize the FTI binding and therefore the chain rearrangement.

Such an accommodation could also better explain why the isoquinoline derivatives are totally inert, and if such an inactivity is related or not to their steric hindrance.

## Experimental section

**<sup>1</sup>H-NMR spectra** were performed at 300 MHz, using a Varian 300 Mercury instrument; the chemical shifts are reported in ppm;

**Thermal analyses** were performed on two different instruments:

- DCS 2010 TA INSTRUMENTS, using 1-3 mg of each sample, and a heating speed of 5 °C/ min;
- Büchi Melting Point B-540;

**TLC** were performed on standard analytical silica gel layers (thickness 0.20 mm; Macherey-Nagel ALUGRAM SIL G/UV254)

**Chromatographic purifications** were performed, in normal phase, using Biotage instruments ( Horizon or SP1) over different Biotage SNAP Ultra flash chromatography cartridges, filled of Merck Silica Gel 60 (0.040-0.063 µm)

The analysis by reverse phase was accomplished using an VWR-Hitachi LaChrom Elite® HPLC System, with diode array detector (190–400 nm), over a Water XBridge™ C18 column (5µm, 4.6x150mm); the purification by reverse phase was performed on VWR-Hitachi LaPrep® HPLC System, over a Water XBridge™ Prep C18 column (5µm, 19x150mm).

The **ionization constants** and **lipophilicity parameters** of the dipeptides were determined at 25 °C by potentiometric titration with the GlpKa apparatus (Sirius Analytical Instruments Ltd., Forest, Row, East Sussex, UK).

All experiments were carried out under a slow nitrogen flow to avoid CO<sub>2</sub> absorption. A weighted sample (5–10 mg) was supplied manually, whereas the diluent and all the other reagents were added automatically.

**Acronyms used:**

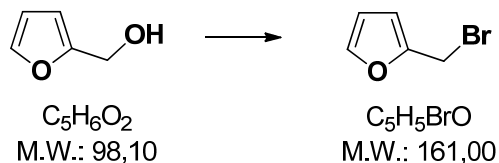
(NMR): s (*singlet*); bs (*broad singlet*); d (*doublet*); t (*triplet*); m (*multiplet*)

(Solvents and Reactives): ACN (*Acetonitrile*); DCM (*Dichloromethane*); DIPEA (*N,N-Diisopropylethylamine*); DME (*1,2-dimethoxyethane*); DMF (*Dimethylformamide*); DMSO (*Dimethylsulphoxide*); EDAC (*N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride*); HOBt (*1-Hydroxybenzotriazole hydrate*); TFA (*Trifluoroacetic acid*); THF (*Tetrahydrofuran*);





## 2-(bromomethyl)furan



To a solution of furan-2-methanol (10.00 g, 101.94 mmol) in 100 mL of dry THF, at -5 °C under a nitrogen atmosphere, is slowly dropped  $PBr_3$  (3.19 mL, 33.98 mmol)

The temperature is maintained at 0 °C and the reaction progression is followed by TLC<sup>65</sup>; at completion, the solution is poured in 240 mL of an iced mixture of Ethyl ether/ Water= 2/1.

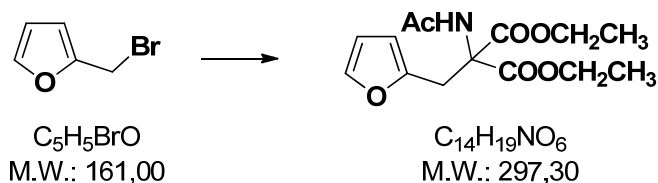
The two layers are separated; the organic phase is washed with  $NaHCO_3$  and brine, dried over  $Na_2SO_4$ , decolorized over charcoal and used for the successive reaction without further purification.

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 7.41 (m, 1H), 6.35 (m, 2H), 4.45 (s, 2H).

<sup>65</sup> TLC conditions: Cyclohexane / Ethyl Acetate= 60/ 40  
Rf: 0.60



## Diethyl 2-acetamido-2-(furan-2-ylmethyl)malonate



A suspension of potassium *tert*-butoxide (9.15 g, 81.55 mmol) in 100 mL of THF, under a nitrogen atmosphere, is added of diethyl acetamidomalonate (17.71 g, 81.55 mmol) and stirred for 20 minutes. Then the ethereal solution of the 2-(bromomethyl)furan (101.94 mmol) is dropped; the ether is removed and the mixture is refluxed.

After 2 hours, monitoring the progression of the reaction by TLC<sup>66</sup>, the suspension is concentrated under vacuum, resumed with Ethyl Acetate, washed with HCl 10%, NaHCO<sub>3</sub> 10% and brine.

The organic phase is dried over Na<sub>2</sub>SO<sub>4</sub>, decolorized on carchoal, filtered and concentrated under reduced pressure; the crude is a sticky solid that is further crystallized in isopropyl ether yielding 10.91 g (36.70 mmol) of a white solid.

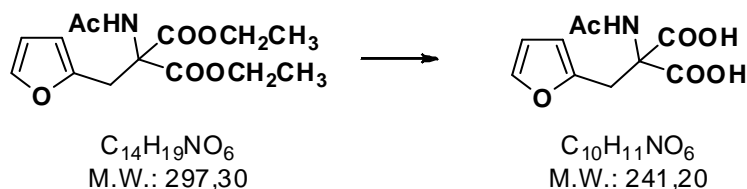
Yield: 45%

Melting point: 81.81 °C

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 7.27 (m, 1H), 6.65 (bs, 1H), 6.26 (dd, 1H, *J* = 1.92 Hz, *J* = 3.30 Hz), 6.03 (dd, 1H, *J* = 0.55 Hz, *J* = 3.30 Hz), 4.28 (q, 4H, *J* = 7.16 Hz), 3.72 (s, 2H), 2.01 (s, 3H), 1.29 (t, 6H, *J* = 7.16 Hz).

<sup>66</sup> TLC conditions: Cyclohexane/ Ethyl Acetate = 60/ 40  
Rf: 0.23

## 2-acetamido-2-(furan-2-ylmethyl)malonic acid



To a solution of diethyl 2-acetamido-2-(furan-2-ylmethyl)malonate (10.91 g, 36.70 mmol) in 100 mL of Dioxane are added dropwise 75 mL of NaOH 4M and the mixture is refluxed for 3 hours.

The reaction is monitored by NMR, at completion, the mixture is slowly poured in a flask, containing 300 mL of Ethyl Acetate/ HCl 10%= 1/1.

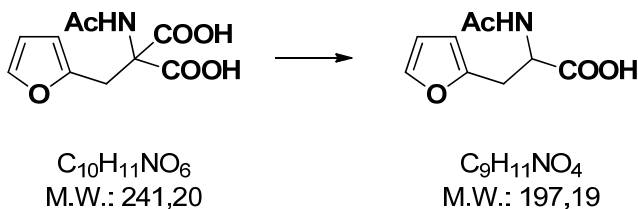
The two layers are separated and the aqueous phase is extracted twice more with Ethyl Acetate; the organic phases are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure, yielding 7.44 g (30.83 mmol) of a white solid.

Yield: 84%

Melting point: 106.0 °C

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 7.81 (bs, 1H), 7.45 (dd, 1H, *J* = 0.55 Hz, *J* = 1.92 Hz), 6.32 (dd, 1H, *J* = 1.92 Hz, *J* = 3.30 Hz), 6.03 (dd, 1H, *J* = 0.55 Hz, *J* = 3.30 Hz), 3.48 (s, 2H), 2.01 (s, 3H).

## 2-acetamido-3-(furan-2-yl)propanoic acid



A solution of 7.44 g (30.83 mmol) of 2-acetamido-2-(furan-2-ylmethyl)malonic acid in 75 mL of Dioxane is acidified with Acetic Acid and refluxed for 2 ½ hours, monitoring the completion of the reaction by NMR.

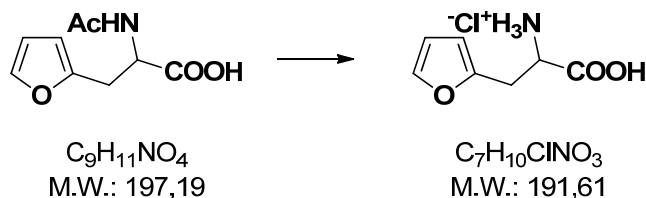
The removal of the solvents yields 4.86 g (24.66 mmol) of a pale brown solid.

Yield: 80%

Melting point: 156.78 °C

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 8.20 (d, 1H, *J* = 7.70 Hz), 7.50 (dd, 1H, *J* = 0.55 Hz, *J* = 1.92 Hz), 6.33 (dd, 1H, *J* = 1.92 Hz, *J* = 3.07 Hz), 6.13 (d, 1H, *J* = 0.55 Hz, *J* = 3.07 Hz), 4.49 (m, 1H), 3.04 (dd, 1H, *J* = 5.27 Hz, *J* = 10.11 Hz), 2.91 (dd, 1H, *J* = 8.79 Hz, *J* = 10.11 Hz), 1.85 (s, 3H).

## 1-carboxy-2-(furan-2-yl)ethanaminium chloride



A solution of 4.86 g (24.66 mmol) of 2-acetamido-3-(furan-2-yl)propanoic acid in 95 mL of NaOH 4 M is refluxed for 2 ½ hours, monitoring the reaction by NMR.

At completion, the mixture is cooled to 0 °C and acidified with HCl 37%, the solvents are removed and the crude is resumed with Isopropanol, heated for 1 hour and filtered.

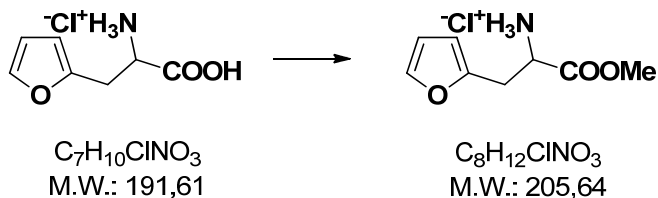
The resulting solution is concentrated under vacuum, yielding 4.06 g (21.18 mmol) of a pale grey solid.

Yield: 85.9 %

Melting point: 222.04 °C

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 8.71 (bs, 3H), 7.58 (dd, 1H, *J* = 0.55 Hz, *J* = 1.92 Hz), 6.39 (dd, 1H, *J* = 1.92 Hz, *J* = 3.30 Hz), 6.26 (d, 1H, *J* = 0.55 Hz, *J* = 3.30 Hz), 4.10 (t, 1H, *J* = 5.90 Hz), 3.22 (d, 2H, *J* = 5.90 Hz).

### 3-(furan-2-yl)-1-methoxy-1-oxopropan-2-aminium chloride



Thionyl chloride (1.55 mL, 21.18 mmol) is slowly dropped to a solution of 1-carboxy-2-(furan-2-yl)ethanaminium chloride 4.06 g (21.18 mmol) and trimethyl orthoformate (2.32 mL, 21.18 mmol) in anhydrous methanol (50 mL) at 0°C under a nitrogen atmosphere.

The resulting mixture is warmed to RT, refluxed for 1 h, monitoring the reaction by NMR; at completion the solvent is removed, the crude is resumed with Methanol, decolorized on carchoal, filtered and concentrated under reduce pressure.

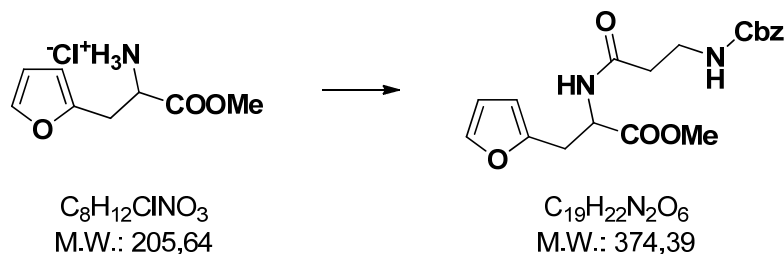
Crystallization in Acetonitrile yields 3.00 g of a white solid (14.61 mmol).

Yield: 69%

Melting point: 152.70 °C

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 8.56 (bs, 3H), 7.58 (dd, 1H, *J* = 0.55 Hz, *J* = 1.92 Hz), 6.39 (dd, 1H, *J* = 1.92 Hz, *J* = 3.30 Hz), 6.26 (d, 1H, *J* = 0.55 Hz, *J* = 3.30 Hz), 4.25 (t, 1H, *J* = 5.90 Hz), 3.68 (s, 3H), 3.20 (d, 2H, *J* = 5.90 Hz).

## Methyl 2-(3-(((benzyloxy)carbonyl)amino)propanamido)-3-(furan-2-yl)propanoate



A solution of EDAC (3.36 g, 17.53 mmol) and DIPEA (3.05 mL, 17.53 mmol) in 15 mL of DCM is added dropwise to a solution of 3-(((benzyloxy)carbonyl)amino)propanoic acid (3.91 g, 17.53 mmol), HOBT (2.37 g, 17.53 mmol) in 30 mL of DCM under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of 3-(furan-2-yl)-1-methoxy-1-oxopropan-2-aminium chloride (3.00 g, 14.61 mmol) and DIPEA (2.54 mL, 14.61 mmol) in 15 mL of DCM is added dropwise.

The reaction mixture is stirred at RT for 2 hours and monitored by TLC<sup>67</sup>.

At completion, the mixture is treated with DCM and HCl 10%; the organic layer is washed with NaHCO<sub>3</sub> and brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated.

The crude product is purified by column chromatography on silica gel, using Toluene/Ethyl Acetate = 60/40 as elution solvent, to give 3.50 g (9.35 mmol) of the desired product, as pale yellow solid.

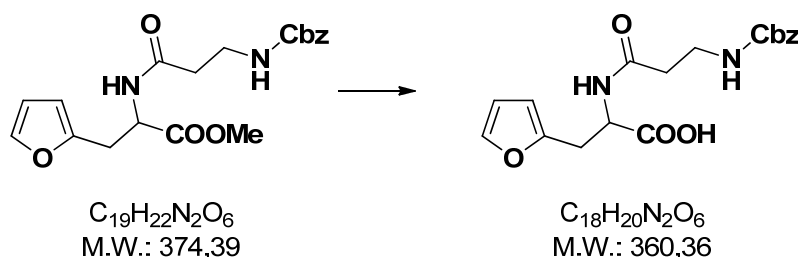
Yield: 64 %

Melting point: 89.79 °C

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 8.39 (d, 1H, *J*= 7.71 Hz), 7.50 (dd, 1H, *J*= 0.55 Hz; *J*=1.92 Hz), 7.33 (m, 5H), 7.17 (m, 1H), 6.32 (dd, 1H, *J*= 1.92 Hz, *J*= 2.48 Hz), 6.13 (dd, 1H, *J*= 0.55 Hz, *J*= 2.48 Hz), 5.00 (s, 2H), 4.50 (m, 1H), 3.59 (s, 3H), 3.14 (m, 2H), 3.00 (m, 2H), 2.28 (m, 2H).

<sup>67</sup> TLC conditions: Chloroform/ Methanol= 95/5 + TEA 1%  
 R<sub>f</sub>= 0.56

## 2-(3-(((benzyloxy)carbonyl)amino)propanamido)-3-(furan-2-yl)propanoic acid



3.50 g (9.35 mmol) of Methyl 2-(3-(((benzyloxy)carbonyl)amino)propanamido)-3-(furan-2-yl)propanoate are dissolved in 35 mL of Methanol; 7 of NaOH 4M are dropped and the resulting mixture is stirred at RT for 1 hour.

After completion of the reaction, followed by TLC<sup>68</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with HCl 10% and extracted three times with Ethyl Acetate.

The organic phases are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated, yielding 3.27 g (9.07 mmol) of a white solid.

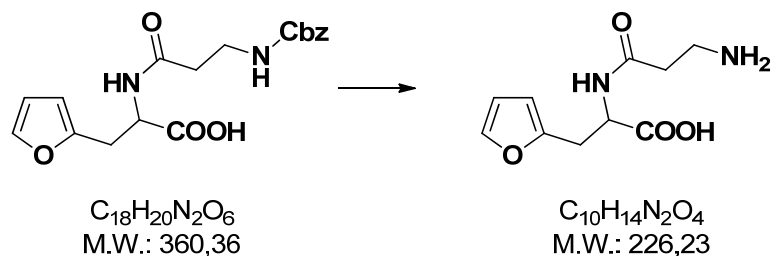
Yield: 97%

Melting point: 136.22 °C

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 8.22 (d, 1H, *J*=7.71 Hz), 7.50 (dd, 1H, *J*=0.55 Hz, *J*=1.92 Hz), 7.33 (m, 5H), 7.17 (m, 1H), 6.32 (dd, 1H, *J*=1.92 Hz, *J*=3.30 Hz), 6.13 (dd, 1H, *J*=0.55 Hz, *J*=3.30 Hz), 5.00 (s, 2H), 4.43 (m, 1H), 3.14 (m, 2H), 3.00 (m, 2H), 2.28 (m, 2H).

<sup>68</sup> TLC conditions: Toluene/ Ethyl Acetate= 60/40  
R<sub>f</sub> = basal

## 2-(3-aminopropanamido)-3-(furan-2-yl)propanoic acid



3.27 g (9.07 mmol) of the 2-(3-(((benzyloxy)carbonyl)amino)propanamido)-3-(furan-2-yl)propanoic acid undergo hydrogenolysis with Pd/C 10% in 50 mL of dry Methanol. The reaction is monitored by TLC<sup>69</sup>; at completion, catalyst filtration and solvent evaporation gives 1.31 g (5.80 mmol) of a sticky white solid.

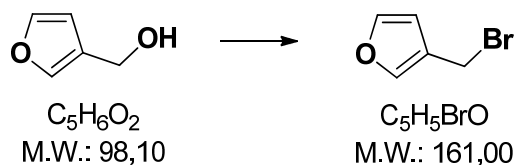
Yield: 64%

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 7.32 (dd, 1H,  $J=0.55$  Hz,  $J=1.92$  Hz), 6.28 (dd, 1H,  $J=1.92$  Hz,  $J=3.30$  Hz), 6.09 (dd, 1H,  $J=0.55$  Hz,  $J=3.30$  Hz), 4.38 (m, 1H), 3.08 (m, 3H), 2.94 (m, 1H), 2.55 (m, 2H).

<sup>69</sup> TLC conditions: Toluene/ Ethyl Acetate= 60/40 + Acetic Acid 10%  
Rf= basal



### 3-(bromomethyl)furan



To a solution of furan-3-methanol (10.00 g, 101.94 mmol) in 100 mL of dry THF, at -5 °C under a nitrogen atmosphere, is slowly dropped  $PBr_3$  (3.19 mL, 33.98 mmol)

The temperature is maintained at 0 °C and the reaction progression is followed by TLC<sup>70</sup>; at completion, the solution is poured in 250 mL of an iced mixture of Ethyl ether/ Water= 2/1.

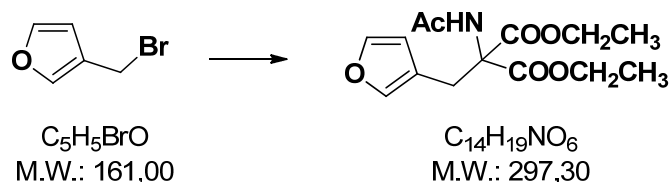
The two layers are separated; the organic phase is washed with  $NaHCO_3$  and brine, dried over  $Na_2SO_4$ , decolorized over charcoal and used for the successive reaction without further purification.

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 7.41 (m, 1H), 6.35 (m, 2H), 4.45 (s, 2H).

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<sup>70</sup> TLC conditions: Cyclohexane / Ethyl Acetate= 60/ 40  
Rf: 0.58

## Diethyl 2-acetamido-2-(furan-3-ylmethyl)malonate



A suspension of potassium *tert*-butoxide (9.15 g, 81.55 mmol) in 100 mL of THF, under a nitrogen atmosphere, is added of diethyl acetamidomalonate (17.71 g, 81.55 mmol) and stirred for 20 minutes. Then the ethereal solution of the 3-(bromomethyl)furan (101.94 mmol) is dropped; the ether is removed and the mixture is refluxed.

After 2 hours, monitoring the progression of the reaction by TLC<sup>71</sup>, the suspension is concentrated under vacuum, resumed with Ethyl Acetate, washed with HCl 10%, NaHCO<sub>3</sub> 10% and brine.

The organic phase is dried over Na<sub>2</sub>SO<sub>4</sub>, decolorized on carchoal, filtered and concentrated under reduced pressure; the crude is a sticky solid that is further crystallized in Isopropyl ether yielding 11.64 g (39.14 mmol) of a white solid.

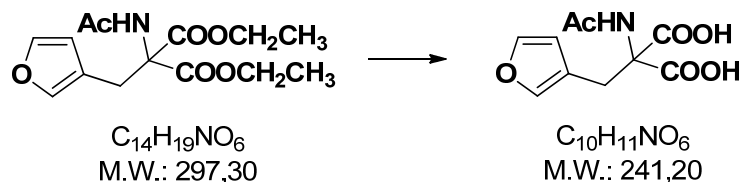
Yield: 48%

Melting point: 89.18 °C

<sup>1</sup>H-NMR (CDCl<sub>3</sub>): 7.81 (s, 1H), 7.53 (m, 1H), 7.35 (m, 1H), 6.18 (dd, 1H, *J* = 0.83 Hz, *J* = 1.65 Hz), 4.28 (q, 4H, *J* = 7.16 Hz), 3.24 (s, 2H), 2.01 (s, 3H), 1.29 (t, 6H, *J* = 7.16 Hz).

<sup>71</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 60/ 40  
Rf: 0.23

## 2-acetamido-2-(furan-3-ylmethyl)malonic acid



To a solution of diethyl 2-acetamido-2-(furan-3-ylmethyl)malonate 11.64 g (39.14 mmol) in 100 mL of Dioxane are added dropwise 78 mL of NaOH 4M and the mixture is refluxed for 1 hour.

The reaction is monitored by NMR, at completion, the mixture is slowly poured in a flask, containing 300 mL of a mixture of Ethyl Acetate/ HCl 10%= 1/ 1.

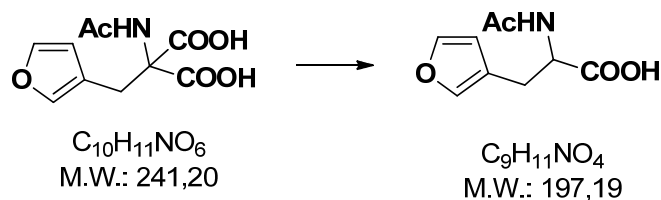
The two layers are separated and the aqueous phase is extracted twice more with Ethyl Acetate; the organic phases are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure, yielding 7.84 g (32.49 mmol) of a beige solid.

Yield: 83%

Melting point: 130.07 °C

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 7.81 (bs, 1H), 7.53 (m, 1H), 7.35 (m, 1H), 6.18 (dd, 1H, *J* = 0.83 Hz, *J* = 1.65 Hz), 3.24 (s, 2H), 1.95 (s, 3H).

## 2-acetamido-3-(furan-3-yl)propanoic acid



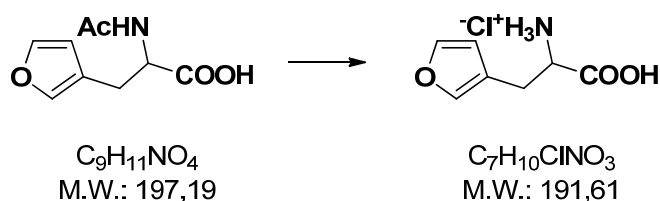
A solution of 7.84 g (32.49 mmol) of 2-acetamido-2-(furan-3-ylmethyl)malonic acid in 80 mL of Dioxane is acidified with Acetic Acid and refluxed for 2 hours, monitoring the completion of the reaction by NMR.

The removal of the solvents yields 5.12 g (25.99 mmol) of a orange oil.

Yield: 80%

**$^1H$ -NMR ( $d_6$ DMSO):** 8.14 (d, 1H,  $J = 7.70$  Hz), 7.53 (m, 1H), 7.44 (m, 1H), 6.37 (m, 1H), 4.31 (m, 1H), 2.70 (m, 1H), 2.81 (m, 1H), 1.90 (s, 3H).

## 1-carboxy-2-(furan-3-yl)ethanaminium chloride



A solution of 5.12 g (25.99 mmol) of 2-acetamido-3-(furan-3-yl)propanoic acid in 100 mL of NaOH 4M is refluxed for 2 hours, monitoring the reaction by NMR.

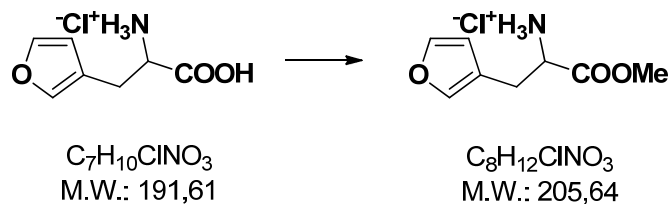
At completion, the mixture is cooled to 0°C and acidified with HCl 37%, the solvents are removed and the crude is resumed with Methanol, heated for 1 hour and filtered.

The resulting solution is concentrated under vacuum, yielding 4.48 g (23.39 mmol) of beige solid.

Yield: 90 %

$^1H$ -NMR ( $d_6$ DMSO): 7.30 (m, 1H), 7.22 (m, 1H), 6.23 (m, 1H), 3.26 (m, 1H), 2.59 (m, 2H).

### 3-(furan-3-yl)-1-methoxy-1-oxopropan-2-aminium chloride



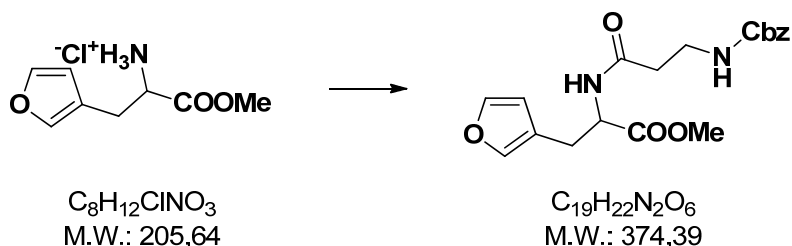
Thionyl chloride (1.71 mL, 23.39 mmol) is slowly dropped to a solution of 1-carboxy-2-(furan-3-yl)ethanaminium chloride 4.48 g (23.39 mmol) and trimethyl orthoformate (2.56 mL, 23.39 mmol) in anhydrous methanol (50 mL) at 0°C under a nitrogen atmosphere; the resulting mixture is warmed to RT and refluxed for 1 h.

The reaction is monitored by NMR; at completion the solvent is removed, the crude is resumed with Methanol and decolorized on carchoal, filtered and concentrated under reduce pressure, yielding 4.43 g (21.52 mmol) of a brown oil.

Yield: 92%

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 7.43 (m, 1H), 7.40 (m, 1H), 6.28 (m, 1H), 4.31 (m, 1H), 3.78 (s, 3H), 3.10 (m, 2H).

## Methyl 2-(3-(((benzyloxy)carbonyl)amino)propanamido)-3-(furan-3-yl)propanoate



A solution of EDAC (4.94 g, 25.82 mmol) and DIPEA (4.50 mL, 25.82 mmol) in 20 mL of DCM is added dropwise to a solution of 3-(((benzyloxy)carbonyl)amino)propanoic acid (5.76 g, 25.82 mmol), HOBT (3.49 g, 25.82 mmol) in 50 mL of DCM under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of 3-(furan-3-yl)-1-methoxy-1-oxopropan-2-aminium chloride (4.43 g, 21.52 mmol) and DIPEA (3.75 mL, 21.52 mmol) in 30 mL of DCM is added dropwise.

The reaction mixture is stirred at RT for 1 hour and monitored by TLC<sup>72</sup>.

At completion, the mixture is treated with DCM and HCl 10%; the organic layer is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated.

The crude product is purified by column chromatography on silica gel, using Toluene/Ethyl Acetate = 60/40 as elution solvent, to give 3.55 g (9.47 mmol) of the desired product, as a yellow solid.

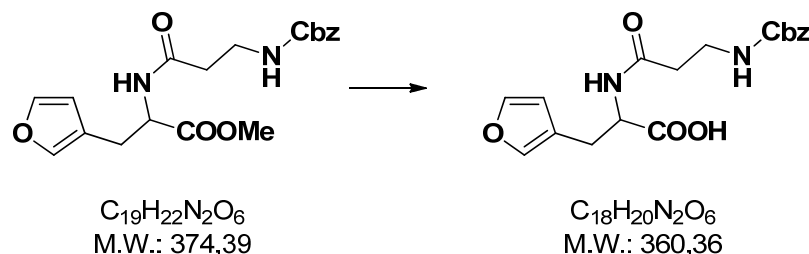
Yield: 44 %

Melting point: 81.03 °C

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 8.21 (d, 1H, *J* = 7.43 Hz), 7.53 (m, 1H), 7.42 (m, 1H), 7.32 (m, 1H), 7.19 (m, 1H), 6.40 (m, 1H), 5.02 (s, 2H), 4.50 (m, 1H), 3.78 (s, 3H), 3.19 (m, 2H), 2.80 (m, 2H), 2.30 (m, 2H).

<sup>72</sup> TLC conditions: Toluene/ Ethyl Acetate= 60/40  
 R<sub>f</sub>= 0.23

## 2-(3-(((benzyloxy)carbonyl)amino)propanamido)-3-(furan-3-yl)propanoic acid



3.55 g (9.47 mmol) of Methyl 2-(3-(((benzyloxy)carbonyl)amino)propanamido)-3-(furan-2-yl)propanoate are dissolved in 40 mL of Methanol; 7 mL of NaOH 4M are dropped and the resulting mixture is stirred at RT for 1 hour.

After completion of the reaction, followed by TLC<sup>73</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with HCl 10% and extracted three times with Ethyl Acetate.

The organic phases are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated, yielding 3.14 g (8.71 mmol) of a white solid.

Yield: 92%

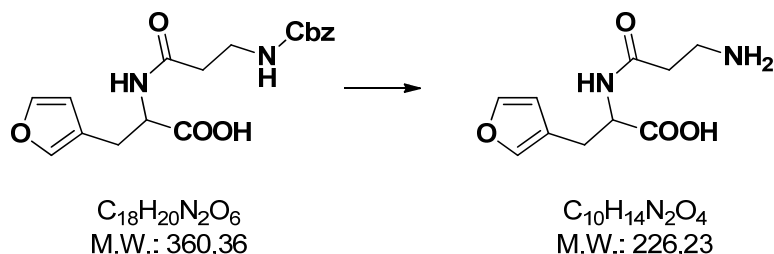
Melting point: 131.98 °C

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 8.21 (d, 1H, *J*=7.71 Hz), 7.53 (m, 1H), 7.42 (m, 1H), 7.32 (m, 5H), 7.19 (m, 1H), 6.40 (m, 1H), 5.02 (s, 2H), 4.33 (m, 1H), 3.19 (m, 2H), 3.00 (m, 2H), 2.90 (m, 1H), 2.70 (m, 1H), 2.32 (m, 2H).

<sup>73</sup> TLC conditions: Toluene/ Ethyl Acetate= 60/40  
R<sub>f</sub> = basal



## 2-(3-aminopropanamido)-3-(furan-3-yl)propanoic acid



3.14 g (8.71 mmol) of the 2-(3-(((benzyloxy)carbonyl)amino)propanamido)-3-(furan-2-yl)propanoic acid undergo hydrogenolysis with Pd/C 5% in 50 mL of dry Methanol. The reaction is monitored by TLC<sup>74</sup>, at completion, catalyst filtration and solvent evaporation gives a white solid that is further crystallized from Water yielding 0.89 g (3.92 mmol) of the final product.

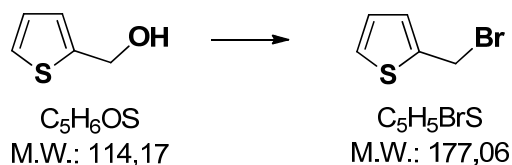
Yield: 45%

Melting point: 252.30 °C

<sup>1</sup>H-NMR (d<sub>6</sub>DMSO): 7.33 (m, 1H), 7.26 (m, 1H), 6.28 (m, 1H), 4.31 (m, 1H), 3.11 (m, 2H), 3.00 (m, 1H), 2.70 (m, 1H), 2.53 (m, 2H).

<sup>74</sup> TLC conditions: Toluene/ Ethyl Acetate= 60/40 + Acetic Acid 10%  
Rf= basal

## 2-(bromomethyl)thiophene



To a solution of thiophen-2-methanol (10.00 g, 87.59 mmol) in 100 mL of dry THF, at -5 °C under a nitrogen atmosphere, is slowly dropped PBr<sub>3</sub> (2.74 mL, 29.20 mmol)

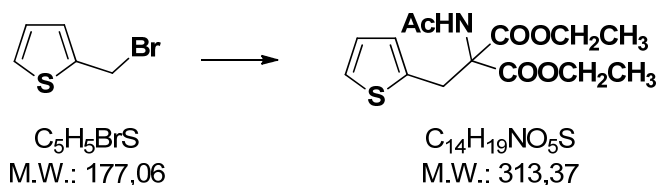
The temperature is maintained at 0 °C and the reaction progression is followed by TLC<sup>75</sup>; at completion, the solution is poured in 250 mL of an iced mixture of Ethyl ether/ Water= 2/1.

The two layers are separated; the organic phase is washed with NaHCO<sub>3</sub> and brine, dried over Na<sub>2</sub>SO<sub>4</sub>, decolorized over charcoal and used for the successive reaction without further purification.

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 7.33 (m, 1H), 7.12 (m, 1H), 6.97 (m, 1H), 4.78 (s, 2H).

<sup>75</sup> TLC conditions: Cyclohexane / Ethyl Acetate= 60/ 40  
Rf: 0.60

## Diethyl 2-acetamido-2-(thiophen-2-ylmethyl)malonate



A suspension of potassium *tert*-butoxide (7.86 g, 70.07 mmol) in 80 mL of THF, under a nitrogen atmosphere, is added of diethyl acetamidomalonate (15.22 g, 70.07 mmol) and stirred for 20 minutes. Then the ethereal solution of the 2-(bromomethyl)thiophene (87.59 mmol) is dropped; the ether is removed and the mixture is refluxed.

After 2 hours, monitoring the progression of the reaction by TLC<sup>76</sup>, the suspension is concentrated under vacuum, resumed with Ethyl Acetate, washed with HCl 10%, NaHCO<sub>3</sub> 10% and brine.

The organic phase is dried over Na<sub>2</sub>SO<sub>4</sub>, decolorized on carchoal, filtered and concentrated under reduced pressure; the crude is a sticky solid that is further crystallized in isopropyl ether yielding 9.88 g (31.53 mmol) of a white solid.

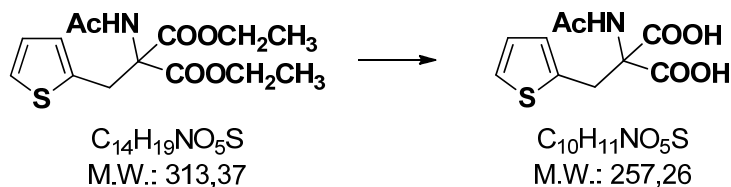
Yield: 45%

Melting point: 118.09 °C

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 7.18 (m, 1H), 6.91 (m, 1H), 6.70 (m, 2H), 4.25 (q, 4H, *J*= 7.15 Hz), 3.88 (s, 2H), 2.06 (s, 3H), 1.29 (t, 6H, *J*= 7.15 Hz).

<sup>76</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 60/ 40  
Rf: 0.23

## 2-acetamido-2-(thiophen-2-ylmethyl)malonic acid



To a solution of diethyl 2-acetamido-2-(thiophen-2-ylmethyl)malonate (9.88 g, 31.53 mmol) in 100 mL of Dioxane are added dropwise 63 mL of NaOH 4M, and the mixture is refluxed for 1 hour.

The reaction is monitored by NMR, at completion, the mixture is slowly poured in a flask, containing 150 mL of a mixture of Ethyl Acetate/ HCl 10%=1/ 1.

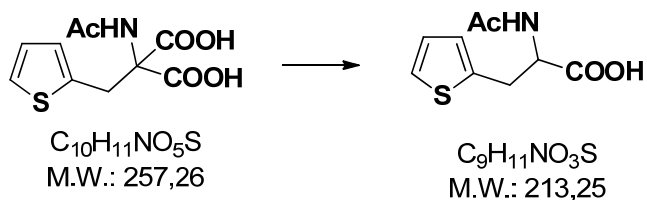
The two layers are separated and the aqueous phase is extracted twice more with Ethyl Acetate; the organic phases are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure, yielding 6.49 g (25.22 mmol) of a beige solid.

Yield: 80%

Melting point: 117.04 °C

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 7.84 (bs, 1H), 7.33 (m, 1H), 6.91 (m, 1H), 6.74 (m, 1H), 3.64 (s, 2H), 1.95 (s, 3H).

## 2-acetamido-3-(thiophen-2-yl)propanoic acid



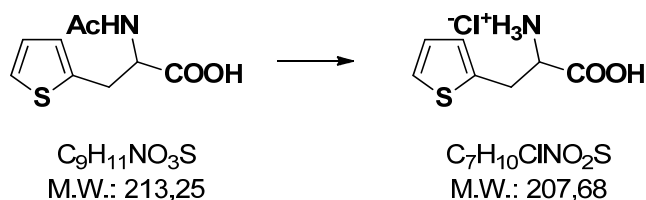
A solution of 6.49 g (25.22 mmol) of 2-acetamido-2-(thiophen-2-ylmethyl)malonic acid in 65 mL of Dioxane is acidified with Acetic Acid and refluxed for 2 hours, monitoring the completion of the reaction by NMR.

The removal of the solvents yields 4.84 g (22.70 mmol) of beige oil.

Yield: 90%

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 8.21 (d, 1H, *J*= 7.70 Hz), 7.33 (m, 1H), 6.90 (m, 2H), 4.37 (m, 1H), 3.30 (m, 1H), 3.11 (m, 1H), 1.82 (s, 3H).

## 1-carboxy-2-(thiophen-2-yl)ethanaminium chloride



A solution of 4.84 g (22.70 mmol) of 2-acetamido-3-(thiophen-2-yl)propanoic acid in NaOH 2.5M is refluxed for 2 ½ hours, monitoring the reaction by NMR.

At completion, the mixture is cooled to 0°C and acidified with HCl 37%, the solvents are removed and the crude is resumed with Isopropanol, heated for 1 hour and filtered.

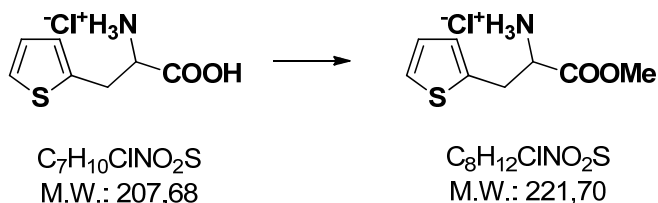
The resulting solution is concentrated under vacuum, yielding 3.58 g (17.25 mmol) of a pale grey solid.

Yield: 76 %

Melting point: 248.30 °C

$^1H$ -NMR ( $d_6$ DMSO): 8.65 (bs, 3H), 7.40 (m, 1H), 7.00 (m, 2H), 4.10 (m, 1H), 3.37-3.48 (m, 2H).

### 3-(thiophen-2-yl)-1-methoxy-1-oxopropan-2-aminium chloride



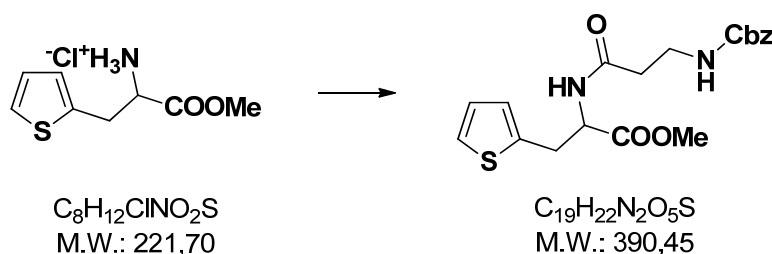
Thionyl chloride (1.26 mL, 17.25 mmol) is slowly dropped to a solution of 1-carboxy-2-(thiophen-2-yl)ethanaminium chloride (3.58 g, 17.25 mmol) and trimethyl orthoformate (1.89 mL, 17.25 mmol) in anhydrous methanol (40 mL) at 0 °C under a nitrogen atmosphere; the resulting mixture is warmed to RT and refluxed for 1 h.

The reaction is monitored by NMR; at completion the solvent is removed, yielding 3.67 g (16.56 mmol) of sticky beige solid.

Yield: 96%

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 7.27 (m, 1H), 6.92 (m, 2H), 4.64 (m, 1H), 3.67 (s, 3H), 3.43 (m, 2H).

## Methyl 2-(3-(((benzyloxy)carbonyl)amino)propanamido)-3-(thiophen-2-yl)propanoate



A solution of EDAC (3.81 g, 19.87 mmol) and DIPEA (3.46 mL, 19.87 mmol) in 12 mL of DCM is added dropwise to a solution of 3-(((benzyloxy)carbonyl)amino)propanoic acid (4.44 g, 19.87 mmol), HOBT (2.68 g, 19.87 mmol) in 20 mL of DCM under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of 3-(thiophen-2-yl)-1-methoxy-1-oxopropan-2-aminium chloride (3.67 g, 16.56 mmol) and DIPEA (2.88 mL, 16.56 mmol) in 30 mL of DCM is added dropwise.

The reaction mixture is stirred at RT for 1 hour and monitored by TLC<sup>77</sup>.

At completion, the mixture is treated with DCM and HCl 10%; the organic layer is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated.

The crude product is purified by column chromatography on silica gel, using Toluene/Ethyl Acetate = 60/40 as elution solvent, to give 1.94 g (4.97 mmol) of the desired product, as a yellow solid.

Yield: 36 %

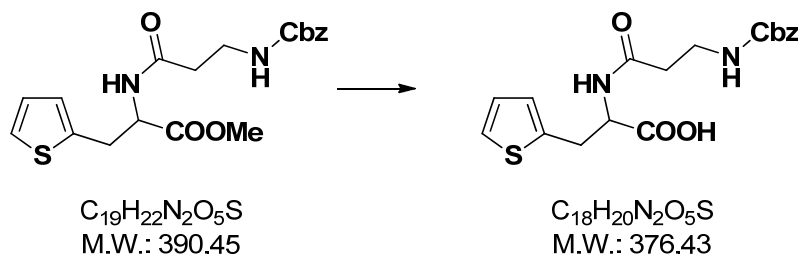
Melting point: 103.89 °C

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 8.45 (d, 1H, *J* = 7.43 Hz), 7.32 (m, 6H), 7.19 (m, 1H), 6.90 (m, 2H), 5.00 (s, 2H), 4.45 (m, 1H), 3.61 (s, 3H), 3.08-3.32 (m, 4H), 2.30 (m, 2H).

<sup>77</sup> TLC conditions: Toluene/ Ethyl Acetate= 60/40  
 R<sub>f</sub>= 0.31



## 2-(3-(((benzyloxy)carbonyl)amino)propanamido)-3-(thiophen-2-yl)propanoic acid



1.94 g (4.97 mmol) of Methyl 2-(3-(((benzyloxy)carbonyl)amino)propanamido)-3-(thiophen-2-yl)propanoate are dissolved in 25 mL of Methanol; 6 mL of NaOH 2.5M are dropped and the resulting mixture is stirred at RT for 1 hour.

After completion of the reaction, followed by TLC<sup>78</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with HCl 10% and extracted three times with Ethyl Acetate.

The organic phases are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated, yielding 1.80 g (4.77 mmol) of a white solid.

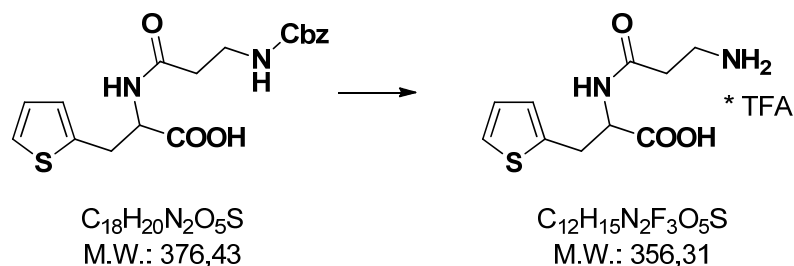
Yield: 96%

Melting point: 149.81 °C

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 8.29 (d, 1H, *J*=7.43 Hz), 7.30 (m, 6H), 7.18 (m, 1H), 6.90 (m, 2H), 5.00 (s, 2H), 4.40 (m, 1H), 3.07-3.32 (m, 4H), 2.30 (m, 2H).

<sup>78</sup> TLC conditions: Toluene/ Ethyl Acetate= 60/40  
R<sub>f</sub> = basal

## 2-(3-aminopropanamido)-3-(thiophen-2-yl)propanoic acid



1.80 g (4.77 mmol) of the 2-(3-(((benzyloxy)carbonyl)amino)propanamido)-3-(thiophen-2-yl)propanoic acid is dissolved in 20 mL of TFA, in presence of anisole (1.08 mL, 9.54 mmol).

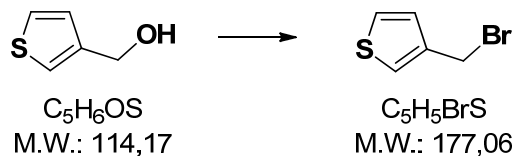
The reaction is monitored by TLC<sup>79</sup>, at completion, the solvent is removed, the residue is treated with Ethyl Acetate and Water, the aqueous layer is concentrated under reduced pressure, yielding 1.12 g (3.15 mmol) of the final product, as sticky yellow solid.

Yield: 66%

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 8.52 (d, 1H, *J*=7.70 Hz), 7.78 (bs, 3H), 7.32 (m, 2H), 6.90 (m, 1H), 4.42 (m, 1H), 3.32 (m, 2H), 3.10 (m, 2H), 2.95 (m, 2H).

<sup>79</sup> TLC conditions: DCM/ Methanol= 90/10  
R<sub>f</sub>= 0.02

### 3-(bromomethyl)thiophene



To a solution of thiophen-3-methanol (10.00 g, 87.59 mmol) in 100 mL of dry THF, at -5 °C under a nitrogen atmosphere, is slowly dropped PBr<sub>3</sub> (2.74 mL, 29.20 mmol)

The temperature is maintained at 0 °C and the reaction progression is followed by TLC<sup>80</sup>; at completion, the solution is poured in 200 mL of an iced mixture of Ethyl ether/ Water= 2/1.

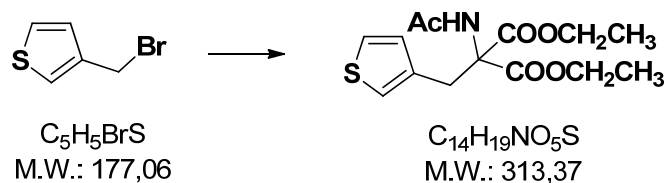
The two layers are separated; the organic phase is washed with NaHCO<sub>3</sub> and brine, dried over Na<sub>2</sub>SO<sub>4</sub>, decolorized over charcoal and used for the successive reaction without further purification.

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 7.21 (m, 2H), 7.02 (m, 1H), 4.43 (s, 2H).

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<sup>80</sup> TLC conditions: Cyclohexane / Ethyl Acetate= 60/ 40  
Rf: 0.60

## Diethyl 2-acetamido-2-(thiophen-3-ylmethyl)malonate



A suspension of potassium *tert*-butoxide (7.86 g, 70.07 mmol) in 80 mL of THF, under a nitrogen atmosphere, is added of diethyl acetamidomalonate (15.22 g, 70.07 mmol) and stirred for 20 minutes. Then the ethereal solution of the 3-(bromomethyl)thiophene (87.59 mmol) is dropped; the ether is removed and the mixture is refluxed.

After 2 hours, monitoring the progression of the reaction by TLC<sup>81</sup>, the suspension is concentrated under vacuum, resumed with Ethyl Acetate, washed with HCl 10%, NaHCO<sub>3</sub> 10% and brine.

The organic phase is dried over Na<sub>2</sub>SO<sub>4</sub>, decolorized on carchoal, filtered and concentrated under reduced pressure; the crude is a sticky solid that is further crystallized in Isopropyl ether yielding 13.61 g (43.44 mmol) of a white solid.

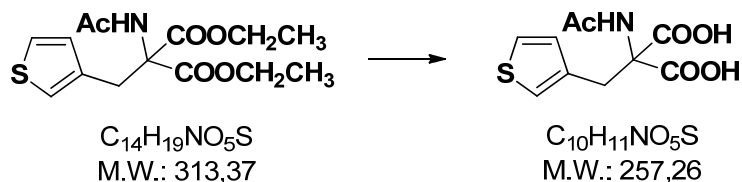
Yield: 62%

Melting point: 66.91 °C

<sup>1</sup>H-NMR (CDCl<sub>3</sub>): 7.21 (m, 1H), 7.15 (m, 1H), 6.84 (m, 1H), 6.69 (bs, 1H), 4.19 (q, 4H, *J*= 7.04 Hz), 3.71 (s, 2H), 1.97 (s, 3H), 1.22 (t, 6H, *J*= 7.04 Hz).

<sup>81</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 60/ 40  
Rf: 0.23

## 2-acetamido-2-(thiophen-3-ylmethyl)malonic acid



To a solution of diethyl 2-acetamido-2-(thiophen-3-ylmethyl)malonate 13.61 g (43.44 mmol) in 150 mL of Dioxane are added dropwise 87 mL of NaOH 4M and the mixture is refluxed for 1 hour.

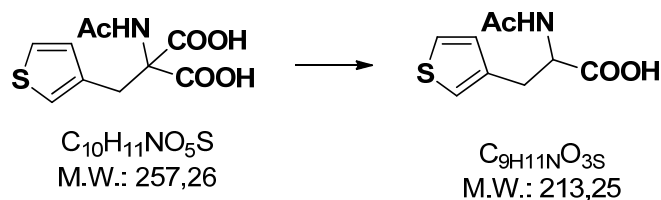
The reaction is monitored by NMR, at completion, the mixture is slowly poured in a flask, containing 300 mL of a mixture of Ethyl Acetate/ HCl 10%= 1/ 1.

The two layers are separated and the aqueous phase is extracted twice more with Ethyl Acetate; the organic phases are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure, yielding 9.83 g (38.23 mmol) of a sticky beige solid.

Yield: 88%

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 7.76 (bs, 1H), 7.41 (m, 1H), 7.05 (m, 1H), 6.78 (m, 1H), 3.44 (s, 2H), 1.92 (s, 3H).

## 2-acetamido-3-(thiophen-3-yl)propanoic acid



A solution of 9.83 g (38.23 mmol) of 2-acetamido-2-(thiophen-3-ylmethyl)malonic acid in 100 mL of Dioxane is acidified with Acetic Acid and refluxed for 2 hours, monitoring the completion of the reaction by NMR.

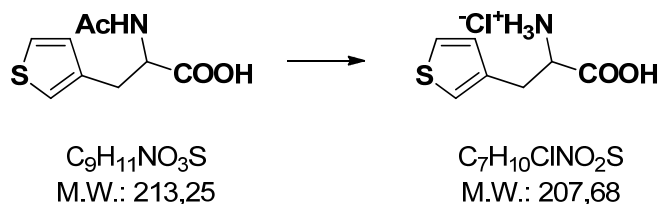
The removal of the solvents yields 7.10 g (33.26 mmol) of beige oil.

Yield: 87%

Melting point: 147 °C

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 8.17 (d, 1H, *J* = 7.70 Hz), 7.41 (m, 1H), 7.20 (m, 1H), 6.98 (dd, 2H, *J* = 1.32 Hz, *J* = 4.48 Hz), 4.45 (m, 1H), 3.07 (m, 1H), 2.83 (m, 1H), 1.80 (s, 3H).

## 1-carboxy-2-(thiophen-3-yl)ethanaminium chloride



A solution of 7.10 g (33.26 mmol) of 2-acetamido-3-(thiophen-3-yl)propanoic acid in NaOH 2.5M is refluxed for 2 ½ hours, monitoring the reaction by NMR.

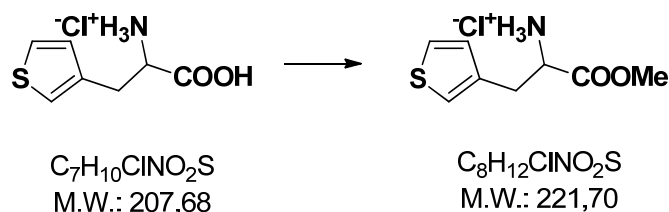
At completion, the mixture is cooled to 0°C and acidified with HCl 37%, the solvents are removed and the crude is resumed with Methanol, heated for 1 hour and filtered.

The resulting solution is concentrated under vacuum, yielding 6.08 g (29.27 mmol) of a white sticky solid.

Yield: 88 %

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 8.55 (bs, 3H), 7.50 (dd, 1H, *J* = 0.55 Hz, *J* = 4.48 Hz), 7.34 (dd, 1H, *J* = 0.55 Hz, *J* = 1.32 Hz), 7.03 (d, 1H, *J* = 1.32 Hz, *J* = 4.48 Hz), 4.09 (t, 1H, *J* = 5.77 Hz), 3.19 (d, 2H, *J* = 5.77 Hz).

### 3-(thiophen-3-yl)-1-methoxy-1-oxopropan-2-aminium chloride



Thionyl chloride (2.14 mL, 29.27 mmol) is slowly dropped to a solution of 1-carboxy-2-(thiophen-3-yl)ethanaminium chloride (6.08 g, 29.27 mmol) and trimethyl orthoformate (3.20 mL, 29.27 mmol) in anhydrous methanol (70 mL) at 0 °C under a nitrogen atmosphere; the resulting mixture is warmed to RT and refluxed for 3 h.

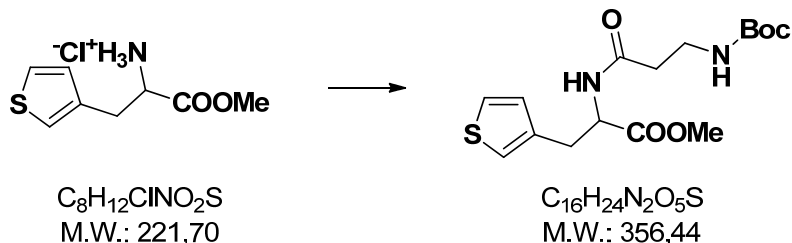
The reaction is monitored by NMR; at completion the solvent is removed, yielding 6.10 g (27.51 mmol) of beige oil.

Yield: 94%

$^1\text{H-NMR}$  ( $\text{d}_6\text{DMSO}$ ): 8.81 (bs, 3H), 7.49 (d, 1H,  $J = 0.55$  Hz,  $J = 4.48$  Hz), 7.34 (m, 1H), 6.99 (d, 1H,  $J = 4.48$  Hz), 4.22 (m, 1H), 3.66 (s, 3H), 3.21 (m, 2H).



## Methyl 2-(3-((tert-butoxycarbonyl)amino)propanamido)-3-(thiophen-3-yl)propanoate



A solution of EDAC (6.32 g, 33.01 mmol) and DIPEA (5.75 mL, 33.01 mmol) in 20 mL of DCM is added dropwise to a solution of 3-((tert-butoxycarbonyl)amino)propanoic acid (6.25 g, 33.01 mmol), HOBT (4.46 g, 33.01 mmol) in 30 mL of DCM under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of 3-(thiophen-3-yl)-1-methoxy-1-oxopropan-2-aminium chloride (6.10 g, 27.51 mmol) and DIPEA (4.79 mL, 27.51 mmol) in 20 mL of DCM is added dropwise.

The reaction mixture is stirred at RT for 2 hours and monitored by TLC<sup>82</sup>.

At completion, the mixture is treated with DCM and HCl 10%; the organic layer is washed with NaHCO<sub>3</sub> and brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated.

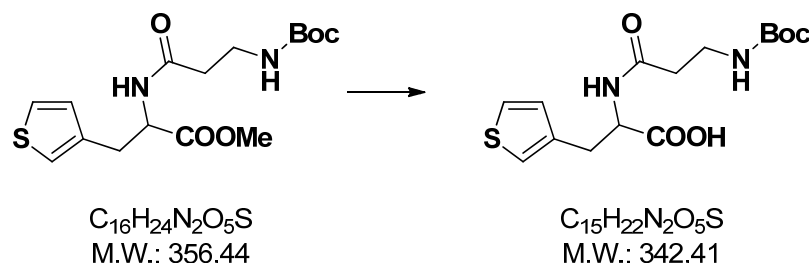
The crude product is purified by column chromatography on silica gel, using Toluene/Ethyl Acetate = 60/40 as elution solvent, to give 5.55 g (15.57 mmol) of the desired product, as pale yellow oil.

Yield: 56.6 %

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 8.35 (d, 1H, *J* = 7.42 Hz), 7.42 (d, 1H, *J* = 4.95 Hz), 7.20 (d, 1H, *J* = 1.50 Hz), 6.97 (dd, 1H, *J* = 1.50 Hz, *J* = 4.95 Hz), 6.67 (m, 1H), 4.43 (m, 1H), 3.59 (s, 3H), 3.08 (m, 2H), 2.88 (m, 2H), 2.24 (m, 2H), 1.36 (s, 9H).

<sup>82</sup> TLC conditions: Toluene/ Ethyl Acetate= 60/40  
Rf= 0.31

## 2-(3-((tert-butoxycarbonyl)amino)propanamido)-3-(thiophen-3-yl)propanoic acid



5.55 g (15.57 mmol) of Methyl 2-(3-(((benzyloxy)carbonyl)amino)propanamido)-3-(thiophen-3-yl)propanoate are dissolved in 50 mL of Methanol; 12 mL of NaOH 4M are dropped and the resulting mixture is stirred at RT for 1 hour.

After completion of the reaction, followed by TLC<sup>83</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with HCl 10% and extracted three times with Ethyl Acetate.

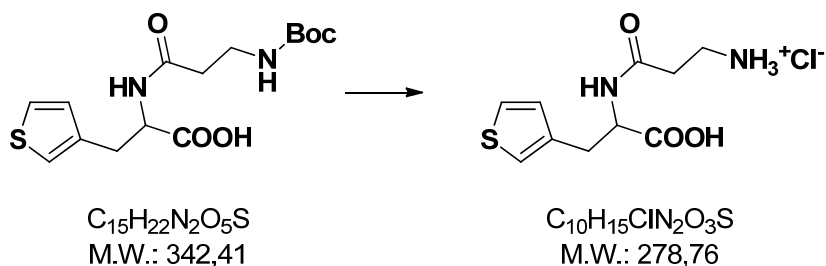
The organic phases are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated, yielding 4.95 g (14.48 mmol) of a sticky white solid.

Yield: 93%

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 8.21 (d, 1H, *J*=7.43 Hz), 7.42 (dd, 1H, *J*=0.55 Hz, *J*=4.95 Hz), 7.20 (d, 1H, *J*= 1.50 Hz), 6.97 (dd, 1H, *J*= 1.50 Hz, *J*= 4.95 Hz), 6.67 (bs, 1H), 4.45 (m, 1H), 3.10 (m, 2H), 2.90 (m, 2H), 2.24 (m,2H), 1.36 (s, 9H).

<sup>83</sup> TLC conditions: Toluene/ Ethyl Acetate= 60/40  
R<sub>f</sub> = basal

## 2-(3-aminopropanamido)-3-(thiophen-3-yl)propanoic acid



A solution of 4.95 g (14.48 mmol) of the 2-(3-(((benzyloxy)carbonyl)amino)propanamido)-3-(thiophen-3-yl)propanoic acid in 50 mL of Ethyl Acetate is acidified with HCl 10% and heated at 50 °C .

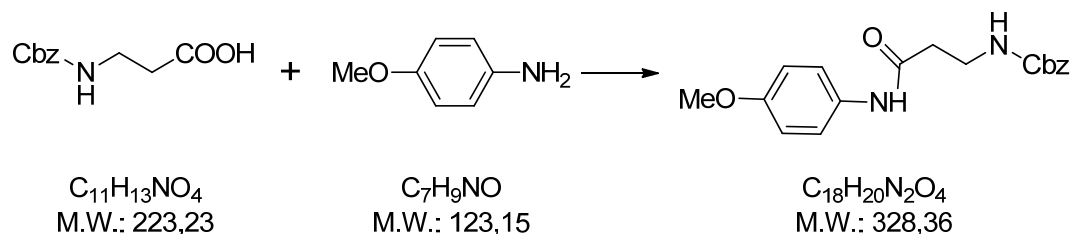
The reaction is monitored by TLC<sup>84</sup>, at completion, the solvent evaporation yields 3.75 g (13.47 mmol) of a sticky white solid.

Yield: 93%

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 8.21 (d, 1H, *J* = 7.70 Hz), 7.42 (dd, 1H, *J* = 0.55 Hz, *J* = 4.95 Hz), 7.20 (dd, 1H, *J* = 0.55 Hz, *J* = 1.50 Hz), 6.97 (dd, 1H, *J* = 1.50 Hz, *J* = 4.95 Hz), 4.43 (m, 1H), 3.08 (m, 2H), 2.94 (m, 2H), 2.24 (m, 2H).

<sup>84</sup> TLC conditions: DCM/ Methanol= 90/10  
R<sub>f</sub>= basal

## Benzyl (3-((4-methoxyphenyl)amino)-3-oxopropyl)carbamate



A solution of EDAC (0.86 g, 4.48 mmol) and DIPEA (0.78 mL, 4.48 mmol) in 3 mL of DCM is added dropwise to a solution of 3-((tert-butoxycarbonyl)amino)propanoic acid (1.00 g, 4.48 mmol), HOBT (0.61 g, 4.48 mmol) in 10 mL of DCM under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of 4-methoxyaniline (0.46 g, 3.73 mmol) in 2 mL of DCM is added dropwise.

The reaction mixture is stirred at RT for 3 hours and monitored by TLC<sup>85</sup>.

At completion, the mixture is treated with DCM and HCl 10%; the organic layer is washed with NaHCO<sub>3</sub> and brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated.

The crude product is crystallized from Isopropanol, yielding 0.69 g (2.10 mmol) of the desired product, as a white solid.

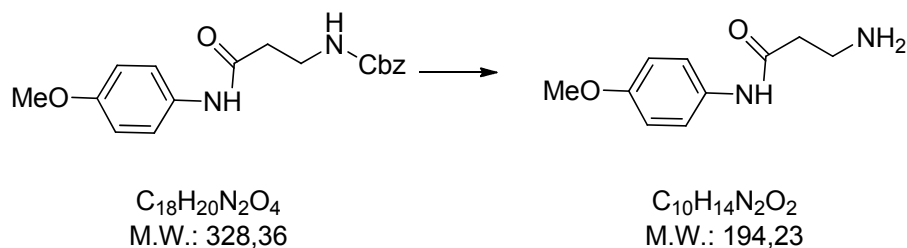
Yield: 56.4 %

Melting point: 163.78 °C

**<sup>1</sup>H NMR (CDCl<sub>3</sub>):** 7.34-7.39 (m, 9H), 6.83 (d, 1H, *J*= 7.70 Hz), 5.10 (s, 2H), 3.79 (s, 3H), 3.56 (t, 2H, *J*= 6.05 Hz), 2.59 (t, 2H, *J*= 6.05 Hz).

