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TESI DI DOTTORATO DI RICERCA

SYNTHESIS AND BIOLOGICAL ACTIVITY EVALUATION OF NATURAL ANTIFUNGALS AND THEIR ANALOGUES

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ANNO ACCADEMICO 2011/2012

Dedicated to
my beloved
parents

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled **"Synthesis and biological activity evaluation of natural antifungals and their analogues"** which is being submitted to the **University of Milan** for the award of **Doctor of Philosophy** in Chemistry, Biochemistry and Ecology of pesticides by **Sachin Tanaji Aiwale** was carried out by him under my supervision at **University of Milan, Milan**. This work is original and has not been submitted in part or full, for any degree or diploma to this or any other University.

Prof. Sabrina Dallavalle

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Candidate's Declaration

I hereby declare that the thesis entitled **"Synthesis and biological activity evaluation of natural antifungals and their analogues"** submitted for the award of degree of **Doctor of Philosophy** in Chemistry, Biochemistry and Ecology of pesticides to the **University of Milan, Milan**. This work is original and has not been submitted in part or full, for any degree or diploma to this or any other University. This work was carried out by me at the **Organic Chemistry Department, Faculty of Agriculture, University of Milan, Milan, Italy**.

Sachin Tanaji Aiwale January 03, 2013

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1. NMR Spectras

^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ solutions (where not otherwise stated) at room temperature on a Bruker AMX-300 spectrometer operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C. Chemical shifts are reported as d values in parts per million (ppm), and are indirectly referenced to tetramethylsilane (TMS) via the solvent signal (7.26 for ^{1}H, 77.0 for ^{13}C) in $CDCl_3$. Coupling constant (*J*) are given in Hz.

2. Solvents

Solvents were routinely distilled prior to use; anhydrous tetrahydrofuran (THF) and ether (Et_2O) were obtained by distillation from sodium-benzophenone ketyl; dry methylene chloride (CH_2Cl_2) and toluene were obtained by distillation from $CaCl_2$. All reactions requiring anhydrous conditions were performed under a positive nitrogen flow, and glassware was oven-dried.

3. Flash chromatography

Isolation and purification of the compounds were performed by flash column chromatography on silica gel 60 (230-400 mesh); when necessary deactivated silica gel was used.

4. Analytical thin-layer chromatography

Analytical thin-layer chromatography (TLC) was conducted on Fluka TLC plates (silica gel 60 F254, aluminium foil), and spots were visualized by UV light and/or by means of dyeing reagents.

5. Melting points

Melting points were determined on a Stuart Scientific SMP3 instrument and are uncorrected.

6. HPLC analysis

HPLC analyses were recorded on a Varian Prostar liquid chromatography Column description – Column size 250 x 4.6 mm (Lx I.D) Chiral Daicel OD lot no. 555-024-50303.

7. Reverse phase chromatography

Reverse phase silica RP-18 was used for isolation and purification of the compounds.

Abbreviations

Ac	-	Acetyl
AcOH	-	Acetic acid
Ac2O	-	Acetic anhydride
Ar	-	Aryl
Bn	-	Benzyl
BnBr	-	Benzyl bromide
Brs	-	Broad singlet
Boc	-	*tert*-Butoxy carbonyl
(Boc)2O	-	Di-*tert*-butyl dicarbonate
Bu	-	Butyl
t-Bu	-	*tert*-Butyl
BuLi	-	Butyl Lithium
Cat.	-	Catalytic/Catalyst
Cbz	-	Carbobenzyloxy
$CDCl_3$	-	Deuterated chloroform
DCM	-	Dichloromethane
d	-	Doublet
dd	-	Doublet of doublet
DBU	-	1,8-Diazabicyclo [5.4.0] undec-7-ene
DCC	-	Dicyclohexylcarbodiimide
DEAD	-	Diethyl azodicarboxylate
DIAD	-	Diisopropyl azodicarboxylate
DIBAL-H	-	Diisobutylaluminium hydride
DIPEA	-	Diisopropylethylamine

DIPT	-	Diisopropyltartrate
DMA	-	*N, N*-Dimethylacetamide
DMAP	-	*N, N'*-Dimethylaminopyridine
DMF	-	*N, N'*-Dimethylformamide
DMSO	-	Dimethyl sulfoxide
DMPU	-	1,3-Dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone
Et	-	Ethyl
EtBr	-	Ethyl bromide
Et3N	-	Trimethyl amine
EtOAc	-	Ethyl acetate
EtOEt, Et2O	-	Diethylether
EtOH	-	Ethanol
g	-	grams
h	-	hours
HBTU	-	2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HPLC	-	High performance liquid chromatography
Hz	-	Hertz
Im	-	Imidazole
IBX	-	2-Iodoxybenzoic acid
LAH	-	Lithium aluminium hydride
LDA	-	Lithiumdiisopropylamide
LiHMDS	-	Lithium bis(trimethylsilyl)amide
m	-	Multiplate
Me	-	Methyl
MEM-Cl	-	2-Methoxyethoxymethyl chloride

mg	-	Miligram
MIC	-	Minimum inhibitory concentration
min	-	Minutes
MeOH	-	Methanol
mmol	-	mmol
m.p	-	Melting point
NaOMe	-	Sodium methoxide
NMR	-	Nuclear magnetic resonance
NOESY	-	Nuclear overhauser effect spectroscopy
NMM	-	N- methyl morpholine
PCC	-	Pyridiniumclorochromate
PDC	-	Pyridiniumdichromate
Ph	-	Phenyl
Pd/C	-	Palladium on carbon
ppm	-	Parts per million
*p*TSA	-	*para*-Toluenesulfonic acid
Py	-	Pyridine
RED-Al	-	Sodium bis(2-methoxyethoxy)aluminium hydride
rt	-	Room temperature
s	-	Singlet
SEM-Cl	-	2-(Trimethylsilyl)ethoxy methylchloride
TBAI	-	Tetra-*n*-butylammonium iodide
TBAF	-	Tetrabutylammonium fluoride
TBDMS-Cl	-	*tert*-Butyldimethylchlorosilane
TBDPS-Cl	-	*tert*-Butyldiphenylsilylchloride
TBHP	-	*tert*-Butyl hydroperoxide

TBS	-	*tert*-Butyldimethylsilyl
TBSOTf	-	*tert*-Butyldimethylsilyl triflate
TEA	-	Triethyl amine
TEMPO	-	2,2,6,6-Tetramethylpiperidine-1-oxyl
THF	-	Tetrahydrofuran
TFA	-	Trifluoroacetic acid
TFFA	-	Trifluoroacetic anhydride
TLC	-	Thin layer chromatography
Ts	-	Tosyl

CHAPTER 1

General Introduction

1. Background

The birth of civilization can be traced back to the time when humans first began to cultivate crops – the so-called Neolithic revolution. This caused a dramatic increase in the number of people that could be supported in a given environment, but it also brought problems that farmers have had to contend with ever since. The devastation to crops mainly formed by weeds and pests (fungi, bacteria etc). Farmers all over the world, however, are on the frontline of the fight against these forces of destruction and some of their most important weapons are agrochemicals, which help to protect against the worst crop losses. In the absence of the remarkable levels of growth in the yields of important crops, neither the rapid increase in living standards in industrialized countries nor the adequate standard of nutrition for the greater part of the world's population would have been possible. Alongside high-yielding varieties, improved agricultural techniques, and rapid mechanization, the chemical industry has also contributed substantially to progress in agriculture since roughly the middle of the nineteenth century. From the chemists 'Kitchens' came two 'magic weapons': artificial fertilisers and chemical agents for crops protection. Today both have become indispensable to modern yield and quality orientated agriculture. The word 'agrochemicals' has often assumed a pejorative (expressing disapproval) character in the public mind.

Feeding the world is an increasing challenge, on the other hand the size of arable land is limited and cannot be expanded without further inroads into vital natural habitats like the rain forests. As more people need more food per head from less land, the yields need to be increased by a combination of methods which is known as 'Integrated Crop Management'. Chemical crop protection products with activity against weeds, plant diseases, insects and mites help the farmer to assure or increase yields.

In modern days, we have become habitual to a wide variety of cheap, nutritious food which is one the basic needs of living organisms, while plants are the major source of food. Rapidly growing world population put tremendous pressure to increase and preserve the food supply, Additionally, in recent years public pressure to reduce the use of synthetic fungicides in agriculture has increased. Concerns have been raised about both the environmental impact and the potential health risk related to the use of these pesticides.

Throughout history, various types of pests, such as insects, fungi, weeds, bacteria, rodents and other biological organisms, have bothered humans or threatened human health. A large number of volumes and reviews have been written about the use of naturally products as pesticides,[1-9] each having strengths and weakness when considering the vast array of compounds that have found use or are currently being used in crop protection. The chemical protection of crops probably began in pre-Roman times with the application of elemental sulphur (the Greek author Homer mention 'pest-averting sulphur' see Williams and Cooper [10]). Progress for the next few hundred years was unspectacular, burning bitumen, cow dung, tobacco, and mercury being some typical examples of protection methods. For controlling pests the era of synthetic pesticides really got going in the 1950s with the carbamate insecticides, and 2,4-dichlorophenoxyacetic acid (2,4 D). These pesticides can be defined as a substance or mixture of substance used to kill a pest. A pesticide may be a chemical substance, biological agent (such as a virus or bacterium), antimicrobial, disinfectant or device used against any pest.

Fungicides are one of the classes of pesticides. Fungicides are chemical compounds, biological organisms to kill or inhibit fungi or fungal spores. Fungi are capable of causing serious damage in agriculture, resulting in critical losses of yield, quality and profit. Fungi are the number one cause of crop loss worldwide and cause about 70% of all major crop diseases. Although similar, Oomycetes are not fungi. However, they use the same mechanisms to infect plants (some of important diseases of crop plant are mentioned in table 1.1 with their common name and scientific

name). Consequently, in the study of plant disease (phytopathology), chemicals used to control oomycetes are also referred to as fungicides. As well as in agriculture, fungicides are used to fight fungal infections in animal tissues. Fungicides can be either contact or systemic. A contact fungicide kills fungi when sprayed on its surface; a systemic fungicide has to be absorbed by the plant. Although there are benefits to the use of fungicides, there are also drawbacks, such as potential toxicity to humans and other animals. Regarding these drawbacks, the agrochemical industry deserve for safer and more effective agrochemicals with reduced environmental and/or mammalian toxicity. Essential to these efforts is identification of new lead candidates possessing high levels of desirable biological activities, reduced unwanted toxicities, new structural types, and perhaps different modes of action, thereby providing protection from cross-resistance to currently used agrochemicals.

Table 1.1 ***Important Diseases of Crop Plants***

Fungal Class	Pathogan	
	Scientific Name	**Common Name**
Phycomycetes subclass oomycetes	Phytophthora infestans Plasmopara viticola Pseudoperonospora Cubensis	potato late blight downy mildew of grape cucumber downy mildew
Ascomycetes	Pythium spp. Erysiphe graminis Gaeumannomyces graminis Podosphaera leucotricha Pyrenophora teres Pyricularia oryzae Rhynchosporium secalis Sclerotinia Spp. Sphaerotheca fuliginea Uncinula necator Venturia inaequalis Mycosphaerella fijiensis	damping off diseases powdery mildew of wheat/barley take-all of oats and wheat apple powdery mildew net blotch of barley rice blast leaf scald of barley, brown rot of pome fruit Leaf Spot of brassicas and legumes cucurbit powdery mildew grape powdery mildew scab of apple sigatoka disease of bananas
Basidiomycetes	Puccinia Spp. Rhizoctonia Spp. Tilletia Spp. Uromyces Spp. Ustilago Spp.	Leaf rust of wheat and oats black scurf of potato Sheath blight of rice sharp eyespot of wheat Bunts of wheat Beans rust Smuts of wheat, barley, oat and maize, early blight of potato
Deuteromycetes	Alternaria Spp. Botrytis.Spp Cercospora Spp. Fusarium Spp. Helminthosporium Spp. Pseudocercosporella herpotrichoides Septoria nodorum, Septoria tritici	early blight tobacco brown spot leaf of brassicas grey mold of grape and other crops leaf spot of sugarbeet, brown eyespot of coffee wilts, broad range of hosts blight of wheat root and foot rot of wheat leaf spor of maize eye spot of wheat glume blotch of wheat

1.1 Why are Fungicides Needed.

Diseases are a common occurrence on plants, often having a significant economics impact on yield and quality, thus managing disease is an essential component of production for most crops. Broadly, there are main reasons why fungicides are used.

(a) To control a disease during the establishment and development of crop.

(b) To increase the productivity of a crop and to reduce blemishes. Diseased food crops may produce less because their leaves, which are needed for photosynthesis, are affected by the disease (Fig.1.) Blemishes can affect the edible part of crop or, in the case of ornamentals, their attractiveness, that can affect the market value of the crop.

(c) To improve the storage life and quality of harvested plants and produce. Some of the great disease losses occur post-harvest. Fungi often spoil sorted fruits, vegetables, tubers, and seeds. A few which infect grains produce toxins (mycotoxins) capable of causing severe illness or even death in humans and animals when consumed. Fungicides developed so far have not been sufficiently effective to be useful for managing mycotoxins associated with other disease.

Fig. 1

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1.2 A Short History of Fungicides

Farmers have been at the mercy of plant diseases since plants were first domesticated. The mysterious appearance of blights and mildews, apparently coming form nowhere, led to the theories of gods, vapors, demons, an decay as causes of disease. Beginning in the early 1800s, plant scientists and chemists began the long journey to discover and invent fungicides that would reduce disease losses. Many discoveries were made and summarized the exceptional few that irrevocably changed agriculture. (summarized in Table 1.2).

1.2.1. 1807-The first fungicide

B.Prevost discovered the first chemical means for controlling disease in a practical way in 1807. Bunts and smuts of cereals had been a key limitation to cereal cultivation for centuries, appearing unexpectedly on healthy-looking plants that should have been producing grain. Prevost was the first to observe that spores, which grew into tiny germinating "plants," caused wheat bunt. He then made the serendipitous observation that a weak copper solution (generated when he held the spore suspension in a copper vessel) prevented their growth. Through experimentation, he demonstrated that farmers could control bunt by wetting wheat kernels with a copper sulphate solution. Previous methods of bunt control required steeping the seeds in salt water and lime of putrefied urine, which were not very effective.[11] Copper-based seed treatments remained popular in some countries, including France, through the end of the 20th century.

1.2.2. 1885- The First Foliar Fungicide.

Eight decades passed before a method of controlling foliar disease was discovered in 1885. P. M. A. Millardet described the effective use of a mixture of copper sulphate and lime for controlling the downy mildew on grapevines. A farmer in the Bordeaux region of France had mixed the unattractive concoction to discourage university students from pilfering grapes on their way to

class, and Millardet noticed that the sprayed vines retained their leaves while unsprayed plants had been defoliated by downy mildew.[12] This unexpected discovery, thereafter known as Bordeaux mixture, stimulated research into other possible methods of controlling fungal diseases, a search that gained momentum during the 20th century. To this day, copper-based foliar fungicides are used to control a variety of fungal disease, particularly on fruits and vegetables, and for suppression of bacterial diseases. Many have new-found utility as disease control agents in organic food production.

1.2.3. 1915-Broad-Spectrum Control of Seed-Borne Disease.

The first organic (carbon-based) fungicides synthesized in a laboratory were the organomercurial seed treatments. Even though copper seed treatments had been used on cereals for 100 years, they controlled only bunt and could be phytotoxic. Early in the 20th century, discoveries made by fledging pharmaceutical companies studying the medicinal aspects of compounds made from metals and dyestuff intermediates stimulated plant pathologists to look for compounds that could control plant disease. The organomercurial seed treatment, chloro(2-hydroxyphenyl)mercury was introduced in Germany in 1913.[13] Research on organomercurials continued through the 1920s and 1930s, leading to commercialization of the 2-methoxyethyl silicate and acetate salt of 2-hydroxyphenyl mercury, among others. These seed treatments were a breakthrough for cereal farmers because the treatments had good seed safety and controlled mycelia of seed-borne fungi such as *Fusarium* and *Dreschlera* as well as bunt. They also provided protection against soil-borne *Fusarium* species, resulting in improved stand establishment[14] and their vapor activity helped overcome incomplete coverage of the seed surface.[15]

The organomercurial seed treatments had some flaws. They were not deeply systemic, so loose smuts were not controlled. Despite their multi-site-mode of action and use only once per year, resistance eventually developed in some populations of *Dreschlera* on barley and oats.

Environmental toxicity and the persistence of mercury led most countries to ban the organomercurials when safer alternative became available. Despite the availability of alternative treatments, organomercurial seed treatments were not banned in the UK until 1992.[14]

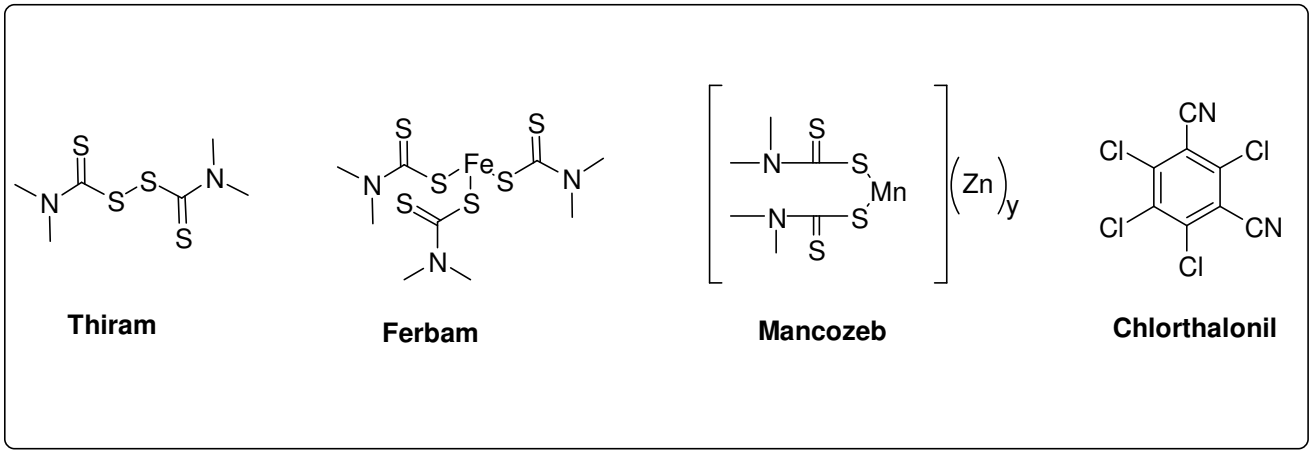


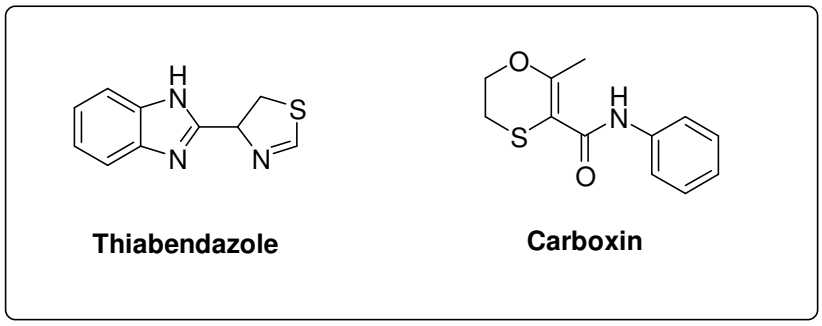
Table 1.2. Milestone fungicides.

Common name	Trade name (example)[x]	Class of chemistry	Year of launch	Mode of action	WHO toxicity class[y]
Copper sulfate		Inorganic	1873	General toxicant	II
Chloro (2-hydroxy phenyl) mercury	UPSULAM	Organo-mercurial	1913	General toxicant	
Thiram		Dithio-carbamate	1940	General toxicant	III
Ferbam		Dithio-carbamate	1943	General toxicant	U
Mancozeb	DITHANE™	Dithio-carbamate	1961	General toxicant	U
Carboxin	VITAVAX	Carboxamide	1969	Mitochondrial electron transport Complex II	U
Benomyl	BENLATE	Benzimidazole	1970	β-tubulin synthesis	U
Triadimefon	BAYLETON	Triazole	1976	Ergosterol biosynthesis	III
Metalaxyl	RIDOMIL, APRON	Phenylamide	1977	RNA transferase	III
Fosetyl-Aluminum	ALIETTE	Phosphonate	1977	Not conclusively determined	U
Azoxystrobin	QUADRIS, AMISTAR, HERITAGE	Strobilurin	1996	Mitochondrial electron transport Complex III	U
Tricyclazole	BEAM™	Benzothiazole	1976	Melanin biosynthesis inhibitor	II
Probenazole	ORYZEMATE	Benzisothiazole	1979	Systemic acquired resistance	U
Acibenzolar-S-methyl	BION, ACTIGARD	Benzo-thiadiazole	1996	Systemic acquired resistance	III

Quinoxyfen	**FORTRESS™** **QUINTEC™**	**Quinoline**	**1997**	**Signal transduction**	**U**

[x] DITHANE™, BEAM™, FORTRESS™, and QUINTEC™ are trademarks of Dow AgroSciences LLC.

[y] World Heath Organization classification for estimating acute toxicity of pesticides (25): II = Moderately hazardous; III = Slightly hazardous; and U = Product unlikely to present acute hazard in normal use.



1.2.4. 1969-The First Systemic Seed Treatment.

The first systemic fungicide was a milestone both because it had true redistribution in the plant and because it had the potential to replace organomercurial cereal seed treatments. Carboxin, described in 1966 and commercialized in 1969, not only controlled surface-borne bunts and smuts but also penetrated deeply in the seed embryo, where it eradicated loose smut infection. Carboxin also gave excellent control of early season rust and *Rhizoctonia* damping off, although it was less effective on seed-borne *Fusarium* and *Dreschlera* disease than organomercurials.[16] Additionally, carboxin had good plant safety as a seed treatment of row crops, particularly cotton and canola. Resistance

development has been slow, although field resistance was eventually documented in some populations of *Ustilago nuda* after many years of continuous use.[17] Despite their excellent fit in limited markets, the utility of carboxin and its derivative of foliar application, oxycarboxin, was never broad because of their specificity for Basidiomycetous diseases.

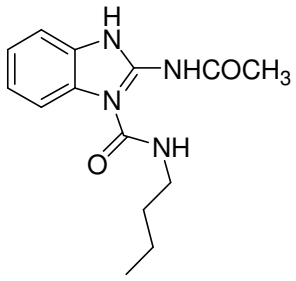
1.2.5. 1970- The First Broad-Spectrum Foliar Systemic Fungicide.

The first fungicide with the broader spectrum typical of dithiocarbamates and the systemic activity of organophosphate insecticides was benomyl. This benzimidazole fungicide was launched by DuPont in 1970 and provided systemic and curative activity at low rates, with excellent plant and mammalian safety. For the first time, farmers were able to cure existing infections, extend intervals between sprays, and not worry about perfect coverage. These characteristics made benomyl extremely popular from its introduction.[18] The list of fungi controlled by benomyl and other benzimidazole fungicides is extensive. Most Ascomycetes with light-colored spores are controlled, including numerous types of leaf spots, fruit rots caused by *Botrytis* and *Penicillium*, powdery mildews, and stem diseases such as eyespot. Some Basidiomycetes, such as selected anastamosis groups of *Rhizoctonia solani*, are controlled, but most are not. Diseases caused by Oomycetes and by Ascomycetes with dark spores (such as *Alternaria* and *Helminthosporium*) are also not controlled.[19] Additional benzimidazole fungicides launched after the introduction of benomyl include thiophanate-methyl (1971) and carbendazim (1974).

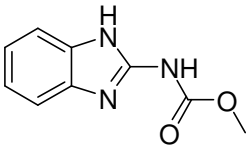
The characteristics that made benomyl so popular and effective also had a troubling aspect. Repeated, exclusive use on polycyclic diseases led to rapid development of resistant fungal populations. Within three years of introduction, resistance was reported in field and/or greenhouse populations of *Erysiphe*, *Botrytis*, *Penicillium*, and *Cercospora*.[18] Benomyl's single-site mode-of-

action could be bypassed by the fungus with a single mutation. Resistant strains could be equal in fitness to their susceptible counterparts, resulting in persistence of some resistant populations even when the benzimidazole fungicides were discontinued.[20] The agrochemical industry learned an important lesson about fungicides with specific modes-of-action from the benzimidazole experience, and now begins assessment of resistance risk early in fungicide development so that resistance management plans are in place at product launch.[20]

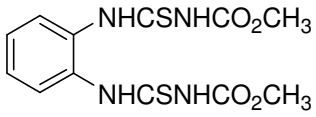
The benzimidazole fungicides were very successful on fruits and vegetables but had less utility for cereal diseases, since they gave no control of rusts or *Dreschlera* species. Further, the cereal diseases that were controlled, in particular the powdery mildews, rapidly became resistant.[18] A systemic, broad-spectrum fungicide with a new mode-of-action was still needed for foliar disease control in cereals.



Benomyl



Carbendazim



Thiophanate

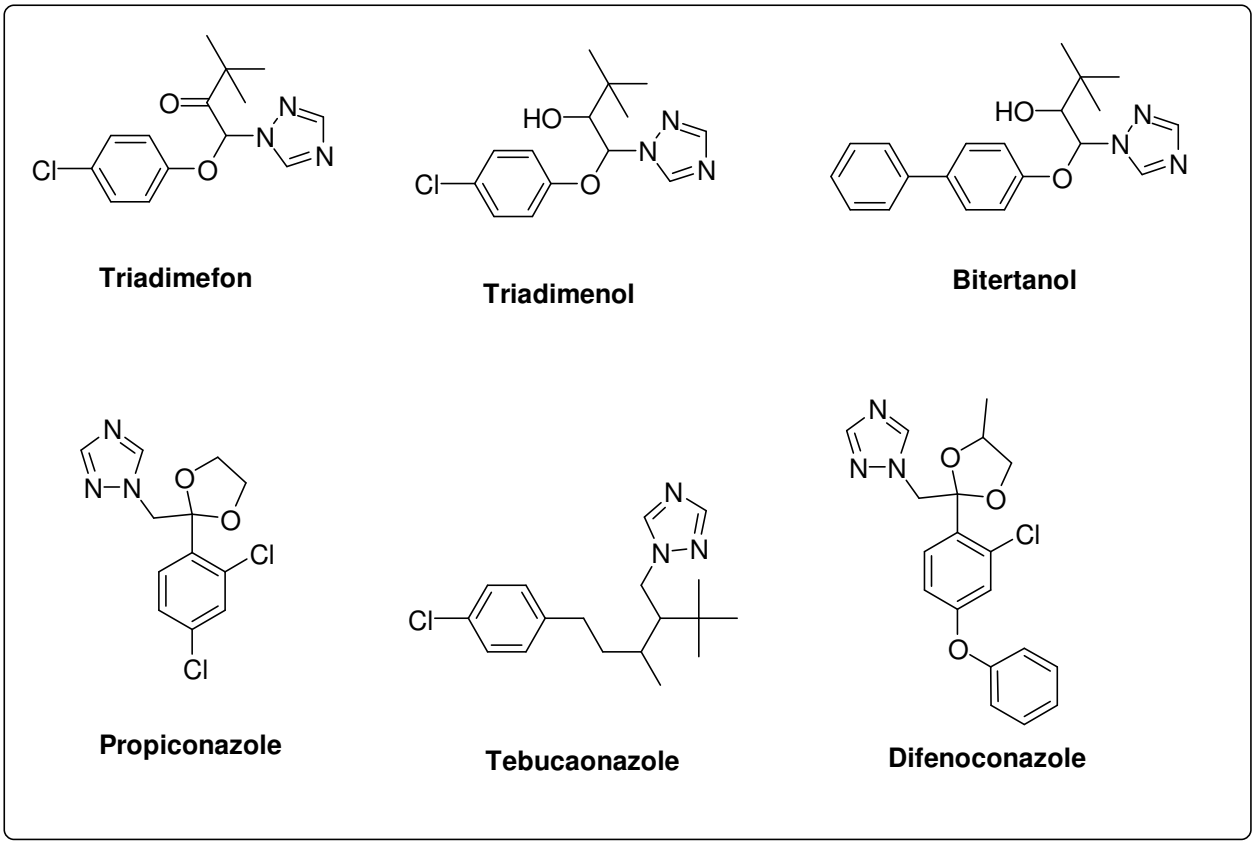
1.2.6. 1976-A Systemic, Curative Foliar Fungicide for Cereals.

The breakthrough for cereal disease management came in 1976 with the introduction of the triazole fungicide triadimefon by Bayer.[21] Triadimefon provided curative as well as protectant activity, low application rates, and excellent redistribution in the plant. The spectrum of control covered all major cereal diseases and included most Ascomycetes and Basidiomycetes (but not Oomycetes).

Additional triazole fungicides were introduced over the next two decades with improved potency and plant safety on cereals (e.g., epoxiconazole), a broader effective spectrum (e.g., propiconazole, tebuconazole), or specialized applications (e.g., difenoconazole and triticonazole for seed treatment)[21]. The triazole fungicides significantly increased farmers' expectations for fungicides, particularly for reach-back (curative) activity and redistribution to unsprayed growth.

The revolutionary triazoles have not been immune to challenges in their development and maintenance. They have well-documented side effects on plants. Application to shoots and roots often reduces elongation and causes leaves to be smaller, thicker, and greener. Treated plants may be delayed in senescence, which can impede harvest or improve yields, depending on the crop.[22] A larger concern has been resistance development, since the triazoles have many of the same properties as the benzimidazoles (curative activity, single-site MOA, multiple applications per season). Resistance to the triazole fungicides (and other inhibitors of C14-demethylase in ergosterol biosynthesis) developed first in the powdery mildews and has been observed (but is less problematic) on other diseases.[21] Unlike resistance to the benzimidazoles, resistance to the triazoles involves multiple genes with intermediate levels of resistance and incomplete cross-resistance between different fungicides.[22] The use of mixtures has been remarkably successful in maintaining useful activity against most fungal targets for three decades.

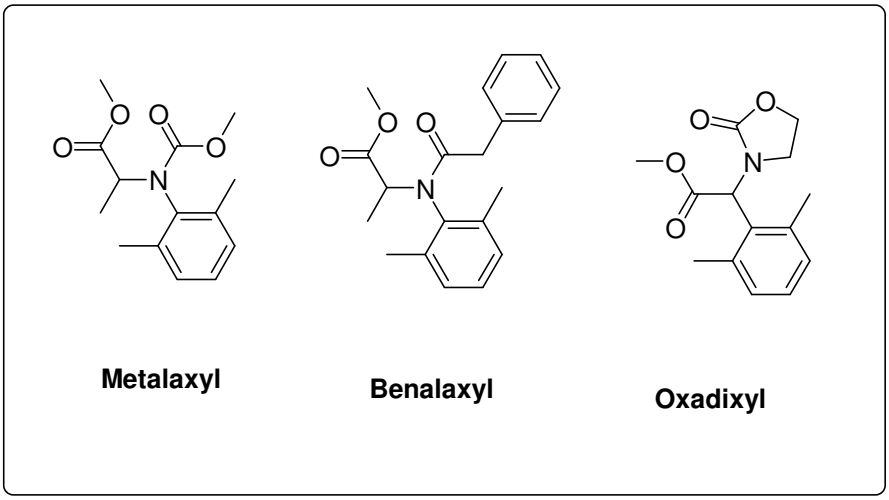
The launches of benzimidazole and triazole fungicides provided potent, systemic fungicide solutions for Ascomycete and Basidiomycete diseases, but control of devastating Oomycete diseases such as potato late blight and grape downy mildew was limited to frequent sprays of protectant fungicides. Root rots of established plants (caused by *Phytophthora* and *Pythium*) and systemic downy mildews could not be controlled at all, and took an unknown toll on crop yield.



1.2.7. 1977-The First Systemic Oomycete Fungicides

The launch of the phenylamide fungicide metalaxyl in 1977 by Ciba-Geigy changed farmers' expectations for control of Oomycete diseases.[23] This fungicide was an immediate success because of its outstanding properties: high potency; excellent curative and protectant activity; excellent redistribution and protection of new growth; control of all members of the order Peronosporales (including *Pythium*); and flexible application methods including foliar spray, seed treatment, and root drench.[24] As with the benzimidazoles, the phenomenal success and overuse of the phenylamide fungicides led to rapid resistance development. Significant resistance to metalaxyl was first described in 1980 on cucumber downy mildew and late blight.[23] Resistance developed more rapidly where metalaxyl was used alone, disease pressure was very high, and applications were made curatively. Ciba-Geigy responded with the development of fungicide prepacks containing metalaxyl and protectant fungicides, such as mancozeb, which extended the product life significantly.[23,24] The

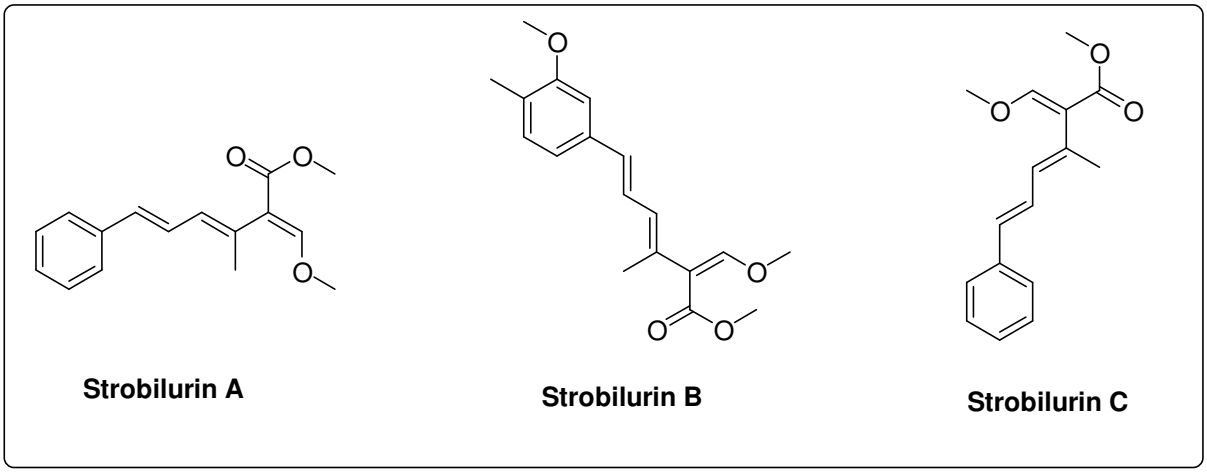
phenylamide experience was pivotal in the formation of the Fungicide Resistance Action Committee (FRAC), which developed a coordinated strategy across rival companies to limit the number of recommended phenylamide applications per season.[25] Despite a coordinated effort, susceptibility to phenylamides gradually eroded in populations of many foliar pathogens, and foliar uses of metalaxyl are now met by other fungicides in many markets. Soil and seed applications of metalaxyl (or its active enantiomer, mefenoxam) have generally retained their effectiveness, particularly for control of *Pythium* and the root-infecting species of *Phytophthora*.

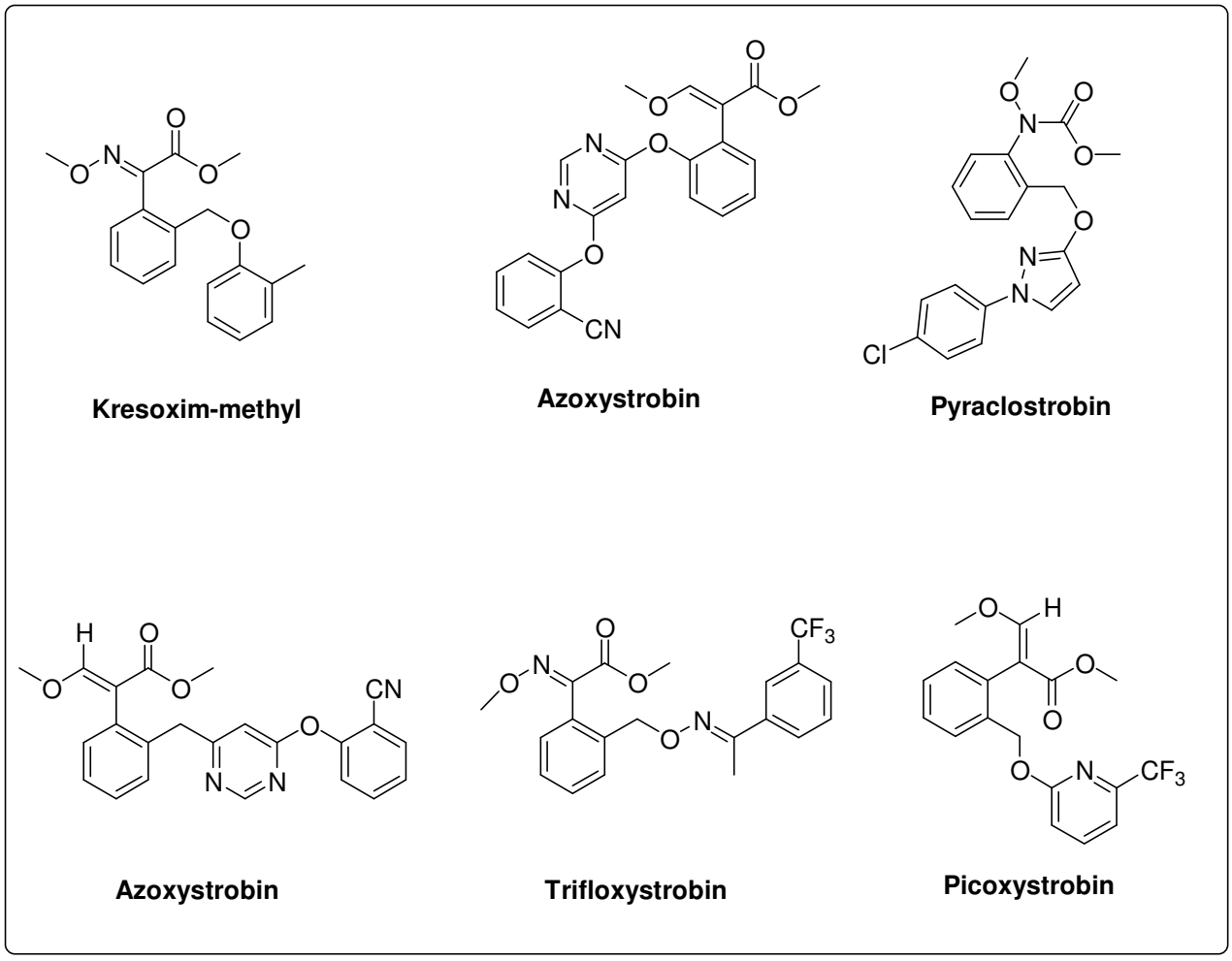


1.2.8. 1996-Broad-Spectrum Fungicides with Novel Spectrum and New Mode-of-Action

The natural products strobilurin A and oudemansin had been isolated from a saprophytic fungus in the late 1970s and demonstrated excellent broad-spectrum control of fungal growth. Parallel research programs at ICI and BASF in the early 1980s were focused on invention of synthetic analogues with improved UV stability and spectrum.[25] These strobilurins differed from previous fungicides in combining an unusually broad spectrum (including control of Oomycetes, Ascomycetes, and Basidiomycetes) with a site-specific mode-of-action. The first strobilurin products were launched in 1996; kresoxim-methyl from BASF had strong utility on cereals, and azoxystrobin from Zeneca was suitable for a variety of crops due to its plant safety and strong redistribution. Additional strobilurins, including trifloxystrobin, picoxystrobin, and pyraclostrobin, have been launched by a number of companies. These compounds became popular in many markets because of their versatility at controlling diseases from different taxonomic classes, such as powdery and downy mildew on vines, and sheath blight and blast on rice.[26] An additional benefit came from the physiological response of the plant to the fungicide; as with the triazoles, strobilurins often enhanced plant greening and delayed senescence, leading to improved yields even in the absence of significant disease pressure.[26,27] Some of the strobilurin fungicides commercialized after azoxystrobin were tailored to the cereal market rather than the vegetable and fruit market, with attributes of long residual protection, vapor phase activity, and moderate redistribution. Widespread use of strobilurins has already led to the development of resistance for several diseases, including wheat, barley, and cucumber powdery mildew, grape and cucumber downy mildew, apple scab, black sigatoka on bananas,[27] and *Septoria* blotch on wheat.[28] Resistance is typically caused by single base pair mutations in the mitochondrial gene encoding cytochrome b.[27] Current recommendations for use of strobilurin fungicides limit the number of applications per season,

suggest alternation of application with fungicides that have different modes-of-action, and recommend mixtures for many markets.[27]





1.2.9. 1976-1996-Fungicides with indirect Modes-of-Action

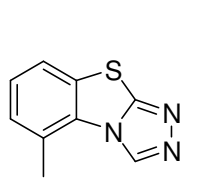
Increasing environmental and regulatory pressures built interest in fungicides that act on the plant-pathogen interaction rather than the fungus.[29] These compounds are not toxic to the isolated fungus, and should be more environmentally benign. It is not even clear if they should be called fungicides since they do not kill fungi directly. The first compound developed was tricyclazole, introduced in 1976. This systemic fungicide (as well as the newer carpropamid) inhibits melanin biosynthesis, which is required for penetration of the leaf by the appressorium of some fungi.[30] Utility is limited mainly to rice blast. Quinoxyfen, a compound from Dow AgroSciences that is highly specific for powdery mildews, also acts by inhibiting the fungus' ability to initiate infection. Molecular studies suggest that quinoxyfen disrupts the infection process by inhibiting early fungus-plant signaling events and interfering with the fungus' ability to make the morphological changes necessary for infection.[31]

Other fungicides have been commercialized that act through stimulation of the plant's natural defence response. Probenazole is a systemic compound that indirectly controls rice blast and some bacterial rice diseases. It stimulates the accumulation of toxins and enzymes associated with systemic acquired resistance in rice, but is ineffective in other cereals.[14,32] Acibenzolar-S-methyl has the widest spectrum of activity among the non-fungitoxic compounds developed to date. It is active against various fungi, bacteria, and viruses and is highly mobile, with both acropetal and basipetal transport, but is rapidly metabolized.[26] It stimulates the plant's natural defense system, and must be applied as a protectant treatment several days before infection. It has been developed for use against powdery mildews in cereals, rice blast, sigatoka diseases of banana, and blue mold of tobacco.[26]

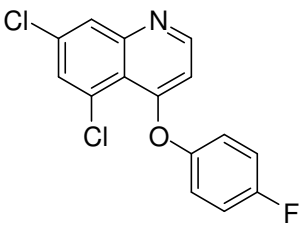
A challenge for treatments that elicit resistance responses in plants is the potential reduction of yield. Alteration of the production of secondary metabolites in plants has a demonstrated fitness

cost in some cases, resulting in diminished plant growth.[33] This yield drag in the absence of disease may limit the future development of compounds that alter plant metabolism, especially if the compound must be applied before disease pressure is significant.

