A Non-coded β2,2-Amino Acid with Isoxazoline Core Able to Stabilize Peptides Folding Through an Unprecedented Hydrogen Bond

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Dedicated to Prof. Cesare Gennari on the occasion of his 70th birthday.

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Abstract: New peptidomimetics containing a β2,2-isoxazoline amino acid, i.e. 5-(aminomethyl)-3-phenyl-4,5-dihydroisoxazole-5-carboxylic acid (Isox-β2,2AA), were prepared and studied by NMR and theoretical calculation. Although similar amino acid derivatives have already been prepared via 1,3-dipolar cycloaddition reaction, neither experimental details nor characterization were found and they were never used for peptide synthesis. Both enantiomers were inserted in peptide sequences to verify their ability to induce a secondary structure. We found that an unexpected conformation is given by R-Isox-β2,2AA, inducing the folding of short peptides thanks to an unprecedented H-bond involving C=N of the isoxazoline side chain of our β2,2-AA.

Introduction

In billions years of evolution, Nature has produced a basic set of 'molecular bricks', such as 20 amino acids (AAs) that are at the basis of the living world.[1] More exotic extraterrestrial amino acids, such as β-(aminomethyl)succinic acid, were found in the Murchison meteorite.[2] The continuous search for new non-

natural amino acidic systems to prepare novel bio-inspired molecules has always been a long-standing goal for scientists.

Although they are such simple molecules, peptides have unique properties, such as biocompatibility, electronic conduction, chemical and structural diversity, robustness and ease of large-scale synthesis. Their great versatility makes them very attractive tools for disparate applications, from catalysis[3–5] to electrochemistry,[5] from nanotechnology[6–11] to biology.

In biological context the use of non-coded AAs is of paramount importance. Their insertion in peptide sequences can give new functionalities to the system, improving also conformational[12,13] and proteolytic stability.[8,14,15] For this reason, the preparation of peptidomimetics is a promising strategy for the obtainment of next-generation therapeutics.[16] Indeed, having more stable bioactive conformation in comparison with natural peptides, peptidomimetics are considered interesting candidates for protein-protein interactions modulations.[17]

The use of α,α-disustituted AAs or β-homologues of natural amino acids have been extensively studied to stabilize peptide conformation.[8,18,19] On the other hand, few examples of the more complex and not easily accessible β2,2-AAs are reported in literature,[20–24] giving interesting stable conformations.[25,26]

Here, we present the use of a non-coded β2,2-amino acid with an isoxazoline core, i.e. 5-(aminomethyl)-3-phenyl-4,5-dihydroisoxazole-5-carboxylic acid (Isox-β2,2-AA; Figure 1), for the preparation of peptidomimetics.

Similar derivatives are presented in literature having an interesting potential in biological application.[27] However, they were never used for the preparation of peptidomimetics.

The presence of isoxazoline core in peptidomimetics, found in several biologically active compounds,[28,29] could be particularly useful. Moreover, the oxygen/nitrogen atoms, as well as the aromatic moiety, make Isox-β2,2AA appealing for possible intermolecular H-bonds and π-interactions. Considering all the above features, we indeed envisaged that Isox-β2,2AA could induce a stable conformation when inserted in model peptides.

Moreover, the isoxazoline C=N bond is a isostere of the carbonyl moiety,[30] making Isox-β2,2AA an analogue of β-(aminomethyl)succinic acid mentioned above (Figure 1).

Recently, we reported on the synthesis of peptidomimetics containing both a bicyclic isoxazoline scaffold and a ɣ-AA possessing an isoxazoline core, able to stabilize a hairpin motif and a α-turn respectively. [31,32]

Among all the secondary structures, turns are of paramount importance because they can direct protein globular folding. Moreover, they are usually located at protein surface, where binding normally occurs.[33]

α-Turn, that are less frequently found as isolated motif in protein compared with β- and ɣ-turns, involves five residue, forming a 13-membered pseudo ring thanks to a H-bond between the i and i+4 AAs.[14,34] This structural motif occurs in many key sites of proteins, such as enzyme active site, and metal binding domains.[35] On the other hand, only few molecules are known to mimic or stabilize it on isolated peptides.[32,36–39]

Here we report on the synthesis of tripeptides N-Boc-[Val-Leu-Isox-β2,2-AA]-OMe, containing both R- and S-Isox-β2,2AA.

The use of the Val-Leu motif is dictated by the known extended conformational behaviour of this dipeptide in solution.[40,41] By this way, it is indeed easy to certify the ability of Isox-β2,2AA to induce any particular folding when inserted in a peptide chain.

Interestingly, we found that R-Isox-β2,2AA can induce a very stable conformation even at the tripeptide level. A 13-membered pseudo ring is formed by an unprecedented H-bond involving C=N of the isoxazoline side chain, as confirmed with theoretical calculation and NMR analyses.

Considering that R-Isox-β2,2AA can stabilize this particular conformation, the hexapeptide N-Boc-[Val-Leu-Isox-R-β2,2-AA]2-OMe was prepared (Figure 1). While also the hexapeptide folds in two consecutive 13-membered pseudo ring, mimicking two α-turn-like motifs (the bending involves the side chain and not the peptide backbone), the S-isomer favors an extended conformation.