<sup>85</sup> TLC conditions: Chloroform/ Methanol= 90/10  
R<sub>f</sub>= 0.50

### 3-amino-N-(4-methoxyphenyl)propanamide



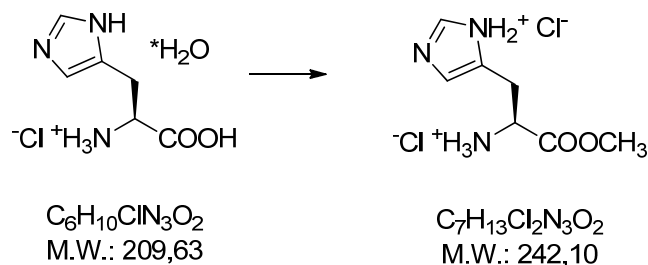
0.69 g (2.10 mmol) undergo hydrogenolysis with Pd/C 5% in 10 mL of Methanol. The reaction is monitored by TLC<sup>86</sup>, at completion, catalyst filtration and solvent evaporation gives a sticky white solid, that is further crystallized from Isopropanol/isopropyl ether = 1/1, yielding 0.15 g (0.76 mmol) of the final product.

Yield: 36%

Melting point: 103.70 °C

**<sup>1</sup>H NMR (D<sub>2</sub>O):** 7.21 (d, 1H, *J* = 7.07 Hz), 6.83 (d, 1H, *J* = 7.07 Hz), 3.70 (s, 3H), 2.82 (t, 2H, *J* = 6.05 Hz), 2.41 (t, 2H, *J* = 6.05 Hz).

<sup>86</sup> TLC conditions: Chloroform/ Methanol= 90/10  
R<sub>f</sub>= basal

**(L)-Histidine-methyl ester dihydrochloride**

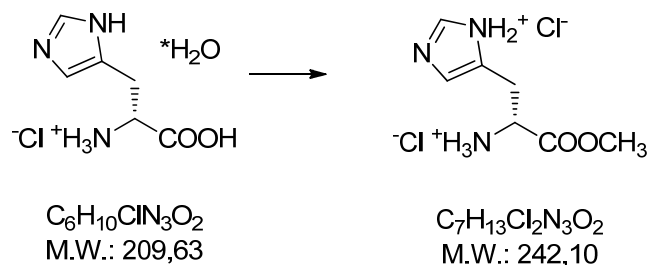
Thionyl chloride (13.84 mL, 190.81 mmol) is added dropwise to a suspension of *L*-histidine monochloride monohydrate (40.0 g, 190.81 mmol) and trimethyl orthoformate (20.87 mL, 190.81 mmol) in anhydrous methanol (400 mL) at 0 °C under a nitrogen atmosphere, and the resulting mixture is refluxed for 16 h.

The reaction is monitored by NMR; at completion the mixture is cooled down to RT and concentrated, yielding 46.10 g (190.43 mmol) of a white solid.

Yield: 98.8 %

Melting point: 200 °C

$^1\text{H NMR}$  ( $\text{d}_6\text{DMSO}$ ): 9.1 (s, 1H), 7.50 (s, 1H), 4.46 (t, 1H,  $J=7.15$  Hz), 3.70 (s, 3H), 3.30 (d, 2H,  $J=7.15$  Hz).

**(D)-Histidine-methyl ester dihydrochloride**

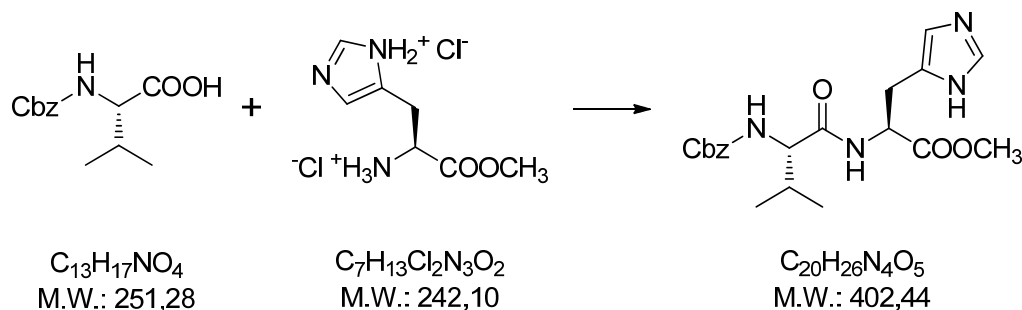
Thionyl chloride (13.84 mL, 190.81 mmol) is added dropwise to a suspension of *D*-histidine monochloride monohydrate (40.0 g, 190.81 mmol) and trimethyl orthoformate (20.87 mL, 190.81 mmol) in anhydrous methanol (400 mL) at 0°C under a nitrogen atmosphere, and the resulting mixture is refluxed for 16 h.

The reaction is monitored by NMR, at completion the mixture is cooled down to RT and concentrated, yielding 45.83 g of a white solid (189.28 mmol).

Yield: 99.2 %

Melting point: 197°C

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 9.05 (s, 1H), 7.50 (s, 1H), 4.46 (t, 1H, *J*=7.15 Hz), 3.69 (s, 3H), 3.31 (d, 2H, *J*=7.15 Hz).

**(L)-(benzyloxy)carbonyl-Valine-(L)-Histidine-methyl ester**

A solution of EDAC (0.95 g, 4.96 mmol) and DIPEA (0.86 mL, 4.96 mmol) in 2 mL of dry DMF is added dropwise to a solution of (benzyloxy)carbonyl-(L)Valine (1.04 g, 4.13 mmol), HOBT (0.67 g, 4.96 mmol) in 8 mL of dry DMF under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of (L)-Histidine methyl ester dihydrochloride (1.00 g, 4.13 mmol) and DIPEA (1.44 mL, 8.26 mmol) in 5 mL of dry DMF is added dropwise.

The reaction mixture is stirred at RT overnight and monitored by TLC<sup>87</sup>.

After completion, the solvent is removed under vacuum and the residue is treated with Ethyl Acetate and washed first with a solution of NaHCO<sub>3</sub> and then with brine.

The organic phase is dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and evaporated.

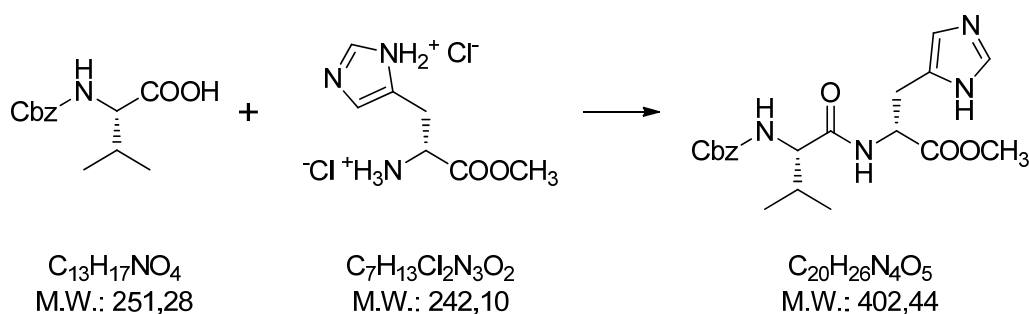
The crude product is purified by column chromatography on silica gel, using DCM/Methanol = 90/10 as elution solvent, to give 1.00g (2.48 mmol) of the desired dipeptide.

Yield: 60 %

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 11.84 (bs, 1H), 8.33 (d, 1H, *J*=7.15 Hz), 7.51 (s, 1H), 7.25-7.35 (m, 6H), 6.81 (s, 1H), 5.01 (s, 2H), 4.46 (m, 1H), 3.86 (m, 1H), 3.55 (s, 3H), 2.84-2.90 (m, 2H), 1.91 (m, 1H), 0.83 (d, 3H, *J*= 6.88 Hz), 0.80 (d, 3H, *J*= 6.88 Hz).

<sup>87</sup> TLC conditions: DCM/ Methanol= 90/10  
R<sub>f</sub>= 0.34



**(L)-(benzyloxy)carbonyl-Valine-(D)-Histidine-methyl ester**

A solution of EDAC (0.95 g, 4.96 mmol) and DIPEA (0.86 mL, 4.96 mmol) in 2 mL of dry DMF is added dropwise to a solution of (benzyloxy)carbonyl-(L)Valine (1.04 g, 4.13 mmol), HOBT (0.67 g, 4.96 mmol) in 8 mL of dry DMF under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of (D)-Histidine methyl ester dihydrochloride (1.00 g, 4.13 mmol) and DIPEA (1.44 mL, 8.26 mmol) in 5 mL of dry DMF is added dropwise.

The reaction mixture is stirred at RT overnight and monitored by TLC<sup>88</sup>.

After completion, the solvent is removed under vacuum and the residue is treated with Ethyl Acetate and washed first with a solution of NaHCO<sub>3</sub> and then with brine.

The organic phase is dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and evaporated.

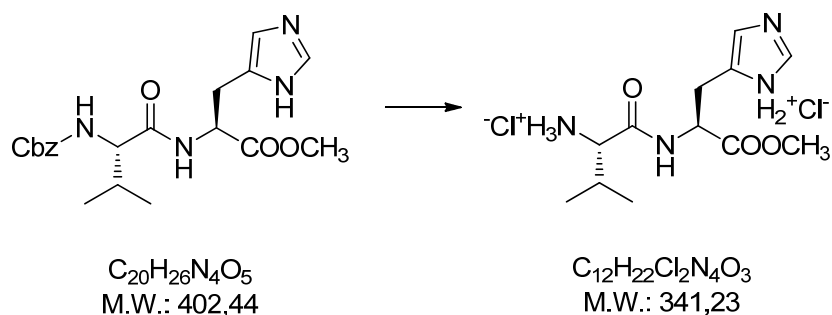
The crude product is purified by column chromatography on silica gel, using DCM/Methanol = 90/10 as elution solvent, to give 0.76 g (1.90 mmol) of the desired dipeptide.

Yield: 46%

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 11.80 (bs, 1H), 8.33 (d, 1H, *J*=7.15 Hz), 7.48 (s, 1H), 7.22-7.40 (m, 6H), 6.78 (s, 1H), 5.01 (s, 2H), 4.43 (m, 1H), 3.84 (m, 1H), 3.58 (s, 3H), 2.80-2.92 (m, 2H), 1.86 (m, 1H), 0.76 (s, 6H).

<sup>88</sup> TLC conditions: DCM/ Methanol= 90/10  
Rf= 0.30

## (L)-Valine-(L)-Histidine-methyl ester dihydrochloride

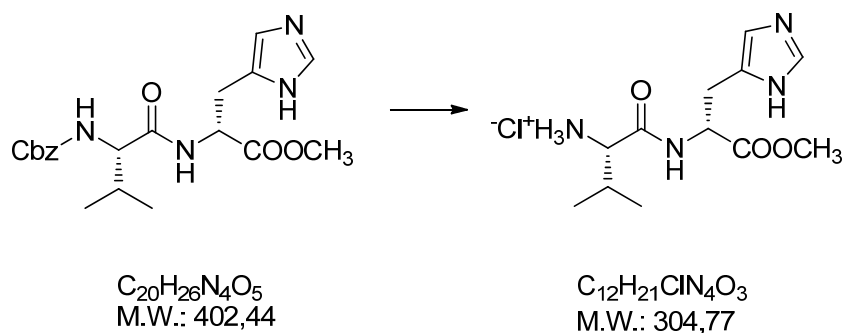


1.00g (2.48 mmol) of the protected dipeptide undergo hydrogenolysis with Pd/C 10% in a mixture of 15 mL of dry Methanol and 4 mL of 1.25 M HCl Methanol solution. The reaction is monitored by TLC<sup>89</sup>, at completion, achieved after stirring for 18 hours at RT, the solvents are evaporated giving 0.85 g of a sticky white solid in quantitative yield.

Yield: Quantitative

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 9.25 (d, 1H, *J* = 7.15 Hz), 8.92 (s, 1H), 8.0-8.9 (bs, 5H), 7.48 (s, 1H), 4.68 (m, 1H), 3.71 (d, 1H, *J* = 5.5 Hz), 3.64 (s, 3H), 3.13-3.20 (m, 2H), 2.10 (m, 1H), 0.94 (s, 3H), 0.92 (s, 3H).

<sup>89</sup> TLC conditions: DCM/ Methanol= 90/10  
R<sub>f</sub>= basal

**(L)-Valine-(D)-Histidine-methyl ester dihydrochloride**

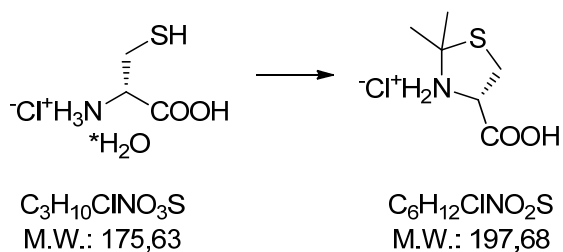
0.76 g (1.90 mmol) of the protected dipeptide undergo hydrogenolysis with Pd/C 10% in a mixture of 15 mL of dry Methanol and 3 mL of 1.25 M HCl Methanol solution. The reaction is monitored by TLC<sup>90</sup>, at completion, achieved after stirring for 18 hours at RT, the solvents are evaporated giving 0.55 g of a sticky white solid in quantitative yield.

Yield: Quantitative

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 8.99 (d, 1H, *J* = 7.97 Hz), 7.77 (s, 1H), 6.93 (s, 1H), 4.62 (m, 1H), 3.63 (s, 3H), 3.60 (d, 1H, *J* = 5.13 Hz), 3.01 (dd, 1H, *J* = 14.58 Hz, *J* = 4.68 Hz), 2.89 (dd, 1H), 1.96 (m, 1H, *J* = 14.58 Hz, *J* = 9.63 Hz), 0.78 (d, 3H, *J* = 6.88 Hz), 0.70 (d, 3H, *J* = 6.78 Hz).

<sup>90</sup> TLC conditions: DCM/ Methanol= 90/10  
R<sub>f</sub>= basal

## (L)-2,2-dimethylthiazolidine-4-carboxylic acid hydrochloride



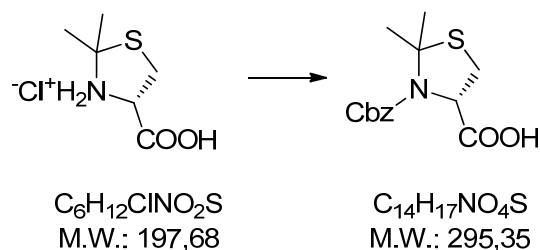
(L)-Cysteine hydrochloride monohydrate (5.0 g, 28.47 mmol) is dissolved in 200 mL of Acetone and then refluxed for 2 hours; the reaction is monitored by NMR analysis. At completion, the mixture is cooled and concentrated, obtaining 5.06 g (25.62 mmol) of the desired product, as white solid.

Yield: 90%

Melting point: 168 °C

**$^1\text{H}$  NMR ( $\text{d}_6\text{DMSO}$ ):** 7.60 (bs, 3H), 3.96 (dd, 1H,  $J=7.15$  Hz,  $J=8.53$  Hz), 3.32 (dd, 1H,  $J=7.15$  Hz,  $J=11.17$  Hz), 2.93 (dd, 1H,  $J=8.53$  Hz,  $J=11.17$  Hz), 1.58 (s, 3H), 1.41 (s, 3H).

## (L)-3-((benzyloxy)carbonyl)-2,2-dimethylthiazolidine-4-carboxylic acid



(L)-2,2-dimethylthiazolidine-4-carboxylic acid hydrochloride (5.06 g, 25.62 mmol) is dissolved in 100 mL of Acetonitrile and added of DIPEA (5.80 mL; 33.31 mmol), under nitrogen condition.

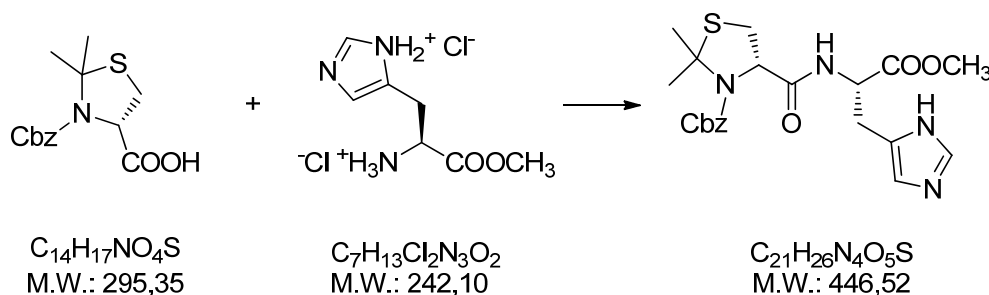
The mixture is treated with benzyl chloroformate (4.75 mL; 33.31 mmol) at 0 °C.

The reaction is then warmed to RT and monitored by NMR. After 3 hours the solvent is removed, the residue treated with Ethyl Acetate and washed with HCl 10% and brine. The organic phase is dried over  $\text{Na}_2\text{SO}_4$ , filtered, and concentrated under reduced pressure, giving 7.57 g of the desired product, in quantitative yield.

Yield: Quantitative

$^1\text{H NMR}$  ( $\text{d}_6\text{DMSO}$ ): 9.40 (bs, 1H), 7.22-7.46 (m, 5), 4.92-5.32 (m, 3H), 3.18-3.38 (m, 2H), 1.92 (s, 3H), 1.83 (s, 3H).

**(L)-3-((benzyloxy)carbonyl)-2,2-dimethylthiazolidine-4-  
(L)Histidine-methyl ester**



A solution of EDAC (0.95 g, 4.96 mmol) and DIPEA (0.86 mL, 4.96 mmol) in 2 mL of dry DMF is added dropwise to a solution of (L)-3-((benzyloxy)carbonyl)-2,2-dimethylthiazolidine-4-carboxylic acid (1.22 g, 4.13 mmol), HOBT (0.67 g, 4.96 mmol) in 8 mL of dry DMF under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of (L)-Histidine methyl ester dihydrochloride (1.00 g, 4.13 mmol) and DIPEA (1.44 mL, 8.26 mmol) in 5 mL of dry DMF is added dropwise.

The reaction mixture is stirred at RT overnight and monitored by TLC<sup>91</sup>.

After completion, the solvent is removed under vacuum and the residue is treated with Ethyl Acetate and washed first with a solution of NaHCO<sub>3</sub> and then with brine.

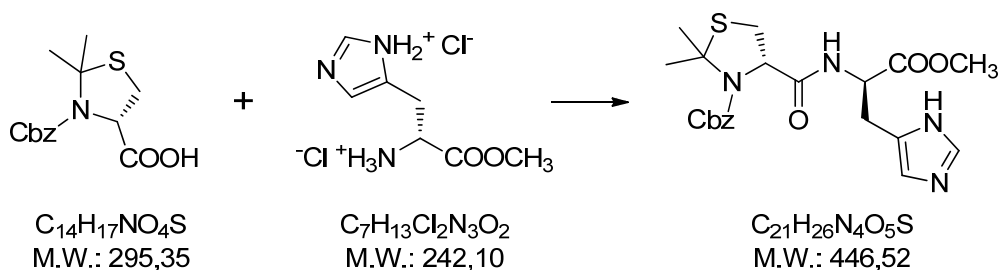
The organic phase is dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and evaporated. The crude product is purified by column chromatography on silica gel, using DCM/Methanol = 90/10 as elution solvent, to give 0.94 g (2.11 mmol) of the desired dipeptide.

Yield: 51 %

<sup>1</sup>H NMR (d<sub>6</sub>DMSO): 11.83 (bs, 1H), 8.40 (d, 1H, *J*=5.57 Hz), 7.50 (s, 1H), 7.26-7.49 (m, 6H), 6.76 (s, 1H), 5.05 (s, 2H), 4.78 (m, 1H), 4.47 (m, 1H), 3.52 (s, 3H), 3.37 (m, 2H), 2.97 (m, 2H), 1.78 (s, 3H), 1.71 (s, 3H).

<sup>91</sup> TLC conditions: DCM/ Methanol= 90/10  
R<sub>f</sub>= 0.37

**(L)-3-((benzyloxy)carbonyl)-2,2-dimethylthiazolidine-4-  
(D)Histidine-methyl ester**



A solution of EDAC (0.95 g, 4.96 mmol) and DIPEA (0.86 mL, 4.96 mmol) in 2 mL of dry DMF is added dropwise to a solution of (L)-3-((benzyloxy)carbonyl)-2,2-dimethylthiazolidine-4-carboxylic acid (1.22 g, 4.13 mmol), HOBT (0.67 g, 4.96 mmol) in 8 mL of dry DMF under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of (D)-Histidine methyl ester dihydrochloride (1.00 g, 4.13 mmol) and DIPEA (1.44 mL, 8.26 mmol) in 5 mL of dry DMF is added dropwise.

The reaction mixture is stirred at RT overnight and monitored by TLC<sup>92</sup>.

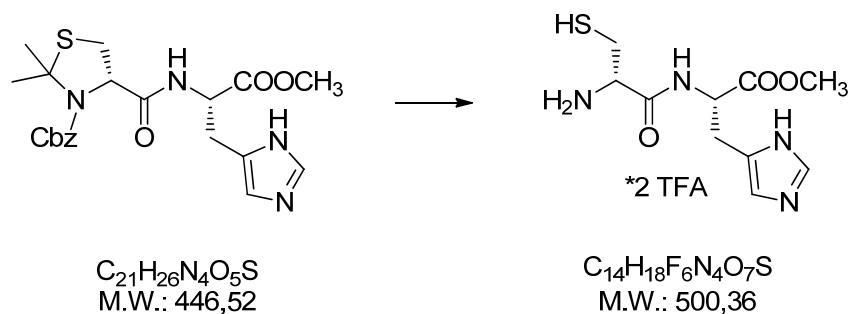
After completion, the solvent is removed under vacuum and the residue is treated with Ethyl Acetate and washed first with a solution of NaHCO<sub>3</sub> and then with brine.

The organic phase is dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and evaporated. The crude product is purified by column chromatography on silica gel, using DCM/Methanol = 90/10 as elution solvent, to give 1.07 g (2.40 mmol) of the desired dipeptide.

Yield: 58 %

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 7.69 (d, 1H, *J*=7.90 Hz), 7.24-7.33 (m, 6H), 7.26 (d, 1H, *J*=8.48 Hz), 6.66 (s, 1H), 4.96 (s, 2H), 3.90 (m, 1H), 3.77 (m, 3H), 3.46 (m, 1H), 3.31 (m, 2H), 3.01 (m, 2H), 2.03 (s, 3H), 1.99 (s, 3H).

<sup>92</sup> TLC conditions: DCM/ Methanol= 90/10  
Rf= 0.36

**(L)-Cysteine-(L)-Histidine-methyl ester di-trifluoroacetate**

0.94 g (2.11 mmol) of the (L)-3-((benzyloxy)carbonyl)-2,2-dimethylthiazolidine-4-(L)-Histidine methyl ester is stirred in TFA for 24 hours in presence of anisole (0.46 mL, 4.22 mmol).

At completion of the reaction, monitored by TLC<sup>93</sup>, the solvent is removed and the residue rinsed and washed three times with Ethyl ether.

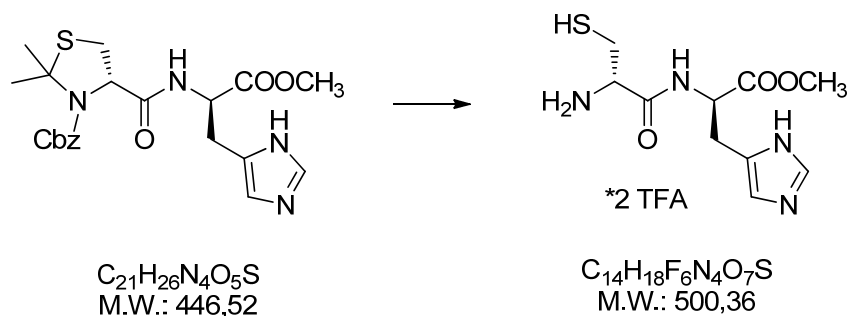
Simply water removal under vacuum gives 1.06 g of the final product in quantitative yield.

Yield: Quantitative

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 9.04 (d, 1H, *J* = 7.70 Hz), 8.99 (s, 1H), 8.26 (bs, 5H), 7.42 (s, 1H), 4.69 (m, 1H), 4.04 (m, 1H), 3.65 (s, 3H), 2.94-3.22 (m, 4H).

<sup>93</sup> TLC conditions: DCM/ Methanol= 90/10  
R<sub>f</sub>= basal



**(L)-Cysteine-(D)-Histidine-methyl ester di-trifluoroacetate**

1.07 g (2.40 mmol) of the (L)-3-((benzyloxy)carbonyl)-2,2-dimethylthiazolidine-4-(L)-Histidine methyl ester is stirred in TFA for 24 hours in presence of anisole (0.46 mL, 4.22 mmol).

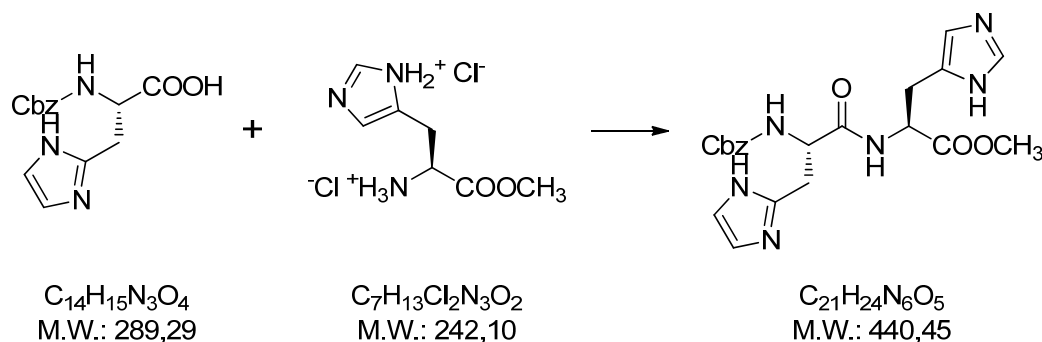
At completion of the reaction, monitored by TLC<sup>94</sup>, the solvent is removed and the residue rinsed and washed three times with Ethyl ether.

Simply water removal under vacuum gives 1.20 g of the final product in quantitative yield.

Yield: Quantitative

<sup>1</sup>H NMR (**-d<sub>6</sub>DMSO**): 9.11 (d, 1H, *J* = 7.70 Hz), 8.98 (s, 1H), 8.26 (bs, 5H), 7.41 (s, 1H), 4.73 (m, 1H), 4.03 (m, 1H), 3.66 (s, 3H), 3.20 (dd, 1H, *J* = 15.13 Hz, *J* = 4.95 Hz), 3.04 (dd, 1H, *J* = 15.13 Hz, *J* = 9.63 Hz), 2.85 (m, 2H).

<sup>94</sup> TLC conditions: DCM/ Methanol= 90/10  
 Rf= basal

**(L)-(benzyloxy)carbonyl-Histidine-(L)-Histidine-methyl ester**

A solution of EDAC (0.95 g, 4.96 mmol) and DIPEA (0.86 mL, 4.96 mmol) in 2 mL of dry DMF is added dropwise to a solution of (L)-(benzyloxy)carbonyl-Histidine (1.19 g, 4.13 mmol), HOBT (0.67 g, 4.96 mmol) in 8 mL of dry DMF under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of (L)-Histidine methyl ester dihydrochloride (1.00 g, 4.13 mmol) and DIPEA (1.44 mL, 8.26 mmol) in 5 mL of dry DMF is added dropwise.

The reaction mixture is stirred at RT overnight and monitored by TLC<sup>95</sup>.

After completion, the solvent is removed under vacuum and the residue is treated with Ethyl Acetate and washed first with a solution of NaHCO<sub>3</sub> and then with brine.

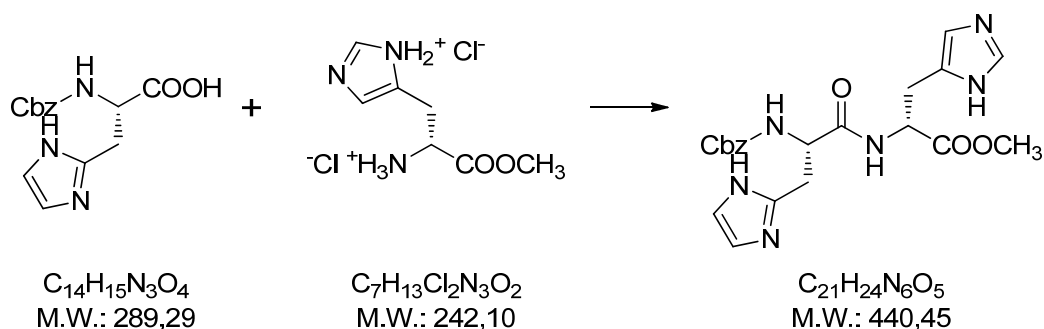
The organic phase is dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and evaporated. The crude product is purified by column chromatography on silica gel, using Ethyl Acetate/Methanol = 95/5 as elution solvent, to give 0.85 g (1.94 mmol) of the desired dipeptide.

Yield: 47 %

<sup>1</sup>H NMR (d<sub>6</sub>DMSO): 11.81 (bs, 2H), 8.34 (d, 1H, *J*=6.96 Hz), 7.50 (s, 1H), 7.42 (d, 1H, *J*=8.43 Hz), 7.30-7.39 (m, 6H), 6.79 (s, 1H), 6.76 (s, 1H), 4.97 (s, 2H), 4.44 (m, 1H), 4.24 (m, 1H), 3.57 (s, 3H), 2.67-2.91 (m, 4H).

<sup>95</sup> TLC conditions: Ethyl Acetate/ Methanol= 95/5  
R<sub>f</sub>= 0.30

**(L)-(benzyloxy)carbonyl-Histidine-(D)-Histidine-methyl ester**



A solution of EDAC (0.95 g, 4.96 mmol) and DIPEA (0.86 mL, 4.96 mmol) in 2 mL of dry DMF is added dropwise to a solution of (L)-(benzyloxy)carbonyl-Histidine (1.19 g, 4.13 mmol), HOBT (0.67 g, 4.96 mmol) in 8 mL of dry DMF under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of (D)-Histidine methyl ester dihydrochloride (1.00 g, 4.13 mmol) and DIPEA (1.44 mL, 8.26 mmol) in 5 mL of dry DMF is added dropwise.

The reaction mixture is stirred at RT overnight and monitored by TLC<sup>96</sup>.

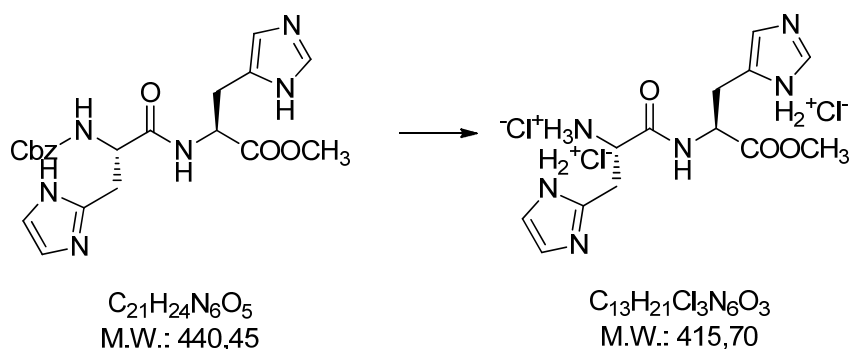
After completion, the solvent is removed under vacuum and the residue is treated with Ethyl Acetate and washed first with a solution of NaHCO<sub>3</sub> and then with brine.

The organic phase is dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and evaporated. The crude product is purified by column chromatography on silica gel, using Ethyl Acetate/Methanol = 95/5 as elution solvent, to give 0.91 g (2.07 mmol) of the desired dipeptide.

Yield: 50 %

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 11.80 (bs, 2H), 8.53 (d, 1H, *J*=6.96 Hz), 7.49 (s, 1H), 7.41 (s, 1H), 7.29-7.39 (m, 6H), 6.76 (s, 1H), 6.67 (s, 1H), 4.97 (s, 2H), 4.44 (m, 1H), 4.24 (m, 1H), 3.57 (s, 3H), 2.67-2.91 (m, 4H).

<sup>96</sup> TLC conditions: Ethyl Acetate/ Methanol= 95/5  
R<sub>f</sub>= 0.28

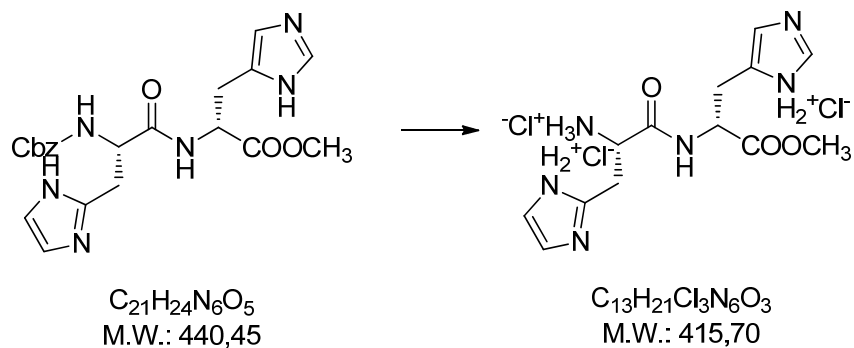
**(L)-Histidine-(L)-Histidine-methyl ester trihydrochloride**

0.85 g (1.94 mmol) of the protected dipeptide undergo hydrogenolysis with Pd/C 10% in a mixture of 10 mL of dry Methanol and 4.7 mL of 1.25 M HCl Methanol solution. The reaction is monitored by TLC<sup>97</sup>, at completion, achieved after stirring for 18 hours at RT, the solvents are evaporated giving 0.81 g of a sticky white solid in quantitative yield.

Yield: Quantitative

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 9.43 (d, 1H, *J* = 7.47 Hz), 9.05 (s, 1H), 9.03 (s, 1H), 8.65 (bs, 7H), 7.54 (s, 1H), 7.47 (s, 1H), 4.69 (m, 1H), 4.31 (m, 1H), 3.62 (s, 3H), 3.11-3.25 (m, 4H).

<sup>97</sup> TLC conditions: Ethyl Acetate/ Methanol= 95/5  
 R<sub>f</sub>= basal

**(L)-Histidine-(D)-Histidine-methyl ester trihydrochloride**

0.91 g (2.07 mmol) of the protected dipeptide undergo hydrogenolysis with Pd/C 10% in a mixture of 15 mL of dry Methanol and 3 mL of 1.25 M HCl Methanol solution.

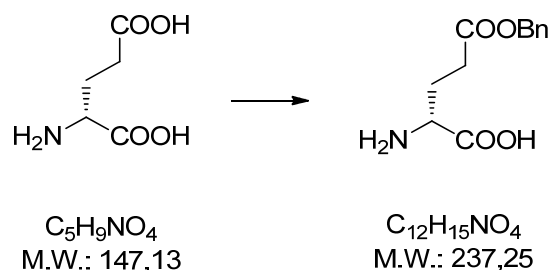
The reaction is monitored by TLC<sup>98</sup>, at completion, achieved after stirring for 18 hours at RT, the solvents are evaporated giving 0.86 g of a sticky white solid in quantitative yield.

Yield: Quantitative

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 9.32 (m, 1H), 8.99 (s, 1H), 8.94 (s, 1H), 7.42 (s, 1H), 7.36 (s, 1H), 4.60 (m, 1H), 4.20 (m, 1H), 3.62 (s, 3H), 3.03-3.17 (m, 4H).

<sup>98</sup> TLC conditions: Ethyl Acetate/ Methano I= 95/5  
 Rf= basal

## (L)-Glutamic acid- $\gamma$ -benzyl ester



(L)-Glutamic acid (5.0 g, 34.0 mmol) is added portionwise to a solution of benzyl alcohol (5.30 mL, 51.0 mmol) in 5 mL of Toluene.

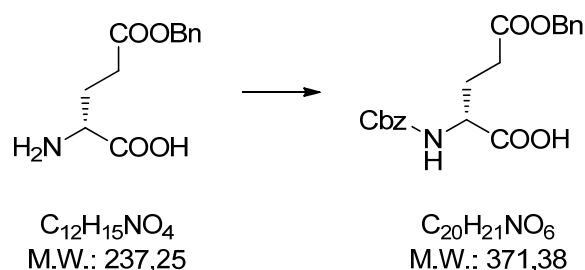
The mixture is heated to 45°C and sequentially 2.64 mL (40.8 mmol) of methanesulfonic acid are slowly dropped in. The temperature is maintained at 45°C for 2 hours and then at 32°C for further 3 hours.

After 5 hours the solution is cooled to RT and 10 mL of Water are added; after separation of the two phases, the aqueous layer is amounted of 15 mL of Ethanol and 20%NH<sub>3</sub>, reaching a neutral pH.

The mixture is heated at 60°C for 2 hours and then cooled to 5°C, till the formation of a white precipitate. The solid is filtered and washed three times with Ethanol and twice with iced Water, obtaining 4.27 g (18.00 mmol) of the desired product.

Yield: 52%

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 7.33 (m, 5H), 5.00 (s, 2H), 4.04 (m, 1H), 2.31 (m, 1H), 2.12 (m, 2H), 1.95 (m, 1H).

**(L)-(benzyloxy)carbonyl-Glutamic acid- $\gamma$ -benzyl ester**

A solution of 2.70 mL (18.9 mmol) of benzyl chloroformate in 40 mL of THF is dropped, at 0 °C, to a white suspension of (L)-Glutamic acid- $\gamma$ -benzyl ester (4.27 g, 18.00 mmol) and NaHCO<sub>3</sub> (3.10 g, 36.9 mmol) in 40 mL of Water.

The mixture is then warmed to RT and monitored by TLC<sup>99</sup>; at completion the THF is removed and the aqueous layer is firstly washed with 30 mL of Ethyl Ether, secondly acidified with 10% Citric Acid and finally extracted three times with Ethyl Acetate.

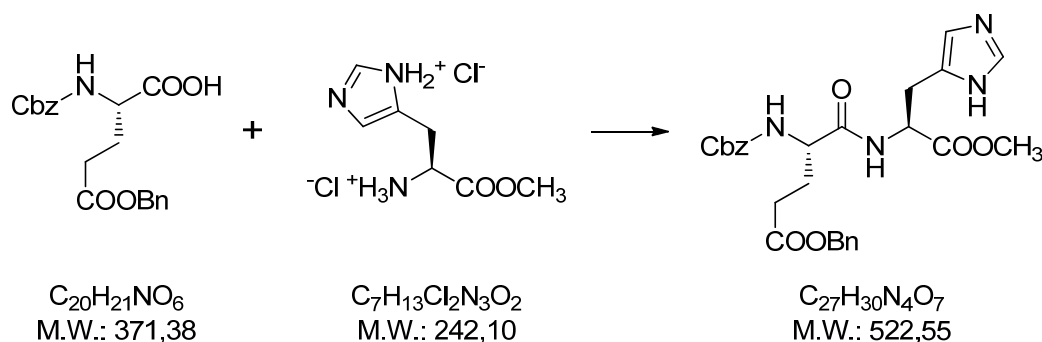
The organic phases are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated, yielding 6.48 g (17.46 mmol) of the protected amino acid, as colourless oil.

Yield: 97%

**<sup>1</sup>H NMR (CDCl<sub>3</sub>):** 7.31 (m, 10H), 5.51 (d, 1H, *J* = 7.98 Hz), 5.10 (s, 4H), 4.44 (m, 1H), 2.50 (m, 2H), 2.27 (m, 1H), 2.02 (m, 1H).

<sup>99</sup> TLC conditions: DCM/ Methanol/ Acetic acid = 90/8 /2  
R<sub>f</sub> = 0.52

**(L)-(benzyloxy)carbonyl-Glutammate-γ-benzyl ester-(L)-  
Histidine-methyl ester**



A solution of EDAC (2.17 g, 11.34 mmol) and DIPEA (1.97 mL, 11.34 mmol) in 4 mL of dry DMF is added dropwise to a solution of (L)-(benzyloxy)carbonyl-Glutamic acid-γ-benzyl ester (3.24 g, 8.72 mmol), HOBT (1.53 g, 11.34 mmol) in 16 mL of dry DMF under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of (L)-Histidine methyl ester dihydrochloride (2.11 g, 8.72 mmol) and DIPEA (3.04 mL, 17.44 mmol) in 5 mL of dry DMF is added dropwise.

The reaction mixture is stirred at RT overnight and monitored by TLC<sup>100</sup>.

After completion, the solvent is removed under vacuum and the residue is treated with Ethyl Acetate and washed first with a solution of NaHCO<sub>3</sub> and then with brine.

The organic phase is dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and evaporated. The crude product is purified by column chromatography on silica gel, using Ethyl Acetate/Methanol = 95/5 as elution solvent, to give 2.23 g (4.27 mmol) of the desired dipeptide.

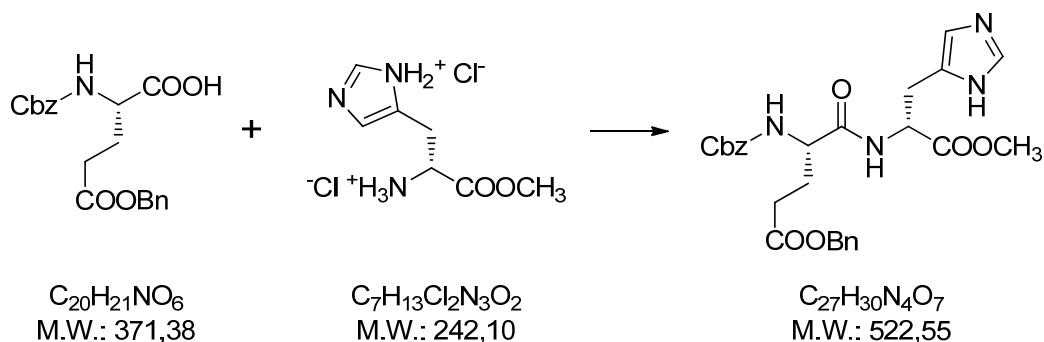
Yield: 49 %

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 11.81 (bs, 1H), 8.35 (d, 1H, *J*=7.43 Hz), 7.49 (s, 1H), 7.28-7.38 (m, 11H), 6.81 (s, 1H), 5.07 (s, 2H), 5.00 (s, 2H), 4.46 (m, 1H), 4.04 (m, 1H), 3.58 (s, 3H), 2.87-2.90 (m, 2H), 2.39 (m, 2H), 1.94 (m, 1H), 1.83 (m, 1H).

<sup>100</sup> TLC conditions: Ethyl Acetate/ Methanol= 95/5  
R<sub>f</sub>= 0.32



**(L)-(benzyloxy)carbonyl-Glutammate-γ-benzyl ester-(D)-  
Histidine-methyl ester**



A solution of EDAC (2.17 g, 11.34 mmol) and DIPEA (1.97 mL, 11.34 mmol) in 4 mL of dry DMF is added dropwise to a solution of (L)-(benzyloxy)carbonyl-Glutamic acid-γ-benzyl ester (3.24 g, 8.72 mmol), HOBT (1.53 g, 11.34 mmol) in 16 mL of dry DMF under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of (D)-Histidine methyl ester dihydrochloride (2.11 g, 8.72 mmol) and DIPEA (3.04 mL, 17.44 mmol) in 5 mL of dry DMF is added dropwise.

The reaction mixture is stirred at RT overnight and monitored by TLC<sup>101</sup>.

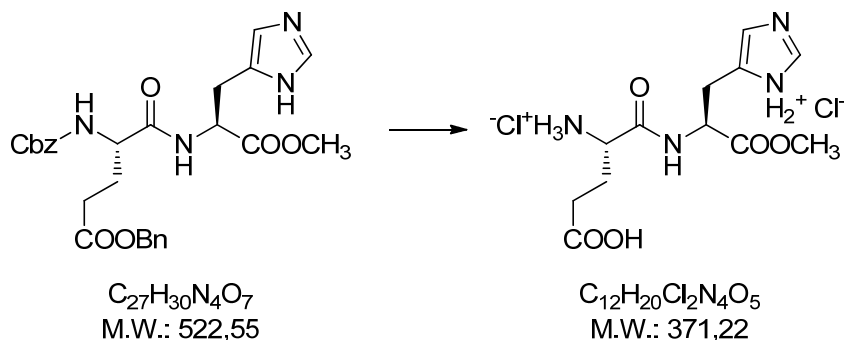
After completion, the solvent is removed under vacuum and the residue is treated with Ethyl Acetate and washed first with a solution of NaHCO<sub>3</sub> and then with brine.

The organic phase is dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and evaporated. The crude product is purified by column chromatography on silica gel, using Ethyl Acetate/Methanol = 95/5 as elution solvent, to give 3.01 g (5.76 mmol) of the desired dipeptide.

Yield: 66 %

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 11.77 (bs, 1H), 8.34 (d, 1H, *J*=7.43 Hz), 7.44 (s, 1H), 7.22-7.30 (m, 11H), 6.78 (s, 1H), 5.05 (s, 2H), 5.00 (s, 2H), 4.46 (m, 1H), 4.04 (m, 1H), 3.57 (s, 3H), 2.88 (m, 2H), 2.30 (m, 2H), 1.64-1.96 (m, 2H).

<sup>101</sup> TLC conditions: Ethyl Acetate/ Methanol= 95/5  
R<sub>f</sub>= 0.30

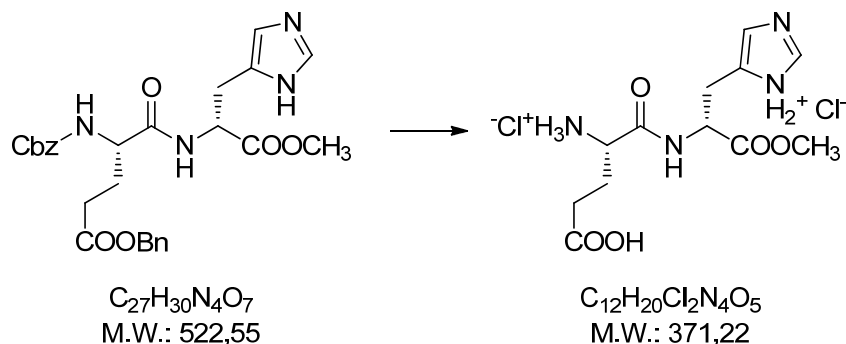
**(L)-Glutamate-(L)-Histidine-methyl ester dihydrochloride**

2.23 g (4.27 mmol) of the protected dipeptide undergo hydrogenolysis with Pd/C 10% in a mixture of 25 mL of dry Methanol and 6.8 mL of 1.25 M HCl Methanol solution. The reaction is monitored by TLC<sup>102</sup>, at completion, achieved after stirring for 18 hours at RT, the solvents are evaporated giving 1.59 g of a sticky white solid in quantitative yield.

Yield: Quantitative

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 9.30 (d, 1H, *J*= 7.43 Hz), 9.02 (s, 1H), 8.39 (bs, 5H), 7.52 (s, 1H), 4.69 (m, 1H), 3.92 (m, 1H), 3.65 (s, 3H), 3.20 (dd, 1H, *J*= 15.68 Hz, *J*= 5.22 Hz), 3.13 (dd, 1H, *J*= 15.68 Hz, *J*= 6.33 Hz), 2.38 (m, 1H), 1.95 (m, 3H).

<sup>102</sup> TLC conditions: Ethyl Acetate/ Methanol= 95/5  
Rf= basal

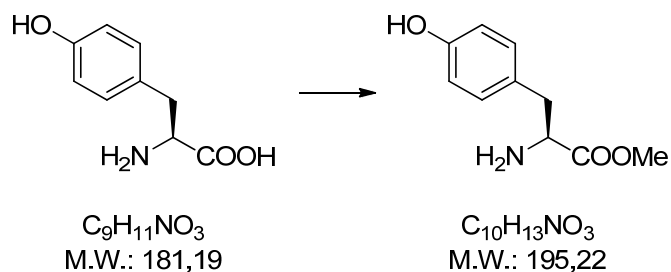
**(L)-Glutamate-(D)-Histidine-methyl ester dihydrochloride**

3.01 g (5.76 mmol) of the protected dipeptide undergo hydrogenolysis with Pd/C 10% in a mixture of 30 mL of dry Methanol and 9.2 mL of 1.25 M HCl Methanol solution. The reaction is monitored by TLC<sup>103</sup>, at completion, achieved after stirring for 18 hours at RT, the solvents are evaporated giving 2.14 g of a sticky white solid in quantitative yield.

Yield: Quantitative

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 9.39 (d, 1H, *J*= 7.15 Hz), 8.93 (s, 1H), 8.46 (bs, 5H), 7.49 (s, 1H), 4.52 (m, 1H), 3.84 (m, 1H), 3.63 (s, 3H), 3.12-3.19 (m, 2H), 2.21 (m, 2H), 1.91 (m, 2H).

<sup>103</sup> TLC conditions: Ethyl Acetate/ Methanol= 95/5  
 R<sub>f</sub>= basal

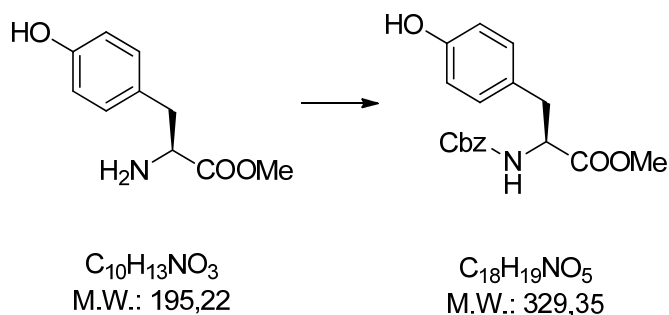
**(L)-Tyrosine-methyl ester**

Thionyl chloride (1.60 mL, 22.08 mmol) is added dropwise to a suspension of L-tyrosine (2.0 g, 11.04 mmol) in 30 mL of anhydrous methanol at 0°C, the resulting mixture is warmed to RT and then refluxed for 16 h.

The reaction is monitored by NMR, at completion the mixture is cooled down to RT and concentrated, yielding 2.16 g (11.04 mmol) of a white solid.

Yield: Quantitative

**$^1H$  NMR ( $D_2O$ ):** 7.03 (d, 1H,  $J = 7.97$  Hz), 6.97 (d, 1H,  $J = 7.97$  Hz), 4.25 (m, 1H), 3.71 (s, 3H), 3.05-3.12 (m, 2H).

**(L)-(benzyloxy)carbonyl-Tyrosine-methyl ester**

To a solution of (L)-Tyrosine-methyl ester (2.16 g, 11.04 mmol) and  $Na_2CO_3$  (1.52 g, 14.38 mmol) in 50 mL of Water/Acetone =1/1 is slowly added Benzyl chloroformate (1.74 mL, 12.17 mmol), at 0°C.

The reaction mixture is then warmed to RT and stirred for 18 hours, monitoring the reaction progression by TLC<sup>104</sup>.

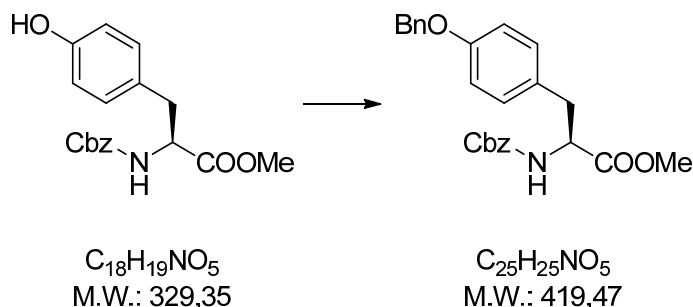
At completion, the suspension is treated with Ethyl Acetate and Water, the organic phase is washed with brine, dried over  $Na_2SO_4$ , filtered and concentrated under vacuum.

The crude is purified on silica gel, using Cyclohexane / Ethyl Acetate= 80/20 as elution solvent, yielding 2.65 g (8.05 mmol) of the desired product.

Yield: 73%

**<sup>1</sup>H NMR (CDCl<sub>3</sub>):** 7.38 (m, 5H), 6.96 (d, 1H,  $J= 7.97$  Hz), 6.67 (d, 1H,  $J= 7.97$  Hz), 5.24 (d, 1H,  $J= 7.05$  Hz), 5.10 (s, 2H), 4.60 (m, 1H), 3.71 (s, 3H), 2.95-3.10 (m, 2H).

<sup>104</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 70/30  
Rf= 0.33

**(L)-(benzyloxy)carbonyl-O-benzyl-Tyrosine-methyl ester**

A solution of (L)-(benzyloxy)carbonyl-Tyrosine-methyl ester (2.65 g, 8.05 mmol) in 25 mL of Acetone is amount of solid  $Cs_2CO_3$  (2.75 g, 8.85 mmol) and stirred for 30 minutes.

To the resulting suspension, at 0 °C, is dropped benzyl bromide (1.05 mL, 8.85 mmol), the mixture is then heated to reflux and monitored by TLC<sup>105</sup>.

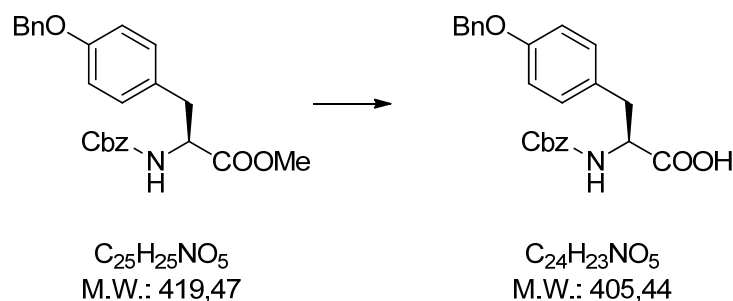
At completion, the suspension is cooled to RT and filtered over Celite; the filtrate is concentrated under reduced pressure.

The crude is purified on silica gel, using Cyclohexane/ Ethyl Acetate = 80/20 as elution solvent, yielding 2.20 g (5.24 mmol) of a colourless oil, corresponding to the desired product.

Yield: 65 %

**<sup>1</sup>H NMR (CDCl<sub>3</sub>):** 7.38 (m, 10H), 7.02 (d, 1H, *J* = 7.97 Hz), 6.92 (d, 1H, *J* = 7.97 Hz), 5.24 (d, 1H, *J* = 7.05 Hz), 5.10 (s, 2H), 5.00 (s, 2H), 4.61 (m, 1H), 3.71 (s, 3H), 3.00-3.14 (m, 2H).

<sup>105</sup> TLC condition: Cyclohexane / Ethyl Acetate = 70/30  
Rf: 0.41

**(L)-(benzyloxy)carbonyl-O-benzyl-Tyrosine**

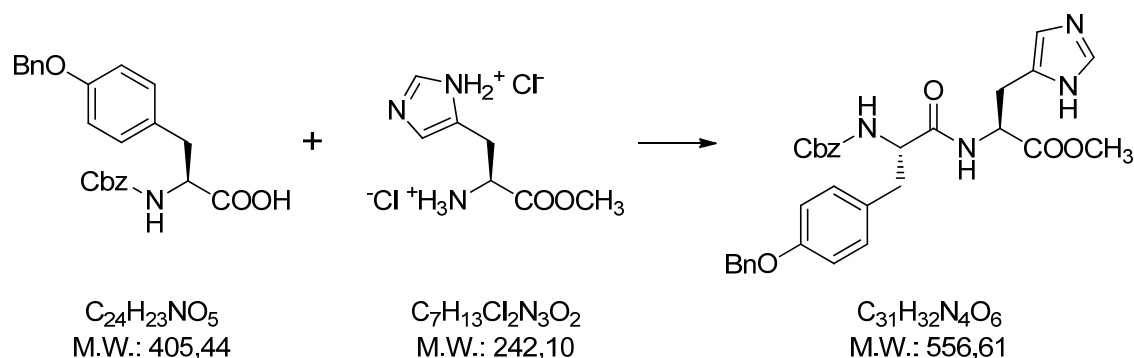
2.20 g (5.24 mmol) of (L)-(benzyloxy)carbonyl-O-benzyl-tyrosine-methyl ester are dissolved in 25 mL of Methanol and amounted, dropwise, of 2.5 mL of NaOH 2.5 M. The reaction is stirred at RT for 18 hours and monitored by TLC<sup>106</sup>; at completion, after solvent removal, the residue is rinse and washed with Ethyl ether, the aqueous layer is then acidified with HCl 10% and extraxted three times with Ethyl Acetate. The organic phases are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated, yielding 2.12 g of the free carboxylic acid, in quantitative yield.

Yield: Quantitative

**<sup>1</sup>H NMR (CDCl<sub>3</sub>):** 9.08 (bs, 1H), 7.33-7.43 (m, 10H), 7.07 (d, 1H, *J* = 8.53 Hz), 6.92 (d, 1H, *J* = 8.53 Hz), 5.24 (d, 1H, *J* = 7.98 Hz), 5.10 (s, 2H), 5.02 (s, 2H), 4.78 (m, 1H), 3.08-3.14 (m, 2H).

<sup>106</sup> TLC condition: Cyclohexane / Ethyl Acetate = 70/30  
Rf: 0.10

## (L)-(benzyloxy)carbonyl-O-benzyl-Tyrosine-(L)-Histidine-methyl ester



A solution of EDAC (0.65 g, 3.41 mmol) and DIPEA (0.59 mL, 3.41 mmol) in 4 mL of dry DMF is added dropwise to a solution of (L)-(benzyloxy)carbonyl-O-benzyl-Tyrosine (1.06 g, 2.62 mmol), HOBT (0.46 g, 3.41 mmol) in 16 mL of dry DMF under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of (L)-Histidine methyl ester dihydrochloride (0.63 g, 2.62 mmol) and DIPEA (0.91 mL, 5.24 mmol) in 5 mL of dry DMF is added dropwise.

The reaction mixture is stirred at RT overnight and monitored by TLC<sup>107</sup>.

After completion, the solvent is removed under vacuum and the residue is treated with Ethyl Acetate and washed first with a solution of NaHCO<sub>3</sub> and then with brine.

The organic phase is dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and evaporated. The crude product is purified by column chromatography on silica gel, using DCM/Methanol = 90/10 as elution solvent, to give 0.74 g (1.34 mmol) of the desired dipeptide.

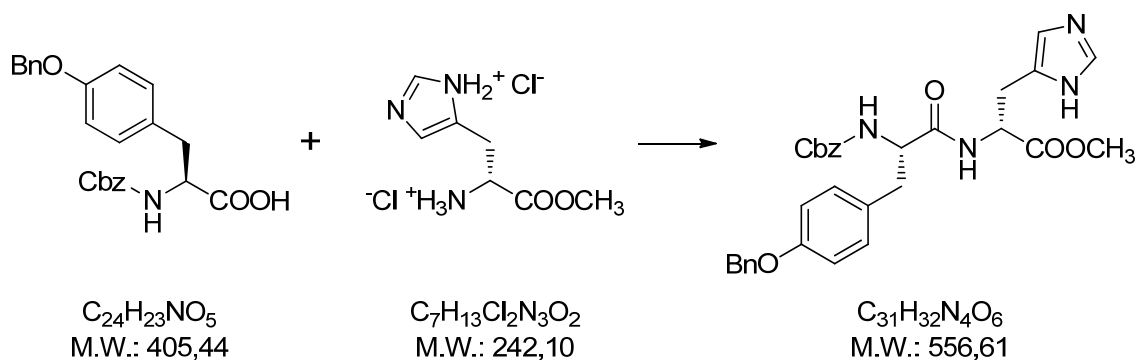
Yield: 51 %

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 7.47 (s, 1H), 7.22-7.41 (m, 10 H), 7.21 (bs, 1H), 7.05 (d, 2H, *J*=8.34 Hz), 6.85 (d, 2H, *J*=8.34 Hz), 6.69 (s, 1H), 5.57 (bs, 1H), 5.04 (d, 2H, *J*=4.68 Hz), 4.99 (s, 2H), 4.76 (dd, 1H, *J*=4.98 Hz, *J*=12.28 Hz), 4.38 (dd, 1H, *J*=7.02 Hz, *J*=7.32 Hz), 3.67 (s, 3H), 3.10 (m, 2H), 3.03 (dd, 1H, *J*=15.58 Hz, *J*=4.98 Hz), 2.90 (dd, 1H, *J*=15.58 Hz, *J*=12.28 Hz).

<sup>107</sup> TLC conditions: DCM/ Methanol= 90/10  
 R<sub>f</sub>= 0.32



**(L)-(benzyloxy)carbonyl-O-benzyl-Tyrosine-(D)-Histidine-methyl ester**



A solution of EDAC (0.65 g, 3.41 mmol) and DIPEA (0.59 mL, 3.41 mmol) in 4 mL of dry DMF is added dropwise to a solution of (L)-(benzyloxy)carbonyl-O-benzyl-Tyrosine (1.06 g, 2.62 mmol), HOBT (0.46 g, 3.41 mmol) in 16 mL of dry DMF under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of (D)-Histidine methyl ester dihydrochloride (0.63 g, 2.62 mmol) and DIPEA (0.91 mL, 5.24 mmol) in 5 mL of dry DMF is added dropwise.

The reaction mixture is stirred at RT overnight and monitored by TLC<sup>108</sup>.

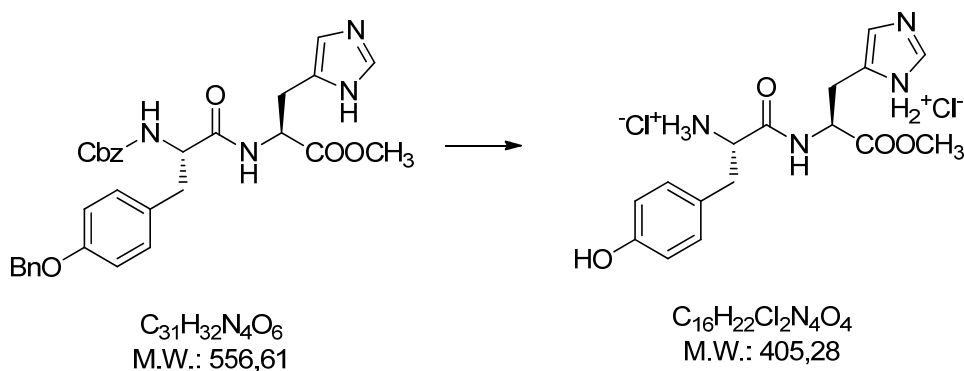
After completion, the solvent is removed under vacuum and the residue is treated with Ethyl Acetate and washed first with a solution of NaHCO<sub>3</sub> and then with brine.

The organic phase is dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and evaporated. The crude product is purified by column chromatography on silica gel, using DCM/Methanol = 90/10 as elution solvent, to give 0.71 g (1.28 mmol) of the desired dipeptide.

Yield: 49 %

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 11.80 (bs, 1H), 8.47 (d, 1H, *J*=7.33 Hz), 7.51 (s, 1H), 7.19-7.49 (m, 10 H), 7.12 (d, 2H, *J*=8.80 Hz), 7.07 (d, 2H, *J*=8.43 Hz), 6.86 (d, 1H, *J*=8.80 Hz), 6.81 (s, 1H), 5.03 (s, 2H), 4.94 (s, 2H), 4.48 (ddd, 1H, *J*=7.33 Hz, *J*=5.36 Hz, *J*=8.43 Hz), 4.19 (ddd, 1H, *J*=8.43 Hz, *J*=6.97 Hz, *J*=8.80 Hz), 3.61 (s, 3H), 2.75-2.91 (m, 4H).