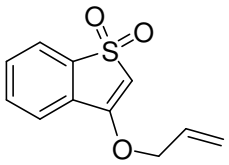
Because these compounds do not place selection pressure directly on fungal growth, they were expected to be more durable than conventional fungicides and unlikely to stimulate resistance development. Resistance has developed, however, for some compounds with indirect modes of action. Tricyclazole has remained effective for three decades, but resistance rapidly developed to carpropamid,[30] despite both being inhibitors of melanin biosynthesis. Resistance to quinoxyfen developed in the wheat powdery mildew population in Europe after more than five years of intensive use.[34] On the other hand, the compounds which act by stimulating host resistance have remained effective.



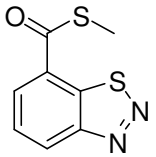
Tricyclazole



Quinoxyfen



Probenazole



Acibenzolar-s-methyl

1.3 Application Methods

Fungicides are applied as dust, granules, gas and most commonly, liquid. They are applied to

1. Seed, bulbs, roots of transplants and other propagative organs. These treatments are usually done by the seed company. Some treatments need to be done by the grower on-site at the time of planting. The goal is to kill pathogens that are on the planting material or to protect the young plant from pathogens in the soil.
2. Soil either in-furrow at planting. After planting as a soil drench (including through drip irrigation), or as a directed spray around the base of the plant.
3. Foliage and other aboveground parts of plants by means of sprayer.
4. Inside of trees via trunk injection.
5. Air in enclosed areas such as greenhouse and covered soil. Fungicides are called fumigants when applied as a vapour-active chemical in the gaseous phase. Some fumigants are also active against nematodes, insects and weed seeds.
6. Harvested produces, as a dip or spray in the packinghouse.

Fungicides are used as a formulated products consisting of an active ingredient plus inert ingredients that improve the performance of the products. Fungicides are typically mixed with water then applied by spraying. Application equipments ranges from small hand held and back-pack sprayers to large spray units carried by tractors or aircraft. A few fungicides are applied as a dust. Fungicides can also be applied in greenhouse as smoke. Mist fog or aerosol.

A look at the history of fungicides gave us some idea of what to expect in the future. The major changes in fungicide use have usually been associated with changes in the spectra of pathogens as well as in crop intensities, practices of prices.

In the above section historical perspective clearly reflects applications, advantages, and limitations of mostly each commercialized groups of fungicides. Clearly understanding of historical development of fungicides proves the major contribution of natural products in crop protection such as strobilurins. Our special orientation towards the natural products considers them in terms of safety of both, environment and human health. Additionally, successful story of strobilurins in crop protection motivated us to work in the area of natural products synthesis, which should contribute in the research of crop protection. At the end, we should try to meet the aspects and challenges of fungicide research.

Nature is a rich source of compounds exhibiting biological activity against weeds, plans diseases, insects and mites. Natural products, with their tremendous structural diversity, are an important source of new toxophores. Many of these natural products have complex structures, insufficient biological activity and low persistence under field condition. Thus the share of natural products being used as active ingredients per se in today's crop protection market is relatively small. In some cases the natural products have been further modified to provide semi-synthetics derivatives with improved biological properties. More importantly, natural products served as lead structures inspiring chemists to prepare new synthetic analogues with often improved biological activity, simplified structure, increased safety towards human and the environment an optimized persistence.

1.4 What is a natural substance

A scientist and a lay-person perhaps have a different understanding of what the term 'natural' conveys. Similarly, the motivation for investigating the use of natural products in plant protection might also be different. By definition, a natural or biogenic substance is either synthetized directly by a living organism or is derived form substance of biogenic origin by chemical reaction occurring without human intervention; for example by decomposition of biological materials. Thus, humus, or in the wider sense coal, oil and limestone, are examples of

natural or biogenic substance. A natural products is viewed as a physiologically active chemical which is synthetized by plants, animals or microbes. In contrast, a synthetic chemical is one which does not occur naturally and must be synthetized from other substance by human intervention. Of course, many naturally occurring substance can also be synthetized in the laboratory, and indeed the use of a pure, chemically synthetized molecule in laboratory tests in usually a pre-requisite for the acceptance of biological activity attributed to a particular substance in complex mixture form a natural source.

1.5 Natural products in crop protection

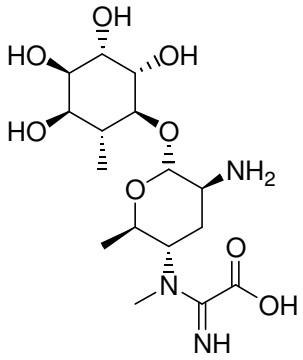
Natural products have been used for the benefit of humankind for many thousands of years, be it for food, clothing, cosmetics, construction of shelters and traps, tools and weapons, poisons for game and fish, medicines or crop protection agents. Where a physiological effect on a pest was required, early compounds were simply extracted from a source and used as an impure mixture of chemicals, one or more of which gave the required response. The science of natural products has advanced significantly in recent times, and these compounds are being used as products in their own right as pure (or at least characterised) compounds, as new chemical skeletons that can be modified by the ingenious synthesis chemist or as indicators of new, effective biochemical modes of action (increasingly important in a world of high-through put in vitro screening). A large number of volumes and reviews have been written about the use of natural products as pesticides. Most of these literature deals with compounds with promising activity that are not commercially available.

1.5.1 Kasugamycin

Kasugamycin and kasugamycin hydrochloride hydrate were isolated form the soli actinomycete *Streptomyces kasuganesis* Hamad et al. and were first described by Umezawa eta al. in 1965[35] and introduced by Hokko Chemical Industry Co. Ltd. The product is sold as a hydrochloride. The biological activity of these compounds was first described by Hamada et al.[36] in 1965. Kasugamycin is recommended for the control of rice blast (*P. oryzae*; perfect stage *M. grisea*) in the rice, leaf spot in sugar beet and celery (*Cercospora spp.*), bacterial disease in rice and vegetables and scab (*Venturia spp.*) in apples and pears. Kasugamycin is a systemic fungicide and bactericide with both protectant and curative properties. Resistance to kasugamycin was detected within 3 years of its introduction in 1965, and by 1972 it had become a serious problem in Japanese rice field. Today, mixture of kasugamycin with other fungicides with different mode of action are used. Kasugamycin inhibits hyphal growth of *P.oryzae* on rice, preventing lesion development; it is a comparatively weak inhibitor of spore germination, appressorium formation and penetration into the epidermal cells. In contrast, against *Fulvia fulva* (Cooeek) Cif. on tomatoes, inhibition of sporulation is high, but its effects on hyphal growth are poor. Kasugamycin is taken up by plan tissue and is translocated.

Kasugamycin hydrochloride hydrate is sold as WP, DP, soluble concentrates (SL) and granule (GR) formulations under the trade names Kasugamin, Kasumin and Kasu-rab-valida-sumi (plus phthalide plus validamycine plus fenitrothion) from Hokko. It is applied as a foliar spray, a dust or a seed treatment at rates from 20g AIL-1 . There has been evidence of slight phytotoxicity on crops such as peas, beans, soybeans, grapes, citrus and apples. No injury has been found on rice, tomatoes, sugar beet, potatoes and many other vegetables. The use of kasugamycin has declined following the rapid onset of resistance and the release of new, disease-specific chemical fungicides for rice blast control. As with other aminoglycoside antibiotics, Kasugamycin is not considered to

be toxic to mammals and there is no evidence that it has had any adverse effects on non-target organisms or on the environment.

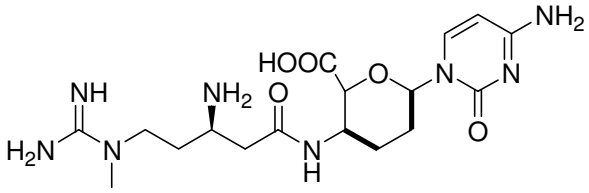


Kasugamycin

1.5.2 Blasticidin S

Blasticidin S was isolated from the soil actinomycete, *Streptomyces griseochromogenes* Fukunaga in 1955 by Fukunaga et al,[37] and later by Takeuchi et al,[38] in 1958. Its fungicidal properties were first described by Misato et al.[39] It is usually sold as benzylaminobenzenesulfonic acid salt. Blasticidin S is used to control the rice blast [*Pyricularia oryzae* Cavara; perfect stage *Magnaporthe grisea* (Hebert) Barr] by foliar application. Blasticidin-S inhibits protein biosynthesis by binding to the 50S ribosome in prokaryotes (at the same site as gougerotin), leading to the inhibition of peptidyl transfer and protein chain elongation. It is a contact fungicide with protective and curative action, exhibiting a wide range of inhibitory activity on the growth of bacterial and fungal cells. In addition, it has been shown to have antiviral and antitumour activity. It inhibits spore germination and mycelial growth of *P. oryzae* in the laboratory at rates below 1mgmL-1. It is sold as dustable powder (DP), emulsifiable concentrate (EC) and wettable powder (WP) formulations under the trade name Bla-S by Kaken, Kumiai and Nihon Noyaku. For control for rice blast it is used at rates between 1000 and 300 g Alha-1 by foliar application. Damage can be caused

to alfalfa, aubergines, clover, potatoes, soybean, tobacco and tomatoes. Excessive application produces yellow spot on rice leaves. In recent years, its significance as a fungicide has decreased following the introduction of new lower-toxicity, pathogen-specific, synthetic rice blast products. Blasticidin-S is relatively toxic to mammals, with the acute oral LD_{50} to rodents being below 100 $mgkg^{-1}$. It is also a severe eye irritant. It is relatively non-hazardous to non-target organisms when used as labelled and has no deleterious effect on the environment.

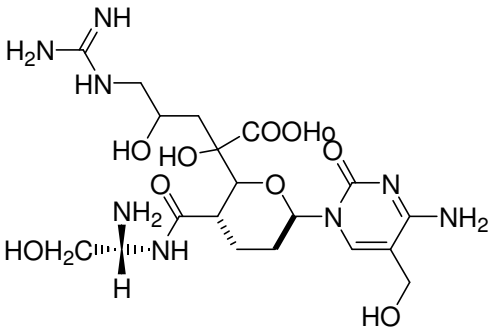


Blasticidin S

1.5.3 Mildiomycin

Mildiomycin was isolated from the soil actinomycete *Streptoverticillium rimofaciens* strain B-98 891 and first reported by Harada and Kishi;[40] its fungicidal properties were reported by Kusaka et al. in 1979.[41] Mildiomycin used to control powdery mildews [*Erysiphe spp,* Uncinula necator (Schwein) Burrill, *Podosphaera spp*. And *Sphaerotheca spp*.] in ornamentals. The mode of action of mildiomycin is believed to be inhibition of protein biosynthesis in fungi by blocking peptidyl-transferase.[42] In the field, it is effective as an eradicant, with some systemic activity. Mildiomycin is specifically active against the pathogens that cause bacteria.

It is sold as a WP formulation under the trade name Mildiomycin by Sumitomo Chemical Takeda. Rates of use to eradicate infection and offer subsequent protection are from 50 to 100 mgL^{-1}.Mildiomycin has not been widely used for disease control outside Japan. Mildiomycin has very low mammalian toxicity and it has not been shown to have any adverse effects on non-target organisms or the environment.

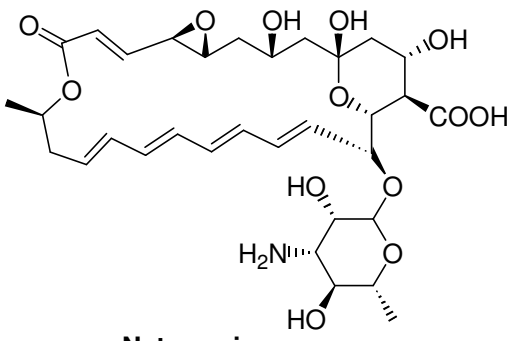


Mildiomycin

1.5.4 Natamycin

Natamycin (also known as myprozine, pimaricin and tennecin) is a secondary metabolite of the actinomycetes *Streptomyces natalensis* Struyk, Hoette, Drost, Waisvisz, Van Edk & Hoogerheide and S. Chattanoogensis Burns & Holtman. Its structure was established by Golding et al[44] and Meyer,[44] and its sterochemistry was revised by Lancelin and Beau,[45] and Duplantier and Masamune.[46] It was introduced as a fungicide by Gist-Brocades N V. Natamycin is used to contol various fungal disease, but especially basal rots on ornamental bulbs such as daffodils that are caused by *Fusarium oxysporum*. Schlecht. It is usually applied as a dip in combination with a hot water treatment prior to planting. Its precise mode of action is not known. Natamycin was sold as WP under the trade name Delvolan, but it is now withdrawn. Natamycin is not toxic to mammals or fish and is readily biodegradable.[47,48] No adverse effects have been observed on non-target organisms or on the environment.

Plant diseases are known from times preceding the earliest writings. Fossile evidence indicates that plants were affected by disease 250 million years ago. The Bible and other early writings mention diseases, such as rusts, mildews, blights, and blast that have caused famine and other drastic changes in the economy of nations. Some of the plant disease out breaking with similar far-reaching effects in more recent times include.



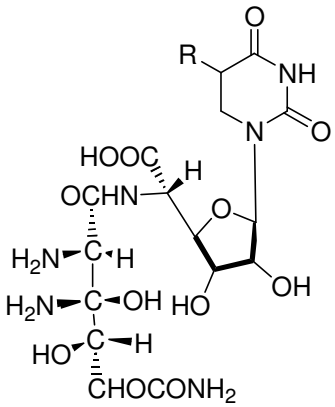
Natamycin

1.5.6 The polyoxins

The fungicidal polyxins are polyoxin B and polyoxorim (BSI, pa ISO). Polyoxin B is one of the secondary metabolites produced by fermentation of the soil actinomcete *Streptomyces cacoai* var *asoensis* Isoono te al. It was first isolated by Isono et al,[49] in 1965 and was introduced as a commercial fungicide by Hokko Chemial Industry Company Ltd, Kaken Pharmaceutical company Ltd, Kumiai Chemical Industry Company Ltd, and Nihon Nohyaku Company Ltd. Polyoxorim (polyoxin D) was isolated by Suzuki et al[50] and Isono et al.[51] The zinc salt was introduced as a fungicide by Kaken Pharmaceutical Company Ltd, Kumiai Chemical Industry Company Ltd and Nihon Nohyaku Company Ltd. Polyoxin B is used to contol a range of plant pathogenic fungi such as *Sphaerotheca* spp. and other powdery mildews, *Botrytis cinerea* Pers., *Sclerotinia sclerotiorum* De Bary, *Corynespora melonis* Lindau, *Cochliobolus miyabeanus* (Ito & Kuribay) Drechsler ex Dastur, *Alernaria alternate* (Fr.) Keissler and other *Alternaria* species in vines, apples, pears, vegetables and ornamentals. The primary use of polyoxorim is for the control of rice sheath blight, *R solani*. It is also effective against apple and pear canker [*Nectria galligena Bresadola* (*Diplodia pseudodiplodia* Fuckel)] and *Drechslera* spp., *Bipolaris* spp., *Curvularia* spp. and *Helminthosporium* spp. with the major use being in rice, although it also has application in pome fruit and turf. Polyoxins cause a marked abnormal swelling on the germ tuves of spores and hyphal tips of the pathogen, rendering the treated fungus non-pathogenic. In addition, the incorporation of [^{14}C] gulcosamine into cell wall chitin of *C. miyabeanus* is inhibited. Polyoxins apparently exert

their effects through inhibition of cell wall biosynthesis.[53,54] They are systemic fungicides with protective action. Polyoxin B is effective at controlling a variety of fungal pathogens, but is ineffective against pear black spot and apple cork spot (*Alternaria* Spp.), grey moulds (*B. cinerea*) an other sclerotia-forming plant pathogens. Resistance to polyoxin B has been found in *A alternata* in some orchards in Japan following intensive treatment. Polyoxin has good activity against rice sheath blight when applied as a foliar spray at rates of about 2 gL-1. It can be used to control apple and pear canker when applied as a paste. It is also ineffective against bacteria and yeasts.

The polyoxins are sold as WP, EC, water soluble granule (SG) and paste (PA) formulations. Polyoxin B is sold under a variety of trade names including Polyoxin AL by Kaken, Kumaiai, Nihon Nohyaku and Hokko and Polybelin by Kumiai. Polyoxorim trade names include Endorse, Polyoxin A and Stopit as symptoms appear.

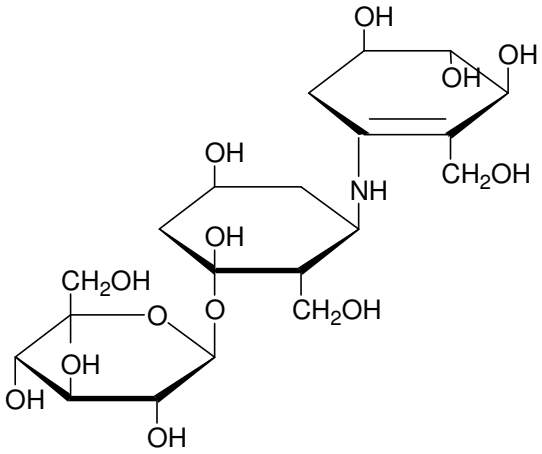


Poloxycin B, R =CH_2OH
Polyoxorim, R = COOH

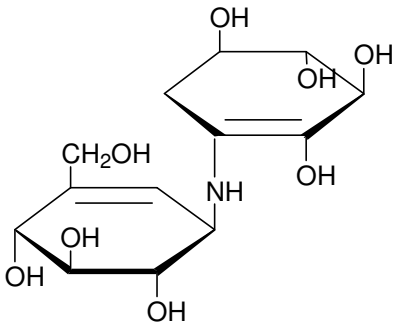
1.5.7. Validamycin

Validamycin (also known as validamycin A) was isolated from the soil actinomycete *Streptomyces hygroscopicus* (Jensen) Waskman & Henrici isolate *limoneus*. Its biological activity was described by Horii et al.[54] Validamycin is used for the control of *R solani* and other *Rhizoctonia* species in rice, potatoes, vegetables, strawberries, tobacco, ginger, cotton, rice, sugar beet and other crops. Validamycin is non-systemic, but has a fungistatic action, showing non fungicidal action

against *R solani*, but causing abnormal branching of the tips of pathogen followed by a cessation of further development.[55] Validamycin has a potent inhibitory activity against trehalase in *R solani* AG-1, without any significant effects on other glycohydrolytic enzymes tested.[56] Trehalose is well known as a storage carbohydrate in the pathogen, and trehalase is believed to play an essential role in the digestion of trehalose and transport of glucose to the hyphal tips. Very low rates of use give excellent control of *R solani* in various crops, and rates of 0.3g AIL-1 give effective control of rice sheath blight. It is applied as a foliar spray, a soil drench, a seed treatment or by soil incorporation. Validamycin is sold as DP, DL, powder seed treatment (DS) and liquid formulations under a wide variety of trade names including Validacin, Valimun, Dantotsupadanvalida (plus captan hydrochloride plus clothianidin) and Hustler (plus captan hydrochloride plus clothianidin plus ferimzone plus phthalide) by Sumitomo Chemical Takeda, Sheathmar by Sumitomo Chemical Takeda and Dhanuka, Mycin by Sanonda, Rhizocin by Nagarjuna Agrichem, Solacol by Bayer Crop Science, Valida by Nichimen, Kasu-rab-valida-sumi (plus fenitrothion plus kasugamycin plus phthalide) and kasurabvadlidtrebon [plus etofenprox plus kasugamycin hydrochloride hydrate plus phthalide)] by Hokko and Vivadamy and Vimix (plus 1-naphthylacetic acid plus 2-naphththyloxyacetic acid) by Vipesco. Concentrations as high as 1 gL-1 showed no phytotoxicity to over 150 different target crops. Validamycin continues to find wide range of application in a wide variety of crops, particularly in Japan. Validamycin is not considered to be toxic to mammals and has no adverse effects on non-target organisms or on the environment.



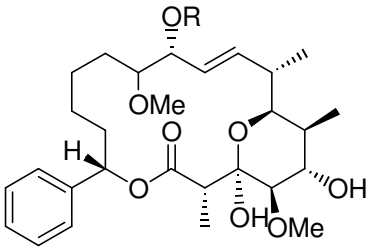
Validamycin A



Validoxylamine A

1.5.8 Soraphen A

Soraphen A was discovered by the research groups of Reichenbach and Hofle at GBF[57], In their screening of extract form myxobacteria, a sample from *Sorangium cellulosum* stain So ce26 showed broad antifungal *in vitro* activity. The new metabolite soraphen A which was mainly responsible for the activity, was isolated and fully characterized by NMR and X-ray crystallography.[57] The total synthesis of soraphen A was achieved by Giese and coworkers.[58] Greenhouse tests at Ciba-Geigy soon revealed the high potential of soraphen as a plant protection agent against fungal pathogens. Field tests met the high hopes generated by the greenhouse results, and economical application rates seemed feasible.



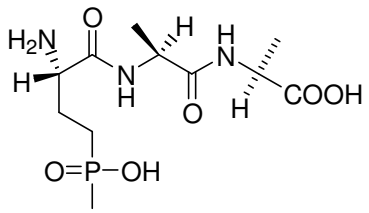
Soraphen A R = CH_3

1.5.9 Bilanafos

The tripeptide bilanafos was originally isolated from the soil-inhibiting actinomycyete *S. hygroscopicus*, and was introduced as a herbicide produced by fermentation by Meiji Seika[59,60], it is also produced by *S. viridochromeogenes* (Krainsky) Waksman & Henrict.[61]

Bilanafos is used post-emergence in vines, apples, brassicas, cucurbits, mulberries, azaleas, rubber and many other annual weeds in crop situations and for control of annual and perennial weeds on uncultivated land. Bilanafos itself is not directly phytotoxic but must be metabolically converted by the target plant to the actual phytotoxin, phosphinothricin {4-[hydroxy(methyl)phosphinoyl]-L-homoalanine}, a potent irreversible inhibitor of glutamine synthesize.

Bilanafos is sold as SP and liquid formulations under the trade name Herbiace (introduced by Meiji Seika in 1988). It is applied post-emergence at rates of 0.9-1.8 kgAIha-1 for control of annual weeds and at higher rates for control of perennial weeds. When applied post weed emergence in crop situations, it must be directed at the weed and away from the crop. Bilanafos is relatively non-toxic to mammals and other non-target organisms.[62,63] They have very little activity in soil, mainly owing to rapid microbial degradation. They are considered to be low environmental impact herbicides.



Bilanafos

1.6 The Future of Fungicides

Fungicides have changed the nature of agriculture. Each key invention was rapidly incorporated into farming practice and raised farmers' expectations for the next breakthrough in performance. The success of these breakthroughs has been attenuated in some cases by development of resistance in the targeted fungi. Experiences with fungicide resistance have spurred improvements in fungicide stewardship.

The report titled "Global Crop Protection Industry Outlook to 2016-Bio-pesticides: The Next Generation Crop Protection products" provides a comprehensive analysis on the size of agrochemical market, fungicides, herbicides, insecticides and bio-pesticides, micro-nutrients and adjuvant segments. The report covers the sales of crop protection products across all the regions in the world with detailed coverage on Europe, Asia and NAFTA region and 9 countries including the US, Canada, India, China, Japan, Germany, France, Italy and Spain. Talk among the watchers of worldwide crops indicates the global use of fungicides will continue to outpace. The global agrochemical market will keep growing in the future around 2% annually between 2010 and 2015, according to "Crop protection & Agriculture Biotechnology Consultant" head Phillips McDougall. However, fungicides are expected to have a higher growth rate than insecticides, so in the mid term (by 2015) fungicides will have a higher market value than insecticides. Fungicides are primarily a crop-driven market that drives yield.

Significantly, high soft commodity process also plays a key role in fungicides usage. A primary indicator of the soft commodity market is cotton. For example, the US market saw historic levels of increases in 2010 with prices spiking 80% in summer months to the $1.30-a-pound range. "Due to high soft commodity prices, farmers have been willing to invest in their crops and apply fungicides even in situations with less disease pressure," says Elise Kisling , BASF head of media relations, crop protection.

Five products currently drive the fungicide market.

- **Azoxystrobin ($1 billion annually)**
- **Pyraoxystrobin ($700 million)**
- **Mancozeb ($600 million)**
- **Chlorothalonil ($535 million)**
- **Copper-based fungicides ($450 million)**

A new trend emerging among fungicide manufacturers is treating "disease complexes" with chemicals that stem more than one disease state. These inhibitors "kill a whole trend of diseases.

The main requirements for fungicides today are effective disease control, broad spectrum control, physiological effects, use restriction due to the regulatory profile, maximum residue levels, resistance management, and easy-to-use formulations.

BASF is planning to address the needs of farmers with several new technologies. In fungicides, the main innovations will be Xemium, which has an active ingredient for broad-spectrum disease control in a wide range of crops for reliable and flexible use, which provides higher yield and improved quality, and Initium, which has an environmentally friendly chemistry with a new mode of action for vine and vegetable growers.

An increase in worldwide demand for high-quality food is perhaps the most compelling factor driving fungicide use. This is particularly true of driving the yield of Europe's wheat and barley, fruits and vegetables, soybeans, rice, and maize.

Feeding a growing world population is daunting to today's farmers. "Today's growers face a huge challenge: They will be instrumental in feeding a growing global population," says Pete Thomas, global portfolio manager, DuPont Crop Protection. "We will need to double food production by 2050 if we are to meet this escalating need. Clearly, improving crop productivity and protecting food quality is part of that equation."

People in rapidly developing countries including China and India are demanding higher volumes of food, and seeking more nutritious food. Those desires are increasing demand for many commodities, from grains to protein sources, which has in turn put upward pressure on commodity prices.

As growers begin to see the benefits of fungicides in helping them achieve this goal, they are more willing to invest in the technology. The more willing they are to invest, the more valuable crop protection is to the marketplace.

Thus the major multinational companies will be focusing their future on an integration of genetic traits and agrochemicals. Projected growth of the agrochemical market is 3.4% over the next 5 years, whereas traits are forecast to grow at 7%. The average current (2005) R & D expenditures of the six major Agro companies are 64.5% for chemistry and 35.5% for seed and traits. This endures that new fungicides will continue to be developed to protect the ever more precious cultivars, where they do not have sufficient genetic disease resistance. This balance between genetic resistances and disease control products of chemical, biochemical or biological nature will reamin and is not likely to change dramatically in the near future. Equally important for sustainable disease control will be the intelligent integration of these technologies with sound cultural and sanitation measures.

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Chapter 2

Scope of the thesis

New fungicides, including natural product based fungicides, are being discovered and developed to replace the compounds lost due to the new registration requirements. A new product needs to be highly active against the target species and needs to have the desired biological spectrum which is defined by the farmer's needs and the company's product portfolio. It should not be cross-resistant to existing products and therefore new modes of action are desirable. Furthermore, it should not cause damage to the crop, in which it is used, and should be safe for the farmer, the consumer, and the environment.

Natural products have been used for the benefit of humankind for many thousands of year, be it for food, clothing, cosmetics, construction of shelters and traps, tools and weapons, poisons for game and fish, medicines or crop protection agents. Where a physiological effect on a pest was required, early compounds were simply extracted from a source and used as an impure mixture of chemicals, one or more of which gave the required response. The science of natural products has advanced significantly in recent times, and these compounds are being used as products in their own right as pure (or at least characterised) compounds, as new chemical skeletons that can be modified by chemists or as indicators of new, effective biochemical modes of action (increasingly important in a world of high-through put in vitro screening). An important benefit of natural product based fungicides is their relatively short environmental half-lives, which is due to the fact that they do not possess 'unnatural' ring structures and contain relatively few halogen substituents, offer advantages in that they can sometime be specific to a target species and often have unique modes of action with little mammalian toxicity. Moreover these compounds are perceived to be environmentally benign.

A large number of volumes and reviews have been written about the use of natural products as pesticides. Most of these literature deals with compounds with promising activity that are not commercially available. A survey of recent literature supplies lists of novel bioactive compounds, which have been reported very recently and show promising biological activity. Among a number of possible candidates we have selected three natural products, Farinomalein, Epicoccamide, and Harzianic acid as a synthetic targets. Thus, the aim of our thesis is to synthesize these natural products, their analogues in order to study the SAR and evaluation of their biological activity.

In chapter 3, we discuss Farinomalein with respect to the isolation, structure elucidation, antifungal activity, study towards the synthesis and biological results. We also discuss previously reported synthesis of farinomalein and its drawbacks. We have overcome this drawbacks and report a different synthetic approach to farinomalein, developed in four steps with high yield and without using any hazardous chemical.

In chapter 4, we describe the efforts towards the first total synthesis of Epicoccamide D. Epicoccamid D is quite unusual since it is composed of three biosynthetically distinct subunits; glycosidic, fatty acid and tetramic acid. The epicoccamide D has significant antifungal and plant growth promotion effect, which makes it a potential lead for the design of new antifungal. Its biological activity and novel chemical structure has made this compound an attractive target for chemical synthesis.

In chapter 5, we describe the efforts towards the first total synthesis of Harzianic acid. Harzianic acid has an intriguing structure, which includes a tetramic acid group in its N-methylserine-derived heterocycle.

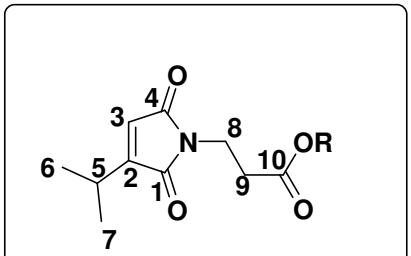
In chapter 6, we discuss about a synthetic path of new heterocyclic scaffolds as structural analogues of natural nucleic bases. In particular, we focus on 1,4 dihydro[1,2,4]triazolo[5,1-

c][1,2,4]triazine as a core structure and we develop a general method for the synthesis of this ring system that might be amenable to the preparation of 3,7-diaryl-substituted derivatives.

CHAPTER 3

IMPROVED SYNTHESIS OF FARINOMALEIN AND ITS ANALOGUES AND THEIR EVALUATION FOR BIOLOGICAL ACTIVITY

3.1 *Introduction*



1. R=H
2. R=CH_3

Farinomalein (1)

Farinomalein **(1)** 3-(3-Isopropyl-2,5-dioxo-2,5-dihydropyrrol-1-yl)-propionic acid is a relatively structurally simple maleimide, recently isolated in 2009 from the entomopathogenic fungus *Paecilomyces farinosusn* HF599, by Nihira et.al.[1] Entomopathogenic fungi are well known for their ability to produce various compounds during infection and proliferation in insects[2-4] and thus are considered as potential sources of novel bioactive compounds.[5-7] Those belonging to the genus *Paecilomyces* have been the source of wide range of bioactive metabolites, including the antimalarial and antitumor cyclohexadepsipeptide paecilodepsipeptide A from *P.cinnamomeus*,[8] an antibiotic containing a tetramic acid moiety (paecilosetin from *P. farinosus*[9]) and neuritogenic pyridine alkaloids (farinosones A-C and militarinones A-D form *P.militaris,* respectively[10,11]). During a screening for a compound active against plant pathogenic oomycetes, the EtOAc extract of *P. farinosus* HF599 showed strong inhibitory activity against *Phytophthora sojae.*[12] Detailed investigation of the fungal extract led tot the identification of the new maleimide compound

farinomalein (**1**). Reports of natural compounds bearing maleimide rings are very limited. Examples include showdomycin from *Streptomyces showdoensis*, pencolide from *Penicillium multicolor,* and turrapubesin from the twigs and leaves of *Turraea pubescens*.[13-15]

The structure of farinomalein contains two functional groups: -COOH and -$N(CO)_{2;}$ it shows five aliphatic carbons (one of them bonded to nitrogen), two carbon in the olefinic region, and two carbonyl carbon. C-2 and C-3 complete the maleimide core which is substituted with an isopropyl group at C-2. High resolution ESITOFMS revealed an $[M + Na]^+$ at *m/z* 234.0710, corresponding to the molecular formula $C_{10}H_{13}NO_4$. The ^1H and ^{13}C NMR spectroscopic data were in agreement with the assigned molecular formula. The ^{13}C NMR spectrum of farinomalein showed 10 signals, indicating five aliphatic carbons (one of them bonded to nitrogen), two carbons in the olefinic region, and two carbonyl carbons.

3.2 *Biological activity*

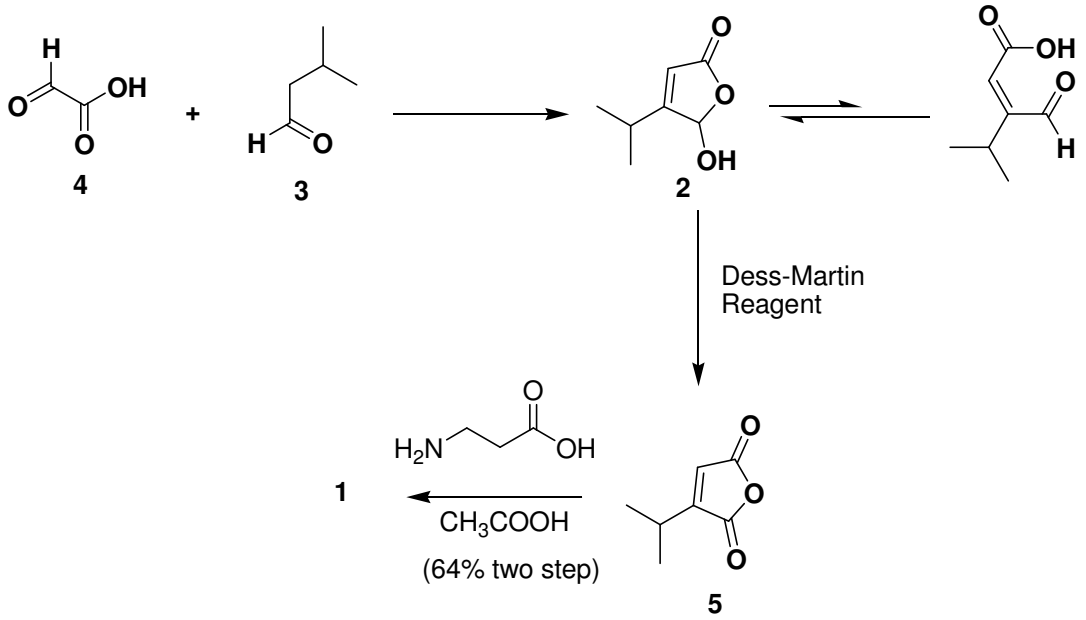
Farinomalein shows potent inhibition of *Phytophthora sojae*, a plant pathogen that causes every year enormous damage to soybean crops,[2] with an MIC value of 5µg/disk, whereas the MIC of the antifungal agent amphotericin B is 10µg/disk. Recently, maleimides such as N-methylmaleimide, N-ethylmaleimide, and phenylmaleimide PM-20 have attracted the interest of many researchers due to their cytotoxicity towards tumor cell lines through the inhibition of human topoisomerase II[16] or the inhibition of Cdc25A.[17] These facts suggested that, in addition to its potent antioomycete activity, farinomalein could also possess other biological functions, such as cytotoxicity toward tumor cell lines.

3.3 *Reported Synthesis of farinomalein*

Recently, a three-step synthesis of Farinomalein has been reported by Miles and Yan.[18] The sequence is based on the reaction of isovaleraldehyde **3** and glyoxylic acid **4** to give a γ-hydroxybutenolide **2** (Scheme 1). Successive oxidation to the corresponding anhydride **5** and treatment with β-alanine afforded Farinomalein. However, the Authors themselves state that their synthesis is difficult to scale-up [18] due to the use of the hazardous and expensive oxidant Dess-Martin periodinane. The use of this reagent was necessary to obtain a reasonable yield, because some of the more common oxidants (PCC and KMnO4) typically gave only moderate yields of anhydride **5** which was difficult to purify. On the contrary, the Dess-Martin periodinane oxidation proceeded cleanly and the removal of the excess Dess-Martin periodinane and of its side products was readily accomplished by a simple hexane extraction.

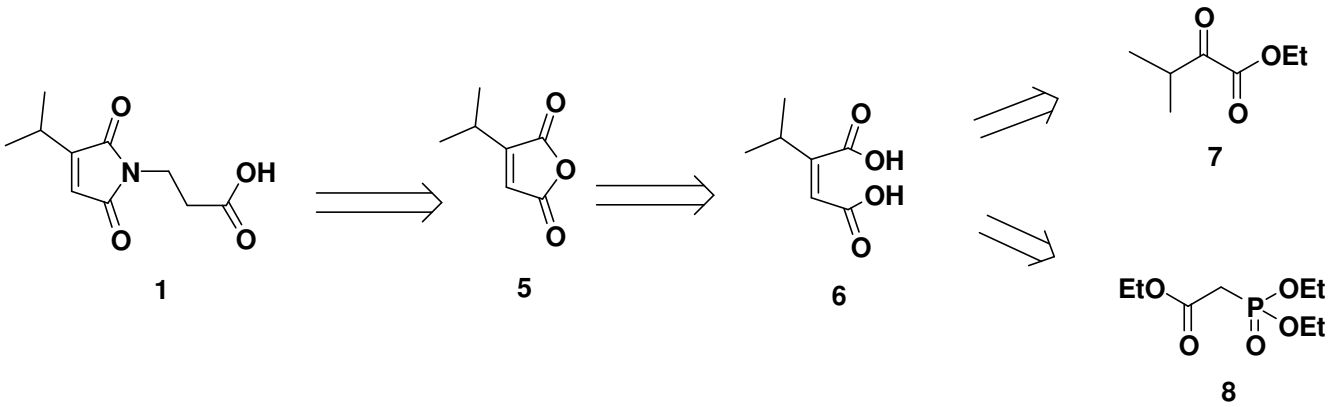
Herein we report a different synthetic approach to Farinomalein, developed in four steps with high yield and without using any hazardous chemical. Our retrosynthetic approach is shown is Scheme **2**, The key intermediate **5** can be constructed by dehydration of *cis* -diacid **6**, on its turn obtained by condensation of commercially available ethyl 3-methyl-2-oxobutyrate **7** and triethyl phosphonoaceate **8** in the Horner Wadsworth-Emmons conditions.

