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# A Non-coded $\beta^{2,2}$ -Amino Acid with Isoxazoline Core Able to Stabilize Peptides Folding through an Unprecedented Hydrogen Bond

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Gianluigi Brogginì,<sup>[b]</sup> Leonardo Lo Presti,<sup>[c]</sup> Alessandro Contini,<sup>[a]</sup> and Maria Luisa Gelmi\*<sup>[a]</sup>Dedicated to Prof. Cesare Gennari on the occasion of his 70<sup>th</sup> birthday

New peptidomimetics containing a  $\beta^{2,2}$ -isoxazoline amino acid, *i.e.* 5-(aminomethyl)-3-phenyl-4,5-dihydroisoxazole-5-carboxylic acid (Isox- $\beta^{2,2}$ AA), 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- $\beta^{2,2}$ AA, inducing the folding of short peptides thanks to an unprecedented H-bond involving C=N of the isoxazoline side chain of our  $\beta^{2,2}$ -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.<sup>[1]</sup> More exotic extraterrestrial amino acids, such as  $\beta$ -(aminomethyl)succinic acid, were found in the Murchison meteorite.<sup>[2]</sup> 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<sup>[3–5]</sup> to electrochemistry,<sup>[5]</sup> from nanotechnology<sup>[6–11]</sup> 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<sup>[12,13]</sup> and proteolytic stability.<sup>[8,14,15]</sup> For this reason, the preparation of peptidomimetics is a promising strategy for the obtainment of next-generation therapeutics.<sup>[16]</sup> Indeed, having more stable bioactive conformation in comparison with natural peptides, peptidomimetics are considered interesting candidates for modulations of protein-protein interactions modulations.<sup>[17]</sup>

The use of  $\alpha,\alpha$ -disubstituted AAs or  $\beta$ -homologues of natural amino acids have been extensively studied to stabilize peptide conformation.<sup>[8,18,19]</sup> On the other hand, few examples of the more complex and not easily accessible  $\beta^{2,2}$ -AAs are reported in literature,<sup>[20–24]</sup> giving interesting stable conformations.<sup>[25,26]</sup>

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

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

The presence of isoxazoline core in peptidomimetics, found in several biologically active compounds,<sup>[28,29]</sup> could be particularly useful. Moreover, the oxygen/nitrogen atoms, as well as the aromatic moiety, make Isox- $\beta^{2,2}$ AA appealing for possible intermolecular H-bonds and  $\pi$ -interactions. Considering all the

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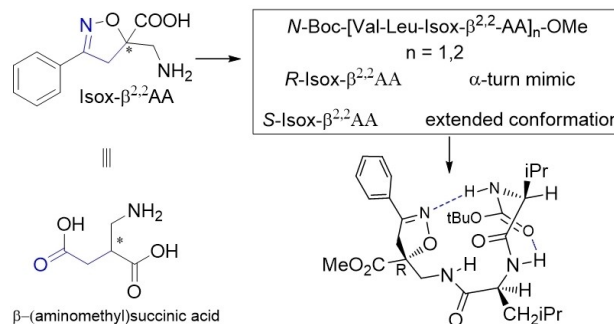


Figure 1. General structure of Isox- $\beta^{2,2}$ AA, an analogue of the extra-terrestrial  $\beta$ -(aminomethyl)succinic acid, and the prepared peptidomimetics

above features, we indeed envisaged that Isox- $\beta^{2,2}$ AA could induce a stable conformation when inserted in model peptides.

Moreover, the isoxazoline C=N bond is an isostere of the carbonyl moiety,<sup>[30]</sup> making Isox- $\beta^{2,2}$ AA an analogue of  $\beta$ -(aminomethyl)succinic acid mentioned above (Figure 1).

Recently, we reported on the synthesis of peptidomimetics containing both a bicyclic isoxazoline scaffold and a  $\gamma$ -AA possessing an isoxazoline core, able to stabilize a hairpin motif and a  $\alpha$ -turn, respectively.<sup>[31,32]</sup>

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.<sup>[33]</sup>

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

Here we report on the synthesis of tripeptides *N*-Boc-[Val-Leu-Isox- $\beta^{2,2}$ -AA]-OMe, containing both *R*- and *S*-Isox- $\beta^{2,2}$ AA.