<sup>108</sup> TLC conditions: DCM/ Methanol= 90/10  
R<sub>f</sub>= 0.30

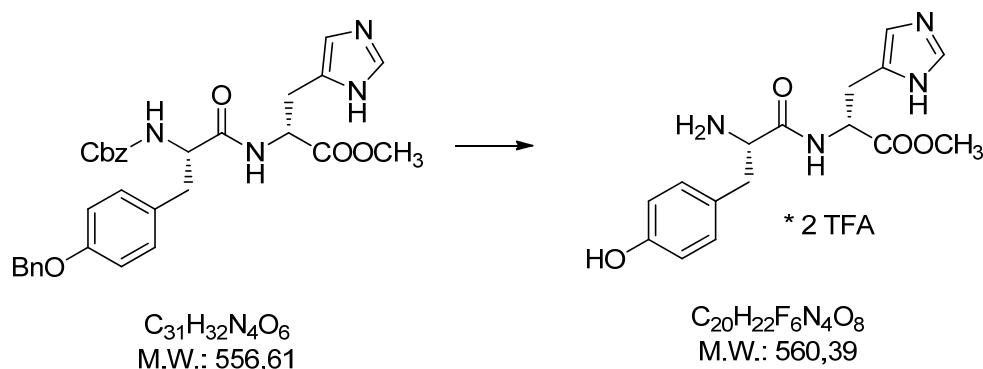
**(L)-Tyrosine-(L)-Histidine-methyl ester dihydrochloride**

0.74 g (1.34 mmol) of the protected dipeptide undergo hydrogenolysis with Pd/C 10% in a mixture of 25 mL of dry Methanol and 2.14 mL of 1.25 M HCl Methanol solution. The reaction is monitored by TLC<sup>109</sup>, at completion, achieved after stirring for 18 hours at RT, the solvents are evaporated giving 0.54 g of a sticky white solid in quantitative yield.

Yield: Quantitative

<sup>1</sup>H NMR (d<sub>6</sub>DMSO): 8.45 (s, 1H), 7.07 (s, 1H), 6.96 (d, 2H, *J* = 7.15 Hz), 6.66 (d, 2H, *J* = 7.15 Hz), 4.65 (m, 1H), 4.04 (m, 1H), 3.58 (s, 3H), 2.83-3.17 (m, 4H).

<sup>109</sup> TLC conditions: DCM/ Methanol= 90/10  
R<sub>f</sub>= basal

**(L)-Tyrosine-(D)-Histidine-methyl ester di-trifluoroacetate**

0.71 g (1.28 mmol) of the protected dipeptide undergo hydrogenolysis with Pd/C 10% in a mixture of 10 mL of dry Methanol and 2.1 mL of 1.25 M HCl Methanol solution.

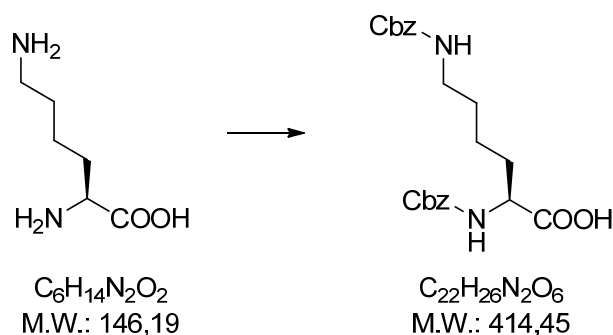
The reaction is monitored by TLC<sup>110</sup>, at completion, achieved after stirring for 18 hours at RT, the solvents are evaporated.

The residue is further purified by reverse phase preparative HPLC, according to the gradient reported in Table 3, giving 0.56 g (1.01 mmol) of a sticky white solid.

Yield: 75%

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 9.41 (d, 1H, *J* = 7.70 Hz), 9.04 (s, 1H), 8.19 (bs, 5H), 7.48 (s, 1H), 6.95 (d, 2H, *J* = 8.53 Hz), 6.68 (d, 2H, *J* = 8.53 Hz), 4.56 (m, 1H), 3.95 (m, 1H), 3.64 (s, 3H), 2.98-3.21 (m, 2H), 2.91 (m, 1H), 2.70 (m, 1H).

<sup>110</sup> TLC conditions: DCM/ Methanol= 90/10  
R<sub>f</sub>= basal

**(L)-di-(benzyloxy)carboxyl-Lysine**

To a solution of (*L*)-Lysine hydrochloride (3.0 g, 16.42 mmol) in NaOH, at 0 °C, is slowly dropped benzyl chloroformate (5.16 mL, 36.12 mmol).

The reaction is warmed to RT and monitored by TLC<sup>111</sup>.

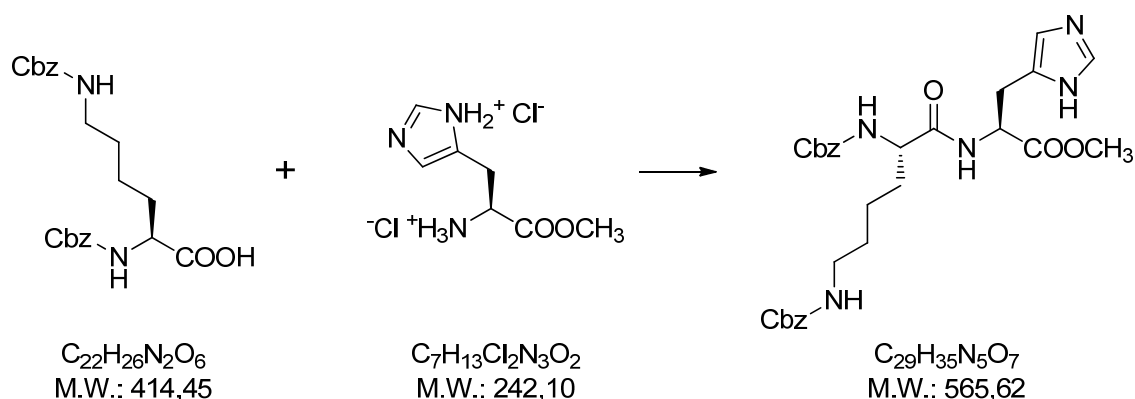
At completion, the mixture is washed with Ethyl ether, acidified with HCl 10% and extracted three times with Ethyl Acetate.

The organic phases are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated, yielding 6.80 g of a colorless oil.

Yield: Quantitative

**<sup>1</sup>H NMR (CDCl<sub>3</sub>):** 7.35 (m, 10H), 5.58 (m, 1H), 5.09 (s, 2H), 5.06 (s, 2H), 4.93 (m, 1H), 4.38 (m, 1H), 3.17 (m, 2H), 1.86 (m, 1H), 1.73 (m, 1H), 1.47 (m, 2H), 1.39 (m, 2H).

<sup>111</sup> TLC conditions: DCM/ Methanol/ Acetic Acid= 90/9/1  
R<sub>f</sub>= 0.15

**(L)-di-(benzyloxy)carboxyl-Lysine-(L)-Histidine-methyl ester**

A solution of EDAC (1.11 g, 5.79 mmol) and DIPEA (1.00 mL, 5.79 mmol) in 4 mL of dry DMF is added dropwise to a solution of (L)-di-(benzyloxy)carbonyl-Lysine (2.00 g, 4.83 mmol), HOBT (0.78 g, 5.79 mmol) in 16 mL of dry DMF under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of (L)-Histidine methyl ester dihydrochloride (1.17 g, 4.83 mmol) and DIPEA (0.84 mL, 4.83 mmol) in 5 mL of dry DMF is added dropwise.

The reaction mixture is stirred at RT overnight and monitored by TLC<sup>112</sup>.

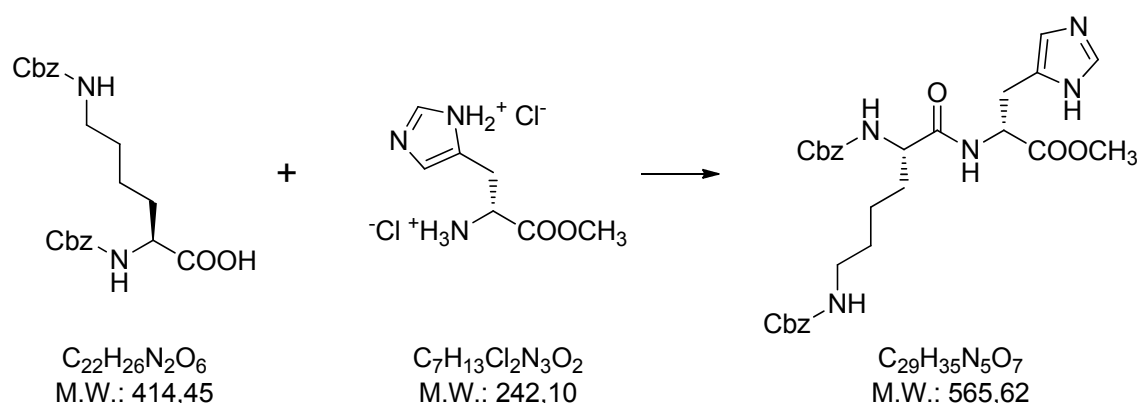
After completion, the solvent is removed under vacuum and the residue is treated with Ethyl Acetate and washed first with a solution of  $\text{NaHCO}_3$  and then with brine.

The organic phase is dried over  $\text{Na}_2\text{SO}_4$ , filtered, and evaporated. The crude product is purified by column chromatography on silica gel, using DCM/Methanol = 90/10 as elution solvent, to give 1.50 g (2.66 mmol) of the desired dipeptide.

Yield: 55 %

**<sup>1</sup>H NMR (CD<sub>3</sub>OD):** 7.52 (s, 1H), 7.25-7.32 (m, 10 H), 6.86 (s, 1H), 5.08 (s, 2H), 5.04 (s, 2H), 4.66 (m, 1H), 4.09 (m, 1H), 3.66 (s, 3H), 3.00-3.14 (m, 4H), 1.69 (m, 1H), 1.62 (m, 1H), 1.46 (m, 2H), 1.38 (m, 2H).

<sup>112</sup> TLC conditions: DCM/ Methanol= 90/10  
 Rf= 0.34

**(L)-di-(benzyloxy)carboxyl-Lysine-(D)-Histidine-methyl ester**

A solution of EDAC (0.65 g, 3.41 mmol) and DIPEA (0.59 mL, 3.41 mmol) in 4 mL of dry DMF is added dropwise to a solution of (L)-(benzyloxy)carbonyl-O-benzyl-Tyrosine (1.06 g, 2.62 mmol), HOBT (0.46 g, 3.41 mmol) in 16 mL of dry DMF under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of (D)-Histidine methyl ester dihydrochloride (1.06 g, 2.62 mmol) and DIPEA (0.91 mL, 5.24 mmol) in 5 mL of dry DMF is added dropwise.

The reaction mixture is stirred at RT overnight and monitored by TLC<sup>113</sup>.

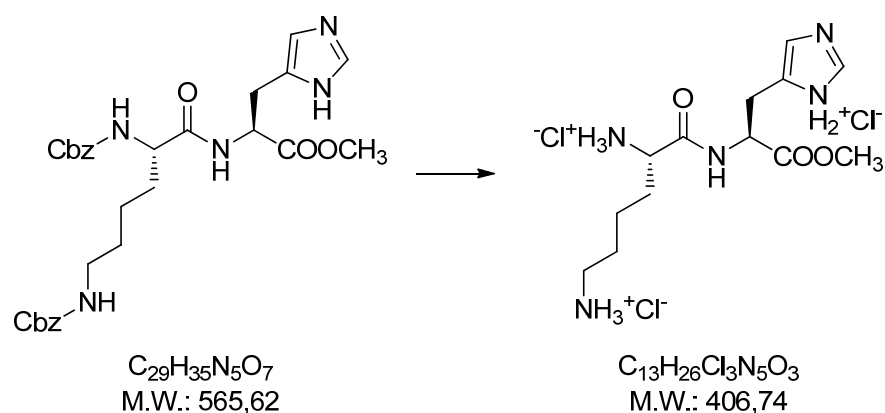
After completion, the solvent is removed under vacuum and the residue is treated with Ethyl Acetate and washed first with a solution of  $\text{NaHCO}_3$  and then with brine.

The organic phase is dried over  $\text{Na}_2\text{SO}_4$ , filtered, and evaporated. The crude product is purified by column chromatography on silica gel, using DCM/Methanol = 90/10 as elution solvent, to give 0.74 g (1.31 mmol) of the desired dipeptide.

Yield: 50 %

**<sup>1</sup>H NMR (CDCl<sub>3</sub>):** 7.42 (s, 1H), 7.24-7.32 (m, 11H), 6.73 (s, 1H), 5.80 (bs, 1H), 5.09 (s, 2H), 5.06 (s, 2H), 4.76 (m, 1H), 4.18 (m, 1H), 3.70 (s, 3H), 3.09-3.13 (m, 4H), 1.79 (m, 1H), 1.61 (m, 1H), 1.43 (m, 2H), 1.25 (m, 2H).

<sup>113</sup> TLC conditions: DCM/ Methanol= 90/10  
 Rf= 0.33

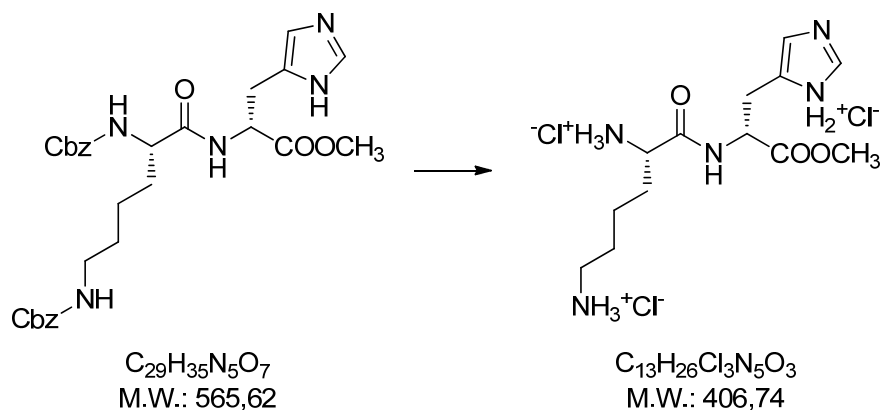
**(L)-Lysine-(L)-Histidine-methyl ester tri-hydrochloride**

1.50 g (2.66 mmol) of the protected dipeptide undergo hydrogenolysis with Pd/C 10% in a mixture of 25 mL of dry Methanol and 6.38 mL of 1.25 M HCl Methanol solution. The reaction is monitored by TLC<sup>114</sup>, at completion, achieved after stirring for 18 hours at RT, the solvents are evaporated giving 1.08 g of a sticky white solid in quantitative yield.

Yield: Quantitative

**<sup>1</sup>H NMR (D<sub>2</sub>O):** 8.53 (s, 1H), 7.24 (s, 1H), 4.78 (dd, J= 5.77 Hz, J= 8.25 Hz, 1H), 3.95 (dd, J= 6.6 Hz, J= 6.32 Hz, 1H), 3.67 (s, 3H), 3.28 (dd, J= 15.68 Hz, J= 5.77 Hz, 1H), 3.15 (dd, J= 15.68 Hz, J= 8.25 Hz, 1H), 2.90 (m, 2H), 1.83 (m, 2H), 1.61 (m, 2H), 1.37 (m, 2H).

<sup>114</sup> TLC conditions: DCM/ Methanol= 90/10  
 Rf= basal

**(L)-Lysine-(D)-Histidine-methyl ester tri-hydrochloride**

0.74 g (1.31 mmol) of the protected dipeptide undergo hydrogenolysis with Pd/C 10% in a mixture of 25 mL of dry Methanol and 3.14 mL of 1.25 M HCl Methanol solution. The reaction is monitored by TLC<sup>115</sup>, at completion, achieved after stirring for 18 hours at RT, the solvents are evaporated giving 0.53 g of a sticky white solid in quantitative yield.

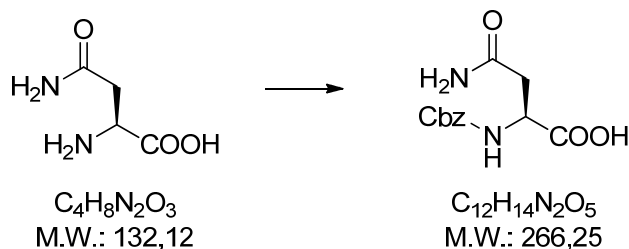
Yield: Quantitative

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 9.10 (d, 1H, *J* = 7.69 Hz), 8.31 (s, 1H), 8.12 (bs, 8H), 7.16 (s, 1H), 4.62 (m, 1H), 3.77 (m, 1H), 3.65 (s, 3H), 3.07 (m, 1H), 2.92 (m, 1H), 2.69 (m, 2H), 1.60 (m, 2H), 1.47 (m, 2H), 1.19 (m, 1H), 1.03 (m, 1H).

<sup>115</sup> TLC conditions: DCM/ Methanol= 90/10  
 R<sub>f</sub>= basal



## (L)-(benzyloxy)carbonyl-Asparagine



2.0 g (15.14 mmol) of (L)-Asparagine are dissolved in 30 mL of 10% aqueous solution of  $Na_2CO_3$  and 18 mL of Dioxane.

The solution is cooled to  $0^\circ C$ , 2.59 mL (18.17 mmol) of benzyl chloroformate are slowly dropped and then the mixture is warmed to RT and monitored by NMR.

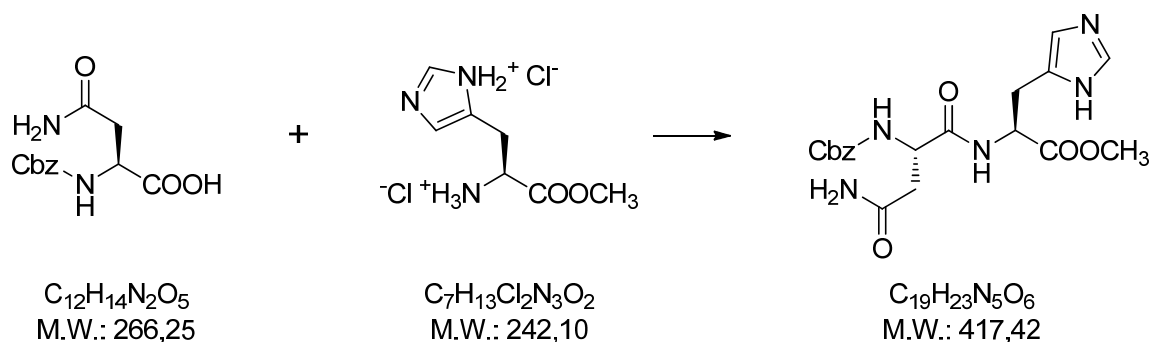
At completion, the reaction is poured in water, washed with Ethyl ether, acidified with HCl 10% till the precipitation of a white solid.

The precipitate is filtered and washed three times with Ethyl ether, obtaining 3.02 g (11.36 mmol) of the protected amino acid.

Yield: 75%

**$^1H$ -NMR ( $CDCl_3$ ):** 7.44 (d, 1H,  $J = 8.19$  Hz), 7.34 (m, 6H), 6.91 (bs, 1H), 5.01 (s, 2H), 4.32 (m, 1H), 2.43-2.52 (m, 2H).

## (L)-(benzyloxy)carbonyl-Asparagine-(L)-Histidine-methyl ester



A solution of EDAC (0.86 g, 4.51 mmol) and DIPEA (0.79 mL, 4.51 mmol) in 3 mL of dry DMF is added dropwise to a solution of (L)-(benzyloxy)carbonyl-Asparagine (1.00 g, 3.76 mmol), HOBT (0.61 g, 4.51 mmol) in 5 mL of dry DMF under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of (L)-Histidine methyl ester dihydrochloride (0.91 g, 3.76 mmol) and DIPEA (1.31 mL, 7.52 mmol) in 3 mL of dry DMF is added dropwise.

The reaction mixture is stirred at RT overnight and monitored by TLC<sup>116</sup>.

After completion, the solvent is removed under vacuum and the residue is treated with Ethyl Acetate and washed first with a solution of  $\text{NaHCO}_3$  and then with brine.

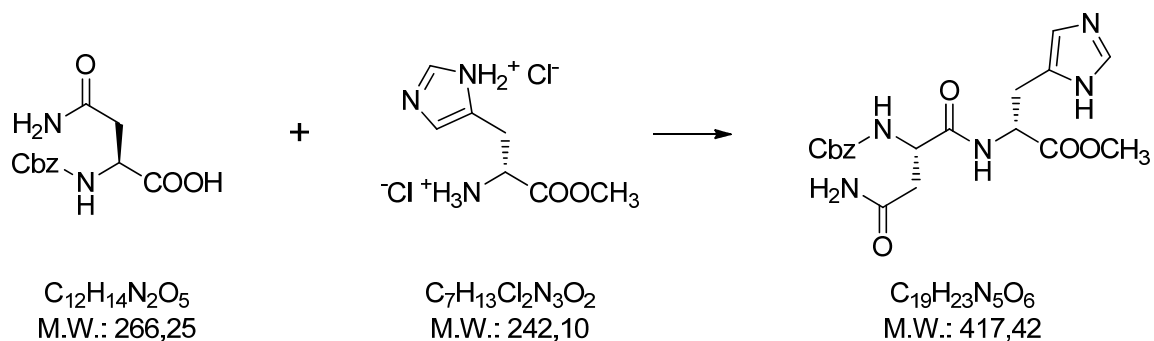
The organic phase is dried over  $\text{Na}_2\text{SO}_4$ , filtered, and evaporated. The crude product is purified by column chromatography on silica gel, using DCM/Methanol = 96/4 as elution solvent, to give 0.57 g (1.35 mmol) of the desired dipeptide.

Yield: 36 %

<sup>1</sup>H NMR ( $\text{d}_6\text{DMSO}$ ): 11.78 (bs, 1H), 8.54 (d, 1H,  $J=7.33$  Hz), 7.80 (d, 1H,  $J=8.43$  Hz), 7.49 (s, 1H), 7.21-7.42 (m, 7H), 6.81 (s, 1H), 5.05 (s, 4H), 4.44 (m, 2H), 3.57 (s, 3H), 2.90 (m, 2H), 2.66 (m, 2H).

<sup>116</sup> TLC conditions: DCM/ Methanol= 96/4  
 Rf= 0.24

## (L)-(benzyloxy)carbonyl-Asparagine-(D)-Histidine-methyl ester



A solution of EDAC (0.86 g, 4.51 mmol) and DIPEA (0.79 mL, 4.51 mmol) in 3 mL of dry DMF is added dropwise to a solution of (L)-(benzyloxy)carbonyl-Asparagine (1.00 g, 3.76 mmol), HOBT (0.61 g, 4.51 mmol) in 5 mL of dry DMF under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of (D)-Histidine methyl ester dihydrochloride (0.91 g, 3.76 mmol) and DIPEA (1.31 mL, 7.52 mmol) in 3 mL of dry DMF is added dropwise.

The reaction mixture is stirred at RT overnight and monitored by TLC<sup>117</sup>.

After completion, the solvent is removed under vacuum and the residue is treated with Ethyl Acetate and washed first with a solution of  $\text{NaHCO}_3$  and then with brine.

The organic phase is dried over  $\text{Na}_2\text{SO}_4$ , filtered, and evaporated. The crude product is purified by column chromatography on silica gel, using DCM/Methanol = 96/4 as elution solvent.

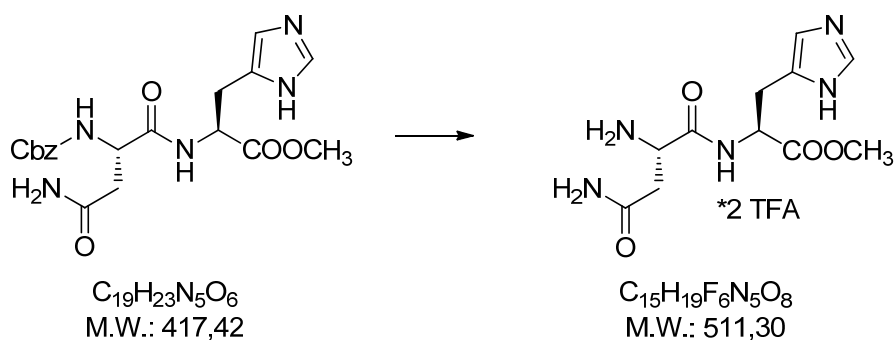
The compound undergoes further purification by reverse phase preparative HPLC, according to the gradient reported in Table 2, giving 0.60 g (1.43 mmol) of the desired dipeptide.

Yield: 38 %

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 8.96 (s, 1H), 8.40 (d, 1H,  $J=7.98$  Hz), 7.44 (d, 1H,  $J=7.98$  Hz), 7.24-7.40 (m, 7H), 6.90 (s, 1H), 5.01 (s, 2H), 4.99 (s, 2H), 4.56 (m, 1H), 4.28 (m, 1H), 3.62 (s, 3H), 3.12 (dd, 1H,  $J=4.68$  Hz,  $J=16.78$  Hz), 3.07 (dd, 1H,  $J=9.07$  Hz,  $J=16.78$  Hz), 2.33-2.47 (m 2H).

<sup>117</sup> TLC conditions: DCM/ Methanol= 96/4  
 R<sub>f</sub>= 0.24

## (L)-Asparagine-(L)-Histidine-methyl ester di-trifluoroacetate



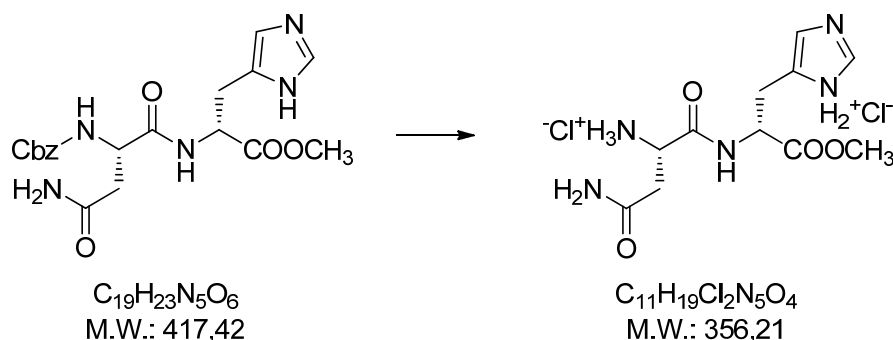
0.57 g (1.35 mmol) of the protected dipeptide undergo hydrogenolysis with Pd/C 10% in a mixture of 10 mL of dry Methanol and 2.16 mL of 1.25 M HCl Methanol solution. The reaction is monitored by TLC<sup>118</sup>, at completion, achieved after stirring for 18 hours at RT, the solvents are evaporated.

The residue is further purified by reverse phase preparative HPLC, according to the gradient reported in Table 3, giving 0.69 g of a sticky white solid.

Yield: Quantitative

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 9.23 (d, 1H, *J*= 7.43 Hz), 8.83 (s, 1H), 7.80 (s, 1H), 7.40 (s, 1H), 7.21 (s, 1H), 4.63 (m, 1H), 4.11 (m, 1H), 3.63 (s, 3H), 3.06-3.19 (m, 2H), 2.74 (dd, 1H, *J*= 4.13 Hz, *J*= 16.76 Hz), 2.62 (dd, 1H, *J*= 7.98 Hz, *J*= 16.76 Hz).

<sup>118</sup> TLC conditions: DCM/ Methanol= 96/4  
R<sub>f</sub>= basal

**(L)-Asparagine-(D)-Histidine-methyl ester dihydrochloride**

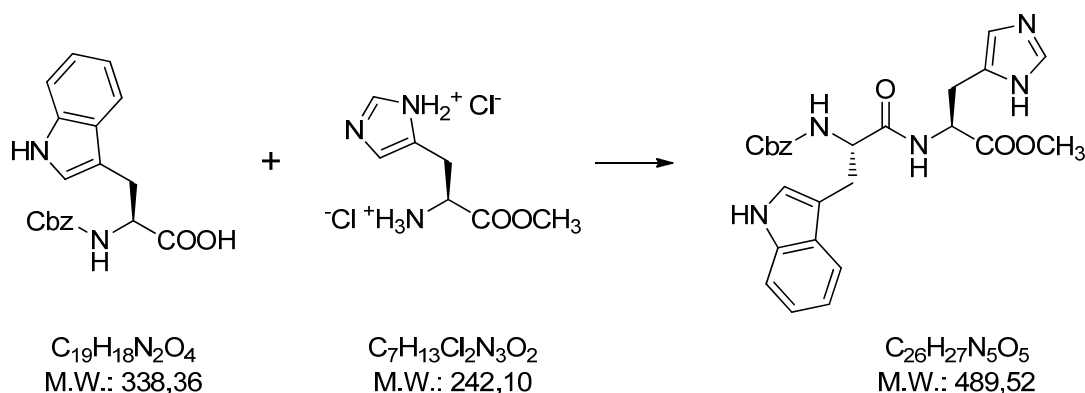
0.60 g (1.43 mmol) of the protected dipeptide undergo hydrogenolysis with Pd/C 10% in a mixture of 10 mL of dry Methanol and 2.3 mL of 1.25 M HCl Methanol solution. The reaction is monitored by TLC<sup>119</sup>, at completion, achieved after stirring for 18 hours at RT, the solvents are evaporated giving 0.73 g of a sticky white solid in quantitative yield.

Yield: Quantitative

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 9.13 (d, 1H, *J* = 8.20 Hz), 9.03 (s, 1H), 8.20 (bs, 5H), 7.76 (s, 1H), 7.46 (s, 1H), 7.21 (s, 1H), 4.60 (m, 1H), 4.04 (m, 1H), 3.60 (s, 3H), 3.22 (m, 1H), 3.16 (m, 1H), 2.68 (dd, 1H, *J* = 4.76 Hz, *J* = 16.87 Hz), 2.56 (dd, 1H, *J* = 7.68 Hz, *J* = 16.87 Hz).

<sup>119</sup> TLC conditions: DCM/ Methanol= 96/4  
 R<sub>f</sub>= basal

## (L)-(benzyloxy)carbonyl-Tryptophan-(L)-Histidine-methyl ester



A solution of EDAC (0.68 g, 3.55 mmol) and DIPEA (0.62 mL, 3.55 mmol) in 3 mL of dry DMF is added dropwise to a solution of (L)-(benzyloxy)carbonyl-Tryptophan (1.00 g, 2.95 mmol), HOBT (0.48 g, 3.55 mmol) in 5 mL of dry DMF under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of (L)-Histidine methyl ester dihydrochloride (0.71 g, 2.95 mmol) and DIPEA (1.03 mL, 5.90 mmol) in 3 mL of dry DMF is added dropwise.

The reaction mixture is stirred at RT overnight and monitored by TLC<sup>120</sup>.

After completion, the solvent is removed under vacuum and the residue is treated with Ethyl Acetate and washed first with a solution of NaHCO<sub>3</sub> and then with brine.

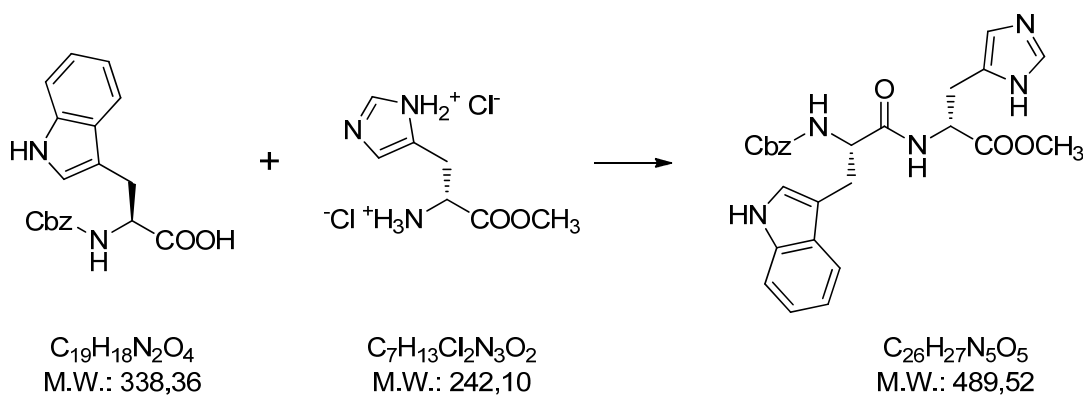
The organic phase is dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and evaporated. The crude product is purified by column chromatography on silica gel, using Ethyl Acetate/Methanol = 95/5 as elution solvent, to give 1.33 g (2.71 mmol) of the desired dipeptide.

Yield: 92 %

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 11.80 (bs, 1H), 10.81 (s, 1H), 8.43 (d, 1H, *J*=7.52 Hz), 7.62 (d, 1H, *J*=7.7 Hz), 7.51 (s, 1H), 7.22-7.35 (m, 7H), 7.08-6.95 (m, 3H), 6.81 (bs, 1H), 4.92 (s, 2H), 4.52 (m, 1H), 4.28 (m, 1H), 3.56 (s, 3H), 2.81-3.06 (m, 4H).

<sup>120</sup> TLC conditions: Ethyl Acetate/ Methanol= 90/10  
 R<sub>f</sub>= 0.28

## (L)-(benzyloxy)carbonyl-Tryptophan-(D)-Histidine-methyl ester



A solution of EDAC (0.68 g, 3.55 mmol) and DIPEA (0.62 mL, 3.55 mmol) in 3 mL of dry DMF is added dropwise to a solution of (L)-(benzyloxy)carbonyl-Tryptophan (1.00 g, 2.95 mmol), HOBT (0.48 g, 3.55 mmol) in 5 mL of dry DMF under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of (D)-Histidine methyl ester dihydrochloride (0.71 g, 2.95 mmol) and DIPEA (1.03 mL, 5.90 mmol) in 3 mL of dry DMF is added dropwise.

The reaction mixture is stirred at RT overnight and monitored by TLC<sup>121</sup>.

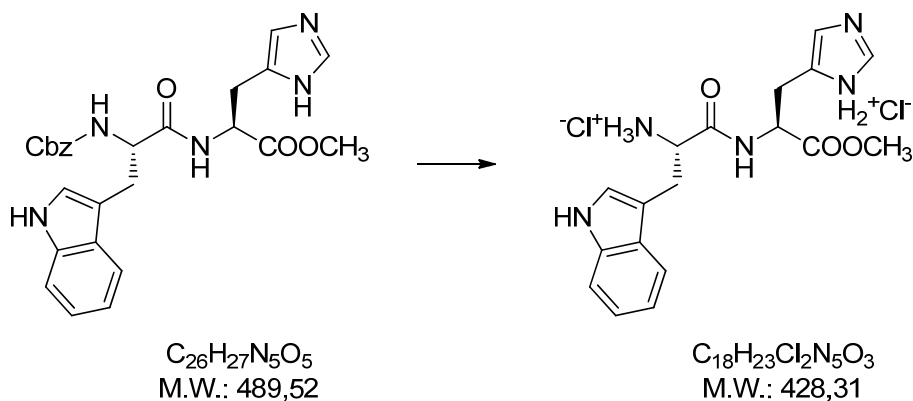
After completion, the solvent is removed under vacuum and the residue is treated with Ethyl Acetate and washed first with a solution of NaHCO<sub>3</sub> and then with brine.

The organic phase is dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and evaporated. The crude product is purified by column chromatography on silica gel, using Ethyl Acetate/Methanol = 95/5 as elution solvent, to give 1.27 g (2.60 mmol) of the desired dipeptide.

Yield: 88 %

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 11.80 (bs, 1H), 10.76 (s, 1H), 8.49 (d, 1H, *J*=7.7 Hz), 7.60 (d, 1H, *J*=7.7 Hz), 7.49 (s, 1H), 7.22-7.35 (m, 7H), 7.03 (m, 2H), 6.95 (m, 1H), 6.82 (bs, 1H), 4.92 (s, 2H), 4.50 (m, 1H), 4.27 (m, 1H), 3.61 (s, 3H), 2.71-2.96 (m, 4H).

<sup>121</sup> TLC conditions: Ethyl Acetate/ Methanol= 90/10  
 R<sub>f</sub>= 0.30

**(L)-Tryptophan-(L)-Histidine-methyl ester dihydrochloride**

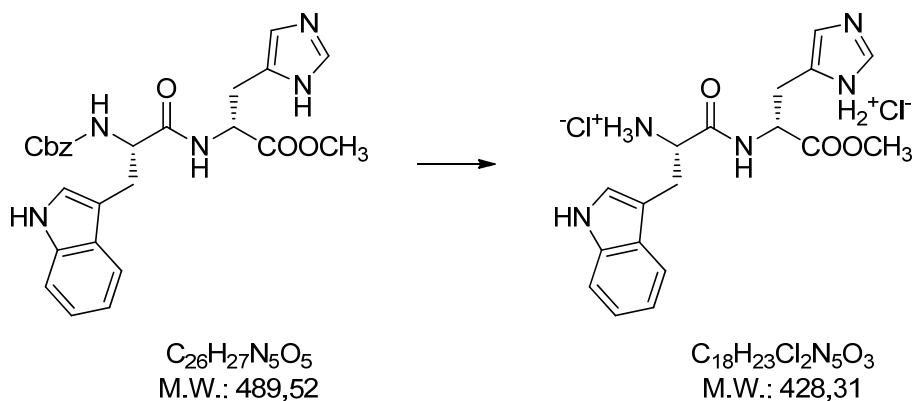
1.33 g (2.71 mmol) of the protected dipeptide undergo hydrogenolysis with Pd/C 10% in a mixture of 20 mL of dry Methanol and 4.34 mL of 1.25 M HCl Methanol solution. The reaction is monitored by TLC<sup>122</sup>, at completion, achieved after stirring for 18 hours at RT, the solvents are evaporated giving 1.16 g of a sticky white solid in quantitative yield.

Yield: Quantitative

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 11.06 (s, 1H), 9.35 (d, 1H, *J* = 7.62 Hz), 8.92 (s, 1H), 7.73 (d, 1H, *J* = 7.63 Hz), 7.44 (s, 1H), 7.34 (d, 1H, *J* = 7.92 Hz), 7.21 (s, 1H), 7.06 (dd, 1H, *J* = 7.92 Hz, *J* = 7.04 Hz), 6.96 (dd, 1H, *J* = 7.63 Hz, *J* = 7.04 Hz), 4.68 (m, 1H), 4.09 (m, 1H), 3.62 (s, 3H), 2.87-3.28 (m, 4H).

<sup>122</sup> TLC conditions: Ethyl Acetate/ Methanol= 90/10  
R<sub>f</sub>= basal



**(L)-Tryptophan-(D)-Histidine-methyl ester dihydrochloride**

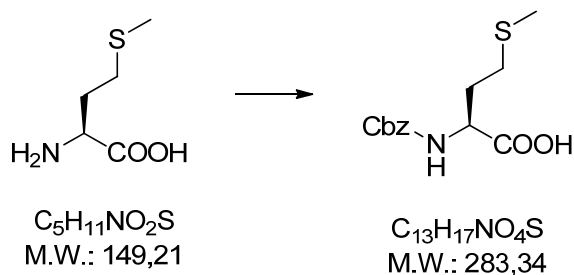
1.27 g (2.60 mmol) of the protected dipeptide undergo hydrogenolysis with Pd/C 10% in a mixture of 20 mL of dry Methanol and 4.16 mL of 1.25 M HCl Methanol solution. The reaction is monitored by TLC<sup>123</sup>, at completion, achieved after stirring for 18 hours at RT, the solvents are evaporated giving 1.11 g of a sticky white solid in quantitative yield.

Yield: Quantitative

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 11.02 (s, 1H), 9.26 (d, 1H, *J*= 7.70 Hz), 8.25 (s, 1H), 7.61 (d, 1H, *J*= 7.71 Hz), 7.34 (d, 1H, *J*= 8.25 Hz), 7.12 (s, 2H), 7.07 (dd, 1H, *J*= 8.25 Hz, *J*= 7.15 Hz), 6.97 (dd, 1H, *J*= 7.71 Hz, *J*= 7.15 Hz), 4.56 (m, 1H), 4.02 (m, 1H), 3.63 (s, 3H), 2.87-3.17 (m, 4H).

<sup>123</sup> TLC conditions: Ethyl Acetate/ Methanol= 90/10  
 R<sub>f</sub>= basal

## (L)-(benzyloxy)carbonyl-Methionine-methyl ester



2.0 g (13.40 mmol) of (*L*)-Methionine are dissolved in 20 mL of 10% aqueous solution of  $\text{NaHCO}_3$  and cooled to 0 °C.

The solution is amounted, dropwise, of 2.10 mL (14.74 mmol) of benzyl chloroformate and then the mixture is warmed to RT and monitored by TLC<sup>124</sup>.

At completion, the reaction is poured in washed with Ethyl ether, acidified with HCl 10% and extracted three times with Ethyl Acetate.

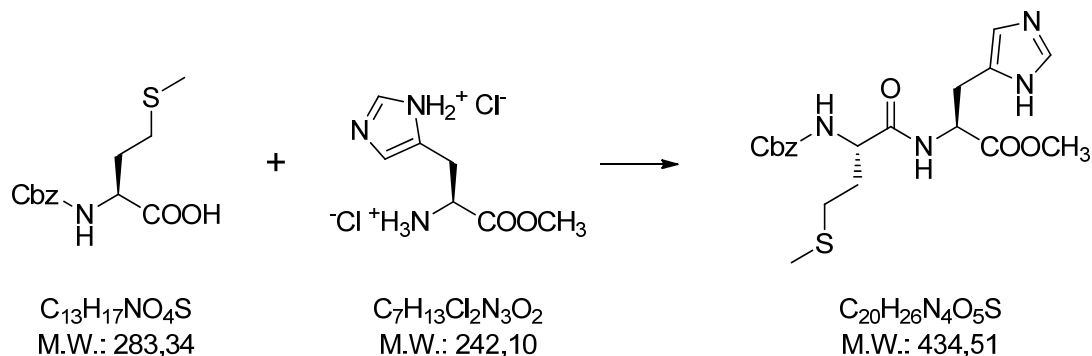
The organic phases are collected, dried over  $\text{Na}_2\text{SO}_4$ , filtered and concentrated under reduce pressure, yielding 2.39 g (8.44 mmol) of the desired product, as colourless oil.

Yield: 63%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 7.75 (bs, 1H), 7.37 (m, 5H), 5.44 (d, 1H, *J* = 7.43 Hz), 5.14 (s, 2H), 4.58 (m, 1H), 2.58 (t, 2H, *J* = 6.60 Hz), 2.19 (m, 1H), 2.04 (s, 3H), 1.98 (m, 1H).

<sup>124</sup> TLC conditions: DCM/ Methanol= 95/ 5  
Rf: 0.32

## (L)-(benzyloxy)carbonyl-Methionine-(L)-Histidine-methyl ester



A solution of EDAC (0.97 g, 5.08 mmol) and DIPEA (0.88 mL, 5.08 mmol) in 5 mL of dry DMF is added dropwise to a solution of (L)-(benzyloxy)carbonyl-Methionine (1.2 g, 4.24 mmol), HOBT (0.69 g, 5.08 mmol) in 7 mL of dry DMF under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of (L)-Histidine methyl ester dihydrochloride (1.03 g, 4.24 mmol) and DIPEA (1.48 mL, 8.48 mmol) in 3 mL of dry DMF is added dropwise.

The reaction mixture is stirred at RT overnight and monitored by TLC<sup>125</sup>.

After completion, the solvent is removed under vacuum and the residue is treated with Ethyl Acetate and washed first with a solution of NaHCO<sub>3</sub> and then with brine.

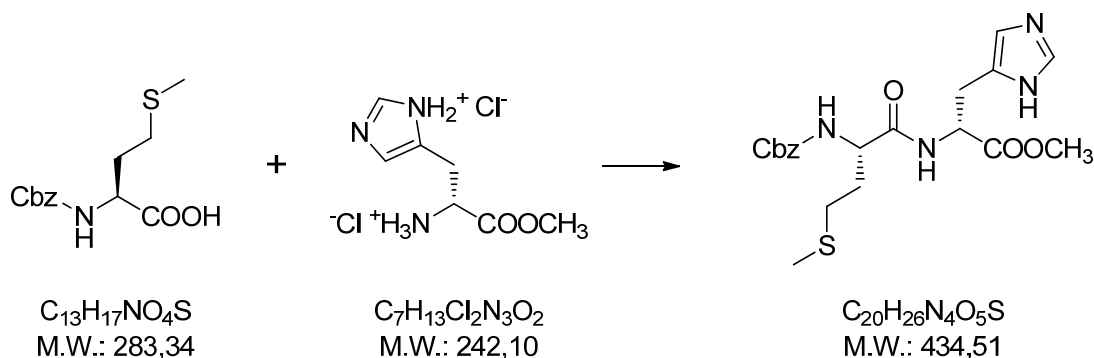
The organic phase is dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and evaporated. The crude product is purified by column chromatography on silica gel, using DCM/Methanol = 90/10 as elution solvent, to give 0.92 g (2.12 mmol) of the desired dipeptide.

Yield: 50 %

<sup>1</sup>H NMR (d<sub>6</sub>DMSO): 11.80 (bs, 1H), 8.34 (d, 1H, *J*=7.43 Hz), 7.49 (s, 1H), 7.26-7.39 (m, 6H), 6.81 (s, 1H), 5.01 (s, 2H), 4.44 (m, 1H), 4.08 (m, 1H), 3.56 (s, 3H), 2.88 (m, 2H), 2.48 (m, 2H), 1.72-1.94 (m, 2H).

<sup>125</sup> TLC conditions: DCM/ Methanol= 90/10  
 R<sub>f</sub>= 0.33

## (L)-(benzyloxy)carbonyl-Methionine-(D)-Histidine-methyl ester



A solution of EDAC (0.97 g, 5.08 mmol) and DIPEA (0.88 mL, 5.08 mmol) in 5 mL of dry DMF is added dropwise to a solution of (L)-(benzyloxy)carbonyl-Methionine (1.2 g, 4.24 mmol), HOBT (0.69 g, 5.08 mmol) in 7 mL of dry DMF under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of (D)-Histidine methyl ester dihydrochloride (1.03 g, 4.24 mmol) and DIPEA (1.48 mL, 8.48 mmol) in 3 mL of dry DMF is added dropwise.

The reaction mixture is stirred at RT overnight and monitored by TLC<sup>126</sup>.

After completion, the solvent is removed under vacuum and the residue is treated with Ethyl Acetate and washed first with a solution of  $\text{NaHCO}_3$  and then with brine.

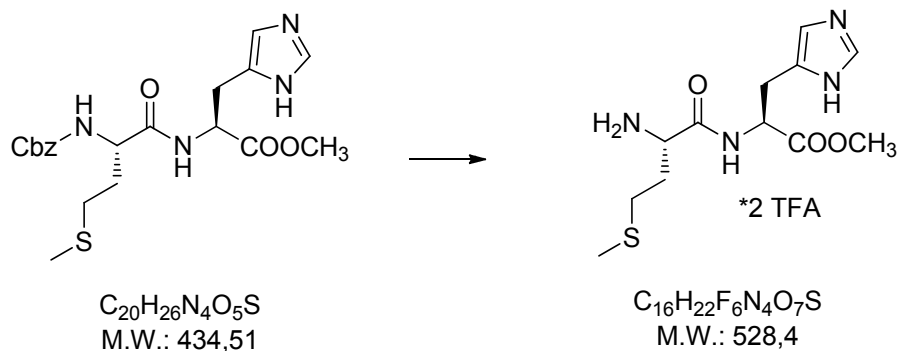
The organic phase is dried over  $\text{Na}_2\text{SO}_4$ , filtered, and evaporated. The crude product is purified by column chromatography on silica gel, using DCM/Methanol = 90/10 as elution solvent, to give 1.16 g (2.67 mmol) of the desired dipeptide.

Yield: 63 %

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 11.82 (bs, 1H), 8.32 (d, 1H,  $J=7.68$  Hz), 7.48 (s, 1H), 7.26-7.40 (m, 6H), 6.78 (s, 1H), 5.01 (s, 2H), 4.48 (m, 1H), 4.09 (m, 1H), 3.59 (s, 3H), 2.88 (m, 2H), 2.34 (m, 2H), 1.72 (m, 2H).

<sup>126</sup> TLC conditions: DCM/ Methanol= 90/10  
 Rf= 0.32

**(L)-Methionine-(L)-Histidine-methyl ester di-trifluoroacetate**



0.92 g (2.12 mmol) of the protected dipeptide undergo hydrogenolysis with Pd/C 10% in a mixture of 10 mL of dry Methanol and 3.39 mL of 1.25 M HCl Methanol solution. The reaction is monitored by TLC<sup>127</sup>, at completion, achieved after stirring for 18 hours at RT, the solvents are evaporated.

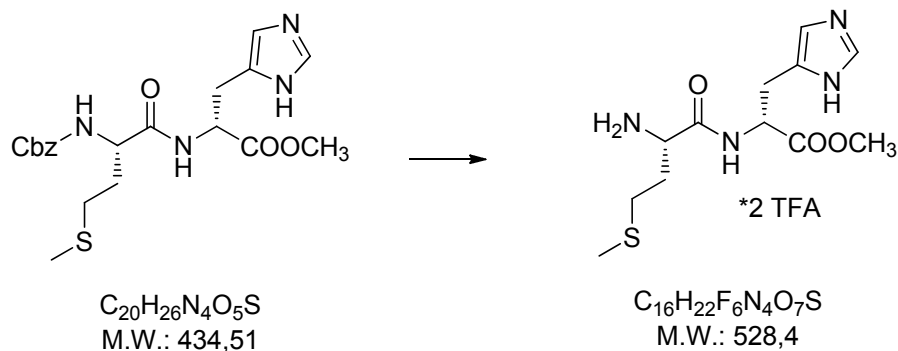
The residue is further purified by reverse phase preparative HPLC, according to the gradient reported in Table 3. **L'origine riferimento non è stata trovata.**, giving 1.12 g of a sticky white solid.

Yield: Quantitative

<sup>1</sup>H NMR (d<sub>6</sub>DMSO): 8.52 (s, 1H), 7.22 (s, 1H), 4.75 (m, 1H), 4.03 (dd, 1H, *J* = 6.6 Hz, *J* = 6.32 Hz), 3.67 (s, 3H), 3.28 (dd, 1H, *J* = 15.5 Hz, *J* = 5.4 Hz), 3.15 (dd, 1H, *J* = 15.5 Hz, *J* = 8.25 Hz), 2.46-2.52 (m, 2H), 2.02-2.10 (m, 2H), 2.00 (s, 3H).

<sup>127</sup> TLC conditions: DCM/ Methanol= 96/4  
 R<sub>f</sub>= basal

## (L)-Methionine-(D)-Histidine-methyl ester di-trifluoroacetate



1.16 g (2.67 mmol) of the protected dipeptide undergo hydrogenolysis with Pd/C 10% in a mixture of 15 mL of dry Methanol and 4.27 mL of 1.25 M HCl Methanol solution. The reaction is monitored by TLC<sup>128</sup>, at completion, achieved after stirring for 18 hours at RT, the solvents are evaporated.

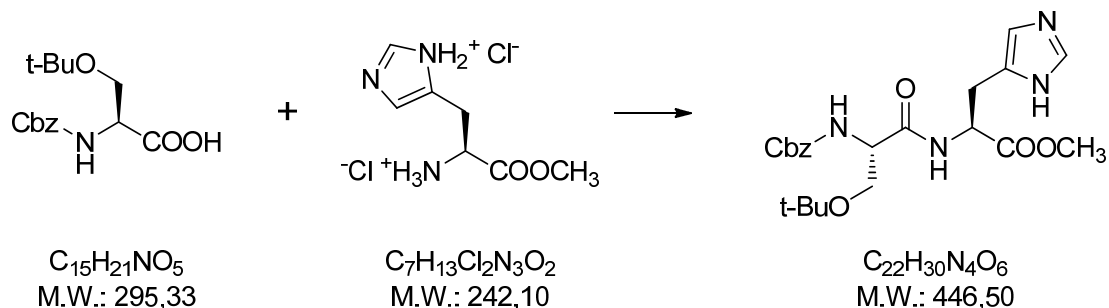
The residue is further purified by reverse phase preparative HPLC, according to the gradient reported in Table 3, giving 1.41 g of a sticky white solid.

Yield: Quantitative

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 8.53 (s, 1H), 7.22 (s, 1H), 4.75 (dd, 1H, J= 4.68 Hz, J= 10.17 Hz), 3.97 (dd, 1H, J= 6.6 Hz, J= 6.32 Hz), 3.65 (s, 3H), 3.30 (dd, 1H, J= 15.68 Hz, J= 4.68 Hz), 3.08 (dd, 1H, J= 15.68 Hz, J= 10.17 Hz), 2.10-2.28 (m, 2H), 1.86-1.93 (m, 2H), 1.91 (s, 3H).

<sup>128</sup> TLC conditions: DCM/ Methanol= 96/4  
Rf= basal

## (L)-(benzyloxy)carbonyl O-tert-Butyl Serine-(L)-Histidine-methyl ester



A solution of EDAC (0.78 g, 4.06 mmol) and DIPEA (0.71 mL, 4.06 mmol) in 5 mL of dry DMF is added dropwise to a solution of (L)-(benzyloxy)carbonyl-O-tert-butyl-Serine (1.0 g, 3.39 mmol), HOBT (0.55 g, 4.06 mmol) in 8 mL of dry DMF under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of (L)-Histidine methyl ester dihydrochloride (0.82 g, 3.39 mmol) and DIPEA (1.18 mL, 6.78 mmol) in 3 mL of dry DMF is added dropwise.

The reaction mixture is stirred at RT overnight and monitored by TLC<sup>129</sup>.

After completion, the solvent is removed under vacuum and the residue is treated with Ethyl Acetate and washed first with a solution of NaHCO<sub>3</sub> and then with brine.

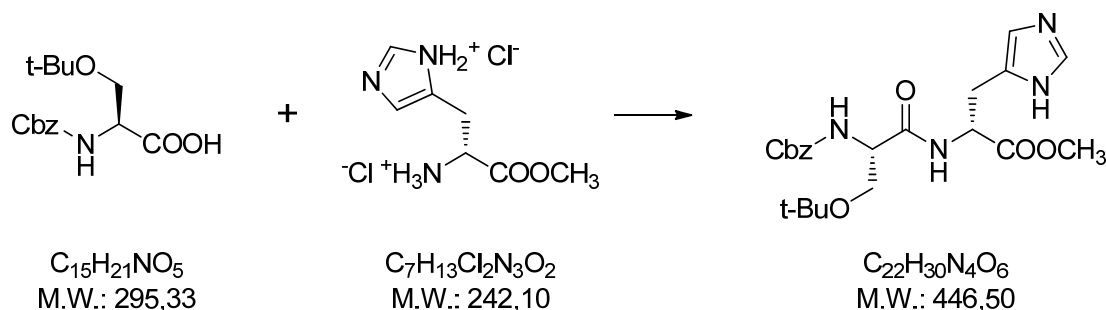
The organic phase is dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and evaporated. The crude product is purified by column chromatography on silica gel, using Ethyl Acetate/Methanol = 95/5 as elution solvent, to give 1.10 g (2.47 mmol) of the desired dipeptide.

Yield: 73 %

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 11.80 (bs, 1H), 8.26 (d, 1H, *J*=7.43 Hz), 7.48 (s, 1H), 7.22-7.33 (m, 6H), 6.80 (s, 1H), 5.02 (s, 2H), 4.49 (m, 1H), 4.10 (m, 1H), 3.55 (s, 3H), 3.35-3.48 (m, 2H), 2.88 (m, 2H), 1.07 (s, 9H).

<sup>129</sup> TLC conditions: Ethyl Acetate/ Methanol= 95/5  
 R<sub>f</sub>= 0.30

## (L)-(benzyloxy)carbonyl-O-*tert*-Butyl Serine-(D)-Histidine-methyl ester



A solution of EDAC (0.78 g, 4.06 mmol) and DIPEA (0.71 mL, 4.06 mmol) in 5 mL of dry DMF is added dropwise to a solution of (L)-(benzyloxy)carbonyl-O-*tert*-butyl-Serine (1.0 g, 3.39 mmol), HOBT (0.55 g, 4.06 mmol) in 8 mL of dry DMF under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of (D)-Histidine methyl ester dihydrochloride (0.82 g, 3.39 mmol) and DIPEA (1.18 mL, 6.78 mmol) in 3 mL of dry DMF is added dropwise.

The reaction mixture is stirred at RT overnight and monitored by TLC<sup>130</sup>.

After completion, the solvent is removed under vacuum and the residue is treated with Ethyl Acetate and washed first with a solution of NaHCO<sub>3</sub> and then with brine.

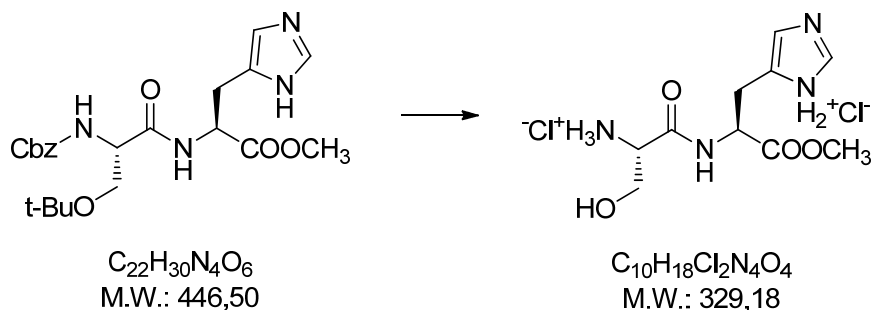
The organic phase is dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and evaporated. The crude product is purified by column chromatography on silica gel, using Ethyl Acetate/Methanol = 95/5 as elution solvent, to give 1.21 g (2.71 mmol) of the desired dipeptide.

Yield: 80 %

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 11.78 (bs, 1H), 8.30 (d, 1H, *J*=7.42 Hz), 7.49 (s, 1H), 7.17-7.37 (m, 6H), 6.78 (s, 1H), 5.02 (s, 2H), 4.48 (m, 1H), 4.10 (m, 1H), 3.58 (s, 3H), 3.32-3.45 (m, 2H), 2.71-2.87 (m, 2H), 1.04 (s, 9H).

<sup>130</sup> TLC conditions: Ethyl Acetate/ Methanol= 95/5  
 R<sub>f</sub>= 0.30



**(L)-Serine-(L)-Histidine-methyl ester dihydrochloride**

1.10 g (2.47 mmol) of the full protected dipeptide are stirred 10 mL of DCM/TFA = 7/3 for 3 hours in presence of anisole (0.54 mL, 4.94 mmol).

The solvent is removed and the residue rinsed and washed three times with Ethyl ether; simply water removal under vacuum giving the (L)-(benzyloxy)carbonyl-Serine-(L)-Histidine-methyl ester, in quantitative yield.

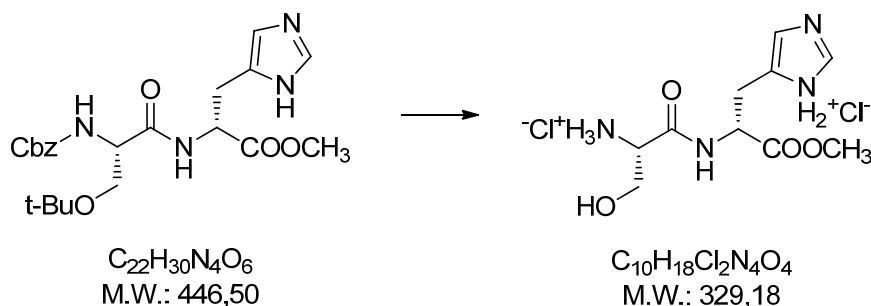
The dipeptide undergoes hydrogenolysis with Pd/C 10% in a mixture of 15 mL of dry Methanol and 3.95 mL of 1.25 M HCl Methanol solution.

The reaction is monitored by TLC<sup>131</sup>, at completion, achieved after stirring for 18 hours at RT, the solvents are evaporated, giving 0.81 g of a sticky white solid.

Yield: Quantitative

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 9.10 (d, 1H, *J* = 7.69 Hz), 8.99 (s, 1H), 8.22 (bs, 5H), 7.45 (s, 1H), 5.50 (bs, 1H), 4.67 (m, 1H), 3.88 (m, 1H), 3.76 (dd, 1H, *J* = 3.66 Hz, *J* = 11.37 Hz), 3.5-3.7 (m, 1H), 3.64 (s, 3H), 3.04-3.22 (m, 2H).

<sup>131</sup> TLC conditions: DCM/ Methanol= 96/4  
 R<sub>f</sub>= basal

**(L)-Serine-(D)-Histidine-methyl ester dihydrochloride**

1.21 g (2.71 mmol) of the full protected dipeptide are stirred 10 mL of DCM/TFA =7/3 for 3 hours in presence of anisole (0.59 mL, 5.42 mmol).

The solvent is removed and the residue rinsed and washed three times with Ethyl ether; simply water removal under vacuum gives (L)-(benzyloxy)carbonyl-Serine-(D)-Histidine-methyl ester, in quantitative yield.

The dipeptide undergoes hydrogenolysis with Pd/C 10% in a mixture of 15 mL of dry Methanol and 4.34 mL of 1.25 M HCl Methanol solution.

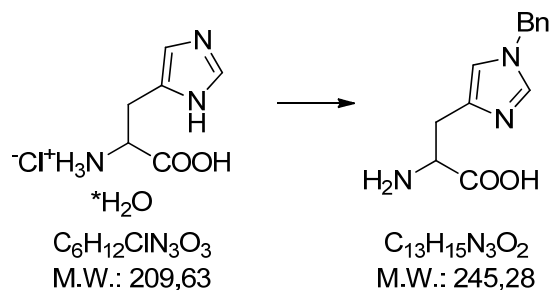
The reaction is monitored by TLC<sup>132</sup>, at completion, achieved after stirring for 18 hours at RT, the solvents are evaporated, giving 0.89 g of a sticky white solid.

Yield: Quantitative

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 9.13 (d, 1H, *J* = 7.69 Hz), 8.98 (s, 1H), 8.21 (bs, 5H), 7.44 (s, 1H), 5.54 (bs, 1H), 4.67 (m, 1H), 3.85 (m, 1H), 3.77 (dd, 1H, *J* = 4.33 Hz, *J* = 11.37 Hz), 3.57-3.70 (m, 1H), 3.65 (s, 3H), 3.03-3.22 (m, 2H).

<sup>132</sup> TLC conditions: DCM/ Methanol= 96/4  
Rf= basal

## 2-amino-3-(1-benzyl-1H-imidazol-4-yl)propanoic acid



75 mL of dry liquid ammonia are added, at  $-76^\circ\text{C}$ , to 10.0 g (47.40 mmol) of (L)-Histidine monohydrochloride monohydrate; the mixture is amount of small pieces of Na (4.39 g, 190.80 mmol) and then benzyl chloride (5.58 mL, 52.47 mmol) is slowly dropped.

The reaction is stirred for 2 hours and monitored by NMR.

At completion, ammonia is removed, 70 mL of iced Water are added and the solution is washed with Ethyl ether.

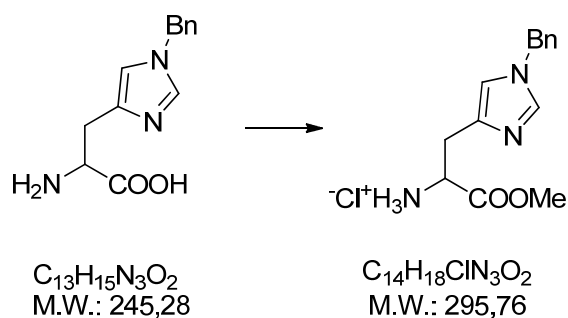
The aqueous layer is acidified with  $\text{H}_2\text{SO}_4$  until precipitation begins; the filtration of the white solid and the further crystallization with Ethanol/ Water= 7/3 let the obtainment of 6.16 g (25.12 mmol) of the desired product.