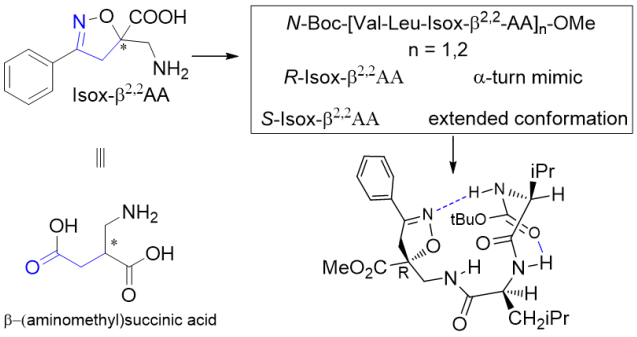


Figure 1. General structure of Isox-β2,2AA, an analogue of the extra-terrestrial β-(aminomethyl)succinic acid, and the prepared peptidomimetics

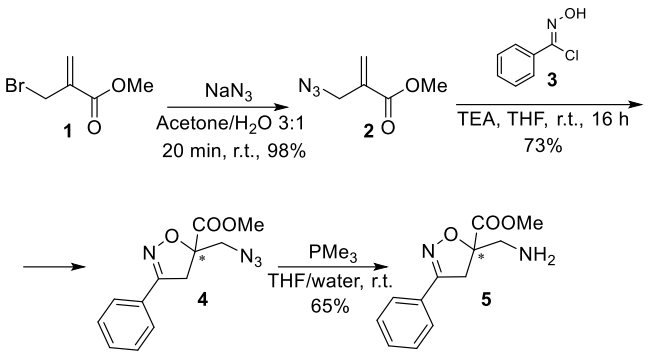
Results and Discussion

Isox-β2,2-AA is a very expensive commercially available compound which synthesis and characterization is not reported in literature. The syntheses of similar derivatives are present in literature, but without details. As key step, a [3+2]-cycloaddition reaction between methyl 2-(bromomethyl)acrylate 1 or the azido-derivative 2 and the nitriloxides derived from the corresponding benzaldoximes is used.[27,42,43]

To give experimental details of this reaction as well as the spectroscopic data for the protected Isox-β2,2-AA, we performed the cycloaddition between 2 and the chloroxime 3, the precursor of the nitriloxide (Scheme 1).

Compound 2 was prepared from bromo-derivative 1 by nucleophilic substitution with sodium azide (2 eq., acetone/H2O: 3/1, r.t., 20 minutes, 98%).

As expected, the 1,3-dipolar cycloaddition, performed under overnight reaction at r.t. in the presence of TEA (2 eq.) and THF as solvent, was proven to be regioselective, affording compound 4 with high yield (73%), having the carboxylic function in position 5 of the isoxazoline ring. The azido derivative 4 was reduced to the corresponding amino ester 5 using the Staudinger protocol. A significant improvement in terms of yield was observed switching from PPh3 to PMe3 (48% and 65%, respectively). This finding could be ascribed to the different hydrolysis rate of imino-phosphorane intermediates to the free amine derivative. Since it is known that electronic and steric effects play an important role on Staudinger reduction rate and yield,[44] we hypothesize that π-interactions between the aromatic group of the isoxazoline moiety and the aryl directly attached to the phosphorus, made in the first case the imino-phosphorane intermediate too stable.



Scheme 1. Synthesis of compound 5

The above synthesis gave amino acid 5 in racemic form. This allows to prepare diastereoisomeric peptidomimetics, starting from both Isox-β2,2-AA enantiomers, and to investigate about their ability to induce a particular secondary structure in a peptide chain.

In order to resolve the racemic mixture of 5, its coupling with N-Boc-(L)-Leu-OH was performed (Scheme 2). Propylphosphonic anhydride (T3P) was used in presence of N,N-Diisopropylethylamine (DIEA), and chosen as the best coupling reagent with respect to the conventional ones. The mixture of the diasteroisomers 6 (65%) were not separable by flash chromatography. On the other hand, after the deprotection of the N-terminus [trifluoroacetic acid (TFA), CH2Cl2], pure diasteroisomes 7a and 7b were isolated (quantitative yields) after flash chromatography [CH2Cl2/MeOH 10:1 + 0.1% triethylamine (TEA)].

X-Ray analysis of a 7a sample single crystal was performed and the absolute R-stereochemistry of C5 of the isooxazoline ring was found (Figure 2, for further details see SI).