Scheme 1 Reported Synthetic route



3.3.1.Retrosynthetic Approach

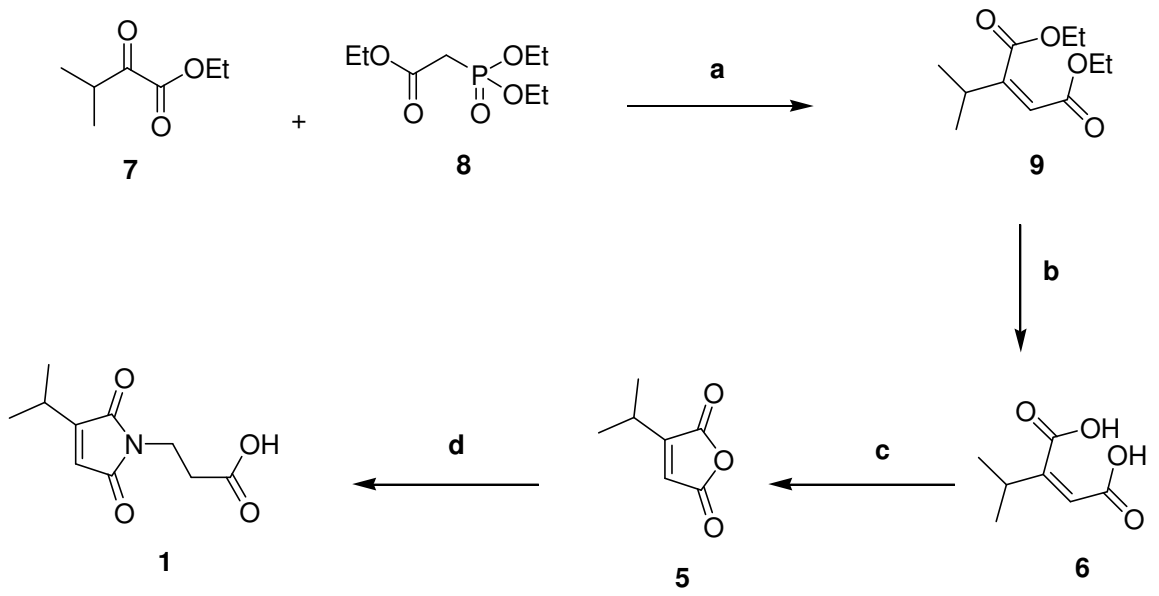
Scheme 2 Retro Synthesis



3.3.2 Synthesis of Farinomalein

For the preparation of 3-isopropyl-furan-2,5-dione (**5**) we used the procedures described by Watanabe.[19] In this synthetic path we condensed commercially available ethyl 3-methyl-2-oxobutyrate **7** with triethyl phosphonoacetate **8** in the Horner Wadsworth-Emmons[19] conditions, with NaH as a base and dry THF as a solvent. The reaction was carried out at 0 ^{o}C for 5 min and then the reaction mixture was slowly warmed to 50 ^{o}C for 2 h. After purification 80 % of the desired product 2-isopropyl-but-2-enedioic acid diethyl ester **9**[20] was obtained as a faint yellow oil. The product was fully characterized by ^{1}H-NMR, and ^{13}C-NMR. The olefinic proton at C-3 resonates at δ 5.8, whereas the coupling constant (*J*) of 1.17 Hz confirmed the *cis* configuration. Hydrolysis of diester **9** was carried out by using 2N LiOH in the presence of THF as a solvent. The reaction mixture was stirred for 8h at rt, then acidic workup gave crude 2-isopropyl-but-2-enedioic acid[21] **6** as a colourless oil in 96 % yield. Analysis of ^{1}H-HMR, and ^{13}C-NMR confirmed the structure of diacid. The crude diacid without further purification was treated with 10 eq of trifluoroacetic acid by overnight stirring at rt, to provide 3-isopropyl-furan-2,5-dione **5**[22] as a shiny blackish brown oil in quantitative yield. Disappearance of the protons of diacid in ^{1}H-NMR spectra confirmed the formation of an anhydride. Finally **5** was refluxed with 1 eq of *β*-alanine in the presence of AcOH[19,23] for 2 h. After ethyl acetate workup and column chromatography purification by using diethyl ether and hexane as eluent, **1** was obtained as a white solid in 60 % yield. The spectroscopic data, including ^{1}H-NMR, ^{13}C-NMR and MS Spectra of the synthetic farinomalein, matched with those reported in the literature for the natural compound, thus confirming its structure.

Scheme 2 Improved synthetic route



Reagents and conditions :

a) NaH, THF, 0 ^{o}C-50 ^{o}C, 1 h, 80%; b) 2N LiOH, THF, rt, 8h, 96%; c) TFAA, rt, overnight, quant; d) β-Alanine, AcOH, reflux, 1.5h, 60%

3.4 Synthesis of analogues

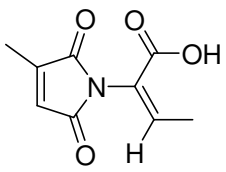
We scaled up the developed synthetic route up to 10 g batches, which appeared less expensive and non hazardous. Having the final product farinomalein in our hands, we prepared a number of analogues which can be useful for determination of structure-activity relationship (SAR) for these compounds. Also the optimised reaction conditions might be helpful for the modification of natural farinomalein into new synthetic fungicides.

In order to modify the natural farinomalein it was important to take an overview on the development of new natural compounds bearing maleimide rings.

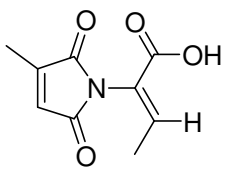
3.4.1 Natural compounds bearing maleimide rings.

3.4.2 Pencolide from Penicillium multicor

A new nitrogen-containg product, pencolide,[24] obtained from the culture fluid of a strain of *Penicillium multicolor* Grigorieva Manilova and Poradielova, grown on Raulin Thom medium, was reported by Birkinshaw[14]et al. in 1963 (**A**). The E configuration was subsequently assigned to the double bond on the basis of NMR arguments (**B**); however Olsen and collaborators have revised the geometry to Z in a recent publication. It was suggested (**A**) that biosynthetic precursor of pencolide might be citraconic acid and threonine or a related amino acid.



(A)



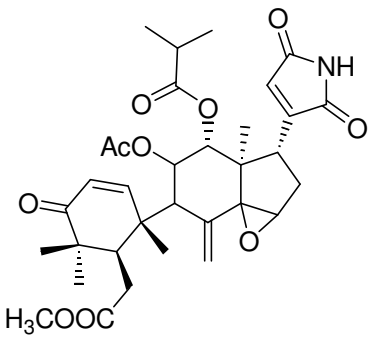
(B)

Fig. 2.1

Pencolide was first synthesized by Strungz and Ren[25] in 1975 from citraconic anhydride and threonine. Evidence is presented which corroborates the Z configuration.

3.4.3 Turrapubesin B

Turrapubesin B,[26] (fig 2.2), a maleimide bearing limonoid, was isolated from the twigs and leaves of *Turraea pubescens* by Yue and et al, in 2006. Limonoids are a class of highly oxygenated nortriterpenoids, either containing or derived from a precursor with a 4,4,8-trimethyl-17-furanylsteroid skeleton, and present a wide range of biological activities, such as insect antifeeding, antibacterial, antifungal, antiviral, antimalarial and anticancer properties. [27] The plants belonging to the families of Meliaceae and Rutaceae are rich sources of these fascinating metabolites. Previous studies on the genus of *Turraea* have afforded a series of protolimonoids and limonoids.[28] The plant material of *T. pubescens* has been used in the remedies of dysentery, pharyngolaryngitis, and traumatic hemorrhage[29]. Turrapubesin B is the first example of maleimide bearing limonoids in nature.

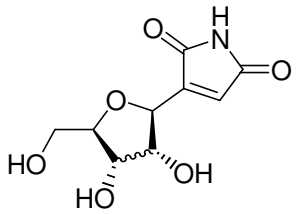


Turrapubesin B

Fig. 2.2

3.4.4 Showdomycin

Showdomycin[30] (fig. 2.3) is a broad spectrum antibiotic, first isolated from *Streptomyces showdoensis* by Nishimura and his coworkers[31], in 1964 (1). The antibiotic has been found to exhibit definite activity against Ehrlich ascites tumor in vivo and against cultured HeLa cells. The structure has now been elucidated as a new class of C-nucleoside. Showdomycin consist of a maleimide portion and a furanose group bearing two cis vicinal hydroxyl groups. Both the sugar and the base moieties are linked with C-C bond.



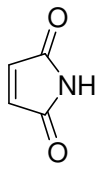
(1)

Showdomycin

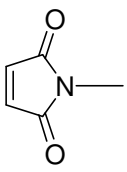
Fig. 2.3

3.4.5 *N*-methylmaleimide, *N*-ethylmaleimide and PhenylmaleimidePM 20.

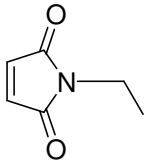
Maleimide, *N*-ethyl-maleimide (NME), and N-methyl-maleimide (NMM) (fig. 2.4) were identified as potent catalytic inhibitors of purified human topoisomerase IIα[32], whereas the ring analog succinimide was completely inactive. Catalytic inhibition was not abrogated by topoisomerase II mutations that totally abolish the effect of bisdioxopiperazine compounds on catalytic inhibition, suggesting a different mode of action by these maleimides. Furthermore, in DNA cleavage assay maleimide and NEM could antagonize etoposide-induced DNA double strand breaks. In a clonogenic assay maleimide antagonized the cytotoxicity of etoposide and daunorubicin on four different cell lines of human and murine origin. NMM and NEM have been extensively used for enzyme modification and labelling by reaction with the SH group of cysteine residues by Michael addition, thereby alkylating these.



maleimide



N-methyl-maleimide



N-ehtyl-maleimide

Fig .2. 4

In short, the knowledge of the structure, shape and physical properties of farnomalein and related compounds has allowed us to design the synthetic analogues in which fungicidal activity and stability could be improved.

3.5 Structure-Activity Relationship

To determine the structure activity relationship (SAR) in farinomalein , first we decided to vary the side chain. Natural farinomalein possesses a side chain most probably derived from *β*-alanine. To elucidate the role of side chain in the antifungal activity we have synthesized unnatural farinomalein analogues using different amino acid as side chain (Part A).

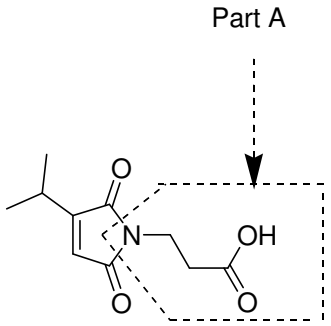


Fig .2. 5

Accordingly, we have chosen *L*-leucine, *L*-phenylalanine, *L*-valine and glycine acids to build the side chain. The common intermediate **5** was reacted with these commercially available amino acids to obtained analogues **10**, **11**, **12** and **13** with different alkyl chains on the maleimide skeleton (fig. 2.6).

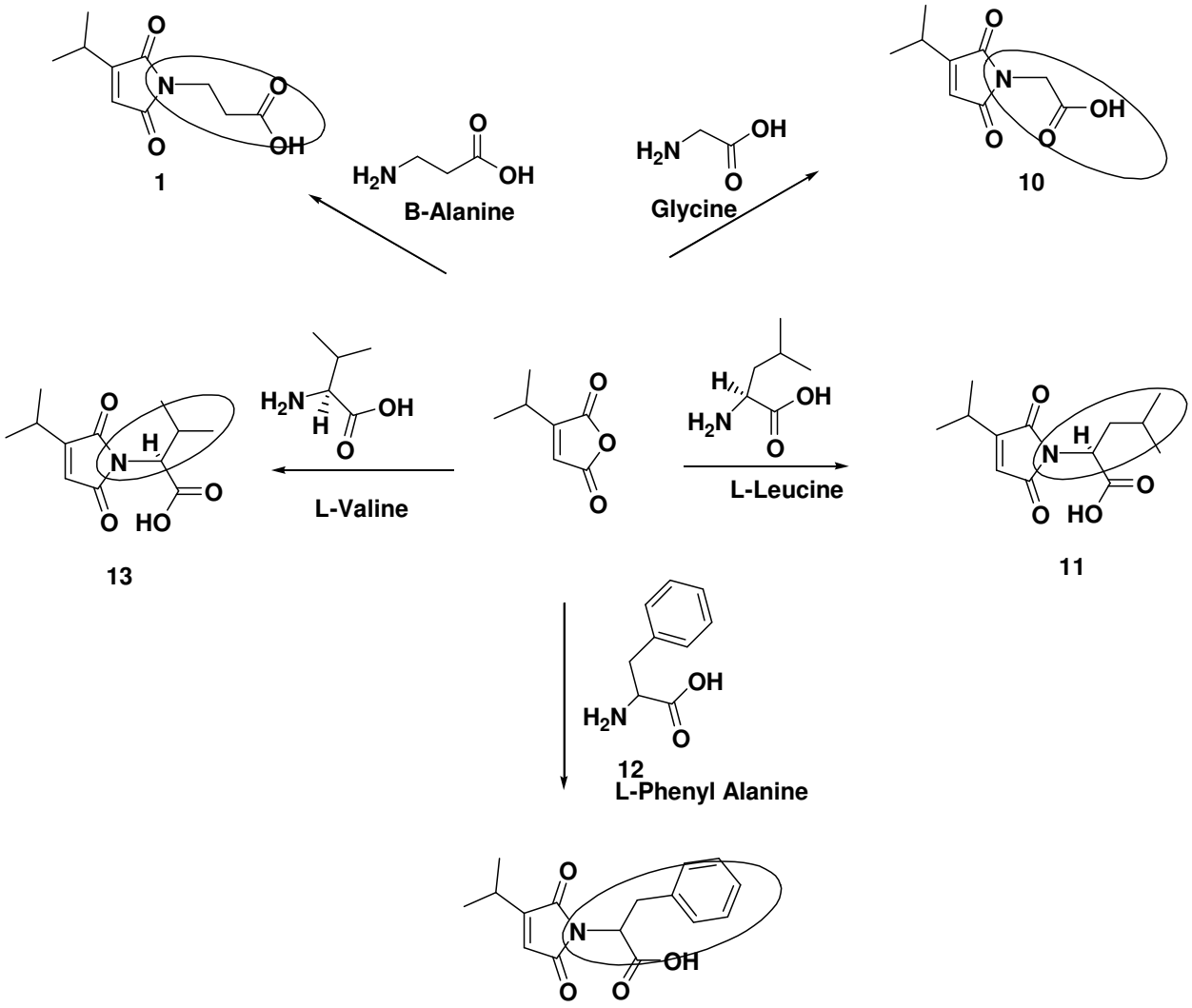
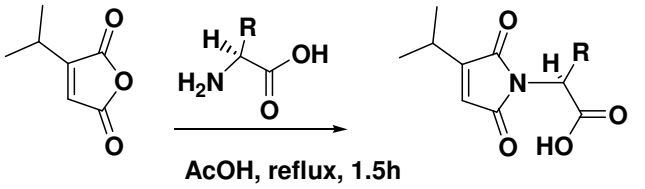

1
B-Alanine
Glycine
10
13
L-Valine
L-Leucine
11
12
L-Phenyl Alanine

Fig . 2. 6

3.6 Synthesis of analogues

For the synthesis of compound **10**, we used the synthetic route already described in scheme 2. The common intermediate **5** was treated with glycine using AcOH and refluxed for 1.5 h, to give (3-Isopropyl-2,5-dioxo-2,5-dihydropyrrol-1-yl)-acetic acid **(10)** in 57 % yield (Scheme 3). Similarly other analogues, 2-(3-Isopropyl-2,5-dioxo-2,5-dihydropyrrol-1-yl)-3-methyl-butyric acid (**13),** 2-(3-Isopropyl-2,5-dioxo-2,5-dihydro-pyrrol-1-yl)-4-methyl-pentanoic acid **(11),** 2-(3-Isopropyl-2,5-dioxo-2,5-dihydropyrrol-1-yl)-3-phenyl-propionic acid **(12)**, were synthesized by using *L*-valine, *L*-leucine, and *L*-phenylalanine respectively in 55 -60 % yields (Scheme3)

Scheme 3



5

10 R = H
13 R = $CH(CH_3)_2$
11 R = $CH_2CH(CH_3)_2$
12 R = CH_2Ph

3.7 Evaluation of Fungicidal Activity

3.7.1 Bioassay evaluation

As the activity of Farinomalein against *Phytophthora sojae* is already documented in the literature[1], we decided to assess the activity of farinomalein against *Cladosporium cladosporioides*, a fungal plant pathogen that affects wheat, using the method of bioautography.[33,34] (Table 2.1).

For the antifungal assay 10.0 μL of solutions corresponding to 50.0, 40.0, 30., 20.0, 10.0, 5.0, 1.0, 0.5, and 0.1 μg were applied to precoated Si gel TLC plates. The chromatograms were sprayed with a spore suspension of *Cladosporium cladosporioides* in CZAPEK broth and incubated for 72h in darkness in a moistened chamber at 25 ^{o}C, following a previously reported procedure.[34,35] Fungal growth inhibition appeared as clear zones against a dark background (fig 8), indicating the minimum amount of compounds required for it (table). Prochloraz was used as control.

3.7.2 Results and discussion

Antifungal activities of compounds **1**, **10**, **11**, **12**, and **13**, were evaluated against *Cladosporium cladosporioides*.

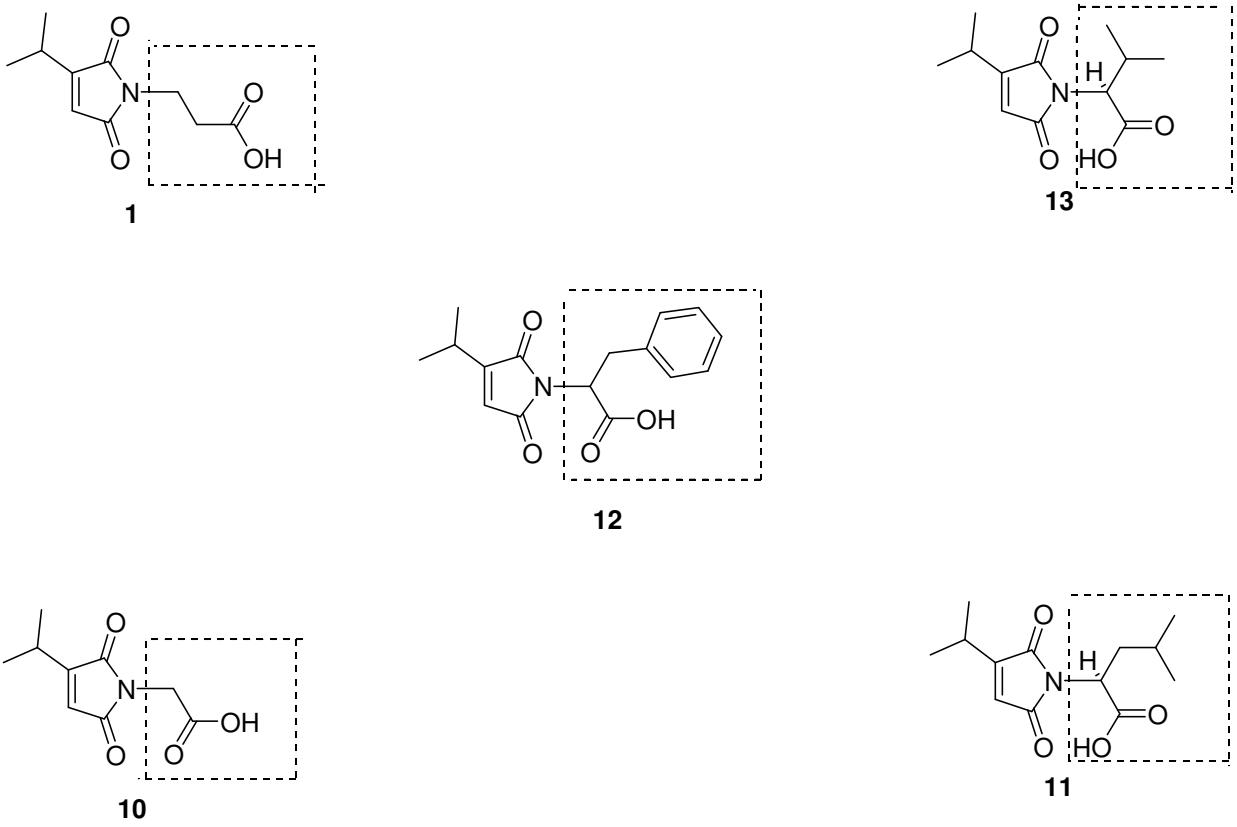


Fig . 2.7

Fig. 2.7 Farinomalein and analogues

The antifungal test result of all the tested compounds and their structural characteristics clearly offered a structure-activity relationship which is summarized as follows.(table 2.1)

1. The bioassay results confirmed that Farinomalein is endowed with antifungal activity also on *Cladosporium cladosporioides* and likely on other plant pathogens.
2. The minimum amount required for the inhibition of fungal growth on thin-layer chromatography plates was 5µg.

3. Moreover, the results clearly indicate that the introduction of different chains was detrimental for activity. In fact, the minimum amount of compounds **10**, **13**, **12** required for fungal growth inhibition was 6-10 fold higher than for **1** (30-50 µg). Branched amino acid are not crucial for antifungal activity.
4. The compound **11**, the analogue with *L*-leucine, had a moderate activity at 20 µg, showing that increasing the no. of carbons in the side chain may give good activity.

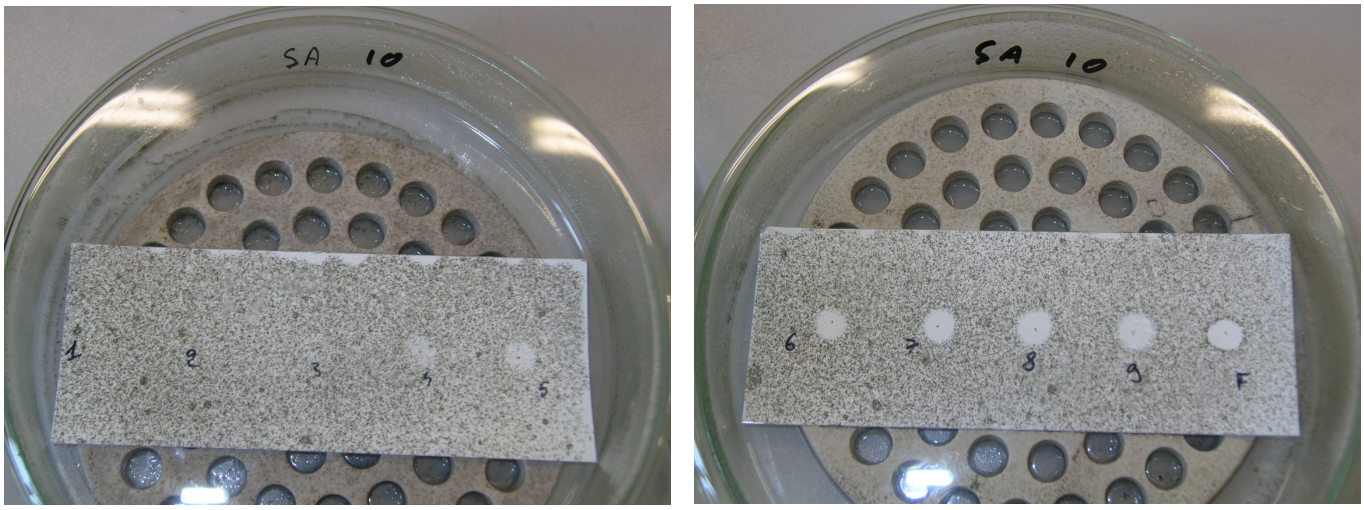


Fig . 2.8 Fungal growth inhibition as clear zones.

Table 2.1. Antifungal activity of Farinomalein and analogues

Compound	Antifungal activity (μg)[a] *C. cladosporioides*
1	5
10	50
12	50
11	20
13	50
Prochloraz	0.1

[a] Minimum amount required for the inhibition of fungal growth on thin-layer chromatography plates (TLC)

3.8 Conclusion

A practical and convenient synthesis of the fungicidal natural compound Farinomalein was described starting from readily available ethyl 3-methyl-2-oxobutyrate and triethyl phosphonoacetate, employing a Horner-Wadsworth-Emmons condensation as the key step. The antifungal activities of a series of analogues of farinomalein were evaluated against *Cladosporium cladosporioides*. Changing the linear side chain into a branched one are not crucial for antifungal activity, but increasing the no. of side chain carbon may be effective. Taking into consideration the result of this study, new synthetic analogues can be designed and synthesized for improving the antifungal activity of farinomalein.

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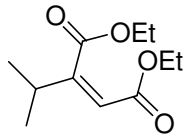
3.9 Experimental

2-Isopropyl-but-2-enedioic acid diethyl ester (9)

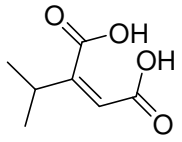


9

To a stirred solution of NaH (60 %, 0.138 g 3.4 mmole 1eq) in THF (5 mL), a solution of triethyl phosphonoacetate **8** (0.77 g 3.4 mmol, 1eq) in THF(15.5 mL) was added at 0 ^{o}C, stirred for 30 min at 0 ^{o}C. To this reaction mixture ethyl 3-methyl-2-oxobutyrate **7** (0.51 ml 3.4 mmole) was added at 0 ^{o}C and stirred for 5 min at 0 ^{o}C, then the reaction mixture was slowly warmed to 50 ^{o}C for 2 h. Saturated aqueous NH_4Cl (10 mL) was added and the aqueous phase was extracted with diethyl ether (3 x 30 mL). The combined organic layers were dried (Na_2SO_4), filtered, and concentrated. The residue was purified by flash column chromatography (7 % EtOAc in hexane), to afford **9** (0.590 g 79.%) as a faint yellow oil. R*f* = 0.42 (EtOAc / hexane 7:93),

^{1}H NMR (300 MHz, $CDCl_3$) δ: 5.80 (d, J = 1.2 Hz, 1H), 4.12 (q, J = 7.2 Hz, 2H), 4.31 (q, J = 7.2 Hz, 2H), 2.65 (m, 1H), 1.32 (t, J = 7.2 Hz, 3H), 1.25 (t, J = 7.2 Hz, 3H), 1.15 (d, J = 7.0 Hz, 6H).

2-Isopropyl-but-2-enedioic acid (6)

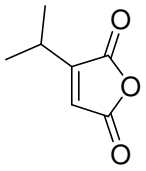


6

To the stirred solution of **9** (0.405 g, 1.8 mmole) in THF (21 mL) was added 2N LiOH (16 eq, 0.634 g in 7 mL H_2O) at 0 ^oC, then the reaction mixture was stirred for 8 h at RT. THF was removed under vacuum, the aqueous reaction mixture was acidified with 1N HCl (~30 mL) at 0 ^oC. The aqueous phase was extracted with ethyl acetate (3 x 40 mL). The combined organic layers were dried (Na_2SO_4), filtered, and concentrated, to obtain **6** as a colourless oil (0.286 g, 96 %). This crude product was used for the next reaction. R*f* = 0.43 (EtOAc / hexane 50:50),

^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.60 (brs, 2H), 5.85 (s, 1H), 2.65-2.80 (m, 1H), 1.20 (d, J = 7.0 Hz, 6H).

3-Isopropyl-furan-2,5-dione (5)

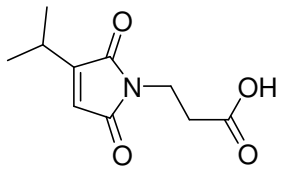


5

In trifluoroacetic anhydride (2.56 mL, 10 eq), **6** (0.289 g) was stirred overnight at RT. The resulting solution was evaporated under vacuum to provide **5** as a blackish brown oil (0.245 g, 98 %), R*f* = 0.34 (EtOAc/hexane 7:93). The crude product was used for the next reaction.

^{1}H NMR (300 MHz, $CDCl_3$) δ 6.51 (s, 1H), 2.80-3.00 (m, 1H), 1.2 (d, J = 7.0 Hz, 6H).

3-(3-Isopropyl-2,5-dioxo-2,5-dihydro-pyrrol-1-yl)-propionic acid (1)



1

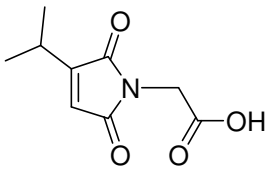
To the stirred solution of **5** (0.100 g 0.71 mmol) in AcOH (0.5 mL) β-alanine (0.063 g, 0.71 mmol) was added at room temperature. The reaction mixture was refluxed for 2 h, then cooled to 60 ^{o}C and the solvent was evaporated under vacuum. To the crude product EtOAc (10 mL) was added and the organic layer was washed with 2N HCl (5 mL) and brine, then dried over Na_2SO_4 and concentrated. Purification by flash column chromatography afforded **1** as a white solid (0.089 g, 60 %). m.p. 76 °C, Rf = 0.36 (diethyl ether/hexane 50:50).

^{1}H NMR (300 MHz, $CDCl_3$) δ: 6.25 (d, J = 1.8 Hz, 1H), 3.80 (t, J = 7.0 Hz, 2H), 2.80-2.95 (m, 1H), 2.70 (t, J = 7.0 Hz, 2H), 1.21 (d, J = 6.9 Hz, 6H).

^{13}C NMR (CD_3OD)) δ: 173.2, 171.0, 170.8, 155.7, 124.5, 33.3, 32.2, 25.6, 19.7.

Anal. Calcd for $C_{10}H_{13}NO_4$: C 56.86; H 6.20; N 6.63. Found: C 56.98; H 6.10; N 6.51.

(3-Isopropyl-2,5-dioxo-2,5-dihydro-pyrrol-1-yl)-acetic acid (10)



10

To the stirred solution of **5** (0.050 g 0.35 mmol) in AcOH (0.5 mL) glycine (0.026 g, 0.35 mmol) was added at room temperature. The reaction mixture was refluxed for 2 h, then cooled to 60 ^{o}C and the solvent was evaporated under vacuum. To the crude product EtOAc (10 mL) was added and the organic layer was washed with 2N HCl (5 mL) and brine, then dried over Na_2SO_4 and concentrated. Purification by flash column chromatography afforded **10** as a oil.

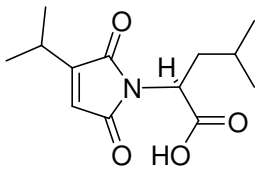
Yield 57 %, R*f* = 0.4 (diethyl ether/hexane 50:50).

^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.80 (brs, 1H), 6.30 (s, 1H), 4.28 (s, 2H), 2.75-2.95 (m, 1H), 1.20 (J = 7.0 Hz, 6H)

^{13}C NMR (75 MHz, $CDCl_3$) δ: 172.5, 170.3, 169.9, 156.5, 125.0, 38.3, 26.0, 20.8.

Anal. Calcd for $C_9H_{11}NO_4$: C 54.82; H 5.62; N 7.10. Found: C 55.01; H 5.41; N 7.01.

2*S*-(3-Isopropyl-2,5-dioxo-2,5-dihydro-pyrrol-1-yl)-4-methyl-pentanoic acid (11)



11

To the stirred solution of **5** (0.100 g 0.71 mmol) in AcOH (0.5 mL) *L*-leucine (0.093 g, 0.71 mmol) was added at room temperature. The reaction mixture was refluxed for 2 h, then cooled to 60 ^{o}C and the solvent was evaporated under vacuum. To the crude product EtOAc (10 mL) was added and the organic layer was washed with 2N HCl (5 mL) and brine, then dried over Na_2SO_4 and concentrated. Purification by flash column chromatography afforded **11** as an oil.

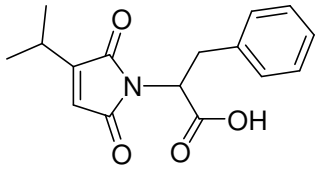
Yield 53 %, R*f* = 0.3 (diethyl ether/hexane 50:50).

^{1}H NMR (300 MHz, $CDCl_3$) δ: 6.28 (s, 1H), 4.75 (dd, J = 4.6, 11.3 Hz, 1H), 2.80-2.95 (m, 1H), 2.20-2.32 (m, 1H), 1.80-1.95 (m, 1H), 1.35-1.50 (m, 1H), 1.20 (d, J = 7.0 Hz, 6H), 0.90 (d, J = 7.0 Hz, 6H).

^{13}C NMR (75 MHz, $CDCl_3$) δ: 175.9, 170.6, 170.3, 156.1, 124.9, 50.3, 37.0, 25.9, 25.2, 23.1, 21.0, 20.8 (x 2).

Anal. Calcd for $C_{13}H_{19}NO_4$: C 61.64; H 7.56; N 5.53. Found: C 61.39; H 7.87; N 5.31.

2-(3-Isopropyl-2,5-dioxo-2,5-dihydro-pyrrol-1-yl)-3-phenyl-propionic acid (12)



12

To a stirred solution of **5** (0.05 g 0.35 mmol) in AcOH (0.5 mL) *L*-phenylalanine (0.06 g, 0.35 mmol) was added at room temperature. The reaction mixture was refluxed for 2 h, then cooled to 60 ^{o}C and the solvent was evaporated under vacuum. To the crude product EtOAc (10 mL) was added and the organic layer was washed with 2N HCl (5 mL) and brine, then dried over Na_2SO_4 and concentrated. Purification by flash column chromatography afforded **12** as an oil.

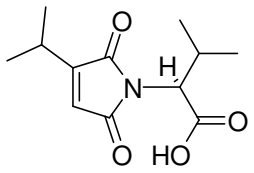
Yield 64 %**,** R*f* = 0.6 (diethyl ether/hexane 50:50).

^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.10-7.30 (m, 5H), 6.12 (s, 1H), 4.98 (dd, J = 6.3, 10.5 Hz,1H), 3.40-3.55 (m, 2H), 2.68-2.80 (m, 1H), 1.18 (d, J = 7.0 Hz, 3H), 1.09 (d, J = 7.0 Hz, 3H).

^{13}C NMR (75 MHz, $CDCl_3$) δ: 174.5, 170.2, 169.9, 155.9, 136.5, 129.0 (x 2), 128.7 (x 2), 127.0, 124.5, 52.9, 34.4, 25.8, 20.7.

Anal. Calcd for $C_{16}H_{17}NO_4$: C 66.89; H 5.96; N. 4.88. Found: C 66.55; H 6.12; N. 4.65.

2-(3-Isopropyl-2,5-dioxo-2,5-dihydropyrrol-1-yl)-3-methyl-butyric acid (13)



13

To the stirred solution of **5** (0.05 g 0.35 mmol) in AcOH (0.5 mL) *L*-valine (0.041 g, 0.35 mmol) was added at room temperature. The reaction mixture was refluxed for 2 h, then cooled to 60 ^{o}C and the solvent was evaporated under vacuum. To the crude product EtOAc (10 mL) was added and the organic layer was washed with 2N HCl (5 mL) and brine, then dried over Na_2SO_4 and concentrated. Purification by flash column chromatography afforded **13** as an oil.

Yield 41 %, *Rf* = 0.5 (diethyl ether/hexane 50:50).

^{1}H NMR (300 MHz, $CDCl_3$) δ: 9.00 (brs, 1H), 6.25 (s, 1H), 4.40 (d, J = 8.4 Hz, 1H), 2.80-2.95 (m, 1H), 2.60-2.75 (m, 1H), 1.20 (d, J = 7.0 Hz, 6H), 1.10 (d, J = 7.0 Hz, 3H), 0.85 (d, J = 7.0 Hz, 3H).

^{13}C NMR (75 MHz, $CDCl_3$) δ: 174.3, 170.6, 170.3, 156.0, 124.7, 57.6, 28.4, 25.9, 20.8 (x3), 19.5. Anal. Calcd for $C_{12}H_{17}NO_4$: C 60.24; H 7.16; N 5.85. Found: C 60.66; H 7.00; N 5.57.

3.10 References

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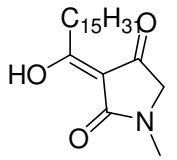
CHAPTER 4

Epicoccamide D

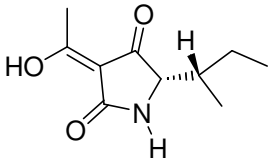
4.1 Introduction

3-Acyltetramic acids[1,2] (3-acylpyrrolidine-2,4-diones) are core structural skeletons that have been found in various naturally occurring biologically active products. In recent years, the chemistry of tetramates has experienced a renaissance instigated by a steadily increasing number of isolated natural products of this type with distinct biological activities.[5] The structurally complex[6] 3-acyltetramic acids are among the most commonly found biologically active products such as magnesidein A (antibiotic activity),[7a] reutericyclin (antibiotic activity),[7b] tenuazonic acid (antiviral and antitumor activity),[7c] Sch 213766 (anti-HIV activity),[7d] and the melophins (antitumor),[7e] and the macrocidins (herbicidal activity)[7f] (Fig.1)

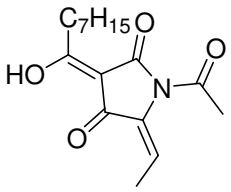
Fig 1. Examples of naturally occurring 3-acyltetramic acids.



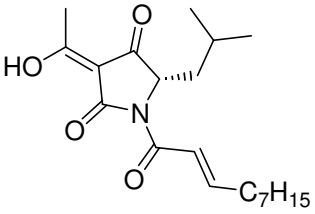
Melophin A



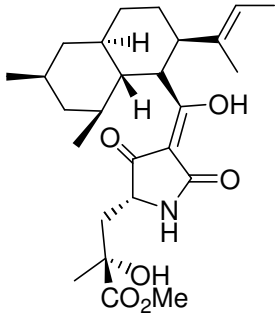
Tenuazonic acid



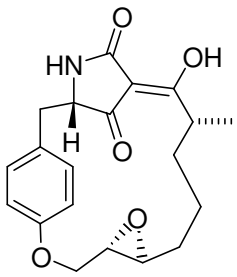
Magnesidin A



Reutericyclin

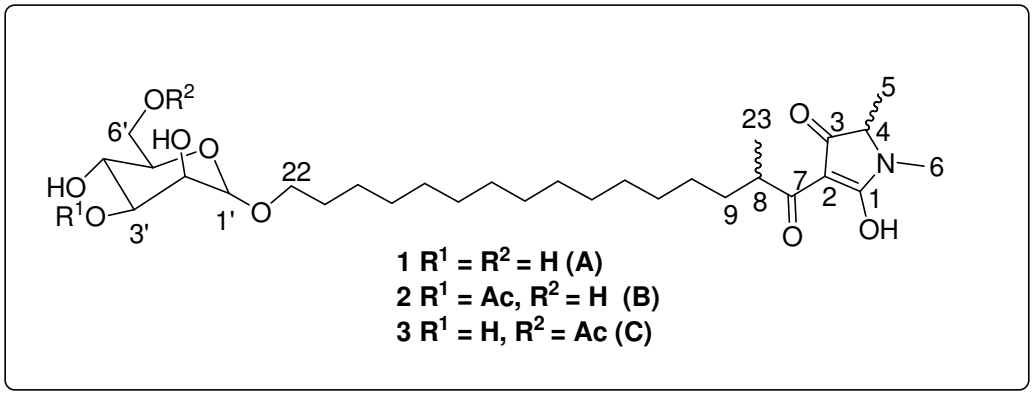


Sch 213766

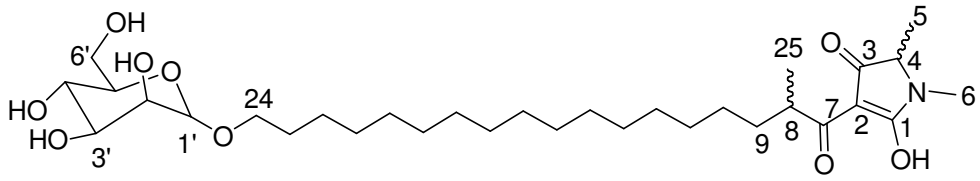


Macrocidin A

Three new tetramic acid derivatives, epicoccamides B-D, have recently been isolated from an *Epicoccum sp*. associated with the tree fungus *Pholiota squarrosa*, in 2007 by Hertweck et al.[3] In 2003 Konig[4] and co-workers isolated epicoccamide (**A**) from the inner tissue of the jellyfish *Aurelia aurita,* a marine strain of the fungus *Epicoccum purpurascens.*



1 $R^1 = R^2 = H$ (A)
2 R^1 = Ac, R^2 = H (B)
3 R^1 = H, R^2 = Ac (C)



4 (D)

Epicoccamides are quite unusual since they are composed of three biosynthetically distinct subunits; glycosidic, fatty acid and tetramic acid. The structures of the new compounds were elucidated on the basis of their physical data and chemical degradation. Epicoccamides B-D, differ in substitution pattern and in the size of the central carbon chain. The main metabolite "epicoccamide A" (**1**) proved to be identical with an unusual tetramic acid glycoside.[4] The ^{1}H-NMR spectrum of **2** was similar to that of **1**, except for the presence of an additional singlet at δ 2.16 (3H, s, H-8'). Also in the ^{13}C NMR spectrum additional resonances were observed at δ 21.0 (CH3) and 171.2 (C=O), suggesting that **2** is an *O*-acetyl derivative of **1**. The sugar moiety was identified as β-mannose with D configuration. Thus, **2** was identified as 5-hydroxy-1,2-dimethyl-4-[2-methyl-16-(3-*O*-acetyl-β-D-mannopyranosyl)hexadecanoyl]-1*H* –pyrrol-3(2H)-one (epicoccamide B). Its structure was confirmed by MS/MS data, which showed the cleavage of the acyl moiety followed by dehydration. The molecular formula of Epicoccamide C (**3)** was determined by HREIMS, indicating that **3** is an isomer of **2**. Its ^{1}H NMR and ^{13}C NMR spectra were almost identical with those of **2**. The structure of **3** was elucidated as 5-hydroxy-1,2-dimethyl-4-[2-methyl-16-(6-*O*-acetyl-β-D-mannopyranosyl)hexadecanoyl]-1*H*–pyrrol-3(2H)-one by HMBC. The UV spectrum of

epicoccamide D (compound **4)** as well as the MS/MS fragmentation patterns suggested that **4** is a homologue of **1** with a mass difference of 28 units. Two ion fragments at m/z = 422.2 and 125.9 indicated the loss of the sugar moiety and that the aliphatic chain consists of two additional methylene groups, a result supported by NMR data. The structure of **4** was thus elucidated as 5-hydroxy-1,2-dimethyl-4-[2-methyl-18-β-D-mannopyranosyloctadecanoyl]-1*H*–pyrrol-3(2H)-one.