Yield: 53%

Melting point:  $248^\circ\text{C}$

**$^1\text{H}$  NMR ( $\text{D}_2\text{O}$ ):** 7.62 (s, 1H), 7.26-7.33 (m, 5H), 6.91 (s, 1H), 5.06 (s, 2H), 3.82 (dd, 1H,  $J=4.68$  Hz,  $J=7.98$  Hz), 3.03 (dd, 1H,  $J=4.68$  Hz,  $J=15.40$  Hz), 2.91 (dd, 1H,  $J=7.98$  Hz,  $J=15.40$  Hz).

## Methyl 2-amino-3-(1-benzyl-1H-imidazol-4-yl)propanoate hydrochloride



25 mL of 1.25 M HCl Methanol solution are dropped to a solution of 6.16 g (25.12 mmol) in 60 mL of Methanol.

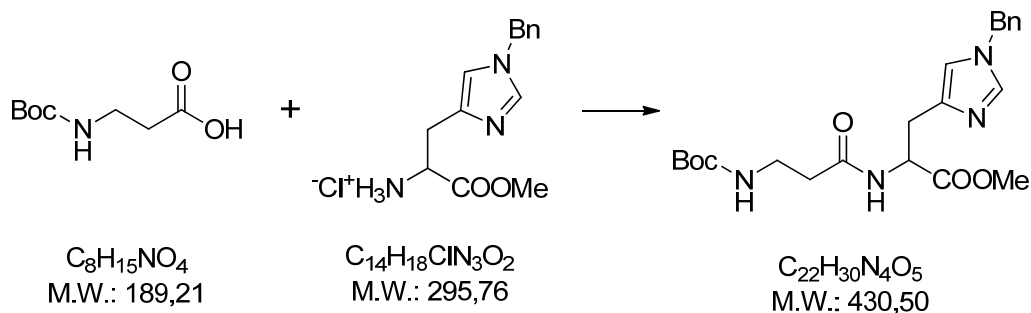
The solution is refluxed for 18 hours, monitoring the progression by NMR.

At completion, the simply solvents removal yields 7.54 g (25.12 mmol) of a dense oil.

Yield: Quantitative

**$^1H$  NMR ( $d_6$ DMSO):** 9.31 (s, 1H), 8.88 (bs, 3H), 7.61 (s, 1H), 7.35-7.41 (m, 5H), 5.40 (s, 2H), 4.44 (m, 1H), 3.64 (s, 3H), 3.27 (m, 2H).

## Methyl 3-(1-benzyl-1H-imidazol-4-yl)-2-(3-(((benzyloxy)carbonyl)amino)propanamido)propanoate



A solution of EDAC (6.25 g, 32.66 mmol) and DIPEA (5.69 mL, 32.66 mmol) in 30 mL of DCM is added dropwise to a solution of 3-((tert-butoxycarbonyl)amino)propanoic acid (5.70 g, 30.14 mmol), HOBT (4.41 g, 32.66 mmol) in 30 mL of DCM under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of Methyl 2-amino-3-(1-benzyl-1H-imidazol-4-yl)propanoate hydrochloride (7.54 g, 25.12 mmol) and DIPEA (27.63 mL, 25.12 mmol) in 30 mL of DCM is added dropwise.

The reaction mixture is stirred at RT overnight and monitored by TLC<sup>133</sup>.

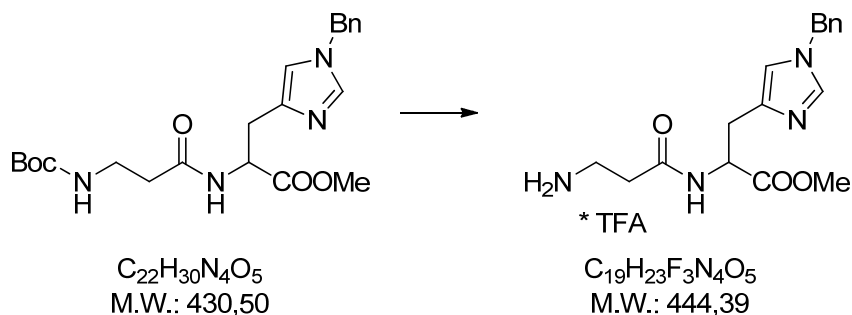
After completion, the mixture is treated with DCM and a solution of  $\text{NaHCO}_3$  and then the organic phase is washed with brine, dried over  $\text{Na}_2\text{SO}_4$ , filtered, and evaporated. The crude product is purified by column chromatography on silica gel, using DCM/Methanol = 94/4 as elution solvent, to give 6.81 g (15.82 mmol) of the dense oil.

Yield: 63 %

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 8.21 (d, 1H,  $J=7.7$  Hz), 7.62 (s, 1H), 7.17-7.38 (m, 5H), 6.88 (s, 1H), 6.72 (bs, 1H), 5.11 (s, 2H), 4.43 (m, 1H), 3.57 (s, 3H), 3.05 (dt, 1H,  $J= 7.15$  Hz,  $J= 13.20$  Hz), 2.70-2.85 (m, 2H), 2.21 (t, 2H,  $J= 7.15$  Hz), 1.35 (s, 9H).

<sup>133</sup> TLC conditions: DCM/ Methanol= 95/5  
Rf= 0.17

## Methyl 2-(3-aminopropanamido)-3-(1-benzyl-1H-imidazol-4-yl)propanoate trifluoroacetate



6.81 g (15.82 mmol) of the Methyl 3-(1-benzyl-1H-imidazol-4-yl)-2-(3-(((benzyloxy)carbonyl)amino)propanamido)propanoate are dissolved in 70 mL of DCM and treated with 13 mL of TFA.

The reaction is stirred for 1<sup>1/2</sup> hours, evaluating the completion by TLC<sup>134</sup>.

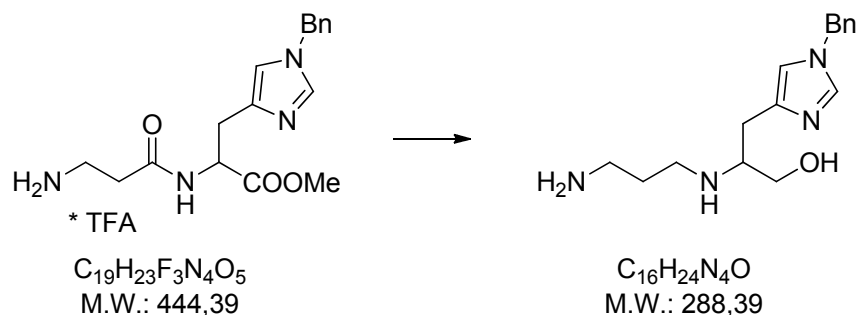
The simply removal of the solvents gives 7.03 g of a dense oil, in quantitative yield.

Yield: Quantitative

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 9.18 (s, 1H), 8.68 (d, 1H, *J*= 7.7 Hz), 7.79 (bs, 3H), 7.50 (s, 1H), 7.34-7.42 (m, 5H), 5.38 (s, 2H), 4.58 (m, 1H), 3.57 (s, 3H), 3.07 (m, 1H), 3.02 (m, 1H), 2.87-2.94 (m, 2H), 2.42-2.50 (m, 2H).

<sup>134</sup> TLC condition: DCM / Methanol= 90 / 10  
R<sub>f</sub>: 0.12

## 2-((3-aminopropyl)amino)-3-(1-benzyl-1H-imidazol-4-yl)propan-1-ol



7.03 g (15.82 mmol) of the Methyl 2-(3-aminopropanamido)-3-(1-benzyl-1H-imidazol-4-yl)propanoate trifluoroacetate in 50 mL of dry THF are slowly dropped, at  $-10^\circ\text{C}$ , to a suspension of  $\text{LiH}_4$  (4.20 g, 110.74 mmol) in 20 mL of dry THF, under a nitrogen atmosphere.

The mixture is warmed to RT and then refluxed, monitoring the progression by NMR.

At completion, the mixture is cooled to RT, 40 ml of Water are firstly added, followed by 50 mL of Ethanol.

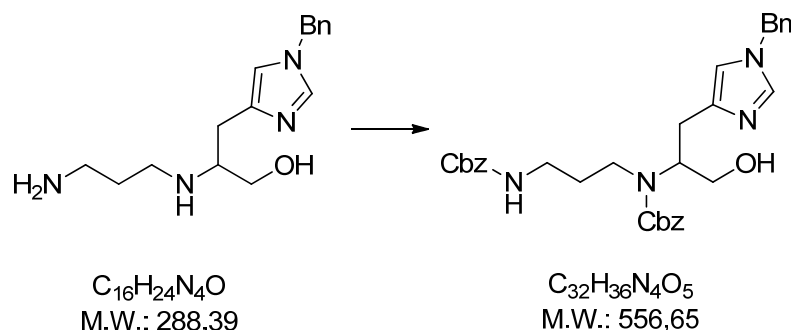
The suspension is stirred at  $60^\circ\text{C}$  for 2 hours, cooled to RT and filtered over Celite.

The solution is dried over  $\text{Na}_2\text{SO}_4$ , filtered and concentrated under vacuum, yielding 4.11 g of the dense oil (14.34 mmol).

Yield: 90%

**$^1\text{H}$  NMR (TFA+ $\text{D}_2\text{O}$ ):** 7.65-7.80 (m, 5H), 7.64 (s, 1H), 7.49 (s, 1H), 5.49 (s, 1H), 4.20 (m, 2H), 3.92 (m, 2H), 3.47-3.63 (m, 5H), 2.58 (m, 2H).

## Benzyl (1-(1-benzyl-1H-imidazol-4-yl)-3-hydroxypropan-2-yl)(3-(((benzyloxy)carbonyl)amino)propyl)carbamate



A solution of 2-((3-aminopropyl)amino)-3-(1-benzyl-1H-imidazol-4-yl)propan-1-ol (4.11 g, 14.34 mmol) and DIPEA (5.00 mL, 28.68 mmol) in 40 mL of THF is heated at 60 °C, under a nitrogen atmosphere.

Benzyl chloroformate (4.09 mL, 28.68 mmol) is then slowly dropped, and the progression of the reaction is monitored by TLC<sup>135</sup>

At completion, the mixture is cooled to RT, the solvent is removed and the residue is resumed with Ethyl Acetate; the organic layer is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure.

Purification on silica gel, using Ethyl Acetate as elution solvent, yields 3.83 g (6.88 mmol) of the di-protected compound, as yellow oil.

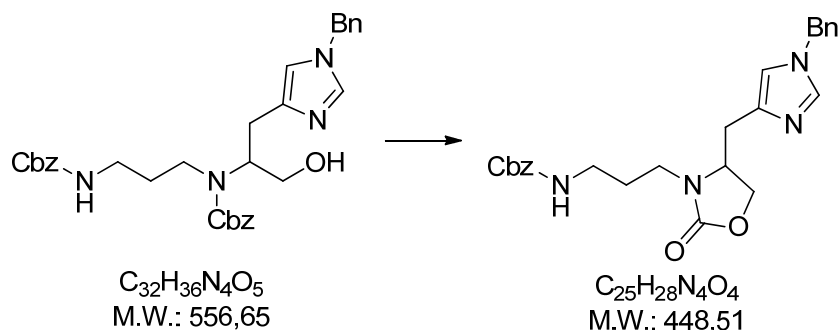
Yield: 48 %

**<sup>1</sup>H NMR (d<sub>6</sub>DMSO):** 7.63 (s, 1H), 7.14-7.34 (m, 16H), 6.77 (d, 1H, *J* = 13.46 Hz), 5.08 (s, 2H), 4.99 (s, 2H), 4.97 (s, 2H), 4.35 (m, 1H), 4.19 (bs, 1H), 3.06 (m, 2H), 2.87 (m, 2H), 2.66 (m, 2H), 1.50 (m, 4H).

<sup>135</sup> TLC conditions: Ethyl Acetate 100%  
R<sub>f</sub>: 0.30



## Benzyl (3-(4-((1-benzyl-1H-imidazol-4-yl)methyl)-2-oxooxazolidin-3-yl)propyl)carbamate



To a suspension of NaH (0.20 g, 8.26 mmol) in 10 mL of THF, at 0°C, is dropped a solution of Benzyl (1-(1-benzyl-1H-imidazol-4-yl)-3-hydroxypropan-2-yl)(3-(((benzyloxy)carbonyl)amino)propyl)carbamate (3.83 g, 6.88 mmol) in 20 mL of THF.

The reaction is refluxed, monitoring the progression by TLC<sup>136</sup>; after 1 hour, the solvent is removed and the residue is treated with Ethyl Acetate and brine.

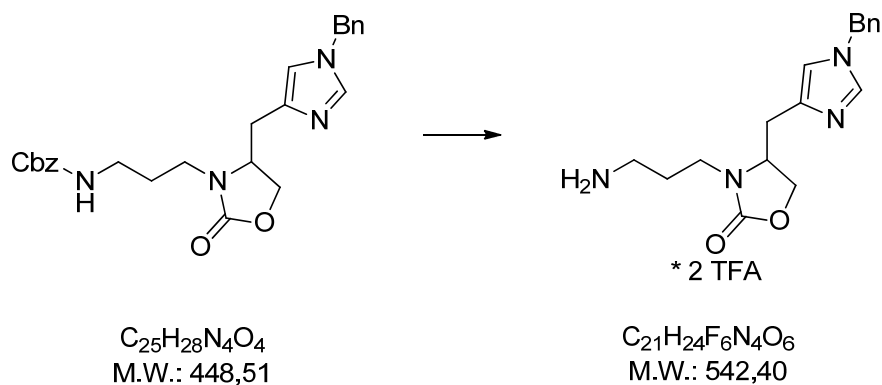
The organic phase is dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure; the crude is purified on silica gel, using Ethyl Acetate/ Methanol= 90/10 as elution solvent, obtaining 2.05.g (4.59 mmol) of a pale yellow oil..

Yield:67%

**<sup>1</sup>H NMR (CDCl<sub>3</sub>):** 7.45 (s, 1H), 7.26-7.39 (m, 9H), 7.14 (s, 1H), 7.11 (s, 1H), 6.67 (s, 1H), 5.57 (m, 1H), 5.08 (s, 2H), 5.03 (s, 2H), 4.29 (m, 1H), 4.11 (m, 1H), 3.46 (m, 1H), 3.07-3.30 (m, 3H), 2.96 (dd, 1H, *J* = 3.51 Hz, *J* = 14.33 Hz), 2.66 (dd, 1H, *J* = 7.60 Hz, *J* = 14.33 Hz), 1.71 (m, 1H).

<sup>136</sup> TLC conditions: Ethyl Acetate/ Methanol= 90/ 10  
Rf: 0.14

### 3-(3-aminopropyl)-4-((1-benzyl-1H-imidazol-4-yl)methyl)oxazolidin-2-one di-trifluoroacetate



2.05.g (4.59 mmol) of Benzyl (3-(4-((1-benzyl-1H-imidazol-4-yl)methyl)-2-oxooxazolidin-3-yl)propyl)carbamate are dissolved in a solution of 20 mL of Methanol and 7.34 mL of 1.25 M HCl Methanol solution.

The mixture undergoes hydrogenolysis with Pd/C 10% at RT; the progression of the reaction is monitored by TLC<sup>137</sup>.

At completion, the solvents are removed and the crude is further purified by reverse phase preparative HPLC, according to the gradient reported in Table 3, with a flowrate of 7 mL/min, yielding 1.49 g (2.75 mmol) of a sticky white solid.

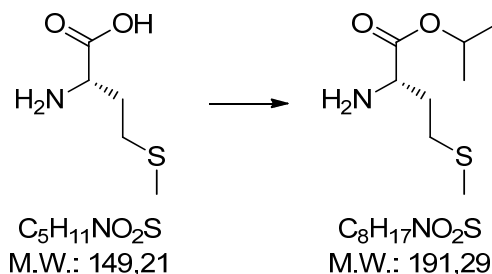
Yield: 60%

<sup>1</sup>H NMR (D<sub>2</sub>O): 8.48 (s, 1H), 7.17 (s, 1H), 4.30 (t, 1H, *J* = 8.8 Hz), 4.19 (m, 1H), 4.02 (dd, 1H, *J* = 4.12 Hz, *J* = 8.8 Hz), 3.37 (m, 1H), 3.16 (m, 1H), 2.98 (m, 2H), 2.86 (m, 2H), 1.81 (m, 2H).

<sup>13</sup>C-NMR (D<sub>2</sub>O): 160.22, 133.99, 127.44, 117.36, 67.53, 54.07, 38.91, 36.99, 26.21, 25.19.

<sup>137</sup> TLC conditions: Ethyl Acetate/ Methanol = 80 /20  
 Rf: basal

## (L)-Methionine isopropyl ester



125 mL of Isopropyl alcohol, at 0 °C, are slowly amount of 16.66 mL (234.0 mmol) of Acetylchloride; the mixture is maintained cold and stirred for 30 minutes.

L-Metionine (10.0 g, 67.0 mmol) is sequentially added portionwise as solid, afterwards the reaction is warmed to RT and then refluxed for 4 hours.

After completion of the reaction, followed by TLC<sup>138</sup>, the mixture is concentrated, treated with 100 mL of DCM and carefully washed with 100 mL of  $NaHCO_3$ .

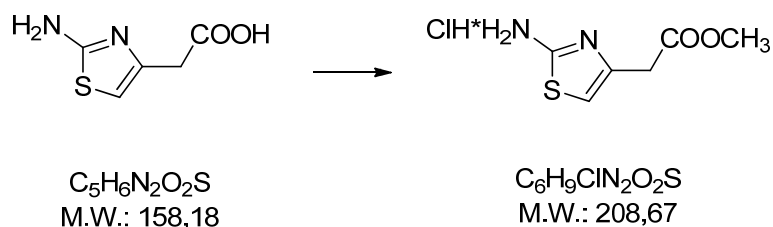
The two layers are separated; the organic phase is washed with brine, dried over  $Na_2SO_4$ , filtered and concentrated under reduced pressure, giving 12.82 g (67.0 mmol) of the desired product as pale yellow oil.

Yield: quantitative

<sup>1</sup>H-NMR ( $CDCl_3$ ): 5.05 (m, 1H), 4.59 (bs, 1H), 3.77 (dd, 1H,  $J=5.22$  Hz,  $J=7.7$  Hz), 2.67 (m, 2H), 2.10 (s, 3H), 1.97-2.16 (m, 2H).

<sup>138</sup> Cyclohexane / Ethyl Acetate= 1/1 + TEA 0.25%  
Rf= 0.3

## Methyl 2-(2-aminothiazol-4-yl)acetate hydrochloric acid



10.0 g (63.22 mmol) of 2-amino-4-thiazolylacetic acid are dissolved in 100 mL of methanol; the solution is cooled to  $-15^{\circ}C$  and slowly added of 5.04 mL (60.54 mmol) of  $SOCl_2$ .

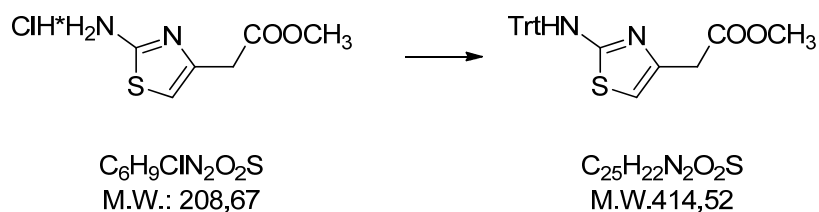
The reaction is monitored by NMR analysis; at the completion of the reaction, the mixture is concentrated under reduce pressure, obtaining 13.19 g of the desired product, as a white solid.

Yield: quantitative

Melting point:  $173.84^{\circ}C$

**$^1H$ -NMR ( $CDCl_3$ ):** 6.51 (s, 1H), 4.66 (bs, 3H), 3.64 (s, 2H), 3.60 (s, 3H).

## Methyl 2-(2-(tritylamino)thiazol-4-yl)acetate



13.19 g (63.22 mmol) of methyl 2-(2-aminothiazol-4-yl)acetate hydrochloric acid are dissolved in 100 mL of DCM and added of 19.39 mL (139.08 mmol) of TEA.

The reaction mixture is cooled to  $-10^\circ\text{C}$ , slowly amounted of 21.15 g (75.86 mmol) of Trityl chloride in 160 mL of DCM and then warmed to RT.

The reaction is monitored by TLC<sup>139</sup>; after completion, the mixture is treated with 100 mL of DCM and quenched with 300 mL of  $\text{NaHCO}_3$  10%, the organic layer is washed with brine, dried over  $\text{Na}_2\text{SO}_4$ , filtered and concentrated under reduced pressure.

The crude is crystallized by Diethyl ether, giving 23.58 g (56.90 mmol) of the desired product, as a white solid.

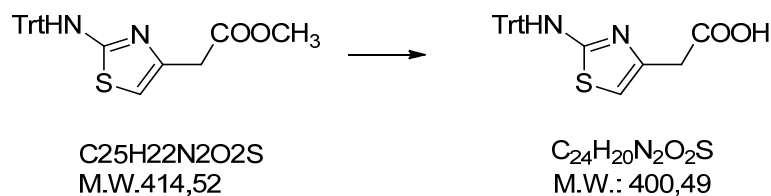
Yield: 90%

Melting point:  $190.22^\circ\text{C}$

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 7.26-7.35 (m, 15H), 6.71 (bs, 1H), 6.17 (s, 1H), 3.70 (s, 3H), 3.54 (s, 2H).

<sup>139</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
Rf= 0.60

## 2-(2-(tritylamino)thiazol-4-yl)acetic acid



A solution of 23.58 g (56.90 mmol) of the methyl 2-(2-(tritylamino)thiazol-4-yl)acetate in 230 mL of Methanol is treated with 21.5 mL of 2.5 M NaOH and refluxed.

The reaction is monitored by TLC<sup>140</sup>; after completion, achieved in 2 hours, the solvent is removed under vacuum, the residue is resumed with 250 mL of Water and acidified using HCl 37%.

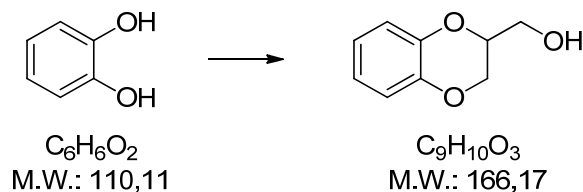
The white precipitate is filtered and dried under reduce pressure, giving 21.65 g (54.06 mmol) of the free carboxylic acid.

Yield: 95%

Melting point: 152 °C

<sup>1</sup>H-NMR (CDCl<sub>3</sub>): 7.25-7.34 (m, 15H), 6.05 (s, 1H), 3.58 (s, 2H).

<sup>140</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
R<sub>f</sub>= 0.15

**(2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methanol**

A red solution of Catechol (10.0 g, 90.82 mmol) in 100 mL of Methanol is added of 27.61 g of  $K_2CO_3$  (200 mmol) as solid, forming a suspension that is stirred for 30 minutes.

21.31 mL (272.46 mmol) of Epichlorohydrin are then dropped and the resulting mixture is refluxed for 8 hours, monitoring the completion by TLC<sup>141</sup>.

After evaporating the solvent, the residue is resumed with 80 mL of DCM, washed with brine, dried over  $Na_2SO_4$ , filtered and concentrated under vacuum.

The crude undergoes crystallization with Isopropyl ether, giving 8.15 g (49.0 mmol) of a white solid.

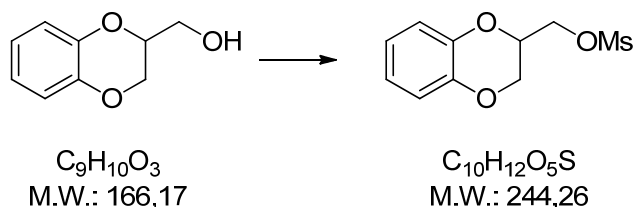
Yield: 54%

Melting point: 86.32 °C

**<sup>1</sup>H-NMR ( $CDCl_3$ ):** 6.83-6.92 (m, 4H), 4.24-4.32 (m, 2H), 4.07-4.14 (m, 1H), 3.81- 3.93 (m, 2H), 2.03 (bs, 1H).

<sup>141</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
Rf= 0.33

## (2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methyl methanesulfonate



A solution of 7.51 ml of TEA (53.9 mmol) in 8 mL of DCM is dropped to the reaction mixture, containing (2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methanol (8.15 g, 49.0 mmol) in 32 mL of DCM, the resulting pale yellow solution is then cooled to  $-15^{\circ}C$  and amount of 4.17 mL of Methansulfonyl chloride (53.9 mmol), over 15 minutes.

The reaction is monitored by TLC<sup>142</sup>; after completion, the mixture is treated with 50 mL of DCM and quenched with 80 mL of HCl 10%, the organic layer is washed with brine, dried over  $Na_2SO_4$ , filtered and concentrated under reduced pressure.

The crude is crystallized from Isopropanol, obtaining 10.77 g (44.1 mmol) of the desired product, as a white solid.

Yield: 90%

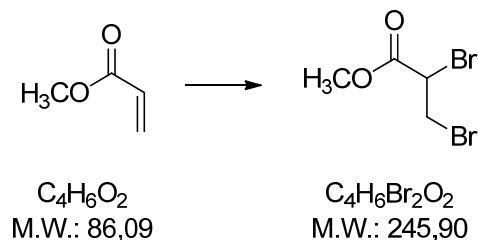
Melting point: 62.7%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 7.00 (m, 1H), 6.89-6.96 (m, 3H), 4.43-4.55 (m, 3H), 4.32 (dd, 1H,  $J=1.82$  Hz,  $J=11.82$  Hz), 4.20 (dd, 1H,  $J=4.95$  Hz,  $J=11.82$  Hz), 3.09 (s, 3H).

<sup>142</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
Rf= 0.30



## Methyl 2,3-dibromopropanoate



A solution of methyl acrylate (10.46 mL, 116.16 mmol) in 50 mL of DCM is refluxed and sequentially added of bromine (5.95 mL, 116.16 mmol) in 50 mL of DCM.

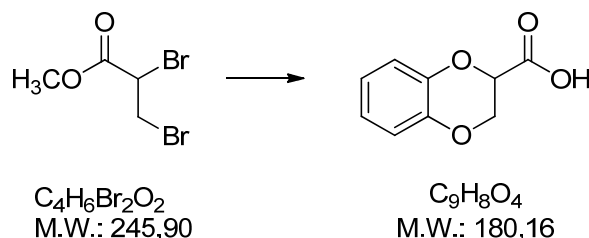
The reaction is monitored by the disappearing of the orange coloration and by NMR analysis; the completion is achieved after 30 minutes.

The reaction mixture is then cooled, washed with 80 mL of 10%  $Na_2S_2O_5$  in water, dried over  $Na_2SO_4$ , filtered and concentrated under reduced pressure, obtaining 28.01 g (113.93 mmol) of the desired product, ad a colourless oil.

Yield: 98%

**$^1H$ -NMR ( $CDCl_3$ ):** 4.45 (dd, 1H,  $J=2.18$  Hz,  $J=5.45$  Hz), 3.93 (dd, 1H,  $J=4.36$  Hz,  $J=5.45$  Hz), 3.84 (s, 3H), 3.67(dd, 1H,  $J=2.18$  Hz,  $J=4.36$  Hz).

## 2,3-dihydrobenzo[b][1,4]dioxine-2-carboxylic acid



A suspension of  $K_2CO_3$  (47.24 g, 341.79 mmol) in 125 mL of Isopropanol is slowly added of solid catechol (12.54g, 113.93 mmol) and then stirred for 30 minutes.

After that period, a solution of Methyl 2,3-dibromopropanoate (28.01 g, 113.93 mmol) in 250 mL of Isopropanol is dropped, and all the reaction mixture is refluxed overnight.

The mixture is concentrated, resumed with 200 mL of Methanol and then added of 5.45 g of NaOH in pellets. The reaction is refluxed for 2 hours and monitored by TLC<sup>143</sup>; after evaporating the solvent, the residue is treated with water, acidified with HCl 10% and extracted three times with 70 mL of DCM.

The organic layers are collected, dried over  $Na_2SO_4$ , filtered and concentrated under reduced pressure; the crude obtained is then crystallized by Toluene, giving 16.00 g (88.86 mmol) of the desired product, as white solid.

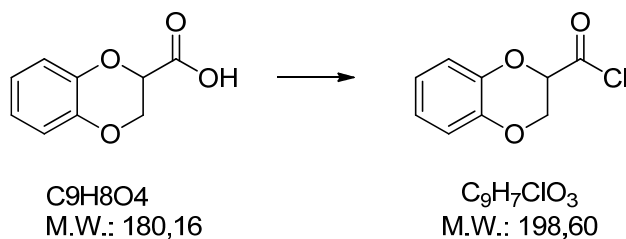
Yield: 78%

Melting Point: 114.69 °C

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 8.51 (bs, 1H), 7.00 (m, 1H), 6.87-6.94 (m, 3H), 4.90 (dd, 1H,  $J= 3.02$  Hz,  $J= 4.4$  Hz), 4.44 (dd, 1H,  $J= 4.4$  Hz,  $J= 11.28$  Hz), 4.38 (dd, 1H,  $J=3.02$  Hz,  $J= 11.28$  Hz).

<sup>143</sup> TLC conditions: DCM/ Methanol= 1/1 + 10% Acetic Acid  
Rf= 0.20

## 2,3-dihydrobenzo[b][1,4]dioxine-2-carbonyl chloride



16.00 g of 2,3-dihydrobenzo[b][1,4]dioxine-2-carboxylic acid (88.86 mmol) are suspended in 160 mL of Toluene, cooled at  $-10^{\circ}\text{C}$  and amount of 9.67 mL (133.29 mmol) of pure  $\text{SOCl}_2$ .

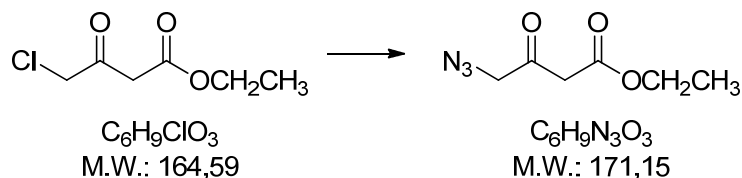
The mixture is then warmed to RT and sequentially refluxed for 3 hours, following the completion by NMR.

The evaporation of the solvents let the obtainment of 17.65 g of the desired product, as a sticky solid, in quantitative yields.

Yield: quantitative

**$^1\text{H-NMR}$  ( $\text{CDCl}_3$ ):** 7.00 (m, 1H), 6.89-6.96 (m, 3H), 5.09 (dd, 1H,  $J = 2.75$  Hz,  $J = 2.82$  Hz), 4.74 (dd, 1H,  $J = 2.82$  Hz,  $J = 11.82$  Hz), 4.34 (dd, 1H,  $J = 2.75$  Hz,  $J = 11.82$  Hz).

## Ethyl 4-azido-3-oxobutanoate



A solution of freshly distilled ethyl 4-chloro-3-oxo-butanoate (19.50 g, 118.48 mmol) in 35 mL of THF is slowly dropped, at 4 °C, to a mixture of NaN<sub>3</sub> (17.72 g, 272.50 mmol) in Water.

The reaction is stirred at RT for 18 hours, monitoring the progression by TLC<sup>144</sup>.

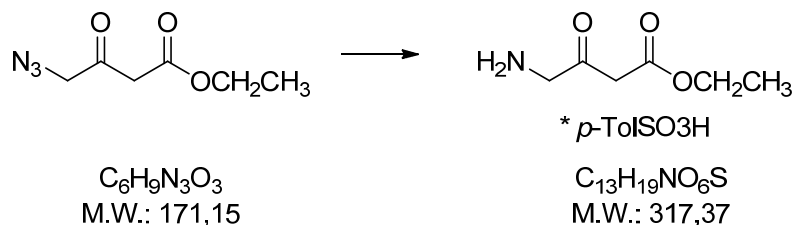
At completion, 20 mL of Ethyl Acetate are added to the mixture, the aqueous layer is extracted twice more with Ethyl Acetate, the organic phases are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under vacuum, yielding 19.35 g (112.35 mmol) of a brown oil.

Yield: 94.83 %

<sup>1</sup>H-NMR (d<sub>6</sub>DMSO): 4.26 (s, 2H), 4.07 (q, 2H, J=3.58 Hz), 3.60 (s, 2H), 1.17 (t, 3H, J=3.58 Hz).

<sup>144</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 80/20  
R<sub>f</sub>= 0.36

## Ethyl 4-amino-3-oxobutanoate *p*-toluenesulfonate



A solution of ethyl 4-azido-3-oxobutanoate (19.35 g, 112.35 mmol) and *p*-toluenesulfonic acid (19.23 g, 112.35 mmol) in 100 mL of Ethanol undergoes hydrogenolysis with Pd/C 10% at RT, the progression of the reaction is monitored by TLC<sup>145</sup>.

At completion, catalyst filtration and solvent removal give a residue that is further crystallized from Isopropanol, yielding 10.1 g (31.82 mmol) of a white solid.

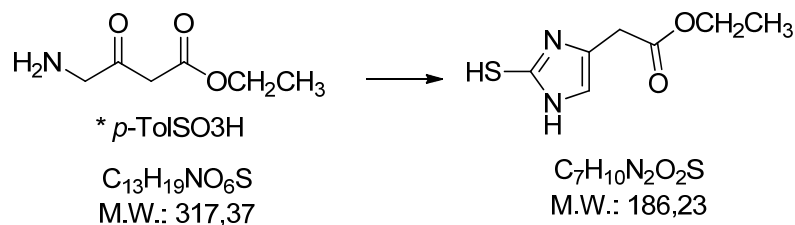
Yield: 28.33 %

Melting point: 111.05 °C

<sup>1</sup>H-NMR (**d<sub>6</sub>**DMSO): 8.07 (s, 3H), 7.46 (d, 2H, *J* = 7.98 Hz), 7.10 (d, 2H, *J* = 7.98 Hz), 4.10 (q, 2H, *J* = 7.15 Hz), 3.98 (s, 2H), 3.70 (s, 2H), 2.15 (s, 3H), 1.10 (t, 3H).

<sup>145</sup> TLC conditions: Cyclohexane/ Ethyl Acetate = 80 / 20  
Rf: 0.05

## Ethyl 2-(2-mercapto-1H-imidazol-4-yl)acetate



A solution of 10.1 g (31.82 mmol) of ethyl 4-amino-3-oxobutanoate *p*-toluenesulfonate in 17.3 mL of Water is refluxed; 5.27 g (54.26 mmol) of KNCS are slowly added to the mixture portionwise.

The reaction is monitored by TLC<sup>146</sup>; at completion, the mixture is warmed to RT and the pH is moved to 5, adding NaHCO<sub>3</sub>.

The suspension is cooled to 4 °C, until the precipitation of a white solid is achieved; its filtration yields 1.84 g (9.88 mmol) of the desired product.

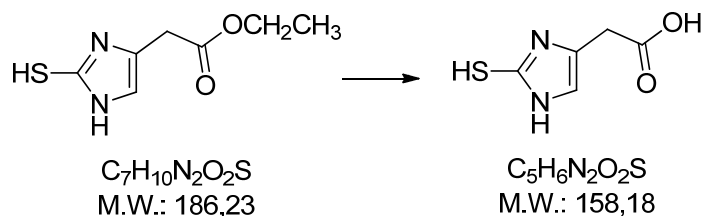
Yield: 31.05 %

Melting point: 152.18 °C

<sup>1</sup>H-NMR (d<sub>6</sub>DMSO): 11.86 (s, 1 H), 11.75 (s, 1H), 6.63 (s, 1H), 4.07 (q, 2H, *J*= 7.16 Hz), 3.47 (s, 2H), 1.17 (t, 3H, *J*= 7.16 Hz).

<sup>146</sup> TLC conditions: DCM/ Methanol= 95/ 5  
R<sub>f</sub>: 0.27

## 2-(2-mercapto-1H-imidazol-4-yl)acetic acid



1.84 g (9.88 mmol) of ethyl 2-(2-mercapto-1H-imidazol-4-yl)acetate are dissolved in a mixture of 15 mL of Methanol and 8 mL of 2.5M of NaOH.

The solution is refluxed for 1 hour, monitoring the progression by TLC<sup>147</sup>; at completion, the solvents are removed, the crude is resumed with Ethanol and acidified with an ethanolic H<sub>2</sub>SO<sub>4</sub> solution.

The suspension is then filtered and the solid is further crystallized from Isopropanol, yielding 0.750 g (4.74 mmol) of the desired product.

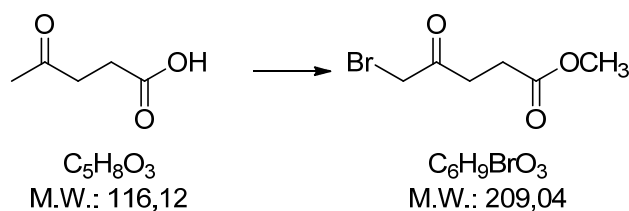
Yield:48 %

Melting point:

<sup>1</sup>H-NMR (d<sub>6</sub>DMSO): 11.82 (bs, 1H), 11.70 (bs, 1H), 6.60 (s, 1H), 3.38 (s, 2H).

<sup>147</sup> TLC conditions: DCM/ Methanol= 95/ 5  
Rf: 0.6

## Methyl 5-bromo-4-oxo-pentanoate



Br<sub>2</sub> (17.70 mL, 344.47 mmol) is slowly dropped to a solution of levulinic acid (40.00 g, 344.47 mmol) in Methanol (340 ml), at 3 °C.

The reaction is warmed to RT and monitored by TLC<sup>148</sup>.

At completion, after 2 ½ days, the Methanol is removed, the residue is treated with 140 mL of DCM and with 10% NaHCO<sub>3</sub>, neutralizing the aqueous pH.

The two layers are discarded, the aqueous layer is extracted ones more with DCM, and the organic phases are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure.

The residue is purified on silica gel, using Cyclohexane / Ethyl Acetate= 80 / 20 as elution solvent, yielding 36.76 g (175.85 mmol) of a colourless oil.

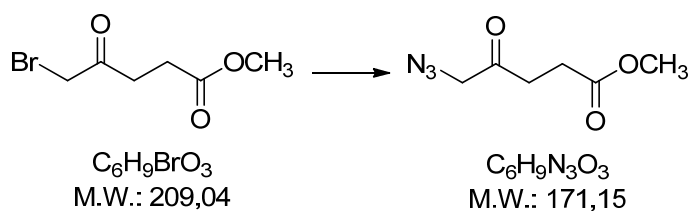
Yield : 51.05 %

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 3.94 (s, 2H), 3.65 (s, 3H), 2.93 (t, 2H, *J*= 6.32 Hz), 2.63 (t, 2H, *J*= 6.32 Hz).

<sup>148</sup> TLC conditions : Cyclohexane/ Ethyl Acetate = 80/ 20  
Rf: 0.36



## Methyl-5-azido-4-oxo-pentanoate



A solution of methyl-5-bromo-4-oxo-pentanoate (36.76 g, 175.85 mmol) in 70 mL of THF is slowly dropped, at 2 °C, to a mixture of NaN<sub>3</sub> (26.29 g, 404.45 mmol) in 40 mL of Water.

The reaction is stirred at RT for 3 hours, monitoring the progression by TLC<sup>149</sup>.

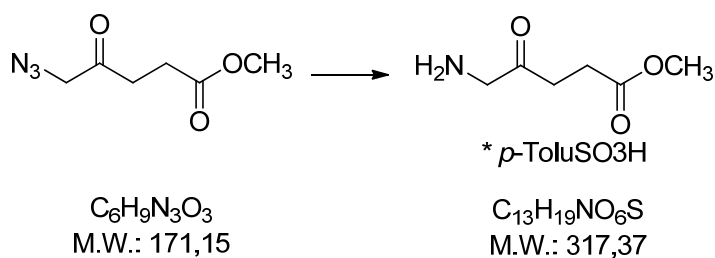
At completion, 80 mL of Ethyl Acetate are added to the mixture, the aqueous layer is extracted twice more with Ethyl Acetate, the organic phases are collected, washed with Water and brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under vacuum, yielding 27.73 g (162.03 mmol) of a yellow oil.

Yield: 92.14 %

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 4.00 (s, 2H), 3.64 (s, 3H), 2.69 (m, 2H), 2.64 (m, 2H).

<sup>149</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 80/20  
Rf= 0.32

## Methyl-5-amino-4-oxo-pentanoate *p*-toluenesulfonate



A solution of methyl 5-azido-4-oxo pentanoate (27.73 g, 162.03 mmol) and *p*-toluenesulfonic acid (27.90 g, 162.03 mmol) in 200 mL of Methanol undergoes hydrogenolysis with Pd/C 10% at RT, the progression of the reaction is monitored by TLC<sup>150</sup>.

At completion, after 24 hours, catalyst filtration and solvent removal give a residue that is further crystallized from Isopropanol, yielding 42.88 g (135.11 mmol) of a white solid.

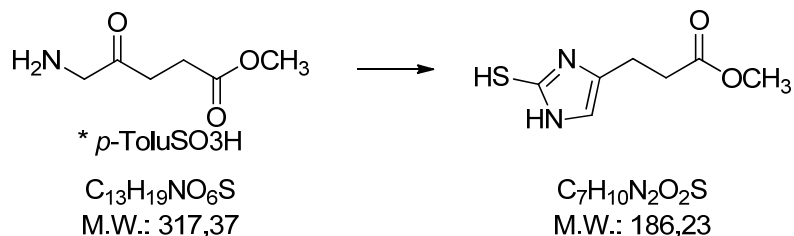
Yield: 83.38%

Melting point= 130.43 °C

<sup>1</sup>H-NMR (CDCl<sub>3</sub>): 4.00 (s, 2H), 3.64 (s, 3H), 2.69 (m, 2H), 2.64 (m, 2H).

<sup>150</sup> TLC conditions: Cyclohexane/ Ethyl Acetate = 80 /20  
Rf: 0.05

## Methyl 3-(2-mercapto-1H-imidazol-4-yl)propanoate



A solution of 42.88 g (135.11 mmol) of methyl 5-amino-4-oxopentanoate *p*-toluenesulfonate in 80 mL of Water is refluxed; 22.32 g (229.72 mmol) of KNCS are slowly added to the mixture portionwise.

The reaction is monitored by TLC<sup>151</sup>; at completion, the mixture is warmed to RT and the pH is moved to 5, adding NaHCO<sub>3</sub>.

The suspension is cooled to 4 °C, until the precipitation of a pale grey solid is achieved; its filtration yields 13.57 g (72.88 mmol) of the desired product.

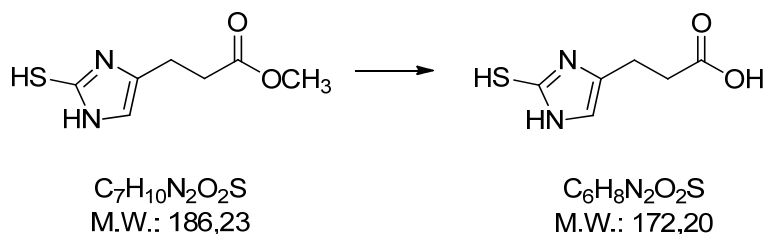
Yield: 53.94 %

Melting point = 161.46 °C

**<sup>1</sup>H-NMR (D<sub>2</sub>O):** 6.57 (s, 1H), 3.56 (s, 3H), 2.70 (m, 2H), 2.57 (m, 2H).

<sup>151</sup> TLC conditions: DCM/ Methanol= 95/ 5  
Rf: 0.23

### 3-(2-mercapto-1H-imidazol-4-yl)propanoic acid



13.57 g (72.88 mmol) of methyl 3-(2-mercapto-1H-imidazol-4-yl)propanoate are suspended in a mixture of 15 mL of Water and 40 mL of 4M NaOH.

The solution is heated at 55°C for 2 hours, monitoring the progression by TLC<sup>152</sup>; at completion, the mixture is cooled to 4°C and acidified with HCl 10%, until the precipitation of a grey solid.

The suspension is then filtered and the solid is further crystallized from Isopropanol, yielding 9.02 g (52.39 mmol) of the desired product.

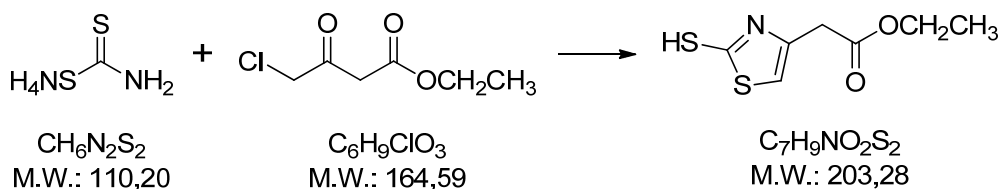
Yield: 71.88%

Melting point: 203.14 °C

<sup>1</sup>H-NMR (D<sub>2</sub>O): 6.60 (s, 1H), 2.70 (t, 2H, J= 7.42 Hz), 2.58 (t, 2H, J= 7.42 Hz).

<sup>152</sup> TLC conditions: DCM/ Methanol= 95/ 5  
Rf: 0.06

## Ethyl 2-(2-mercaptothiazol-4-yl)acetate



9.00 g of ethyl-4-chloro-3-oxo butanoate (54.81 mmol) are added dropwise to a solution of ammonium dithiocarbamate (6.03 g, 54.81 mmol) in 80 mL of water.

The reaction mixture is refluxed overnight and monitored by TLC<sup>153</sup>; at completion, the suspension is warmed to RT, until the precipitation of a solid that is filtered and crystallized from Toluene, yielding 5.12 g (25.19 mmol) of the desired product.

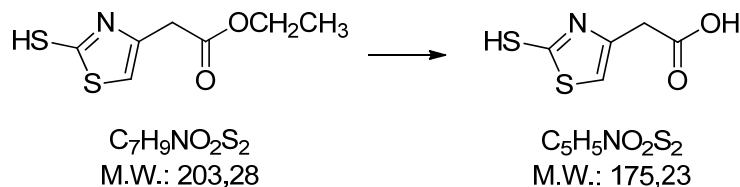
Yield: 45.95 %

Melting point: 143.01 °C

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 6.46 (s, 1H), 4.23 (q, 2H, *J*= 3.30 Hz), 3.58 (s, 2H), 1.29 (t, 3H, *J*= 3.30 Hz).

<sup>153</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 80/ 20  
Rf: 0.17

## 2-(2-mercaptothiazol-4-yl)acetic acid



A solution of ethyl 2-(2-mercaptothiazol-4-yl)acetate (5.12 g, 25.19 mmol) in 25 mL of 2.5M NaOH is heated at 55°C for 1 hour, monitoring the hydrolysis progression by TLC<sup>154</sup>.

At completion, the reaction mixture is warmed to RT and then cooled to 4°C, before acidification with HCl 10% and formation of a precipitate.

The suspension filtration yields 3.59 g (20.50 mmol) of a pale brown solid, corresponding to the desired product.

Yield: 81.39 %

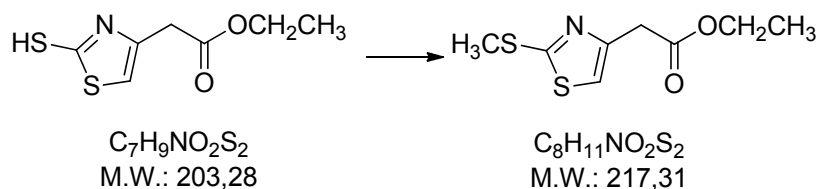
Melting point: 165.88 °C

**<sup>1</sup>H-NMR (d<sub>6</sub> DMSO):** 6.70 (s, 1H), 3.52 (s, 2H), 3.29 (s, 1H).

<sup>154</sup> Eluente : Cicloesano : Acetato di etile 70 : 30 Rf part = 0,17 Rf prod = 0,05  
KMnO<sub>4</sub>

Evidenziatore :

## Ethyl 2-(2-(methylthio)thiazol-4-yl)acetate



A solution of ethyl 2-(2-mercaptothiazol-4-yl)acetate (2.50 g, 12.30 mmol) in 20 mL of dry DME is added dropwise to a suspension of NaH (0.30 g, 12.30 mmol) in 10 mL of dry DME, under a nitrogen atmosphere.

The mixture is stirred for 10 minutes and then a solution of methyl iodide (1.00 mL, 14.80 mmol) is slowly dropped; the reaction is stirred RT and monitored by TLC<sup>155</sup>.

At completion, 10 ml of Water and 20 ml of Ethyl Acetate are added, the two phases are separated and the aqueous layer is extracted twice with Ethyl Acetate.

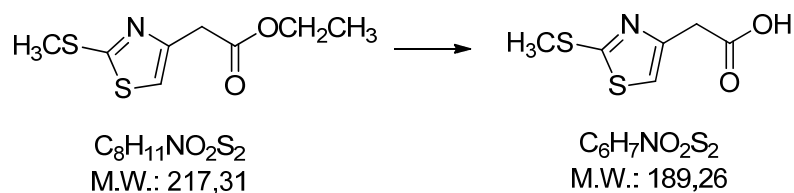
The organic phases are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduce pressure, yielding 2.48 g (11.41 mmol) of a pale yellow oil.

Yield: 92.77 %

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 7.08 (s, 1H), 4.18 (q, 2H, *J*= 3.85 Hz), 3.78 (s, 2H), 2.68 (s, 3H), 1.24 (t, 3H, *J*= 3.85 Hz).

<sup>155</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 70/ 30  
Rf: 0.50

## 2-(2-(methylthio)thiazol-4-yl)acetic acid



2.48 g (11.41 mmol) of ethyl 2-(2-(methylthio)thiazol-4-yl)acetate are dissolved in 15 mL of Methanol; 6 mL of NaOH 2.5 M are dropped and the resulting mixture is heated at 60 °C for 18 hours.

After completion of the reaction, followed by TLC<sup>156</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with 10% HCl, and then extracted three times with 15 mL of Ethyl Acetate.

The organic phases are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure, obtaining a pale grey solid (0.64 g, 2.58 mmol), corresponding to the desired compound.

Yield: 97.05 %

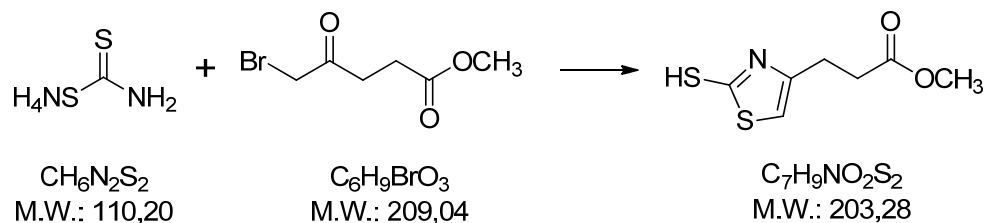
Melting point: 122.01 °C

**<sup>1</sup>H-NMR (d<sub>6</sub> DMSO):** 7.34 (s, 1H), 3.65 (s, 2H), 2.64 (s, 3H).

<sup>156</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 70/ 30  
Rf: 0.10



## Methyl 3-(2-mercaptothiazol-4-yl)propanoate



13.05 g of methyl-5-bromo-4-oxo pentanoate (62.43 mmol) are added dropwise to a solution of ammonium dithiocarbamate (6.88 g, 62.43 mmol) in 60 mL of water.

The reaction mixture is refluxed for 3 hours and monitored by TLC<sup>157</sup>; at completion, the suspension is warmed to RT, until the precipitation of a solid that is filtered and crystallized from Isopropyl ether, yielding 6.31 g (25.19 mmol) of a pale yellow solid, corresponding to the desired product.

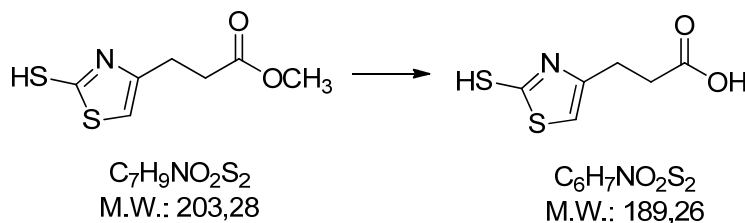
Yield: 49.71 %

Melting point: 126.54 °C

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 11.80 (s, 1H), 6.28 (s, 1H), 3.71 (s, 3H), 2.84 (t, 2H, *J* = 6.60 Hz), 2.66 (t, 2H, *J* = 6.60 Hz).

<sup>157</sup> TLC conditions: Cyclohexane/ Ethyl Acetate = 70/ 30  
Rf: 0.25

### 3-(2-mercaptothiazol-4-yl)propanoic acid



6.31 g (25.19 mmol) of methyl 3-(2-mercaptothiazol-4-yl)propanoate are dissolved in 12.5 mL of NaOH 4 M; the resulting mixture is heated at 55 °C for 1 hour.

After completion of the reaction, followed by TLC<sup>158</sup>, the reaction mixture is acidified with 10% HCl, and then extracted three times with 10 mL of Ethyl Acetate.

The organic phases are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure, obtaining a beige solid (4.35 g, 23.00 mmol), corresponding to the desired compound.

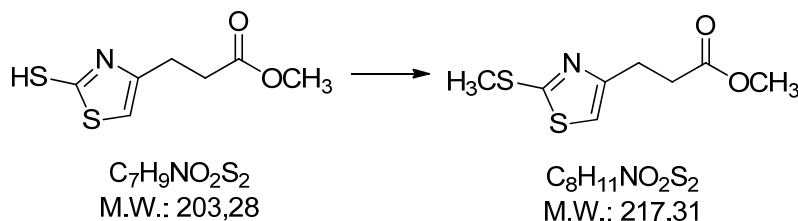
Yield: 91.30 %

Melting point: 191.92 °C

<sup>1</sup>H-NMR (D<sub>2</sub>O): 6.57 (s, 1H), 2.78 (t, 2H, J= 7.15 Hz), 2.62 (t, 2H, J=7.15 Hz).

<sup>158</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 70/ 30  
Rf: 0.05

## Methyl 3-(2-(methylthio)thiazol-4-yl)propanoate



A solution of methyl 3-(2-mercaptothiazol-4-yl)propanoate (2.50 g, 12.30 mmol) in 20 mL of DME/ THF =1/ 1 is added dropwise to a suspension of NaH (0.30 g, 12.30 mmol) in 5 mL of dry DME, under a nitrogen atmosphere.

The mixture is stirred for 10 minutes and then a solution of methyl iodide (1.00 mL, 14.80 mmol) is slowly dropped; the reaction is stirred RT and monitored by TLC<sup>159</sup>.

At completion, 20 ml of Water and 20 ml of Ethyl Acetate are added, the two phases are separated and the aqueous layer is extracted twice with Ethyl Acetate.

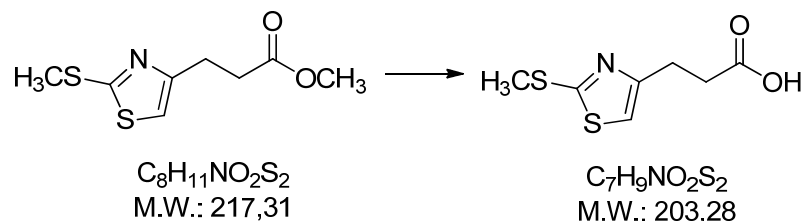
The organic phases are collected, dried over  $Na_2SO_4$ , filtered and concentrated under reduce pressure, yielding 2.54 g (11.69 mmol) of a brown oil.

Yield: 95.04 %

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 6.83 (s, 1H), 3.67 (s, 3H), 3.03 (t, 2H,  $J = 7.70$  Hz), 2.73 (t, 2H,  $J = 7.70$  Hz), 2.66 (s, 3H).

<sup>159</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 70/ 30  
Rf: 0.58

### 3-(2-(methylthio)thiazol-4-yl)propanoic acid



2.54 g (11.69 mmol) of methyl 3-(2-(methylthio)thiazol-4-yl)propanoate are dissolved in 15 mL of Methanol; 7.9 mL of NaOH 2.5 M are dropped and the resulting mixture is stirred at RT for 18 hours.

After completion of the reaction, followed by TLC<sup>160</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with 10% HCl, and then extracted three times with 15 mL of Ethyl Acetate.

The organic phases are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure, obtaining a pale yellow solid (2.27 g, 11.17 mmol), corresponding to the desired compound.

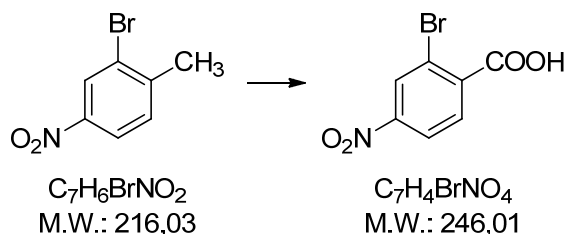
Yield: 95.43 %

Melting point: 96.69 °C

**<sup>1</sup>H-NMR (d<sub>6</sub> DMSO):** 12.14 (s, 1H), 7.18 (s, 1H), 2.85 (t, 2H, *J* = 7.43 Hz), 2.63 (s, 3H), 2.56 (t, 2H, *J* = 7.43 Hz).

<sup>160</sup> TLC conditions: Cyclohexane/ Ethyl Acetate = 70/ 30  
Rf: 0.08

## 2-bromo-4-nitrobenzoic acid



To a stirring solution of 2-bromo-4-nitro-toluene (30 g, 138.9 mmol) in a mixture of Pyridine/ Water 1:1 (300 mL), at 80 °C, it is added solid  $KMnO_4$  (118.9g, 752.3 mmol), in portions of 7 grams, during a period of 3 days.

The progress and conclusion of the reaction is defined using NMR experiment.

The heterogeneous mixture reaction is then cooled, the black solid is filtered and washed with Ethyl Acetate (50 mL); the filtered solution is acidified with HCl 37% and extracted three times with Ethyl Acetate (3\*150 mL).

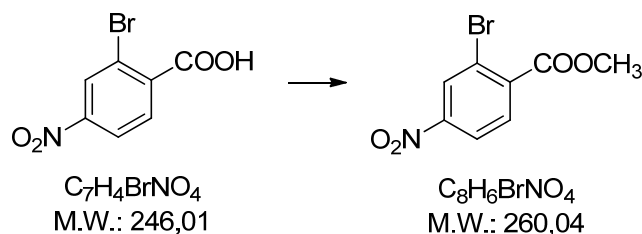
The organic layers are combined, dried over  $Na_2SO_4$ , filtered and concentrated under reduced pressure. The residue is crystallized from isopropyl ether, giving 25.6 g of a white solid, referring to the desired compound.

Yield: 75%

Melting Point: 165.08 °C

**$^1H$ -NMR ( $d_6$ DMSO):** 14.07 (bs, 1H), 8.44 (d, 1H,  $J=2.2$  Hz), 8.26 (dd, 1H,  $J=2.2$  Hz,  $J=8.43$  Hz), 7.92 (d, 1H,  $J=8.43$  Hz).

## Methyl 2-bromo-4-nitrobenzoate



2-bromo-4-nitrobenzoic acid (10.00 g, 40.65 mmol) is dissolved in 35 mL of Methanol, trimethyl orthoformate (7.56 mL, 69.13 mmol) is added and then 1 mL of concentrated H<sub>2</sub>SO<sub>4</sub> is dropped to the reaction.

The mixture is refluxed for 12 hours, monitoring the reaction progress by TLC<sup>161</sup>; the reaction is then cooled, till the desired product precipitate.

The white solid is filtered, washed with fresh Methanol and dried, obtaining 9.40 g of methyl 2-bromo-4-nitrobenzoate.

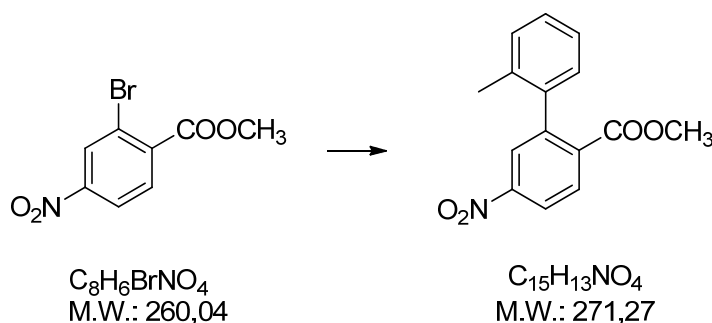
Yield: 89%

Melting Point: 80.77 °C

<sup>1</sup>H-NMR (CDCl<sub>3</sub>): 8.49 (d, 1H, J=2.2 Hz), 8.19 (dd, 1H, J=2.2 Hz, J=8.79 Hz), 7.91 (d, 1H, J=7.89 Hz), 3.98 (s, 3H)

<sup>161</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
Rf= 0.56

## Methyl 2'-methyl-5-nitro-[1,1'-biphenyl]-2-carboxylate



To a sonicated solution of the methyl 2-bromo-4-nitrobenzoate (1.00 g, 3.85 mmol) in 6 mL of Toluene, under a nitrogen atmosphere,  $Pd(PPh_3)_4$  (0.44 g, 0.385 mmol) is added as solid.

The solution is stirred for 10 minutes and then amount of a solution of  $K_2CO_3$  (1.60 g, 11.55 mmol) in 2 mL of Water and then of a solution of o-tolylboronic acid (0.63 g, 4.62 mmol) in 1 mL of Methanol.

The reaction mixture is heated at 80°C for 18 hours and monitored by TLC<sup>162</sup>; at completion it is cooled to RT.

The mixture is treated with Ethyl Acetate and brine, the organic phase is dried over  $Na_2SO_4$ , filtered and concentrated under reduced pressure.

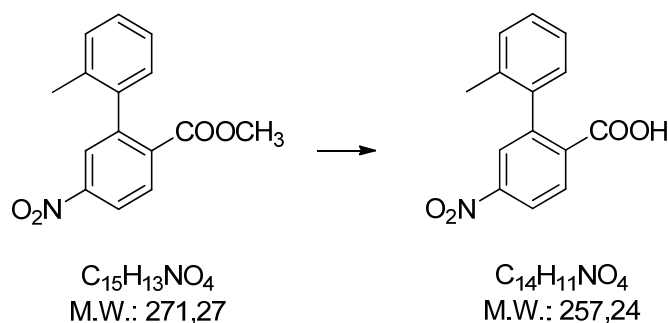
Purification by chromatography on silica gel, through elution with Cyclohexane/ Ethyl Acetate= 90/ 10, yields 0.86 g (3.16 mmol) of the desired product, as yellow solid.

Yield: 82%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 8.26 (dd, 1H,  $J=2.47$  Hz,  $J=8.52$  Hz), 8.15 (m, 2H), 7.26 (m, 3H), 7.07 (d, 1H,  $J=7.70$  Hz), 3.65 (s, 3H), 2.13 (s, 3H).

<sup>162</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 80/ 20  
Rf= 0.57

## 2'-methyl-5-nitro-[1,1'-biphenyl]-2-carboxylic acid



0.86 g (3.16 mmol) of methyl 2'-methyl-5-nitro-[1,1'-biphenyl]-2-carboxylate are dissolved in 10 mL of Methanol; 2.5 mL of NaOH 2.5 M are dropped and the resulting mixture is stirred at RT for 18 hours.

After completion of the reaction, followed by TLC<sup>163</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with 10% HCl, and then extracted three times with 10 mL of Ethyl Acetate.

The organic phases are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure, obtaining a pale green solid (0.78 g, 3.03 mmol), corresponding to the desired compound.