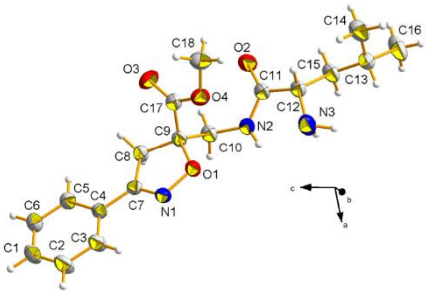
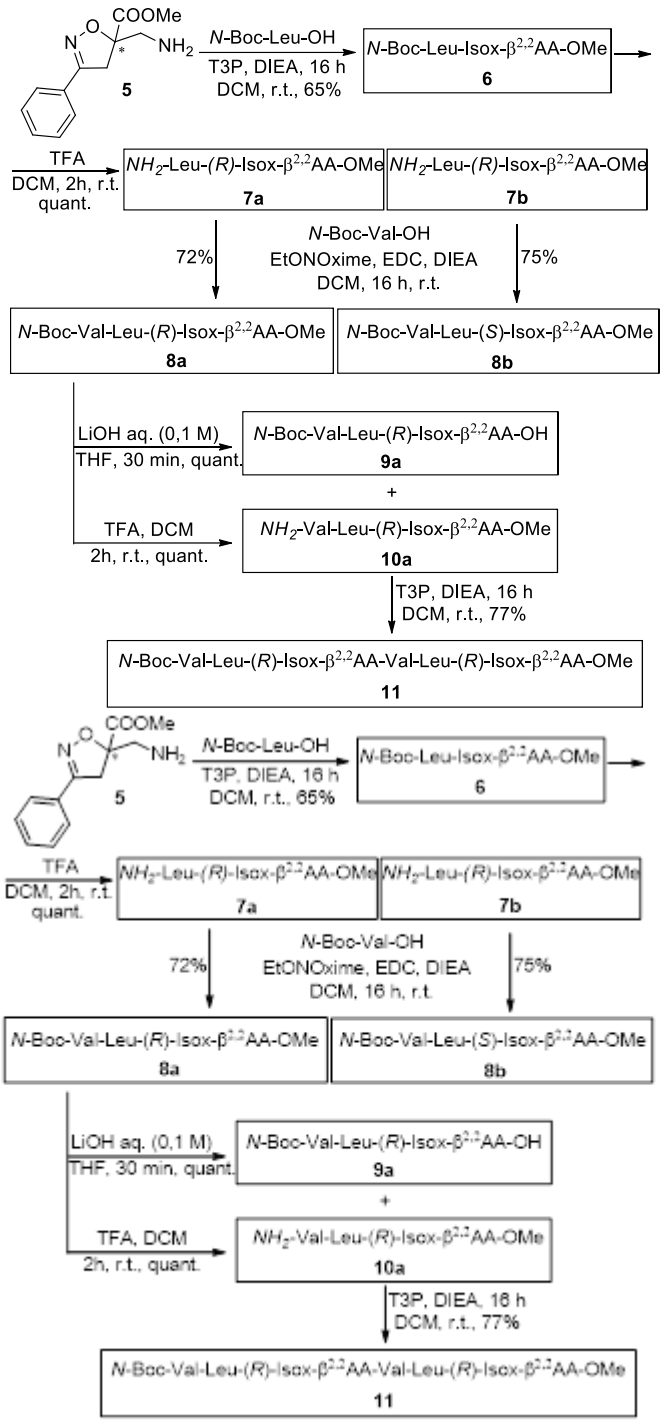


Figure 2: Asymmetric unit of 7a at RT, with the atom-numbering scheme. Thermal ellipsoids of non-H atoms were drawn at the 30 % probability level. The usual colour code was employed for atoms (grey: C; white: H; blue: N; red: O). CCDC 2172521 contains the supplementary crystallographic data for this paper. The data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/structures.

Tripeptides 8a (72%) and 8b (75%) were synthesized starting from 7a and 7b (1 eq.), respectively, by reaction with N-Boc-(L)-Val-OH (1.1 eq.), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (ECD, 1.1 eq.) and ethyl 2-cyano-2-(hydroxyimino)acetate (EtONOxime) (1.1 eq.) as coupling reagent and DIEA (2.2 eq.) as base (Scheme 2).



Scheme 2. Synthesis of peptides 8 and 11

Since NMR analyses and theoretical calculation showed that 8a is able to induce a stable turn when inserted in a small tripeptide (see below), longer hexapeptide 11, containing two R-isox-β2,2AA moieties, was synthesized. First, tripeptides 9a, with free C-terminus (LiOH 0,1 M, Scheme 2) and 10a, having the free N-terminus (TFA in CH2Cl2) were prepared in near quantitative yield from 8a. Their reaction gave hexapeptide 11. Also in this case, different coupling reagents were tried, proving T3P as the best one and giving 11 in very high yield (77%).

Computational Analysis

A conformational search (CS) was preliminary done on tripeptide 8a at the molecular mechanics level to generate several possible conformations. All conformers within the range of 3.0 kcal/mol (36 geometries) were then optimized by DFT. Energies were then computed on the optimized structures including solvent effects for chloroform and empirical corrections for dispersive interactions (Table S1, SI). Two low-energy conformations, namely 8a-C11 and 8a-C13 were found, differing only for the orientation of the -COOMe group. The former resulted the most stable when considering enthalpy ($\Delta\Delta H$ = 0.5 kcal/mol); the latter was found more stable when considering free energy ($\Delta\Delta G$ = 0.3 kcal/mol) Both 8a-C11 and 8a-C13 geometries, that can be considered equivalent, are shown as superposed structures in Figure 3A. Tripeptide 8a secondary structure seems to be stabilized by at least two H-bonds: the first is observed between the C=OBoc and the NHLeu (C=O···HN distance = 2.1 Å); the second H-bond can be detected between the C=NIsox and the NHVal (C=N···HN distance = 2.2 Å). Additionally, a CH/π interaction was also found possible between the phenyl moiety and the Me2CHVal. Indeed, 2.7 Å were measured between the phenyl centroid and the Me2CHVal. This H-bond network, together with the contribution of dispersive interactions between the Val-1 sidechain and the phenyl group, is probably responsible of the secondary structure assumed by the tripeptide (Figure 3). Indeed, ϕ1 and Ψ1 dihedrals suggest the ability of an inverse ɣ-turn at the N-termini,[13] while ϕ2 and Ψ2 values suggest the propensity toward a left-handed α-helix.[45]