All the epicoccamides have two stereogenic centres in the aglycone portion of the molecule, but the absolute configuration at C-8 and C-4 could not be solved so far.

4.2 Biological activity

Microorganisms that live in symbiosis have proven to be a rich source of biologically active natural products.[8] There is growing evidence that many metabolites isolated form plant and animals are in fact produced by microorganisms.[9] The crude extract of *Epicoccum* sp. exhibited notable antiproliferative and cytotoxic activities, then all four epicoccamides were individually probed. Among these four compounds the derivative with the longest chain, epicoccamide D proved to be the most active . It exhibits weak to moderate cytotoxicity towards HeLa a cell lines (CC_{50} 17.0µM) and good antiproliferative effects on mouse fibroblast (L-929) and human leukaemia cell lines (K-562) with inhibition of growth (GI_{50}) of 50.5 and 33.3µM, respectively. Furthermore, epicoccamide D induces morphogenesis and pigment formation in surface cultures of the fungus *Phoma destructive* at a concentration of 1.7 mM.

4.3 Synthesis of Epicoccamide D (4)

Compound **4** has an intriguing structure, which includes a tetramic acid group in its *N*-methylalanine derived heterocycle. Numerous biologically active natural products contain the tetramic acid (pyrrolidin-2, 4-dione) ring system. The range of biological activities possessed by

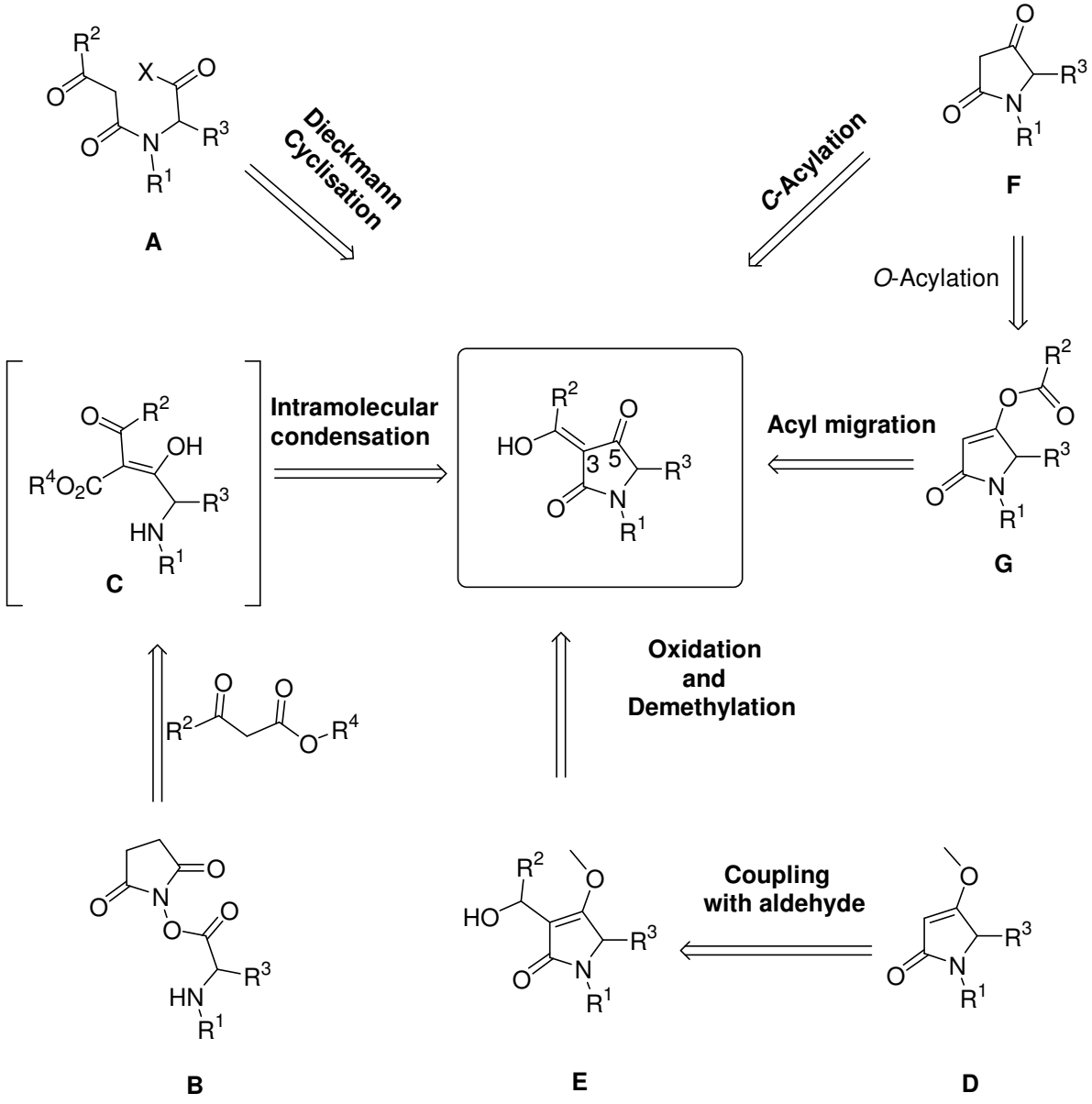
individual members of this series includes potent antibiotic, antiviral, and antiulcerative properties, cytotoxicity and mycotoxicity, the inhibition of tumors as well as fungicidal action . Moreover, certain members of this class are responsible for pigmentation of some sponges and molds.[1,10] The structural complexity, together with potent biological activity, of many tetramic acids makes the total synthesis of these compounds an attractive goal for organic chemists. The epicoccamide D has significant antifungal and plant growth promotion effect, which makes it a potential lead for the design of new antifungal. Herein, we describe the efforts towards the first total synthesis of epicoccamide D (**4**).

4.4 Reported synthetic methodologies for 3-acyltetramic acids.

Given the biological importance of this motif, various synthetics methodologies for 3-acyltetramic acids[1,11,12] as well as tetramic acids themselves[13] have been developed, and there are two main routes for their synthesis (fig 2). One is to incorporate the acyl group (R^2) before the construction of the tetramic acid core,[12-16] and the most common approach is by Dieckmann-type cyclization of β-keto amide **A** under basic conditions.[12-15] However, even though such cyclization, including solid phase protocols,[16] has been widely applied for the synthesis of natural[12-14] and unnatural[15] analogues, only *N*-unsubstituted and *N*-alkyl 3-acyltetramic acid (R^1 = H, alkyl) have been prepared in this way, but critically not the *N*-acyl system found in magnesidin **A** and reutericyclin (R^1 = acyl). This is likely to be due to the difficulty of accessing the required *N*-diacyl precursor **A** (R^1 = acyl). Importantly, the Markopoulou group has elegantly circumvented this difficulty to some extent by *in situ C*-acylation of β-keto esters and malonates with activated ester **B** to give precursors **C** followed by intramolecular condensation under basic condition, which established access not only to *N*-substitued and N-methyl but also some *N*-acyl and *N*-alkoxycarbonly 3-acyltetramic acids.[16]

The second alternative route is to incorporate the acyl group (R^2) after the construction of the tetramic acid main core.[17-19] In this matter, Jones' group has developed a methodology involving base mediated (n-BuLi) condensation of 4-*O*-methyl tetramic acid **D** with aldehydes (R^2CHO) to give hydroxyl adducts **E**, and oxidation followed by demethylation, under basic conditions to give the desired 3-acyltetramic acid.[17] This method was particularly efficient for 1,5,5-trisubstituted tetramates **D** but less substituted tetramates suffered from a side reaction caused by lithiation at both N(1) and C(5).[17b] More conveniently, direct acylation at the 3-position of tetramic acid **F** could be achieved with acid chlorides (R^2COCl) activated by Lewis acid catalysts such as $BF_3.OEt_2$ and $TiCl_4$,[18] although with 5-unsubstituted tetramic acids, self-condensation under these conditions has been reported.[18d] Finally, their synthesis via base-induced acyl migration of *O*-acyltetramic acids **G**, themselves readily prepared from tetramic acids **F** using Kech coupling conditions in the presence of *N*, *N*'-dicyclohexylcarbodiimide (DCC) with a catalytic amount of 4-dimethylaminopyridine (DMAP),[19a] has been reported; for example, the in situ or stepwise acyl-migration of *O*-acyltetramic acids **G** induced by triethylamine,[19b,c] triethylamine with hydroxybenzotriazole,[19d] or acetone cyanohydrin [19c] is possible. Schobert et al[19c] reported the synthesis of tetramic acid main core from α-aminoesters or their ammonium salts; cyclization is brought about by a domino addition-Wittig alkenylation reaction with the cumulated phosphorus ylide (triphenylphosphoranylidene)ketene, Ph_3PCCO, under neutral non-racemizing conditions.

Fig 2. Synthetic approaches to 3-acyltetramic acid



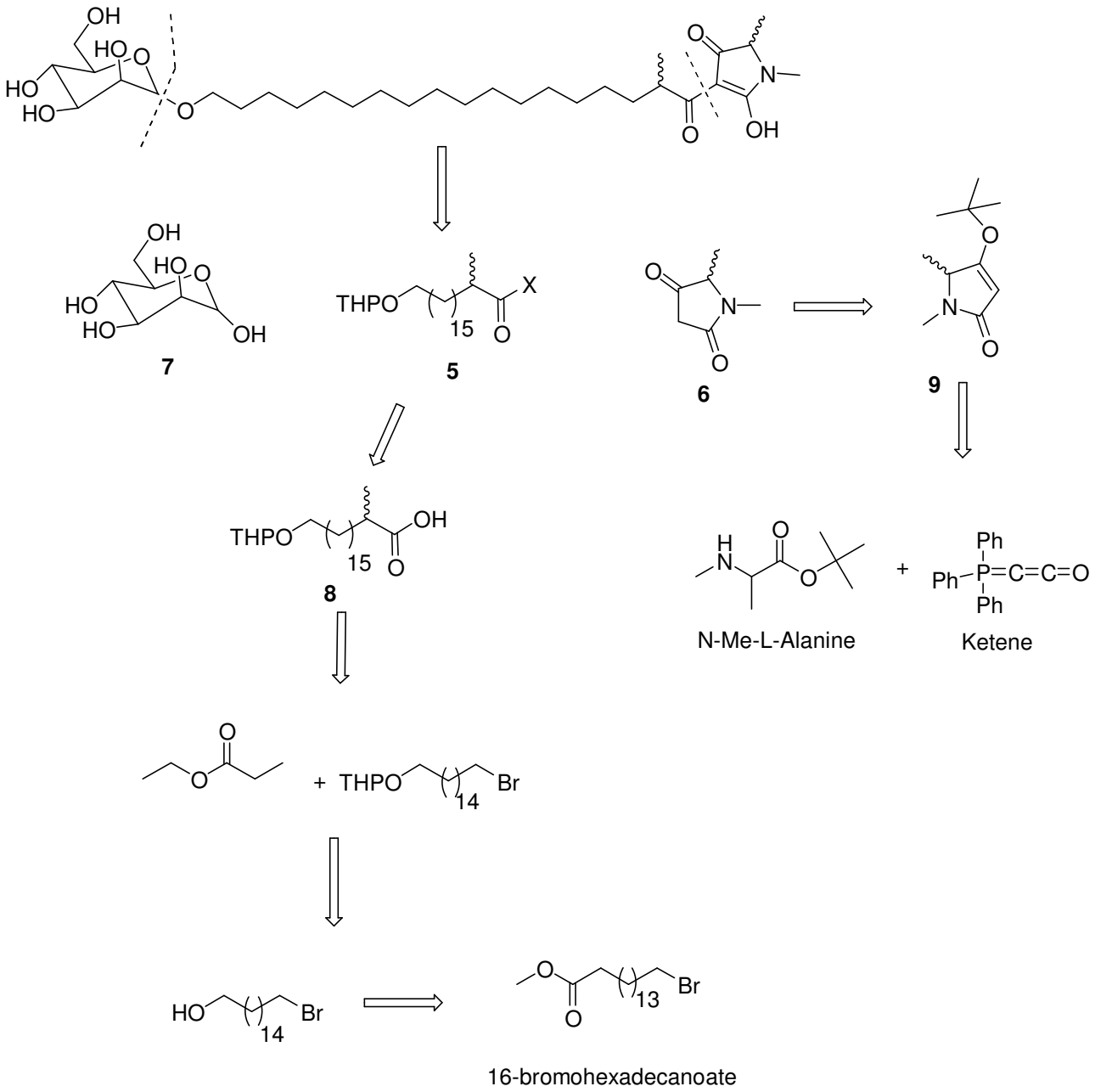
4.5 Convergent synthetic strategy

4.5a Retrosynthetic Approach

Our initial retrosynthetic analysis was based upon the construction of the acyltetramic nucleus. In the beginning we envisioned that epicoccamide D could be constructed by the convergent synthetic strategy which has been proposed by Schobert et.al.[19c] Our first strategy for the synthesis of **4** was based on the coupling of two domains: a side chain subunit **5** and N-methyl pyrrolidine-2,4-dione subunit **6** (Scheme 1).

We envisaged that the preparation of the subunit **5** could start from the *O*-protected long carbon chain acid **8** which could be easily obtained by manipulation of the commercially available methyl 16-bromohexadecanoate. Whereas the tetramic acid moiety, subunit **6,** could be obtained by Wittig alkenylation reaction between phosphorus ylide (triphenylphosphoranylidene)ketene, Ph_3PCCO and commercially available *N*-methyl *L*-alanine ammonium salt.

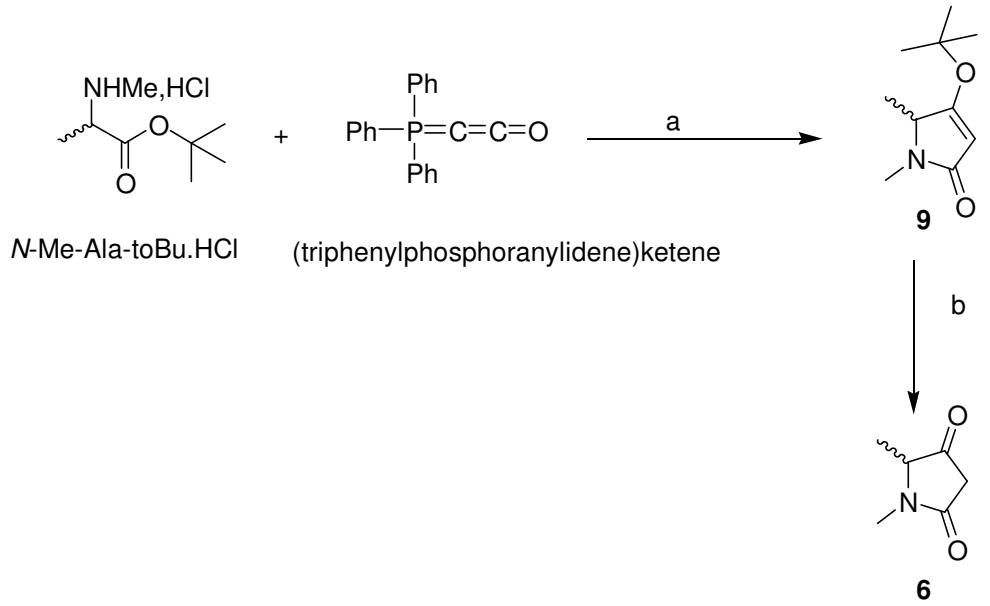
Scheme 1


OH
HO
HO
HO
O
O
N
OH
O
7
THPO
15
X
O
5
6
9
8
N-Me-L-Alanine
Ketene
Ph
P=C=C=O
+ THPO
Br
14
13
16-bromohexadecanoate

4.6 Synthesis of subunit 6

For the preparation of the tetramic acid main core, subunit **6** (Scheme 2), we used procedures described by Schobert et.al.[19c] In this synthetic path we carried out a microwave reaction of *N*-methyl L-alanine t-butyl ester, and Ph_3PCCO ketene in presence of THF as a solvent, for 30 min at 90 ^oC. After purification, 50 % of the intermediate 4-tert-butoxy-1,5-dimethyl-1,5-dihydropyrrol-2-one **9** was obtained as a white solid. The product was fully characterized by ^1H-NMR and ^{13}C-NMR. Cyclization is confirmed by the presence of an olefinic proton at δ 5.00 (s). This reaction suffers from a drawback, as it was difficult to remove the side product triphenylphosphane oxide ($Ph_3P=O$). After column chromatography the product was washed with diethyl ether to remove triphenylphosphane oxide. The detert-butoxylation of intermediate **9** was carried out by using trifluroaceticacid (TFA) as a dehydrating agent, to quantitatively yield 1,5-dimethyl-pyrrolidine-2,4-dione **6**. (Scheme 2)**.** The spectroscopic data, including ^1H NMR and ^{13}C NMR confirmed the structure. Disappearance of the protons of tert-butoxy group and appearance of a broad peak of keto-enol proton in 1H NMR confirmed the formation of a tetramic acid.

Scheme 2



N-Me-Ala-toBu.HCl (triphenylphosphoranylidene)ketene

Reagents and conditions :

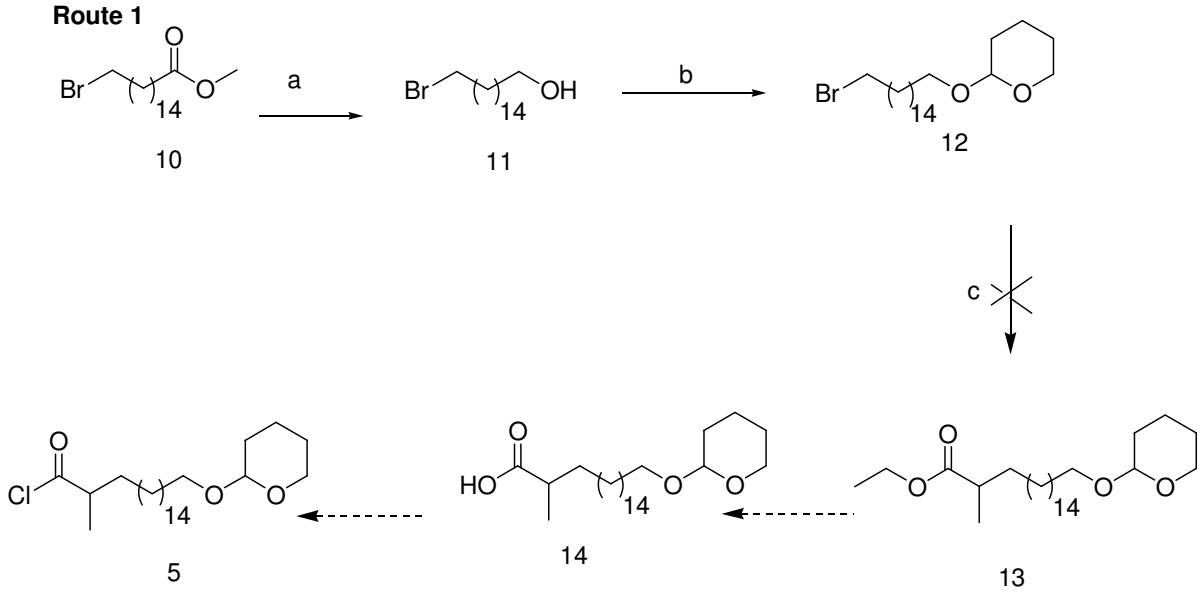
a) Microwave, THF, 120 ^{o}C, 30 min, 50 %; b) TFA, rt, 3 h, 99 %.

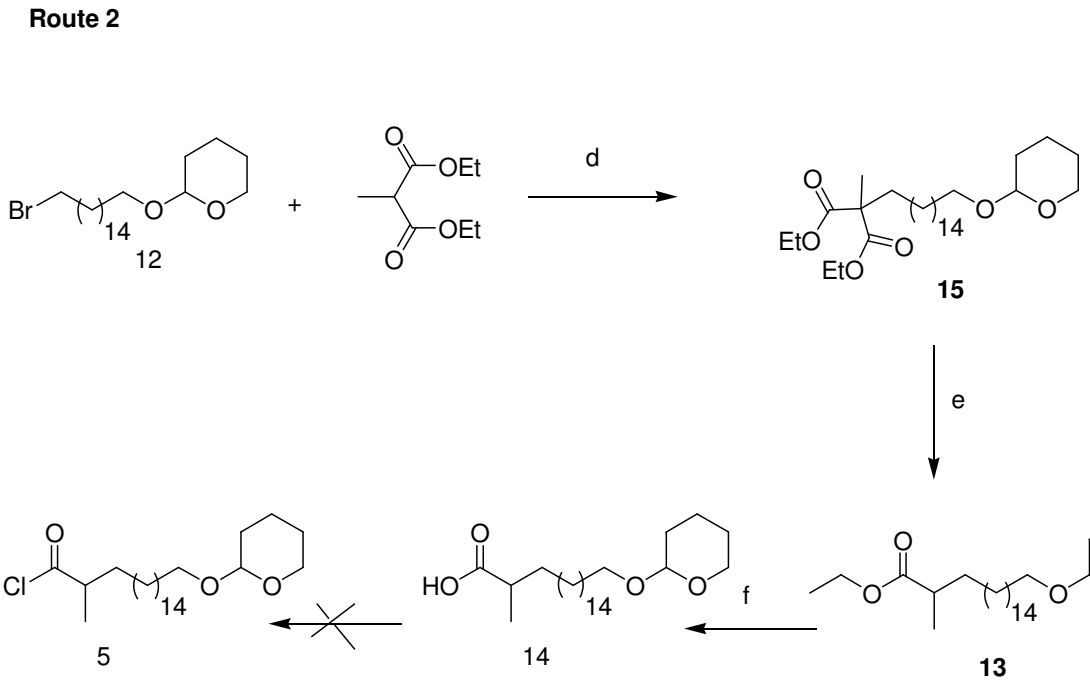
4.7 Synthesis of subunit 5

For the synthesis of fatty acid subunit **5**, we planned to use a commercially available and cheap 16-bromo hexadecanoate as a starting material**.** Synthesis of subunit **5** is outlined in scheme 3. Methyl 16-bromohexadecanoate **10** was successively reduced by 2M solution of lithium aluminium hydride in THF, at 0 ^{o}C in the presence of diethyl ether as a solvent, to give 16-bromo hexadecanol **11** in almost 85 % yield. The primary alcohol **11** was protected with tetrahydropyran, reaction was carried out at rt for 8 h by using 3,4-dihydro-2-H pyran and catalytic amount of PTSA in the presence of THF as a solvent, to obtain **12** in 90 % yield. The product was fully characterized by ^{1}H

NMR and ^{13}C NMR, where the protection of the primary alcohol was confirmed by the presence of the proton of tetrahydropyran at δ 4.50 (m). In order to have a methyl group on C-8 in epicoccamide D, at the beginning we used ethyl propionate to introduce the methyl group and LDA as a base following a literature procedure.[20] We carried out this reaction in all possible conditions using THF, HMPA as solvents but we were unable to obtain satisfactory results presumably due to unstable Li-enolate of ethyl propionate and solidification of **12** at cooling conditions (Scheme 3 route 1). Therefore, in the second approach we used a procedure described by Omura Satoshi[21] and co-workers, who used diethyl methylmalonate as a methyl source, with sodium hydride as a base and DMF as a solvent. The intermediate **15** was thus obtained in 85 % yield as an oil. The spectroscopic data, including ^{1}H-NMR and ^{13}C-NMR confirmed the structure. Appearance of a new peak at δ 4.10 (4H, q) indicated the presence of the methylene group of two ethyl ester group. Further one ethyl ester group was successively removed by Krapcho decarboxylation reaction, using 2eq of lithium chloride and DMSO as a solvent. We were able to obtain intermediate **13** in 50 % yield as an oil (Scheme 3, route 2). The product was fully characterized by ^{1}H-NMR and ^{13}C-NMR, where the decreased number of protons in ^{1}H-NMR at δ 4.10 (2H, q) confirmed the structure of **13**. Further hydrolysis of ester **13** was carried out by using 5eq of potassium hydroxide in ethanol-water at 80 ^{o}C for 5 h, to give after acidic workup **14** as a colourless oil in 80 % yield. Analysis of ^{1}H-HMR, and ^{13}C-NMR confirmed the structure. In the next step for conversion of acid **14** to acyl chloride **5** we used either thionyl chloride or oxalyl chloride, but unfortunately after several attempts we were not able to obtain acyl chloride **5**. Instead, we obtained a *O*-deprotected side product with a number of other impurities, presumably due to the use of acidic condition. So we were unable to use the acyl chloride **5** for further transformation in order to complete the total synthesis of epicoccamide. Work is still in progress to obtain the conversion of **14** to **5**.

Scheme 3





Reagents and conditions :

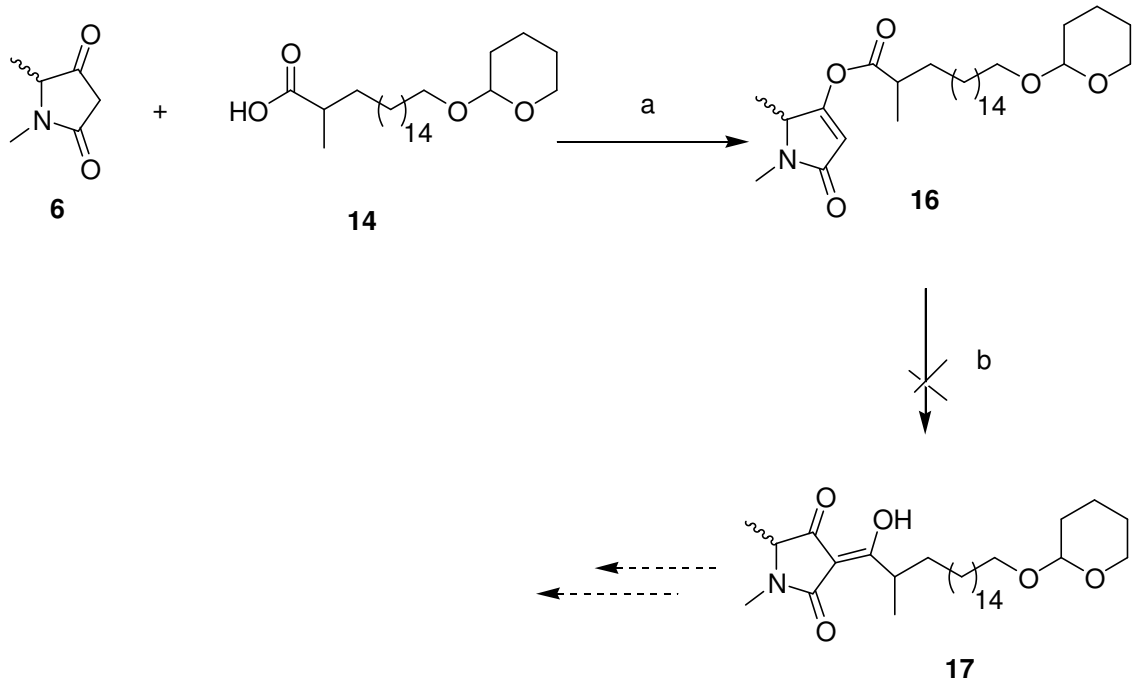
a) 2M LAH in THF, diethyl ether , 0^{o} C, 45 min, 85 %; b) 3,4-dihydro-2H-pyran, THF, PTSA, rt, 8 h, 90 %; c) 1.8M LDA, ethyl propionate, THF, -78^{o}C to -30 ^{o}C; d) NaH, Diethyl methylmalonate,

DMF, 0°C to rt, 4 h, 81 %; e) LiCl, DMSO, H_2O, reflux, 12 h, 55.5 %; f) KOH, EtOH, reflux, 5 h, 70 %.

Looking for a more direct approach, we decided to follow the base-induced acyl migration of *O*-acyltetramic acids **G** (fig 2), readily prepared from tetramic acids **F** using Keck coupling condition in the presence of *N*, *N*'-dicyclohexylcarbodiimide (DCC) with a catalytic amount of 4-dimethylaminopyridine (DMAP).[19a] Moreover, the in situ or stepwise acyl-migration of *O*-acyl tetramic acids **G** induced by triethylamine,[19b,c] triethylamine with hydroxybenzotriazole,[19d] or acetone cyanohydrin [19c] has been reported. Both the subunits **14** and **6** were in our hand. Coupling of the two halves of the molecule was achieved by Keck coupling. We carried our this reaction at rt for 5h in the presence of DCC with a catalytic amount (0.1 equiv) of DMAP.[19c] We obtained **16** (Scheme 4) in 40 % yield, and spectroscopic data including ^{1}H-NMR and ^{13}C-NMR confirmed the structure. The presence of an olefinic proton at δ 6.00 (1H, s) of C-2 confirmed the coupling. With the *O*-acyl derivative **16** in hand, Fries-type migration of the *O*-acyltetramic acid using a catalytic amount (0.5 eq.) of acetone cyanohydrin in the presence of triethylamine (2.0 eq) in acetonitrile, according to the previous reported[19e] was then tried. Unfortunately, by this method we were not able to obtain the migrated product **17,** thus we decided to carry out the procedure reported by Schobert.[19c] Using triethylamine as a base in DCM, after overnight stirring at rt we were able to see a new polar spot on TLC together with unreacted starting material. When for completion of the reaction we stirred the reaction mixture for another 12 h, after concentration of reaction mixture, the new polar spot on TLC had disappeared and converted to starting material. Therefore, for confirmation of this reversible reaction we decided to use other bases such as DIPEA, DMAP, but we observed the same reaction pattern. The yield with this method is strongly dependent on the identity of the carboxylic acid (R^2 group) but not on the nature of R^1 substituent at N(1) (fig 1), thus the reactions with aromatic and α-olefinic carboxylic acids are better.[22] This Fries-type

rearrangement approach has been widely used in tetronic acid synthesis,[24] but it has been much less widely applied in tetramates[23,19c] and in particular its suitability for *N*-acyltetramic acids has not been studied. Moreover, Hori[19b] indicates that although this reaction in a stepwise manner in one pot by formation of the kinetic *O*-acylated enol followed by acyl migration after the addition of triethylamine gives very high yields of 3-alkanoylation for *N*-unsubstituted and *N*-alkyl system, it is less efficient for unsubstituted acids. In fact, the effect of the R^1 and R^2 substituent's on this reaction sequence has not been studied in detail. Unfortunately, we were not able to obtain the migrated product **17** from **16** after several attempts using different base and reaction condition.

Scheme 4

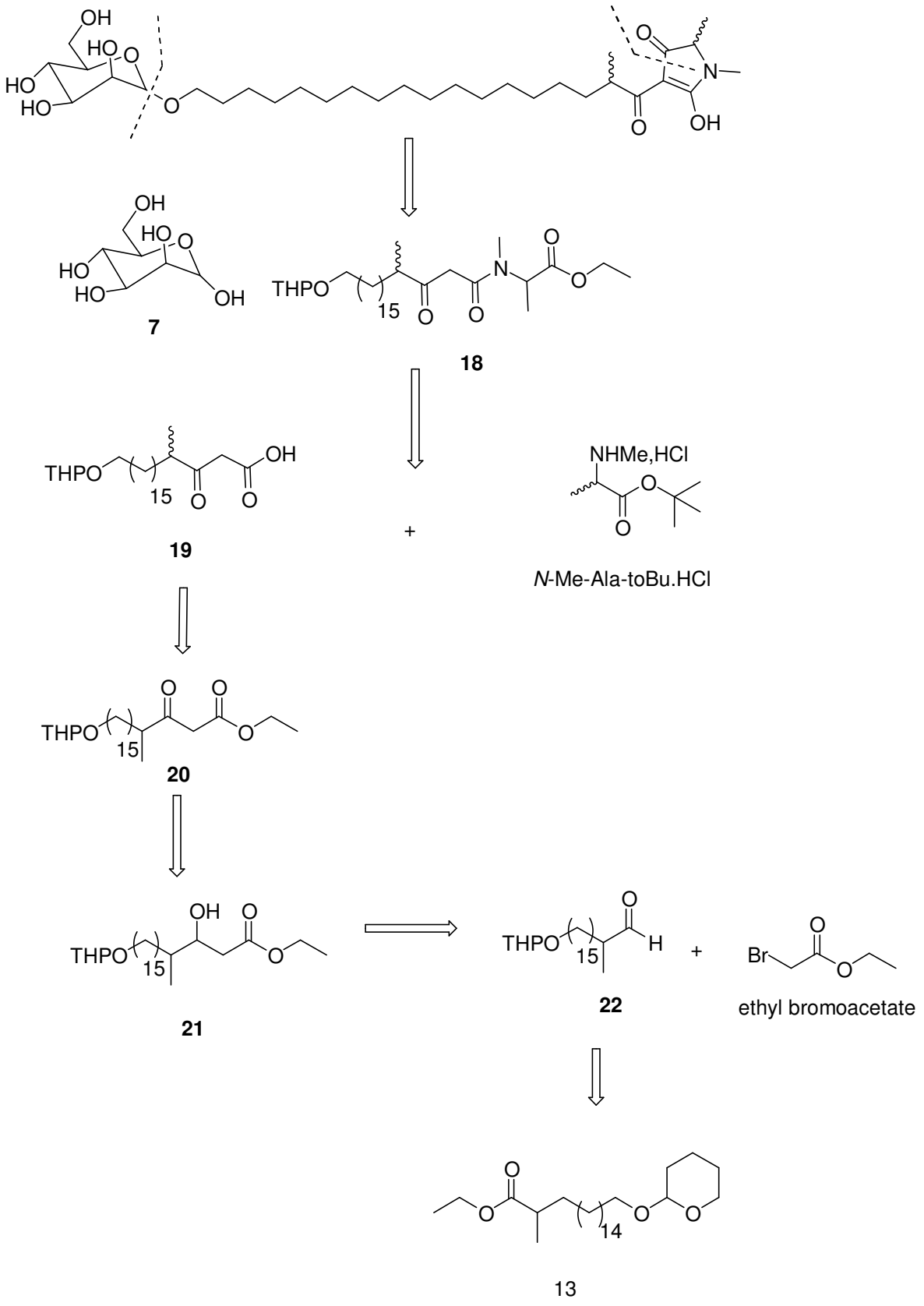


Reagents and conditions :

a) DCC, DMAP, DCM, 0 ^{o}C to rt , 5 h, 40 %; b) TEA, DCM.

As we were unable to obtain intermediate **17,** we decided to follow one of the synthetic approaches discussed in the "reported synthetic methodologies for 3-acyltetramic acids (Fig 1)". Epicoccamide D could be synthesized by a linear synthetic strategy, the most common approach of which is a Dieckmann-type cyclization of β-keto amide **A** (fig. 1) under basic conditions.[12-16] Considering the above fact, we revised the retrosynthesis again, as depicted in Scheme **4**. The disconnection identified fragment **18**, which could be constructed by acid-amine coupling of intermediate β-ketoacid **19** and *N*-methyl *L*-alanine t-butyl ester. The β-keto acid **19** can be obtained by hydrolysis of β-keto ester **20,** which could be constructed by oxidation of β-hydroxy ester **21**. On its turn **21** could be constructed by Zn-activated Reformatsky coupling of aldehydes **22** and ethyl bromoacetate.

Scheme 4


7
18
19
+
NHMe,HCl
N-Me-Ala-toBu.HCl
20
21
22
+
ethyl bromoacetate
13

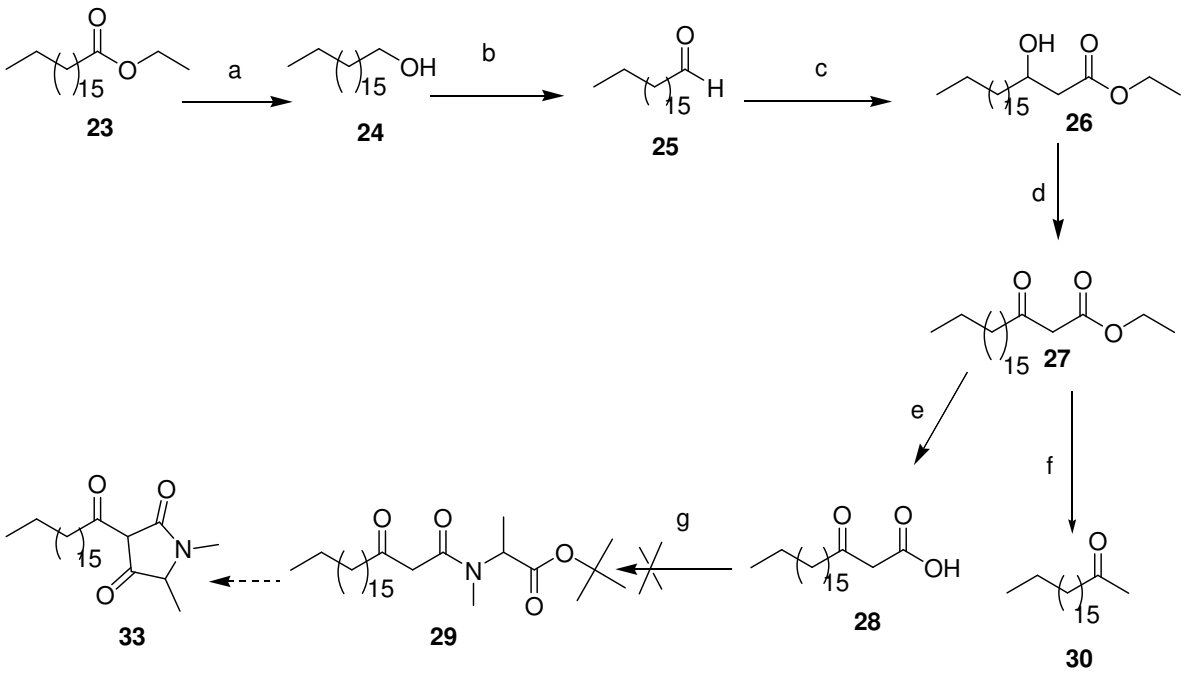
4.8 Liner synthetic strategy

To follow the linear synthetic strategy we decided to carry out the above reaction sequence with another substrate which structurally resembles **22** in order to set the reaction conditions and to avoid loss of aldehyde **22** (Scheme **5**). Considering the above facts we started with commercially available ester **23** which is a model for 16-bromohexadecanoate **10**. Reduction of ester **23** with 2M LAH in THF and diethyl ether as solvent for 1 h at 0 ^{o}C gave the hydroxy intermediate **24**, which was then oxidized by PCC in DCM after overnight stirring at rt to provide aldehyde **25**. All these products were purified by column chromatography and fully characterized by ^{1}H-NMR. Finally the condensation of aldehyde **25** with ethyl bromoacetate using metallic zinc[25] in presence of benzene as a solvent afforded β-hydroxyester **26**, whose structure was confirmed by spectroscopic data including ^{1}H-NMR. Further oxidation of **26** with PCC in DCM afforded β-keto-ester **27**. Further we wanted to hydrolyze ester **27** to β-keto-acid **28**, in order to couple it with *N*-methyl L-alanine t-butyl ester. For this hydrolysis we tried KOH and LiOH and other reaction conditions and solvents but unfortunately we were not able to obtain intermediate **28**. Conversely, the decarboxylated intermediate **30** was obtained. After several attempts, we used AcOH and few drops of HCl for hydrolysis of **27**. After 48 h stirring at rt and workup with diethyl ether we were able to obtain β-keto-acid **28,** structurally confirmed by ^{1}H NMR. This result indicated that some changes had to be made in the synthetic strategy, as in the desired compound we have the acid sensitive OTHP group and we cannot use such strong acidic condition for hydrolysis.