Yield: 96%

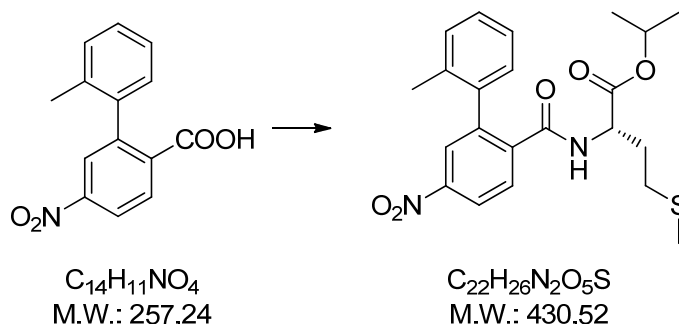
Melting point: 166.30 ° C

<sup>1</sup>H-NMR (CDCl<sub>3</sub>): 8.26 (dd, 1H, *J*=2.2 Hz, *J*=8.52 Hz), 8.14 (m, 2H), 7.27 (m, 3H), 7.07 (d, 1H, *J*=7.42 Hz), 2.09 (s, 3H).

<sup>163</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 80/ 20  
R<sub>f</sub>= 0.13



**(S)-isopropyl 2-(2'-methyl-5-nitro-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoate**



A solution of EDAC (0.69 g, 3.63 mmol) and DIPEA (0.63 mL, 3.63 mmol) in 5 mL of dry DMF is added dropwise to a solution of 2'-methyl-5-nitro-[1,1'-biphenyl]-2-carboxylic acid (0.78 g, 3.03 mmol), HOBT (0.49 g, 3.63 mmol) in 8 mL of dry DMF, under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of L-Methionine isopropyl ester (0.58 g, 3.03 mmol) in 5 mL of dry DMF is dropped.

The reaction mixture is stirred at RT overnight; after completion, monitored by TLC<sup>164</sup>, the mixture is concentrated, resumed with Ethyl Acetate, washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

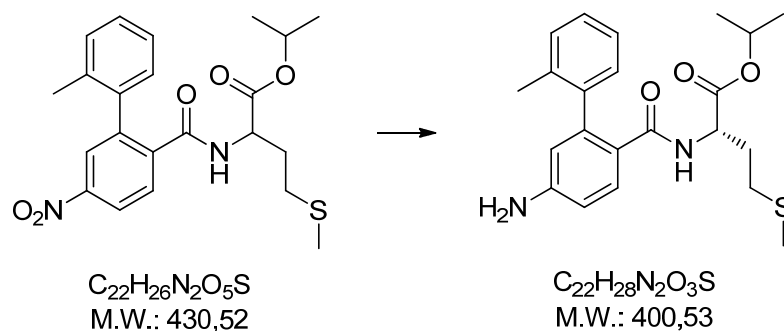
The crude product is purified by column chromatography on silica gel, using Cyclohexane/ Ethyl Acetate= 70/30 as elution solvent, to give 1.23 g (2.85 mmol) of the desired product, as yellow oil.

Yield: 94%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 8.27 (dd, 1H, *J*=2.48 Hz, *J*=8.53 Hz), 8.05 (m, 2H), 7.30 (m, 3H), 7.17 (d, 1H, *J*=6.61 Hz), 6.03 (d, 1H, *J*=7.43 Hz), 4.94 (m, 1H), 4.54 (m, 1H), 2.20 (s, 1H), 2.09 (s, 1H), 2.01 (s, 3H), 1.85 (m, 1H), 1.60 (m, 1H), 1.41 (s, 3H), 1.18 (m, 6H).

<sup>164</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 60/ 40  
 R<sub>f</sub> = 0.69

**(S)-isopropyl 2-(5-amino-2'-methyl-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoate**



1.23 g of (S)-isopropyl-2-(2'-methyl-5-nitro-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoate (2.85 mmol) are dissolved in 37 mL of Ethyl Acetate and 1 mL of Water.

Stannous chloride (2.70 g, 14.25 mmol) is added and the mixture is refluxed for 3 hours.

After completion of the reaction, defined by TLC<sup>165</sup>, the mixture reaction is firstly cooled and poured into 100 mL of iced 5% NaHCO<sub>3</sub>, then it is filtered over Celite and the two layers are separated.

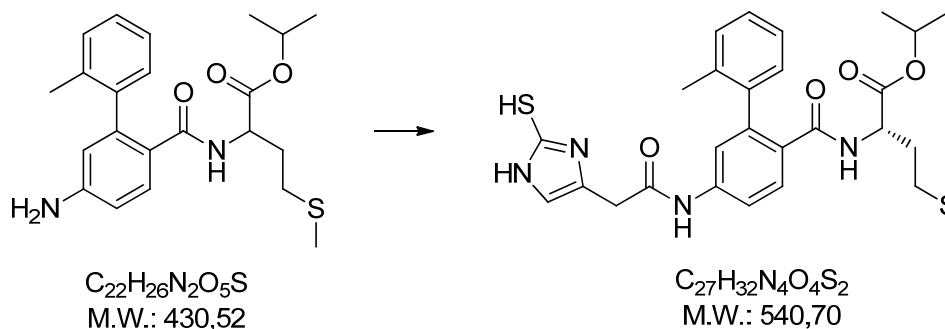
The organic layer is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure, giving 1.08 g (2.71 mmol) of a colourless oil.

Yield: 95%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 7.90 (m, 1H), 7.28 (m, 3H), 7.15 (d, 1H, *J* = 7.88 Hz), 6.95 (d, 1H, *J* = 8.53 Hz), 6.78 (s, 1H), 5.85 (m, 1H), 4.95 (m, 1H), 4.55 (m, 1H), 2.20 (s, 2H), 2.04 (s, 3H), 2.02 (s, 3H), 1.80 (m, 1H), 1.57 (m, 1H), 1.20 (m, 6H).

<sup>165</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
 R<sub>f</sub>= 0.35

**(S)-isopropyl 2-(5-(2-(2-mercapto-1H-imidazol-4-yl)acetamido)-2'-methyl-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoate**



A solution of EDAC (0.31 g, 1.60 mmol) and DIPEA (0.29 mL, 1.60 mmol) in 2 mL of dry DMF is added dropwise to a solution of 2-(2-mercapto-1H-imidazol-4-yl)acetic acid (0.21 g, 1.33 mmol), HOBT (0.22 g, 1.60 mmol) in 3 mL of dry DMF, under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then (S)-isopropyl-2-(5-amino-2'-methyl-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoate (0.53 g, 1.33 mmol) in 3 mL of dry DMF is dropped.

The reaction mixture is stirred at RT overnight; after completion, monitored by TLC<sup>166</sup>, the DMF is removed, the residue is resumed with Ethyl Acetate, washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

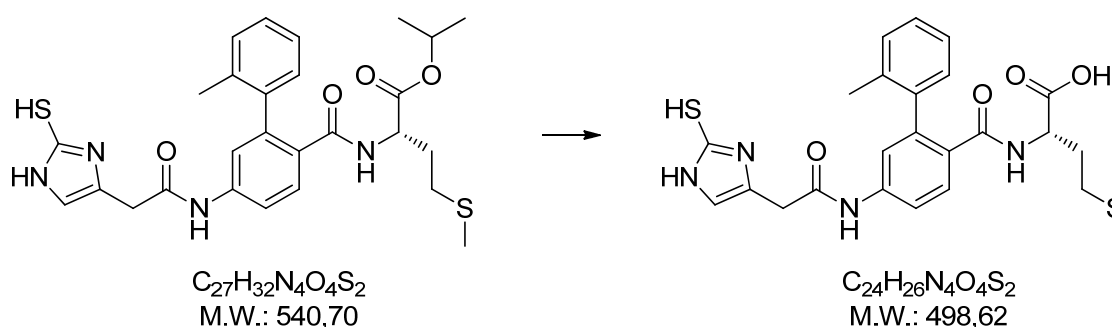
The crude product is purified by column chromatography on silica gel, using DCM/ Methanol= 95/ 5 as elution solvent, to give 0.29 g (0.53 mmol) of the desired product, as brown oil.

Yield: 40%

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 11.87 (bs, 1H), 11.73 (bs, 1H), 10.23 (bs, 1H), 8.07 (d, 1H, *J*=7.15 Hz), 7.61 (dd, 1H, *J*=3.30 Hz, *J*=5.50 Hz), 7.45 (m, 2H), 7.14 (m, 4H), 6.64 (s, 1H), 4.85 (m, 1H), 4.19 (m, 1H), 3.51 (s, 2H), 2.12 (m, 1H), 2.09 (s, 3H), 1.94 (s, 3H), 1.75 (m, 2H), 1.21 (s, 1H), 1.14 (m, 6H).

<sup>166</sup> TLC conditions: DCM/ Methanol= 90/ 10  
 R<sub>f</sub> = 0.60

**(S)-2-(5-(2-(2-mercapto-1H-imidazol-4-yl)acetamido)-2'-methyl-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoic acid**



0.29 g (0.53 mmol) of (S)-isopropyl 2-(5-(2-(2-mercapto-1H-imidazol-4-yl)acetamido)-2'-methyl-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoate are dissolved in 5 mL of Methanol; 0.53 mL of NaOH 1 M are dropped and the resulting mixture is stirred at RT for 15 hours.

After completion of the reaction, followed by TLC<sup>167</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with Formic Acid, till a white solid starts precipitation.

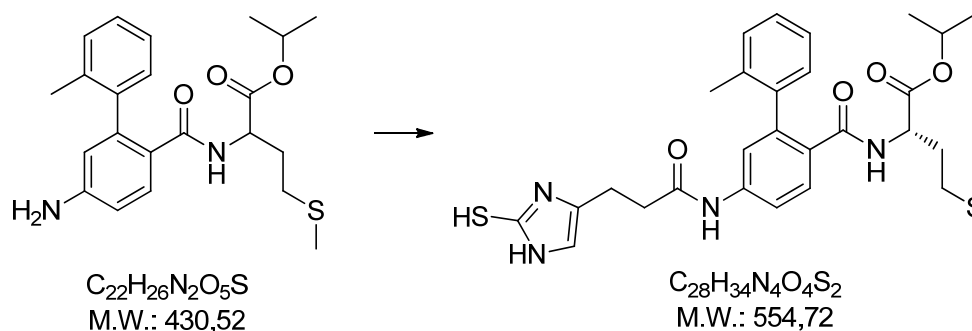
The filtration yields a sticky solid (0.26 g, 0.53 mmol), corresponding to the desired compound.

Yield: quantitative

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 11.89 (bs, 1H), 11.74 (bs 1H), 10.28 (s, 1H), 7.81–7.77 (m, 1H), 7.60–7.58 (m, 1H), 7.51–7.49 (m, 2H), 7.22–7.01 (m, 4 H), 6.60 (s, 1H), 4.18–4.10 (m, 1H), 3.76 (s, 2H), 2.20–1.88 (m, 6H), 1.76–1.60 (m, 4H).

<sup>167</sup> TLC conditions: DCM/ Methanol= 90/ 10  
Rf= basal

**(S)-isopropyl 2-(5-(3-(2-mercapto-1H-imidazol-4-yl)propanamido)-2'-methyl-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoate**



A solution of EDAC (0.63 g, 3.28 mmol) and DIPEA (0.57 mL, 3.28 mmol) in 4 mL of dry DMF is added dropwise to a solution of 2-(2-mercapto-1H-imidazol-4-yl)propanoic acid (0.47 g, 2.73 mmol), HOBT (0.44 g, 3.28 mmol) in 5 mL of dry DMF, under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then (S)-isopropyl-2-(5-amino-2'-methyl-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoate (0.47 g, 2.73 mmol) in 5 mL of dry DMF is dropped.

The reaction mixture is stirred at RT overnight; after completion, monitored by TLC<sup>168</sup>, the DMF is removed, the residue is resumed with Ethyl Acetate, washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

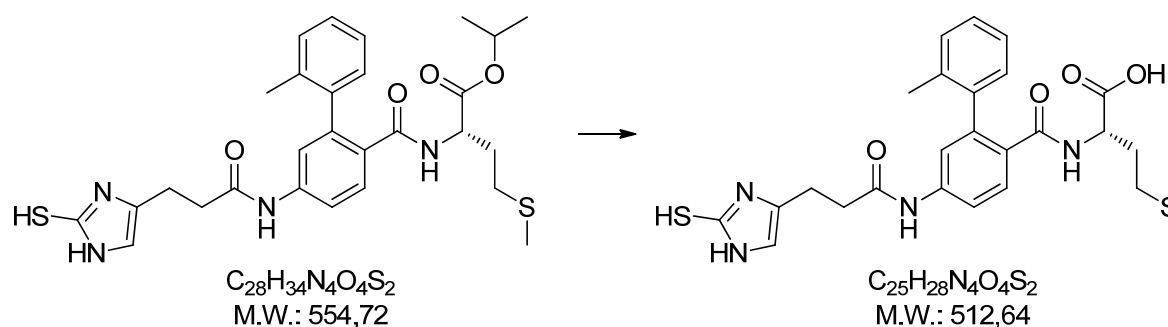
The crude product is purified by column chromatography on silica gel, using DCM/ Methanol= 95/ 5 as elution solvent, to give 0.45 g (0.82 mmol) of the desired product, as pink oil.

Yield: 30%

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 11.85 (bs, 1H), 11.61 (bs, 1H), 10.10 (bs, 1H), 8.02 (d, 1H, *J*=7.25 Hz), 7.93 (d, 1H, *J*=8.25 Hz), 7.65 (d, 1H, *J*= 8.25 Hz), 7.45 (m, 2H), 7.16 (m, 3H), 6.48 (s, 1H), 4.83 (m, 1H), 4.19 (m, 1H), 2.61 (m, 4H), 2.04 (m, 5H), 1.94 (s, 3H), 1.75 (m, 2H), 1.14 (m, 6H).

<sup>168</sup> TLC conditions: DCM/ Methanol= 95/ 5  
 R<sub>f</sub> = 0.13

**(S)-2-(5-(3-(2-mercapto-1H-imidazol-4-yl)propanamido)-2'-methyl-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoic acid**



0.45 g (0.82 mmol) of (S)-isopropyl 2-(5-(3-(2-mercapto-1H-imidazol-4-yl)propanamido)-2'-methyl-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoate are dissolved in 5 mL of Methanol; 0.82 mL of NaOH 1 M are dropped and the resulting mixture is stirred at RT for 15 hours.

After completion of the reaction, followed by TLC<sup>169</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with Formic Acid, till a white solid starts precipitation.

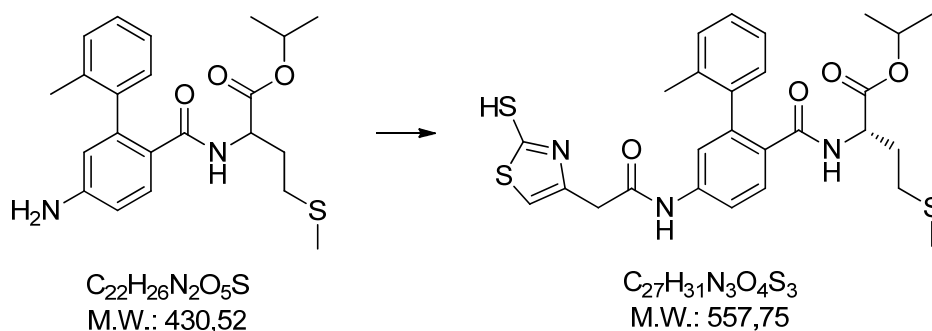
The filtration yields a sticky solid (0.42 g, 0.82 mmol), corresponding to the desired compound.

Yield: quantitative

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 11.88 (bs, 1H), 11.64 (1H), 10.15 (s, 1H), 7.59–7.63 (m, 4H), 7.22–7.05 (m, 4H), 6.49 (s, 1H), 4.10–3.98 (m, 1H), 2.66–2.58 (m, 4H), 2.20–1.88(m, 6H), 1.76–1.58 (m, 4H).

<sup>169</sup> TLC conditions: DCM/ Methanol= 95/ 5  
 Rf= basal

**(S)-isopropyl 2-(5-(2-(2-mercaptothiazol-4-yl)acetamido)-2'-methyl-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoate**



A solution of EDAC (0.26 g, 1.36 mmol) and DIPEA (0.24 mL, 1.36 mmol) in 2 mL of dry DMF is added dropwise to a solution of 2-(2-mercaptothiazol-4-yl)acetic acid (0.20 g, 1.13 mmol), HOBT (0.18 g, 1.36 mmol) in 3 mL of dry DMF, under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then (S)-isopropyl-2-(5-amino-2'-methyl-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoate (0.49 g, 1.13 mmol) in 3 mL of dry DMF is dropped.

The reaction mixture is stirred at RT overnight; after completion, monitored by TLC<sup>170</sup>, the DMF is removed, the residue is resumed with Ethyl Acetate, washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

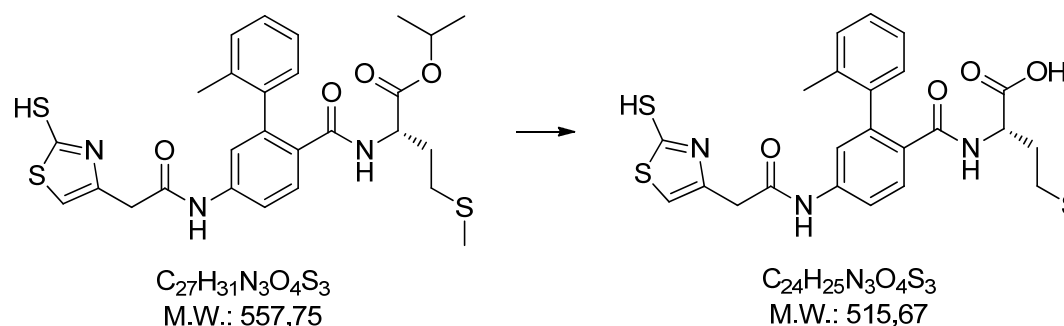
The crude product is purified by column chromatography on silica gel, using Cyclohexane/ Ethyl Acetate= 1/ 1 as elution solvent, to give 0.29 g (0.52 mmol) of the desired product, as red oil.

Yield: 46%

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 10.32 (bs, 1H), 8.08 (d, 1H, *J*=7.97 Hz), 7.62 (d, 1H, *J*=1.93 Hz), 7.52 (m, 2H), 7.16 (m, 4H), 6.73 (s, 1H), 4.84 (m, 1H), 4.19 (m, 1H), 3.65 (s, 2H), 2.15 (m, 2H), 2.12 (s, 3H), 1.94 (s, 3H), 1.76 (m, 2H), 1.14 (m, 6H).

<sup>170</sup> TLC conditions: DCM/ Methanol= 95/ 5  
 R<sub>f</sub> = 0.30

**(S)-2-(5-(2-(2-mercaptothiazol-4-yl)acetamido)-2'-methyl-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoic acid**



0.29 g (0.52 mmol) of (S)-isopropyl 2-(5-(2-(2-mercaptothiazol-4-yl)acetamido)-2'-methyl-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoate are dissolved in 5 mL of Methanol; 0.52 mL of NaOH 1 M are dropped and the resulting mixture is stirred at RT for 18 hours.

After completion of the reaction, followed by TLC<sup>171</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with Formic Acid, till a white solid starts precipitation.

The filtration yields a sticky solid (0.27 g, 0.52 mmol), corresponding to the desired compound.

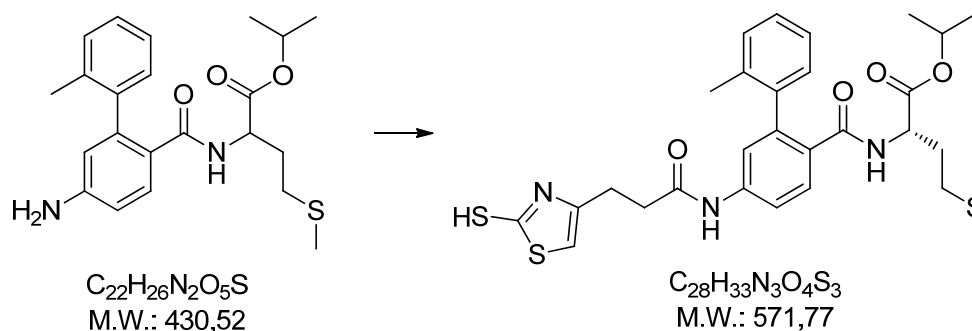
Yield: quantitative

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 10.35 (s, 1H), 7.62–7.51 (m, 2H), 7.42 (s, 1H), 7.19–7.03 (m, 4H), 6.63–6.58 (m, 2H), 4.14 (m, 1H), 3.60 (s, 2H), 2.21–1.98 (m, 6H), 1.82–1.58 (m, 4H).

<sup>171</sup> TLC conditions: DCM/ Methanol= 95/ 5  
R<sub>f</sub>= basal



**(S)-isopropyl 2-(5-(3-(2-mercaptothiazol-4-yl)propanamido)-2'-methyl-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoate**



A solution of EDAC (0.38 g, 1.98 mmol) and DIPEA (0.34 mL, 1.98 mmol) in 3 mL of dry DMF is added dropwise to a solution of 2-(2-mercaptothiazol-4-yl)propanoic acid (0.32 g, 1.65 mmol), HOBT (0.27 g, 1.98 mmol) in 5 mL of dry DMF, under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then (S)-isopropyl-2-(5-amino-2'-methyl-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoate (0.66 g, 1.65 mmol) in 3 mL of dry DMF is dropped.

The reaction mixture is stirred at RT overnight; after completion, monitored by TLC<sup>172</sup>, the DMF is removed, the residue is resumed with Ethyl Acetate, washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

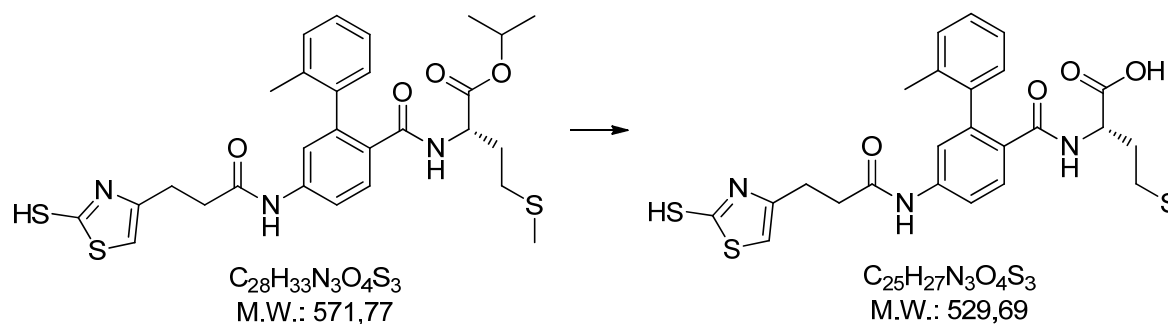
The crude product is purified by column chromatography on silica gel, using Cyclohexane/ Ethyl Acetate= 1/ 1 as elution solvent, to give 0.31 g (0.54 mmol) of the desired product, as yellow oil.

Yield: 33%

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 10.16 (bs, 1H), 8.04 (d, 1H, *J*=6.87 Hz), 7.60 (dd, 1H, *J*=1.93 Hz, *J*=8.53 Hz), 7.46 (m, 2H), 7.15 (m, 4H), 6.54 (s, 1H), 4.84 (m, 1H), 4.19 (m, 1H), 2.74 (m, 2H), 2.66 (m, 2H), 2.10 (s, 3H), 2.06 (m, 1H), 1.98 (s, 3H), 1.75 (m, 2H), 1.14 (m, 6H).

<sup>172</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/ 1  
 R<sub>f</sub> = 0.15

**(S)-2-(5-(3-(2-mercaptothiazol-4-yl)propanamido)-2'-methyl-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoic acid**



0.31 g (0.54 mmol) of (S)-isopropyl 2-(5-(3-(2-mercaptothiazol-4-yl)propanamido)-2'-methyl-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoate are dissolved in 5 mL of Methanol; 0.54 mL of NaOH 1 M are dropped and the resulting mixture is stirred at RT for 18hours.

After completion of the reaction, followed by TLC<sup>173</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with Formic Acid, till a white solid starts precipitation.

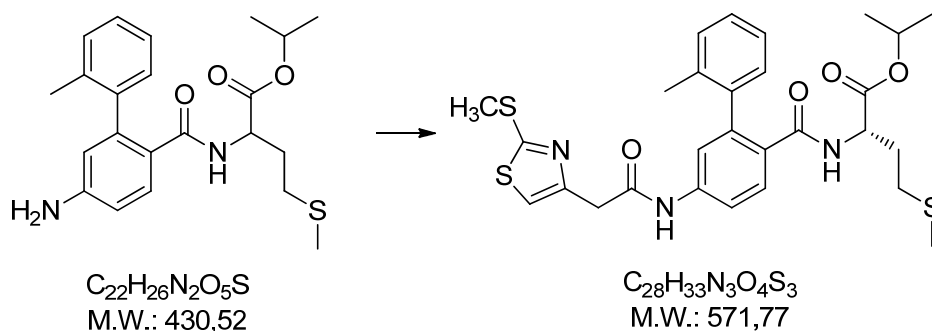
The filtration yields a sticky solid (0.29 g, 0.54 mmol), corresponding to the desired compound.

Yield: quantitative

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 10.21 (s, 1H), 7.97–7.85 (m, 1H), 7.60–7.52 (m, 1H), 7.50–7.39 (m, 1H), 7.22–7.01 (m, 4H), 6.54 (s, 1H), 6.22 (br s, 1H), 4.21–4.12 (m, 1H), 2.81–2.62 (m, 4H), 2.16–1.63 (m, 10H).

<sup>173</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/ 1  
 R<sub>f</sub>= basal

**(S)-isopropyl 2-(2'-methyl-5-(2-(2-(methylthio)thiazol-4-yl)acetamido)-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoate**



A solution of EDAC (0.55 g, 2.86 mmol) and DIPEA (0.50 mL, 2.86 mmol) in 5 mL of dry DMF is added dropwise to a solution of 2-(2-(methylthio)thiazol-4-yl)acetic acid (0.45 g, 2.38 mmol), HOBT (0.38 g, 2.86 mmol) in 8 mL of dry DMF, under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then (S)-isopropyl-2-(5-amino-2'-methyl-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoate (1.02 g, 2.38 mmol) in 8 mL of dry DMF is dropped.

The reaction mixture is stirred at RT overnight; after completion, monitored by TLC<sup>174</sup>, the DMF is removed, the residue is resumed with Ethyl Acetate, washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

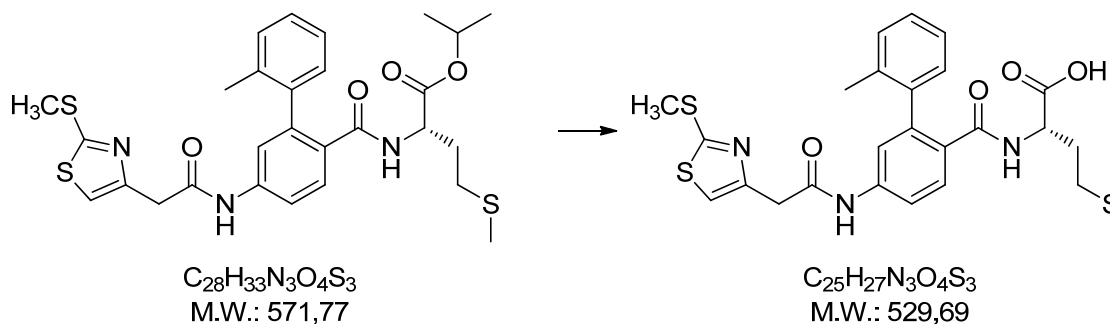
The crude product is purified by column chromatography on silica gel, using Cyclohexane/ Ethyl Acetate= 1/ 1 as elution solvent, to give 0.30 g (0.52 mmol) of the desired product, as red oil.

Yield: 22%

<sup>1</sup>H-NMR (d<sub>6</sub>DMSO): 10.35 (bs, 1H), 8.08 (d, 1H, *J*=6.72 Hz), 7.62 (m, 1H), 7.49 (m, 2H), 7.36 (m, 1H), 7.15 (m, 4H), 4.84 (m, 1H), 4.18 (m, 1H), 3.76 (s, 2H), 2.63 (s, 3H), 2.05 (s, 3H), 2.03 (m, 2H), 2.02 (s, 3H), 1.75 (m, 2H), 1.14 (m, 6H).

<sup>174</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/ 1  
 R<sub>f</sub> = 0.29

**(S)-2-(2'-methyl-5-(2-(2-(methylthio)thiazol-4-yl)acetamido)-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoic acid**



0.30 g (0.52 mmol) of (S)-isopropyl 2-(2'-methyl-5-(2-(2-(methylthio)thiazol-4-yl)acetamido)-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoate are dissolved in 5 mL of Methanol; 0.52 mL of NaOH 1 M are dropped and the resulting mixture is stirred at RT for 18 hours.

After completion of the reaction, followed by TLC<sup>175</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with Formic Acid, till a white solid starts precipitation.

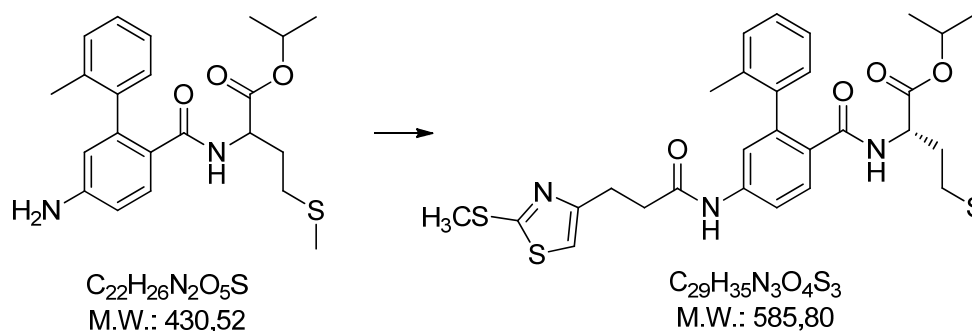
The filtration yields a sticky solid (0.28 g, 0.52 mmol), corresponding to the desired compound.

Yield: quantitative

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 10.38 (s, 1H), 7.78–7.68 (br s, 1H), 7.59–7.51 (m, 1H), 7.48–7.41 (m, 1H), 7.36 (s, 1H), 7.18–7.03 (m, 4H), 4.09 (m, 1H), 3.76 (s, 2H), 2.63 (s, 3H), 2.20–1.98 (m, 6H), 1.85–1.58 (m, 4H).

<sup>175</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/ 1  
Rf= basal

**(S)-isopropyl 2-(2'-methyl-5-(3-(2-(methylthio)thiazol-4-yl)propanamido)-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoate**



A solution of EDAC (0.42 g, 2.18 mmol) and DIPEA (0.38 mL, 2.18 mmol) in 5 mL of dry DMF is added dropwise to a solution of 3-(2-(methylthio)thiazol-4-yl)propanoic acid (0.37 g, 1.82 mmol), HOBT (0.30 g, 2.18 mmol) in 8 mL of dry DMF, under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then (S)-isopropyl-2-(5-amino-2'-methyl-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoate (0.78 g, 1.82 mmol) in 5 mL of dry DMF is dropped.

The reaction mixture is stirred at RT overnight; after completion, monitored by TLC<sup>176</sup>, the DMF is removed, the residue is resumed with Ethyl Acetate, washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

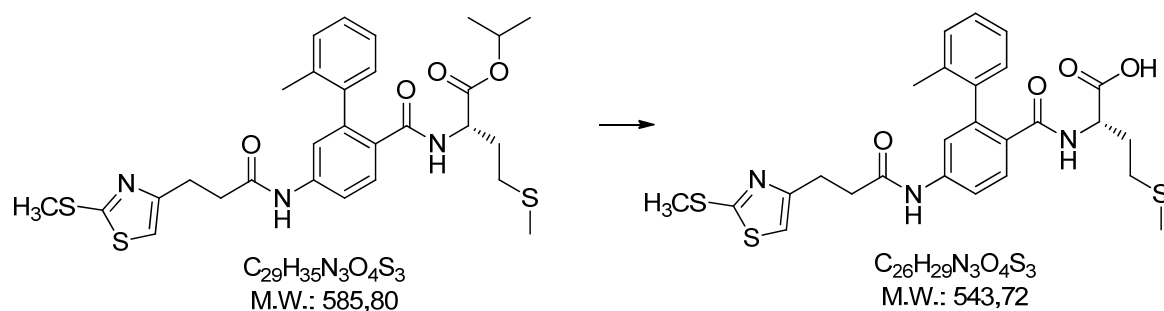
The crude product is purified by column chromatography on silica gel, using Cyclohexane/ Ethyl Acetate= 60/ 40 as elution solvent, to give 0.16 g (0.27 mmol) of the desired product, as yellow oil.

Yield: 15%

<sup>1</sup>H-NMR (d<sub>6</sub>DMSO): 10.13 (bs, 1H), 8.03 (d, 1H, *J*=6.88 Hz), 7.61 (d, 1H, *J*=6.90 Hz), 7.46 (m, 2H), 7.15 (m, 5H), 4.83 (m, 1H), 4.18 (m, 1H), 2.95 (t, 2H, *J*= 7.15 Hz), 2.66 (t, 2H, *J*= 7.15 Hz), 2.62 (s, 3H), 2.07 (m, 8H), 1.74 (m, 2H), 1.15 (m, 6H).

<sup>176</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 60/ 40  
 R<sub>f</sub> = 0.19

**(S)-2-(2'-methyl-5-(3-(2-(methylthio)thiazol-4-yl)propanamido)-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoic acid**



0.16 g (0.27 mmol) of (S)-isopropyl 2-(2'-methyl-5-(2-(3-(methylthio)thiazol-4-yl)propanamido)-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoate are dissolved in 5 mL of Methanol; 0.27 mL of NaOH 1 M are dropped and the resulting mixture is stirred at RT for 18 hours.

After completion of the reaction, followed by TLC<sup>177</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with Formic Acid, till a white solid starts precipitation.

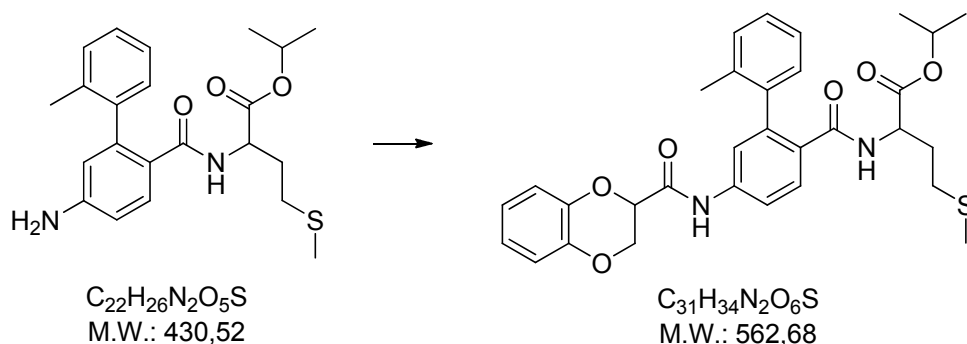
The filtration yields a sticky solid (0.15 g, 0.27 mmol), corresponding to the desired compound.

Yield: quantitative

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 10.17 (s, 1H), 8.27 (s, 1H), 7.60–7.57 (m, 1H), 7.50–7.44 (m, 2H), 7.33 (br s, 1H), 7.18–7.12 (m, 4H), 3.94–3.85 (m, 1H), 2.96–2.91 (m, 2H), 2.71–2.63 (m, 2H), 2.62 (s, 3H), 2.10–1.91 (m, 6H), 1.70–1.58 (m, 4H).

<sup>177</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 60/ 40  
Rf= basal

## Isopropyl 2-(5-(2,3-dihydrobenzo[b][1,4]dioxine-2-carboxamido)-2'-methyl-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoate



A solution of ((S)-isopropyl-2-(5-amino-2'-methyl-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoate (0.62 g, 1.56 mmol) and DIPEA (0.35 mL, 2.03 mmol) in 6 mL of DCM is stirred under a nitrogen atmosphere.

The resulting mixture is cooled to 0°C and then amount of a solution of 2,3-dihydrobenzo[b][1,4]dioxine-2-carbonyl chloride (0.40 g, 2.03 mmol) in 3 mL of DCM.

The reaction mixture is stirred at RT for 1 hour; after completion, monitored by TLC<sup>178</sup>, the mixture is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

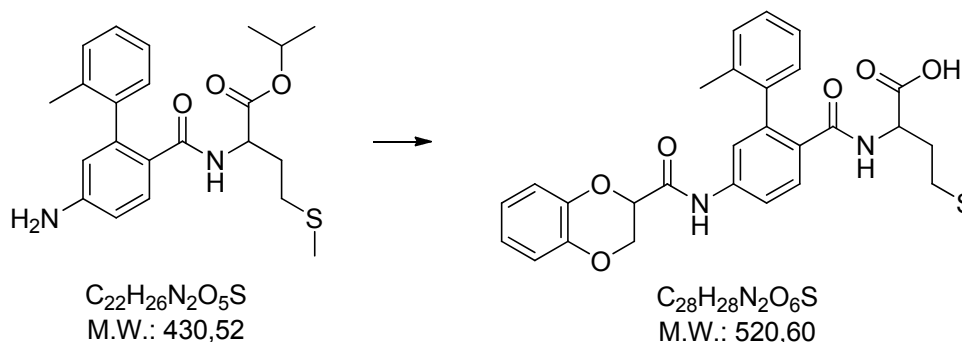
The crude is purified on silica gel, using Cyclohexane/ Ethyl Acetate= 85/15 as elution solvent, obtaining 0.82 g (1.45 mmol) of the desired product, as sticky white solid.

Yield: 93%

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 10.30 (bs, 1H), 8.16 (bs, 1H), 7.70 (dd, 1H, *J*=2.2 Hz, *J*=8.53 Hz), 7.50 (m, 2H), 6.99-7.19 (m, 5H), 6.87 (m, 1H), 4.99 (dd, 1H, *J*=2.75 Hz, *J*=5.5 Hz), 4.84 (m, 1H), 4.44 (dd, 1H, *J*=2.75 Hz, *J*=11.55 Hz), 4.35 (dd, 1H, *J*=5.5 Hz, *J*=11.55 Hz), 4.20 (m, 1H), 2.17 (m, 2H), 1.94 (s, 3H), 1.76 (m, 2H), 1.14 (m, 6H).

<sup>178</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 70/30  
R<sub>f</sub> = 0.44

**2-(5-(2,3-dihydrobenzo[b][1,4]dioxine-2-carboxamido)-2'-methyl-[1,1'-biphenyl]-2-ylcarboxamido)-4-(methylthio)butanoic acid**



0.82 g of (2S)-isopropyl 2-(4-(2,3-dihydrobenzo[b][1,4]dioxine-2-carboxamido)-2-(thiophen-2-yl)benzamido)-4-(methylthio)butanoate (1.45 mmol) are dissolved in 10 mL of Methanol; 1.45 mL of NaOH 1M are dropped and the resulting mixture is stirred at RT overnight.

After completion of the reaction, followed by TLC<sup>179</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with Formic acid till a white precipitate is formed.

The solid filtration yields 0.53 g (1.02 mmol) of the final product, as a sticky solid.

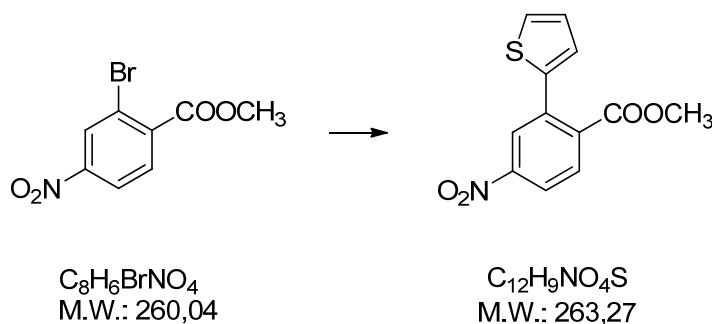
Yield: 70%

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 12.61 (bs, 1H), 10.30 (s, 1H), 8.05 (bs, 1H), 7.70 (dd, 1H, *J*=2.2 Hz, *J*=8.26 Hz), 7.51 (m, 2H), 7.00-7.53 (m, 4H), 6.86 (m, 3H), 4.99 (dd 1H, *J*=2.75 Hz, *J*=5.5 Hz), 4.44 (dd, 1H, *J*=2.75 Hz, *J*=11.55 Hz), 4.35 (dd 3H, *J*=5.5 Hz, *J*=11.55 Hz), 4.21 (m, 1H), 2.14 (m, 2H), 1.94 (s, 3H), 1.76 (m, 2H).

<sup>179</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 70/30  
R<sub>f</sub> = basal



## Methyl 4-nitro-2-(thiophen-2-yl)benzoate



To a sonicated solution of the methyl 2-bromo-4-nitrobenzoate (1.00 g, 3.85 mmol) in 5 mL of dry DMF, under a nitrogen atmosphere,  $Pd(PPh_3)_4$  (0.44 g, 0.385 mmol) is added as solid.

The solution is stirred for 10 minutes and then amount of  $Na_3PO_4 \cdot 12H_2O$  (4.39 g, 11.55 mmol) and of a solution of 2-thienylboronic acid (0.59 g, 4.62 mmol) in 5 mL of dry DMF.

The reaction mixture is heated at 100 °C for 18 hours, monitored by TLC<sup>180</sup> and then concentrated.

The crude is resumed with Ethyl Acetate, washed with brine, dried over  $Na_2SO_4$ , filtered and concentrated under reduced pressure.

Purification by chromatography on silica gel, through elution with Cyclohexane/ Ethyl Acetate= 78/24, yields 0.68 g (2.58 mmol) of the desired product, as yellow solid.

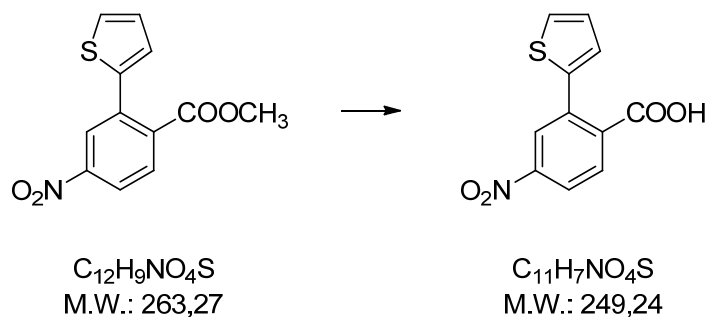
Yield: 67%

Melting point: 98.81 ° C

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 8.35 (d, 1H,  $J=2.2$  Hz), 8.22 (ddd, 1H,  $J=0.55$  Hz,  $J=2.20$  Hz,  $J=8.53$  Hz), 7.83 (d, 1H,  $J=8.53$  Hz), 7.45 (ddd, 1H,  $J=0.55$  Hz,  $J=1.38$  Hz,  $J=4.95$  Hz), 7.09-7.15 (m, 1H), 3.80 (s, 3H).

<sup>180</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
Rf= 0.49

## 4-nitro-2-(thiophen-2-yl)benzoic acid



0.68 g of methyl 4-nitro-2-(thiophen-2-yl)benzoate (2.58 mmol) are dissolved in 10 mL of Methanol; 2 mL of NaOH 2.5 M are dropped and the resulting mixture is heated at 60 °C for 2 hours.

After completion of the reaction, followed by TLC<sup>181</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with 10% HCl, and then extracted three times with 10 mL of Ethyl Acetate.

The organic phases are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure, obtaining a yellow solid (0.64 g, 2.58 mmol), corresponding to the desired compound.

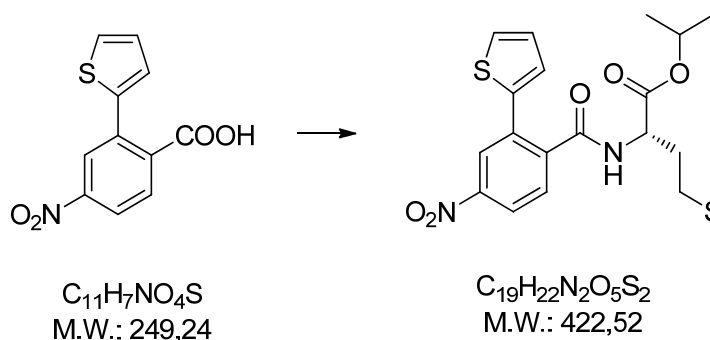
Yield: quantitative

Melting point: 136.19 ° C

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 8.26 (d, 1H, *J*=0.83 Hz), 8.25 (m, 1H), 8.01 (d, 1H, *J*=8.52 Hz), 7.46 (m, 1H), 7.20 (dd, 1H, *J*=0.83 Hz, *J*=2.75 Hz), 7.12 (dd, 1H, *J*=0.83 Hz, *J*=3.56 Hz).

<sup>181</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
R<sub>f</sub> = 0.26

**(S)-isopropyl 4-(methylthio)-2-(4-nitro-2-(thiophen-2-yl)benzamido)butanoate**



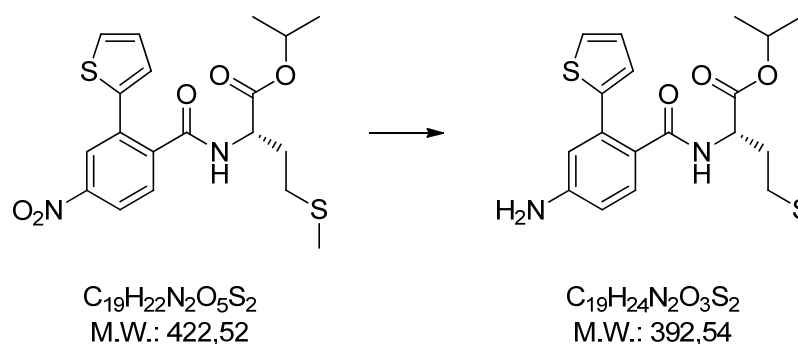
A solution of EDAC (0.59 g, 3.10 mmol) and DIPEA (0.54 mL, 3.10 mmol) in 2 mL of DCM is added dropwise to a solution of 4-nitro-2-(thiophen-2-yl)benzoic acid (0.64 g, 2.58 mmol), HOBT (0.42 g, 3.10 mmol) in 3 mL of DCM, under a nitrogen atmosphere. The resulting mixture is stirred for 30 minutes at RT and then a solution of L-Methionine isopropyl ester (0.49 g, 2.58 mmol) in 3 mL of DCM is dropped. The reaction mixture is stirred at RT overnight; after completion, monitored by TLC<sup>182</sup>, the mixture is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated. The crude product is purified by column chromatography on silica gel, using Cyclohexane/ Ethyl Acetate= 70/30 as elution solvent, to give 0.76 g (1.81 mmol) of the desired product, as yellow oil.

Yield: 70%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 8.33 (d, 1H, *J*=1.1 Hz), 8.22 (dd, 1H, *J*=2.2 Hz, *J*=8.53 Hz), 7.76 (dd, 1H, *J*=1.1 Hz, *J*=8.53 Hz), 7.46 (dd, 1H, *J*=1.37 Hz, *J*=5.22 Hz), 7.27 (d, 1H, *J*=1.37 Hz), 7.12 (dd, 1H, *J*=3.57 Hz, *J*=5.22 Hz), 6.33 (d, 1H, *J*=7.42 Hz), 5.02 (m, 1H), 4.72 (m, 1H), 2.24 (t, 2H, *J*=7.15 Hz), 2.06 (m, 1H), 2.04 (s, 3H), 1.91 (m, 1H), 1.26 (d, 3H, *J*=6.60 Hz), 1.24 (d, 3H, *J*=6.60 Hz).

<sup>182</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
 R<sub>f</sub> = 0.54

**(S)-isopropyl 2-(4-amino-2-(thiophen-2-yl)benzamido)-4-(methylthio)butanoate**



(S)-isopropyl 4-(methylthio)-2-(4-nitro-2-(thiophen-2-yl)benzamido)butanoate (0.76 g, 1.81 mmol) is dissolved in 23 mL of Ethyl Acetate and 1 mL of Water. Stannous chloride (1.72 g, 9.05 mmol) is added and the mixture is refluxed for 3 hours.

After completion of the reaction, defined by TLC<sup>183</sup>, the mixture reaction is firstly cooled and poured into 50 mL of iced 5% NaHCO<sub>3</sub>, then it is filtered over Celite and the two layers are separated.

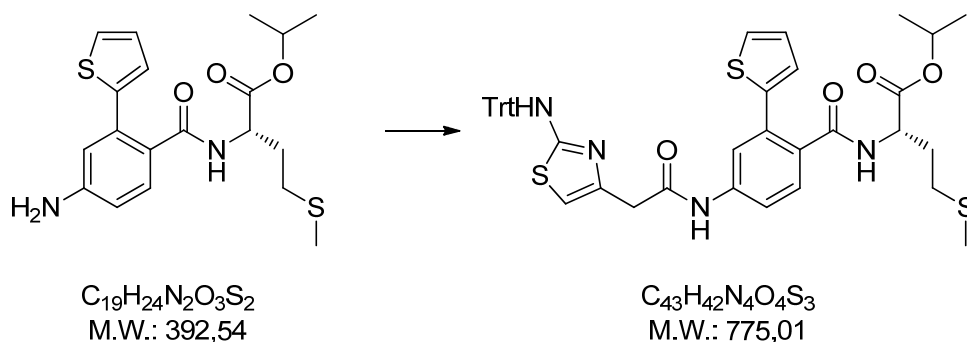
The organic layer is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure, giving 0.61 g (1.56 mmol) of a colourless oil.

Yield: 86%

<sup>1</sup>H-NMR (CDCl<sub>3</sub>): 7.56 (dd, 1H, *J*=1.1 Hz, *J*=9.08 Hz), 7.35 (dd, 1H, *J*=1.1 Hz, *J*=4.96 Hz), 7.04-7.12 (m, 1H), 6.66 (m, 2H), 6.08 (d, 1H, *J*=7.43 Hz), 4.98 (m, 1H), 4.63 (m, 1H), 3.90 (bs, 2H), 2.23 (t, 2H, *J*=7.7 Hz), 2.04 (s, 3H), 2.02 (m, 1H), 1.80 (m, 1H), 1.23 (d, 3H, *J*=6.33 Hz), 1.21 (d, 3H, *J*=6.05 Hz).

<sup>183</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
R<sub>f</sub>= 0.23

**(S)-isopropyl 2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(thiophen-2-yl)benzamido)-4-(methylthio)butanoate**



A solution of EDAC (0.36 g, 1.87 mmol) and DIPEA (0.33 mL, 1.87 mmol) in 2 mL of DCM is added dropwise to a solution of (S)-isopropyl 2-(4-amino-2-(thiophen-2-yl)benzamido)-4-(methylthio)butanoate (0.61 g, 1.56 mmol), HOBT (0.25 g, 1.87 mmol) in 3 mL of DCM, under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of L-Methionine isopropyl ester (0.30 g, 1.56 mmol) in 3 mL of DCM is dropped.

The reaction mixture is stirred at RT overnight; after completion, monitored by TLC<sup>184</sup>, the mixture is washed firstly with a solution of 10% NaHCO<sub>3</sub>, secondly with HCl 10 % and finally with brine.

The organic phase is then dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

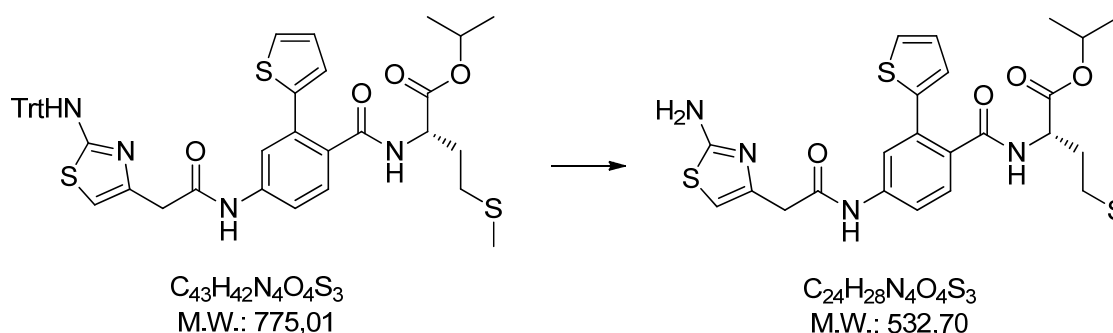
The crude product is purified by column chromatography on silica gel, using Cyclohexane/ Ethyl Acetate= 70/30 as elution solvent, to give 0.74 g (0.95 mmol) of the desired product, as yellow oil.

Yield: 61%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 9.19 (s, 1H), 7.57 (d, 1H, *J*=8.25 Hz), 7.48 (d, 1H, *J*=1.92 Hz), 7.23-7.36 (m, 18H), 7.13 (dd, 1H, *J*=1.38 Hz, *J*=3.58 Hz), 7.06 (dd, 1H, *J*=4.58 Hz, *J*=5.23 Hz), 6.58 (s, 1H), 6.16 (bs, 1H), 5.00 (m, 1H), 4.62 (m, 1H), 3.49 (s, 2H), 2.23 (t, 2H, *J*=7.7 Hz), 2.05 (m, 1H), 2.04 (s, 3H), 1.90 (m, 1H), 1.24 (d, 3H, *J*=7.15 Hz), 1.22 (d, 3H, *J*=6.60 Hz).

<sup>184</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
 R<sub>f</sub> = 0.24

**(S)-isopropyl 2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(thiophen-2-yl)benzamido)-4-(methylthio)butanoate**



0.74 g (0.95 mmol) of (S)-isopropyl 2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(thiophen-2-yl)benzamido)-4-(methylthio)butanoate are dissolved in 9 mL of DCM and then treated with 3 mL of Formic acid.

The reaction is followed by TLC<sup>185</sup>, at completion the reaction mixture is slowly poured in 20 mL of 5% aqueous NaHCO<sub>3</sub>, the two layers are separated; the organic layer is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduce pressure.

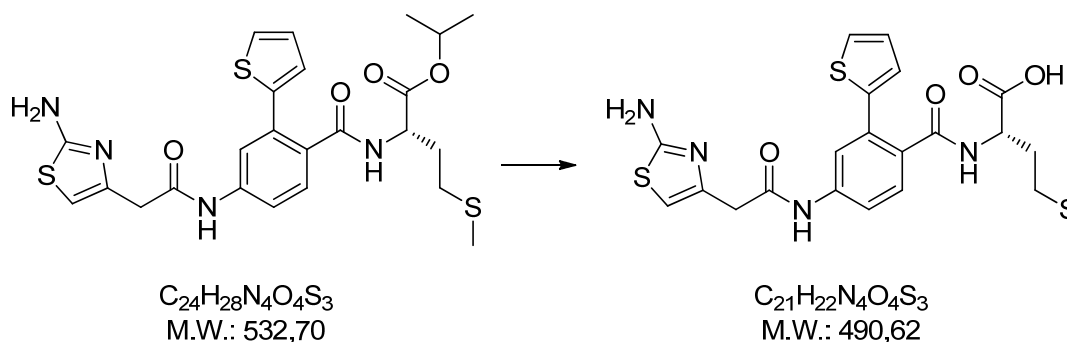
The crude is purified on silica gel, using Ethyl Acetate as elution solvent, yielding 0.34 g of the desired product (0.64 mmol), as sticky solid.

Yield: 67%

<sup>1</sup>H-NMR (CDCl<sub>3</sub>): 9.38 (s, 1H), 7.59 (m, 3H), 7.36 (d, 1H, *J*=4.13 Hz), 7.14 (d, 1H, *J*=2.47 Hz), 7.06 (m, 1H), 6.34 (s, 1H), 6.21 (d, 1H, *J*= 7.7 Hz), 5.13 (bs, 2H), 4.98 (m, 1H), 4.65 (m, 1H), 3.62 (s, 2H), 2.25 (m, 2H), 2.04 (s, 3H), 2.00 (m, 1H), 1.84 (m, 1H), 1.23 (d, 3H, *J*=6.33 Hz), 1.21 (d, 3H, *J*=6.33 Hz).

<sup>185</sup> TLC conditions: Ethyl Acetate= 100 %  
 R<sub>f</sub> = 0.23

**(S)-2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(thiophen-2-yl)benzamido)-4-(methylthio)butanoic acid**



0.34 g of (S)-isopropyl 2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(thiophen-2-yl)benzamido)-4-(methylthio)butanoate (0.64 mmol) are dissolved in 5 mL of Methanol; 1.28 mL of NaOH 1M are dropped and the resulting mixture is heated at 50 °C for 2 hours.

After completion of the reaction, followed by TLC<sup>186</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with Formic acid till a white precipitate is formed.

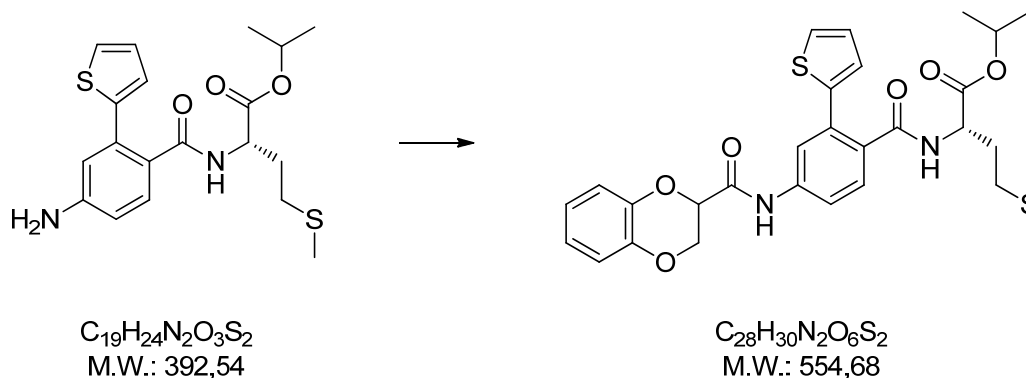
The filtration of the solid yields 0.31 g of the final product, as a sticky solid, in quantitative yield.

Yield: quantitative

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 10.30 (s, 1H), 8.53 (d, 1H, *J* = 7.7 Hz), 7.81 (s, 1H), 7.58 (d, 1H, *J* = 8.35 Hz), 7.53 (d, 1H, *J* = 4.95 Hz), 7.31 (d, 1H, *J* = 8.26 Hz), 7.20 (d, 1H, *J* = 3.3 Hz), 7.00 (m, 2H), 6.89 (s, 2H), 6.30 (s, 1H), 4.34 (m, 1H), 3.48 (s, 2H), 2.36 (m, 2H), 2.00 (s, 3H), 1.87 (m, 2H).

<sup>186</sup> TLC conditions: Ethyl Acetate = 100%  
R<sub>f</sub> = basal

**(2S)-isopropyl 2-(4-(2,3-dihydrobenzo[b][1,4]dioxine-2-carboxamido)-2-(thiophen-2-yl)benzamido)-4-(methylthio)butanoate**



A solution of (S)-isopropyl 2-(4-amino-2-(thiophen-2-yl)benzamido)-4-(methylthio)butanoate (0.61 g, 1.56 mmol) and DIPEA (0.30 mL, 1.72 mmol) in 8 mL of DCM is stirred under a nitrogen atmosphere.

The resulting mixture is cooled to 0°C and then amount of a solution of 2,3-dihydrobenzo[b][1,4]dioxine-2-carbonyl chloride (0.34 g, 1.72 mmol) in 2 mL of DCM.

The reaction mixture is stirred at RT for 2 hours; after completion, monitored by TLC<sup>187</sup>, the mixture is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

The crude is purified on silica gel, using Cyclohexane/ Ethyl Acetate= 75/25 as elution solvent, obtaining 0.73 g (1.31 mmol) of the desired product, as sticky white solid.

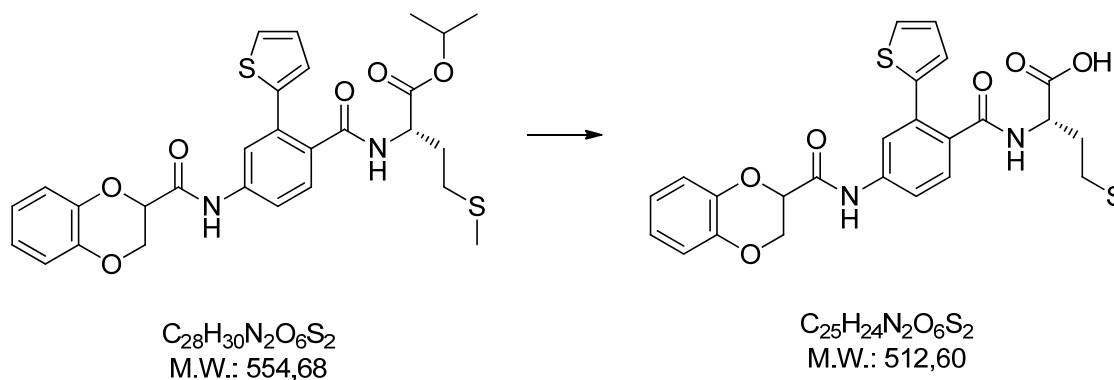
Yield: 85%

<sup>1</sup>H-NMR (CDCl<sub>3</sub>): 8.42 (s,1H), 7.67 (m, 3H), 7.37 (dd, 1H, *J*=1.1 Hz, *J*=4.13 Hz), 7.16 (dd, 1H, *J*=1.1 Hz, *J*=3.57 Hz), 7.02-7.08 (m, 2H), 6.91-6.95 (m, 3H), 6.21 (d, 1H, *J*=7.7 Hz), 5.00 (m, 1H), 4.80 (dd, 1H, *J*=2.75 Hz, *J*=7.43 Hz), 4.68 (dd, 1H, *J*=5.23 Hz, *J*=7.16 Hz), 4.62 (dd, 1H, *J*=2.75 Hz, *J*=11.28 Hz), 4.26 (dd, 1H, *J*=7.43 Hz, *J*=11.26 Hz), 2.23 (t, 2H, *J*=7.7 Hz), 2.04 (s, 3H), 2.01 (m, 1H), 1.87 (m, 1H), 1.24 (d, 3H, *J*=6.6 Hz), 1.22 (d, 3H, *J*=6.33 Hz).

<sup>187</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
 R<sub>f</sub> = 0.44



**(2S)-2-(4-(2,3-dihydrobenzo[b][1,4]dioxine-2-carboxamido)-2-(thiophen-2-yl)benzamido)-4-(methylthio)butanoic acid**



0.62 g of (2S)-isopropyl 2-(4-(2,3-dihydrobenzo[b][1,4]dioxine-2-carboxamido)-2-(thiophen-2-yl)benzamido)-4-(methylthio)butanoate (1.12 mmol) are dissolved in 5 mL of Methanol; 1.12 mL of NaOH 1M are dropped and the resulting mixture is stirred at RT overnight.

After completion of the reaction, followed by TLC<sup>188</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with Formic acid till a white precipitate is formed.

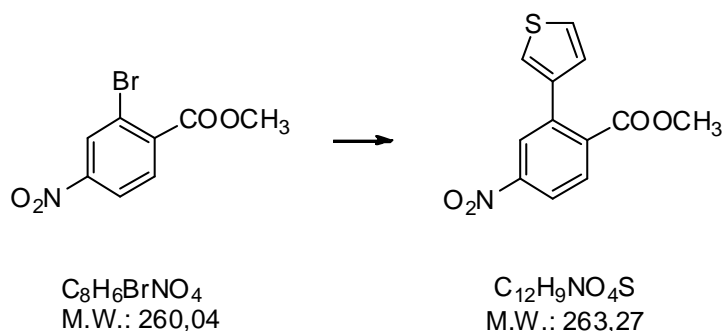
The filtration of the solid yields 0.57 g of the final product, as a sticky solid, in quantitative yield.