To confirm the presence and evaluate the strength of such intramolecular interactions, we performed a Quantum Theory Atom in Molecules (QTAIM) analysis on the DFT-optimized 8a-C11 geometry.[46] Indeed, QTAIM has been successfully used to characterize both intermolecular[47,48] and intramolecular non-covalent interaction,[49] including classic and weak H-bonds in peptides.[45,50] An H-bond, as well as all covalent and non-covalent interactions, can be described by a bond path (BP) and a bond critical point (BCP). The electron density at the BCP (ρ), its Laplacian ($\nabla^2\rho$), and the ellipticity at the BCP (ε) are considered measures of strength and the nature of H-bonds.[47] Moreover, Emamian et al. proposed that the strength of an H-bond in neutral systems could be derived from BCP ρ accordingly to the equation [1] where BE is the energy of the interaction described by the BCP[51]:

$$BE\ (kcal/mol) = -223.08 \cdot \rho(a.u.) + 0.7423 \qquad [1]$$

Figure 3B and Table 1 show that all the H-bonds mentioned above for 8a-C11 are present. Their ρ span from 0.0190 a.u. for the strongest (the C=OBoc NHLeu H-bond, described by BCP1), to 0.0065 a.u. for the weakest (the CH/π interaction between the phenyl group and Val-1, described by BCP3). All the three BCPs show a negative $\nabla^2\rho$, suggesting that their nature is closer to a covalent bond than to an electrostatic interaction.[52] Conversely, ε is low for BCPs 1 and 2 (0.08 and 0.15, respectively), but higher for BCP3 (1.3). Based on equation [1], the estimated energies for the H-Bonds described by BCP1, BCP2 and BCP3 are −3.5, −3.0 and −0.7 kcal/mol, respectively. These values confirm that the two former H-bonds are strong and stable and contribute the most to the stabilization of the secondary structure. Some contribution, but lower, can also be ascribed to the CH/π interaction between Val-1 sidechain and the isoxazoline phenyl group.