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

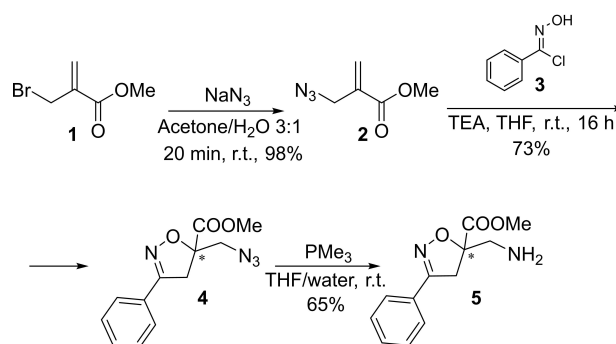
Interestingly, we found that *R*-Isox- $\beta^{2,2}$ AA can induce a very stable conformation even at the tripeptide level. A 13-membered pseudo ring is formed by an unprecedented H-bond involving C=N of the isoxazoline side chain, as confirmed with theoretical calculation and NMR analyses.

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

## Results and Discussion

Isox- $\beta^{2,2}$ 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.<sup>[27,42,43]</sup>

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



Scheme 1. Synthesis of compound 5

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

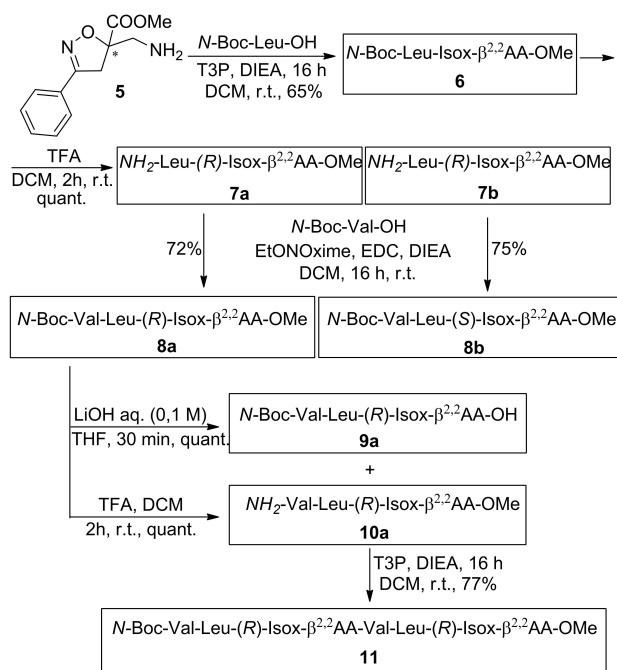
As expected, the 1,3-dipolar cycloaddition, performed under overnight reaction at r.t. in the presence of TEA (2 eq.) and THF as solvent, was proven to be regioselective, affording compound **4** with high yield (73%), having the carboxylic function in position 5 of the isoxazoline ring. The azido derivative **4** was reduced to the corresponding amino ester **5** using the Staudinger protocol. A significant improvement in terms of yield was observed switching from PPh<sub>3</sub> to PMe<sub>3</sub> (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,<sup>[44]</sup> we hypothesize that  $\pi$ -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.

The above synthesis gave amino acid **5** in racemic form. This allows to prepare diastereoisomeric peptidomimetics, starting from both Isox- $\beta^{2,2}$ 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 diastereoisomers **6** (65%) were not separable by flash chromatography. On the other hand, after the deprotection of the *N*-terminus [trifluoroacetic acid (TFA), CH<sub>2</sub>Cl<sub>2</sub>], pure diastereoisomers **7a** and **7b** were isolated (quantitative yields) after flash chromatography [CH<sub>2</sub>Cl<sub>2</sub>/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 isoxazoline ring was found (Figure 2, for further details see SI).