Now both keto-acid **28** and *N*-methyl *L*-alanine t-butyl ester were in our hands. So we tried a acid-amine coupling by using as coupling reagents DCC and HOBT in presence of TEA and DCM as a solvent. Even after overnight stirring at rt we were not able to obtain the desired intermediate **29**. The TLC of this reaction was complex, but purification of the main spot gave the

decarboxylated intermediate **30.** This result means that the hydrolysis of the β-keto-ester favours the decarboxylation.

Scheme 5



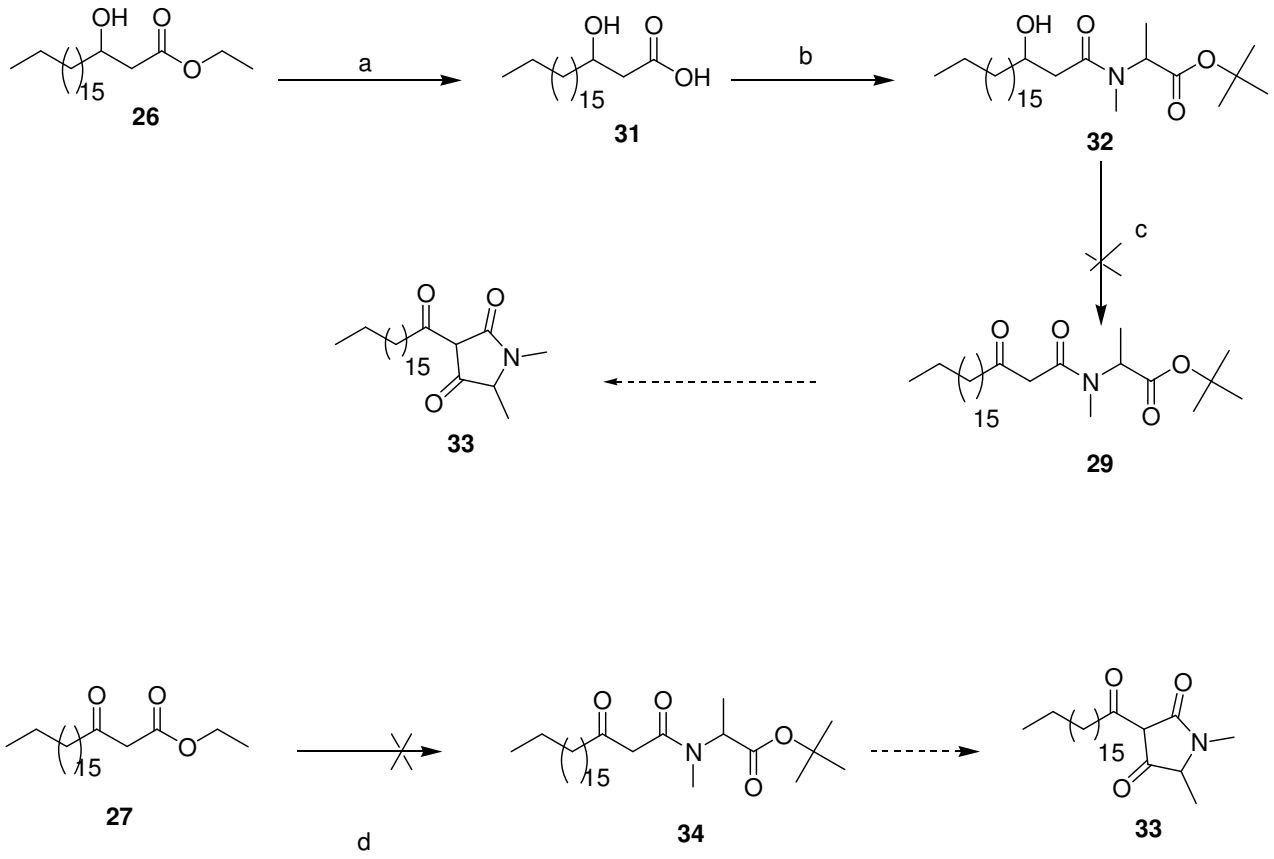
Reagents and conditions :

a) 2M LAH in THF, diethyl ether , 0^{o} C, 45 min, 90 %; b) PCC, DCM, rt, 8 h, 88 %; c) Zn, benzene, ethyl bromoacetate, 80 ^{o}C, 5 h. 88 %; d) PCC, DCM, rt, 7 h, 72 %; e) AcOH, HCl, 48 h, f) KOH, EtOH, reflux, 5 h; g) DCC, HOBT, TEA, DCM, *N*-methyl *L*-alanine t-butyl ester, rt.

As we were unable to obtain the desired intermediates **28** and **29,** we decided to use β-hydroxy-ester **26** for another synthetic approach . The sequence is depicted in scheme **6**. Hydrolysis of intermediate **26** was easily carried out by using KOH in EtOH and H_2O at rt overnight, and after acidic workup we obtained β-hydroxy-acid **31**. Structural confirmation was done by ^{1}H NMR. We successively carried out acid-amine coupling[26]of **31** and *N*-methyl L-alanine t-butyl ester by using

coupling reagent DCC, HOBT and base TEA in DCM to obtain **32**, whose structure was confirmed by ^{1}H-NMR spectra. Oxidation of **32** was attempted with oxidizing agents such as 1) IBX in DMSO 2) supported-TEMPO, KBr, in DCM[27] 3) Dess-Martin[28] in DCM 4) Swern oxidation and 5) Pyridine-SO_3, in DMSO, but none of the above worked. So looking for an ester-amine coupling we found a literature paper[29] mentioning that the ester-amine coupling can be carried out by using DABCO-$(AlMe_3)_2$ and TEA in dry THF at 40 ^{o}C for 1 h. We performed this reaction between β-keto-ester **27** and *N*-methyl L-alanine t-butyl ester but we did not obtain the desired intermediate **34** even after refluxing 20 hours. After all these attempts we came to the conclusion that hydrolysis of β-keto-ester **27** and oxidation of β-hydroxy amide are difficult. To obtain the tetramic acid core **33** we must have β-keto-amide **29** in order to complete synthesis of epicoccamide D.

Scheme 6



Reagents and conditions :

a) KOH, EtOH, rt, overnight, 82 %; b) DCC, HOBT, TEA, DCM, *N*-methyl L-alanine t-butyl ester, rt , overnight, 70 %; c) Oxidation. d) $(AlMe_3)_2$.DABCO, TEA, THF, reflux.

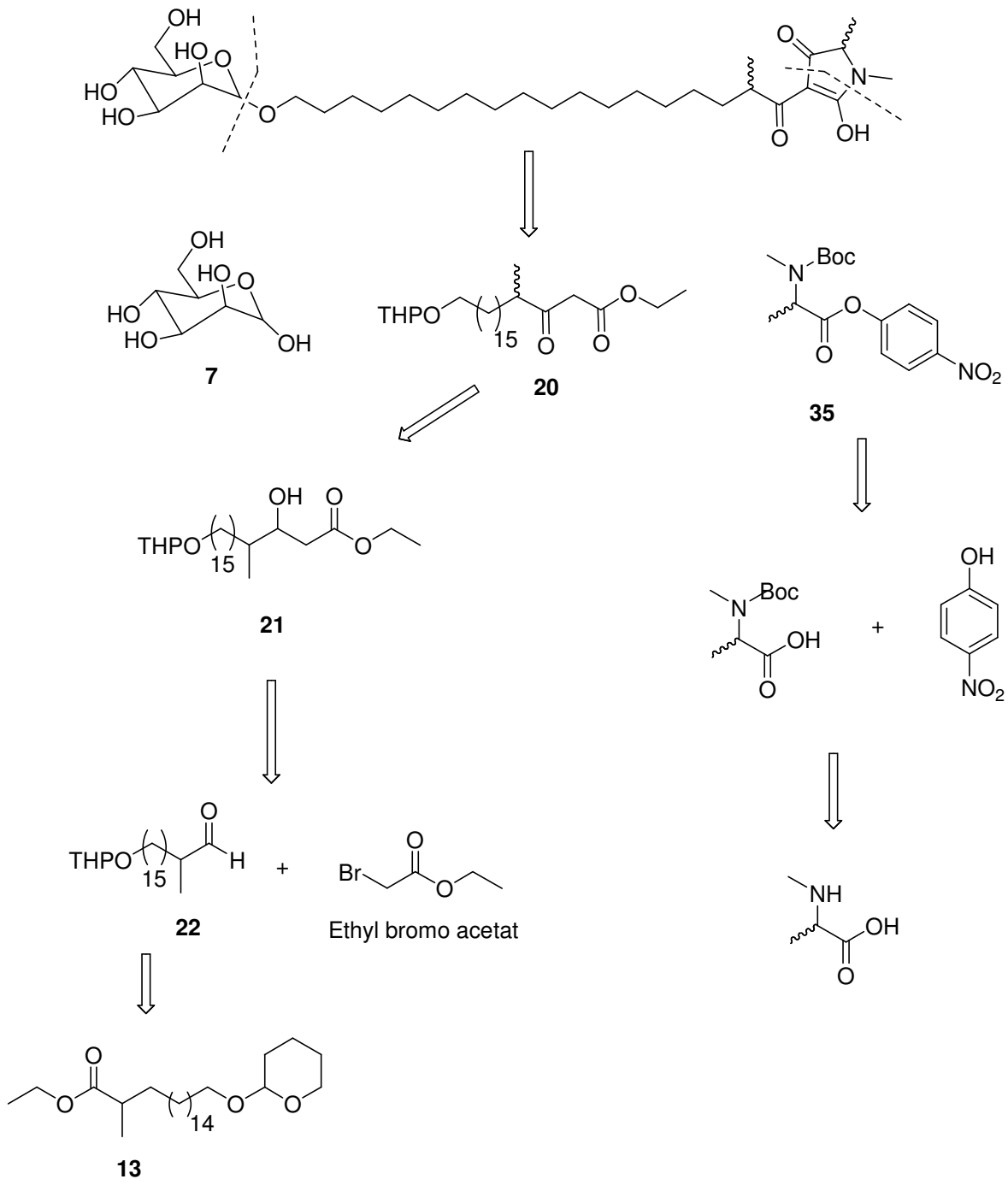
As we were unable to obtain the β-keto-amide **29**, we decided not to carry this synthetic pathway on 16-bromohexadecanoate **10**. So looking for another synthetic approach toward the synthesis of epicoccamide D we found in literature Igglessi-Markopoulou[16d] report on the condensation of *N*-(*N*-acetylglycyloxy)succinimide with a β-keto-ester bearing alkanoyl or dienoyl

groups, that furnishes a 3-substituted *N*-acetyltetramic acids. Herein, we planned to employ the same strategy in our following approach.

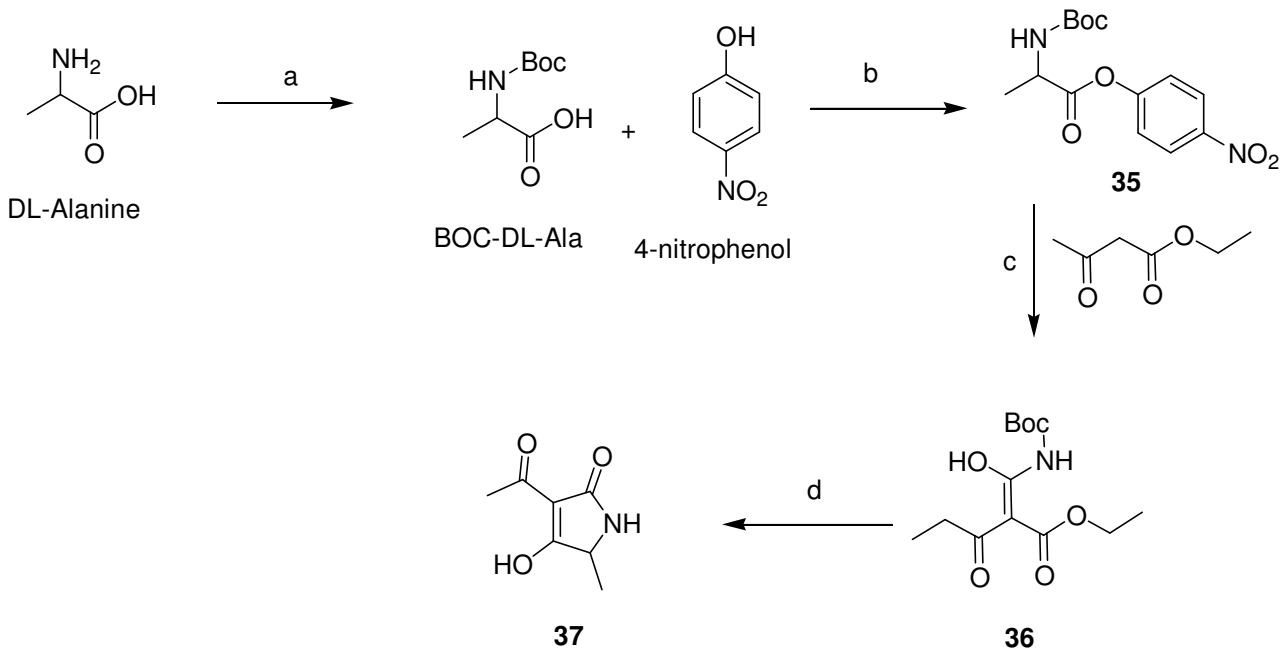
The use of enolic β-dicarbonyl compounds for the synthesis of nitrogen heterocycles[16c,d,e] is another approach to synthesize the tetramic acid core. The proposed methodology is centred on the condensation of *N*-(*N*-acetylglycyloxy)succinimide with an excess of the anion of an appropriate β-keto-ester, generated by the action of a strong base. Considering the above fact, we revised the retrosynthesis again as depicted in scheme 7. The disconnection identified the known fragments β-keto-ester **20** and **35**. The fragment **20** can be constructed by a known synthetic pathway which is described in scheme **5** and fragment **35** could be obtained by partial modification of the literature procedure[16d] where we planned to use 4-nitrophenol instead of N-hydroxysuccinimide.

In order to explore the reaction conditions and to avoid loss of the expensive intermediate **20** we carried out this synthetic sequence by using ethyl acetoacetate instead of β-keto-ester **20** and *DL*-alanine inseated of *N*-methyl *L*-alanine t-butyl ester (Scheme 8). Protection of *DL*-alanine carried out according to a literature procedure[30] by using di-tert-butyldicarbonate in presence of dioxane-water and 1N NaOH provided Boc-protected *DL*-alanine, which was then coupled with 4-nitrophenol by using the coupling reagent EDCI and DCM as solvent to give compound **35,** the structure of which was confirmed by ^{1}H-NMR. Following the same literature procedure,[16d] coupling of **35** and ethyl acetoacetate by using NaH as a base and benzene as a solvent gave the intermediate **36** which was fully characterized by spectroscopic data including ^{1}H-NMR. Finally, cyclization with sodium ethoxide in ethanol at reflux for 3 h afforded the tetramic acid core **37**. Mass spectra of this compound showed a peak at m/z 154 which indicated that the Boc protection had been cleaved.

Scheme 7



Scheme 8

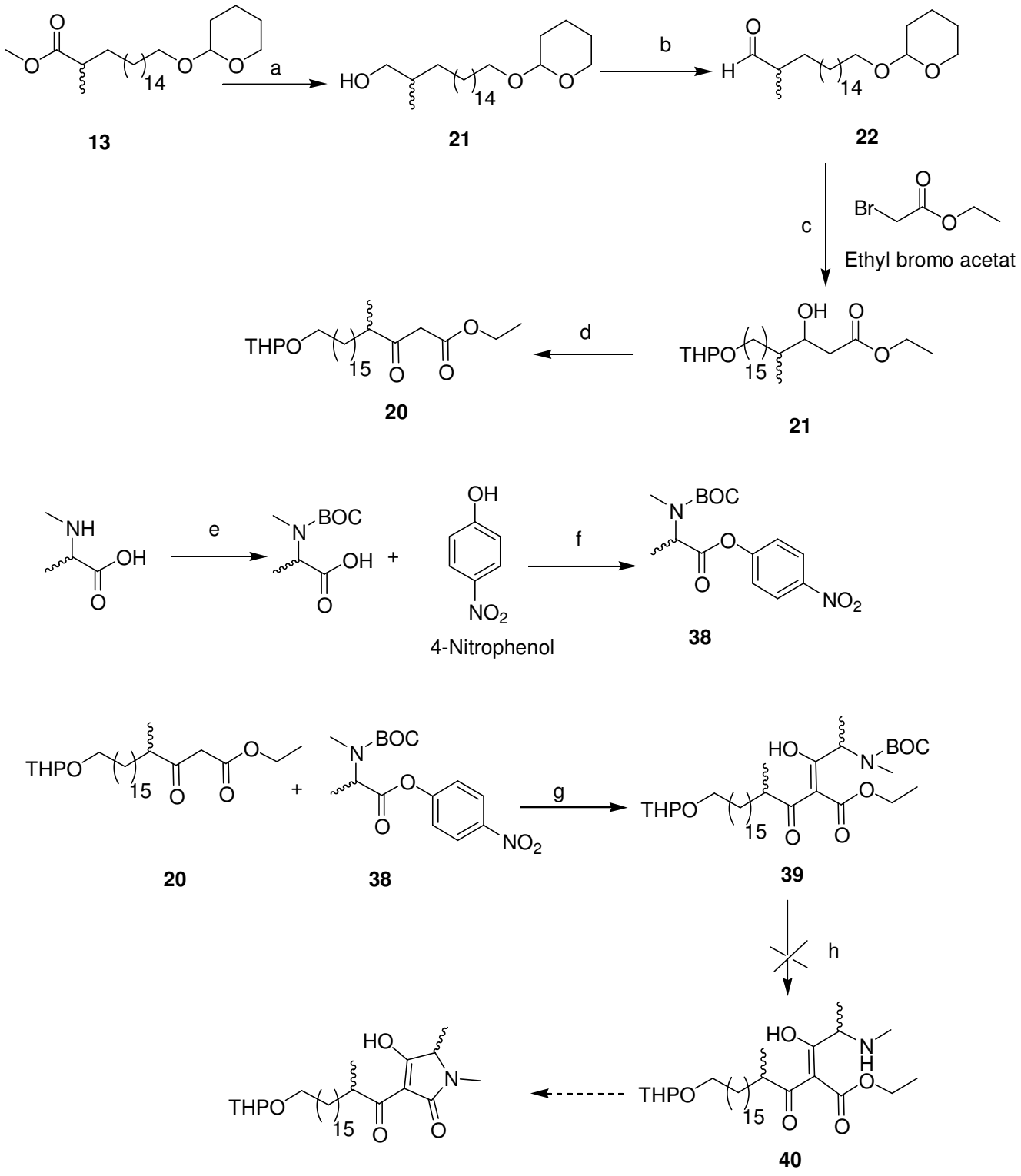


Reagents and conditions :

a) $(Boc)_2O$, 1N NaOH, H_2O, Dioxane, 85 %; b) EDCI, DCM, rt, overnight, 73 %; c) NaH, DMF, ethyl acetoacetate, rt, 2 h, 18 %; d) NaOEt, EtOH, reflux, 3 h.

On the basis of the successful synthesis of tetramic acid core **37,** we applied this synthetic strategy to compound **20,** which is depicted in scheme 9. For the synthesis of the β-keto-ester we followed the same synthetic path which is depicted in scheme 5. We started from known intermediate **13** which had been synthesized from methyl 16-bromohexadecanoate **10** (Scheme 3). Reduction of **13** was carried out by using 2M LAH in THF and diethyl ether as solvent at 0 ^{o}C for 1 h, to give the hydroxy intermediate **21.** Oxidation with PCC in DCM provided aldehyde **22**. The condensation of aldehyde **22** with ethyl bromoacetate using metallic zinc[25] in presence of benzene as a solvent afforded β-hydroxy-ester **21**. Finally, oxidation of **21** was carried out by overnight stirring at rt with PCC in DCM as a solvent to provide β-keto-ester **20** in 80 % yield. Compound **20** was fully characterized by spectroscopic data includes ^{1}H-NMR and ^{13}C-NMR.

Scheme 9


a
b
c
Ethyl bromo acetat
d
e
f
4-Nitrophenol
g
h
13
21
22
21
20
20
38
38
39
40

Reagents and conditions :

a) 2M LAH in THF, diethyl ether , 0^{o} C, 45 min, 95%; b) PCC, DCM, rt, 8 h, 73%; c) Zn, Benzene, ethyl bromoacetate, 80 ^{o}C, 5 h, 80 %; d) PCC, DCM, rt, 7 h, 82%; e) $(Boc)_2O$, 1N NaOH, H_2O, Dioxane; f) EDCI, DCM, rt, overnight, 40 %; g) NaH, Benzene, rt, 2 h, 16 %.

Compound **38** was synthesized from *N*-Me-*L*-alanine. Boc protection of *N*-Me-*L*-alanine using di-tert-butyldicarbonate in presence of dioxane-water and 1N NaOH provided Boc protected L-alanine which was then coupled with 4-nitrophenol by using coupling reagent EDCI in DCM to obtain compound **38**, the structure of which was confirmed by ^{1}H-NMR.

Now both the intermediates **20** and **38** were in our hands. Coupling of the two halves was achieved, according to an extension of the method proposed by Oikawa and Sugano,[31] by condensation of **38** with an excess of the anion of the β-keto-ester **20**, generated by the action of sodium hydride in benzene as a solvent. After purification the coupled compound **39** was obtained in 16 % yield. Compound **39** was fully characterized by ^{1}H-NMR and ^{13}C-NMR.

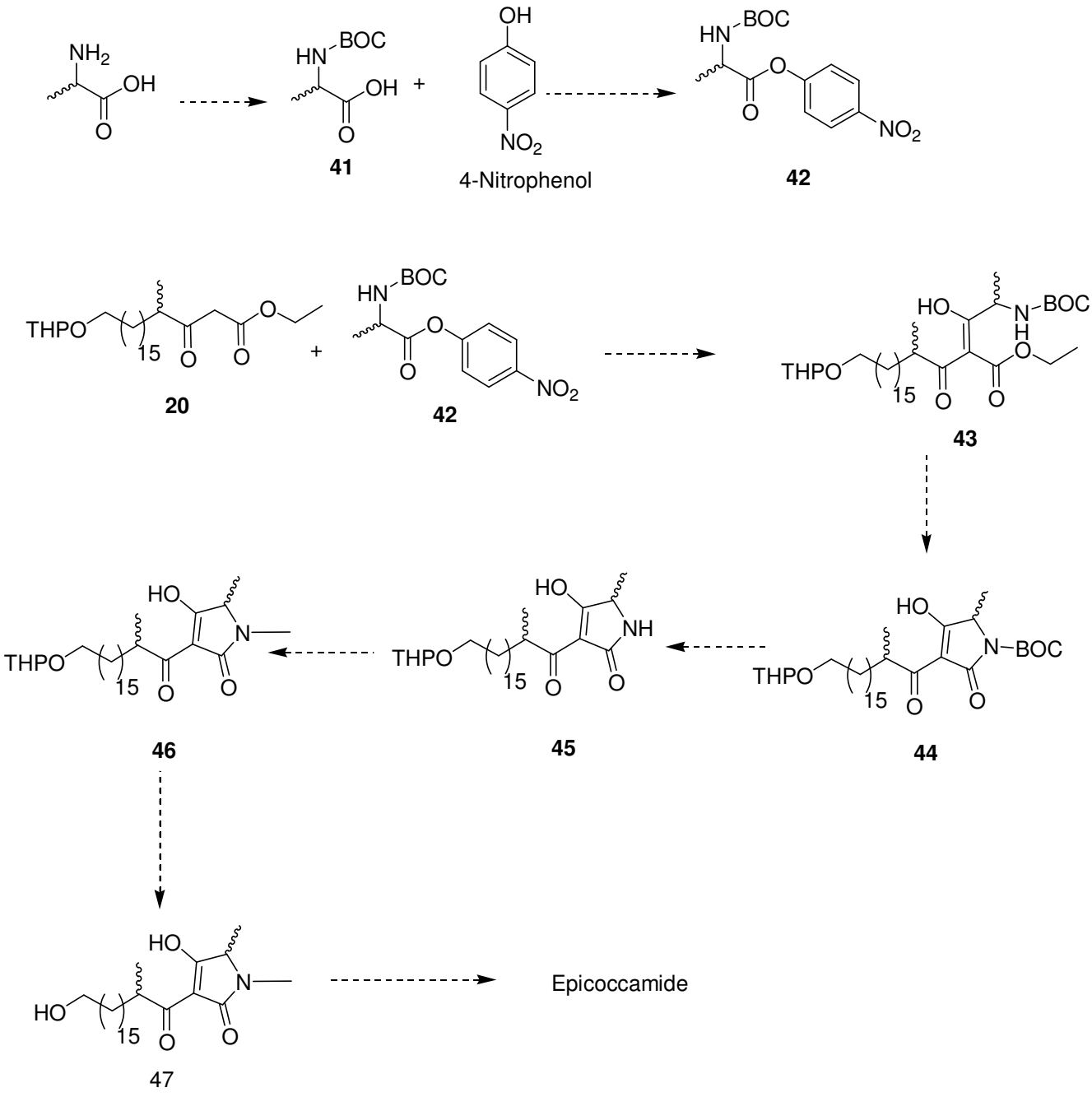
The following crucial step in this synthesis is the cyclization to the desired tetramic acid. Therefore having **39** in our hand we decided to carried out first the deprotection of *N*-Boc and then the cyclization. In the beginning we used $AlCl_3$ in DCM for deprotection of *N*-Boc, but due to the strong acidic nature of $AlCl_3$, starting compound **39** was decomposed. As intermediate **39** is an acid sensitive intermediate we decided to use milder reaction condition for deprotection of N-Boc. Keeping this in mind we followed a literature procedure[32] in which tetraammonium fluoride was used. We carried out this reaction in presence of THF as solvent and 5 eq of tetraammonium fluoride at reflux temperature for more than 7 h but we were not able to see any conversion on TLC. Then we tried another reported procedure[33] in which deprotection of *N*-Boc can be carried out just

by refluxing with water. We performed this reaction by refluxing **39** in water but unfortunately we couldn't see any progress in reaction. Even after several attempts we were unable to obtained deBoc intermediate **40**.

4.9 Future perspectives

Thus the next choice for the cyclization reaction to obtain the tetramic acid core is to couple the Boc protected alanine **41** with 4-nitrophenol by following the same procedure to obtain intermediate **43**, and then condense it with the β-keto-ester **20** followed by the cyclization using sodium ethoxide in ethanol. Finally, deprotection of *N*-Boc could be done by acidic medium followed by methylation can be achieved by using MeI (Scheme 10).

Scheme 10

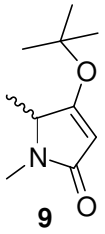

NH2
OH
O
HN
BOC
OH
O
41
OH
NO2
4-Nitrophenol
HN
BOC
O
O
NO2
42
THPO
15
O
O
O
20
+
HN
BOC
O
O
NO2
42
HO
N
BOC
H
THPO
15
O
O
O
43
HO
N
BOC
THPO
15
O
O
44
HO
NH
THPO
15
O
O
45
HO
N
THPO
15
O
O
46
HO
N
HO
15
O
O
47
Epicoccamide

4.10.Conclusion

We have successfully accomplished the synthesis of the two coupling fragments **20** and **38** required for the final stage of the synthesis of epicoccamide **D**. The proposed route is concise and modular, making it convenient for large scale preparation.

4.10 Experimental

4-tert-Butoxy-1,5-dimethyl-1,5-dihydropyrrol-2-one (9)

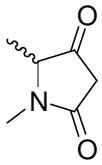


9

Ph_3PCCO (0.309 g 1.02 mmole, 2 eq) was suspended in THF (3 mL) and after 10 min swelling treated with *N*-Me-Ala-tert-butylester(0.100 g, 0.511 mmole). The mixture was irradiated in the microwave synthesizer at 90 ^oC for 30 min. After filtration and washing of keten with (2 x 10 mL) of each THF, DCM and diethyl ether crude product purified by column chromatography using eluent ethyl acetate. Purification by flash column chromatography afforded **9** as a pale yellow oil (0.038 g, 40 %). R*f* = 0.38 (EtOAc),

^{1}H NMR (300 MHz, $CDCl_3$) δ: 5.01 (s, 1H), 3.73 (q, J = 7.2 Hz, 1H), 2.88 (s, 3H), 1.43 (s, 9H), 1.27 (d, J = 7.2 Hz, 3H)

1,5-Dimethyl-pyrrolidine-2,4-dione (6)

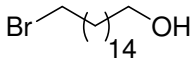


6

Tetramate **9** (0.174 mmole) was dissolved in dry TFA (0.87 mL) and stirred at rt for 3 h. n-hexane was added and all volatiles were removed under reduced pressure on a rotary evaporator. The residue thus obtained was dried on an oil pump to leave a sufficiently pure, bright yellow tetramic acid **6,** (0.038 g, 40%). R*f* = 0.38 (EtOAc).

^{1}H NMR (300 MHz, $CDCl_3$) δ: 3.94 (q, J = 7.2 Hz, 1H), 3.31 (s, 2H), 3.01 (s, 3H), 1.40 (d, J = 7.2 Hz, 3H)

16-bromo hexadecanol (11)

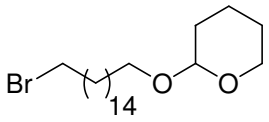


11

To a stirred solution of **10** (0.500 g, 1.43 mmol) in diethyl ether (13 mL) 2M LAH in THF (0.12g, 2 eq, 3.6 mmol) was added at -10 ^{o}C. The reaction mixture was stirred for 1 h at 0 ^{o}C. The reaction was quenched with H_2O at 0 ^{o}C. The aqueous phase was extracted with diethyl ether (3 x 20 mL). The combined organic layers were dried (Na_2SO_4), filtered, and concentrated. Purification by flash column chromatography afforded **11** as a floppy white solid (0.39 g 85%). R*f* = 0.2 (EtOAc / hexane 10:90).

^{1}H NMR (300 MHz, $CDCl_3$) δ: 3.64 (t, J = 6.6 Hz, 2H), 3.41 (t, J = 7.3 Hz, 2H), 1.85 (m, 2H), 1.17-1.63 (m, 26H).

2-(17-Bromo-hexadecyloxy)-tetrahydropyranc (12)

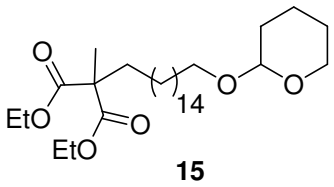


12

To a stirred solution of **11** (0.35 g, 1.1 mmol) in THF (3 mL) 3,4-dihydro 2H pyrane (0.14 g, 1.5eq, 1.6 mmol) was added at rt followed by catalyticale amount of PTSA.H_2O. The reaction mixture was stirred for 8 h at rt. After completion of the reaction, the mixture was evaporated under reduced pressure on a rotary evaporator. Purification by flash column chromatography afforded **12** as a floppy white solid (0.40 g, 90 %). R*f* = 0.8 (EtOAc / hexane 10:90).

^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.51 (t, J = 4.4 Hz, 1H), 3.86 (m,1H), 3.72 (dt, J = 9.1, 7.0 Hz, 1H), 3.31-3.55 (m, 4H), 1.13-1.96 (m, 34H).

2-Methyl-2-[16-(tetrahydropyran-2-yloxy)-pentadecyl]-malonic acid diethyl ester (15)



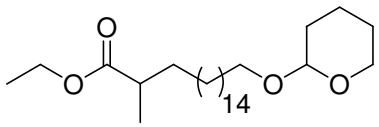
15

To a suspension of NaH (60 %, 0.029 g, 1.2 mmole) in DMF (1 mL) was added dropwise Diethyl methylmalonate (0.077 g, 0.44 mmole) at 0 ^{o}C. After stirring for 30 min, **12** was dissolved in 1 ml DMF and added to above reaction mixture. The mixture was warmed to rt and stirred for 4 h. The reaction mixture was added sat.aq.NH_4Cl solution, and the aqueous layer was extracted with diethyl ether. The combined organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated. Purification by silica gel chromatography afforded **15** as a colourless oil at rt (0.20 g, 81 %). R*f* = 0.5 (EtOAc / hexane 10:90).

^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.57 (m, 1H), 4.17 (q, J = 7.3 Hz, 4H), 3.86 (m, 1H), 3.72 (m, 1H), 3.49 (m, 1H), 3.37 (m, 1H), 0.91-1.89 (m, 43H).

^{13}C NMR (75 MHz, $CDCl_3$): δ: 172.6, 98.9, 67.8, 62.4, 61.4, 61.1, 53.7, 35.5, 30.8, 29.9, 29.7, 29.4, 26.3, 25.8, 25.6, 24.3, 19.9, 19.8, 14.1.

2-Methyl-18-(tetrahydro-pyran-2-yloxy)-heptadecanoic acid ethyl este (13)



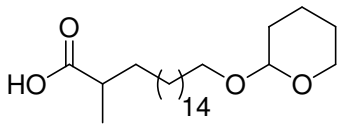
13

A mixture of **15** (0.085 g, 0.174 mmole), lithium chloride (0.018 g, 0.42 mmole), water (5 μL) and DMSO (0.5 mL) were heated at reflux for 12 h. After cooling, the mixture was diluted with brine and the aqueous layer was extracted with diethyl ether. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated. Purification by silica gel chromatography gave **13** (0.040 g, 55.5 %) as an oil. R*f* = 0.58 (EtOAc / hexane 10:90).

^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.58 (t, J = 4.25 Hz, 1H), 4.13 (q, J = 7.04 Hz, 2H), 3.87 (m, 1H), 3.74 (dt, J = 7.0, 9.4 Hz, 1H), 3.50 (m, 1H), 3.38 (dt, J = 6.7, 9.5 Hz, 1H), 2.41 (m, 1H), 1.16-1.90 (m, 37H), 1.14 (d, J = 6.9 Hz, 3H).

^{13}C NMR (75 MHz, $CDCl_3$): δ: 177.1, 98.9, 67.8, 63.2, 62.4, 60.1, 39.6, 33.9, 30.8, 29.7, 29.6, 27.3, 26.3, 25.8, 25.6, 19.8, 17.2, 14.3.

2-Methyl-18-(tetrahydro-pyran-2-yloxy)-heptadecanoic acid (14)



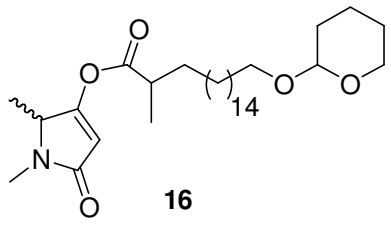
14

To a stirred solution of **13** (0.050 g, 0.117 mmole) in EtOH (0.35 mL) and H_2O (0.35 mL), KOH (0.32 g, 0.58 mmole) was added, the mixture was refluxed for 5 h. The mixture was allowed to cool to rt, then EtOH was evaporated under vacuum. The residue was acidified (pH =2) by 2N HCl and extracted with diethyl ether; the combined organic layers were dried over Na_2SO_4, filtered, and concentrated to obtain **14** as a colourless oil (0.035 g, 70 %) as an oil. R*f* = 0.3 (EtOAc / hexane 10:90).

^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.57 (m, 1H), 3.86 (m, 1H), 3.72 (m, 1H), 3.49 (m, 1H), 2.45 (m, 1H), 1.19-1.90 (m, 34H), 1.16 (d, J = 7.3 Hz, 3H).

^{13}C NMR (75 MHz, $CDCl_3$): δ: 182.3, 98.9, 67.8, 63.2, 62.4, 39.3, 33.6, 30.8, 29.7, 27.2, 26.3, 25.8, 25.5, 19.7, 16.9

2-Methyl-18-(tetrahydro-pyran-2-yloxy)-octadecanoic acid 1,2-dimethyl-5-oxo-2,5-dihydro-1H-pyrrol-3-yl ester(16)



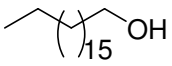
16

A stirred solution of **6** (0.050 g, 0.39 mmole) in dry DCM (3 mL) at 0 ^{o}C was treated with DMAP (0.010 g, 0.08 mmol), **14** (0.156 g, 0.39 mmole) and finally with DCC (0.097 g, 0.47 mmole). Stirring was continued for 10 min at 0 ^{o}C and for another 5 h at rt . The mixture was filtered through a short plug of celite to remove most of the by-product urea; the filtrate was concentrated on a rotary evaporator and the remainder was purified by chromatography on silica gel, obtained **16** (0.070 g, 35 %) as a white solid, R*f* = 0.2 (EtOAc / hexane 50:50).

^{1}H NMR (300 MHz, $CDCl_3$) δ: 6.02 (m, 1H), 4.57 (m, 1H), 4.04 (q, J = 7.0 Hz, 1H), 3.87 (m,1H), 3.73 (m, 1H), 3.49 (m, 1H), 3.38 (m, 1H), 2.93 (s, 3H), 2.62 (m, 1H), 0.98-2.06 (m, 39H).

^{13}C NMR (75 MHz, $CDCl_3$) δ: 177.1, 98.9, 67.8, 63.2, 62.4, 60.1, 39.6, 33.9, 30.8, 29.7, 29.6, 27.3, 26.3, 25.8, 25.6, 19.8, 17.2, 14.3.

Octadecan-1-ol (24)

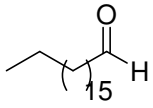


24

To a stirred solution of **23** (0.500 g, 1.6 mmol) in diethyl ether (15 mL) 2M LAH in THF (1.85 ml, 2.2 eq, 3.7 mmol) was added at -10 ^{o}C. The reaction mixture was stirred for 1 h at 0 ^{o}C. The reaction was quenched with H_2O at 0 ^{o}C. The aqueous phase was extracted with diethyl ether (3 x 20 mL). The combined organic layers were dried (Na_2SO_4), filtred and concentrated. Purification by flash column chromatography afforded **24** as a floppy white solid (0.41 g, 90 %). R*f* = 0.3 (EtOAc / hexane 10:90).

^{1}H NMR (300 MHz, $CDCl_3$) δ: 3.64 (t, J = 7.3 Hz, 2H), 1.15-1.62 (m, 32H), 0.88 (t, J = 7.1 Hz, 3H).

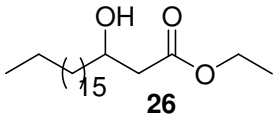
Octadecanal (25)



25

To a stirred solution of **24** (0.410 g, 1.5 mmol) in DMC (15 mL) PCC (0.42 g, 1.9 mmol) was added and mixture was stirred for overnight at rt. Dilute the mixture with diethyl ether (20 mL), decant upper layer. Residue was washed with diethyl ether, combined organic layers filtered, and concentrated, Purification by flash column chromatography afforded **25** as a floppy white solid (0.35 g, 88 %). R*f* = 0.7 (EtOAc / hexane 10:90).

3-Hydroxy-eicosanoic acid ethyl ester (26)

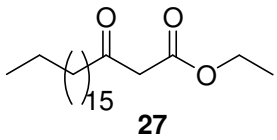


26

A suspension of activated zinc dust (0.22 g, 3.3mmole) in 1 mL benzene was heated up to reflux for 10 min. To the refluxing suspension was added ethyl bromoacetate (0.28 g, 1.6 mmole). After 5 min was added aldehyde **25** (0.300 g, dissolved in 2 ml benzene, 1.1 mmole) and after 2 h the refluxing mixture was cooled to rt then 1N HCl (2 mL) was added. The aqueous mixture was extracted with diethyl ether (3 x 10 mL). The combined organic layers were dried over Na_2SO_4, filtred, and concentrated. Purification by flash column chromatography afforded **26** as a white solid (0.35 g, 88%). R*f* = 0.25 (EtOAc / hexane 10:90).

^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.2 (q, J = 7.2 Hz, 2H), 3.99 (m, 1H), 2.32-2.55 (m, 2H), 0.98-1.70 (m, 32H), 0.87 (t, J = 7.2 Hz, 6H)

3-Oxo-eicosanoic acid ethyl ester (27)

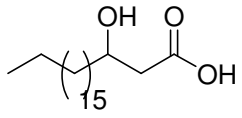


27

To a stirred solution of **26** (0.140 g, 0.39 mmol) in DMC (10 mL) PCC (0.110 g, 0.51mmol) was added and mixture stirred for overnight at rt. Dilute the mixture with diethyl ether (20 mL), decant upper layer. Residue was washed with diethyl ether, combined organic layers filtered, and concentrated. Purification by flash column chromatography afforded **27** as a white solid (0.100 g, 72%). R*f* = 0.73 (EtOAc / hexane 10:90).

^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.19 (q, J = 7.3 Hz, 2H), 3.42 (s, 2H), 2.52 (t, J = 7.2 Hz, 2H), 1.52-1.64 (m, 4H), 0.80-1.35 (m, 32H).