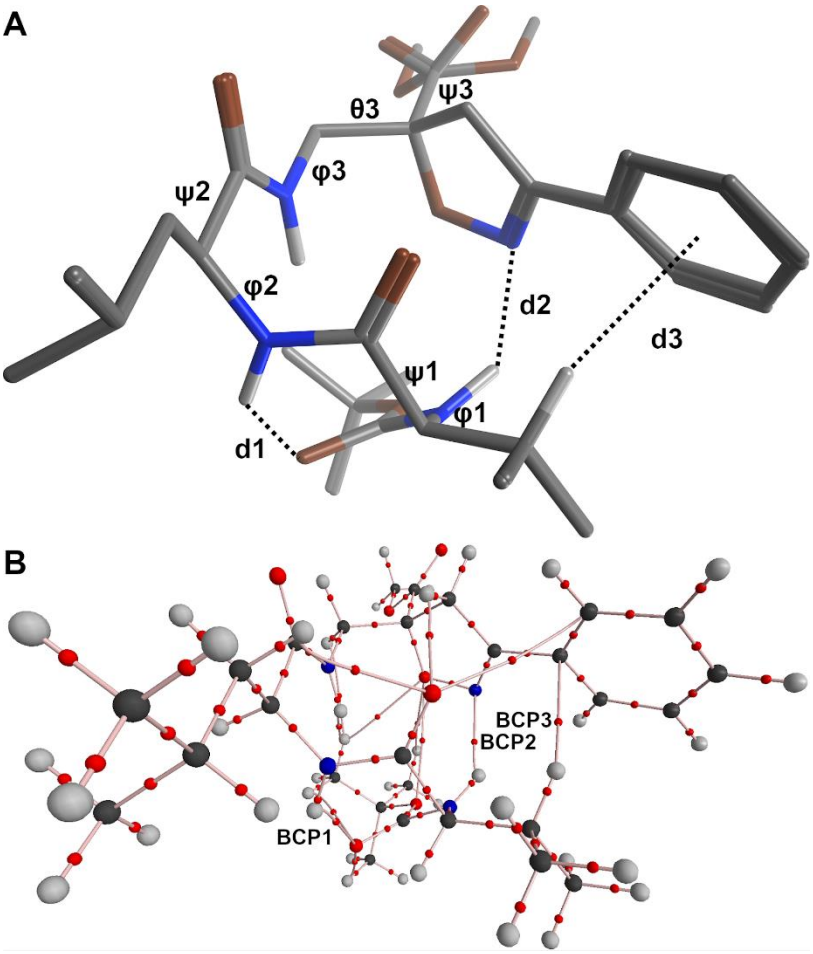


Figure 3. A. Superposed lowest energy geometries C11 and C13 of tripeptide 8a, obtained by CS followed by DFT calculations. Selected dihedrals (in deg.) are ϕ1 (−79.1), Ψ1 (84.1), ϕ2 (57.5), Ψ2 (35.2), ϕ3 (110.0), θ3 (177.7), and Ψ3 (−56.0 and 175.4 for C11 and C13, respectively); selected distances correspond to the H-bonds C=OBoc ···NHLeu (d1 = 2.1 Å), C=NIsox··· NHVal (d2 = 2.2 Å), and the CH/π interaction between phenyl moiety centroid and Me2CHVal (d3 = 2.7 Å). Non-polar hydrogens are not shown, except for Me2CHVal. B. Molecular graph showing bond critical points (BPC) and bond paths (BP) computed for peptide 8a-C11 by QTAIM. Relevant intramolecular interactions are described by BCP1, BCP2 and BCP3 and corresponding BPs.

Table 1. Selected BCPs and Their Features (in a.u.) Computed by QTAIM on 8a_C11

	ρa	∇2ρb	λ1c	λ2c	λ3c	εd	
BCP1	0.0190	–0.0176		–0.0209	–0.0193	0.1106	0.0828
BCP2	0.0167	–0.01468		–0.0188	–0.0164	0.0939	0.1455
BCP3	0.0065	–0.00467		–0.0043	–0.0019	0.0249	1.3151

a. Electron density at the BCP; b. Laplacian of ρ; c. Eigenvalues of the Hessian of ρ; d. ellipticity at the BCP, calculated as λ1/λ2 – 1.

NMR characterization of tripeptides 8 and hexapeptide 11

Tripeptides 8 and hexapeptide 11 are not soluble in water and complete NMR characterization (1H, 13C, COSY, HMBC, HSQC, NOESY/ROESY, 293 K for compounds 8 and at 303K for compound 11; see SI for further details) was performed in CDCl3 since it is well known that peptides can assume a very stable conformation also in organic solvent.

Tripeptides 8a and 8b, which differ for the configuration at isoxazoline C-5, showed different conformational behaviour. The chemical shift of NHs (□□5-7 ppm) and CHα (□□3.5-4.2 ppm) are well dispersed, indicating the presence of structured conformation. In case of 11, NH-differences were detected for Val-Leu-Isox sequences at N-and C-terminus. The amide signals at N-terminus are similar to 8a, except for the deshielded NHIsox3 (□ 7.49), while those of C-terminus resonated in the same region (□□7.22-7-30).

As expected, the most perturbed protons were those of Isox-3 at interface with C-terminus tripeptide sequence. As matter of fact, CH2-4 of the ring (AB system) resonates in 3.73-3.53 ppm region, both for tripeptides 8 and in Isox-6 for 11, while appeared more separated (δ 3.82 and 3.31) in Isox-3.

It is worth to underline the differences of the two diastereomers in terms of Δδ/ΔT. With NMR at variable temperature, it is indeed possible to observe if the amidic protons are blocked in H-bonds or in steric situations, not allowing the shift of the corresponding signals.[53] This analysis is very useful to have a preliminary idea if the peptide can fold. According to these experiments (T: 273-323 K; Figure 4), all the NHs of 8b appeared to be not involved in any H-bonds (NH > -5.5 ppb/K). Compound 8a showed low variation of Δδ/ΔT for NHLeu and NHVal (-3.6 ppb/K and -2.2 ppb/K, respectively), meaning that they are probably involved in strong H-bonds, while the isoxazoline amide proton is not solvent shielded. These data are fully concordant with the computational analysis described above.

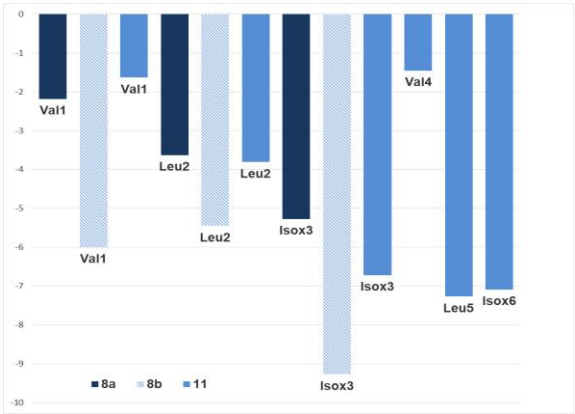


Figure 4. Δδ/ΔT of Tripeptides 8 and hexapeptide 11

The analysis of 11 confirmed a similar trend (T: 273-323 K; Figure 2): NHIsox3/6 are not solvent shielded (Δδ/ΔT > 6.7 ppb/K) but a very low Δδ/ΔT variation was observed for both Val1/4 amide protons (about -1.5 ppb/K), confirming also in this case their involvement in strong H-bonds. While NHLeu2 (-3.81 ppb/K) showed a similar value as in 8a, but NHLeu5 is not involved in a H-bond (-7.3 ppb/K). DMSO-d6 titration was also performed (Figure 6). The tripeptide at C-terminus confirms the above results, being NHVal-4 strongly involved in a H-bond (Δδ NH = 0.22). Furthermore, the chemical shifts of CH2-4 of the ring maintain their values, independently from the amount of DMSO indicating a stable conformation. A different panorama was observed at N-terminus, more exposed to the solvent. The intermediate values for NHAla-1 and NHIsox-3 (Δδ NH = 0.58 and 0.41, respectively) suggest that these NHs are partially solvated and an equilibrium in the formation of two different H-bonds could occur.