Yield: quantitative

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 10.39 (s, 1H), 8.54 (d, 1H, *J*=1.1 Hz), 7.84 (dd, 1H, *J*=3.58 Hz, *J*=5.2 Hz), 7.66 (dd, 1H, *J*=2.2 Hz, *J*=8.25 Hz), 7.54 (dd, 1H, *J*=1.1 Hz, *J*=5.23 Hz), 7.35 (d, 1H, *J*=8.25 Hz), 7.22 (dd, 1H, *J*=1.1 Hz, *J*=3.58 Hz), 7.01-7.04 (m, 2H), 6.84-6.91 (m, 3H), 5.00 (m, 1H), 4.46 (dd, 1H, *J*=2.48 Hz, *J*=11.55 Hz), 4.31-4.44 (m, 3H), 2.35 (m, 2H), 2.00 (s, 3H), 1.91 (m, 2H).

<sup>188</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
Rf= basal

## Methyl 4-nitro-2-(thiophen-3-yl)benzoate



To a sonicated solution of the methyl 2-bromo-4-nitrobenzoate (1.00 g, 3.85 mmol) in 5 mL of dry DMF, under a nitrogen atmosphere,  $Pd(PPh_3)_4$  (0.44 g, 0.385 mmol) is added as solid.

The solution is stirred for 10 minutes and then amount of  $Na_3PO_4 \cdot 12H_2O$  (4.39 g, 11.55 mmol) and of a solution of 3-thienylboronic acid (0.59 g, 4.62 mmol) in 5 mL of dry DMF.

The reaction mixture is heated at  $120^\circ C$  for 18 hours, monitored by TLC<sup>189</sup> and then concentrated.

The crude is resumed with Ethyl Acetate, washed with brine, dried over  $Na_2SO_4$ , filtered and concentrated under reduced pressure.

Purification by chromatography on silica gel, through elution with Cyclohexane/ Ethyl Acetate= 70/30, yields 0.64 g (2.42 mmol) of the desired product, as yellow solid.

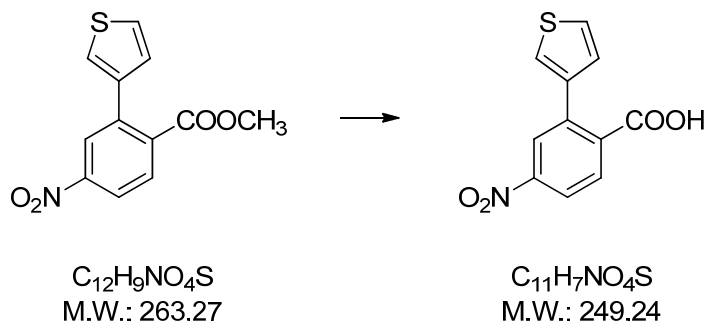
Yield: 63%

Melting point:  $112.93^\circ C$

**$^1H$ -NMR ( $CDCl_3$ ):** 8.29 (d, 1H,  $J=2.2$  Hz), 8.21 (dd, 1H,  $J=2.2$  Hz,  $J=8.52$  Hz), 7.87 (d, 1H,  $J=8.52$  Hz), 7.42 (dd, 1H,  $J=3.03$  Hz,  $J=4.95$  Hz), 7.37 (dd, 1H,  $J=1.38$  Hz,  $J=3.03$  Hz), 7.12 (dd, 1H,  $J=1.1$  Hz,  $J=4.95$  Hz), 3.77 (s, 3H).

<sup>189</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
Rf= 0.60

## 4-nitro-2-(thiophen-3-yl)benzoic acid



0.64 g of methyl 4-nitro-2-(thiophen-3-yl)benzoate (2.42 mmol) are dissolved in 10 mL of Methanol; 2 mL of NaOH 2.5 M are dropped and the resulting mixture is heated at 60 °C for 1 hour.

After completion of the reaction, followed by TLC<sup>190</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with 10% HCl, and then extracted three times with 10 mL of Ethyl Acetate.

The organic phases are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure, obtaining a yellow solid (0.60 g, 2.42 mmol), corresponding to the desired compound.

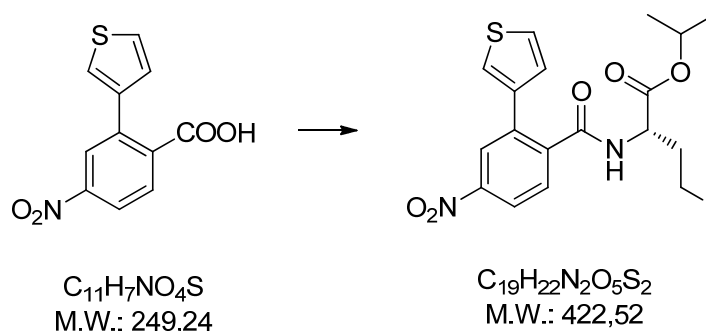
Yield: quantitative

Melting point: 159.28 °C

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 8.29 (d, 1H, *J*=2.47 Hz), 8.24 (dd, 1H, *J*= 2.47 Hz, *J*= 8.53 Hz), 8.03 (d, 1H, *J*=8.53 Hz), 7.40 (m, 2H), 7.16 (dd, 1H, *J*=2.75 Hz, *J*=3.85 Hz).

<sup>190</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
R<sub>f</sub> = 0.20

## (S)-isopropyl 4-(methylthio)-2-(4-nitro-2-(thiophen-3-yl)benzamido)butanoate



A solution of EDAC (0.56 g, 2.90 mmol) and DIPEA (0.50 mL, 2.90 mmol) in 2 mL of DCM is added dropwise to a solution of 4-nitro-2-(thiophen-3-yl)benzoic acid (0.60 g, 2.42 mmol), HOBT (0.39 g, 2.90 mmol) in 3 mL of DCM, under a nitrogen atmosphere. The resulting mixture is stirred for 30 minutes at RT and then a solution of L-Methionine isopropyl ester (0.46 g, 2.42 mmol) in 3 mL of DCM is dropped.

The reaction mixture is stirred at RT overnight; after completion, monitored by TLC<sup>191</sup>, the mixture is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

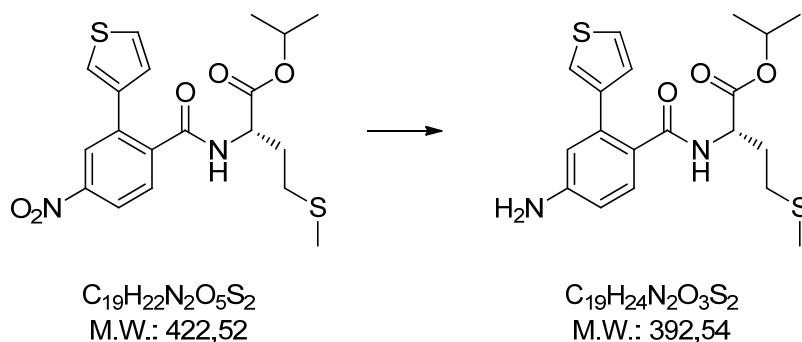
The crude product is purified by column chromatography on silica gel, using Cyclohexane/ Ethyl Acetate= 75/25 as elution solvent, to give 0.84 g (1.98 mmol) of the desired product, as pale yellow oil.

Yield: 82%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 8.29 (d, 1H, *J*=2.48 Hz), 8.22 (ddd, 1H, *J*=0.83 Hz, *J*=2.48 Hz, *J*=8.53 Hz), 7.77 (d, 1H, *J*=8.53 Hz), 7.51 (m, 1H), 7.45 (ddd, 1H, *J*=0.82 Hz, *J*=3.02 Hz, *J*=4.95 Hz), 7.23 (m, 2H), 6.23 (d, 1H, *J*=7.43 Hz), 5.01 (m, 1H), 4.68 (m, 1H), 2.22 (t, 2H, *J*=7.7 Hz), 2.07 (m, 1H), 2.05 (s, 3H), 1.89 (m, 1H), 1.26 (d, 3H, *J*=6.33 Hz), 1.24 (d, 3H, *J*=6.33 Hz).

<sup>191</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
R<sub>f</sub> = 0.50

**(S)-isopropyl 2-(4-amino-2-(thiophen-3-yl)benzamido)-4-(methylthio)butanoate**



(S)-isopropyl 4-(methylthio)-2-(4-nitro-2-(thiophen-3-yl)benzamido)butanoate (0.84 g, 1.98 mmol) is dissolved in 13 mL of Ethyl Acetate and 1 mL of Water. Stannous chloride (1.69 g, 9.9 mmol) is added and the mixture is refluxed for 2 hours.

After completion of the reaction, defined by TLC<sup>192</sup>, the mixture reaction is firstly cooled and poured into 50 mL of iced 5% NaHCO<sub>3</sub>, then it is filtered over Celite and the two layers are separated.

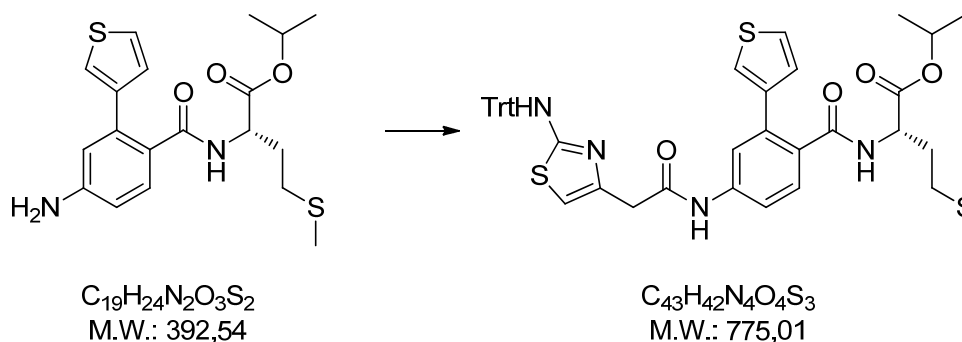
The organic layer is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure, giving 0.39 g (1.78 mmol) of a sticky oil.

Yield: 90%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 7.59 (d, 1H, *J*=8.25 Hz), 7.37 (dd, 1H, *J*=3.03 Hz, *J*=4.95 Hz), 7.33 (dd, 1H, *J*=1.38 Hz, *J*=3.03 Hz), 7.12 (dd, 1H, *J*=1.38 Hz, *J*=4.95 Hz), 6.67 (dd, 1H, *J*=2.48 Hz, *J*=8.25 Hz), 6.60 (d, 1H, *J*=2.48 Hz), 5.97 (d, 1H, *J*=7.42 Hz), 4.98 (m, 1H), 4.60 (m, 1H), 3.90 (bs, 2H), 2.23 (t, 2H, *J*=7.7 Hz), 2.05 (s, 3H), 1.99 (m, 1H), 1.79 (m, 1H), 1.23 (d, 3H, *J*=6.33 Hz), 1.21 (d, 3H, *J*=6.60 Hz).

<sup>192</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
 R<sub>f</sub>= 0.22

**(S)-isopropyl 2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(thiophen-3-yl)benzamido)-4-(methylthio)butanoate**



A solution of EDAC (0.41 g, 2.14 mmol) and DIPEA (0.37 mL, 2.14 mmol) in 2 mL of DCM is added dropwise to a solution of (S)-isopropyl 2-(4-amino-2-(thiophen-3-yl)benzamido)-4-(methylthio)butanoate (0.39 g, 1.78 mmol), HOBT (0.29 g, 2.14 mmol) in 3 mL of DCM, under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of L-Methionine isopropyl ester (0.34 g, 1.78 mmol) in 3 mL of DCM is dropped.

The reaction mixture is stirred at RT overnight; after completion, monitored by TLC<sup>193</sup>, the mixture is washed firstly with a solution of 10% NaHCO<sub>3</sub>, secondly with HCl 10 % and finally with brine.

The organic phase is then dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

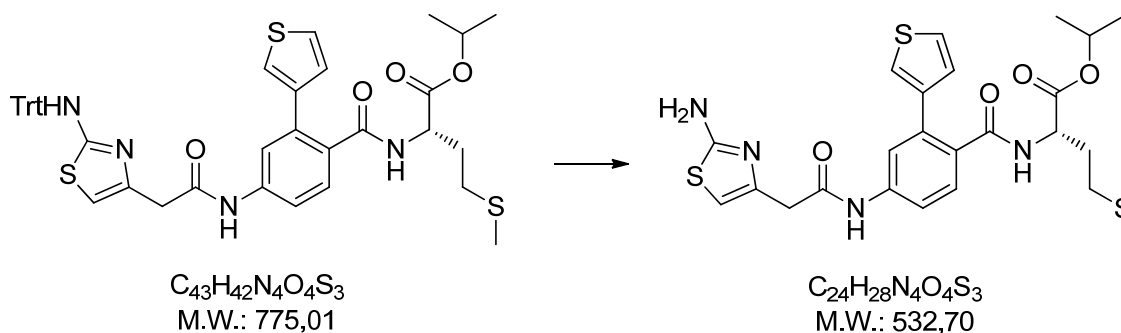
The crude product is purified by column chromatography on silica gel, using Cyclohexane/ Ethyl Acetate= 70/30 as elution solvent, to give 0.99 g (1.28 mmol) of the desired product, as colourless oil.

Yield: 72%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 9.19 (s, 1H), 7.62 (d, 1H, *J*=8.25 Hz), 7.53 (d, 1H, *J*=2.92 Hz), 7.23-7.38 (m, 18H), 7.15 (dd, 1H, *J*=1.1 Hz, *J*=4.68 Hz), 6.70 (bs, 1H), 6.18 (s, 1H), 6.05 (d, 1H, *J*= 7.97 Hz), 4.99 (m, 1H), 4.63 (m, 1H), 3.54 (s, 2H), 2.23 (t, 2H, *J*=7.7 Hz), 2.04 (s, 3H), 2.00 (m, 1H), 1.79 (m, 1H), 1.24 (d, 3H, *J*=7.53 Hz), 1.22 (d, 3H, *J*=6.33 Hz).

<sup>193</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
 R<sub>f</sub> = 0.28

**(S)-isopropyl 2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(thiophen-3-yl)benzamido)-4-(methylthio)butanoate**



0.99 g (1.28 mmol) of (S)-isopropyl 2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(thiophen-3-yl)benzamido)-4-(methylthio)butanoate are dissolved in 6 mL of DCM and then treated with 2 mL of Formic acid.

The reaction is followed by TLC<sup>194</sup>, at completion the reaction mixture is slowly poured in 20 mL of 5% aqueous NaHCO<sub>3</sub>, the two layers are separated; the organic layer is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduce pressure.

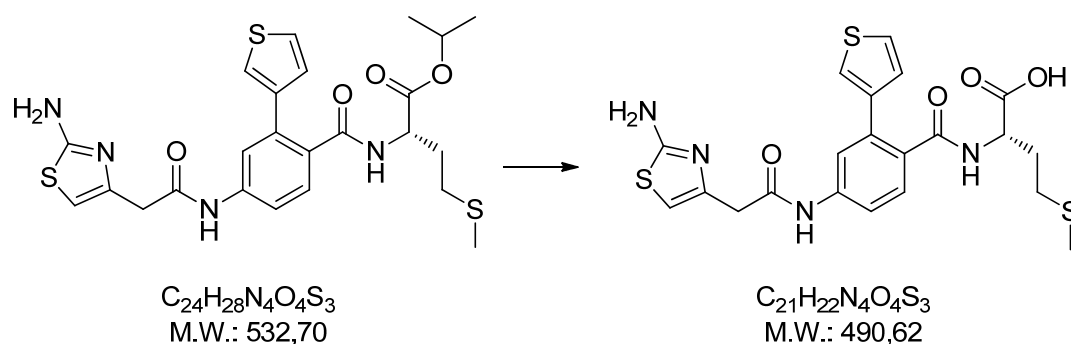
The crude is purified on silica gel, using Ethyl Acetate as elution solvent, yielding 0.44 g of the desired product (0.82 mmol), as sticky solid.

Yield: 64%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 9.34 (s, 1H), 7.64 (d, 1H, *J* = 8.52 Hz), 7.58 (d, 1H, *J* = 1.93 Hz), 7.53 (dd, 1H, *J* = 1.93 Hz, *J* = 8.52 Hz), 7.37 (dd, 1H, *J* = 2.2 Hz, *J* = 3.03 Hz), 7.35 (dd, 1H, *J* = 3.03 Hz, *J* = 4.13 Hz), 7.15 (dd, 1H, *J* = 2.2 Hz, *J* = 4.13 Hz), 6.34 (s, 1H), 6.09 (d, 1H, *J* = 7.42 Hz), 5.01 (bs, 2H), 4.98 (m, 1H), 4.63 (m, 1H), 3.62 (s, 2H), 2.23 (m, 2H), 2.05 (s, 3H), 1.98 (m, 1H), 1.79 (m, 1H), 1.24 (d, 3H, *J* = 7.15 Hz), 1.22 (d, 3H, *J* = 6.6 Hz).

<sup>194</sup> TLC conditions: Ethyl Acetate = 100 %  
 R<sub>f</sub> = 0.25

**(S)-2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(thiophen-2-yl)benzamido)-4-(methylthio)butanoic acid**



0.44 g of (S)-isopropyl 2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(thiophen-3-yl)benzamido)-4-(methylthio)butanoate (0.82 mmol) are dissolved in 5 mL of Methanol; 1.64 mL of NaOH 1M are dropped and the resulting mixture is stirred at RT for 18 hours.

After completion of the reaction, followed by TLC<sup>195</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with Formic acid till a white precipitate is formed.

The filtration of the solid yields 0.40 g of the final product, as a sticky solid, in quantitative yield.

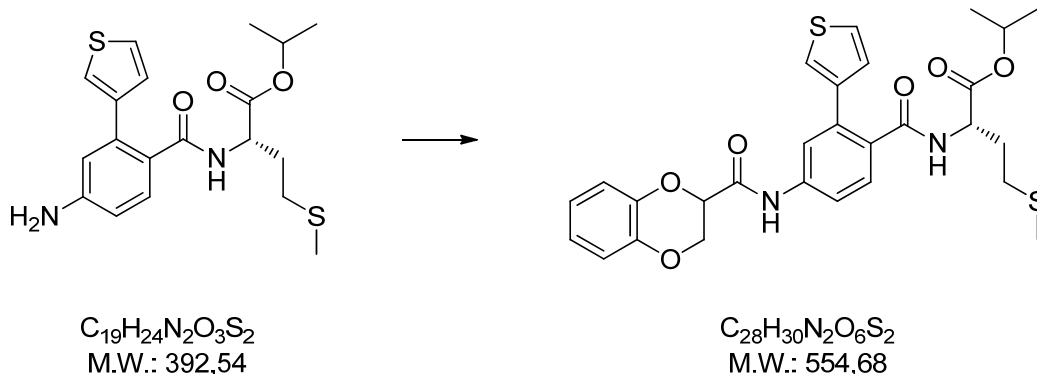
Yield: quantitative

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 10.27 (s, 1H), 8.51 (d, 1H, *J* = 7.7 Hz), 7.74 (d, 1H, *J* = 1.92 Hz), 7.58 (dd, 1H, *J* = 1.92 Hz, *J* = 8.25 Hz), 7.51 (m, 1H), 7.33 (d, 1H, *J* = 8.25 Hz), 7.18 (dd, 1H, *J* = 1.1 Hz, *J* = 6.31 Hz), 6.91 (m, 2H), 6.31 (s, 1H), 4.36 (m, 1H), 3.48 (s, 2H), 3.35 (bs, 2H), 2.41 (m, 2H), 2.01 (s, 3H), 1.88 (m, 2H).

<sup>195</sup> TLC conditions: Ethyl Acetate = 100%  
R<sub>f</sub> = basal



**(2S)-isopropyl 2-(4-(2,3-dihydrobenzo[b][1,4]dioxine-2-carboxamido)-2-(thiophen-3-yl)benzamido)-4-(methylthio)butanoate**



A solution of (S)-isopropyl 2-(4-amino-2-(thiophen-3-yl)benzamido)-4-(methylthio)butanoate (0.39 g, 1.78 mmol) and DIPEA (0.34 mL, 1.72 mmol) in 8 mL of DCM is stirred under a nitrogen atmosphere.

The resulting mixture is cooled to 0°C and then amount of a solution of 2,3-dihydrobenzo[b][1,4]dioxine-2-carbonyl chloride (0.39 g, 1.96 mmol) in 2 mL of DCM.

The reaction mixture is stirred at RT for 2 hours; after completion, monitored by TLC<sup>196</sup>, the mixture is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

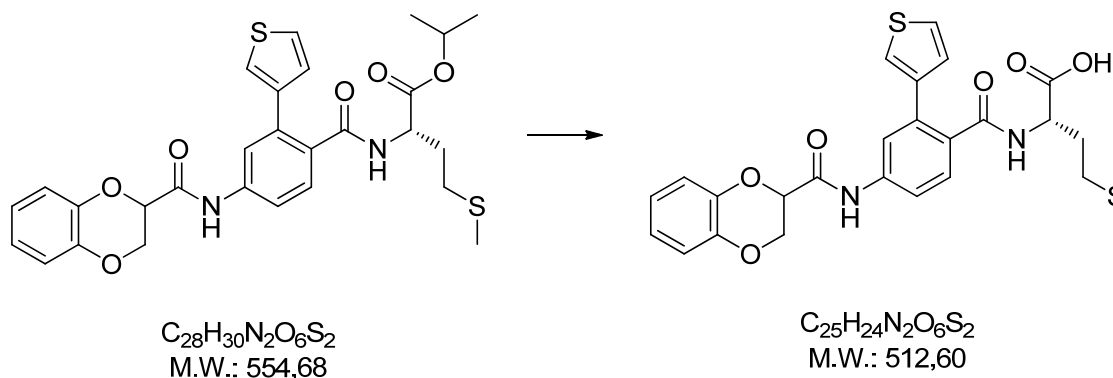
The crude is purified on silica gel, using Cyclohexane/ Ethyl Acetate= 75/25 as elution solvent, obtaining 0.73 g (1.32 mmol) of the desired product, as sticky white solid.

Yield: 74%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 8.38 (s, 1H), 7.70 (d, 1H, *J* = 8.52 Hz), 7.69 (s, 1H), 7.59 (dd, 1H, *J* = 2.2 Hz, *J* = 8.52 Hz), 7.37-7.41 (m, 2H), 7.17 (dd, 1H, *J* = 0.83 Hz, *J* = 5.50 Hz), 6.91-7.06 (m, 3H), 6.09 (d, 1H, *J* = 7.42 Hz), 4.99 (m, 1H), 4.82 (dd, 1H, *J* = 2.75 Hz, *J* = 7.42 Hz), 4.60-4.65 (m, 2H), 4.27 (ddd, 1H, *J* = 1.38 Hz, *J* = 7.7 Hz, *J* = 11.55 Hz), 2.23 (t, 2H, *J* = 7.7 Hz), 2.05 (s, 3H), 1.97 (m, 1H), 1.83 (m, 1H), 1.24 (d, 3H, *J* = 6.33 Hz), 1.22 (d, 3H, *J* = 6.43 Hz).

<sup>196</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
 R<sub>f</sub> = 0.43

**(2S)-2-(4-(2,3-dihydrobenzo[b][1,4]dioxine-2-carboxamido)-2-(thiophen-3-yl)benzamido)-4-(methylthio)butanoic acid**



0.73 g of (2S)-isopropyl 2-(4-(2,3-dihydrobenzo[b][1,4]dioxine-2-carboxamido)-2-(thiophen-3-yl)benzamido)-4-(methylthio)butanoate (1.32 mmol) are dissolved in 5 mL of Methanol; 1.12 mL of NaOH 1M are dropped and the resulting mixture is stirred at RT overnight.

After completion of the reaction, followed by TLC<sup>197</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with Formic acid till a white precipitate is formed.

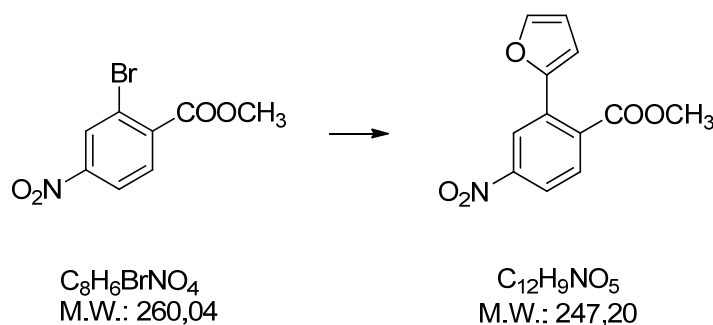
The filtration of the solid yields 0.68 g of the final product, as a sticky solid, in quantitative yield.

Yield: quantitative

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 10.31 (s,1H), 8.47 (d, 1H, *J*=7.7 Hz), 7.77 (s,1H), 7.75 (d, 1H, *J*=9.08 Hz), 7.64 (dd, 1H, *J*=2.2 Hz, *J*=8.52 Hz), 7.49-7.55 (m, 2H), 7.36 (d, 1H, *J*=8.52 Hz), 7.20 (dd, 1H, *J*=0.83 Hz, *J*=4.95 Hz), 7.03 (dd, 1H, *J*= 1.93 Hz, *J*= 7.98 Hz), 6.85-6.90 (m, 3H), 5.00 (m, 1H), 4.45 (dd, 1H, *J*=2.48 Hz, *J*=11.28 Hz), 4.32-4.38 (m, 3H), 2.37 (m, 2H), 2.04 (s, 3H), 2.1 (m, 2H).

<sup>197</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
R<sub>f</sub> = basal

## Methyl 4-nitro-2-(furan-2-yl)benzoate



To a sonicated solution of the methyl 2-bromo-4-nitrobenzoate (1.00 g, 3.85 mmol) in 5 mL of dry THF, under a nitrogen atmosphere,  $Pd(PPh_3)_4$  (0.44 g, 0.385 mmol) is added as solid.

The solution is stirred for 10 minutes and then amount of  $K_2CO_3$  (1.60 g, 11.55 mmol) and of a solution of 2-furanylboronic acid (0.47 g, 4.24 mmol) in 5 mL of dry THF.

The reaction mixture is stirred at RT for 18 hours, monitoring the progression by TLC<sup>198</sup> and then concentrated.

The crude is resumed with Ethyl Acetate, washed with brine, dried over  $Na_2SO_4$ , filtered and concentrated under reduced pressure.

Purification by chromatography on silica gel, through elution with Hexane/ Ethyl Acetate= 90/104, yields 0.70 g (2.85 mmol) of the desired product, as yellow solid.

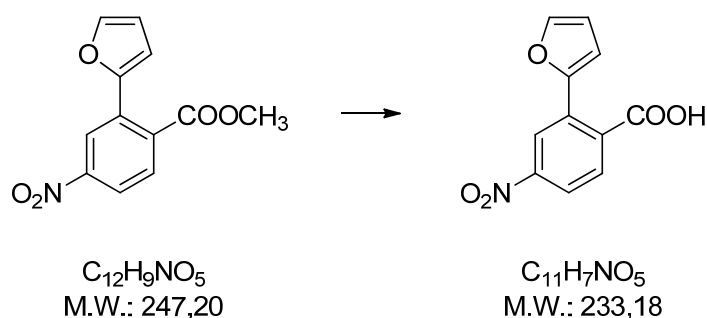
Yield: 74%

Melting point: 67.73° C

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 8.49 (d, 1H,  $J=2.2$  Hz), 8.15 (dd, 1H,  $J=2.20$  Hz,  $J=8.53$  Hz), 7.74 (d, 1H,  $J=8.53$  Hz), 7.55 (dd, 1H,  $J=0.83$  Hz,  $J=1.93$  Hz), 7.75 (dd, 1H,  $J=0.83$  Hz,  $J=3.58$  Hz), 3.91 (s, 3H).

<sup>198</sup> TLC conditions: Hexane/ Ethyl Acetate= 80/20  
Rf= 0.28

### 4-nitro-2-(furan-2-yl)benzoic acid



0.70 g of methyl 4-nitro-2-(furan-2-yl)benzoate (2.85 mmol) are dissolved in 10 mL of Methanol; 2.3 mL of NaOH 2.5 M are dropped and the resulting mixture is stirred at RT for 18 hours.

After completion of the reaction, followed by TLC<sup>199</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with 10% HCl, and then extracted three times with 10 mL of Ethyl Acetate.

The organic phases are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure, obtaining a yellow solid (0.63 g, 2.68 mmol), corresponding to the desired compound.

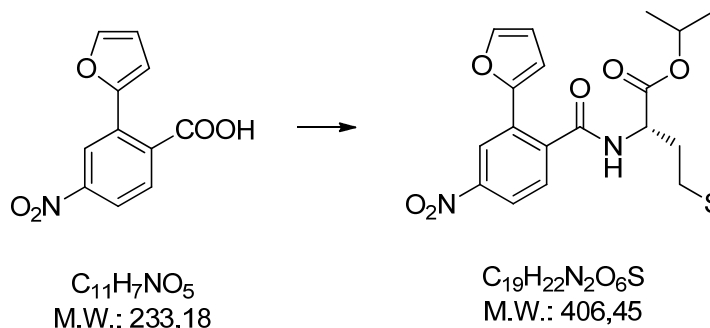
Yield: 94 %

Melting point: 136.19° C

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 8.51 (d, 1H, *J*=2.2 Hz), 8.20 (ddd, 1H, *J*=0.82 Hz, *J*=2.20 Hz, *J*=8.52 Hz), 7.94 (d, 1H, *J*=8.52 Hz), 7.57 (dd, 1H, *J*=0.83 Hz, *J*=1.65 Hz), 6.86 (d, 1H, *J*=3.58 Hz), 6.56 (ddd, 1H, *J*=0.83 Hz, *J*=1.65 Hz, *J*=3.58 Hz).

<sup>199</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
R<sub>f</sub> = 0.20

**(S)-isopropyl 4-(methylthio)-2-(4-nitro-2-(furan-2-yl)benzamido)butanoate**



A solution of EDAC (0.62 g, 3.22 mmol) and DIPEA (0.56 mL, 3.22 mmol) in 2 mL of DCM is added dropwise to a solution of 4-nitro-2-(furan-2-yl)benzoic acid (0.63 g, 2.68 mmol), HOBT (0.44 g, 3.22 mmol) in 3 mL of DCM, under a nitrogen atmosphere. The resulting mixture is stirred for 30 minutes at RT and then a solution of L-Methionine isopropyl ester (0.51 g, 2.68 mmol) in 3 mL of DCM is dropped. The reaction mixture is stirred at RT overnight; after completion, monitored by TLC<sup>200</sup>, the mixture is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated. The crude product is treated with Isopropyl Ether, achieving 1.04 g (2.55 mmol) of the desired product, as white solid.

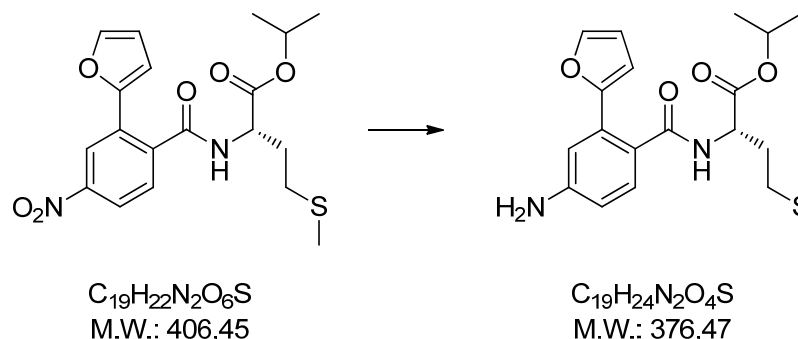
Yield: 95%

Melting point: 140.97 °C

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 8.53 (d, 1H, *J*=2.2 Hz), 8.13 (dd, 1H, *J*=2.2 Hz, *J*=8.25 Hz), 7.62 (dd, 1H, *J*=3.47 Hz, *J*=8.25 Hz), 7.55 (dd, 1H, *J*=0.82 Hz, *J*=2.92 Hz), 6.84 (dd, 1H, *J*=0.82 Hz, *J*=3.67 Hz), 6.50-6.55 (m, 2H), 5.08 (m, 1H), 4.86 (m, 1H), 2.51 (t, 2H, *J*=7.70 Hz), 2.18 (m, 1H), 2.09 (s, 3H), 2.06 (m, 1H), 1.29 (d, 3H, *J*=6.33 Hz), 1.27 (d, 3H, *J*=6.60 Hz).

<sup>200</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
 R<sub>f</sub> = 0.61

**(S)-isopropyl 2-(4-amino-2-(furan-2-yl)benzamido)-4-(methylthio)butanoate**



(S)-isopropyl 4-(methylthio)-2-(4-nitro-2-(furan-2-yl)benzamido)butanoate (1.04 g, 2.55 mmol) is dissolved in 30 mL of Ethyl Acetate. Stannous chloride (2.42 g, 12.75 mmol) is added and the mixture is refluxed for 3 hours.

After completion of the reaction, defined by TLC<sup>201</sup>, the mixture reaction is firstly cooled and poured into 50 mL of iced 5% NaHCO<sub>3</sub>, then it is filtered over Celite and the two layers are separated.

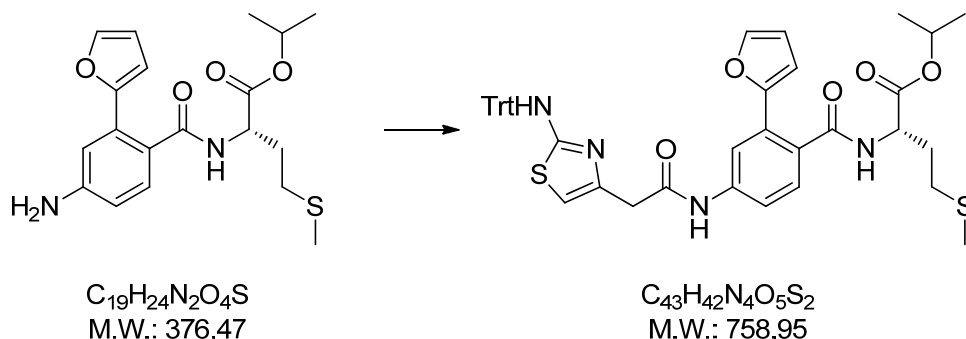
The organic layer is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure, giving 0.84 g (2.22 mmol) of a colourless oil.

Yield: 87

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 7.47 (d, 1H, *J*=0.55), 7.40 (d, 1H, *J*=8.25 Hz), 6.86 (d, 2H, *J*= 2.20 Hz), 6.60-6.64 (m, 2H), 6.45 (dd, 1H, *J*= 1.55 Hz, *J*= 4.4 Hz), 6.25 (d, 1H, *J*= 7.70 Hz), 5.04 (m, 1H), 4.78 (m, 1H), 3.90 (bs, 2H), 2.48 (t, 2H, *J*=7.6 Hz), 2.13 (m, 1H), 2.08 (s, 3H), 1.99 (m, 1H), 1.27 (d, 3H, *J*=6.05 Hz), 1.25 (d, 3H, *J*=5.78 Hz).

<sup>201</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
R<sub>f</sub>= 0.18

**(S)-isopropyl 2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(furan-2-yl)benzamido)-4-(methylthio)butanoate**



A solution of EDAC (0.51 g, 2.66 mmol) and DIPEA (1.97 mL, 2.66 mmol) in 8 mL of dry DMF is added dropwise to a solution of (S)-isopropyl 2-(4-amino-2-(furan-2-yl)benzamido)-4-(methylthio)butanoate (0.84 g, 2.22 mmol), HOBT (0.36 g, 2.66 mmol) in 5 mL of dry DMF, under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of L-Methionine isopropyl ester (0.42 g, 2.22 mmol) in 5 mL of dry DMF is dropped.

The reaction mixture is stirred at RT overnight; after completion, monitored by TLC<sup>202</sup>, the solvents is removed; the residue is resumed with 20 mL of Ethyl Acetate, washed firstly with a solution of 10% NaHCO<sub>3</sub>, secondly with HCl 10 % and finally with brine.

The organic phase is then dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

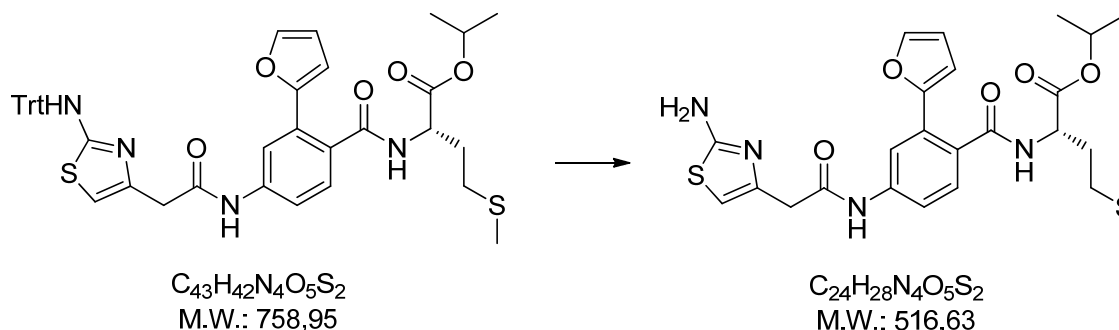
The crude product is purified by column chromatography on silica gel, using Cyclohexane/ Ethyl Acetate= 65/35 as elution solvent, to give 1.08 g (1.42 mmol) of the desired product, as yellow oil.

Yield: 64%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 9.21 (s, 1H), 7.69 (d, 1H, *J*=1.93 Hz), 7.44 (m, 2H), 7.22-7.36 (m, 15H), 6.65 (m, 2H), 6.45 (dd, 1H, *J*=1.93 Hz, *J*=4.68 Hz), 6.32 (d, 1H, *J*=7.98 Hz), 6.19 (s, 1H), 5.05 (m, 1H), 4.80 (m, 1H), 3.53 (s, 2H), 2.49 (t, 2H, *J*=6.86 Hz), 2.17 (m, 1H), 2.09 (s, 3H), 2.00 (m, 1H), 1.28 (d, 3H, *J*=6.05 Hz), 1.26 (d, 3H, *J*=6.24 Hz).

<sup>202</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
 R<sub>f</sub> = 0.48

**(S)-isopropyl 2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(furan-2-yl)benzamido)-4-(methylthio)butanoate**



1.08 g (1.42 mmol) of (S)-isopropyl 2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(furan-2-yl)benzamido)-4-(methylthio)butanoate are dissolved in 12 mL of DCM and then treated with 4 mL of Formic acid.

The reaction is followed by TLC<sup>203</sup>, at completion the reaction mixture is slowly poured in 20 mL of 5% aqueous NaHCO<sub>3</sub>, the two layers are separated; the organic layer is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduce pressure.

The crude is purified on silica gel, using Ethyl Acetate as elution solvent, yielding 0.29 g of the desired product (0.57 mmol), as sticky solid.

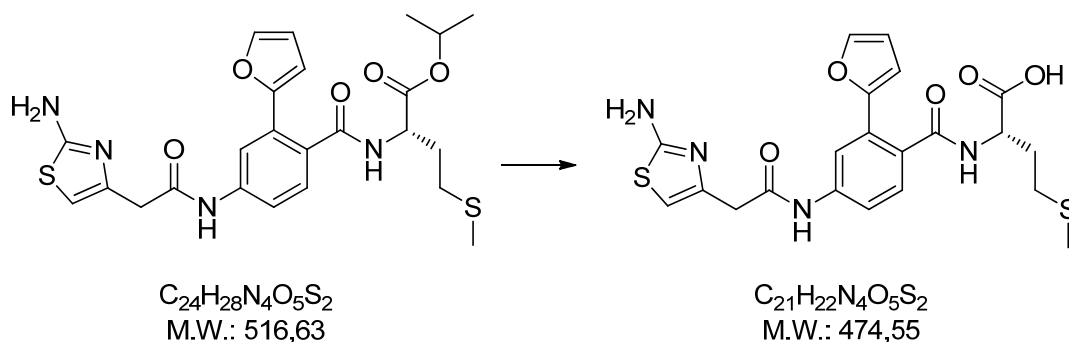
Yield: 40%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 9.37 (s, 1H), 7.76 (d, 1H, *J* = 1.93 Hz), 7.53 (dd, 1H, *J* = 1.93 Hz, *J* = 8.53 Hz), 7.46 (s, 1H), 7.43 (m, 1H), 6.65 (d, 1H, *J* = 3.3 Hz), 6.43 (m, 2H), 6.35 (s, 1H), 5.30 (bs, 2H), 5.05 (m, 1H), 4.79 (m, 1H), 3.63 (s, 2H), 2.49 (t, 2H, *J* = 6.65 Hz), 2.17 (m, 1H), 2.09 (s, 3H), 1.99 (m, 1H), 1.27 (d, 3H, *J* = 6.33 Hz), 1.25 (d, 3H, *J* = 5.75 Hz).

<sup>203</sup> TLC conditions: Ethyl Acetate = 100 %  
 R<sub>f</sub> = 0.30



**(S)-2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(furan-2-yl)benzamido)-4-(methylthio)butanoic acid**



0.29 g of (S)-isopropyl 2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(furan-2-yl)benzamido)-4-(methylthio)butanoate (0.57 mmol) are dissolved in 5 mL of Methanol; 0.57 mL of NaOH 1M are dropped and the resulting mixture is stirred at RT for 18 hours.

After completion of the reaction, followed by TLC<sup>204</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with Formic acid till a white precipitate is formed.

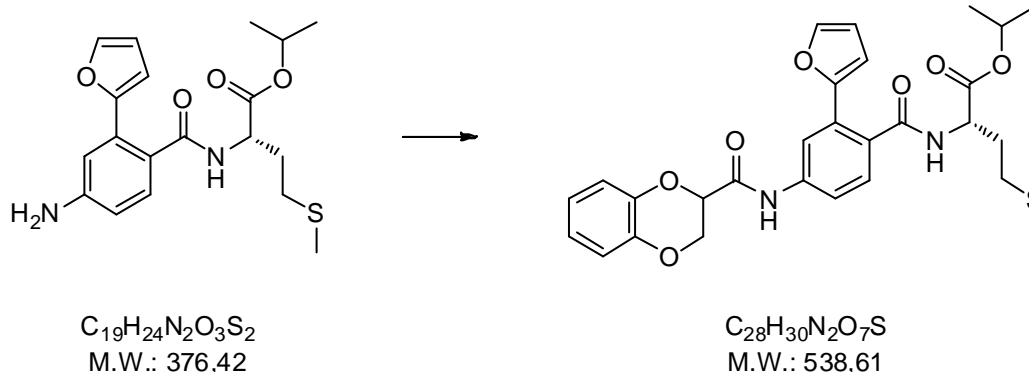
The filtration of the solid yields 0.27 g of the final product, as a sticky solid, in quantitative yield.

Yield: quantitative

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 12.76 (bs, 1H), 10.28 (s, 1H), 8.65 (d, 1H, *J* = 7.65 Hz), 8.01 (d, 1H, *J* = 2.2 Hz), 7.70 (d, 1H, *J* = 1.61 Hz), 7.55 (dd, 1H, *J* = 2.2 Hz, *J* = 8.45 Hz), 7.27 (d, 1H, *J* = 8.45 Hz), 6.89 (s, 1H), 6.74 (d, 1H, *J* = 3.63 Hz), 6.50 (dd, 1H, *J* = 1.61 Hz, *J* = 3.63 Hz), 6.30 (s, 1H), 4.44 (m, 1H), 3.48 (s, 2H), 2.46 (m, 2H), 2.04 (s, 3H), 1.96 (m, 2H).

<sup>204</sup> TLC conditions: Ethyl Acetate = 100%  
R<sub>f</sub> = basal

**(2S)-isopropyl 2-(4-(2,3-dihydrobenzo[b][1,4]dioxine-2-carboxamido)-2-(furan-2-yl)benzamido)-4-(methylthio)butanoate**



A solution of (S)-isopropyl 2-(4-amino-2-(furan-2-yl)benzamido)-4-(methylthio)butanoate (0.84 g, 2.22 mmol) and DIPEA (0.58 mL, 3.33 mmol) in 8 mL of dry THF is stirred under a nitrogen atmosphere.

The resulting mixture is cooled to 0°C and then amount of a solution of 2,3-dihydrobenzo[b][1,4]dioxine-2-carbonyl chloride (0.66 g, 3.33 mmol) in 2 mL of dry THF. The reaction mixture is stirred at RT for 18 hours; after completion, monitored by TLC<sup>205</sup>, the mixture is concentrated.

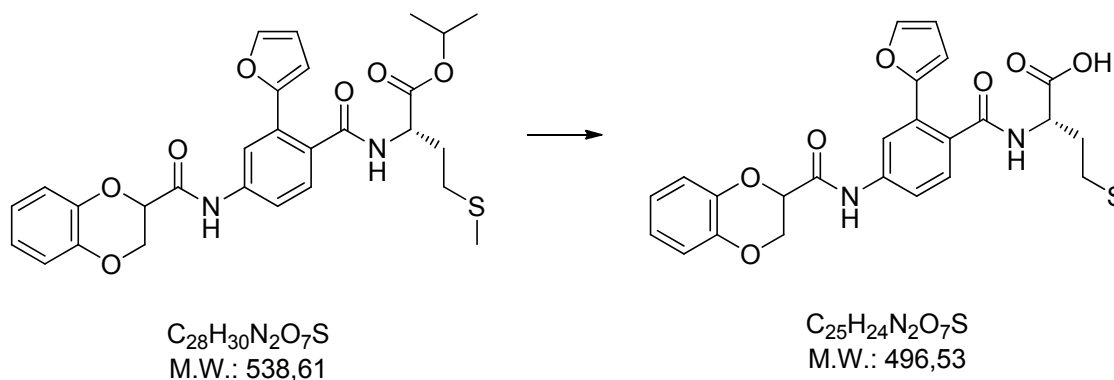
The residue is resumed with 20 mL of DCM, washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated, obtaining 1.19 g (2.21 mmol) of the desired product, as sticky white solid.

Yield: 99.8%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 8.41 (s, 1H), 7.85 (s, 1H), 7.60 (dd, 1H, *J* = 2.2 Hz, *J* = 8.25 Hz), 7.50 (d, 1H, *J* = 8.25 Hz), 7.48 (s, 1H), 7.06 (m, 1H), 6.94 (m, 3H), 6.71 (d, 1H, *J* = 3.58 Hz), 6.46 (dd, 1H, *J* = 1.92 Hz, *J* = 3.58 Hz), 6.38 (d, 1H, *J* = 7.98 Hz), 5.05 (m, 1H), 4.81 (m, 1H), 4.63 (dd, 1H, *J* = 2.75 Hz, *J* = 11.55 Hz), 4.26 (dd, 1H, *J* = 7.70 Hz, *J* = 11.55 Hz), 2.49 (t, 1H, *J* = 6.60 Hz), 2.18 (m, 1H), 2.09 (s, 3H), 1.96 (m, 1H), 1.28 (d, 3H, *J* = 6.05 Hz), 1.26 (d, 3H, *J* = 6.60 Hz).

<sup>205</sup> TLC conditions: Cyclohexane/ Ethyl Acetate = 1/1  
 R<sub>f</sub> = 0.50

**(2S)-2-(4-(2,3-dihydrobenzo[b][1,4]dioxine-2-carboxamido)-2-(furan-2-yl)benzamido)-4-(methylthio)butanoic acid**



1.19 g of (2S)-isopropyl 2-(4-(2,3-dihydrobenzo[b][1,4]dioxine-2-carboxamido)-2-(furan-2-yl)benzamido)-4-(methylthio)butanoate (2.21 mmol) are dissolved in 10 mL of Methanol; 1.19 mL of NaOH 1M are dropped and the resulting mixture is stirred at RT overnight.

After completion of the reaction, followed by TLC<sup>206</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with Formic acid till a white precipitate is formed.

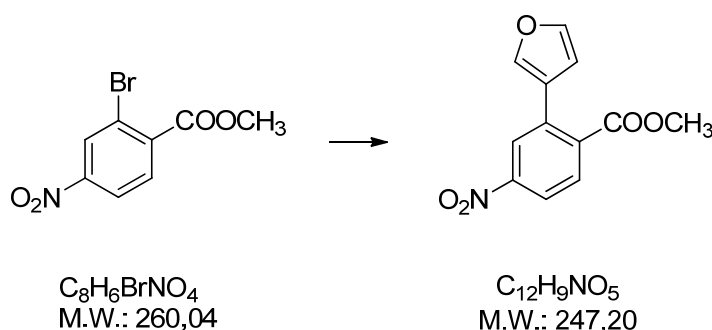
The filtration of the solid yields 1.05 g (2.21 mmol) of the final product, as a sticky solid, in quantitative yield.

Yield: 96%

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 12.72 (bs, 1H), 10.34 (bs, 1H), 8.68 (d, 1H, *J*=7.25 Hz), 8.05 (s, 1H), 7.71 (s, 1H), 7.61 (d, 1H, *J*=7.65 Hz), 7.30 (d, 1H, *J*=8.05 Hz), 7.04 (m, 1H), 6.87 (m, 3H), 6.75 (d, 1H, *J*= 2.82 Hz), 6.51 (d, 1H, *J*= 1.21 Hz), 5.00 (m, 1H), 4.37-4.47 (m, 3H), 2.49 (m, 2H), 2.04 (s, 3H), 1.97 (m, 2H).

<sup>206</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
R<sub>f</sub> = basal

## Methyl 4-nitro-2-(furan-3-yl)benzoate



To a sonicated solution of the methyl 2-bromo-4-nitrobenzoate (1.00 g, 3.85 mmol) in 8 mL of Toluene, under a nitrogen atmosphere,  $Pd(PPh_3)_4$  (0.44 g, 0.385 mmol) is added as solid.

The solution is stirred for 10 minutes and then amount of  $Na_2CO_3$  (1.22 g, 11.55 mmol) in 2 mL of Water and of a solution of 3-furanylboronic acid (0.56 g, 5.00 mmol) in 1 mL of Methanol.

The reaction mixture is heated at 120 °C for 18 hours and monitored by TLC<sup>207</sup>.

At completion, the mixture is treated with DCM and  $NaHCO_3$ , the organic layer is washed with brine and dried over  $Na_2SO_4$ , filtered and concentrated under reduced pressure.

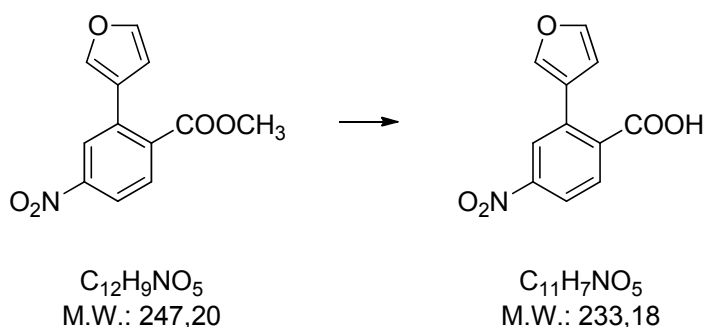
Purification by chromatography on silica gel, through elution with Cyclohexane/ Ethyl Acetate= 80/20, yields 0.76 g (3.08 mmol) of the desired product, as dense yellow oil.

Yield: 80%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 8.26 (d, 1H,  $J=2.2$  Hz), 8.18 (dd, 1H,  $J=2.2$  Hz,  $J=8.53$  Hz), 7.84 (d, 1H,  $J=8.53$  Hz), 7.66 (dd, 1H,  $J=0.82$  Hz,  $J=1.65$  Hz), 7.50 (dd, 1H,  $J=1.38$  Hz,  $J=1.65$  Hz), 6.53 (dd, 1H,  $J=0.82$  Hz,  $J=1.38$  Hz), 3.85 (s, 3H).

<sup>207</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
Rf= 0.51

## 4-nitro-2-(furan-3-yl)benzoic acid



0.76 g of methyl 4-nitro-2-(furan-3-yl)benzoate (3.08 mmol) are dissolved in 10 mL of Methanol; 1.5 mL of NaOH 1 M are dropped and the resulting mixture is heated at 60 °C for 2 hour.

After completion of the reaction, followed by TLC<sup>208</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with 10% HCl, and then extracted three times with 10 mL of Ethyl Acetate.

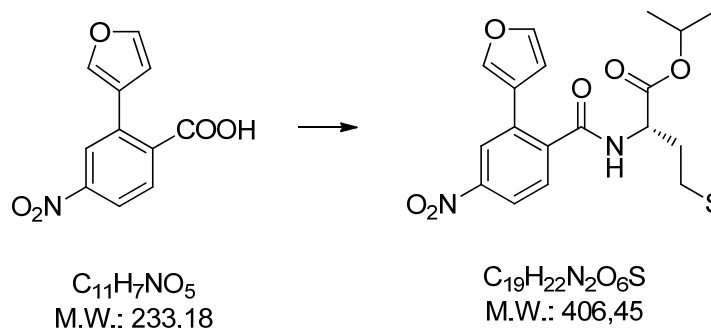
The organic phases are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure, obtaining a dense yellow oil (0.59 g, 2.52 mmol), corresponding to the desired compound.

Yield: 82%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 8.27 (m, 1H), 8.22 (dd, 1H, *J* = 2.2 Hz, *J* = 8.52 Hz), 8.03 (d, 1H, *J* = 8.52 Hz), 7.70 (m, 1H), 7.52 (m, 1H), 6.60 (m, 1H).

<sup>208</sup> TLC conditions: Cyclohexane/ Ethyl Acetate = 1/1  
R<sub>f</sub> = 0.20

**(S)-isopropyl 4-(methylthio)-2-(4-nitro-2-(furan-3-yl)benzamido)butanoate**



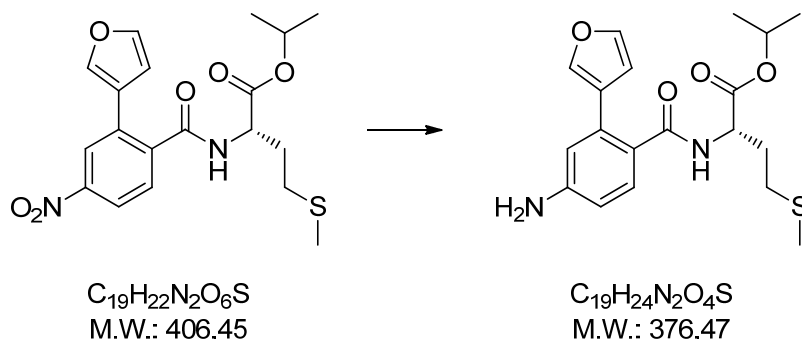
A solution of EDAC (0.58 g, 3.02 mmol) and DIPEA (0.53 mL, 3.02 mmol) in 2 mL of DCM is added dropwise to a solution of 4-nitro-2-(furan-3-yl)benzoic acid (0.59 g, 2.52 mmol), HOBT (0.41 g, 3.02 mmol) in 5 mL of DCM, under a nitrogen atmosphere. The resulting mixture is stirred for 30 minutes at RT and then a solution of L-Methionine isopropyl ester (0.48 g, 2.52 mmol) in 3 mL of DCM is dropped. The reaction mixture is stirred at RT overnight; after completion, monitored by TLC<sup>209</sup>, the mixture is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated. The crude product is purified by column chromatography on silica gel, using Cyclohexane/ Ethyl Acetate= 80/20 as elution solvent, to give 0.77 g (1.89 mmol) of the desired product, as yellow oil.

Yield: 75%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 8.27 (d, 1H, *J*=1.65 Hz), 8.19 (dd, 1H, *J*=2.2 Hz, *J*=8.25 Hz), 7.75 (dd, 1H, *J*=1.1 Hz, *J*=1.65 Hz), 7.71 (dd, 1H, *J*=2.85 Hz, *J*=8.25 Hz), 7.52 (dd, 1H, *J*=2.2 Hz, *J*=2.85 Hz), 6.62 (dd, 1H, *J*=0.83 Hz, *J*=1.61 Hz), 6.41 (d, 1H, *J*=7.7 Hz), 5.06 (m, 1H), 4.87 (m, 1H), 2.37 (t, 2H, *J*=7.7 Hz), 2.16 (m, 1H), 2.07 (s, 3H), 2.03 (m, 1H), 1.28 (d, 3H, *J*=6.60 Hz), 1.26 (d, 3H, *J*=6.60 Hz).

<sup>209</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 80/20  
 R<sub>f</sub> = 0.20

**(S)-isopropyl 2-(4-amino-2-(furan-3-yl)benzamido)-4-(methylthio)butanoate**



(S)-isopropyl 4-(methylthio)-2-(4-nitro-2-(furan-3-yl)benzamido)butanoate (0.77 g, 1.89 mmol) is dissolved in 23 mL of Ethyl Acetate; stannous chloride (1.79 g, 9.45 mmol) is added and the mixture is refluxed for 2 hours.

After completion of the reaction, defined by TLC<sup>210</sup>, the mixture reaction is firstly cooled and poured into 50 mL of iced 5% NaHCO<sub>3</sub>, then it is filtered over Celite and the two layers are separated.

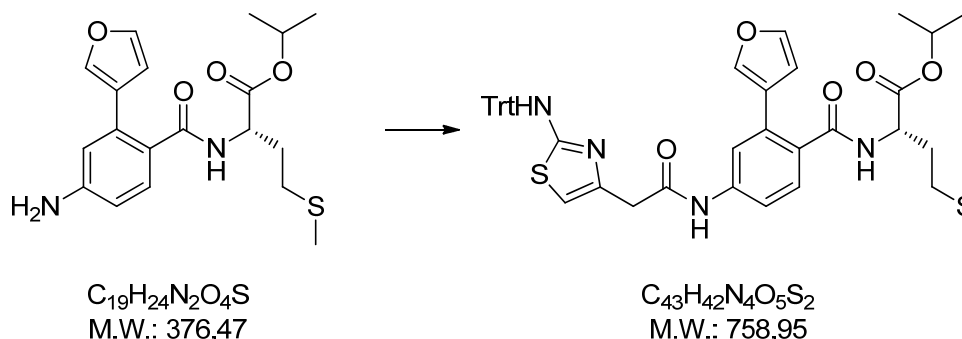
The organic layer is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure, giving 0.67 g (1.78 mmol) of yellow oil.

Yield: 94%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 7.58 (m, 1H), 7.53 (dd, 1H, *J*=8.25 Hz), 7.45 (dd, 1H, *J*=1.38 Hz, 1.65 Hz), 6.65 (dd, 1H, *J*= 2.47 Hz, *J*= 8.25 Hz), 6.61 (d, 1H, *J*=2.2 Hz), 6.52 (d, 1H, *J*= 1.65 Hz), 6.25 (d, 1H, *J*= 7.70 Hz), 5.01 (m, 1H), 4.68 (m, 1H), 2.35 (m, 2H), 2.08 (m, 1H), 2.06 (s, 3H), 1.92 (m, 1H), 1.25 (d, 3H, *J*=6.33 Hz), 1.23 (d, 3H, *J*=6.05 Hz).

<sup>210</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
R<sub>f</sub>= 0.19

**(S)-isopropyl 2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(furan-3-yl)benzamido)-4-(methylthio)butanoate**



A solution of EDAC (0.41 g, 2.14 mmol) and DIPEA (0.37 mL, 2.14 mmol) in 2 mL of dry DMF is added dropwise to a solution of (S)-isopropyl 2-(4-amino-2-(furan-3-yl)benzamido)-4-(methylthio)butanoate (0.67 g, 1.78 mmol), HOBT (0.29 g, 2.14 mmol) in 3 mL of dry DMF, under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of L-Methionine isopropyl ester (0.34 g, 1.78 mmol) in 3 mL of dry DMF is dropped.

The reaction mixture is stirred at RT overnight; after completion, monitored by TLC<sup>211</sup>, the mixture is concentrated, resumed with Ethyl Acetate, washed firstly with a solution of 10% NaHCO<sub>3</sub>, secondly with HCl 10 % and finally with brine.

The organic phase is then dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

The crude product is purified by column chromatography on silica gel, using Cyclohexane/ Ethyl Acetate= 70/30 as elution solvent, to give 0.62 g (0.82 mmol) of the desired product, as colourless oil.

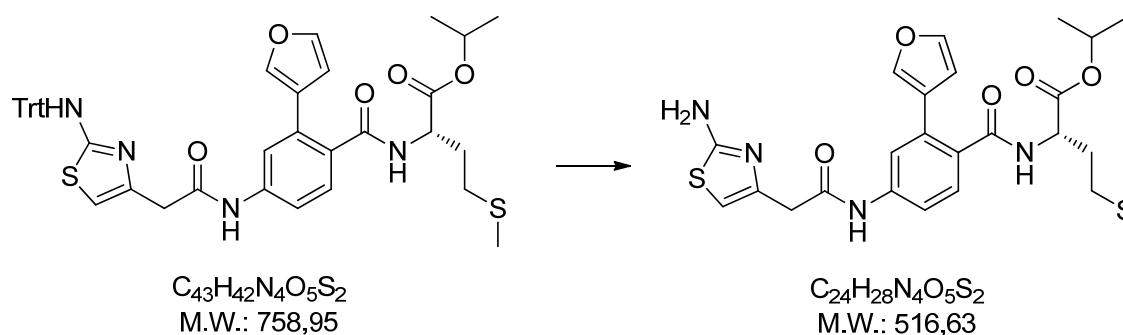
Yield: 46%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 9.50 (bs, 1H), 7.69 (m, 1H), 7.62 (m, 1H), 7.56 (m, 1H), 7.54 (m, 1H), 7.44 (m, 1H), 7.26-7.31 (m, 16H), 6.55 (s, 1H), 6.27 (m, 2H), 5.03 (m, 1H), 4.71 (m, 1H), 3.68 (s, 2H), 2.36 (t, 2H, *J*=6.33 Hz), 2.11 (m, 1H), 2.06 (s, 3H), 1.92 (m, 1H), 1.26 (d, 3H, *J*=6.05 Hz), 1.24 (d, 3H, *J*=6.60 Hz).

<sup>211</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
 R<sub>f</sub> = 0.48



**(S)-isopropyl 2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(furan-3-yl)benzamido)-4-(methylthio)butanoate**



0.62 g (0.82 mmol) of (S)-isopropyl 2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(furan-3-yl)benzamido)-4-(methylthio)butanoate are dissolved in 6 mL of DCM and then treated with 2 mL of Formic acid.

The reaction is followed by TLC<sup>212</sup>, at completion the reaction mixture is slowly poured in 20 mL of 5% aqueous NaHCO<sub>3</sub>, the two layers are separated; the organic layer is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduce pressure.

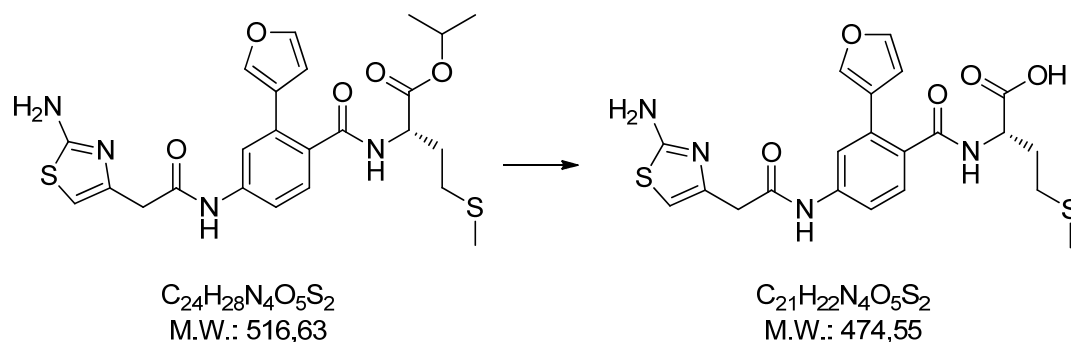
The crude is purified on silica gel, using Ethyl Acetate as elution solvent, yielding 0.31 g of the desired product (0.61 mmol), as sticky solid.