3-Hydroxy-eicosanoic acid (31)

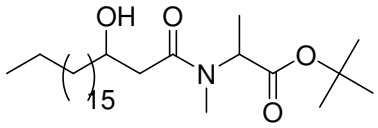


31

To a stirred solution of ester **26** (0.080 g, 0.22 mmole) in EtOH (1 ml) and H_2O (1 mL) KOH (0.063 g, 0.11 mmole) was added, the resulting mixture was stirred at rt for overnight. Ethanol was evaporated under vacuum and the aqueous mixture was acidified with 1N HCl and extracted with diethyl ether (2 x 10 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated, under vacuum to afford **31** as a white solid (0.060 g, 82 %). R*f* = 0.18 (EtOAc / hexane 10:90).

^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.03 (m, 1H), 2.39-2.63 (m, 2H), 1.10-1.60 (m, 32H), 0.88 (t, J = 7.2 Hz, 3H).

2-[(3-Hydroxy-eicosanoyl)-methyl-amino]-propionic acid tert-butyl ester (32)



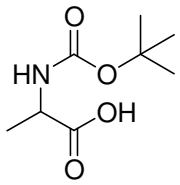
32

To a stirred solution of acid **31** (0.100 g, 0.35 mmole), HCl salt of *N*-methyl *L*-alanine t-butyl ester (0.089 g, 0.45 mmole) in DCM (5 mL) TEA (0.061 g, 0.61 mmole), HOBT (045 g, 0.33 mmol) followed by DCC (0.069 g, 0.33 mmole) was added at 0 ^{o}C. The resulting mixture was stirred for 1 h at 0 ^{o}C and then at rt overnight. The mixture was filtered through a short plug of celite to remove most of the by-product urea, the filtrate was concentrated on a rotary evaporator and the remainder was purified by chromatography on silica gel. Obtained **32** as a white solid (0.109 g, 70 %), R*f* = 0.6 (EtOAc / hexane 50:50).

^{1}H NMR (300 MHz, $CDCl_3$) δ: 5.12 (m, 1H), 3.92-4.2 (m, 2H), 2.91 (s, 3H), 2.23-2.56 (m, 2H), 1.0-1.71 (m, 44H), 0.87 (t, J = 6.6 Hz, 3H).

^{13}C NMR (75 MHz, $CDCl_3$): δ: 173.3, 170.8, 81.6, 68.2, 56.2 52.6, 39.9, 36.4, 31.9, 31.4, 29.6, 29.3, 28.0, 25.6, 22.6, 15.2, 14.5, 14.1.

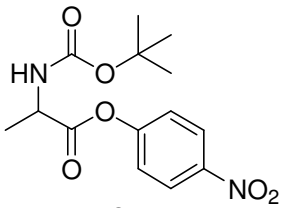
2-tert-Butoxycarbonylamino-propionic acid



Boc-DL-Ala

DL-Alanine (0.5 g, 5.6 mmole) was dissolved in dioxane-H_2O (10 + 5 mL), the mixture was cooled to 0 ^oC and pH was adjusted to 8 by addition of 1N NaOH followed by di-tert-butyldicarbonate (1.34 g, 6.1 mmole) dropwise. The mixture was stirred at rt for 5 h , dioxane was evaporated under vaccum. The oily residue was cooled to 0 ^oC and acidified with 10 % HCl. The aqueous mixture was extracted with EtOAc (3 x 20 ml). The combined organic layers were dried (Na_2SO_4), filtered, and concentrated. Purification by flash column chromatography afforded Boc-DL-Alanine as a oil (0.900 g, 85 %).

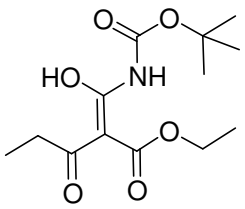
2-tert-Butoxycarbonylamino-propionic acid 4-nitro-phenyl ester (35)



35

To a stirred solution of Boc-DL-Alanine (0.100 g, 0.52mmole) in DCM (5 mL) 4-Nitro phenol (0.88 g, 0.62 mmole) and EDCI (0.21g, 0.62mmole) were added at rt. The resulting mixture was stirred at rt overnight, crude product was purified by column chromatography to obtain **35** (0.120 g, 73 %) as a yellow oil.

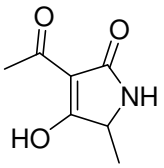
3-tert-Butoxycarbonylamino-3-hydroxy-2-propionyl-acrylic acid ethyl ester (36)



36

The active methylene compound ethyl aceto acetate (0.050 g, 0.38 mmole) was added dropwise to a mixture of sodium hydride (60 % in oil, 0.34 mmole) in anhydrous benzene (0.5 mL) and the thick slurry thus formed was stirred at rt for 1 h. Compound **35** (0.094 g, 0.3 mmole) was then added to the mixture and stirring continued at 10 ^{o}C for 3 h. Water was added to the reaction mixture and the aqueous layer was separated and acidified with 10 % HCl, in an ice water bath and extracted with chloroform. The combined organic layers were dried (Na_2SO_4), filtered, and concentrated. Purification by flash column chromatography gave **36** (0.020 g, 18 %). R*f* = 0.27(EtOAc / hexane 30:70).

3-Acetyl-4-hydroxy-5-methyl-1,5-dihydro-pyrrol-2-one (37)

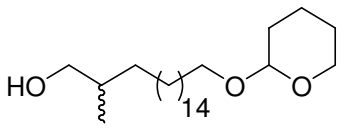


37

To a solution of NaOEt [prepared from Na (0.0018 g, 2.5.eq) in absolute ethanol (0.4 mL)] **36** (0.010 g, 0.03 mole) in benzene (0.4 mL) was added at rt. Then reaction mixture was refluxed for 3 h, ethanol was evaporated under vacuum, in remaining residue H_2O was added 2 mL, and acidified with 10 % HCl in an ice bath. Then aqueous mixture was extracted with chloroform. combined organic layers were dried (Na_2SO_4), filtered, and concentrated, obtained **37** (0.009 g, 18 %). R*f* = 0.22 (MeOH / DCM 10:90),

m/z = 154.

2-Methyl-17-(tetrahydro-pyran-2-yloxy)-heptadecan-1-ol (21)

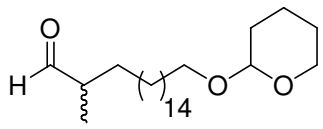


21

To a stirred solution of **13** (0.360 g, 0.84 mmole) in diethyl ether (13 mL) 2M LAH in THF (0.843 ml, 2 eq, 1.6 mmol) was added at -10 ^{o}C. The reaction mixture was stirred for 1 h at 0 ^{o}C. Reaction was quenched with H_2O at 0 ^{o}C, aqueous phase was extracted with diethyl ether (3 x 20 mL). The combined organic layers were dried (Na_2SO_4), filtered, and concentrated. Purification by flash column chromatography afforded **21** as a floppy white solid (0.280 g, 95 %). R*f* = 0.3 (EtOAc/ hexane 10:90).

^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.58 (t, J = 4.0 Hz, 1H), 3.87 (m, 1H), 3.72 (dt, J = 9.2, 7.7 Hz, 1H), 3.32-3.55 (m, 4H), 1.0-1.92 (m, 38H), 0.91 (d, J = 6.75 Hz, 3H).

2-Methyl-17-(tetrahydro-pyran-2-yloxy)-heptadecanal (22)

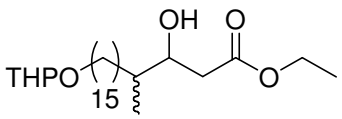


22

To a stirred solution of **21** (0.280 g, 0.72 mmol) in DMC (10 mL) PCC (0.203 g, 0.94 mmol) was added and mixture was stirred for overnight at rt. Dilute the mixture with diethyl ether (20 mL), decant upper layer. Residue was washed with diethyl ether, combined organic layers filtered, and concentrated. Purification by flash column chromatography afforded **22** as a floppy white solid (0.225 g, 73 %). R*f* = 0.6 (EtOAc / hexane 10:90).

^{1}H NMR (300 MHz, $CDCl_3$) δ: 9.60 (d, J = 2.1 Hz, 1H), 4.57 (t, J = 4.4 Hz, 1H), 3.85 (m, 1H), 3.73 (dt, J = 9.5, 6.9 Hz, 1H), 3.49 (m, 1H), 3.38 (dt, J = 9.5, 6.7 Hz, 1H), 2.29 (m, 1H), 1.15-1.80 (m, 36H), 1.08 (d, J = 7.0 Hz, 3H)

3-Hydroxy-4-methyl-19-(tetrahydro-pyran-2-yloxy)-nonadecanoic acid ethyl ester (21)



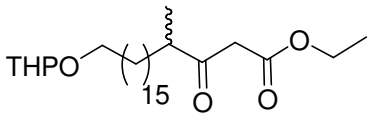
21

A suspension of activated zinc dust (0.115 g, 1.7 mmole) in 1 mL benzene was heated up to reflux for 10 min. To the refluxing suspension was added ethyl bormo acetate (0.147 g, 0.88 mmole); after 5 min was added aldehyde **22** (0.225 g, dissolved in 2 mL benzene, 0.58 mmole). After 2 h of refluxing the mixture was cooled to rt then 1N HCl (2 mL) was added; the aqueous phase was extracted with diethyl ether (3 x 10 mL). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated. Purification by flash column chromatography afforded **21** as a white solid (0.216 g, 80%). R*f* = 0.27 (EtOAc / hexane 10:90).

^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.57 (t, J = 4.0 Hz, 1H), 4.17 (q, J = 7.3 Hz, 2H), 3.66-3.98 (m, 3H), 3.50 (m, 1H), 3.37 (m, 1H), 2.44 (m, 2H), 1.03-1.41 (m, 39H), 0.90 (t, J = 7.3 Hz, 3H)

^{13}C NMR (75 MHz, $CDCl_3$): δ: 173.4, 98.7, 71.7, 71.1, 67.6, 67.3, 62.2, 60.6, 46.4, 42.0, 38.7, 38.0, 37.9, 37.7, 32.6, 32.2, 30.7, 29.8, 29.6, 27.2, 27.0, 26.1.

4-Methyl-3-oxo-19-(tetrahydro-pyran-2-yloxy)-nonadecanoic acid ethyl ester (20)

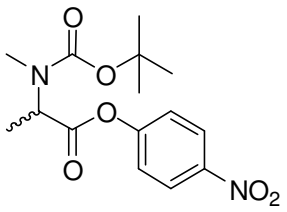


20

To a stirred solution of **21** (0.216 g, 0.45 mmol) in DMC (10 mL) PCC (0.128 g, 0.59 mmol) was added and mixture was stirred for overnight at rt. Dilute the mixture with diethyl ether (20 mL), decant upper layer. Residue was washed with diethyl ether, combined organic layers filtered, and concentrated. Purification by flash column chromatography afforded **20** as a white solid (0.130 g, 82 %). R*f* = 0.73 (EtOAc / hexane 10:90).

^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.55 (t, J = 4.0 Hz, 1H), 4.16 (q, J = 7.1 Hz, 2H), 3.84 (m, 1H), 3.70 (m, 1H), 3.44 (s, 2H), 3.29-3.52 (m, 2H), 2.59 (m, 1H), 0.75-1.87 (m, 42H).

2-(tert-Butoxycarbonyl-methyl-amino)-propionic acid 4-nitro-phenyl ester (38)

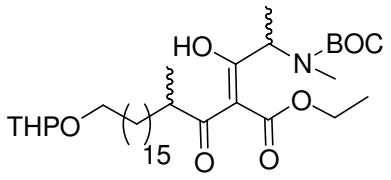


38

To a stirred solution of Boc-*N*-Me-*L*-Alanine (0.215 g, 1.05 mmole) in DCM (5 mL) 4-Nitro phenol (0.146 g, 1.0 mmole) and EDCI (0.242 g, 1.2 mmole) was added at rt, resulting mixture was stirred at rt for overnight, crude product was purified by column chromatography obtained **38** (0.135 g, 40 %) as a yellow oil.

^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.27 (m, 2H), 7.29 (2H, m), 4.77 (m,1H), 2.95 (s, 3H), 1.55 (d, J = 7.3 Hz, 3H), 1.47 (s, 9H).

4-(tert-Butoxycarbonyl-methyl-amino)-3-hydroxy-2-[2-methyl-17-(tetrahydro-pyran-2-yloxy)-heptadecanoyl]-pent-2-enoic acid ethyl ester (39)



39

The active methylene compound **20** (0.050 g, 0.14 mmole) was added dropwise to a mixture of sodium hydride (60 % in oil, 0.08 mmole) in anhydrous benzene (0.5 mL) and the thick slurry thus formed was stirred at rt for 1 h. Compound **35** (0.094 g, 0.3 mmole) was then added to the mixture and stirring continued at 10 ^{o}C for 3 h. Water was added to the reaction mixture and the aqueous layer was separated and acidified with 10 % HCl in an ice water bath and extracted with chloroform The combined organic layers were dried (Na_2SO_4), filtered, and concentrated. Purification by flash column chromatography afforded **39** (0.015 g, 16 %). R*f* = 0.27(EtOAc / hexane 10:90).

^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.57 (m, 1H), 4.02-4.44 (m, 3H), 3.87 (m, 1H), 3.72 (m, 1H), 3.47 (m, 1H), 3.38 (m, 1H), 2.52-2.92 (m, 4H), 0.73-1.93 (m, 54H).

4.11 References

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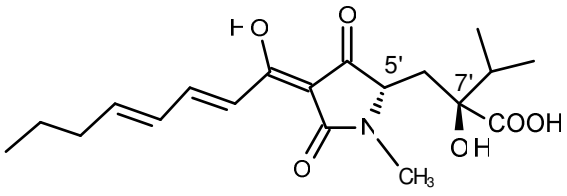
CHAPTER 5

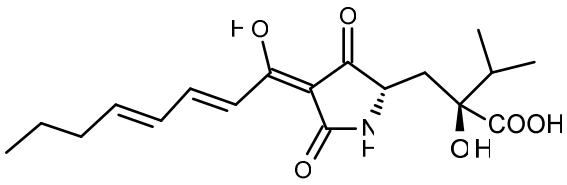
Harzianic Acid

5.1 Introduction

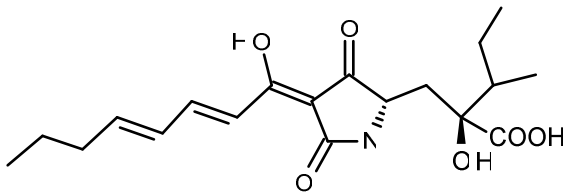
In 1994 Sawa et al.[1] isolated Harzianic acid, a new antimicrobial antibiotic from a *Trichoderma harzianum* strain, and attributed the structure (**1a**), except for the stereochemistry, on the basis of NMR studies. Later, Kawasa et al.[2] reisolated harzianic acid from a fungal strain (F-1531) endowed with inhibiting activity toward serine/threonine phosphatase type 2A (PP2A), together with the analogs demethylharzianic acid (**1b**) and homoharzianic acid (**1c**). Vinale et al.[3] again obtained harzianic acid from a strain of *Trichoderma harzianum,* and established its absolute configuration as 5'S,7'S by X-ray diffraction studies, and found that the compound has antifungal as well as plant growth promoting activity.



1a



1b



1c

5.2 Biological activity

Harzianic acid showed antibiotic activity[3] against *Pythium irregulare, Sclerotinia sclerotiorum, and Rhizoctonia solani*. A plant growth promotion effect is reported at low concentrations of **1a**. Plate antifungal assays with 10 μg of **1a** completely inhibited the growth of the phytopathogenic agents *P. irregulare* and *Sclerotinia sclerotiorum.* Moreover, 62 % and 51 % inhibition was registered using 1 μg of the *Trichoderma* metabolite against *P. irregulare* and *S. sclerotiorum*, respectively. Kawada et al.[2] found that harzianic acid is also a PP2A inhibitor.

The pursuit of compounds containing tetramic acid groups with therapeutic properties has been undertaken for several decades, although comparatively few studies have been devoted toward their potential as anti-infective agents. Harzianic acid and epicoccamide are two quite unusual natural products since they are composed of biosynthetically two distinct subunits: fatty acid and tetramic acid. As we were synthesizing epicoccamide, we decided to develop a synthetic strategy for the preparation of harzianic acid, as both these natural compounds contain the tetramic acid moiety.

5.3 Synthesis of Harzianic Acid

Compound **1a** has an intriguing structure, which includes a tetramic acid[4] group in its N-methylserine-derived heterocycle. Numerous biologically active natural products contain the tetramic acid (pyrrolidine-2,4-dione) ring system as a central element of their structure. The range of biological activities possessed by individual members of this series includes potent antibiotic, antiviral and antiulcerative properties, cytotoxicity and mycotoxicity, the inhibition of tumors as well as fungicidal action. Moreover, certain members of this class are responsible for pigmentation of some sponges and molds.[4] The structural complexity, together with the potent biological activity, of many tetramic acids makes the total synthesis of these compounds an attractive goal for organic chemists. In addition, and differently from other members of the series, harzianic acid comprises a substituted skeleton bearing two stereogenic centers including one which is quaternary, whose

absolute configuration is 5'*S*,7'*S.* Other features are the conjugated double bond (*E*, *E*) and the side chain with the quaternary carbon center bearing –OH and -COOH functional groups. Harzianic acid has significant antifungal and plant growth promotion effect, which makes it a potential lead for the design of new antifungals.

Herein, we describe the efforts towards the first total synthesis of harzianic acid (**1a**).

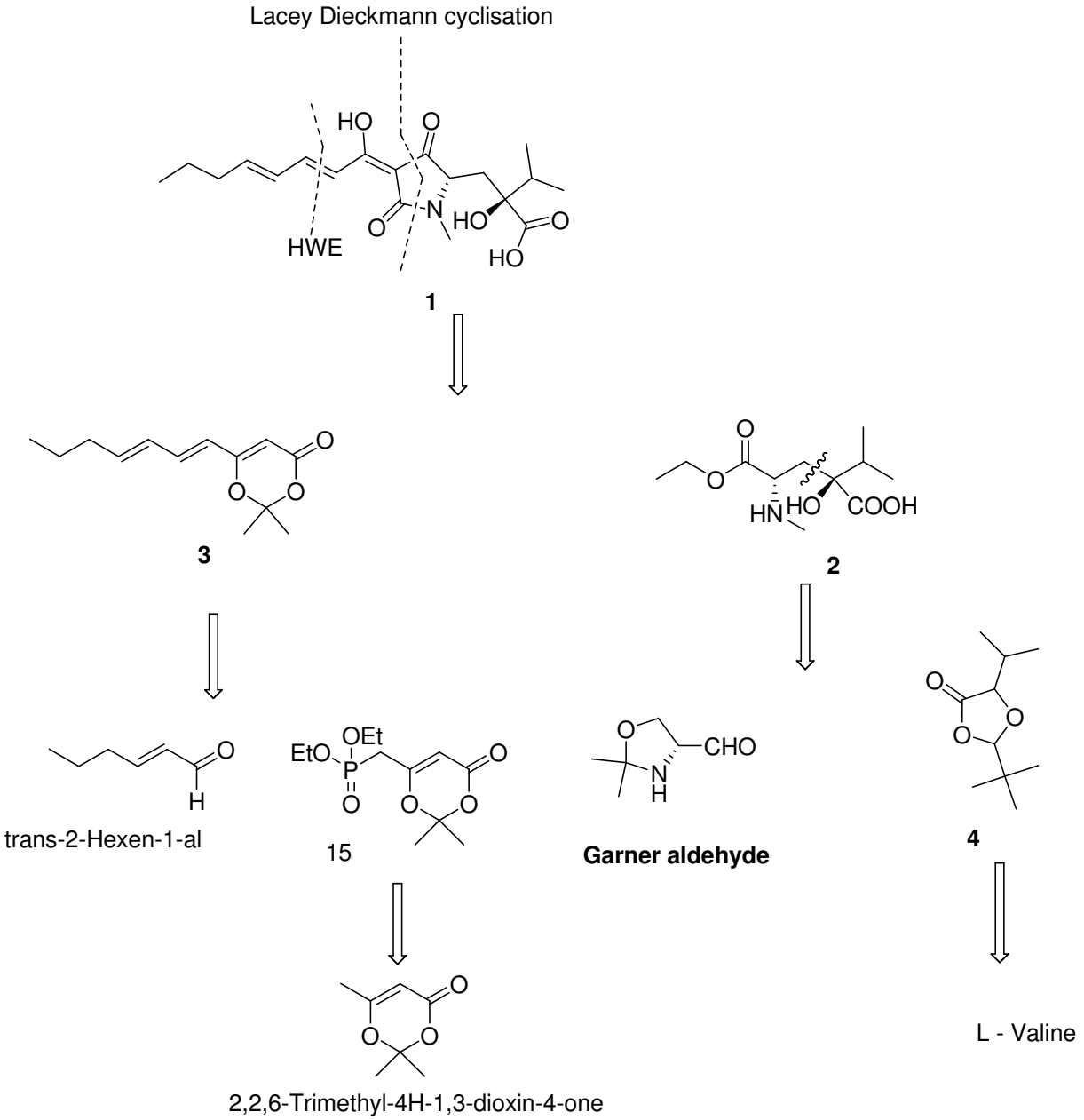
5.3.1 Retrosynthesis

Our retrosynthetic analysis is based upon the construction of the acyl tetramic nucleus. The tetramic acid subunit could be easily obtained by manipulation of an aspartic acid derivative in a Lacey–Dieckmann cyclization.[5] Our first strategy for the synthesis of **1a** is based on the coupling of two domains: a side chain subunit **2** containing the quaternary stereogenic center, and a diene containing subunit **3**; subunit **3** would require a Horner-Wadsworth–Emmons (HWE) reaction of the known phosphonate **15**[6] with the commercially available aldehyde trans-2-hexen-1-al (Scheme 5.1)**.**

For construction of subunit **2**, we found in literature Seebach's report of the enantiospecific alkylation of several 1,3-dioxolanones prepared from α-hydroxycarboxylic acids and pivalaldehyde through the Li-enolate.[7] This reaction has been applied to many total syntheses.[8] Herein, we planned to employ the same strategy in our following approach.

Harzianic acid contains two asymmetric centers at C5' and C7'. The C5' centre has an α -proton which is easily epimerized under basic conditions. Therefore, we planned to use a Garner aldehyde [9] which is commercially available. Considering the above fact, the retrosynthesis approach is depicted in scheme 5.1. The disconnections identified fragment **2**, which can be constructed by treatment of dioxolanone **4**[10] with commercially available (R)-Garner aldehyde.[9] The dioxolanone **4** can be prepared in a stereocontrolled fashion using commercially available *L*-valine.

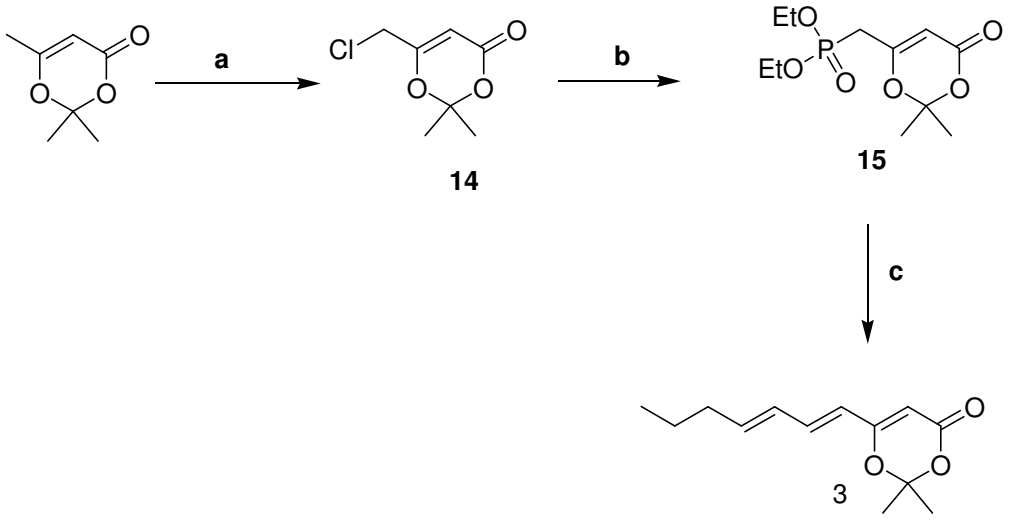
Scheme 5.1


Lacey Dieckmann cyclisation
HO
O
N
HO
O
HO
HWE
1
3
2
trans-2-Hexen-1-al
15
Garner aldehyde
4
L - Valine
2,2,6-Trimethyl-4H-1,3-dioxin-4-one

5.3.2 Synthesis of Subunit 3

Subunit **3** had to be prepared in order to assemble the tetramic acid nucleus via Lackey-Dieckmann cyclisation. So we began the synthesis from commercially available 2,2,6-trimethyl-1,3-dioxen-4-one and prepared known phosphonate **15** using a literature procedure.[6] Horner–Emmons reaction of phosphonate **15** with commercially available trans-2 hexen-1-al using LDA afforded olefin **3** (E/Z=9:1) in 43 % yield. We observed that the use of LiHMDS in this Horner-Emmons reaction does not improve the yield of olefin **3**. (Scheme 5.2)

Scheme 5.2. Synthesis of Subunit 3



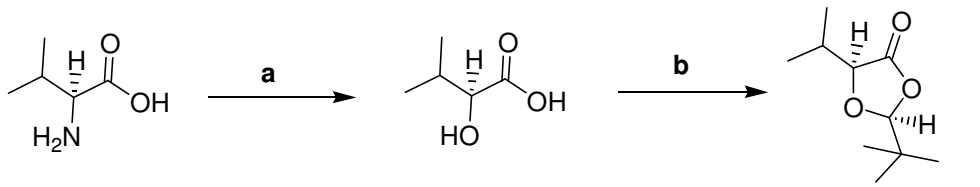
Reagents and conditions :

a) C_2Cl_6, LDA, THF; b) $HP(O)(OEt)_2$, LDA, THF, -78 ^oC; c) trans-2-Hexen-1-al, LDA, -78 ^oC, THF, 3.5 h,43 %.

5.3.3 Synthesis of (2S,5S)-2-(tert-Butyl)-5-isopropyl-1,3- dioxolan-4-one (4)

(*S*)-2-hydroxyisovaleric acid was commercially available only in small laboratory quantities and its relatively high cost raised a barrier to scale up synthesis. Instead, *L*-(+) valine was converted to (S)-2-hydroxyisovaleric acid in 56 % yield.[11] The acid-catalyzed condensation of (*S*)-2-hydroxyisovaleric acid with pivalaldehyde in hexanes produced the crude dioxolane (**4**) as a mixture of cis/trans isomers in a 23:1 ratio. Attempts to remove the undesired trans isomer by a short-path distillation produced a less desirable 5:1 cis/trans mixture. It was then found that the crude dioxolane **4** could be purified by crystallization from an ether/pentane or hexanes solution to provide exclusively the cis isomer. (Scheme 5.3)

Scheme 5.3. Synthesis of (2*S*,5*S*)-2-(*tert*-Butyl)-5-isopropyl-1,3- dioxolan-4-one



L-Valine, *S* — **2-Hydroxyisovaleric acid** — **4** (5*S*, 2*S*)

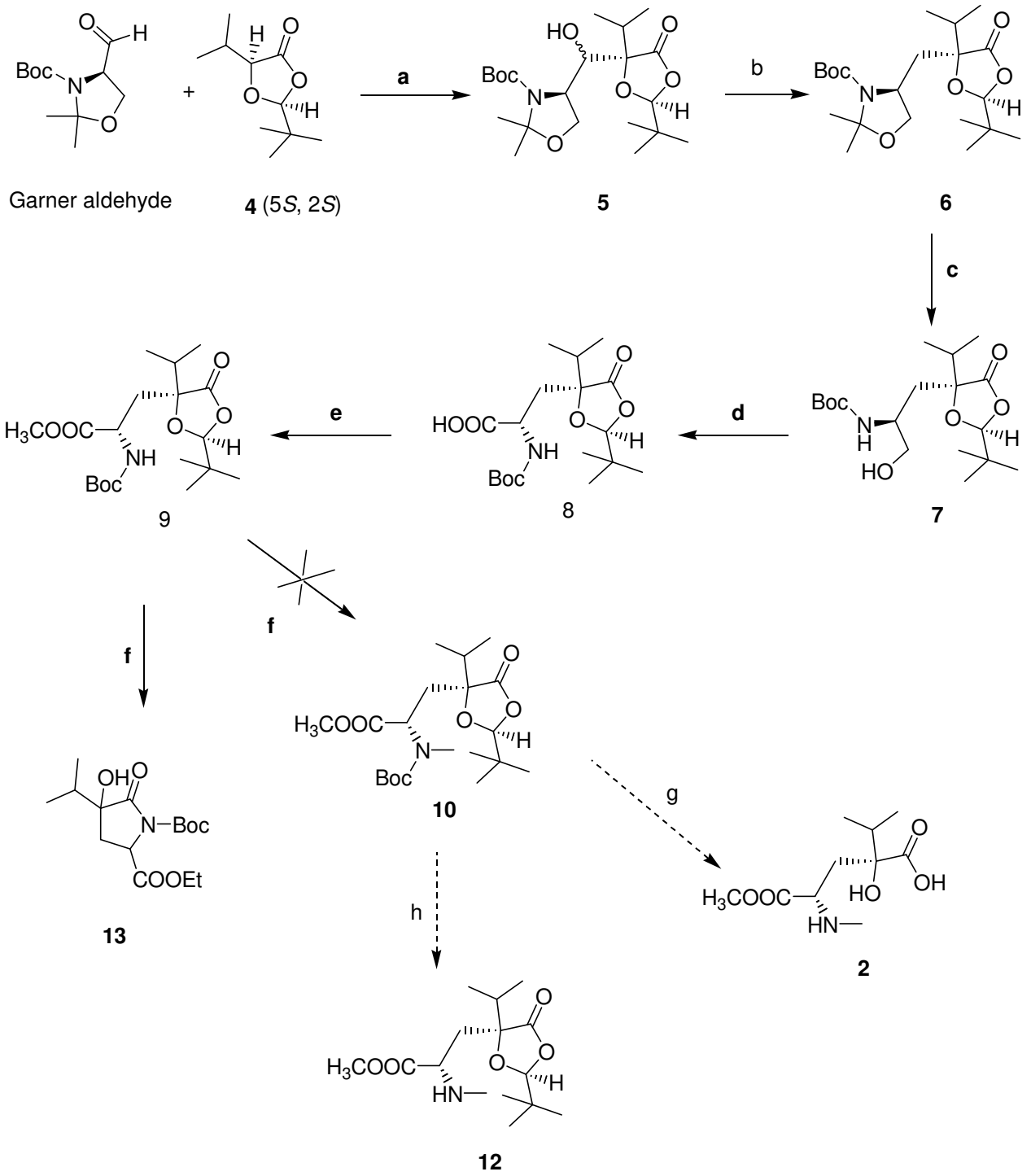
Reagents and conditions :

a)$NaNO_2$, H_2SO_4, H_2O, rt, overnight, 56 %; b) Pivalaldehyde, hexane, *p*-TsOH, 82 %.

Now both the dioxolane **4** and (R)-Garner aldehyde were in our hands. So we tried a steroselective alkylation; the reaction between (R)-Garner aldehyde and enolate of compound **4** was carried out by using the base LDA and THF at -78 ^{o}C for 2 h. After column chromatography purification the condensation product **5** was obtained in 50 % yield, and spectroscopic data including ^{1}H-NMR and ^{13}C-NMR confirmed the structure. This molecule possesses a *β*-hydroxy group which was removed by Barton dexoygenation[12,13] to provide the deoxygenated product **6** in 70 % yield with good

diasteroeoselectivity as determined by ^{1}H-NMR spectroscopy. Selective deprotection of **6** with PPTS in refluxing ethanol[14] for 1 h afford the Boc-protected β-aminoalcohol **7** in 50 % yield. The primary alcohol **7** was oxidized to carboxylic acid **8** with PDC in DMF without epimerization at the C4 position[14.] Esterification of acid **8** carried out by using MeI, K_2CO_3 in DMF afford the ethyl ester **9** in good yield. In order to get subunit **2** we decided to carry out the *N*-methylation of **9** by very mild reaction conditions, and therefore we used MeI and Ag_2O in DMF at 50 ^oC. Unfortunately, we were not able to obtain **10**, as the cyclised compound **13** was formed, as confirmed by ^{1}H-NMR. We tried this reaction in different reaction conditions but we were unable to obtain the desired compound **10**. After all these attempts we concluded that the methylation of intermediate **9** is difficult as the intermediate **9** prefers cyclisation. If we could have obtained intermediate **10** it could have been converted to intermediate **2** by treating with 0.1N HCl/HCO_2H, or selective deprotection of the Boc of intermediate **10** by $AlCl_3$ in DCM should have given compound **12**.

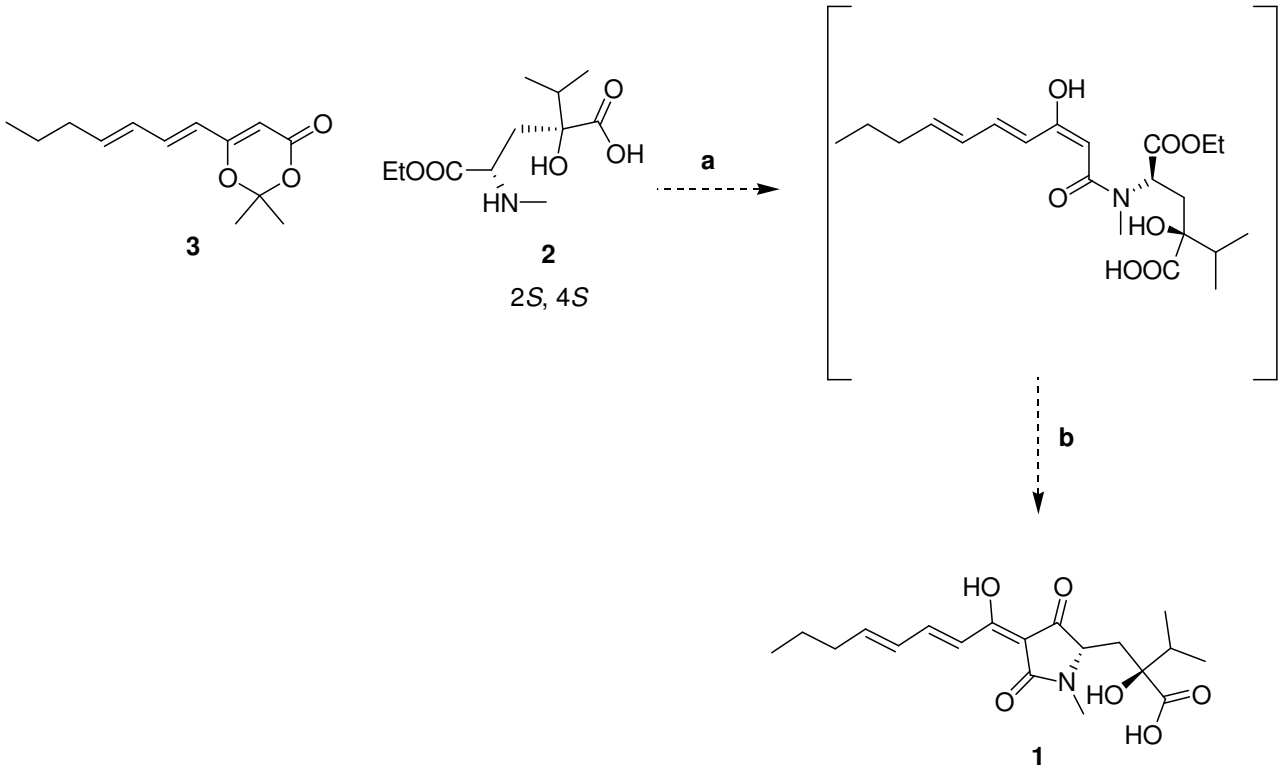
Scheme 5.4. Synthesis of intermediate 2



Reagents and conditions :

a) LDA, THF, -78 ^{o}C, 2 h, 50 %; b) NaH, CS_2, n-Bu_3SnH, AIBN, toluene, reflux, 2 h, 70 %; c) PPTS, EtOH/H_2O (95:5), reflux, 1 h. 50 %; d) PDC, DMF, rt, 48 h, 72 %. e) K_2CO_3, MeI, DMF, rt, overnight; f) Ag_2O, MeI, DMF, 50 ^{o}C, 12 h; g) 0.1N HCl/HCO_2H, reflux, 30 min. h) $AlCl_3$, DCM, rt.

Scheme.5.4.1



1

Reagents and conditions :

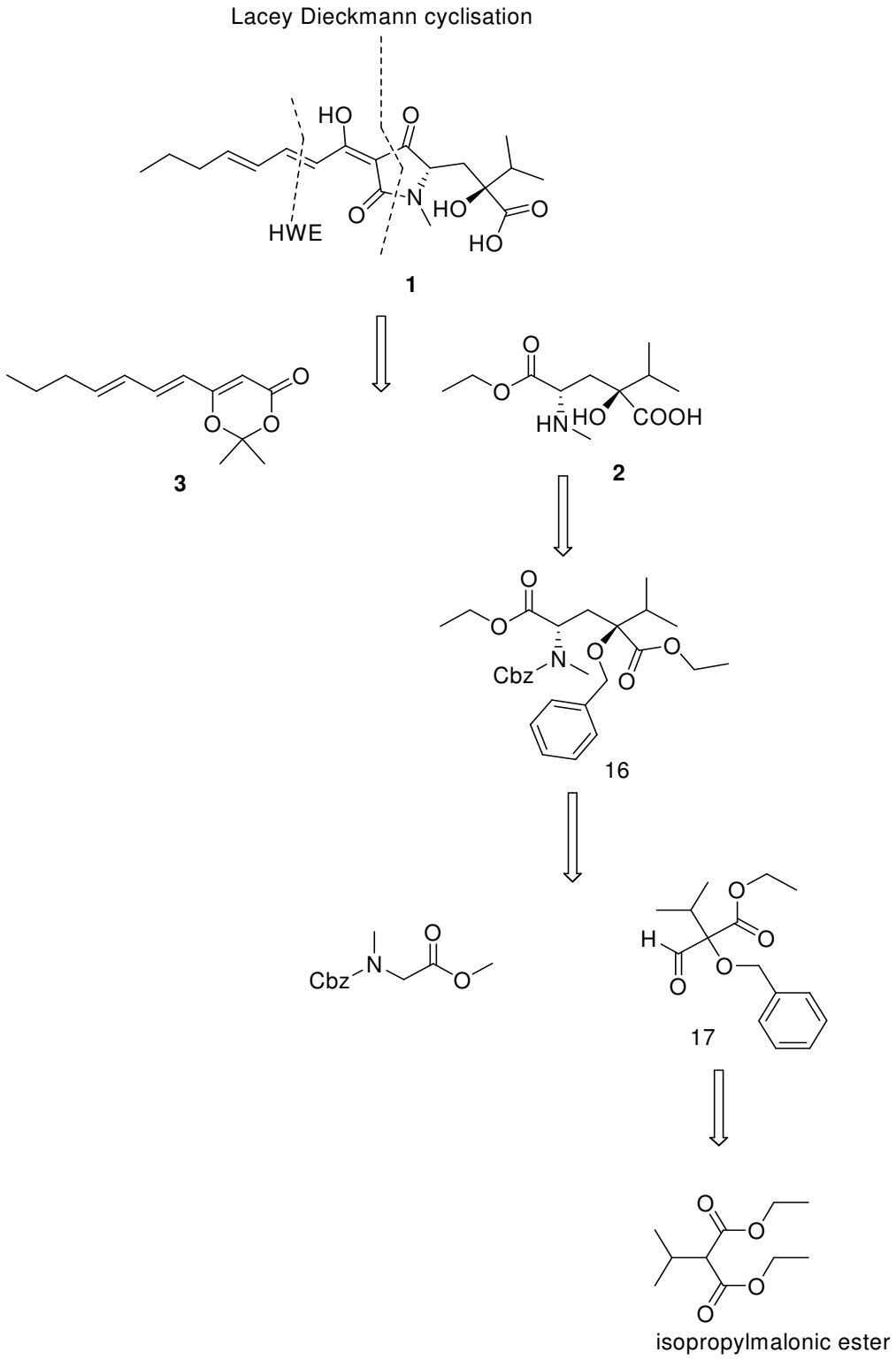
a) Toluene, reflux; b) K^{t}OBu, ^{t}BuOH.