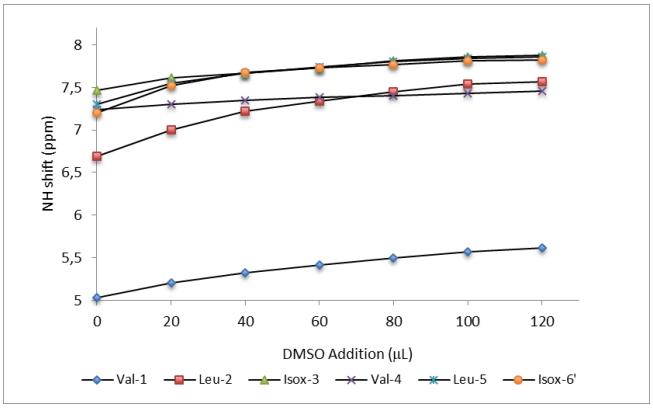


Figure 5. Plots of the chemical shifts of NH groups in the 1H NMR spectra of 11 with the addition of increasing DMSO amounts to the solution of 11 in CDCl3 (v/v; initial peptide concentration: 26 mM).

NOESY experiment on peptide 8b showed the presence of strong dαN(i,i+1) spatial proximities (Figure 6, FS5 and TS3, SI) and absence of dNN(i,i+1). Moreover, considering the absence of H-bonds as well as JCH/NH values for Val (8.5 Hz) and Leu (8.1 Hz) higher than those observed in 8a (see TS2 and TS3, SI), an extended conformation is proposed for this sequence containing S-Isox-β2,2AA.

Tripeptide 8a (Figure 6, FS4 and TS2, SI) showed strong dαN(i,i+1) and a weak spatial proximity between NHIsox and NHLeu suggesting the presence of a turn. ROESY experiment for 11 (303 K) gave similar results (Figure 6, FS6 and TS4, SI). Strong dαN(i,i+1) were detected. Since amide signals resonated in the same region of the aromatic protons, it was difficult to detect dNN except for NHIsox3/NHLeu4 (vw). Of relevance, CHLeu2 showed proximity with NHLeu5 [vw, dαN(i,i+3)] as well as this last with Me2CHVal4.

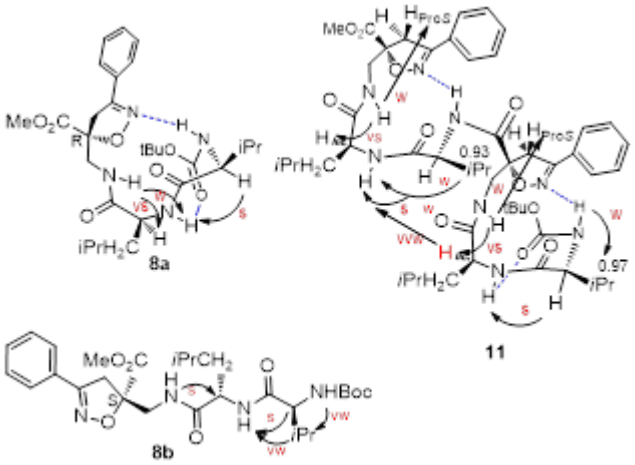


Figure 6. Conformations of the peptides 8a,8b and 11; NOEs (arrows), H-bonds (blue dotted lines)

Taking all this information together, we can assume that R-Isox-β2,2-AA is indeed able to induce the folding of the peptide chain, stabilizing a α-turn-like structure (Figure 6). Of relevance, the H-bond between NHLeu2 with C=OBoc, observed in both 8a and 11, stabilizes an inverse ɣ-turn at N-terminus. Our hypothesis is that this particular turn could induce the start of the peptide folding.

Conclusion

In conclusion, here we detailed the synthesis of methyl 5-(aminomethyl)-3-phenyl-4,5-dihydroisoxazole-5-carboxylic acid 5, reporting its spectroscopic characterization. Moreover, inserting R-Isox-β2,2AA in short tri- and hexapeptide models, we observed an unexpected α-turn-like motif, thanks to an unprecedented strong H-bond between NHVal and the C=N of isoxazoline side chain of our β2,2AA.