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 (EDC, 1.1 eq.) and



Scheme 2. Synthesis of peptides **8** and **11**

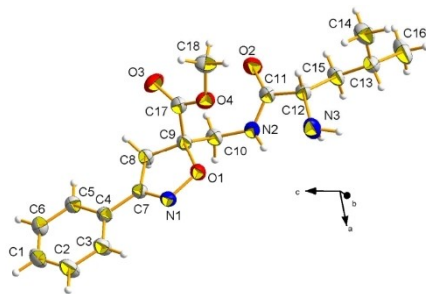


Figure 2. Asymmetric unit of **7a** at RT, with the atom-numbering scheme. Thermal ellipsoids of non-H atoms were drawn at the 30% probability level. The usual colour code was employed for atoms (grey: C; white: H; blue: N; red: O).

ethyl 2-cyano-2-(hydroxyimino)acetate (EtONoxime; 1.1 eq.) as coupling reagent and DIEA (2.2 eq.) as base (Scheme 2).

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-β<sup>2,2</sup>AA 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 CH<sub>2</sub>Cl<sub>2</sub>) 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%).

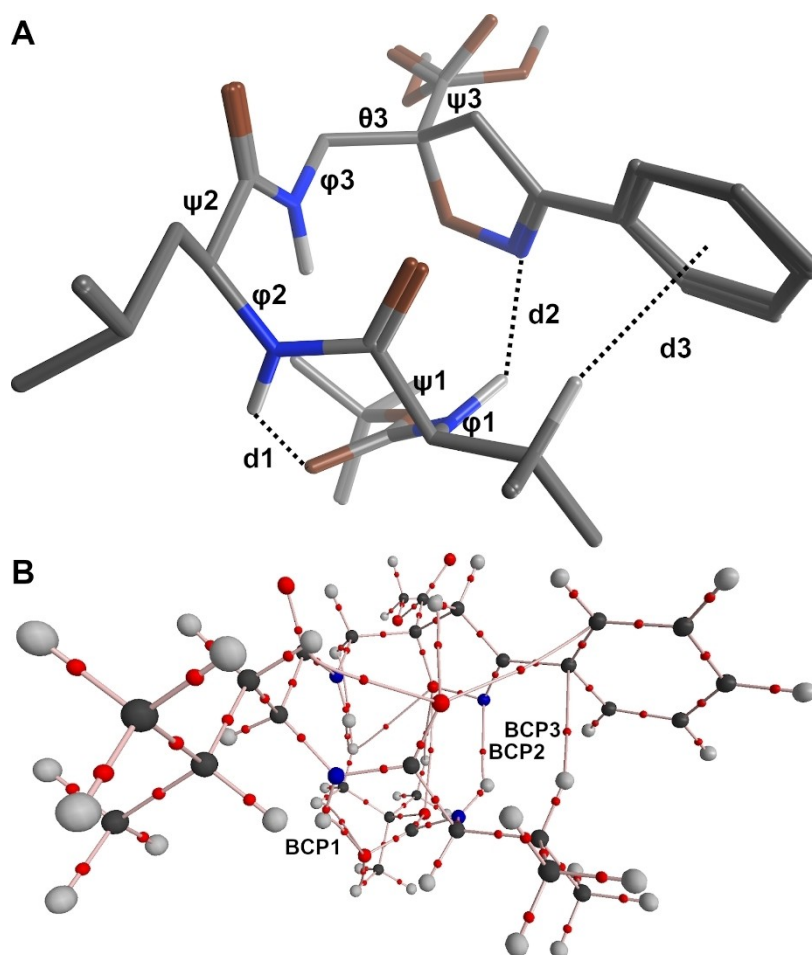
## Computational analysis

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=O<sub>Boc</sub> and the NH<sub>Leu</sub> (C=O...HN distance = 2.1 Å); the second H-bond can be detected between the C=N<sub>isox</sub> and the NH<sub>Val</sub> (C=N...HN distance = 2.2 Å). Additionally, a CH/π interaction was also found possible between the phenyl moiety and the Me<sub>2</sub>CH<sub>Val</sub>. Indeed, 2.7 Å were measured between the phenyl centroid and the Me<sub>2</sub>CH<sub>Val</sub>. 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 γ-turn at the N-terminus,<sup>[13]</sup> while φ2 and Ψ2 values suggest the propensity toward a left-handed α-helix.<sup>[45]</sup>