Yield: 74%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 9.34 (s,1H), 7.62 (s, 1H), 7.57 (dd, 1H, *J*=1.93 Hz, *J*=10.18 Hz), 7.49 (dd, 1H, *J*=1.93 Hz, *J*=8.25 Hz), 7.45 (dd, 1H, *J*=0.83 Hz, *J*=1.65 Hz), 7.10 (m, 1H), 6.55 (m, 1H), 6.35-6.37 (m, 2H), 5.30 (bs, 2H), 5.02 (m, 1H), 4.70 (m, 1H), 3.63 (s, 2H), 2.36 (t, 2H, *J*=7.7 Hz), 2.08 (m, 1H), 2.06 (s, 3H), 1.95 (m, 1H), 1.25 (d, 3H, *J*=5.23 Hz), 1.23 (d, 3H, *J*=7.15 Hz).

<sup>212</sup> TLC conditions: Ethyl Acetate= 100 %  
R<sub>f</sub> = 0.24

**(S)-2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(furan-2-yl)benzamido)-4-(methylthio)butanoic acid**



0.31 g of (S)-isopropyl 2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(furan-3-yl)benzamido)-4-(methylthio)butanoate (0.61 mmol) are dissolved in 5 mL of Methanol; 0.61 mL of NaOH 1M are dropped and the resulting mixture is stirred at RT for 18 hours.

After completion of the reaction, followed by TLC<sup>213</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with Formic acid till a white precipitate is formed.

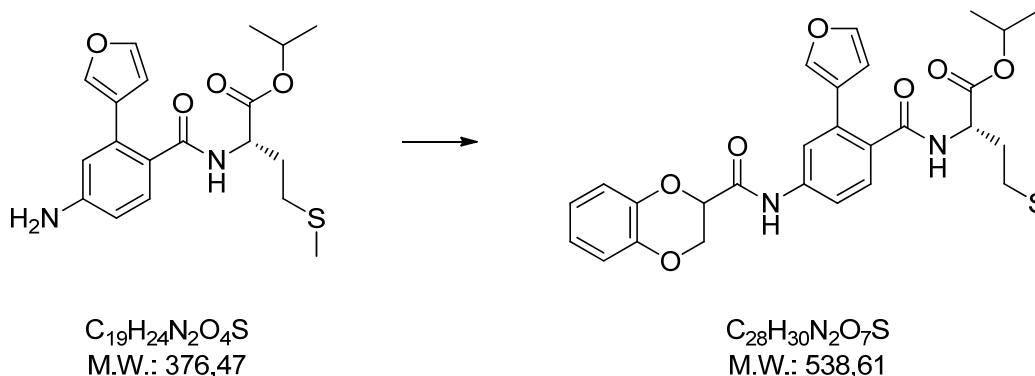
The filtration of the solid yields 0.23 g (0.48 mmol) of the final product, as a sticky solid.

Yield: 78%

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 12.83 (bs, 1H), 10.24 (s, 1H), 8.58 (d, 1H, *J*=7.65 Hz), 7.83 (dd, 3H, *J*=0.80 Hz, *J*=1.61 Hz), 7.71 (d, 1H, *J*=2.01 Hz), 7.65 (dd, 1H, *J*=1.61 Hz, *J*=3.63 Hz), 7.56 (dd, 1H, *J*=2.02 Hz, *J*=8.06 Hz), 7.30 (d, 1H, *J*=8.45 Hz), 7.05 (bs, 1H), 6.89 (bs, 1H), 6.64 (s, 2H), 6.30 (s, 1H), 4.40 (m, 1H), 3.48 (s, 2H), 2.49 (m, 2H), 2.03 (s, 3H), 1.90 (m, 2H).

<sup>213</sup> TLC conditions: Ethyl Acetate= 100%  
R<sub>f</sub> = basal

**(2S)-isopropyl 2-(4-(2,3-dihydrobenzo[b][1,4]dioxine-2-carboxamido)-2-(furan-3-yl)benzamido)-4-(methylthio)butanoate**



A solution of (S)-isopropyl 2-(4-amino-2-(furan-3-yl)benzamido)-4-(methylthio)butanoate (0.67 g, 1.78 mmol) and DIPEA (0.40 mL, 2.31 mmol) in 8 mL of DCM is stirred under a nitrogen atmosphere.

The resulting mixture is cooled to 0°C and then amount of a solution of 2,3-dihydrobenzo[b][1,4]dioxine-2-carbonyl chloride (0.46 g, 2.31 mmol) in 2 mL of DCM.

The reaction mixture is stirred at RT for 2 hours; after completion, monitored by TLC<sup>214</sup>, the mixture is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

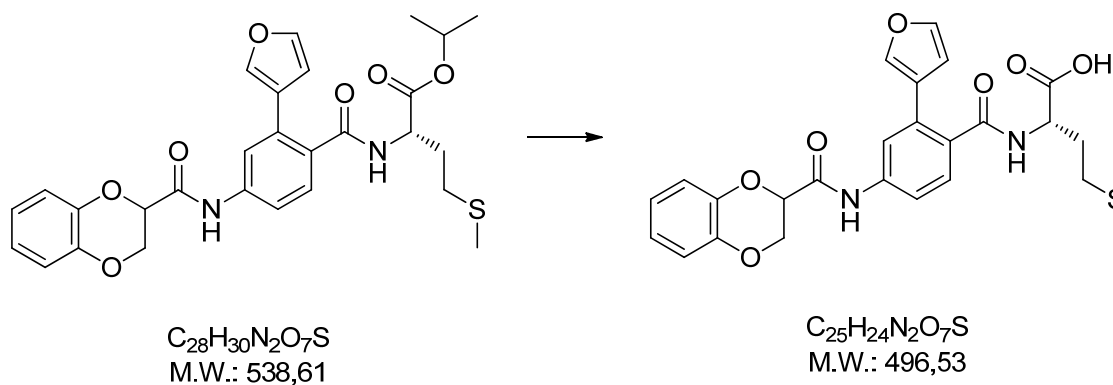
The crude is purified on silica gel, using Cyclohexane/ Ethyl Acetate= 70/30 as elution solvent, obtaining 0.42 g (0.78 mmol) of the desired product, as sticky white solid.

Yield: 44%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 8.37 (s, 1H), 7.55-7.66 (m, 4H), 7.46 (dd, 1H, *J*=1.65 Hz, *J*=1.95 Hz), 7.05 (m, 1H), 6.92-6.96 (m, 3H), 6.57 (dd, 3H, *J*=0.55 Hz, *J*=1.65 Hz), 6.31 (d, 1H, *J*=7.7 Hz), 5.03 (m, 1H), 4.82 (dd, 1H, *J*=2.75 Hz, *J*=7.43 Hz), 4.72 (dd, 1H, *J*=1.93 Hz, *J*=7.43 Hz), 4.63 (dd, 1H, *J*=2.75 Hz, *J*=9.55 Hz), 4.27 (dd, 1H, *J*=7.42 Hz, *J*=11.28 Hz), 2.37 (t, 2H, *J*=7.7 Hz), 2.12 (m, 1H), 2.06 (s, 3H), 1.95 (m, 1H), 1.26 (d, 3H, *J*=6.33 Hz), 1.24 (d, 3H, *J*=6.33 Hz).

<sup>214</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
 R<sub>f</sub> = 0.51

**(2S)-2-(4-(2,3-dihydrobenzo[b][1,4]dioxine-2-carboxamido)-2-(furan-3-yl)benzamido)-4-(methylthio)butanoic acid**



0.42 g of (2S)-isopropyl 2-(4-(2,3-dihydrobenzo[b][1,4]dioxine-2-carboxamido)-2-(furan-3-yl)benzamido)-4-(methylthio)butanoate (0.78 mmol) are dissolved in 5 mL of Methanol; 0.78 mL of NaOH 1M are dropped and the resulting mixture is stirred at RT overnight.

After completion of the reaction, followed by TLC<sup>215</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with Formic acid till a white precipitate is formed.

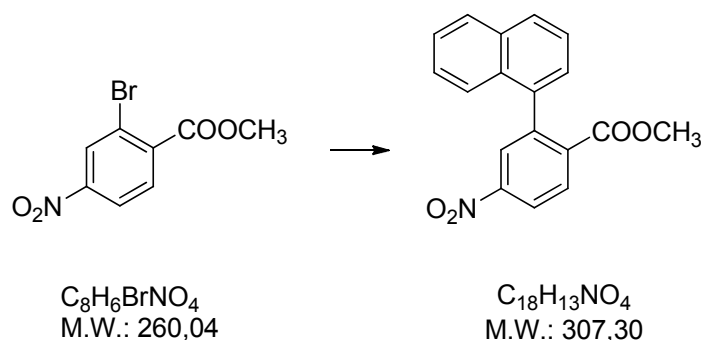
The filtration of the solid yields 0.39 g of the final product, as a sticky solid, in quantitative yield.

Yield: quantitative

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 12.73 (bs, 1H), 10.27 (s, 1H), 8.63 (d, 1H, *J*=6.65 Hz), 7.84 (s, 1H), 7.76 (d, 1H, *J*=1.61 Hz), 7.60-7.66 (m, 2H), 7.34 (dd, 1H, *J*=4.83 Hz, *J*=8.06 Hz), 6.84-7.04 (m, 4H), 6.66 (d, 1H, *J*= 0.81 Hz), 4.99 (m, 1H), 4.42-4.48 (m, 3H), 2.46 (m, 2H), 2.03 (s, 3H), 1.97 (m, 2H).

<sup>215</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
R<sub>f</sub> = basal

## Methyl 2-(naphthalen-1-yl)-4-nitrobenzoate



To a sonicated solution of the methyl 2-bromo-4-nitrobenzoate (1.00 g, 3.85 mmol) in 8 mL of dry DMF, under a nitrogen atmosphere,  $\text{Pd}(\text{PPh}_3)_4$  (0.44 g, 0.385 mmol) is added as solid.

The solution is stirred for 10 minutes and then amount of  $\text{Na}_2\text{CO}_3$  (1.60 g, 11.55 mmol) and of a solution of 1-naphtylboronic acid (0.79 g, 4.62 mmol) in 1 mL of dry DMF.

The reaction mixture is heated at 110 °C for 18 hours and monitored by TLC<sup>216</sup>.

At completion, the solvent is removed and the residue resumed with Ethyl Acetate, washed with brine and dried over  $\text{Na}_2\text{SO}_4$ , filtered and concentrated under reduced pressure.

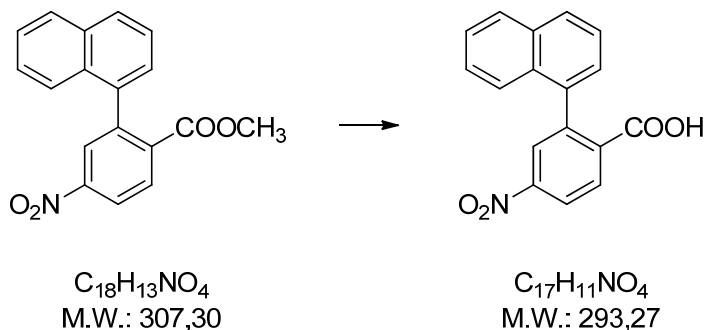
Purification by chromatography on silica gel, through elution with Cyclohexane/ Ethyl Acetate= 85/15, yields 1.15 g (3.73 mmol) of the desired product, as dense yellow oil.

Yield: 97%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 8.36 (dd, 1H,  $J=1.1$  Hz,  $J=8.53$  Hz), 8.31 (dd, 1H,  $J=0.53$  Hz,  $J=1.1$  Hz), 8.15 (dd, 1H,  $J=0.53$  Hz,  $J=8.53$  Hz), 7.93 (dd, 2H,  $J=0.82$  Hz,  $J=7.43$  Hz), 7.33-7.57 (m, 5H), 3.41 (s, 3H).

<sup>216</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
Rf= 0.30

## 4-nitro-2-(naphthalen-3-yl)benzoic acid



1.15 g of methyl 4-nitro-2-(naphthalen-1-yl)benzoate (3.73 mmol) are dissolved in 15 mL of Methanol; 3 mL of NaOH 2.5 M are dropped and the resulting mixture is heated at 60 °C for 3 hour.

After completion of the reaction, followed by TLC<sup>217</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with 10% HCl, and then extracted three times with 15 mL of DCM.

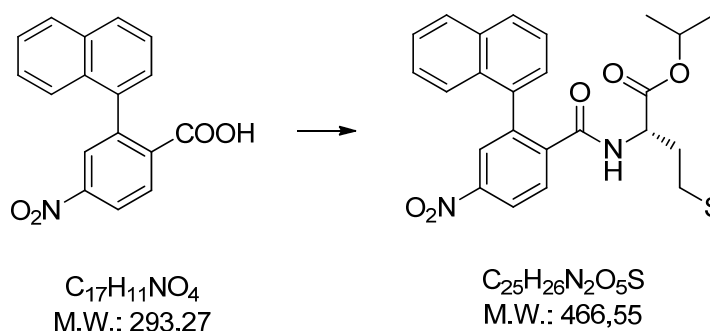
The organic phases are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure, obtaining a dense yellow oil (0.96 g, 3.28 mmol), corresponding to the desired compound.

Yield: 88%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 8.33 (dd, 1H, *J*=2.2 Hz, *J*=8.53 Hz), 8.25 (d, 1H, *J*= 1.93 Hz), 8.17 (d, 1H, *J*=8.53 Hz), 7.91 (d, 2H, *J*=8.25 Hz), 7.50 (m, 2H), 7.29-7.42 (m, 3H).

<sup>217</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
R<sub>f</sub> = 0.30

**(S)-isopropyl 4-(methylthio)-2-(4-nitro-2-(naphthalen-3-yl)benzamido)butanoate**



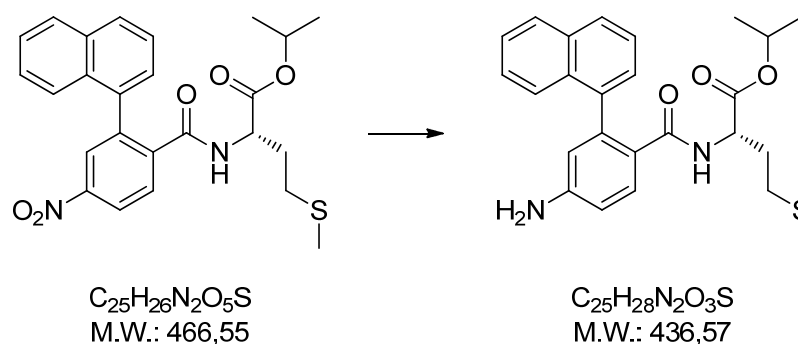
A solution of EDAC (0.75 g, 3.94 mmol) and DIPEA (0.67 mL, 3.94 mmol) in 2 mL of DCM is added dropwise to a solution of 4-nitro-2-(naphthalen-1-yl)benzoic acid (0.96 g, 3.28 mmol), HOBT (0.53 g, 3.94 mmol) in 5 mL of DCM, under a nitrogen atmosphere. The resulting mixture is stirred for 30 minutes at RT and then a solution of L-Methionine isopropyl ester (0.48 g, 2.52 mmol) in 2 mL of DCM is dropped. The reaction mixture is stirred at RT overnight; after completion, monitored by TLC<sup>218</sup>, the mixture is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated. The crude product is purified by column chromatography on silica gel, using Cyclohexane/ Ethyl Acetate= 75/25 as elution solvent, to give 1.36 g (2.92 mmol) of the desired product, as yellow oil.

Yield: 89%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 8.37 (dd, 1H, *J*=2.47 Hz, *J*=8.53 Hz), 8.30 (d, 1H, *J*=1.65 Hz), 8.15 (d, 1H, *J*=8.53 Hz), 7.93-8.00 (m, 2H), 7.42-7.66 (m, 5H), 5.98 (d, 1H, *J*=7.7 Hz), 4.86 (m, 1H), 4.36 (m, 1H), 1.82 (s, 3H), 1.62 (m, 2H), 1.54 (m, 2H), 1.14 (m, 6H).

<sup>218</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 70/30  
 R<sub>f</sub> = 0.27

**(S)-isopropyl 2-(4-amino-2-(naphthalen-3-yl)benzamido)-4-(methylthio)butanoate**



(S)-isopropyl 4-(methylthio)-2-(4-nitro-2-(naphthalen-1-yl)benzamido)butanoate (1.36 g, 2.92 mmol) is dissolved in 40 mL of Ethyl Acetate; stannous chloride (2.77 g, 14.6 mmol) is added and the mixture is refluxed for 5 hours.

After completion of the reaction, defined by TLC<sup>219</sup>, the mixture reaction is firstly cooled and poured into 100 mL of iced 5% NaHCO<sub>3</sub>, then it is filtered over Celite and the two layers are separated.

The organic layer is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure, giving 1.21 g (2.77 mmol) of yellow oil.

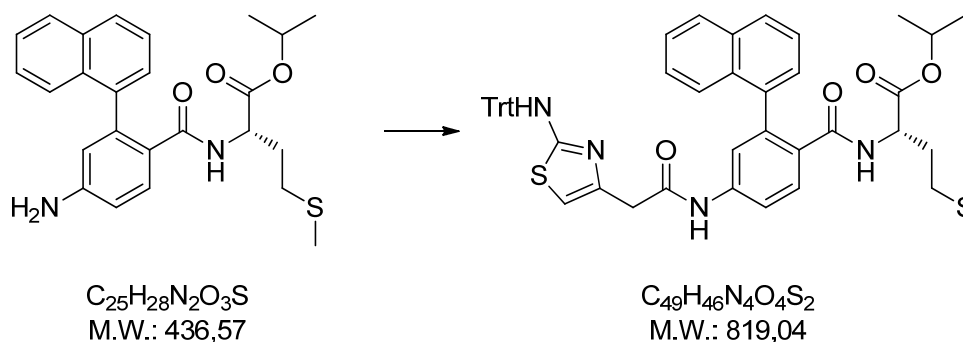
Yield: 95%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 7.90 (m, 2H), 7.74 (dd, 1H, *J*=0.83 Hz, *J*=8.52 Hz), 7.36-7.60 (m, 5H), 6.76 (m, 1H), 6.56 (dd, 1H, *J*= 0.55 Hz, *J*=2.2 Hz), 5.72 (d, 1H, *J*= 7.7 Hz), 4.89 (m, 1H), 4.34 (m, 1H), 1.79 (s, 3H), 1.70 (m, 2H), 1.55 (m, 2H), 1.12 (m, 6H).

<sup>219</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
R<sub>f</sub>= 0.32



**(S)-isopropyl 2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(naphthalen-3-yl)benzamido)-4-(methylthio)butanoate**



A solution of EDAC (0.64 g, 3.32 mmol) and DIPEA (0.43 mL, 3.32 mmol) in 2 mL of dry DMF is added dropwise to a solution of (S)-isopropyl 2-(4-amino-2-(naphthalen-1-yl)benzamido)-4-(methylthio)butanoate (1.21 g, 2.77 mmol), HOBT (0.45 g, 3.32 mmol) in 3 mL of dry DMF, under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of L-Methionine isopropyl ester (0.53 g, 2.77 mmol) in 3 mL of dry DMF is dropped.

The reaction mixture is stirred at RT overnight; after completion, monitored by TLC<sup>220</sup>, the mixture is concentrated, resumed with Ethyl Acetate and washed with brine.

The organic phase is then dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

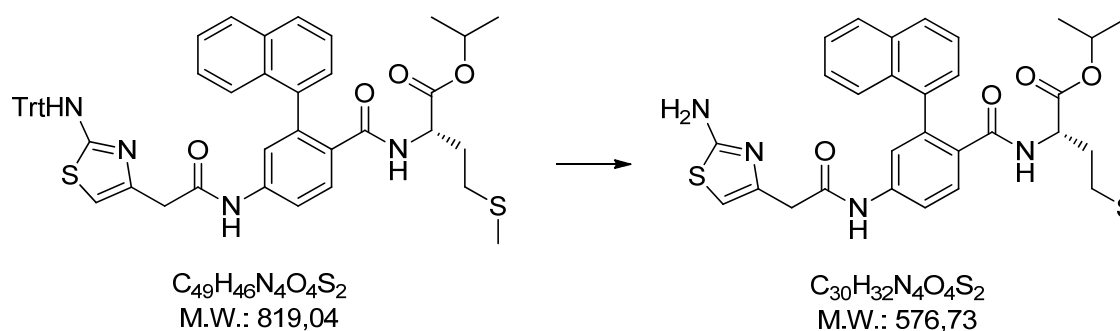
The crude product is purified by column chromatography on silica gel, using Cyclohexane/ Ethyl Acetate= 63/37 as elution solvent, to give 1.64 g (2.00 mmol) of the desired product, as a dense yellow oil.

Yield: 72%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 9.20 (s,1H), 7.94 (m, 3H), 7.16-7.62 (m, 20H), 6.81 (m, 1H), 6.58 (m, 1H), 6.17 (s, 1H), 5.78 (d, 1H, J=7.70 Hz), 4.82 (m, 1H), 4.17 (m, 1H), 3.48 (s, 2H), 1.92 (s, 3H), 1.65 (m, 2H), 1.41 (m, 2H), 1.12 (m, 6H).

<sup>220</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
 Rf = 0.36

**(S)-isopropyl 2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(naphthalen-3-yl)benzamido)-4-(methylthio)butanoate**



1.64 g (2.00 mmol) of (S)-isopropyl 2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(naphthalen-1-yl)benzamido)-4-(methylthio)butanoate are dissolved in 12 mL of DCM and then treated with 4 mL of Formic acid.

The reaction is followed by TLC<sup>221</sup>, at completion the reaction mixture is slowly poured in 30 mL of 5% aqueous NaHCO<sub>3</sub>, the two layers are separated; the organic layer is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduce pressure.

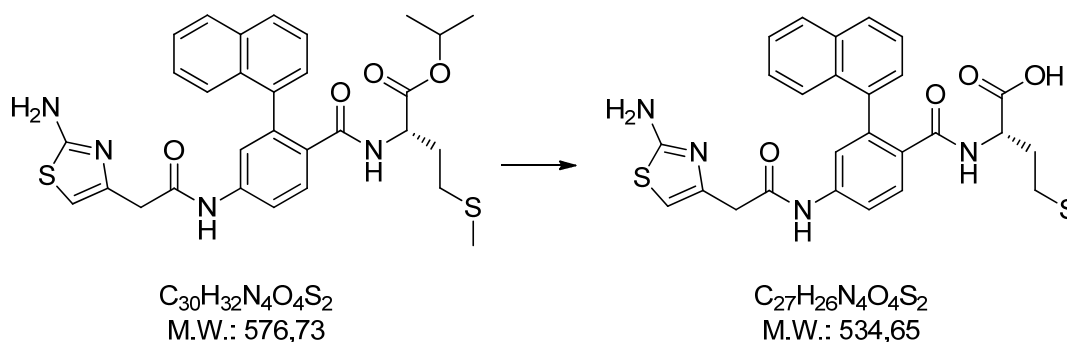
The crude is purified on silica gel, using Ethyl Acetate as elution solvent, yielding 0.69 g of the desired product (1.20 mmol), as sticky solid.

Yield: 60%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 9.42 (d, 1H, *J*=10.73 Hz), 8.02 (d, 3H, *J*=8.53 Hz), 7.89 (m, 2H), 7.77 (m, 1H), 7.36-7.65 (m, 6H), 6.32 (s, 1H), 5.84 (d, 1H, *J*=7.7 Hz), 4.88 (m, 1H), 4.33 (m, 1H), 3.62 (s, 2H), 1.88 (s, 3H), 1.72 (m, 2H), 1.34 (m, 2H), 1.14 (m, 6H).

<sup>221</sup> TLC conditions: Ethyl Acetate= 100 %  
 R<sub>f</sub> = 0.42

**(S)-2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(naphthalen-2-yl)benzamido)-4-(methylthio)butanoic acid**



0.69 g of (S)-isopropyl 2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(naphthalen-1-yl)benzamido)-4-(methylthio)butanoate (1.20 mmol) are dissolved in 10 mL of Methanol; 1.20 mL of NaOH 1M are dropped and the resulting mixture is stirred at RT for 18 hours.

After completion of the reaction, followed by TLC<sup>222</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with Formic acid till a white precipitate is formed.

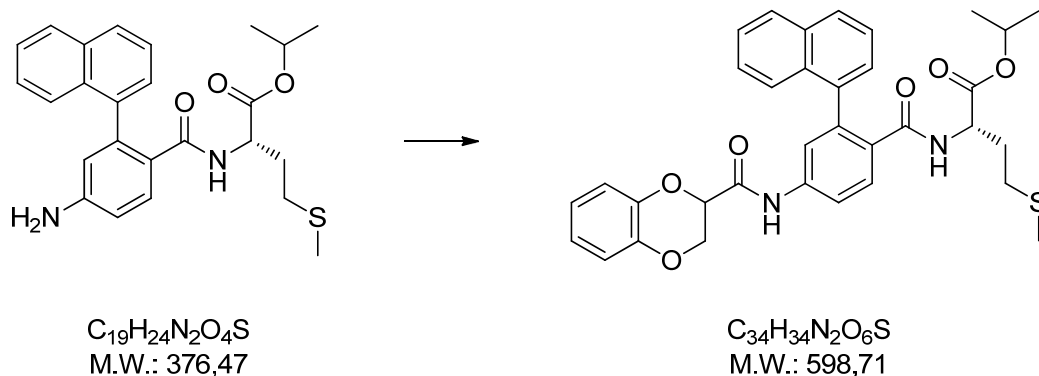
The filtration of the solid yields 0.64 g (1.20 mmol) of the final product.

Yield: Quantitative

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 12.66 (bs, 1H), 10.31 (bs, 1H), 8.22 (d, 1H, *J*=8.45 Hz), 7.90 (m, 2H), 7.74 (d, 1H, *J*=8.52 Hz), 7.30-7.60 (m, 7H), 6.88 (bs, 1H), 6.29 (s, 1H), 4.02 (m, 2H), 3.47 (s, 2H), 2.04 (m, 1H), 1.82 (s, 3H), 1.76 (m, 1H), 1.64 (m, 1H), 1.51 (m, 1H).

<sup>222</sup> TLC conditions: Ethyl Acetate= 100%  
R<sub>f</sub> = basal

**(2S)-isopropyl 2-(4-(2,3-dihydrobenzo[b][1,4]dioxine-2-carboxamido)-2-(naphtalen-3-yl)benzamido)-4-(methylthio)butanoate**



A solution of (S)-isopropyl 2-(4-amino-2-(naphthalen-1-yl)benzamido)-4-(methylthio)butanoate (1.21 g, 2.77 mmol) and DIPEA (0.63 mL, 3.60 mmol) in 8 mL of DCM is stirred under a nitrogen atmosphere.

The resulting mixture is cooled to 0°C and then amount of a solution of 2,3-dihydrobenzo[b][1,4]dioxine-2-carbonyl chloride (0.71 g, 3.60 mmol) in 2 mL of DCM.

The reaction mixture is stirred at RT for 18 hours; after completion, monitored by TLC<sup>223</sup>, the mixture is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

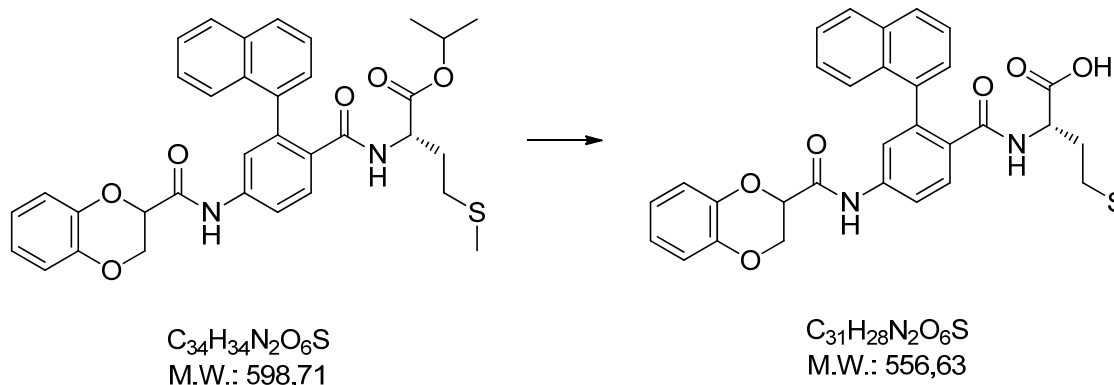
The crude is purified on silica gel, using Cyclohexane/ Ethyl Acetate= 70/30 as elution solvent, obtaining 1.16 g (1.94 mmol) of the desired product, as sticky white solid.

Yield: 70%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 8.42 (m, 2H), 8.08 (dd, 1H, *J*=2.65 Hz, *J*=8.53 Hz), 7.93 (m, 1H), 7.80 (m, 1H), 7.48-7.64 (m, 4H), 7.42 (m, 1H), 6.89-7.03 (m, 4H), 5.84 (d, 1H, *J*=7.98 Hz), 4.79-4.89 (m, 2H), 4.61 (m, 1H), 4.22-4.38 (m, 2H), 1.87 (s, 3H), 1.60 (m, 2H), 1.37 (m, 2H), 1.13 (m, 6H).

<sup>223</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
 R<sub>f</sub> = 0.55

**(2S)-2-(4-(2,3-dihydrobenzo[b][1,4]dioxine-2-carboxamido)-2-(naphthalen-3-yl)benzamido)-4-(methylthio)butanoic acid**



1.16 g of (2S)-isopropyl 2-(4-(2,3-dihydrobenzo[b][1,4]dioxine-2-carboxamido)-2-(naphthalen-1-yl)benzamido)-4-(methylthio)butanoate (1.94 mmol) are dissolved in 15 mL of Methanol; 1.94 mL of NaOH 1M are dropped and the resulting mixture is stirred at RT overnight.

After completion of the reaction, followed by TLC<sup>224</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with Formic acid and extracted three times with Ethyl Acetate.

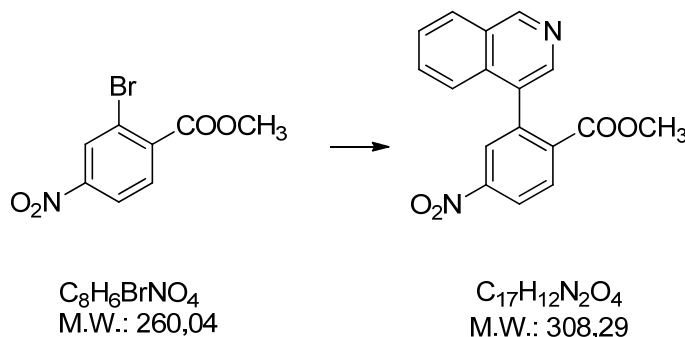
The organic phases are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduce pressure, obtaining 0.93 g (1.67 mmol) of the final product, as a sticky solid.

Yield: 86%

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 12.58 (bs, 1H), 10.35 (s, 1H), 8.29 (d, 1H, *J*=7.76 Hz), 7.79-7.86 (m, 3H), 7.57-7.64 (m, 2H), 7.31-7.50 (m, 5H), 4.98 (m, 1H), 4.35-4.46 (m, 2H), 4.03 (m, 1H), 2.16 (m, 2H), 2.05 (s, 3H), 1.85 (m, 2H).

<sup>224</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
R<sub>f</sub> = basal

## Methyl 2-(isoquinolin-4-yl)-4-nitrobenzoate



To a sonicated solution of the methyl 2-bromo-4-nitrobenzoate (1.00 g, 3.85 mmol) in 10 mL of Toluene, under a nitrogen atmosphere,  $Pd(PPh_3)_4$  (0.44 g, 0.385 mmol) is added as solid.

The solution is stirred for 10 minutes and then amount of  $Na_2CO_3$  (1.02 g, 9.63 mmol) in 2.5 mL of Water and of a solution of 4-isoquinolynboronic acid (0.73 g, 4.24 mmol) in 2.5 mL of Methanol.

The reaction mixture is heated at 80°C for 18 hours and monitored by TLC<sup>225</sup>.

At completion, the mixture is treated with Ethyl Acetate and NaCl 10%, the organic layer is washed with brine and dried over  $Na_2SO_4$ , filtered and concentrated under reduced pressure.

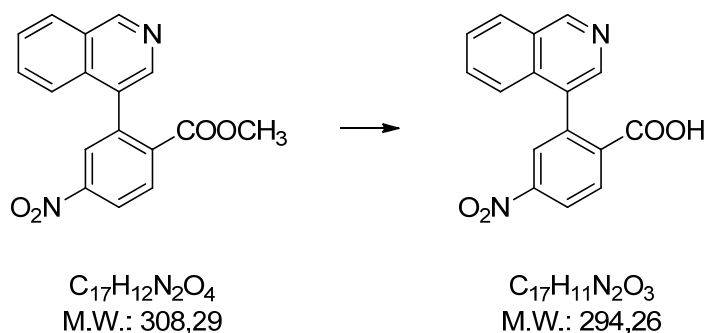
Purification by chromatography on silica gel, through elution with Cyclohexane/ Ethyl Acetate= 60/40, yields 0.66 g (2.16 mmol) of the desired product, as dense red oil.

Yield: 56%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 9.32 (s, 1H), 8.40 (dd, 1H,  $J=3.58$  Hz,  $J=8.53$  Hz), 8.38 (s, 1H), 8.29 (dd, 1H,  $J=1.38$  Hz,  $J=3.58$  Hz), 8.23 (dd, 1H,  $J=1.38$  Hz,  $J=8.53$  Hz), 8.07 (m, 1H), 7.64-7.67 (m, 2H), 7.42 (m, 1H).

<sup>225</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
Rf= 0.25

## 4-nitro-2-(isoquinolin-4-yl)benzoic acid



0.66 g of methyl 4-nitro-2-(isoquinolin-4-yl)benzoate (2.16 mmol) are dissolved in 10 mL of Methanol; 1.3 mL of NaOH 1 M are dropped and the resulting mixture is stirred at RT for 18 hours.

After completion of the reaction, followed by TLC<sup>226</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with 10% HCl, and then extracted three times with 10 mL of Ethyl Acetate.

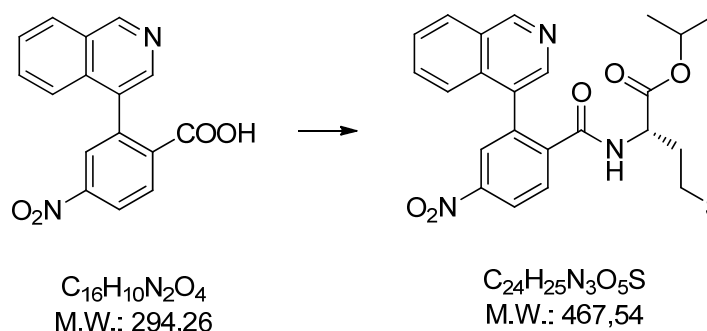
The organic phases are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure, obtaining a dense yellow oil (0.49 g, 1.68 mmol), corresponding to the desired compound.

Yield: 78%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 9.37 (s, 1H), 8.43 (dd, 1H, *J* = 2.48 Hz, *J* = 8.53 Hz), 8.38 (s, 1H), 8.18-8.23 (m, 3H), 7.70-7.75 (m, 2H), 7.45 (m, 1H).

<sup>226</sup> TLC conditions: Cyclohexane/ Ethyl Acetate = 1/1  
R<sub>f</sub> = 0.20

**(S)-isopropyl 4-(methylthio)-2-(4-nitro-2-(isoquinolin-4-yl)benzamido)butanoate**



A solution of EDAC (0.39 g, 2.02 mmol) and DIPEA (0.35 mL, 2.02 mmol) in 2 mL of dry DMF is added dropwise to a solution of 4-nitro-2-(isoquinolin-4-yl) benzoic acid (0.49 g, 1.68 mmol), HOBT (0.27 g, 2.02 mmol) in 5 mL of dry DMF, under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of L-Methionine isopropyl ester (0.32 g, 1.68 mmol) in 3 mL of dry DMF is dropped.

The reaction mixture is stirred at RT overnight; after completion, monitored by TLC<sup>227</sup>, the mixture is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

The crude product is purified by column chromatography on silica gel, using Cyclohexane/ Ethyl Acetate= 60/40 as elution solvent, to give 0.65 g (1.39 mmol) of the desired product, as yellow oil.

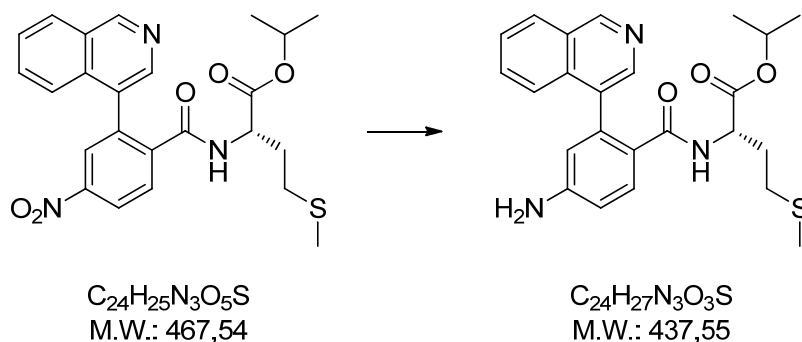
Yield: 83%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 9.36 (d, 1H, *J*=2.48 Hz), 8.52 (d, 1H, *J*=15.4 Hz), 8.41 (m, 1H), 8.30 (d, 1H, *J*= 2.2 Hz), 7.99-8.12 (m, 2H), 7.68-7.74 (m, 2H), 7.50-7.57 (m, 2H), 6.17 (d, 1H, *J*=8.25 Hz), 4.89 (m, 1H), 4.38 (m, 1H), 1.92 (s, 3H), 1.76 (m, 2H), 1.63 (m, 1H), 1.54 (m, 1H), 1.14 (m, 6H).

<sup>227</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
 R<sub>f</sub> = 0.25



**(S)-isopropyl 2-(4-amino-2-(isoquinolin-4-yl)benzamido)-4-(methylthio)butanoate**



(S)-isopropyl 4-(methylthio)-2-(4-nitro-2-(isoquinolin-4-yl)benzamido)butanoate (0.65 g, 1.39 mmol) is dissolved in 20 mL of Ethyl Acetate; stannous chloride (1.79 g, 9.45 mmol) is added and the mixture is refluxed for 1 <sup>1</sup>/<sub>2</sub> hours.

After completion of the reaction, defined by TLC<sup>228</sup>, the mixture reaction is firstly cooled and poured into 50 mL of iced 5% NaHCO<sub>3</sub>, then it is filtered over Celite and the two layers are separated.

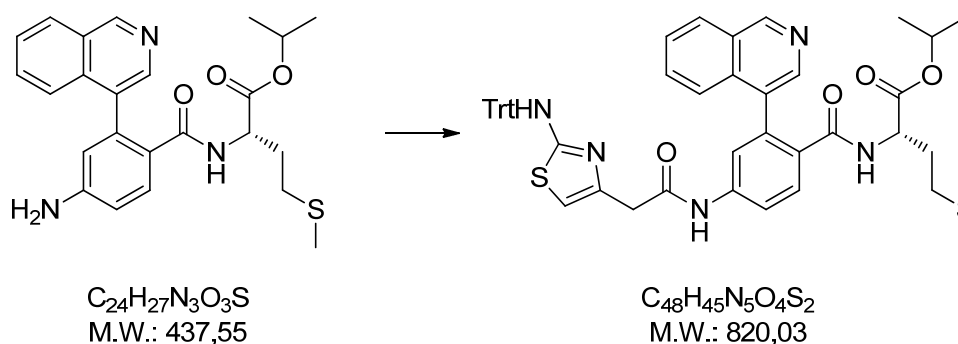
The organic layer is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure, giving 0.55 g (1.27 mmol) of dense white oil.

Yield: 91%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 9.28 (d, 1H, *J*=6.33 Hz), 8.50 (s, 1H), 8.03 (m, 1H), 7.83 (d, 1H, *J*=8.53 Hz), 7.62-7.74 (m, 3H), 6.80 (m, 1H), 6.59 (dd, 1H, *J*=1.1 Hz, *J*=2.2 Hz), 5.80 (d, 1H, *J*=7.7 Hz), 4.86 (m, 1H), 4.34 (m, 1H), 4.10 (bs, 2H), 1.94 (s, 3H), 1.77 (m, 2H), 1.56 (m, 1H), 1.44 (m, 1H), 1.14 (m, 6H).

<sup>228</sup> TLC conditions: Ethyl Acetate= 100 %  
R<sub>f</sub>= 0.30

**(S)-isopropyl 2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(isoquinolin-4-yl) benzamido)-4-(methylthio)butanoate**



A solution of EDAC (0.29 g, 1.52 mmol) and DIPEA (0.26 mL, 1.52 mmol) in 2 mL of dry DMF is added dropwise to a solution of (S)-isopropyl 2-(4-amino-2-(isoquinolin-4-yl)benzamido)-4-(methylthio)butanoate (0.55 g, 1.27 mmol), HOBT (0.21 g, 1.52 mmol) in 2 mL of dry DMF, under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of L-Methionine isopropyl ester (0.24 g, 1.27 mmol) in 2 mL of dry DMF is dropped.

The reaction mixture is stirred at RT overnight; after completion, monitored by TLC<sup>229</sup>, the mixture is concentrated, resumed with Ethyl Acetate, washed firstly with a solution of 10% NaHCO<sub>3</sub>, secondly with HCl 10 % and finally with brine.

The organic phase is then dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

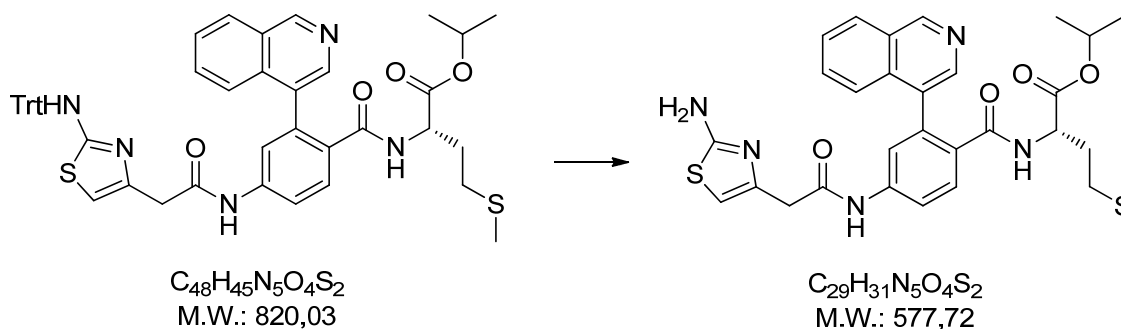
The crude product is purified by column chromatography on silica gel, using Ethyl Acetate= 100% as elution solvent, to give 0.96 g (1.17 mmol) of the desired product, as sticky orange oil.

Yield: 92%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 9.20 (m, 2H), 8.50 (s, 1H), 8.07 (m, 1H), 7.81 (d, 1H, *J*=8.25 Hz), 7.63 (m, 2H), 7.45 (m, 2H), 7.18-7.38 (m, 15H), 6.54 (bs, 1H), 6.19 (s, 1H), 5.90 (d, 1H, *J*=7.43 Hz), 4.82 (m, 1H), 4.35 (m, 1H), 3.54 (s, 2H), 1.84 (s, 3H), 1.78 (m, 2H), 1.56 (m, 2H), 1.12 (m, 6H).

<sup>229</sup> TLC conditions: Ethyl Acetate= 100%  
R<sub>f</sub> = 0.50

**(S)-isopropyl 2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(isoquinolin-4-yl)benzamido)-4-(methylthio)butanoate**



0.96 g (1.12 mmol) of (S)-isopropyl 2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(naphthalen-1-yl)benzamido)-4-(methylthio)butanoate are dissolved in 9 mL of DCM and then treated with 3 mL of Formic acid.

The reaction is followed by TLC<sup>230</sup>, at completion the reaction mixture is slowly poured in 20 mL of 5% aqueous NaHCO<sub>3</sub>, the two layers are separated; the organic layer is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduce pressure.

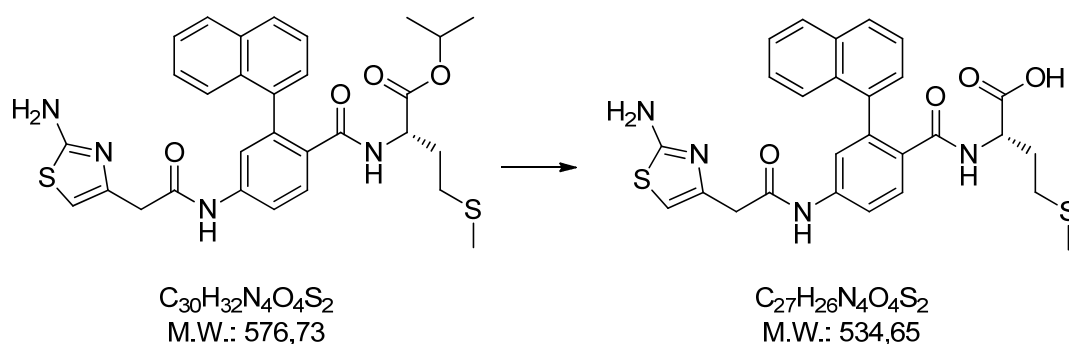
The crude is purified on silica gel, using Ethyl Acetate as elution solvent, yielding 0.65 g of the desired product (1.12 mmol), as sticky solid.

Yield: Quantitative

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 9.52 (d, 1H, *J*=4.13 Hz), 9.28 (d, 1H, *J*=6.05 Hz), 8.53 (s, 1H), 8.03 (m, 1H), 7.91 (d, 1H, *J*=8.53 Hz), 7.81 (m, 1H), 7.51-7.67(m, 4H), 6.33 (s, 1H), 6.06 (d, 1H, *J*=7.43 Hz), 5.21 (bs, 2H), 4.85 (m, 1H), 4.34 (m, 2H), 3.63 (s, 2H), 1.96 (m, 1H), 1.93 (s, 3H), 1.84 (m, 1H), 1.52 (m, 2H), 1.14 (m, 6H).

<sup>230</sup> TLC conditions: Ethyl Acetate/ Methanol= 80/ 25  
 R<sub>f</sub> = 0.55

**(S)-2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(isoquinolin-4-yl)benzamido)-4-(methylthio)butanoic acid**



0.65 g of (S)-isopropyl 2-(4-(2-(2-aminothiazol-4-yl)acetamido)-2-(isoquinolin-4-yl)benzamido)-4-(methylthio)butanoate (1.12 mmol) are dissolved in 6 mL of Methanol; 2.24 mL of NaOH 1M are dropped and the resulting mixture is stirred at RT for 18 hours.

After completion of the reaction, followed by TLC<sup>231</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with Formic acid and extracted three times with Ethyl Acetate.

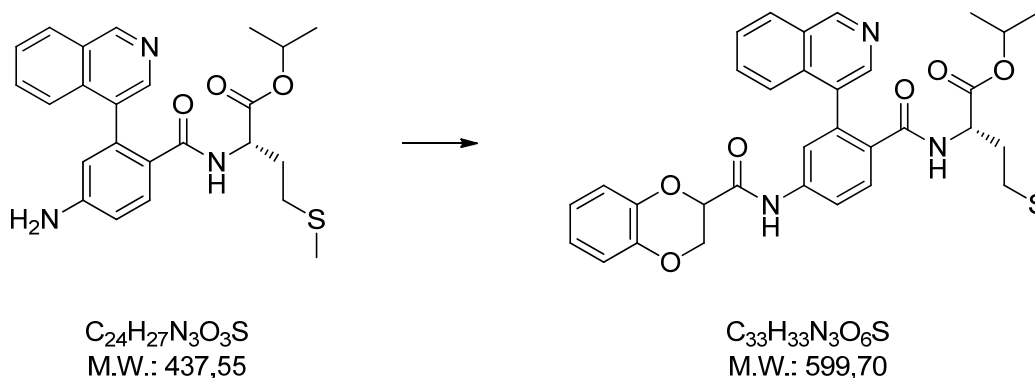
The organic phases are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated, yielding 0.53 g (1.00 mmol) of the final product, as a sticky solid.

Yield: 89%

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 12.61 (bs, 1H), 10.43 (bs, 1H), 9.27 (d, 1H, *J*=6.59 Hz), 8.26-8.39 (m, 4H), 7.57-7.78 (m, 4H), 7.48 (m, 1H), 6.88 (bs, 2H), 6.30 (s, 1H), 3.84 (m, 1H), 3.48 (s, 2H), 1.90 (m, 1H), 1.83 (s, 3H), 1.67 (m, 1H), 1.55 (m, 1H), 1.51 (m, 1H).

<sup>231</sup> TLC conditions: Ethyl Acetate= 100%  
R<sub>f</sub> = basal

**(2S)-isopropyl 2-(4-(2,3-dihydrobenzo[b][1,4]dioxine-2-carboxamido)-2-(isoquinolin-4-yl)benzamido)-4-(methylthio)butanoate**



A solution of (S)-isopropyl 2-(4-amino-2-(isoquinolin-4-yl)benzamido)-4-(methylthio)butanoate (0.55 g, 1.27 mmol) and DIPEA (0.33 mL, 1.91 mmol) in 7 mL of dry DMF is stirred under a nitrogen atmosphere.

The resulting mixture is cooled to 0°C and then amount of a solution of 2,3-dihydrobenzo[b][1,4]dioxine-2-carbonyl chloride (0.38 g, 1.91 mmol) in 3 mL of dry DMF.

The reaction mixture is stirred at RT for 2 hours; after completion, monitored by TLC<sup>232</sup>, the mixture is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

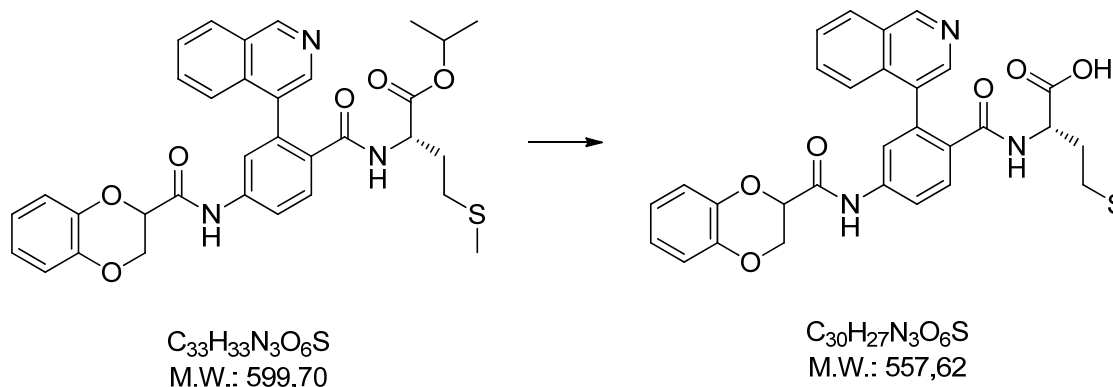
The crude is purified on silica gel, using a gradient of Cyclohexane/ Ethyl Acetate, from 30% to 100% of Ethyl Acetate, as elution solvent, obtaining 0.67 g (1.12 mmol) of the desired product, as sticky white solid.

Yield: 88%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 9.30 (d, 1H, *J*=4.68 Hz), 8.50 (m, 2H), 7.91-8.05 (m, 2H), 7.85 (m, 1H), 7.58-7.67 (m, 3H), 6.90-7.01 (m, 4H), 6.05 (d, 1H, *J*=7.7 Hz), 4.80-4.90 (m, 2H), 4.60 (d, 1H, *J*=11.56 Hz), 4.08-4.31 (m, 2H), 1.92 (s, 3H), 1.84 (m, 2H), 1.56 (m, 2H), 1.12 (m, 6H).

<sup>232</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 30/70  
 R<sub>f</sub> = 0.21

**(2S)-2-(4-(2,3-dihydrobenzo[b][1,4]dioxine-2-carboxamido)-2-(isoquinolin-4-yl)benzamido)-4-(methylthio)butanoic acid**



0.67 g of (2S)-isopropyl 2-(4-(2,3-dihydrobenzo[b][1,4]dioxine-2-carboxamido)-2-(isoquinolin-4-yl)benzamido)-4-(methylthio)butanoate (1.12 mmol) are dissolved in 5 mL of Methanol; 1.12 mL of NaOH 1M are dropped and the resulting mixture is stirred at RT overnight.

After completion of the reaction, followed by TLC<sup>233</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with Formic acid till a white precipitate is formed.

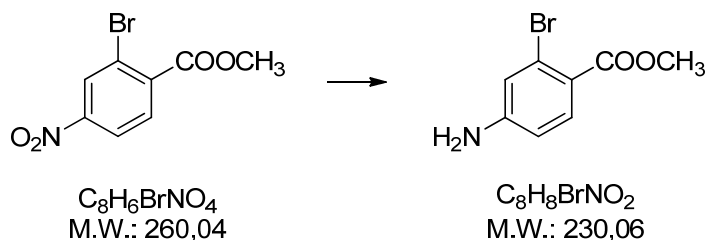
The filtration of the solid yields 0.62 g of the final product, as a sticky white solid, in quantitative yield.

Yield: quantitative

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 12.62 (bs, 1H), 10.41 (bs, 1H), 9.27 (d, 1H,  $J=4.03$  Hz), 8.13-8.36 (m, 3H), 7.86 (dd, 1H,  $J=1.82$  Hz,  $J=8.06$  Hz), 7.50-7.68 (m, 5H), 6.82-7.02 (m, 4H), 5.00 (d, 1H,  $J=2.56$  Hz,  $J=5.5$  Hz), 4.32-4.46 (m, 2H), 4.04 (m, 1H), 2.08 (m, 2H), 1.88 (s, 3H), 1.74 (m, 2H).

<sup>233</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
R<sub>f</sub> = basal

## Methyl 4-amino-2-bromobenzoate



Methyl 2-bromo-4-nitrobenzoate (10.00 g, 38.46 mmol) is dissolved in 300 mL of Ethyl Acetate and 1 mL of Water. Stannous chloride (36.46 g, 192.3 mmol) is added and the mixture is refluxed for 2 hours.

After completion of the reaction, defined by TLC<sup>234</sup>, the mixture reaction is firstly cooled and poured into 600 mL of iced 5% NaHCO<sub>3</sub>, then it is filtered over Celite and the two layers are separated.

The organic layer is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure, giving 7.96 g of methyl 4-amino-2-bromobenzoate, as a white solid.

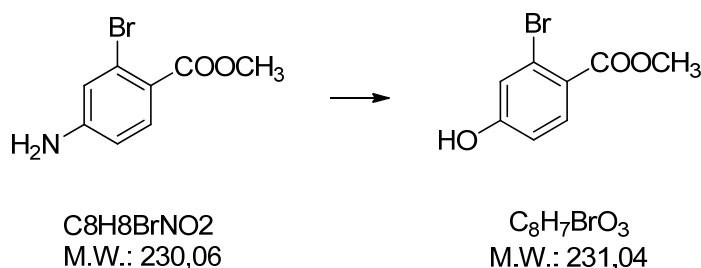
Yield: 90%

Melting Point: 81-83 °C

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 7.76 (dd, 1H, *J*=0.83 Hz, *J*=8.53 Hz), 6.92 (d, 1H, *J*=0.83 Hz, *J*=2.48 Hz), 6.56 (dd, 1H, *J*=2.48 Hz, *J*=8.53 Hz), 4.05 (bs, 2H), 3.86 (s, 3H).

<sup>234</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
R<sub>f</sub>= 0.45

## Methyl 2-bromo-4-hydroxybenzoate



Methyl 4-amino-2-bromobenzoate (1.00 g, 4.35 mmol) is suspended in 15 ml of  $\text{H}_2\text{SO}_4$  20% and then cooled to  $-15^\circ\text{C}$ .

To this mixture an iced solution of  $\text{NaNO}_2$  (0.33g, 4.79 mmol) in 1 mL of Water is slowly dropped; the reaction is maintained at  $-15^\circ\text{C}$  for 30 minutes, then warmed to RT and kept at that temperature for 1 hour.

The yellow solution is lastly heated at  $60^\circ\text{C}$  for 18 hours; the reaction is monitored by TLC<sup>235</sup>; when accomplished, it is cooled and extracted three times with 10 mL of Ethyl Acetate; the organic layers are collected, washed with brine, dried over  $\text{Na}_2\text{SO}_4$ , filtered and concentrated under reduced pressure.

The crude is eventually purified by chromatography on silica gel; elution with Cyclohexane/ Ethyl Acetate= 65/35 yields 0.93 g (4.04 mmol) of the desired product, as yellow solid.

Yield: 94%

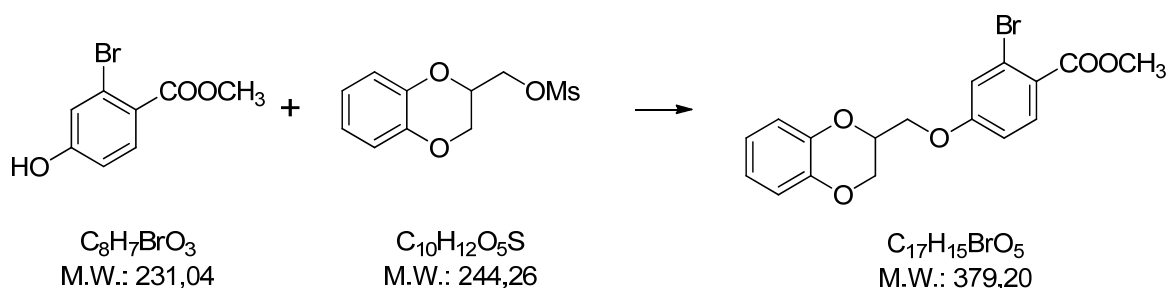
Melting Point:  $152\text{-}155^\circ\text{C}$

**$^1\text{H-NMR}$  ( $\text{CDCl}_3$ ):** 7.82 (d, 1H,  $J=8.53$  Hz), 7.22 (dd, 1H,  $J=0.83$  Hz,  $J=2.48$  Hz), 6.82 (dd, 1H,  $J=2.48$  Hz,  $J=8.53$  Hz), 6.13 (bs, 1H), 3.90 (s, 3H).

<sup>235</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
Rf= 0.54



## Methyl 2-bromo-4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)benzoate



To a suspension of  $\text{K}_2\text{CO}_3$  (0.60 g, 4.33 mmol) in 2 mL of DMF, under a nitrogen atmosphere, is dropped a solution of methyl 2-bromo-4-hydroxybenzoate (1.00 g, 4.33 mmol) in 5 mL of DMF.

The reaction mixture is maintained at RT for 30 minutes and then the 2-mesyloxymethylbenzodioxane (1.06 g, 4.33 mmol), dissolved in 3 mL of DMF, is added in.

The reaction, monitored by TLC<sup>236</sup>, is warmed at 60 °C for 18 hours.

After cooling, the mixture is concentrated and the residue resumed with Ethyl Acetate; the organic layer is washed with brine, dried over  $\text{Na}_2\text{SO}_4$ , filtered and concentrated under reduced pressure.

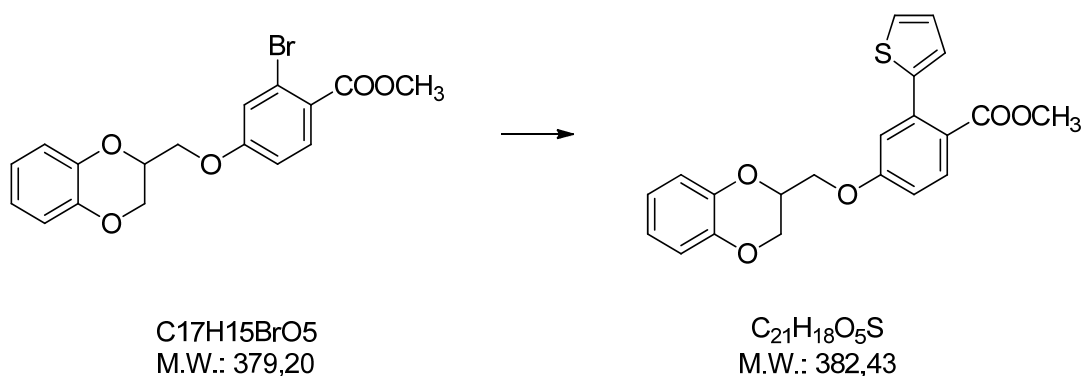
The crude is purified by chromatography on silica gel; elution with Cyclohexane/ Ethyl Acetate= 80/20 yields 1.25 g (3.29 mmol) of the desired product, as yellow oil.

Yield: 76%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 7.86 (d, 1H,  $J=8.8$  Hz), 7.23 (dd, 1H,  $J=0.55$  Hz,  $J=2.75$  Hz), 6.85-6.93 (m, 5H), 4.56 (m, 1H), 4.38 (dd, 1H,  $J=2.48$  Hz,  $J=11.56$  Hz), 4.16-4.30 (m, 3H), 3.90 (s, 3H).

<sup>236</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 70/30  
 Rf= 0.44

## Methyl 4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(thiophen-2-yl)benzoate



To a sonicated solution of the methyl 2-bromo-4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)benzoate (1.00 g, 2.64 mmol) in 5 mL of dry DMF, under a nitrogen atmosphere, Pd(PPh<sub>3</sub>)<sub>4</sub> (0.31 g, 0.264 mmol) is added as solid.

The solution is stirred for 10 minutes and then amount of Na<sub>3</sub>PO<sub>4</sub>·12H<sub>2</sub>O (3.01 g, 7.92 mmol) and of a solution of 2-thienylboronic acid (0.41 g, 3.17 mmol) in 5 mL of dry DMF.

The reaction mixture is heated at 100 °C for 18 hours, monitored by TLC<sup>237</sup> and then concentrated.

The crude is resumed with Ethyl Acetate, washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure.

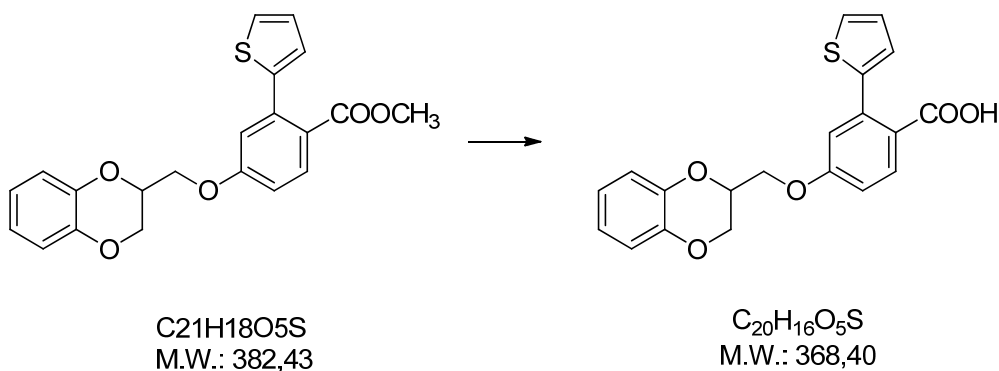
Purification by chromatography on silica gel, through elution with Cyclohexane/ Ethyl Acetate= 80/20, yields 0.81 g (2.11 mmol) of the desired product, as yellow oil.

Yield: 80%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 7.80 (d, 1H, *J*=8.8 Hz), 7.35 (dd, 1H, *J*=1.1 Hz, *J*=4.96 Hz), 7.00-7.06 (m, 3H), 6.85-6.96 (m, 5H), 4.58 (m, 1H), 4.39 (dd, 1H, *J*=2.47 Hz, *J*=11.55 Hz), 4.19-4.33 (m, 3H), 3.71 (s, 3H).