Experimental Section

General information. The NMR spectroscopic experiments were carried out either on a Varian OXFORD 300 MHz (300 and 75 MHz for 1H and 13C, respectively), or Bruker Avance Bruker Avance I 500 MHz spectrometers (500 and 125 MHz for 1H and 13C, respectively). Chemical shifts δ are given in ppm relative to the CHCl3 internal standard, and the coupling constants J are reported in Hertz (Hz). ESI mass spectra were recorded on an LCQESI MS on a LCQ Advantage spectrometer from Thermo Finningan and a LCQ Fleet spectrometer from Thermo Scientific. Optical rotations were measured on a Perkin–Elmer 343 polarimeter at 20°C (concentration in g/100 mL). Synthetic procedure and NMR analysis are in agreement with the literature data for compound 3.[54] The compounds characterization are present in SI

Methyl 2-(azidomethyl)acrylate (2). In a round bottom flask, equipped with magnetic stirrer, methyl 2-(bromomethyl)acrylate (600 mg, 3.51 mmol) was dissolved in a mixture of acetone/H2O (3:1, 12 mL). NaN3 (450 mg, 6.72 mmol) was added to the solution and the mixture was stirred for 20 minutes. The reaction mixture was diluted with CH2Cl2 (20 mL), washed with H2O and s.s. NaCl and dried over Na2SO4. The solvent was removed under reduced pressure, affording a colorless oil. (485.2 mg, 98%).[27]

Methyl 5-(Azidomethyl)-3-phenyl-4,5-dihydroisoxazole-5-carboxylate (4). In a two-necked round-bottom flask, equipped with magnetic stirrer and nitrogen inlet, compound 2 (640 mg, 4.13 mmol) was suspended in dry THF

(5 mL). 4 (580.0 mg, 4.13 mmol), diluted in dry THF (7 mL) and TEA (1.15 mL, 8.26 mmol, 2 eq) were added dropwise to the solution. The reaction was stirred overnight at room temperature under nitrogen atmosphere. The reaction mixture was concentrated under reduced pressure, diluted with AcOEt (10 mL), washed with H2O (15 mL) and finally dried over Na2SO4. The solvent was removed under reduced pressure, affording a dark-yellow oil. Purification of the crude product by flash chromatography (gradient from 100% hexane to mixture hexane/AcOEt, 8:2) afforded compound 4 as a white solid (783.9 mg, 73%).

Methyl 5-(Aminomethyl)-3-phenyl-4,5-dihydroisoxazole-5-carboxylate (5). In a round bottom flask equipped with magnetic stirrer, 4 (771 mg, 2.96 mmol) was suspended in THF (29.6 mL) to obtain a 0.1M solution. Afterwards, H2O (0.37 mL, 20.72 mmol, 7 eq) and a 0,1M solution of PMe3 in toluene (3.26 mL, 3.26 mmol, 1.1 eq) were added to the solution. The reaction mixture turned from colorless to bright yellow. The reaction was stirred for 72 hours at room temperature under nitrogen atmosphere. The mixture was filtered to remove trimethyl-phosphine oxide. The solvent was removed under reduced pressure and the obtained amine 5 (450.4 mg, 65% yield) was immediately used for the following coupling reaction.

N-Boc-Leu-Isoxβ2,2AA-OMe (6). In a round bottom flask equipped with magnetic stirrer, 5 (693 mg, 2.96 mmol) was suspended in dry CH2Cl2 (20 mL), then the solution was cooled to 0 °C. NH-Boc-(L)-Leucine (1.37 g, 5.92 mmol, 2 eq) and T3P (3.2 mL 8.88 mmol, 3 eq) were added. Finally, DIEA was added until pH = 8 (1.5 mL, 8.88 mmol, 3 eq). The reaction was stirred overnight at room temperature. The mixture was washed with KHSO4 (5%, 20 mL), NaHCO3 (20 mL), brine (25 mL), then the organic layer was dried over Na2SO4. The solvent was removed under reduced pressure, affording a pale-yellow oil. Purification of the crude product by flash chromatography (gradient from 100% hexane to mixture hexane/AcOEt, 6:4) afforded compound 6 as a white solid foam (860.47 mg, 65%). Inseparable diastereoisomeric mixture.

NH2-Leu-Isoxβ2,2AA-OMe (7). In a round bottom flask equipped with magnetic stirrer, 6 (850 mg, 1.91 mmol) was suspended in CH2Cl2 (10 mL). The solution was cooled at 0 °C and TFA (10 mL) was added dropwise. The reaction was stirred for 2 hours at room temperature. Then, the reaction mixture was neutralized with NaHCO3 and the organic layer was extracted with CH2Cl2 (10 mL) and dried over Na2SO4. The solvent was removed under reduced pressure, affording compound 7 as a pale-yellow foam (591 mg). The two diastereoisomers were separated through flash chromatography (CH2Cl2/MeOH 10:1 + 0.1% TEA) afforded 7a (266 mg) and 7b (251 mg) as white solids.

N-Boc-Val-Leu-Isoxβ2,2AA-OMe (8). In a round bottom flask equipped with magnetic stirrer, NH-Boc-(L)-Valine (176 mg, 0.811 mmol) was suspended in dry CH2Cl2 (6 mL). Afterwards, the solution was cooled at 0 °C and EtONOxime (115 mg, 0.811 mmol) and EDC (160 mg, 0.811 mmol) were added. The reaction was stirred for 1 hour at 0 °C, then 7 (256 mg, 0.737 mmol) was added. DIEA was added until pH = 8 (0.14 mL, 0.811 mmol, 1.1 eq). The reaction was stirred overnight at room temperature. Then, the reaction mixture was washed with KHSO4 (5%, 20 mL), NaHCO3 (20 mL), brine (25 mL), then the organic layer was dried over Na2SO4. The solvent was removed under reduced pressure, affording 8 as white solid. Complete NMR Characterization are depicted in SI (See TS2 and 3)

N-Boc-Val-Leu-(R)-Isoxβ2,2AA-OH (9a). In a round bottom flask equipped with magnetic stirrer, 8a (277 mg, 0.507 mmol) was suspended in dry THF (10 mL). A 0.1 M solution of LiOH (10.13 mL, 1.01 mmol, 2 eq) was added and the reaction mixture was stirred for 30 min at room temperature. The mixture was washed with KHSO4 (5%, 20 mL) and the organic layer was extracted with CH2Cl2 (10 mL), then dried over Na2SO4. The solvent was removed under reduced pressure, affording 9a as white solid with quantitative yield.