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.<sup>[46]</sup> Indeed, QTAIM has been successfully used to characterize both intermolecular<sup>[47,48]</sup> and intramolecular non-covalent interaction,<sup>[49]</sup> including classic and weak H-bonds in peptides.<sup>[45,50]</sup> A 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 ( $\rho$ ), its Laplacian ( $\nabla^2\rho$ ), and the ellipticity at the BCP ( $\epsilon$ ) are considered measures of strength and the nature of H-bonds.<sup>[47]</sup> Moreover, Emamian *et al.* proposed that the strength of an H-bond in neutral systems could be derived from BCP  $\rho$  accordingly to Equation (1) where BE is the energy of the interaction described by the BCP.<sup>[51]</sup>

$$BE \text{ (kcal/mol)} = -223.08 \cdot \rho(\text{a.u.}) + 0.7423 \quad (1)$$

Figure 3B and Table 1 show that all the H-bonds mentioned above for **8a**-C11 are present. Their  $\rho$  span from 0.0190 a.u. for the strongest (the C=O<sub>Boc</sub> NH<sub>Leu</sub> 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.<sup>[52]</sup> Conversely,  $\epsilon$  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



**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  $\phi 1$  ( $-79.1$ ),  $\Psi 1$  ( $84.1$ ),  $\phi 2$  ( $57.5$ ),  $\Psi 2$  ( $35.2$ ),  $\phi 3$  ( $110.0$ ),  $\theta 3$  ( $177.7$ ), and  $\Psi 3$  ( $-56.0$  and  $175.4$  for C11 and C13, respectively); selected distances correspond to the H-bonds  $C=O_{Boc} \cdots NH_{Leu}$  ( $d1 = 2.1$  Å),  $C=N_{Isox} \cdots NH_{Val}$  ( $d2 = 2.2$  Å), and the  $CH/\pi$  interaction between phenyl moiety centroid and  $Me_2CH_{Val}$  ( $d3 = 2.7$  Å). Non-polar hydrogens are not shown, except for  $Me_2CH_{Val}$ . **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

|      | $\rho$ <sup>[a]</sup> | $\nabla^2 \rho$ <sup>[b]</sup> | $\lambda_1$ <sup>[c]</sup> | $\lambda_2$ <sup>[c]</sup> | $\lambda_3$ <sup>[c]</sup> | $\epsilon$ <sup>[d]</sup> |
|------|-----------------------|--------------------------------|----------------------------|----------------------------|----------------------------|---------------------------|
| 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  $\rho$ ; [c] Eigenvalues of the Hessian of  $\rho$ ; [d] ellipticity at the BCP, calculated as  $\lambda_1/\lambda_2 - 1$ .

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  $CH/\pi$  interaction between Val-1 sidechain and the isoxazoline phenyl group.

## NMR characterization of tripeptides **8** and hexapeptide **11**

Tripeptides **8** and hexapeptide **11** are not soluble in water and complete NMR characterization ( $^1H$ ,  $^{13}C$ , COSY, HMBC, HSQC, NOESY/ROESY, 293 K for compounds **8** and at 303 K for compound **11**; see SI for further details) was performed in  $CDCl_3$  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 ( $\delta$  5–7 ppm) and  $CH\alpha$  ( $\delta$  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*-termini. The amide signals at *N*-terminus are similar to **8a**, except for the deshielded  $NH_{Isox3}$  ( $\delta$  7.49), while those of *C*-terminus resonated in the same region ( $\delta$  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, CH<sub>2</sub>-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 ( $\delta$  3.82 and 3.31) in Isox-3.

It is worth to underline the differences of the two diastereomers in terms of  $\Delta\delta/\Delta T$ . 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.<sup>[53]</sup> 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  $\Delta\delta/\Delta T$  for NH<sub>Leu</sub> and NH<sub>Val</sub> (-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.