5.4.Future perspectives

Once subunit **2** and **3** were in our hand they could be coupled by a Lacey-Dieckmann cyclisation in refluxing toluene, to complete the total synthesis of Harzianic acid **1a** (Scheme 5.4.1) As we were unable to obtain the intermediate **2**, we decided not to carry this synthetic pathway. In order to complete the synthesis of harzianic acid one should protect the side chain subunit **2** containing carboxylic acid, and hydroxyl group and these protection must not interact with nitrogen by intermolecular reaction that leads to cyclisation. So, looking for another synthetic approach toward the synthesis of harzianic acid, we found in literature that the side chain subunit **2** could be constructed by the procedure mentioned by Moldavi.[15] The author protected the acid as ethyl ester and hydroxyl group as a benzyl ether starting from commercial available isopropylmalonic ester. Considering the above facts we revised the retrosynthesis again as depicted in scheme 5.5.

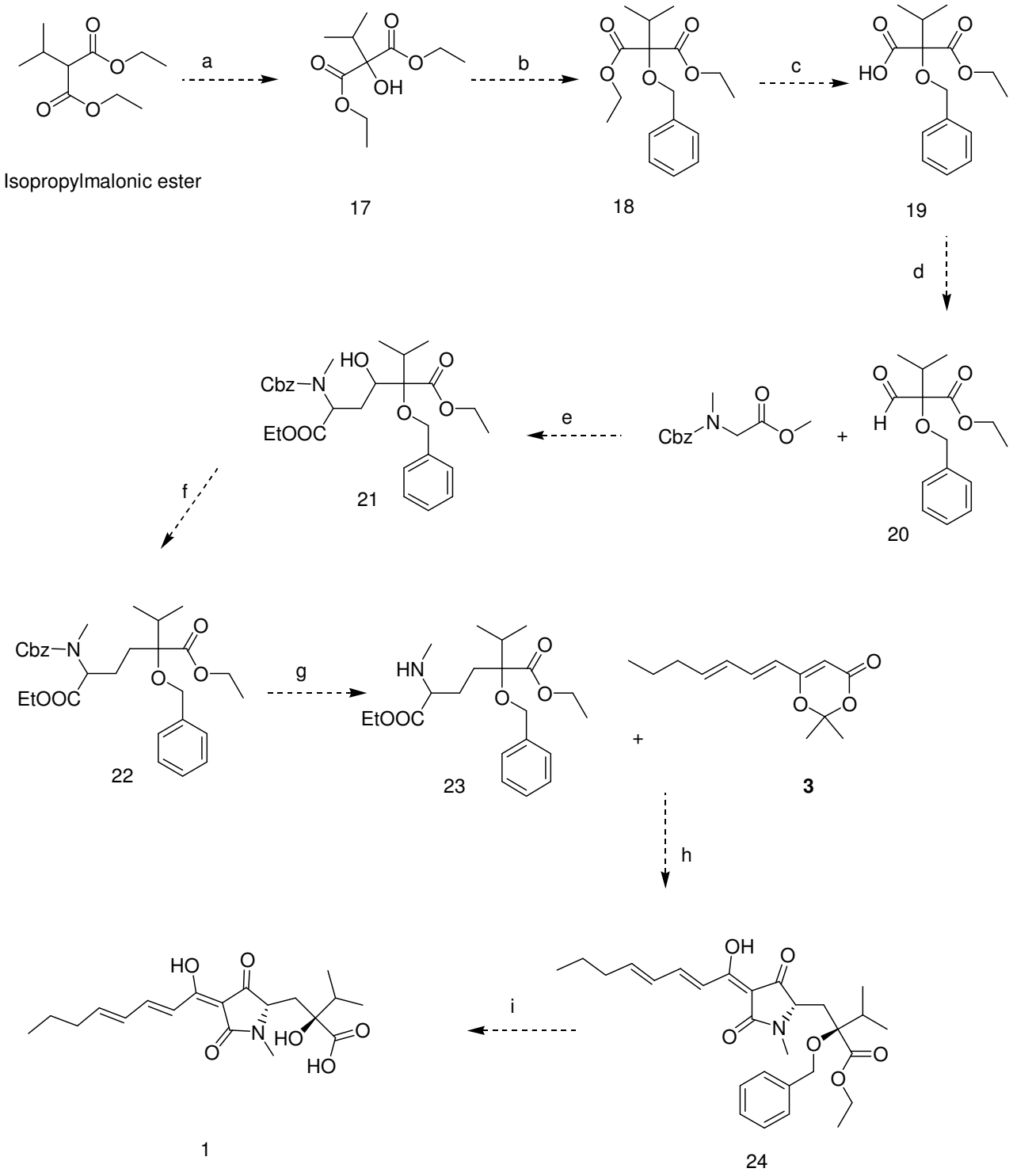
The disconnection identified the known fragments subunit **2** and subunit **3**. The fragment subunit **2** could be constructed by coupling of intermediate **17** and protected glycine, whereas intermediate **17** could be obtained from commercially available isopropylmalonic ester by partial modification of a literature procedure.[15]

Scheme 5.5


Lacey Dieckmann cyclisation
HO
O
N
HO
O
HWE
HO
1
3
2
HN
HO
COOH
Cbz
16
Cbz
17
isopropylmalonic ester

Considering this new retrosynthetic approach, we designed a new synthetic pathway for total synthesis of harzanic acid as a future perspective. Isopropylmalonic ester can be oxidized by benzoylperoxide in presence of $Na_2S_2O_3$ or $NaHSO_3$ to obtain **17** and the hydroxyl group must be protected as the benzyl ether **18** using benzyl chloride. Further partial hydrolysis of diester **18** could be achieved by literature procedure,[15] to obtain **19**. Finally, reduction of acid **19** followed by oxidation must give aldehyde **20** which must be reacted with the enolate of protected glycine[16] to give condensed product **21**. This molecule possesses a β-hydroxyl group which could be removed by Barton deoxygenation[12,13] to provide deoxygenated product **22**, and the diasteroeoselectivity can be determined by ^{1}H-NMR spectroscopy. The following steps include Cbz deprotection by H_2/Pd should give **23**. Once the subunit **23** and **3** are in our hand they can be coupled by a Lacey-Dieckmann cyclisation in refluxing toluene, to complete the total synthesis of Harzianic acid **1** (Scheme 5.6)

Scheme 5.6.

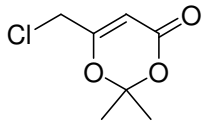

a
b
c
d
e
f
g
h
i
Isopropylmalonic ester
17
18
19
20
21
22
23
3
24
1
Cbz
EtOOC
HO
HN
OH

5.5. Conclusion

We have designed and synthesized the C8-C7' fragment of harzianic acid. The evaluation of the biological activity of this intermediates and completion of total synthesis of harzianic acid is in progress.

5.6 Experimental section

6-(chloromethyl)-2,2-dimethyl-4H-1,3-dioxin-4-one (14)

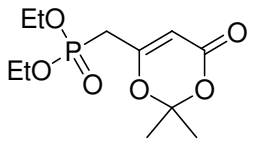


14

LDA (1.8M, 9.04 g, 54 mL) in 40 ml THF was cooled to -78^{o}C and treated with dioxene (7.71 mL 58.0 mmole) in THF (10 mL) over 20 min. During addition a fine yellow suspension formed. The enolate solution was stirred at -78 ^{o}C for an additional 15 min, then transferred to another flask containing hexachloroethane (20 g, 84.4 mmole) in THF (75 mL) at -50 ^{o}C to -55 ^{o}C over 30 min. When the addition was complete, the residual enolate was transferred with an additional portion of THF (10 mL). The resulting reaction mixture allowed to warm slowly at -25 ^{o}C over 30 min and poured into ice-cold aq 10 % HCl (100 mL) and the mixture was briefly shaken to discharge the red color. The organic layer was separated, and the aqueous layer extracted with ether (2 x 50 mL). The combined organic extracts were washed with saturated aqueous sodium bicarbonate solution (50 mL), saturated aqueous sodium chloride (50 mL), dried over Na_2SO_4, concentrated. The crude product was purified by column chromatography to afford **14** (6.0 g, 60 %)

R*f* 0.57 (Ethyl acetate/hexane 2:8).

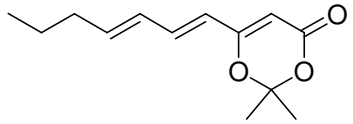
Diethyl (2,2-dimethyl-6-oxo-6H-1,3-dioxin-4-yl)methylphosphonate (15)



15

To a cold solution of tBuOK (11.43 g, 101.9 mmole) in DMF (100 mL) diethyl phosphate (14.59 g, 105.0 mmole) was added dropwise. The resulting solution was stirred in an ice bath for 20- 40 min and then treated dropwise with a solution of **14** (6.0 g, 33.9 mmole) in THF (25 mL) over 20 min. The resulting purple solution was stirred for additional 15 min at 0 ^{o}C and treated with conc. HCl until the purple colour was discharged (approx 3 mL). The resulting mixture was filtered through celite bed, and the collected solid was washed with THF (2 mL). The combined organic portions were treated with several grams of anhydrous potassium carbonate, filtered and the THF was removed with a rotary evaporator. DMF and excess diethyl phosphate were removed by distillation. The residue was diluted with ethyl acetate (100 mL) and placed in the refrigerator at 0 ^{o}C overnight. The solid which precipitated was removed by filtration and the filtrate was concentrated under reduced pressure and purified by column chromatography to afford **15** (4.2 g, 50 %).

6-Hepta-1,3-dienyl-2,2-dimethyl-[1,3]dioxin-4-one (3)

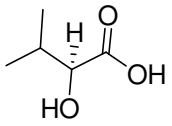


To a solution of **4** (1.50 g, 3.59 mmol) in THF (30 mL) was added LDA (1.8 M in heptane, 2.3 ml, 4.13 mmol) at 0 °C. After stirring for 30 min, the reaction mixture was cooled to –78 ºC. A solution of trans-hexen-1-al (0.528 g, 3.59 mmol) in THF (5 mL) was added slowly to the reaction mixture, and the resulting mixture was stirred at –78 ºC for 3.5 h. The reaction was quenched by adding saturated aqueous NH_4Cl, extracted with ethyl acetate (3 x 40 mL). The combined organic extracts were washed with brine, over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by flash column chromatography (Ethyl acetate/hexane 8:92) to afford olefin **3** (0.55 g**,** 43 %) as a colorless oil. R*f* 0.43 (Ethyl acetate/hexane 2:8).

^{1}H NMR ($CDCl_3$) δ: 6.90 (dd, J = 10.71, J = 15.55, 1H), 6.00- 6.24 (m, 2H), 5.88 (d, J = 5.27 Hz, 1H), 5.27 (s, 1H), 2.14 (m, 2H), 1.69 (s, 6H), 1.44 (m, 2H), 0.90 (t, J = 7.4 Hz, 3H).

^{13}C NMR ($CDCl_3$) δ: 163.8, 162.1, 143.8, 138.7, 129.1, 120. 9, 106.3, 94.0, 35.2, 25.1, 22.7.

(*S*)-(+)-Hydroxyisovaleric Acid

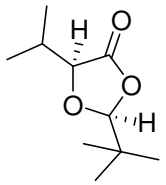


2-Hydroxyisovaleric acid

To a suspension of L-valine (14.2 g, 121.2 mmol) in H_2O (60 mL) conc. H_2SO_4 (3.41 mL, 63.00 mmol) was added slowly, affording a clear solution. Ice (40 g) was added to the solution, cooling the reaction mixture to below 5 °C. Cooling was aided by the use of an external ice bath. A solution of sodium nitrite (10.6 g, 6.2 mol) in water (40 mL) was added slowly, while maintaining the reaction temperature below 5 °C. When the addition of sodium nitrite was complete, the stirred solution was allowed to warm to ambient temperature slowly, overnight. The pH of the reaction mixture was adjusted to 3-4 by the slow addition of solid sodium bicarbonate, and this solution was extracted with ethyl acetate (3 x 40 mL). The combined organic extracts were dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was crystallized from ethyl acetate/hexanes (3:1) to afford (*S*)-(+)-Hydroxyisovaleric Acid (8 g, 56 %).

^{1}H NMR ($CDCl_3$) δ: 4.16 (d, J = 3.5 Hz, 1H), 2.17 (m, 1H), 1.07 (d, J = 6.7 Hz, 3H), 0.93 (d, J = 7.0 Hz, 3H).

(2*S*,5*S*)-2-(*tert*-Butyl)-5-isopropyl-1,3- dioxolan-4-one (4).

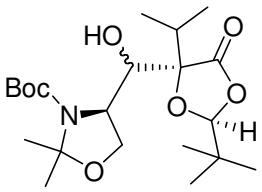


4 (5*S*, 2*S*)

To a solution of (*S*)-(+)-Hydroxyisovaleric acid (4 g, 33.8 mmol) in hexanes (32 mL) trimethylacetaldehyde (4.11 g, 47.0 mmol) and *p*-toluenesulfonic acid (0.041 g, 0.215 mmol) were added. The reaction mixture was heated under reflux until approximately 1.5 mL of water was collected. Heating was discontinued, and the colorless solution was allowed to cool to room temperature. The reaction mixture was poured into saturated aqueous $NaHCO_3$ solution (400 mL), and the layers were mixed well. The layers were separated, and the aqueous layer was extracted with ethyl acetate (3 x 40 mL). The combined organics were dried (Na_2SO_4), filtered, and the solvent was removed in vacuo to afford a colorless oil which consisted of a 20:1 mixture of diastereomers, by NMR analysis. Crystallization from hexanes afforded **4** (5.16 g, 82 %) as white crystals. R*f* 0.71 (Ethyl acetate/hexane 35:65).

^{1}H-NMR ($CDCl_3$) δ: 5.09 (d, J =1.2 Hz, 1H), 4.09 (dd, J =3.7 Hz, 1H), 2.19 (m, 1H),
1.11 (d, J = 7.1 Hz, 3H), 1.01 (d, J = 7.1 Hz, 3H), 0.99 (s, 9H).

(R)-tert-butyl4-(((2S,4R)-2-tert-butyl-4-isopropyl-5-oxo-1,3-dioxolan-4yl)(hydroxy)methyl)-2,2-dimethyloxazolidine-3-carboxylate (5)



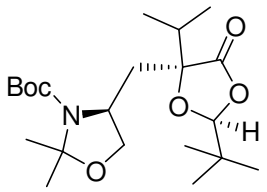
5

Dioxolanone **4** (0.5 g, 2.6 mmole) was dissolved in dry THF (12 mL) and cooled to -78 ^{o}C. LDA (1.8 M, 2.25 mL, 1.5 eq, 4.0 mmole) was added to above solution and reaction mixture stirred for 10 min. (R)-Garner aldehyde (0.615 g, 2.6 mmole in 3 mL THF) was cooled at 0 ^{o}C and added to the above solution at -78 ^{o}C. Then the reaction mixture was stirred continuously for 3 h at -78 ^{o}C, TLC (20 % EtOAc in hexane) showed reaction progress. Then reaction was quenched with 10 mL aq NH_4Cl and stirred for 1 h till rt. The organic mixture was extracted with Et_2O (35 mL x 3) and the combined organic layer dried over Na_2SO_4, filtered and concentrated under vacuum. The residue was purified by flash column chromatography (12 % EtOAc – hexane) to afford **5** (0.45 g, 45 %). R*f* 0.49 (Ethyl acetate/hexane 2:8).

^{1}H-NMR ($CDCl_3$) δ: 5.07 (s, 1H), 4.44 (m, 2H), 4.23(m, 1H), 3.90 (m, 1H), 2.64 (brs, 1H), 2.04 (m, 1H), 1.32-1.66 (m, 15H), 0.96-1.1 (m, 15H).

^{13}C NMR ($CDCl_3$) δ: 174.7, 172.5, 152.4, 110.8, 10.0, 94.2, 93.1, 88.3, 80.8, 70.1, 63.2, 62.8, 61.4, 58.1, 57.4, 35.3, 34.7, 34.3, 33.7, 33.1, 31.6, 31.3, 28.56, 27.0, 26.4, 24.6, 24.0, 22.7, 18.9, 18.5, 17.7, 16.3, 14.2

(S)-tert-butyl4-(((2S,4S)-2-tert-butyl-4-isopropyl-5-oxo-1,3-dioxolan-4-yl)methyl)-2,2 dimethyloxazolidine-3-carboxylate (6)



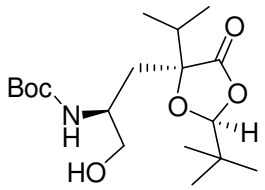
6

To a stirred solution of **5** (0.390 g, 0.93 mmole) at 0 ^{o}C in THF (10 mL) under N_2 was added NaH (0.150 g, 4 eq). The stirred mixture was allowed to warm to rt over 30 min before CS_2 (0.23 mL, 3.7 mmole) was added. After 10 min, methyl iodide (0.23 mL, 3.7 mmole) was added and the solution became yellow. After another 10 min, the yellow color disappeared and the solution was cooled to 0^{o}C. Saturated aqueous NH_4Cl (8 mL), diethyl ether (30 mL) and H_2O (4 mL) were added. The separated aqueous phase was extracted with diethyl ether (15 mL x 2) and the combined organic phases were washed with saturated aqueous NaCl (10 mL), dried over Na_2SO_4, filtered, and concentrated. The residue was azeotropically dried from benzene (20 mL) and then dissolved in toluene (50 mL). After that the resultant solution was deoxygenated with a stream of N_2 for 15 min, AIBN (0.616 g, 3.7 mmole) and Bu_3SnH (1.98 ml, 2.18 g, 7.5 mmole) were added, and the mixture was heated at 80^{o}C for 3 h under N_2. After the solution was cooled to rt it was diluted with ethyl acetate (30 mL) , washed with H_2O (10 mL) and saturated aq NaCl, dried over Na_2SO_4 and concentrated. The crude product purified by column chromatography (10 % EtOAc in Hexane) to afford **6** (0.216 g 70 %).

R*f* 0.5 (Ethyl acetate/hexane 2:8).

^{1}H-NMR ($CDCl_3$) δ: 5.20 (s, 1H), 4.11 (m, 1H), 3.95(m, 2H), 2.25 (m, 1H), 2.10 (m, 2H), 1.50 (s, 9H), 0.95-1.10 (m, 21H).

tert-butyl(S)-3-((2S,4S)-2-tert-butyl-4-isopropyl-5-oxo-1,3-dioxolan-4-yl)-1-hydroxypropan-2-ylcarbamate (7)



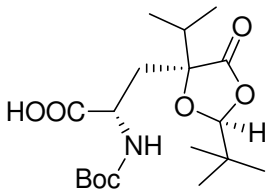
7

To a stirred solution of **6** (0.287 g, 0.72 mmole) in ethanol : H_2O (5 mL : 0.25 mL) and PPTS (0.23 g, 0.92 mmole) were added and the resulting mixture heated to reflux for 3 h. TLC showed the completion of reaction, solvent was evaporated and EtOAc (40 mL) added and washed with H_2O (10 mL). Organic layer dried on Na_2SO_4, filtered and concentrated to afford **7** (0.134g, 52 %)

R*f* 0.2 (Ethyl acetate/hexane 2:8)

^{1}H-NMR ($CDCl_3$) δ: 5.35 (s, 1H), 4.78 (m, 1H), 4.00 (m, 1H), 3.52-3.70 (m, 2H), 2.10 (m, 2H), 1.95 (m, 1H), 1.43 (s, 9H), 0.85-1.10 (m, 15H).

(S)-2-(tert-butoxycarbonyl)-3-((2S,4S)-2-tert-butyl-4-isopropyl-5-oxo-1,3-dioxolan-4-yl)propanoic acid (8)



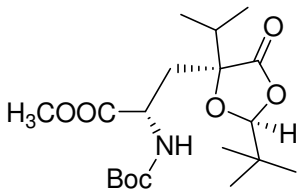
8

To a stirred solution of **7** (0.134 g, 0.34 mmole) in DMF (6 mL) PDC (0.846 g, 2.2 mmole) was added and resulting reaction mixture stirred at rt for 48 h. Then EtOAc (30 mL) was added to reaction mixture and washed with 0.1N HCl. Then organic layer dreed over Na_2SO_4, filtered and concentrated to obtained crude **8** (0.130 g) which was used for next reaction without purification.

R*f* 0.3 (Ethyl acetate/hexane 4:6)

^{1}H-NMR ($CDCl_3$) δ: 5.21 (s, 1H), 4.94 (m, 1H), 4.60 (m, 1H), 2.43 (m, 1H), 2.07 (m, 2H), 1.46 (s, 9H), 0.80-1.18 (m, 15H).

(S)-methyl-2-(tert-butoxycarbonyl)-3-((2S,4S)-2-tert-butyl-4-isopropyl-5-oxo-1,3-dioxolan-4-yl)propanoate (9)



9

To a solution of **8** (0.112 g, 0.3 mmole) in DMF (0.5 mL) cooled to 0 ^{o}C, K_2CO_3 (0.046 g, 0.31 mmole) was added and the reaction mixture stirred for 10 min. MeI (0.037 mL, 0.6 mmole) was added drop wiseand the resulting reaction mixture stirred overnight.TLC showed completion of reaction, reaction mixture was filtered, washed with EtOAc and extracted with EtOAc (15 mL x 3), combined organic layer were washed with H_2O, brine and then dried over Na_2SO_4, and purified by column chromatography, to afford **9** (0.070 g, 60 %) as a yellow oil.

R*f* 0.4 (Ethyl acetate/hexane 2:8)

^{1}H-NMR ($CDCl_3$) δ: 5.23 (s, 1H), 4.86 (m, 1H), 4.58(m, 1H), 3.76 (s, 3H), 2.35 (m, 1H), 2.02 (m, 2H), 1.43 (s, 9H), 1.07 (d, J = 6.4 Hz, 15H).

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CHAPTER 6

Synthesis of 3,7-Diaryl-1,4-dihydro[1,2,4]triazolo[5,1,-C][1,2,4]triazines

6.1 Introduction

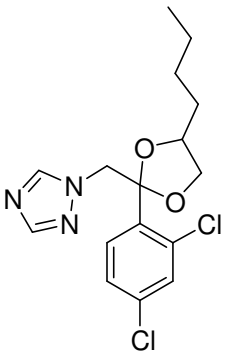
The chemistry of heterocyclic compounds continuous to be an explored field in organic chemistry. Heterocyclic compounds have long played a dominant role in medicinal chemistry and in the science of crop protection.[1] Heterocycles, their preparation, transformation, and properties, are undoubtedly a cornerstone of organic chemistry. Several books not only on heterocyclic chemistry[3-7] but also on some special aspects, such as heterocyclic name reaction, heterocyclic palladium-catalyzed reaction[8], heterocyclic carbene complexes,[9] and fluorinated heterocycles,[10] have been published recently. Approximately more than 70 % of all pharmaceuticals and agrochemicals bear at least one heterocyclic ring. In addition, some of the biggest commercial products to date, such as the broad-spectrum fungicide azoxystrobin (Amistar),[11] currently applied against disease of more than 100 different crops in more than 100 countries, belong to this huge heterocyclic group of active ingredients. There are two major reasons for the tremendous value of heterocycles for the lead optimization of pharmaceuticals and agrochemicals. The heterocyclic scaffold of a drug often has a positive impact on its synthetic accessibility and its physicochemical properties, driving the values of lipophilicity and solubility toward the optimal balanced range regarding uptake and bioavailability. Furthermore, heterocycles seem to be perfect bioisosteres of other iso- or heterocyclic rings as well as of several different functional groups, in most cases delivering through their similarity in structural shape and electronic distribution equal or even better biological efficacy.[12]

Among all these heterocyclic compounds, triazole and triazine derivatives occupy a unique position in heterocyclic chemistry. Substituted triazoles have received considerable attention during

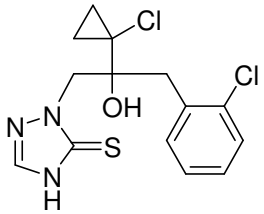
last two decades as they are endowed with a variety of biological activities and have wide range of therapeutic properties. A literature survey indicates that triazole derivatives possess different pharmacological activity and agrochemical activity such as analgesic, antiasthmatic, antihypertensive, fungicide, herbicide, and insecticide.[13-16]

The fungicide group of demethylation inhibitors (DMI), which contains the triazole fungicides, was introduced in the mid-1970s. Triazoles consist of numerous members, of which several are labeled or are in the process of being labeled for use on field crops: cyproconazole, flusilazole, flutriafol, metconazole, myclobutanil, propiconazole, prothioconazole, tebuconazole, and tetraconazole (Fig 1).

Fig. 1



Propiconazole



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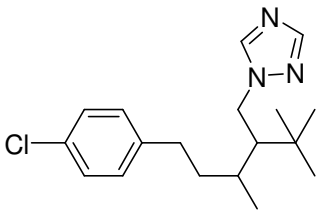
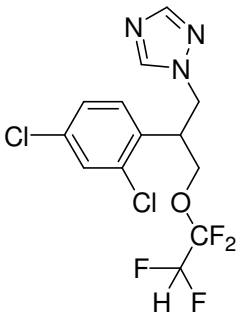
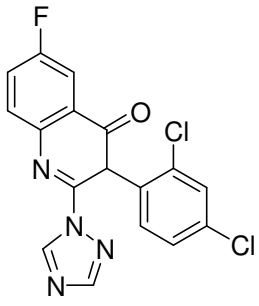


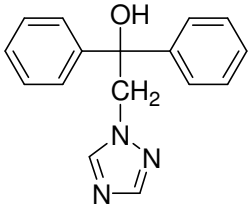
Tebuconazole



Tetraconazole



Fluquiconazole



Flutriafol

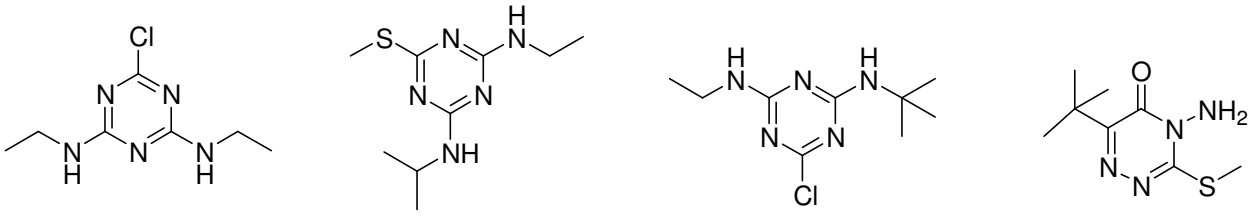
Triazoles are used on many different types of plants including field crops, fruit trees, small fruit, vegetables, and turf. These fungicides are highly effective against many different fungal diseases, especially powdery mildews, rusts, and many leaf-spotting fungi.

Over the past 50 years, triazines have made a great impact on agriculture and world hunger by assisting in the development of new farming methods, providing the greater farming and land use capabilities, and increasing crop yields. The initial discovery and development of triazine herbicides took place between year 1950 and 1970. During this period, a group of leading scientist and agriculture experts of the former chemical company J. R Geigy, Ltd., developed an idea for a new family of herbicides that would support the modern agriculture. A small team of Geigy chemist, biologists and agronomists made excellent progress in turning that initial concept into not only new compounds, but a new field of weed control research. The discovery and development of triazine herbicides were important scientific achievements and a significant example of cooperation among chemists, biologists, and agronomists from around the world. Development of the triazines led to unprecedented success in crop weed management.

Triazines are registered in over 80 countries and save billions of dollars a year. The triazine herbicides is the one book that presents a comprehensive view of the total science and agriculture of these chemicals. Triazines herbicides are commonly used weed killers in crop production lawns. They work by inhibiting the process of photosynthesis in weeds. They are absorbed by weed roots and translocated upward throughout the plant. Triazines are popular because they control a wide range of weeds. Both broad-leaf weeds and grasses are susceptible to herbicides of this chemical family. Most herbicides control either broad-leaf weeds or grasses but not both. The triazine family of herbicides, which includes atrazine, were introduced in the 1950s; they have the current distinction of being the herbicide family of greatest concern regarding groundwater contamination. Atrazine does not break down readily (within a few weeks) after being applied to soils of above neutral pH. Under alkaline soil conditions, atrazine may be carried into the soil profile as far as the

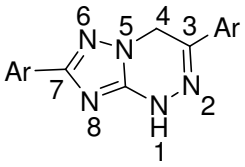
water lavel by soil water following rainfall causing the aforementioned contamination. Atrazine is thus said to have "carryover", a generally undesirable property for herbicides. Triazines consist of numerous members, of which several are labeled or are in the process of being labeled for use on field crops such as Ametryn, Cyanazine, Metribuzin, Bladex and Princep (Fig 2).

Fig. 2.



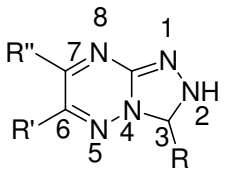
Atrazine **Ametryn** **Cyanazine** **Metribuzin**

Both these heterocycles have unique value in agrochemical industry. As a part of our ongoing studies on compounds endowed with fungicidal activity, we became interested in investigating new heterocyclic scaffolds as structural analogues of natural nucleic bases. In particular we focused on 1,4-dihydro[1,2,4]triazolo[5,1-*c*][1,2,4]triazine (Fig. 3) as a core structure and we planned to develop a general method for the synthesis of this ring system that might be amenable to the preparation of 3,7-diaryl-substituted derivatives **1** of triazole-triazine fused ring system in order to find new fungicides and to study the structure-activity relationship. The triazole-triazine fused ring system occurs in the structures of many pharmaceutical and agrochemical active compounds and other commercial substance. A wide range of synthetic methods, therefore, have been developed for construction of such fused ring systems.



1

Recently some compounds containing triazolo-fused heterocyclic ring such as 1,2,4-triazolo[5,1-*c*][1,2,4]triazin-7-one[17] and 1,2,4-triazolo[1,5-*a*]pyrimidine,[17,18] have shown potent antiviral activity. Triazolotriazine heterocycles are among the least known in the polyazaindolizine series[19-23] and 3,7-diaryl-1,4-dihydro[1,2,4]triazolo[5,1-*c*][1,2,4]triazines **1** have been even less studied. In particular triazolo[2,3-*b*]triazine **3** have never been described and only triazolo[4,3-b]triazines **2**. triazolo[3,4-*c*]triazines **4**, and triazolo[3,2-c]triazines **5** substitued with phenyl, amino, hydroxyl, or mercapto group are known.[19-24] The synthesis and properties of unsbustituted and methyl-substituted triazolo-triazine **2**-**5** were of interest in conjunction with previous investigations in the triazine[20] and azaindolizine series[25].



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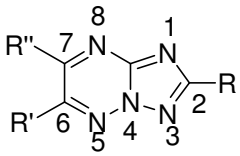

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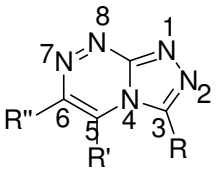

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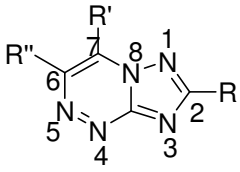


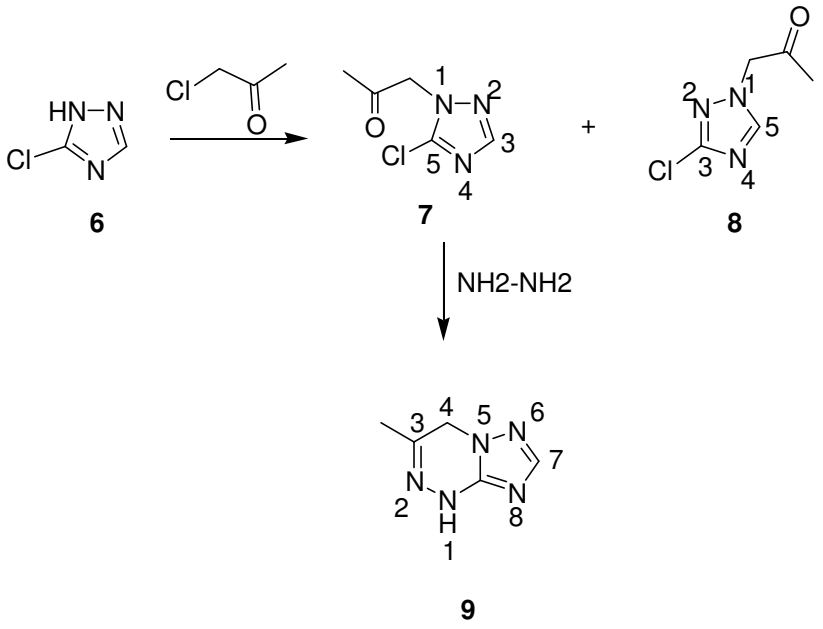
5

6.2 Reported Synthesis

Indeed, to the best of our knowledge, only one method for the synthesis of this class of compounds has been described so far. Namely, Hatzheim and co-workers[26-28] have reported a synthetic pathway from 2-amino-5-aryl-3-phenacyl-1,3,4-oxadiazolium bromides by ring transformation with hydrazine. Low yields and the requirement of multistep synthesis for each building block hamper the use of this approach as a general protocol for the preparation of 3,7-diaryl-1,4-dihydro[1,2,4]triazolo[5,1-*c*][1,2,4]triazines. In 1977 Daunis and Lopez[19] reported a procedure to

obtain 3-methyl-1,4-dihydro[1,2,4]triazolo[5,1-*c*][1,2,4]triazine **9** via reaction of 5-chloro-1*H*-1,2,4-triazole **6** with chloroacetone. After chromatographic separation of the two isomeric 1-(3-chloro-1*H*-1,2,4-triazol-1-yl)- and 1-(5-chloro-1*H*-1,2,4-triazol-1-yl)propan-2-ones, **8** and **7** respectively, the latter was treated with hydrazine to obtain the cyclized triazine in a 14 % overall yield (Fig. 3)

Fig. 3

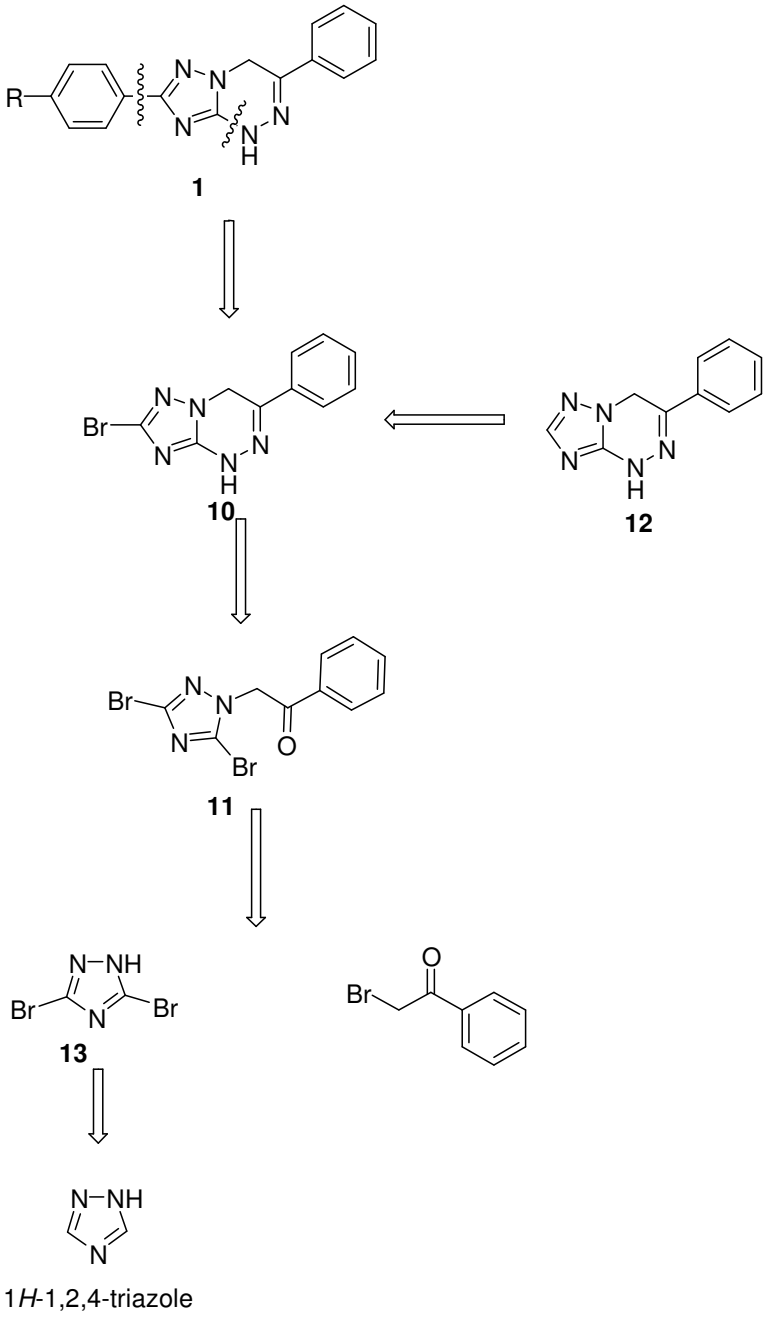


6.3 Retrosynthetic Approach

Treating 5-chloro-1*H*-1,2,4-triazole with chloroacetone by Daunis and Lopez[19] reported method gave a mixture of two regiosomers, due to unsymmetry of the molecule. We envisaged that this strategy could be implemented by alkylating 3,5-dibromo-1*H*-1,2,4-triazole instead of 5-chloro-1*H*-1,2,4-triazole. The use of 3,5-dibromo-1*H*-1,2,4-triazole could give two main advantages. First, the symmetry of the molecule overcomes regiochemical difficulties, as alkylation should occur at N-1 due to the higher nucleophilicity of the N–N system,[29] giving only one product. This would avoid a troublesome separation of isomers. Second, the presence of a further bromine group would allow the subsequent elaboration of the template, by introducing differently substituted aromatic rings via

Suzuki coupling with various commercially available boronic acids. The retrosynthesis is outlined in Scheme 1. The disconnection identified the fragment **11** as a phenacylated 3,5-dibromo[1,2,4]triazole which could be obtained by alkylation of commercially available 3,5-dibromotriazole with phenacyl bromide. Alternatively, the bromine atom could be removed by catalytic hydrogenation to obtain the monoaryl derivatives **12**.