<sup>237</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 70/30  
R<sub>f</sub>= 0.45

## 4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(thiophen-2-yl)benzoic acid



0.81 g of methyl 4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(thiophen-2-yl)benzoate (2.11 mmol) is dissolved in 10 mL of Methanol; 3.17 mL of NaOH 1M are dropped and the resulting mixture is heated at 60 °C for 18 hours.

After completion of the reaction, followed by TLC<sup>238</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether and then acidified with 10% HCl, till a sticky solid precipitate.

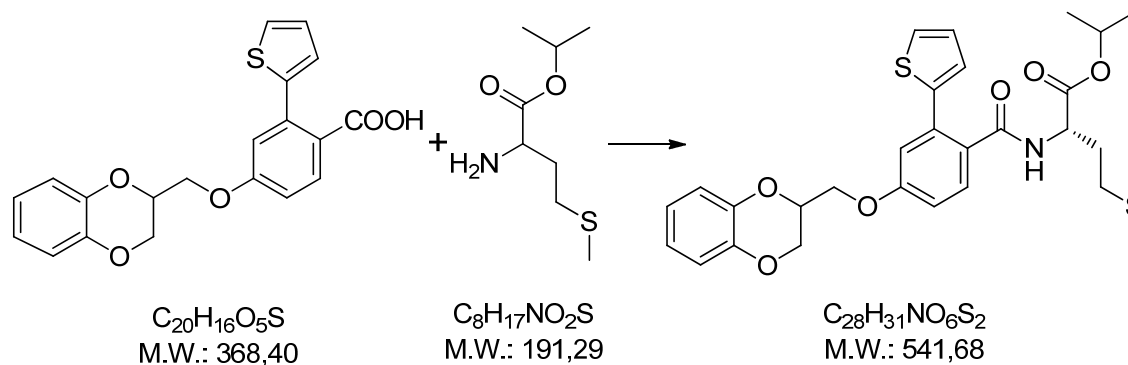
The filtration yields 0.73 g (1.96 mmol) of the desired compound.

Yield: 93%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 7.95 (d, 1H, *J*=8.53 Hz), 7.35 (dd, 1H, *J*=1.65 Hz, *J*=4.67 Hz), 6.85-7.26 (m, 8H), 4.58 (m, 1H), 4.39 (dd, 1H, *J*=2.48 Hz, *J*=11.55 Hz), 4.20-4.34 (m, 3H).

<sup>238</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
R<sub>f</sub> = 0.40

**(2S)-isopropyl 2-(4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(thiophen-2-yl)benzamido)-4-(methylthio)butanoate**



A solution of EDAC (0.45 g, 2.35 mmol) and DIPEA (0.41 mL, 2.35 mmol) in 2 mL of DCM is added dropwise to a solution of 4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(thiophen-2-yl)benzoic acid (0.73 g, 1.96 mmol), HOBT (0.31 g, 2.35 mmol) in 3 mL of DCM, under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of L-Methionine isopropyl ester (0.37 g, 1.96 mmol) in 3 mL of DCM is dropped.

The reaction mixture is stirred at RT overnight; after completion, monitored by TLC<sup>239</sup>, the mixture is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

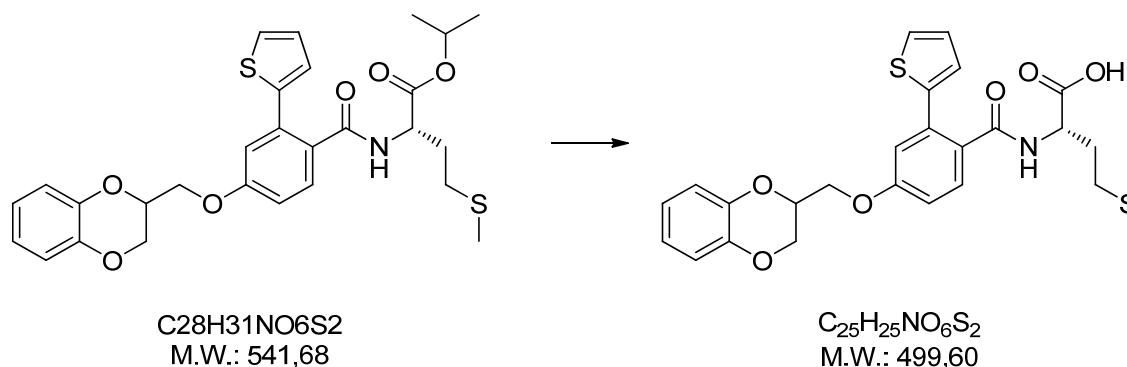
The crude product is purified by column chromatography on silica gel, using Cyclohexane/ Ethyl Acetate= 70/30 as elution solvent, to give 0.88 g (1.63 mmol) of the desired product.

Yield: 83%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 7.65 (dd, 1H, *J*=0.83 Hz, *J*=8.8 Hz), 7.37 (dd, 1H, *J*=1.1 Hz, *J*=5.22 Hz), 7.15 (dd, 1H, *J*=1.1 Hz, *J*=3.58 Hz), 7.08 (dd, 1H, *J*=3.58 Hz, *J*=5.22 Hz), 6.84-6.98 (m, 6H), 6.15 (d, 1H, *J*=7.7 Hz), 5.00 (m, 1H), 4.66 (m, 1H), 4.57 (m, 1H), 4.20-4.42 (m, 3H), 2.23 (t, 2H, *J*=7.7 Hz), 2.04 (s, 3H), 2.00-2.07 (m, 1H), 1.84 (m, 1H), 1.24 (d, 3H, *J*=6.6 Hz), 1.22 (d, 3H, *J*=6.32 Hz).

<sup>239</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
R<sub>f</sub> = 0.54

**(2S)-2-(4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(thiophen-2-yl)benzamido)-4-(methylthio)butanoic acid**



0.88 g of (2S)-isopropyl 2-(4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(thiophen-2-yl)benzamido)-4-(methylthio)butanoate (1.63 mmol) are dissolved in 10 mL of Methanol; 1.63 mL of NaOH 1M are dropped and the resulting mixture is stirred RT for 18 hours

After completion of the reaction, followed by TLC<sup>240</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with 10% HCl and then extracted three times with 10 mL of Ethyl Acetate.

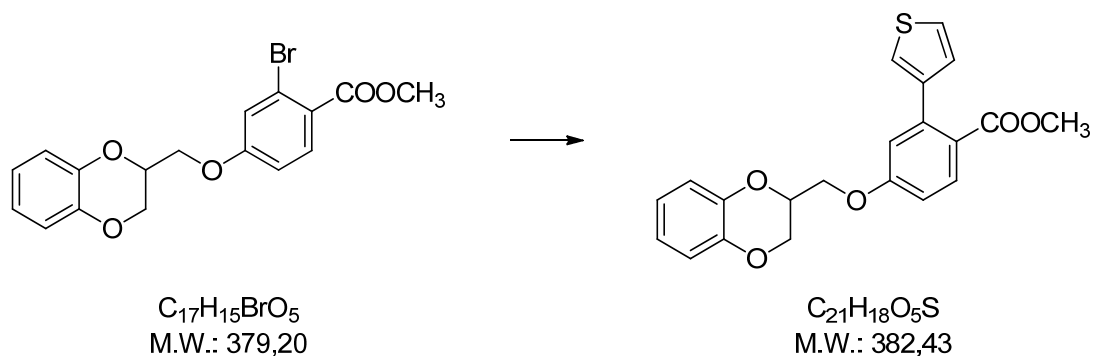
The filtration yields 0.81 g of the final product, as a sticky solid, in quantitative yield.

Yield: quantitative

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 12.60 (bs, 1H), 8.54 (d, 1H, *J*=7.7 Hz), 7.54 (dd, 1H, *J*=1.1 Hz, *J*=5.14 Hz), 7.33 (d, 1H, *J*=9.16 Hz), 7.25 (dd, 1H, *J*=1.11 Hz, *J*=3.3 Hz), 7.03 (m, 3H), 6.81-6.92 (m, 4H), 4.58 (m, 1H), 4.3-4.46 (m, 4H), 4.15 (m, 1H), 2.32-2.40 (m, 2H), 2.00 (s, 3H), 1.86-2.02 (m, 2H).

<sup>240</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
 R<sub>f</sub> = basal

## Methyl 4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)- 2-(thiophen-3-yl)benzoate



To a sonicated solution of the methyl 2-bromo-4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)benzoate (1.00 g, 2.64 mmol) in 5 mL of dry DMF, under a nitrogen atmosphere, Pd(PPh<sub>3</sub>)<sub>4</sub> (0.31 g, 0.264 mmol) is added as solid.

The solution is stirred for 10 minutes and then amount of Na<sub>3</sub>PO<sub>4</sub>·12H<sub>2</sub>O (3.01 g, 7.92 mmol) and of a solution of 3-thienylboronic acid (0.41 g, 3.17 mmol) in 5 mL of dry DMF.

The reaction mixture is heated at 100 °C for 18 hours, monitored by TLC<sup>241</sup> and then concentrated.

The crude is resumed with Ethyl Acetate, washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure.

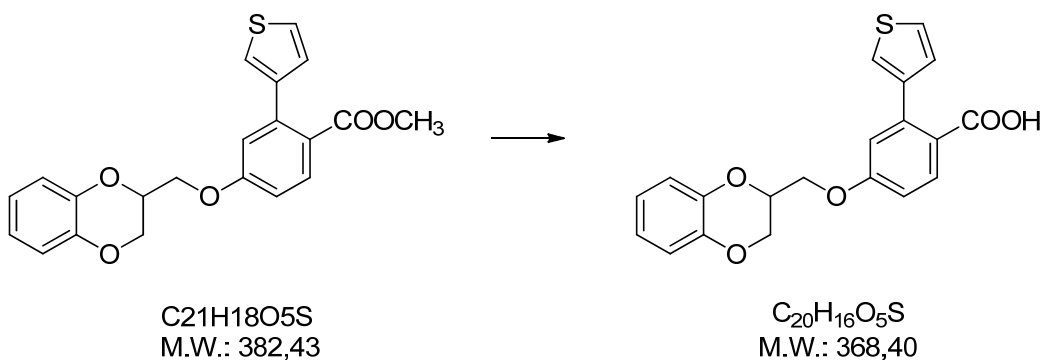
Purification by chromatography on silica gel, through elution with Cyclohexane/ Ethyl Acetate= 80/20, yields 0.84 g (2.19 mmol) of the desired product, as yellow oil.

Yield: 83%

<sup>1</sup>H-NMR (CDCl<sub>3</sub>): 7.85 (d, 1H, *J*=9.08 Hz), 7.32 (dd, 1H, *J*=0.82 Hz, *J*=4.95 Hz), 7.22 (dd, 1H, *J*=0.82 Hz, *J*=2.75 Hz), 7.06 (m, 1H), 6.85-6.95 (m, 6H), 4.59 (m, 1H), 4.40 (dd, 1H, *J*=2.2 Hz, *J*=11.55 Hz), 4.19-4.33 (m, 3H), 3.70 (s, 3H).

<sup>241</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 70/30  
R<sub>f</sub>= 0.46

## 4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(thiophen-3-yl)benzoic acid



0.84 g of methyl 4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(thiophen-3-yl)benzoate (2.19 mmol) is dissolved in 10 mL of Methanol; 5 mL of NaOH 4M are dropped and the resulting mixture is stirred RT for 18 hours.

After completion of the reaction, followed by TLC<sup>242</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with 10% HCl and then extracted three times with Ethyl Acetate.

The organic layers are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure, giving 0.80 g (2.19 mmol) of a white solid, corresponding to the 4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(thiophen-3-yl)benzoic acid.

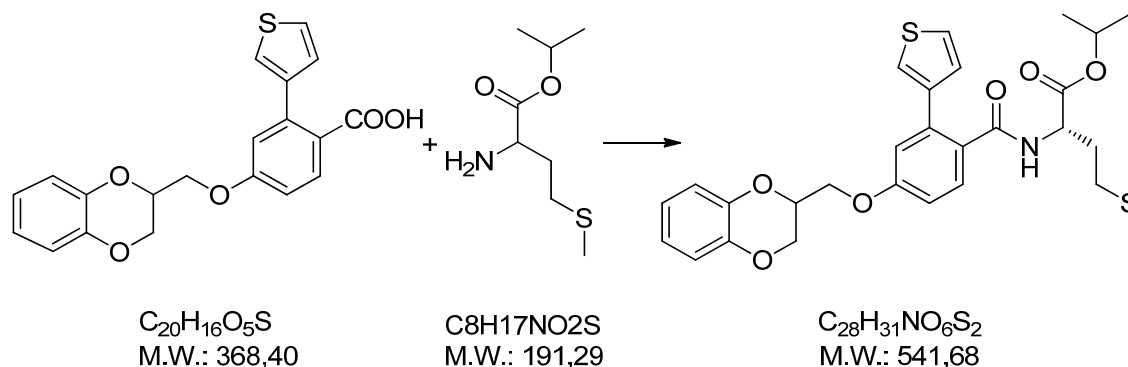
Yield: quantitative

Melting point: 187-190 °C

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 12.55 (bs, 1H), 7.69 (d, 1H, *J*=8.45 Hz), 7.50 (m, 2H), 7.11 (m, 1H), 7.00 (m, 2H), 6.80-6.91 (m, 4H), 4.58 (m, 1H), 4.28-4.44 (dd, 3H), 4.14 (dd, 1H, *J*=6.85 Hz, *J*=11.27 Hz).

<sup>242</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
R<sub>f</sub> = 0.10

**(2S)-isopropyl 2-(4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(thiophen-3-yl)benzamido)-4-(methylthio)butanoate**



A solution of EDAC (0.50 g, 2.63 mmol) and DIPEA (0.461 mL, 2.63 mmol) in 3 mL of DMF is added dropwise to a solution of 4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(thiophen-3-yl)benzoic acid (0.80 g, 2.19 mmol), HOBT (0.36 g, 2.63 mmol) in 4 mL of DMF, under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of L-Methionine isopropyl ester (0.42 g, 2.19 mmol) in 3 mL of DMF is dropped.

The reaction mixture is stirred at RT overnight; after completion, monitored by TLC<sup>243</sup>, the mixture is concentrated, resumed with 20 mL of Ethyl Acetate, washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

The crude product is purified by column chromatography on silica gel, using Cyclohexane/ Ethyl Acetate= 80/20 as elution solvent, to give 1.02 g (1.88 mmol) of the desired product, as a dense milky oil.

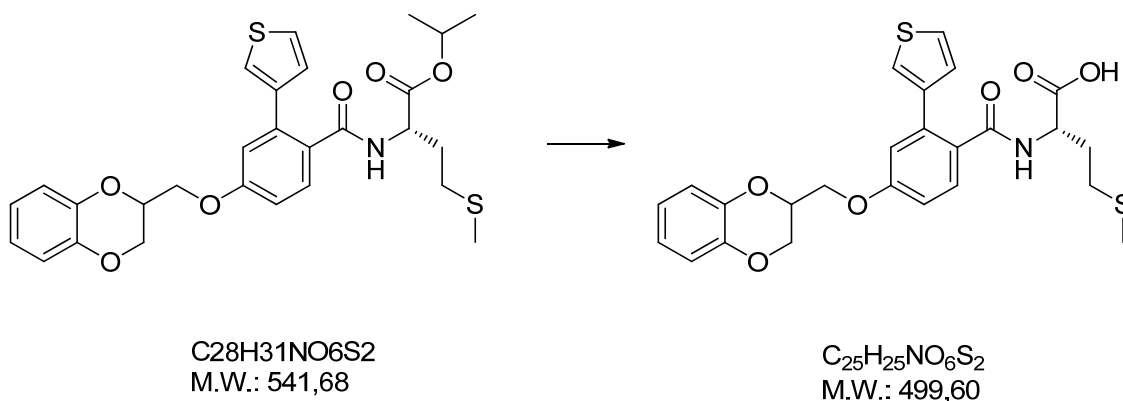
Yield: 86%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 7.68 (d, 1H, *J*=8.25 Hz), 7.39 (m, 2H), 7.15 (ddd, 1H, *J*=0.83 Hz, *J*=1.65 Hz, *J*=4.68 Hz), 6.84-6.97 (m, 6H), 6.04 (d, 1H, *J*=7.7 Hz), 4.99 (m, 1H), 4.55-4.66 (m, 2H), 4.40 (dd, 1H, *J*=2.48 Hz, *J*=11.55 Hz), 4.18-4.32 (m, 3H), 2.24 (t, 2H, *J*=7.7 Hz), 2.05 (s, 3H), 1.99 (m, 1H), 1.82 (m, 1H), 1.24 (d, 3H, *J*=6.87 Hz), 1.22 (d, 3H, *J*=6.32 Hz).

<sup>243</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 70/30  
R<sub>f</sub> = 0.30



**(2S)-2-(4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(thiophen-3-yl)benzamido)-4-(methylthio)butanoic acid**



1.02 g of (2S)-isopropyl 2-(4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(thiophen-3-yl)benzamido)-4-(methylthio)butanoate (1.88 mmol) are dissolved in 10 mL of Methanol; 1.88 mL of NaOH 1M are dropped and the resulting mixture is stirred RT for 18 hours

After completion of the reaction, followed by TLC<sup>244</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with 10% HCl and then extracted three times with 10 mL of Ethyl Acetate.

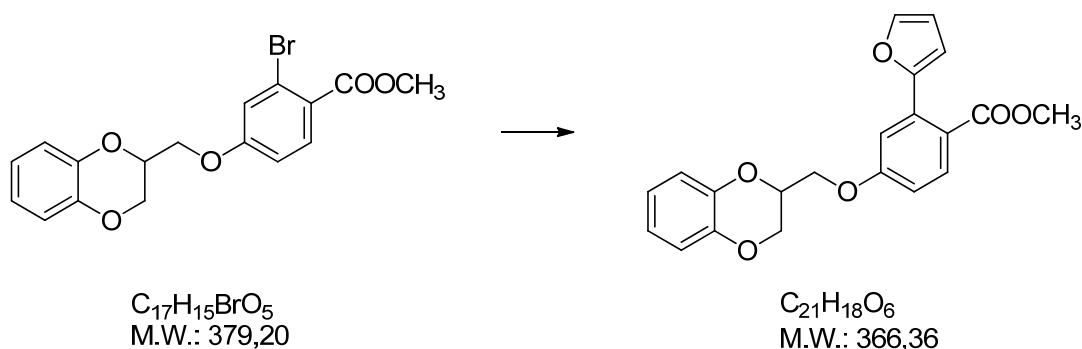
The filtration yields 0.94 g of the final product, as a sticky solid, in quantitative yield.

Yield: quantitative

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 12.50 (bs, 1H), 8.50 (d, 1H, *J*=7.65 Hz), 7.63 (dd, 1H, *J*=1.61 Hz, *J*=3.22 Hz), 7.49 (dd, 1H, *J*=3.22 Hz, *J*=5.23 Hz), 7.33 (d, 1H, *J*=8.45 Hz), 7.27 (dd, 1H, *J*=1.61 Hz, *J*=5.23 Hz), 7.08 (m, 1H), 7.00 (dd, 1H, *J*=2.01 Hz, *J*=8.45 Hz), 6.82-6.92 (m, 4H), 4.59 (m, 1H), 4.4 (d, 1H, *J*=11.67 Hz), 4.31-4.37 (m, 3H), 4.15 (dd, 1H, *J*=7.25 Hz, *J*=11.67 Hz), 2.37-2.43 (m, 2H), 2.00 (s, 3H), 1.86-2.02 (m, 2H).

<sup>244</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
R<sub>f</sub> = basal

## Methyl 4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(furan-2-yl)benzoate



To a sonicated solution of the methyl 2-bromo-4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)benzoate (1.00 g, 2.64 mmol) in 5 mL of dry THF, under a nitrogen atmosphere, Pd(PPh<sub>3</sub>)<sub>4</sub> (0.31 g, 0.264 mmol) is added as solid.

The solution is stirred for 10 minutes and then amount of K<sub>2</sub>CO<sub>3</sub> (1.09 g, 7.92 mmol) in 1 mL of Water and of a solution of 2-furanylboronic acid (0.35 g, 3.17 mmol) in 5 mL of dry THF.

The reaction mixture is stirred RT for 18 hours, monitored by TLC<sup>245</sup> and then concentrated.

The crude is resumed with Ethyl Acetate, washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure.

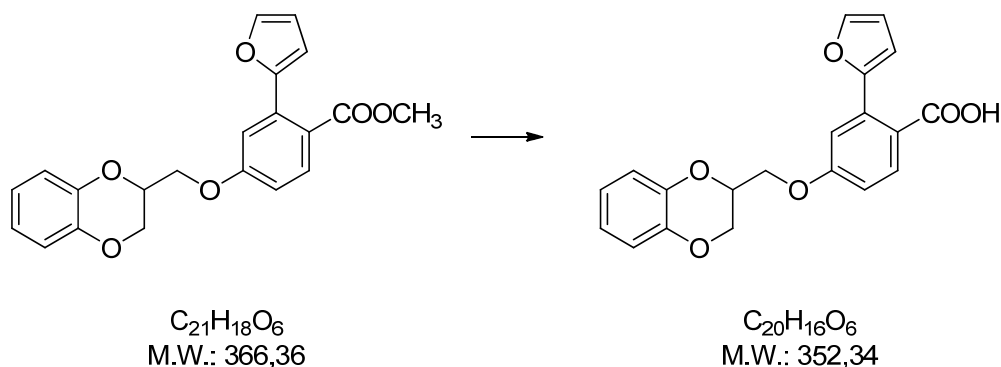
Purification by chromatography on silica gel, through elution with Cyclohexane/ Ethyl Acetate= 80/20, yields 0.92 g (2.51 mmol) of the desired product, as yellow oil.

Yield: 95%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 7.72 (d, 1H, *J*=8.53 Hz), 7.49 (dd, 1H, *J*=0.83 Hz, *J*=1.93 Hz), 7.14 (dd, 1H, *J*=2.75 Hz), 6.85-6.94 (m, 5H), 6.59 (dd, 1H, *J*=0.83 Hz, *J*=3.58 Hz), 6.48 (dd, 1H, *J*=1.93 Hz, *J*=3.58 Hz), 4.59 (m, 1H), 4.40 (dd, 1H, *J*=1.55 Hz, *J*=2.20 Hz), 4.32 (dd, 1H, *J*=4.95 Hz, *J*=9.90 Hz), 4.20-4.27 (m, 2H), 3.81 (s, 3H).

<sup>245</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 70/30  
R<sub>f</sub>= 0.40

## 4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(furan-2-yl)benzoic acid



0.92 g of methyl 4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(furan-2-yl)benzoate (2.51 mmol) is dissolved in 10 mL of Methanol; 4.22 mL of NaOH 1M are dropped and the resulting mixture is heated at 60 °C for 6 hours.

After completion of the reaction, followed by TLC<sup>246</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with 10% HCl and then extracted three times with Ethyl Acetate.

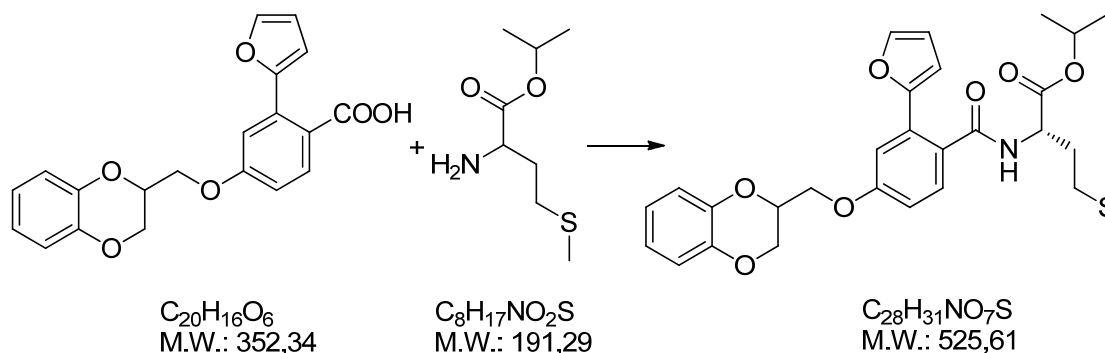
The organic phases are sequentially collected, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under pressure, giving the desired product as sticky yellow solid (0.74 g, 2.10 mmol)

Yield: 84%

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 7.73 (s, 1H), 7.61 (d, 1H, *J*=8.52 Hz), 7.18 (d, 1H, *J*=2.48 Hz), 7.03 (dd, 1H, *J*=2.48 Hz, *J*=8.52 Hz), 6.82-6.92 (m, 5H), 6.77 (dd, 1H, *J*=0.83 Hz, *J*=1.38 Hz), 6.56 (dd, 1H, *J*=1.38 Hz, *J*=3.08 Hz), 4.59 (m, 1H), 4.43 (dd, 1H, *J*=1.55 Hz, *J*=2.20 Hz), 4.34 (m, 2H), 4.16 (dd, 1H, *J*=7.15 Hz, *J*=11.55 Hz).

<sup>246</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
R<sub>f</sub> = basal

**(2S)-isopropyl 2-(4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(furan-2-yl)benzamido)-4-(methylthio)butanoate**



A solution of EDAC (0.48 g, 2.52 mmol) and DIPEA (0.44 mL, 2.52 mmol) in 2 mL of dry DMF is added dropwise to a solution of 4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(furan-2-yl)benzoic acid (0.74 g, 2.10 mmol), HOBT (0.34 g, 2.52 mmol) in 3 mL of dry DMF, under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of L-Methionine isopropyl ester (0.40 g, 2.10 mmol) in 3 mL of dry DMF is dropped.

The reaction mixture is stirred at RT overnight; after completion, monitored by TLC<sup>247</sup>, the mixture is concentrated, resumed with Ethyl Acetate, washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

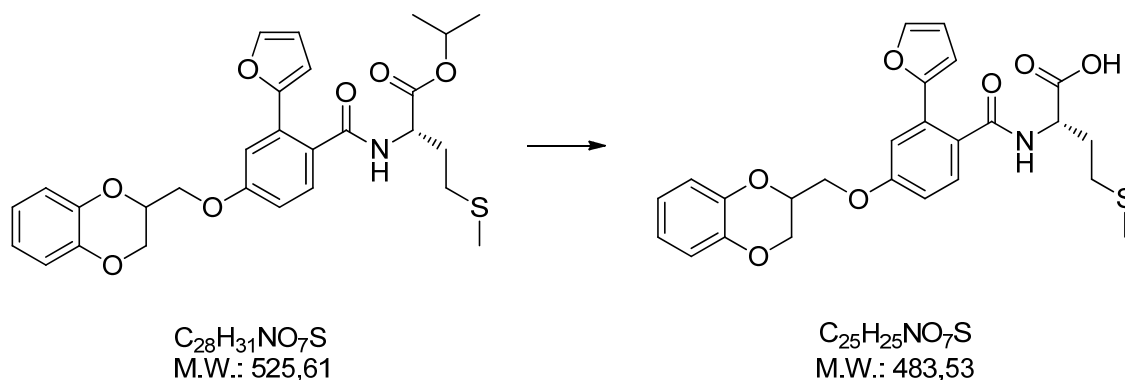
The crude product is purified by column chromatography on silica gel, using Cyclohexane/ Ethyl Acetate= 70/30 as elution solvent, to give 0.79 g (1.51 mmol) of the desired product, as white dense oil.

Yield: 72%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 7.48 (m, 2H), 7.18(d, 1H, *J*=2.48), 6.85-6.94 (m, 5H), 6.67 (dd, 1H, *J*=0.83 Hz, *J*=3.62 Hz), 6.46 (dd, 1H, *J*=1.65 Hz, *J*=2.65 Hz), 6.32 (d, 1H, *J*=7.7 Hz), 5.05 (m, 1H), 4.80 (ddd, 1H, *J*=4.95 Hz, *J*=7.15 Hz, *J*=7.7 Hz), 4.68 (m, 1H), 4.40 (dd, 1H, *J*=2.48 Hz, *J*=11.55 Hz), 4.19-4.34 (m, 3H), 2.49 (t, 2H, *J*=6.68 Hz), 2.19 (m, 1H), 2.09 (s, 3H), 2.03 (m, 1H), 1.28 (d, 3H, *J*=6.05 Hz), 1.26 (d, 3H, *J*=6.05 Hz).

<sup>247</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 70/30  
R<sub>f</sub> = 0.22

**(2S)-2-(4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(furan-2-yl)benzamido)-4-(methylthio)butanoic acid**



0.79 g of (2S)-isopropyl 2-(4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(furan-2-yl)benzamido)-4-(methylthio)butanoate (1.51 mmol) are dissolved in 10 mL of Methanol; 1.27 mL of NaOH 1M are dropped and the resulting mixture is stirred RT for 18 hours

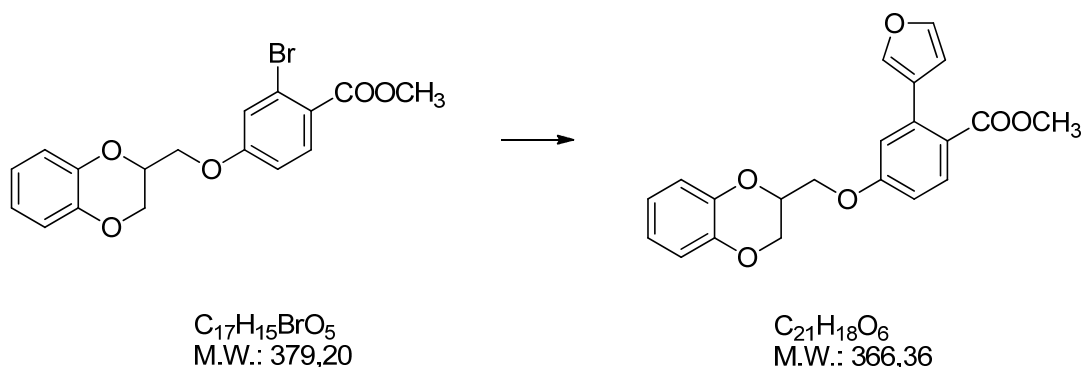
After completion of the reaction, followed by TLC<sup>248</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with 10% HCl and then extracted three times with 10 mL of Ethyl Acetate. The filtration yields 0.73 g (1.51 mmol) of the final product, as a pale pink sticky solid, in quantitative yield.

Yield: quantitative

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 12.68 (bs, 1H), 8.62 (d, 1H, *J*=7.64 Hz), 7.68 (d, 1H, *J*=1.21 Hz), 7.27 (m, 2H), 7.01 (dd, 1H, *J*=1.61 Hz, *J*=4.03 Hz), 6.81-6.99 (m, 5H), 6.51 (t, 1H, *J*=1.61 Hz), 4.58 (m, 1H), 4.42 (m, 2H), 4.31 (m, 2H), 4.15 (m, 1H), 2.47-2.54 (m, 2H), 2.04 (s, 3H), 1.95 (m, 2H).

<sup>248</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
 R<sub>f</sub> (starting compound)= 0.54

## Methyl 4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(furan-3-yl)benzoate



To a sonicated solution of the methyl 2-bromo-4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)benzoate (1.00 g, 2.64 mmol) in 6 mL of Toluene, under a nitrogen atmosphere, Pd(PPh<sub>3</sub>)<sub>4</sub> (0.31 g, 0.264 mmol) is added as solid.

The solution is stirred for 10 minutes and then amount of Na<sub>2</sub>CO<sub>3</sub> (0.84 g, 7.92 mmol) in 3 mL of Water and of a solution of 3-furanylboronic acid (0.35 g, 3.17 mmol) in 1.5 mL of Methanol.

The reaction mixture is heated at 75 °C for 18 hours, monitored by TLC<sup>249</sup> and then cooled.

The residue is treated with 20 mL of DCM and 15 mL of NaHCO<sub>3</sub>, the two layers are separated and the organic one washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure.

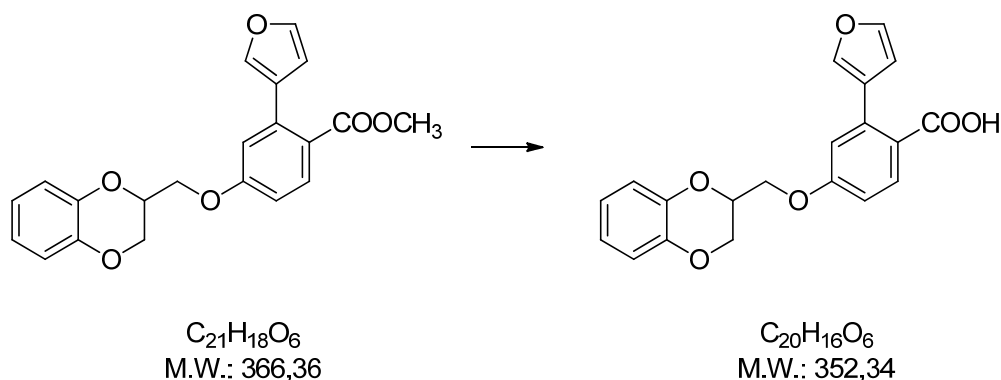
Purification by chromatography on silica gel, through elution with Cyclohexane/ Ethyl Acetate= 80/20, yields 0.69 g (1.87 mmol) of the desired product, as yellow oil.

Yield: 71%

<sup>1</sup>H-NMR (CDCl<sub>3</sub>): 7.96 (dd, 1H, *J*=1.65 Hz, *J*=9.8 Hz), 7.42 (dd, 1H, *J*=0.82 Hz, *J*=9.8 Hz), 6.82-6.96 (m, 7H), 6.41 (d, 1H, *J*=0.82 Hz), 4.59 (m, 1H), 4.39 (dd, 1H, *J*=2.2 Hz, *J*=9.35 Hz), 4.19-4.33 (m, 3H), 3.79 (s, 3H).

<sup>249</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 70/30  
R<sub>f</sub>= 0.44

## 4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(furan-3-yl)benzoic acid



0.69 g of methyl 4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(furan-3-yl)benzoate (1.87 mmol) is dissolved in 10 mL of Methanol; 7.48 mL of NaOH 1M are dropped and the resulting mixture is heated at 60 °C for 18 hours.

After completion of the reaction, followed by TLC<sup>250</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether and then acidified with 10% HCl, till a pale yellow solid precipitate.

The filtration yields 0.73 g (1.96 mmol) of the desired compound.

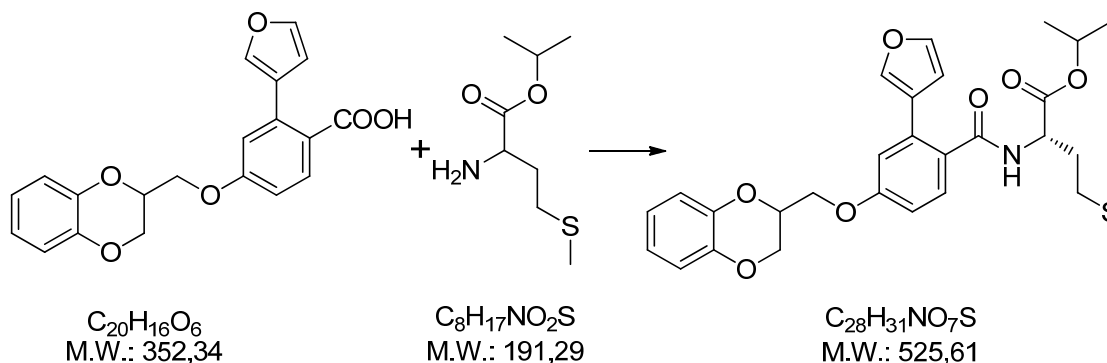
Yield: 85%

Melting Point: 145-149 °C

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 8.04 (m, 1H), 7.56 (d, 1H, *J*=0.83 Hz), 7.44 (t, 2H, *J*=1.65 Hz), 6.85-6.99 (m, 5H), 6.52 (dd, 1H, *J*=0.83 Hz, *J*=1.65 Hz, ), 4.59 (m, 1H), 4.40 (dd, 1H, *J*=2.2 Hz, *J*=9.35 Hz), 4.20-4.38 (m, 3H).

<sup>250</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 70/30  
R<sub>f</sub> = 0.19

**(2S)-isopropyl 2-(4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(furan-3-yl)benzamido)-4-(methylthio)butanoate**



A solution of EDAC (0.45 g, 2.35 mmol) and DIPEA (0.41 mL, 2.35 mmol) in 2 mL of dry DMF is added dropwise to a solution of 4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(furan-3-yl)benzoic acid (0.73 g, 1.96 mmol), HOBT (0.31 g, 2.35 mmol) in 4 mL of DCM, under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of L-Methionine isopropyl ester (0.37 g, 1.96 mmol) in 2 mL of DCM is dropped.

The reaction mixture is stirred at RT overnight; after completion, monitored by TLC<sup>251</sup>, the mixture is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

The crude product is purified by column chromatography on silica gel, using Cyclohexane/ Ethyl Acetate= 70/30 as elution solvent, to give 0.69 g (1.31 mmol) of the desired product, as yellow oil.

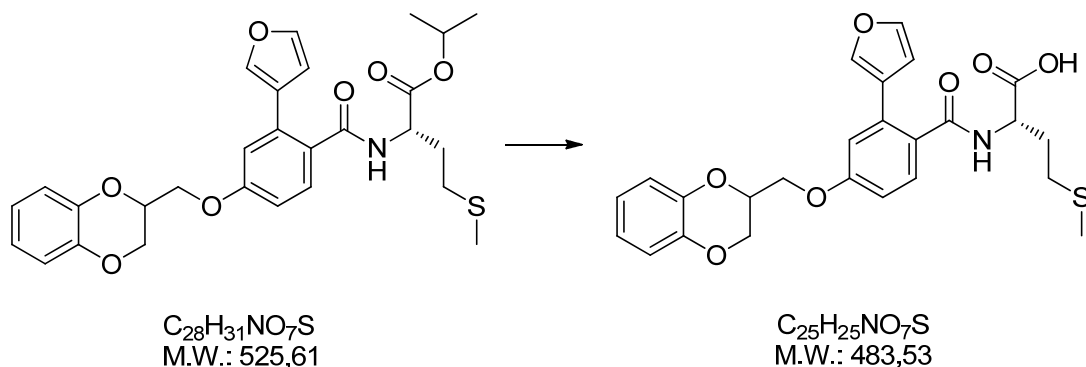
Yield: 67%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 7.79 (d, 1H, *J*=8.8 Hz), 7.62 (s, 1H), 7.61 (d, 1H, *J*=1.1 Hz), 7.47 (t, 1H, *J*=1.65 Hz), 6.85-6.98 (m, 5H), 6.55 (dd, 1H, *J*=0.83 Hz, *J*=1.65 Hz), 6.27 (d, 1H, *J*=7.7 Hz), 5.02 (m, 1H), 4.71 (dd, 1H, *J*=2.2 Hz, *J*=7.15 Hz), 4.58 (dd, 1H, *J*=1.65 Hz, *J*=4.95 Hz), 4.40 (dd, 1H, *J*=2.47 Hz, *J*=11.55 Hz), 4.17-4.32 (m, 2H), 2.39 (t, 2H, *J*=1.65 Hz), 2.07 (s, 3H), 2.08 (m, 1H), 1.94 (m, 1H), 1.26 (d, 3H, *J*=6.33 Hz), 1.24 (d, 3H, *J*=6.33 Hz).

<sup>251</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 70/30  
 R<sub>f</sub> = 0.24



**(2S)-2-(4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(furan-3-yl)benzamido)-4-(methylthio)butanoic acid**



0.69 g of (2S)-isopropyl 2-(4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(furan-3-yl)benzamido)-4-(methylthio)butanoate (1.31 mmol) are dissolved in 10 mL of Methanol; 1.63 mL of NaOH 1M are dropped and the resulting mixture is stirred RT for 18 hours

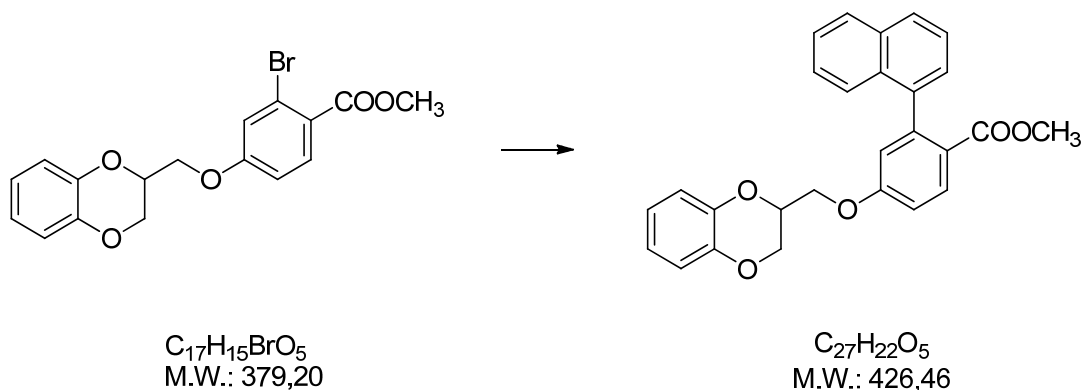
After completion of the reaction, followed by TLC<sup>252</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with 10% HCl and then extracted three times with 10 mL of Ethyl Acetate. The filtration yields 0.79 g of the final product, as a sticky white solid, in quantitative yield.

Yield: quantitative

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 12.67 (bs, 1H), 8.52 (d, 1H, *J*=7.65 Hz), 7.94 (s, 1H), 7.87 (d, 1H, *J*=8.85), 7.64 (t, 1H, *J*=1.61 Hz), 7.30 (d, 1H, *J*=8.45 Hz), 6.78-7.08 (m, 6H), 4.58 (m, 1H), 4.35-4.51 (m, 4H), 4.14 (m, 1H), 2.52 (m, 2H), 2.04 (s, 3H), 1.94 (m, 2H).

<sup>252</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 70/30  
R<sub>f</sub> = basal

## Methyl 4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(naphthalen-1-yl)benzoate



To a sonicated solution of the methyl 2-bromo-4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)benzoate (1.00 g, 2.64 mmol) in 5 mL of dry DMF, under a nitrogen atmosphere, Pd(PPh<sub>3</sub>)<sub>4</sub> (0.31 g, 0.264 mmol) is added as solid.

The solution is stirred for 10 minutes and then amount of Na<sub>3</sub>PO<sub>4</sub>·12H<sub>2</sub>O (3.01 g, 7.92 mmol) and of a solution of 1-naphthylboronic acid (0.41 g, 3.17 mmol) in 5 mL of dry DMF.

The reaction mixture is heated at 100 °C for 18 hours, monitored by TLC<sup>253</sup> and then concentrated.

The crude is resumed with Ethyl Acetate, washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure.

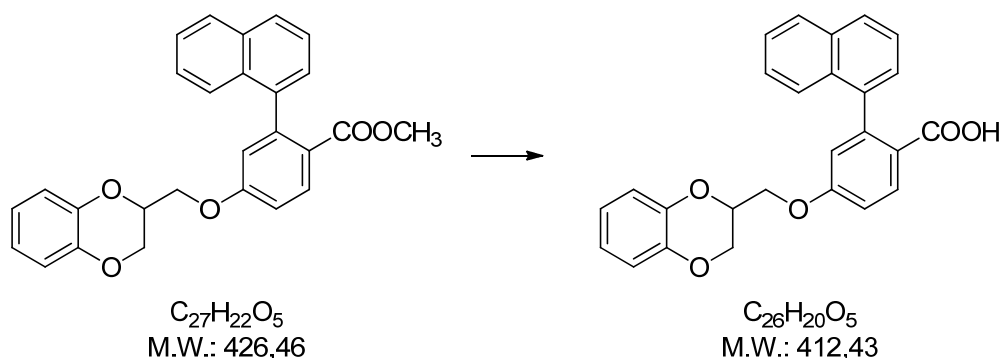
Purification by chromatography on silica gel, through elution with Cyclohexane/ Ethyl Acetate= 85/15, yields 1.02 g (2.35 mmol) of the desired product, as pale pink oil.

Yield: 89%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 8.08 (d, 1H, *J*=8.53 Hz), 7.87 (dd, 1H, *J*=7.88 Hz, *J*=8.52 Hz), 7.29-7.53 (m, 5H), 7.06 (dd, 1H, *J*=2.75 Hz, *J*=8.80 Hz), 6.83-6.93 (m, 5H), 4.58 (m, 1H), 4.40 (dd, 1H, *J*=2.48 Hz, *J*=11.55 Hz), 4.20-4.30 (m, 3H), 3.37 (s, 3H).

<sup>253</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 70/30  
R<sub>f</sub>= 0.45

## 4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(naphthalen-1-yl)benzoic acid



1.02 g of methyl 4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(naphthalen-1-yl)benzoate (2.35 mmol) is dissolved in 10 mL of Methanol; 9.4 mL of NaOH 1M are dropped and the resulting mixture is heated at 60 °C for 18 hours.

After completion of the reaction, followed by TLC<sup>254</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with 10% HCl and then extracted three times with Ethyl Acetate.

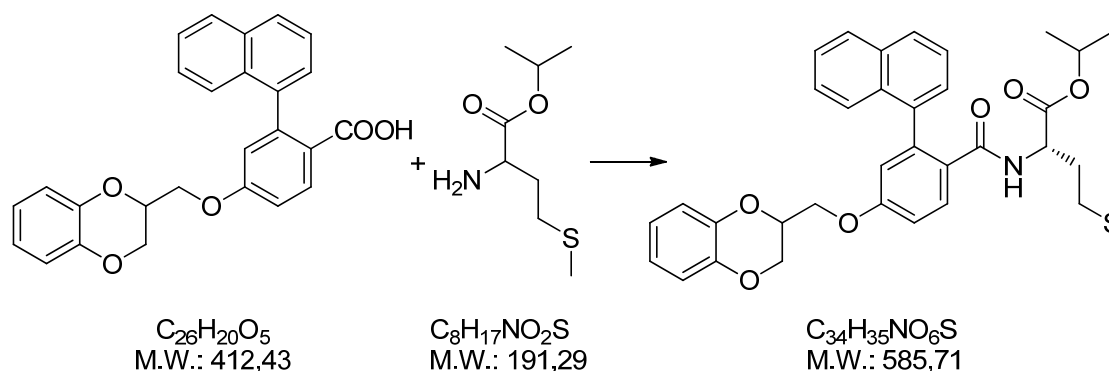
The organic layer is dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure, yielding 0.78 g (1.88 mmol) of the desired compound, as sticky solid.

Yield: 80%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 8.08 (dd, 1H, *J*=0.83 Hz, *J*=8.81 Hz), 7.86 (dd, 1H, *J*=8.25 Hz, *J*=9.35 Hz), 7.40-7.49 (m, 3H), 7.33 (dd, 1H, *J*=7.15 Hz, *J*=7.85 Hz), 7.25 (m, 1H), 6.82-6.90 (m, 5H), 4.56 (m, 1H), 4.37 (dd, 1H, *J*=1.1 Hz, *J*=11.52 Hz), 4.09-4.35 (m, 3H).

<sup>254</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
R<sub>f</sub> = 0.23

**(2S)-isopropyl 2-(4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(naphthalen-1-yl)benzamido)-4-(methylthio)butanoate**



A solution of EDAC (0.43 g, 2.26 mmol) and DIPEA (0.39 mL, 2.26 mmol) in 2 mL of dry DMF is added dropwise to a solution of 4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(naphthalen-1-yl)benzoic acid (0.78 g, 1.88 mmol), HOBT (0.30 g, 2.26 mmol) in 3 mL of dry DMF, under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of L-Methionine isopropyl ester (0.36 g, 1.88 mmol) in 3 mL of dry DMF is dropped.

The reaction mixture is stirred at RT overnight; after completion, monitored by TLC<sup>255</sup>, the mixture is concentrated, resumed with 15 mL of Ethyl Acetate, washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

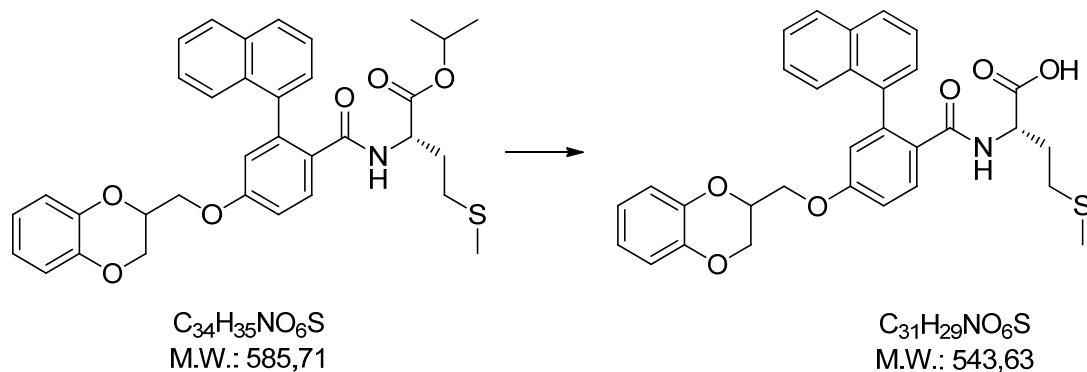
The crude product is purified by column chromatography on silica gel, using Cyclohexane/ Ethyl Acetate= 70/30 as elution solvent, to give 1.00 g (1.71 mmol) of the desired product, as colourless oil.

Yield: 91%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 8.05 (d, 1H, *J*=8.53 Hz), 7.88-7.95 (m, 2H), 7.39-7.63 (m, 5H), 7.09 (m, 1H), 6.83-6.92 (m, 5H), 5.81 (d, 1H, *J*=9.7 Hz), 4.85 (m, 1H), 4.58 (m, 1H), 4.19-4.41 (m, 4H), 1.81 (s, 3H), 1.65 (m, 2H), 1.40 (m, 2H), 1.15 (d, 3H, *J*=6.33 Hz), 1.13(d, 3H, *J*=6.05 Hz).

<sup>255</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 70/30  
R<sub>f</sub> = 0.27

**(2S)-2-(4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(naphthalen-1-yl)benzamido)-4-(methylthio)butanoic acid**



1.00 g of (2S)-isopropyl 2-(4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(naphthalen-1-yl)benzamido)-4-(methylthio)butanoate (1.71 mmol) are dissolved in 10 mL of Methanol; 3.42 mL of NaOH 1M are dropped and the resulting mixture is stirred RT for 3 hours

After completion of the reaction, followed by TLC<sup>256</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with 10% HCl and then extracted three times with 10 mL of Ethyl Acetate.

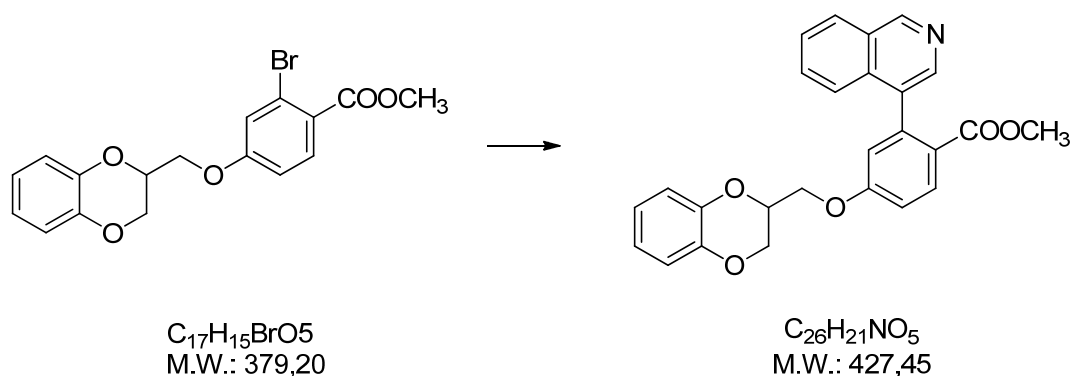
The filtration yields 0.93 g of the final product, as a colourless sticky solid, in quantitative yield.

Yield: quantitative

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 12.51 (bs, 1H), 8.20 (d, 1H, *J*=8. Hz), 7.77-7.94 (m, 2H), 7.57-7.63 (m, 2H), 7.33-7.50 (m, 4H), 7.14-7.19 (m, 1H), 6.80-6.92 (m, 4H), 4.57 (m, 1H), 4.32-4.43 (m, 3H), 4.00-4.15 (m, 2H), 2.02 (m, 1H), 1.97 (m, 1H), 1.84 (s, 3H), 1.76 (m, 1H), 1.72 (m, 1H).

<sup>256</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
R<sub>f</sub> = basal

## Methyl 4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(isoquinolin-4-yl)benzoate



To a sonicated solution of the methyl 2-bromo-4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)benzoate (1.00 g, 2.64 mmol) in 7.5 mL of Toluene/Methanol: 4/1, under a nitrogen atmosphere,  $Pd(PPh_3)_4$  (0.31 g, 0.264 mmol) is added as solid.

The solution is stirred for 10 minutes and then amount of  $Na_2CO_3$  (0.70 g, 6.6 mmol) in 1.5 mL of Water and of solid 4-isoquinolineboronic acid (0.50 g, 2.90 mmol).

The reaction mixture is heated at 80°C for 18 hours, monitored by TLC<sup>257</sup> and, at completion, treated with Ethyl Acetate, washed with brine, dried over  $Na_2SO_4$ , filtered and concentrated under reduced pressure.

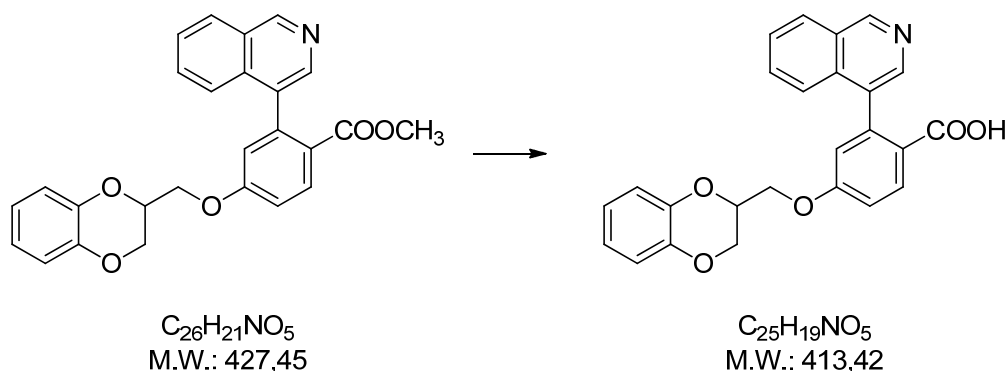
Purification by chromatography on silica gel, through elution with Cyclohexane/ Ethyl Acetate= 60/40, yields 0.65 g (1.53 mmol) of the desired product, as yellow-red oil.

Yield: 58%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 9.33 (s, 1H), 8.35 (s, 1H), 8.15 (d, 1H,  $J=9.08$  Hz), 8.11 (m, 1H), 7.72 (m, 2H), 7.53 (m, 1H), 7.12 (ddd, 1H,  $J=1.1$  Hz,  $J=2.75$  Hz,  $J=8.80$  Hz), 6.83-6.91 (m, 5H), 4.60 (m, 1H), 4.39 (dd, 1H,  $J=2.48$  Hz,  $J=11.55$  Hz), 4.21-4.36 (m, 3H), 3.44 (s, 3H).

<sup>257</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
Rf= 0.24

## 4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(isoquinolin-4-yl)benzoic acid



0.65 g (1.53 mmol) of methyl 4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(isoquinolin-4-yl)benzoate is dissolved in 5 mL of Methanol; 3 mL of NaOH 2.5 M are dropped and the resulting mixture is heated at 60 °C for 18 hours.

After completion of the reaction, followed by TLC<sup>258</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether and then acidified with Formic acid, till a white solid precipitate.

The filtration yields 0.38 g (0.92 mmol) of the desired compound.

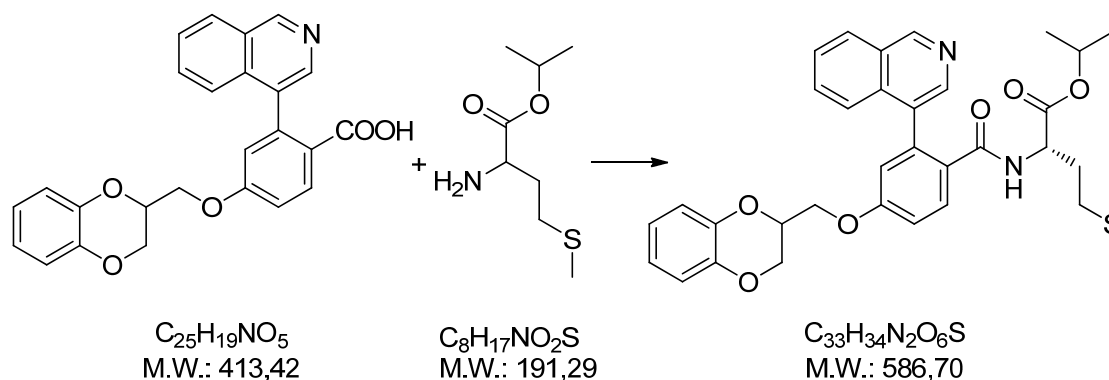
Yield: 60%

Melting point: 235-238 °C

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 9.24 (s, 1H), 8.26 (s, 1H), 8.12 (dd, 1H, *J*=4.02 Hz, *J*=5.32 Hz), 7.96 (d, 1H, *J*=8.46 Hz), 7.62 (m, 2H), 7.43 (dd, 1H, *J*=2.82 Hz, *J*=8.46 Hz), 7.14 (dd, 1H, *J*=2.41 Hz, *J*=8.85 Hz), 6.79-6.88 (m, 5H), 4.56 (m, 1H), 4.24-4.42 (m, 3H), 4.08-4.14 (m, 1H), 4.11 (dd, 1H, *J*=7.25 Hz, *J*=11.27 Hz).

<sup>258</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 1/1  
R<sub>f</sub> = basal

**(2S)-isopropyl 2-(4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(isoquinolin-4-yl)benzamido)-4-(methylthio)butanoate**



A solution of EDAC (0.21 g, 1.11 mmol) and DIPEA (0.19 mL, 1.1 mmol) in 2 mL of DMF is added dropwise to a solution of 4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(isoquinolin-4-yl)benzoic acid (0.38 g, 0.92 mmol), HOBT (0.15 g, 1.11 mmol) in 3 mL of DMF, under a nitrogen atmosphere.

The resulting mixture is stirred for 30 minutes at RT and then a solution of L-Methionine isopropyl ester (0.18 g, 0.92 mmol) in 3 mL of DMF is dropped.

The reaction mixture is stirred at RT overnight; after completion, monitored by TLC<sup>259</sup>, the mixture is washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated.

The crude product is purified by column chromatography on silica gel, using Cyclohexane/ Ethyl Acetate= 1/1 as elution solvent, to give 0.41 g (0.70 mmol) of the desired product.

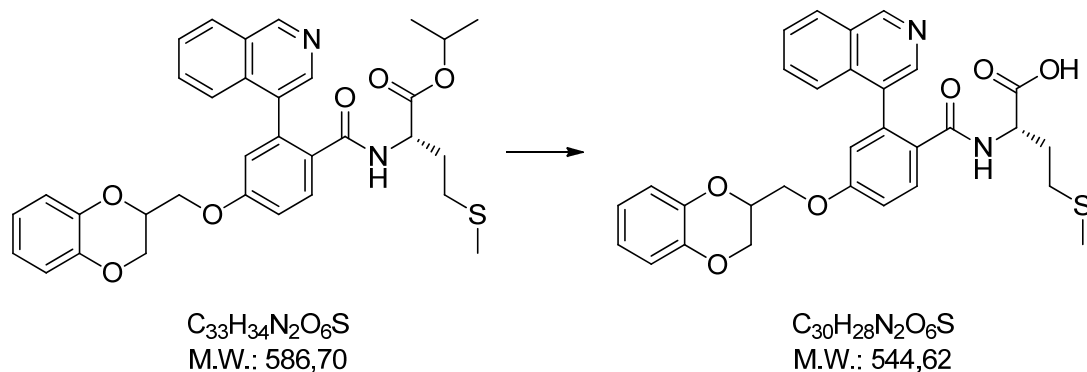
Yield: 76%

**<sup>1</sup>H-NMR (CDCl<sub>3</sub>):** 9.32 (s, 1H), 8.50 (s, 1H), 8.07 (m, 1H), 7.89 (d, 1H, *J*=8.53 Hz), 7.57-7.72 (m, 3H), 7.09-7.14 (m, 1H), 6.83-6.93 (m, 5H), 6.05 (d, 1H, *J*=7.43 Hz), 4.87 (m, 1H), 4.59 (m, 1H), 4.21-4.42 (m, 5H), 2.04 (m, 2H), 1.94 (s, 3H), 1.58 (m, 2H), 1.16 (d, 3H, *J*=4.4 Hz), 1.14 (d, 3H, *J*=4.4 Hz).

<sup>259</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 30/70  
R<sub>f</sub> = 0.46



**(2S)-2-(4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(isoquinolin-4-yl)benzamido)-4-(methylthio)butanoic acid**



0.41 g of (2S)-isopropyl 2-(4-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-2-(isoquinolin-4-yl)benzamido)-4-(methylthio)butanoate (0.70 mmol) are dissolved in 5 mL of Methanol; 0.41 mL of NaOH 1M are dropped and the resulting mixture is stirred RT for 18 hours.

After completion of the reaction, followed by TLC<sup>260</sup>, the Methanol is evaporated and the residue resumed with water; the aqueous layer is washed with Ethyl Ether, acidified with Formic acid till a white precipitate is formed.

The filtration of the solid yields 0.38 g of the final product, as a white solid, in quantitative yield.

Yield: quantitative

Melting point: 117 °C

**<sup>1</sup>H-NMR (d<sub>6</sub>DMSO):** 12.57 (bs, 1H), 9.26 (s, 1H), 8.39 (m, 1H), 8.31 (s, 1H), 8.16 (m, 2H), 7.49-7.67 (m, 4H), 7.21 (dd, 1H, *J*=2.42 Hz, *J*=8.46 Hz), 7.00 (dd, 1H, *J*=1.61 Hz, *J*=3.63 Hz), 6.79-6.90 (m, 3H), 4.58 (m, 1H), 4.28-4.16 (m, 3H), 3.97-4.16 (m, 2H), 2.05 (m, 2H), 1.84 (s, 3H); 1.66 (m, 2H).

<sup>260</sup> TLC conditions: Cyclohexane/ Ethyl Acetate= 30/70  
 R<sub>f</sub> (starting compound)= basal

A tutti quelli che mi sono stati vicino in questi tre anni, lunghi in termini di tempo ma decisamente volati, dedico questa poesia, che sia di RINGRAZIAMENTO ma soprattutto di AUGURIO, per poter camminare insieme tanti giorni ancora!

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Grazie MAMMA e PAPA',

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(ELI, LETY, CE, MONI, ILI, CRI, FRENK, LELE, TIA, STE, VALSE, MAURO, VALTI, FABRI),

Grazie don Marco e Grazie a chiunque mi sia stato vicino,

Ti auguro la FATICA

che farà più grande la gioia che ogni sera proverai  
nel voltarti indietro a guardare il cammino percorso.

Ti auguro il SOLE

che scaldi ogni fibra del tuo corpo  
e ogni sospiro della tua anima.

Ti auguro la PIOGGIA

che rinfreschi e disseti l'arsura  
delle giornate troppo aride  
dei deserti della vita.

Ti auguro il VENTO

che ti accarezzi con brezza leggera il viso  
e riempi con il suo soffio il cuore.

Ti auguro l'AMORE:

è Dio che passo dopo passo  
ti condurrà alla meta.

*Vale*