NH2-Val-Leu-(R)-Isoxβ2,2AA-OMe (10a). In a round bottom flask equipped with magnetic stirrer, 8a (350 mg, 0.64 mmol) was suspended in CH2Cl2 (12 mL). The solution was cooled at 0 °C and TFA (12 mL) was added dropwise. The reaction was stirred for 2 hours at room temperature. Then, the reaction mixture was concentrated under reduced pressure, affording compound 10a as a white solid in quantitative yield (324 mg, quantitative).

N-Boc-Val-Leu-(R)-Isoxβ2,2AA-Val-Leu-(R)-Isoxβ2,2AA-OMe (11). In a round bottom flask equipped with magnetic stirrer, 9a (120 mg, 0.26 mmol) was suspended in dry CH2Cl2 (10 mL), then the solution was cooled to 0 °C. 10a (276,77 mg, 0.52 mmol, 2 eq) and T3P (0,28 mL, 0,78 mmol, 3 eq) were added. Finally, DIEA was added until pH = 8 (0.15 mL, 0,78 mmol, 3 eq). The reaction was stirred overnight at room temperature. The reaction mixture was washed with KHSO4 (5%, 10 mL), NaHCO3 (10 mL), brine (10 mL). The organic layer was dried over Na2SO4. The solvent was removed under reduced pressure, affording a pale-yellow oil. Purification of the crude product by flash chromatography (hexane/AcOEt, 1:2) afforded compound 11 as a white solid (192.3 mg, yield 77%). Complete NMR Characterization are depicted in SI (See TS 4)

Single crystal X-ray diffraction (7a). A X-ray quality single crystal of 7a was mounted on a Bruker AXS Smart three-circle diffractometer equipped with a CCD area detector. A 100 % complete full sphere of diffraction data was recorded at room temperature up to a resolution of 0.7 Å using graphite-monochromated Mo K□ X-rays (□ = 0.71073 Å) with a nominal source power of 50 kV x 30 mA. 7a crystallizes in the monoclinic acentric polar P21 space group with 2 formulae in cell and 1 molecule in the asymmetric unit. The unit cell parameters read a = 12.2315(6) Å, b = 5.3421(3) Å, c = 14.6211(7) Å, □ = 104.470(2) deg, V = 925.06(8) Å3. The structure was solved by direct methods and the final model comes from a least-squares fitting against 5186 symmetry-independent structure factor amplitudes. The shelx suite of programs was used throughout[55]. The overall agreement factor was R(F) = 0.0415 for 3682 reflections with Fo > 4□(Fo), with goodness-of-fit 0.950 and largest Fourier residuals as low as □ 0.13 e· Å3. The absolute structure was secured by the Parson's metod[56] through anomalous dispersion effects, with a final Flack parameter of 0.1(3) from 1274 selected quotients. See Supplementary Information for a full discussion on the chemical structure and crystal packing of 7a.

Theoretical calculations

Peptide 8a was initially constructed using the MOE software.[57] A conformational search (CS) was then performed with the same software. The Low Mode Molecular Dynamics CS method was applied,[58] using the Amber10EHT forcefield.[59,60] The Reaction-Field implicit solvation model,[61] with a dielectric constant set to 4.8, was applied to simulate chloroform solvation. The rejection limit, iteration limit, and MM iteration limit were set to 1000, 100000 and 5000, respectively, to improve accuracy. while other parameters were set as default. All conformation within 3.0 kcal/mol (36 structures) were then subjected to geometry minimization, using the Gaussian16 software.[62] The density functional theory (DFT) was applied in combination with the MPW1B95 functional and the 6-31G(d) basis set.[63] This functional was chosen based on our experience with conformational analyses of non-natural peptides.[50] A vibrational analysis was conducted at the same level to verifying the absence of imaginary frequencies, and to compute thermochemical corrections at standard conditions (298.15 K and 1 atm). Single point energy calculations were then conducted on all the optimized geometries at the MPW1B95/6-311+G(d,p) level, including chloroform solvation effects via the CPCM solvent model[64] and GD3 empirical correction to dispersive interactions.[65] QTAIM analyses were done on the wavefunction obtained from the above calculations for 8a-C11. The AIM2000 software was used by setting all parameters as default,[66] except the maximum number of Newton iterations and the step size that wer set to 400 and 0.5, respectively.

All the Original spectra, X-Ray analysis data, absolute energies, thermochemical corrections, number of imaginary frequencies, Gaussian16 input root section and Cartesian coordinates of computational models discussed herein are provided as Supporting Information

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