Scheme 1

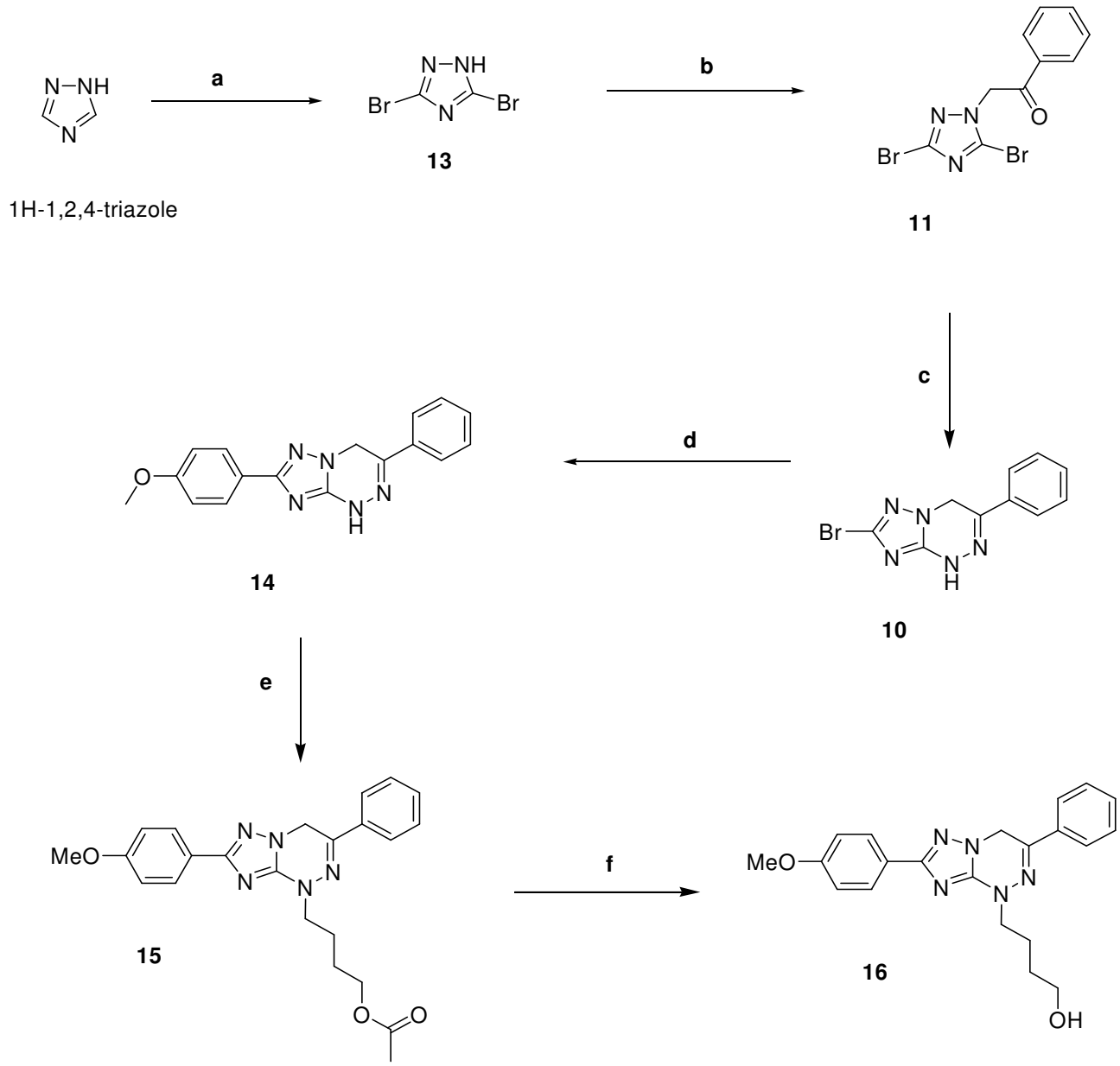


6.4 Synthesis

According to this strategy, 1*H*-1,2,4-triazole was brominated by using bromine with potassium bromide. The reaction was carried out at 110^{o}C for 2 h in the presence of H_2O as a solvent, the excess of bromine was removed by heating reaction mixture for another 15 min, and a solid precipitated out. This was filtered, washed with H_2O and dried under high vacuum to afford an 88 % yield of 3,5-dibromo-1*H*-1,2,4-triazole **13**,[30]which matches with commercial available **13**. Compound **13** was then alkylated with phenacyl bromide by using potassium carbonate as a base and acetone as a solvent, reaction carried out at rt for 4 h. (Scheme 1). As expected, alkylation occurred at N-1 and gave the 3,5-dibromo-1-phenacyltriazole **11** in 82 % yield. The product was fully characterized by ^{1}H NMR and ^{13}C NMR, whereas the alkylation at N-1 was confirmed by the presence of aromatic protons at δ 7-8 and of a methylene proton at δ 5.60 (2H, m). Treatment of **11** with hydrazine hydrate in methanol at reflux temperature for 6 h produced an intermediate that spontaneously cyclized to give 7-bromo-3-phenyl-1,4-dihydro[1,2,4]triazolo[5,1-*c*][1,2,4]triazine **10** in 60 % yield. The spectroscopic data, including ^{1}H-NMR, ^{13}C-NMR and mass spectra confirmed the structure. Finally, reaction with 4-methoxyphenylboronic acid under Suzuki conditions afforded **14** in 60–70% yield. Alternatively, the bromine atom was easily removed from **10** by catalytic hydrogenation in presence of methanol to obtain the monoaryl derivative **12** in 85% yield.[31] Appearance of a new peak at δ 7.80 (1H) in ^{1}H-NMR, and an additional peak at δ 146.3 in ^{13}C-NMR clearly indicated that debromination had occurred. We also investigated the possibility of introducing an alkyl fragment with a terminal hydroxy group on N-1, as this kind of chain seems to play a role in increasing the antiviral activity.[17] Thus, treatment of compound **14** with 4-bromobutyl acetate in presence of aqueous sodium carbonate at rt for 2 h afforded **15** in 70 % yield. Analysis of ^{1}H-HMR, and ^{13}C-NMR confirmed the structure, with the appearance of new peaks in the aliphatic region of ^{1}H-HMR, and ^{13}C-NMR. Further hydrolysis of ester **15** was carried out with sodium

methoxide in methanol[17] at 70 ^{o}C for 0.5 h, to give after acidic workup compound **16** in 42 % yield over two steps as a white solid. (Scheme 2).

Scheme 2



Reagents and conditions :

a) Br_2, KBr, KOH, H_2O, reflux, 1.5 h, 60 %; b) $PhCOCH_2Br$, K_2CO_3, acetone, rt, 4 h, 82 %; c) $NH_2NH_2.H_2O$, MeOH, reflux, 6 h, 60 %; d) 4-Methoxyphenylboronic acid, AcOH, $PdCl_2(PPh_3)_2$,

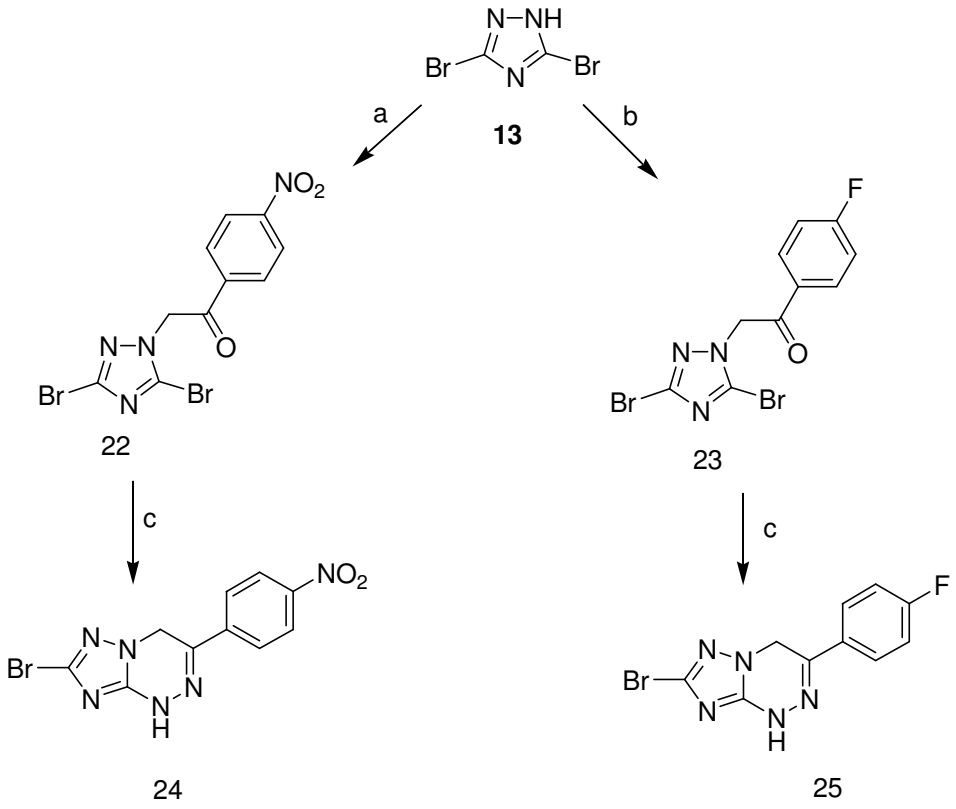
DMF, H_2O, 140 ^{O}C, 2 h, 60 – 70 %; e) $Br(CH_2)_4OAc$, Na_2CO_3, 100 ^{o}C, 2.5 h, 60 %; f) NaOMe, MeOH, reflux, 0.5 h, 70%.

6.5 Synthesis of analogues

Having the 3,7-diaryl-substituted derivatives **1** in our hands, the developed synthetic route allowed the preparation of a number of analogues which could be useful for determination of structure-activity relationship (SAR).

To determine the structure-activity relationship first we decided to replace bromine by differently substituted aromatic rings via Suzuki coupling with various commercially available boronic acids. We wanted to examine the role of different substituted aromatic rings in fungicidal activity. Accordingly, we chose five different aromatic boronic acids, namely, 3-acetlyphenylboronic acid, 3-hydroxypheylboronic acid, 3-pyridineboronic acid, 4-isopropoxyphenylboronic acid, and 4-trifluromehtylphenylboronic acid. We carried out Suzuki coupling between bromo intermediate **10** and boronic acids to obtain analogues **17**, **18**, **19**, **20** and **21** in 60-70 % yield (Scheme 4). We also synthesized the analogues of intermediate **10**. We treated 3,5-dibromo-1*H*-1,2,4-triazole **13**, with two more acyl bromides, namely 2-bromo-4'-nitroacetophenone and 2-chloro-4'-fluoroacetophenone by using potassium carbonate as base and acetone as solvent, reaction carried out at rt for 4 h to obtain intermediates **22** and **23** in 95-98 % yield. Treatment of these intermediate with hydrazine hydrate in methanol at reflux temperature for 6 h produced an intermediate that spontaneously cyclized to give analogue 7-bromo-3-(4-fluorophenyl)-1,4-dihydro-[1,2,4]triazolo[5,1-*c*][1,2,4]triazine **25** and 7-bromo-3-(4-nitrophenyl)-1,4-dihydro-[1,2,4]triazolo[5,1-*c*][1,2,4]triazine **24** (Scheme 3).

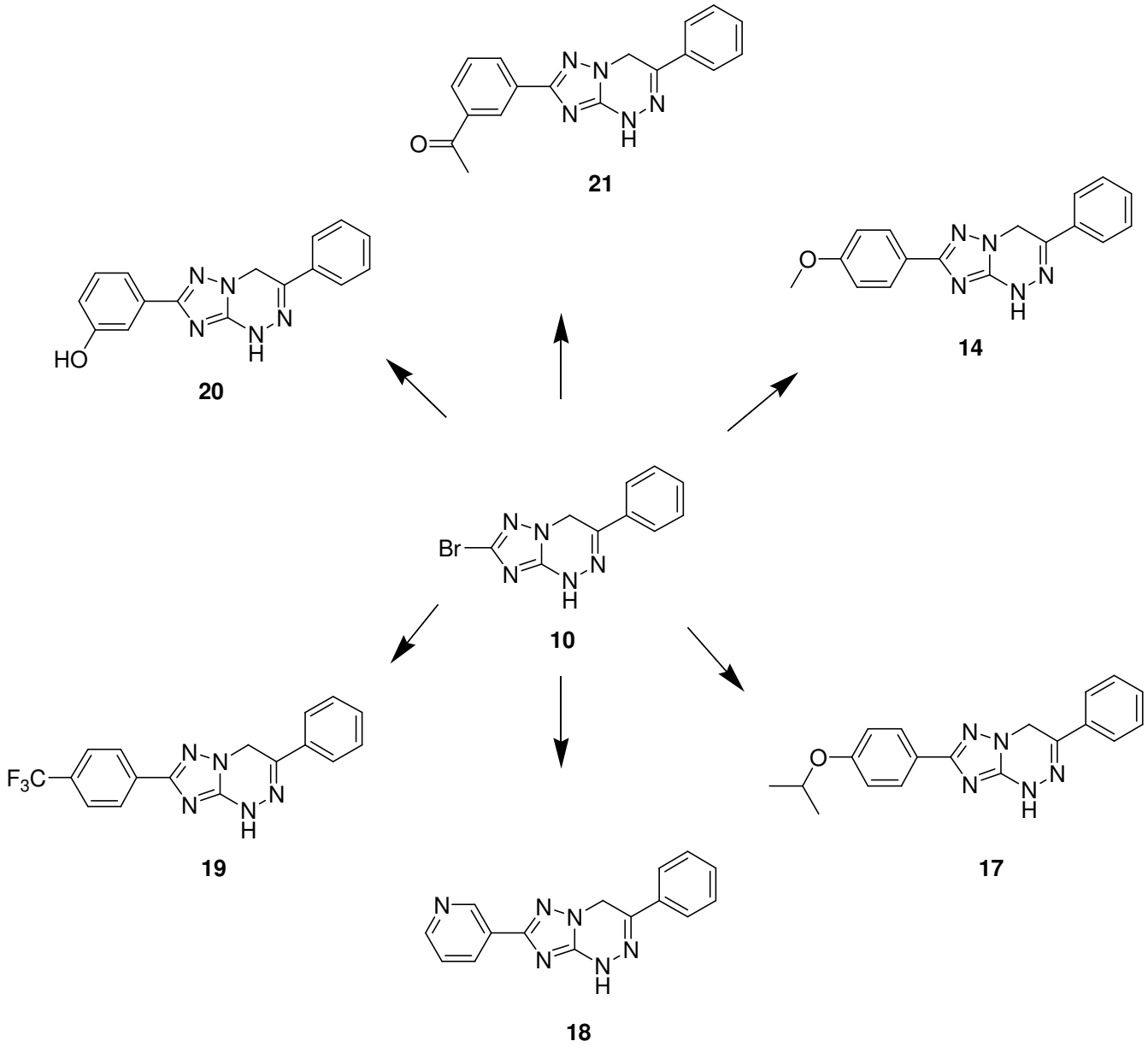
Scheme 3.



Reagents and conditions :

a) 2-Bromo-4'-nitroacetophenone, K_2CO_3, acetone, rt, 4 h, 98 %; b) 2-Chloro-4'-fluoroacetopheone, K_2CO_3, acetone, rt, 4 h, 98 %; c) $NH_2NH_2.H_2O$, MeOH, reflux, 6 h, 60-62 %.

Scheme 4.

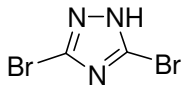


6.6 Conclusion

In conclusion, an expedient synthesis of 3,7-diaryl-1,4-dihydro[1,2,4]triazolo[5,1-*c*][1,2,4]triazines in three steps from 3,5-dibromo-1*H*-1,2,4-triazole has been developed. The reactions were conducted under easy-to-perform, mild conditions with moderate to good yields. The synthesis affords easy entry to previously unreported heterocyclic compounds of potential pharmacological and agrochemical interest.

6.7 Experimental section

3,5-Dbiromo-1H-[1,2,4]triazole (13)

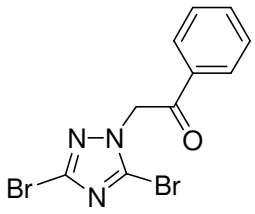


13

To a stirred solution of 1,2,4-triazole (1.38 g, 19.9 mmole) in 20 mL of H_2O KOH (2.80 g, 49.9 mmole, 2.5 eq) was added. This reaction mixture was heated to 80 ^oC for 10 min and then cooled to 30 ^oC, Then Br_2 (2.2 ml, 41.7 mmole, 2.1eq) in KBr-H_2O (1.90 g, 2 mL) solution was added to above reaction mixture drop by drop at 30 ^oC. On refluxing for 1.5 h a solid precipitated, the reaction mixture was cooled and the white solid was filtered through a Buchner funnel, and thoroughly dried to give **13** (2.70 g, 60 %) as a white solid.

m.p. = 215^oC.

2-(3,5-Dibromo-[1,2,4]triazol-1-yl)-1-phenyl-ethanone (11):



11

To the stirred solution of **13** (1.00 g, 4.40 mmole) and K_2CO_3 (0.61 g, 4.40 mmole) in acetone (10 mL) was added phenacyl bromide (0.88 g, 4.40 mmole) at 0°C. Then reaction mixture was stirred for 4 h at rt. The reaction mixture was filtered, the residue washed with acetone. The filtrate was concentrated under vacuum and the crude product crystallized form ethyl acetate and pet. ether, to obtain faint yellow solid **11** (1.23 g, 82 %).

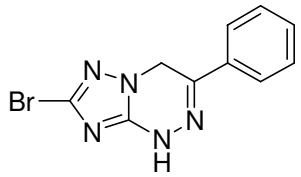
m.p. = 134°C, *Rf* = 0.33 (hexanes–EtOAc, 8:2).

^{1}H NMR (300 MHz, CDCl3): δ = 8.00–7.89 (m, 2H), 7.72–7.61 (m, 1H), 7.60–7.48 (m, 2H), 5.61 (m, 2H).

^{13}C NMR (75 MHz, CDCl3): δ = 188.6, 140.5, 134.7, 133.6, 131.5, 129.2 (2 C), 128.1 (2 C), 55.4.

Anal. Calcd for $C_{10}H_7Br_2N_3O$: C, 34.81; H, 2.05; N, 12.18. Found: C, 35.02; H, 2.33; N, 12.36.

7-Bromo-3-phenyl-1,4-dihydro[1,2,4]triazolo[5,1-*c*][1,2,4]triazine (10)



10

To a stirred solution of **11** (0.50 g, 1.4 mmol) in MeOH (7.5 mL), $H_2NNH_2 \cdot H_2O$ (0.265 g, 5.3 mmol, 3.6 equiv) was added at r.t. The reaction mixture was refluxed for 6 h, then was cooled to r.t. The precipitated white solid was collected by filtration, washed with MeOH to remove side products and unreacted starting material, then dried to obtain **10** as a white shiny solid; yield 0.24 g (60 %); m.p. = 281°C, *Rf* = 0.46 (hexanes–EtOAc, 1:1).

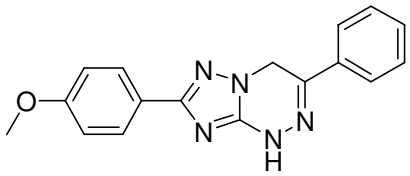
^{1}H NMR (300 MHz, DMSO-d_6): δ = 11.65 (s, 1H), 7.78–7.70 (m, 2H), 7.50–7.39 (m, 3H), 5.21 (s, 2H).

^{13}C NMR (75 MHz, DMSO-d_6): δ = 147.8, 138.3, 138.2, 134.4, 130.1, 129.0 (2 C), 125.4 (2 C), 45.2.

HRMS (ESI+): *m/z* [M + H]+ calcd for $C_{10}H_9BrN_5$: 278.0035; found: 278.0036; *m/z* [M + Na]+ calcd for $C_{10}H_8BrN_5Na$: 299.9855; found: 299.9856.

Anal. Calcd for $C_{10}H_8BrN_5$: C, 43.19; H, 2.90; N, 25.18. Found: C,43.36; H, 2.78; N, 25.22.

7-(4-Methoxyphenyl)-3-phenyl-1,4-dihydro[1,2,4]triazolo[5,1-*c*][1,2,4]triazine (14)



14

To a stirred solution of the bromide **10** (0.05 g, 0.18 mmol) in DMF (1 mL), 4-methoxyphenyl boronic acid (0.032 g, 1.2 equiv) and K_3PO_4 (0.114 g, 3 equiv) were added under nitrogen, followed by H_2O (0.5 mL). The reaction mixture was degassed with nitrogen for 15 min, then $PdCl_2(PPh_3)_2$ (0.006, 0.05 equiv) was added and the mixture was again degassed with nitrogen for 15 min. The reaction mixture was heated to 140 °C for 2 h, then was cooled to rt. and H_2O (5 mL) was added. The aqueous phase was extracted with EtOAc (3 × 10 mL). The combined organic layers were dried (Na_2SO_4), filtered and concentrated. The residue was purified by flash column chromatography (hexanes–EtOAc, 65:35); obtained **14** (0.037 g, 67 %). m.p. = 229–233 °C.

Rf = 0.6 (hexanes–EtOAc, 1:1).

^{1}H NMR (300 MHz, DMSO-d_6): δ = 11.53 (s, 1H), 7.88 (d, *J* = 8.8 Hz, 2H), 7.79–7.70 (m, 2H), 7.49–7.39 (m, 3H), 7.00 (d, *J* = 8.8 Hz, 2H), 5.31 (s, 2H), 3.78 (s, 3H).

^{13}C NMR (75 MHz, DMSO-d_6): δ = 160.4, 159.0, 147.3, 137.3, 134.8, 129.8, 129.0 (2 C), 127.6, 127.3, 125.4, 125.2, 124.0, 114.5, 114.4, 55.6, 45.4.

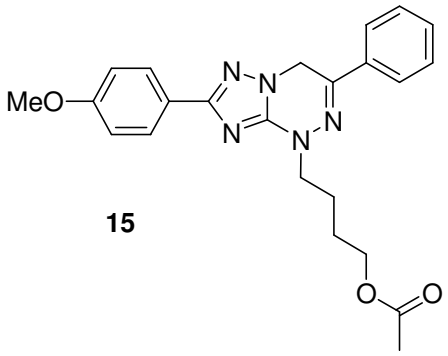
HRMS (ESI+): *m*/*z* [M + H]+ calcd for $C_{17}H_{16}N_5O$: 306.1354;

found: 306.1357; *m*/*z* [M + Na]+ calcd for $C_{17}H_{15}N_5NaO$: 328.1174;

found: 328.1172.

Anal. Calcd for $C_{17}H_{15}N_5O$: C, 66.87; H, 4.95; N, 22.94. Found: C, 67.06; H, 4.75; N, 22.78.

Acetic acid 4-[7-(4-methoxy-phenyl)-3-phenyl-4H-[1,2,4]triazolo[5,1-c]triazin-1-yl]butylester (15)



15

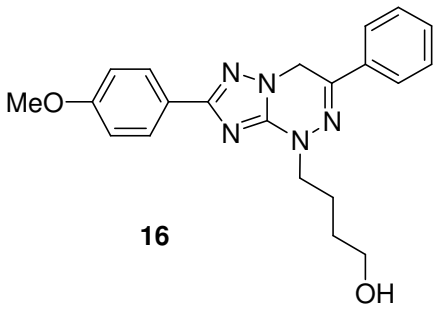
A suspension of 7-(4-methoxy-phenyl)-3-1,4-dihydro-[1,2,4]triazolo[5,1-c]triazine (**14**, 0.050 g, 0.16 mmol) in 17 % aqueous Na_2CO_3 (0.2 mL) was stirred at room temperature for 1.5 h; the precipitate was filtered off, dried and dissolved in DMF (1 mL). 4-Bromobutyl acetate (0.031 g, 0.16 mmol) was added to the resulting solution and the reaction was heated for 2.5 h at 100 ^oC. The mixture was cooled, then water was added. The aqueous solution was extracted with ethyl acetate (3 x 5 mL) and the combined organic layers were dried (Na_2SO_4), filtered and concentrated. The residue was purified by flash column chromatography (hexanes- EtOAc 8:2).to obtain **15** as a solid (0.042 g, 60 %), m.p. = 107-109 °C, R*f* = 0.8 (hexanes- EtOAc 1:1).

^{1}H NMR (300 MHz, DMSO-d*6*) = 7.91 (d, J = 8.5 Hz, 2H), 7.85-7.75 (m, 2H), 7.50-7.40 (m, 3H), 7.02 (d, J = 8.5 Hz, 2H), 5.34 (s, 2H), 4.07 (t, J = 6.4 Hz, 2H), 3.99 (t, J = 6.4 Hz, 2H), 3.80 (s, 3H), 1.98 (s, 3H), 1.90-1.76 (m, 2H), 1.75-1.60 (m, 2H).

^{13}C NMR (75MHz, DMSO-d*6*) = 170.8, 160.5, 158.9, 147.9, 137.4, 134.4, 130.0, 129.0 (x 2), 127.6 (x 2), 125.4 (x 2), 123.8, 114.6, 114.3, 63.8, 55.6, 51.0, 45.4, 25.3, 24.0, 21.0.

Anal Calcd for $C_{23}H_{25}N_5O_3$: C. 65.85; H. 6.01; N. 16.70. Found: C. 65.98; H. 5.95; N. 16.82.

4-[7-(4-Methoxy-phenyl)-3-phenyl-4H-[1,2,4]triazolo[5,1-c][1,2,4]triazin-1-yl]butan-1-ol (16)



16

Acetic acid 4-[7-(4-methoxy-phenyl)-3-phenyl-4H-[1,2,4]triazolo[5,1-c][1,2,4]triazin-1-yl]-butyl ester (**15;** 0.020 g, 0.05 mmol) was added to a solution of sodium methylate prepared from sodium (0.002 g, 0.09 mmol) and methanol (0.5 mL). The reaction mixture was refluxed for 0.5 h, cooled, neutralized with acetic acid and evaporated under vacuum. The products were isolated by column chromatography (EtOAc), to give **16** as a white solid (0.012 g, 70 %), m.p. = 127-130°C.

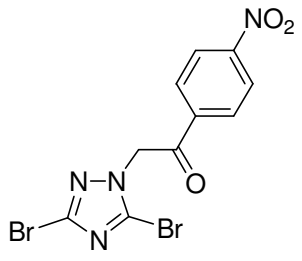
R*f* = 0.27 (hexanes-EtOAc 1:1).

^{1}H NMR (300 MHz, DMSO-d*6*) = 7.91 (d, J = 8.5 Hz, 2H), 7.83-7.73 (m, 2H), 7.53- 7.40 (m, 3H), 7.02 (d, J = 8.5 Hz, 2H), 5.35 (s, 2H), 4.44 (t, J = 4.88 Hz, 1H), 4.01 (t, J = 6.7 Hz, 2H), 3.51-3.40 (m, 2H), 3.90 (s, 3H), 1.98 (s, 3H), 1.89-1.74 (m, 2H), 1.57- 1.48 (m, 2H).

^{13}C NMR (75MHz, DMSO-d*6*) = 160.1, 158.5, 147.6, 136.8, 134.1, 129.6, 128.6 (x 2), 127.2 (x 2), 125.0 (x2), 123.5, 114.1 (x2)

Anal Calcd for $C_{21}H_{23}N_5O_2$: C. 66.83; H. 6.14; N. 18.55. Found: C. 66.53; H. 6.45; N. 18.74.

2-(3,5-Dibromo-1*H*-1,2,4-triazol-1-yl)-1-(4-nitrophenyl)ethanone (22)



22

To the stirred solution of **13** (0.5 g, 2.20 mmole) and K_2CO_3 (0.304 g, 2.20 mmole) in acetone (10 mL) was added 2-bromo-4'-nitroacetophenone (0.537 g, 2.20 mmole) at 0 ^oC. Then reaction mixture was stirred for 4 h at r.t.. The reaction mixture was filtered, the residue washed with acetone. The filtrate was concentrated under vacuum. The product was crystallized (EtOAc–petroleum ether) to give **22** (1.681 g,98 %), as a yellow solid.

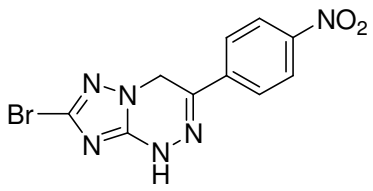
m.p. = 148–153 °C, *Rf* = 0.26 (hexanes–EtOAc, 8:2).

^{1}H NMR (300 MHz, DMSO-*d*6): δ = 8.46–8.36 (m, 2H), 8.35–8.25 (m, 2H), 6.18 (s, 2H).

^{13}C NMR (75 MHz, DMSO-*d*6): δ = 190.6, 150.7, 139.5, 138.1, 132.8, 130.0 (2 C), 124.1 (2 C), 56.5.

Anal. Calcd for $C_{10}H_6Br_2N_4O_3$: C, 30.80; H, 1.55; N, 14.37. Found: C, 31.02; H, 1.21; N, 14.60.

7-Bromo-3-(4-nitro-phenyl)-1,4-dihydro-[1,2,4]triazolo[5,1-c][1,2,4]triazine (24)



24

To a stirred solution of **22** (1 g, 2.5 mmol) in MeOH (15 mL), $H_2NNH_2 \cdot H_2O$ (0.517 g, 9.2 mmol, 3.6 equiv) was added at r.t. The reaction mixture was refluxed for 6 h, then was cooled to r.t. The precipitated white solid was collected by filtration, washed with MeOH to remove side products and unreacted starting material, then dried to obtain **24** as a white shiny solid (0.486 g, 60 %).

m.p. = 291-295 ^{o}C, R*f* = 0.43

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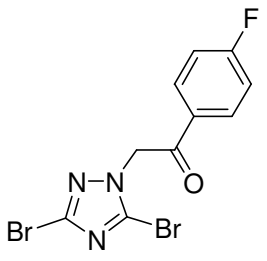
^{1}HMR (300 MHz, DMSO-d*6*) = 12.05 (brs, 1H), 8.31-8.21 (m, 2H), 8.01-7.90 (m, 2H), 5.30 (s, 2H).

^{13}C NMR (75MHZ, DMSO-d6) = 147.6, 146.9, 140.1, 138.1, 136.1, 126.1 (x 2), 123.8 (x 2), 45.1.

Anal Calcd for $C_{10}H_7BrN_6O_2$: C. 37.17, H. 2.18, N. 26.01. Found: C. 37.39, H. 2.01, N. 26.26.

2-(3,5-Dibromo-1*H*-1,2,4-triazol-1-yl)-1-(4-fluorophenyl)ethanone (23)



23

To the stirred solution of **13** (0.5 g, 2.20 mmole) and K_2CO_3 (0.304 g, 2.20 mmole) in acetone (10 mL) was added 2-Bromo-4'-fluoroacetophenone (0.380 g, 2.20 mmole) at 0 ^{o}C. Then reaction mixture was stirred for 4 h at rt. Reaction mixture was filtered, residue washed with acetone, filtrate was concentrated under vacuum. The product was crystallized (EtOAc–petroleum ether) to give a yellow solid **23** (1.681 g, 98 %).

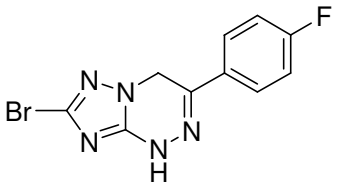
m.p. = 154–159 °C, *Rf* = 0.36 (hexanes–EtOAc, 8:2).

^{1}H NMR (300 MHz, DMSO-*d*6): δ = 8.21–8.11 (m, 2H), 751–7.41 (m, 2H), 6.08 (s, 2H).

^{13}C NMR (75 MHz, DMSO-*d*6): δ = 189.8, 165.8, 139.4, 132.8, 131.6 (2 C), 130.4, 116.2 (2 C), 56.1.

Anal. Calcd for $C_{10}H_6Br_2FN_3O$: C, 33.09; H, 1.67; N, 11.58. Found: C, 33.29; H, 1.41; N, 11.66.

7-Bromo-3-(4-fluoro-phenyl)-1,4-dihydro-[1,2,4]triazolo[5,1-c][1,2,4]triazine (25):



25

To a stirred solution of **23** (0.65 g, 1.7 mmol) in MeOH (9 mL), $H_2NNH_2 \cdot H_2O$ (0.361 g, 6.4 mmol, 3.6 equiv) was added at rt. The reaction mixture was refluxed for 6 h, then was cooled to rt. The precipitated white solid was collected by filtration, washed with MeOH to remove side products and unreacted starting material, then dried to obtain **25** as a white shiny solid (0.354 g, 67 %).

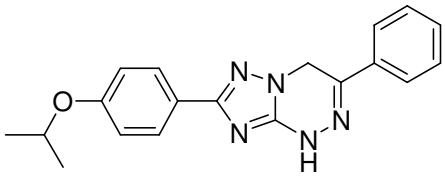
m.p. = 297-300 ^{o}C, R*f* = 0.46

^{1}HMR (300 MHz, DMSO-d*6*) = 11.68 (brs, 1H), 7.84-7.74 (m, 2H), 735-7.25 (m, 2H), 5.24 (s, 2H).

^{13}C NMR (75MHZ, DMSO-d6) = 162.9, 147.3, 138.0, 137.0, 130.6, 127.3 (x 2), 115.6 (x 2), 44.9.

Anal Calcd for $C_{10}H_7BrFN_5$: C. 40.56, H. 2.38, N. 23.65. Found: C. 40.20, H. 2.60, N. 23.45.

7-(4-Isopropoxyphenyl)-3-phenyl-1,4-dihydro-[1,2,4]triazolo[5,1-c][1,2,4]triazine (17)



17

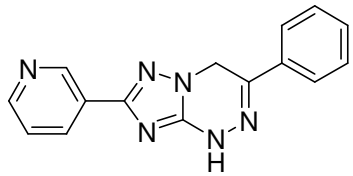
To a stirred solution of the bromide **10** (0.05 g, 0.18 mmol) in DMF (1 mL), 4-isopropoxyphenylboronic acid, (0.032 g, 1.0 equiv) and K_3PO_4 (0.114 g, 3 equiv) were added under nitrogen, followed by H_2O (0.5 mL). The reaction mixture was degassed with nitrogen for 15 min, then $PdCl_2(PPh_3)_2$ (0.006, 0.05 equiv) was added and the mixture was again degassed with nitrogen for 15 min. The reaction mixture was heated to 140 °C for 2 h, then was cooled to rt. and H_2O (5 mL) was added. The aqueous phase was extracted with EtOAc (3 × 10 mL). The combined organic layers were dried (Na_2SO_4), filtered and concentrated. The residue was purified by flash column chromatography (hexane- EtOAc 65:35) to afford **17** (0.035 g 60 %).

m.p. = 258-261 °C, R*f* = 0.3 (hexane- EtOAc 1:1).

^{1}H NMR (300 MHz, DMSO-d*6*) = 11.52 (s, 1H), 7.85 (d, J = 8.8 Hz, 2H), 7.80-7.70 (m, 2H), 7.48-7.37 (m, 3H), 6.96 (d, J = 8.8 Hz, 2H), 5.31 (s, 2H), 4.65 (ept, J 6.2 Hz, 1H), 1.26 (d, J = 6.2 Hz, 6H).

Anal Calcd for $C_{19}H_{19}N_5O$: C. 68.45; H. 5.74; N. 21.01. Found: C. 68.71; H. 5.55; N. 21.30.

3-Phenyl-7-pyridin-3-yl-1,4-dihydro-[1,2,4]triazolo[5,1-c][1,2,4]triazine (6f)



18

To a stirred solution of the bromide **10** (0.2 g, 7.1 mmol) in DMF (2.5 mL), 3-pyridineboronic acid, (0.088 g, 1.0 equiv) and K_3PO_4 (0.459 g, 3 equiv) were added under nitrogen, followed by H_2O (1.5 mL). The reaction mixture was degassed with nitrogen for 15 min, then $PdCl_2(PPh_3)_2$ (0.025g, 0.05 equiv) was added and the mixture was again degassed with nitrogen for 15 min. The reaction mixture was heated to 140 °C for 2 h, then was cooled to rt. and H_2O (20 mL) was added. The aqueous phase was extracted with EtOAc (3 × 30 mL). The combined organic layers were dried (Na_2SO_4), filtered and concentrated. The residue was purified by flash column chromatography (40 % EtOAc in hexane) to afford **18** (0.140 g, 70 %)

m.p. = 295-299°C, R*f* = 0.2 (hexane- EtOAc 1:1).

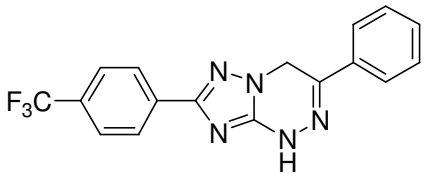
^{1}HMR (300 MHz, DMSO-d*6*) = 11.70 (brs, 1H), 9.18-9.08 (m,1H), 8.67-8.57 (m, 1H), 8.31-8.22 (m, 1H), 7.85-7.71 (m, 2H), 7.58-7.39 (m, 4H), 5.39 (s, 2H).

.

^{13}C NMR (75MHZ, DMSO-d6) = 150.1, 147.3, 146.7, 137.3, 134.3, 132.9, 129.5, 128.6 (x 2), 126.8, 125.0 (x 2), 124.0, 45.3.

Anal Calcd for $C_{15}H_{12}N_6$C. 65.21, H. 4.38, N. 30.42. Found: C. 65.49, H. 4.11, N. 30.13.

3-Phenyl-7-(4-trifluoromethylphenyl)-1,4-dihydro-[1,2,4]triazolo[5,1-c][1,2,4]triazine (19)



19

To a stirred solution of the bromide **10** (0.1 g, 0.36 mmol) in DMF (2 mL), 4-trifluoromehtylphenylboronic acid, (0.068 g, 1.0 equiv) and K_3PO_4 (0.228 g, 3 equiv) were added under nitrogen, followed by H_2O (1 mL). The reaction mixture was degassed with nitrogen for 15 min, then $PdCl_2(PPh_3)_2$ (0.012 g, 0.05 equiv) was added and the mixture was again degassed with nitrogen for 15 min. The reaction mixture was heated to 140 °C for 2 h, then was cooled to rt. and H_2O (20 mL) was added. The aqueous phase was extracted with EtOAc (3 × 15 mL). The combined organic layers were dried (Na_2SO_4), filtered and concentrated. The residue was purified by flash column chromatography (hexane- EtOAc 7:3) to afford **19** (0.075 g, 61 %)

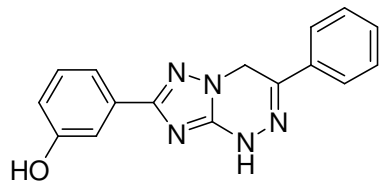
m.p. = 285-288 °C, R*f* = 0.78 (hexane- EtOAc 1:1).

^{1}H NMR (300 MHz, DMSO-d*6*) = 11.64 (s, 1H), 8.15 (d, J = 8.1 Hz, 2H), 7.81 (d, J = 8.1 Hz , 2H), 7.79-7.73 (m, 2H), 7.48-7.40 (m, 3H), 5.37 (s, 2H).

^{13}C NMR (75MHz, DMSO-d*6*) = 157.9, 147.8, 137.6, 135.2, 134.7, 129.8, 128.9, 126.8, 126.4, 125.4, 45.7.

Anal Calcd for $C_{17}H_{12}F_3N_5$: C. 59.48; H. 3.52; N. 20.40. Found: C. 59.75; H. 3.82; N. 20.11.

3-(3-Phenyl-1,4-dihydro-[1,2,4]triazolo[5,1-c][1,2,4]triazin-7-yl)-phenol (20)



20

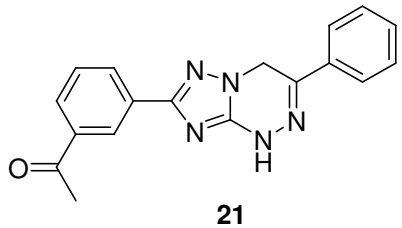
To a stirred solution of the bromide **10** (0.1 g, 0.36 mmol) in DMF (2 mL), 3-hydroxypheylboronic acid, (0.049 g, 1.0 equiv) and K_3PO_4 (0.228 g, 3 equiv) were added under nitrogen, followed by H_2O (1 mL). The reaction mixture was degassed with nitrogen for 15 min, then $PdCl_2(PPh_3)_2$ (0.012 g, 0.05 equiv) was added and the mixture was again degassed with nitrogen for 15 min. The reaction mixture was heated to 140 °C for 2 h, then was cooled to rt. and H_2O (20 mL) was added. The aqueous phase was extracted with EtOAc (3 × 15 mL). The combined organic layers were dried (Na_2SO_4), filtered and concentrated. The residue was purified by flash column chromatography (hexane- EtOAc 6:4) to afford **20** (0.075 g, 61 %).

R*f* = 0.47(hexane- EtOAc 1:1).

^{1}H NMR (300 MHz, DMSO-d*6*) = 11.54 (s, 1H), 9.55 (s, 1H), 7.80-7.67 (m, 2H), 7.50-7.34 (m, 5H), 7.22 (dd, J = 8.07, 8.07 Hz 1H), 6.78 (dd, J = 8.07, 2.2 Hz, 1H), 5.33 (s, 2H).

Anal Calcd for $C_{16}H_{13}N_5O$: C. 65.97; H. 4.50; N. 24.04. Found: C. 66.11; H. 4.21; N. 24.34.

1-[3-(3-Phenyl-1,4-dihydro-[1,2,4]triazolo[5,1-c][1,2,4]triazin-7-yl)-phenyl]-ethanone (21)



21

To a stirred solution of the bromide **10** (0.1 g, 0.36 mmol) in DMF (2 mL), 3-acetylpheylboronic acid, (0.058 g, 1.0 equiv) and K_3PO_4 (0.228 g, 3 equiv) were added under nitrogen, followed by H_2O (1 mL). The reaction mixture was degassed with nitrogen for 15 min, then $PdCl_2(PPh_3)_2$ (0.012 g, 0.05 equiv) was added and the mixture was again degassed with nitrogen for 15 min. The reaction mixture was heated to 140 °C for 2 h, then was cooled to rt. and H_2O (20 mL) was added. The aqueous phase was extracted with EtOAc (3 × 15 mL). The combined organic layers were dried (Na_2SO_4), filtered and concentrated. The residue was purified by flash column chromatography (hexane- EtOAc 6:4). To afford **21** (0.079 g, 70 %)

m.p. = 264-267 °C, R*f* = 0.64 (hexane- EtOAc 1:1).

^{1}H NMR (300 MHz, DMSO-d*6*) = 11.62 (s, 1H), 8.45 (s, 1H), 8.19 (d, J = 8.2 Hz, 1H), 8.00 (d, J = 8.2 Hz, 1H), 7.81-7.70 (m, 2H), 7.62 (dd, J = 8.2, 8.2 Hz, 1H), 7.51- 7.40 (m, 3H), 5.37 (s, 2H), 2.62 (s, 3H).

^{13}C NMR (75MHz, DMSO-d*6*) = 197.6, 158.0, 147.3, 137.2, 134.3, 131.4, 129.9, 129.5, 129.3, 128.9, 128.6 (x 2), 125.0 (x 2), 45.2, 26.8.

Anal Calcd for $C_{18}H_{15}N_5O$: C. 68.13; H. 4.76; N. 22.07. Found: C. 68.42; H.

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