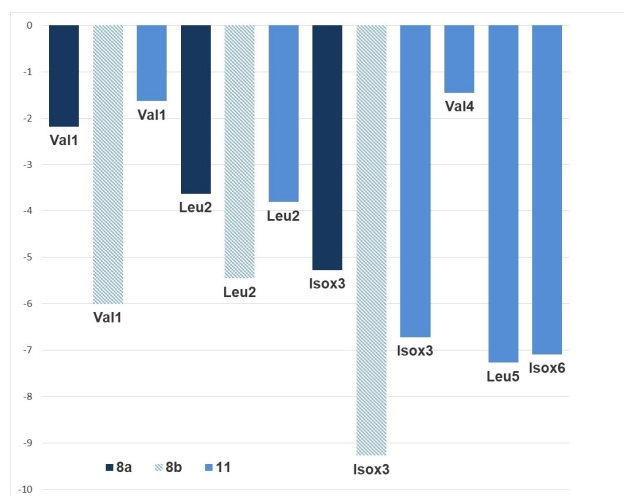


Figure 4.  $\Delta\delta/\Delta T$  of Tripeptides **8** and hexapeptide **11**

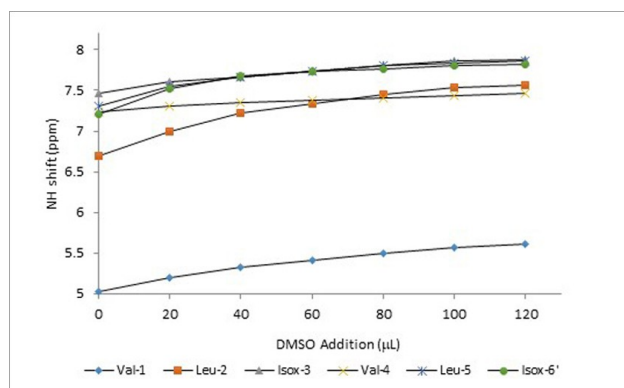


Figure 5. Plots of the chemical shifts of NH groups in the <sup>1</sup>H NMR spectra of **11** with the addition of increasing DMSO amounts to the solution of **11** in CDCl<sub>3</sub> (v/v; initial peptide concentration: 26 mM).

The analysis of **11** confirmed a similar trend (T: 273–323 K; Figure 4): NH<sub>Isox3/6</sub> are not solvent shielded ( $\Delta\delta/\Delta T > 6.7$  ppb/K) but a very low  $\Delta\delta/\Delta T$  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 NH<sub>Leu2</sub> (-3.81 ppb/K) showed a similar value as in **8a**, but NH<sub>Leu5</sub> is not involved in a H-bond (-7.3 ppb/K). DMSO-d<sub>6</sub> titration was also performed (Figure 5). The tripeptide at C-terminus confirms the above results, being NH<sub>Val-4</sub> strongly involved in a H-bond ( $\Delta\delta_{\text{NH}} = 0.22$ ). Furthermore, the chemical shifts of CH<sub>2</sub>-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 NH<sub>Ala-1</sub> and NH<sub>Isox-3</sub> ( $\Delta\delta_{\text{NH}} = 0.58$  and 0.41, respectively) suggest that these NHs are partially solvated and an equilibrium in the formation of two different H-bonds could occur.

NOESY experiment on peptide **8b** showed the presence of strong  $d_{\alpha\text{N}}(i,i+1)$  spatial proximities (Figure 6, FS5 and TS3, SI) and absence of  $d_{\text{NN}}(i,i+1)$ . Moreover, considering the absence of H-bonds as well as  $J_{\text{CH/NH}}$  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- $\beta^{2,2}$ AA.

Tripeptide **8a** (Figure 6, FS4 and TS2, SI) showed strong  $d_{\alpha\text{N}}(i,i+1)$  and a weak spatial proximity between NH<sub>Isox</sub> and NH<sub>Leu</sub> suggesting the presence of a turn. ROESY experiment for **11** (303 K) gave similar results (Figure 6, FS6 and TS4, SI). Strong  $d_{\alpha\text{N}}(i,i+1)$  were detected. Since amide signals resonated in the same region of the aromatic protons, it was difficult to detect  $d_{\text{NN}}$  except for NH<sub>Isox3</sub>/NH<sub>Leu4</sub> (vw). Of relevance, CH<sub>Leu2</sub> showed proximity with NH<sub>Leu5</sub> [vw,  $d_{\alpha\text{N}}(i,i+3)$ ] as well as this last with Me<sub>2</sub>CH<sub>Val4</sub>.

Taking all this information together, we can assume that R-Isox- $\beta^{2,2}$ AA is indeed able to induce the folding of the peptide chain, stabilizing a  $\alpha$ -turn-like structure (Figure 6). Of relevance, the H-bond between NH<sub>Leu2</sub> with C=O<sub>Boc</sub> observed in both **8a** and **11**, stabilizes an inverse  $\gamma$ -turn at N-terminus. Our

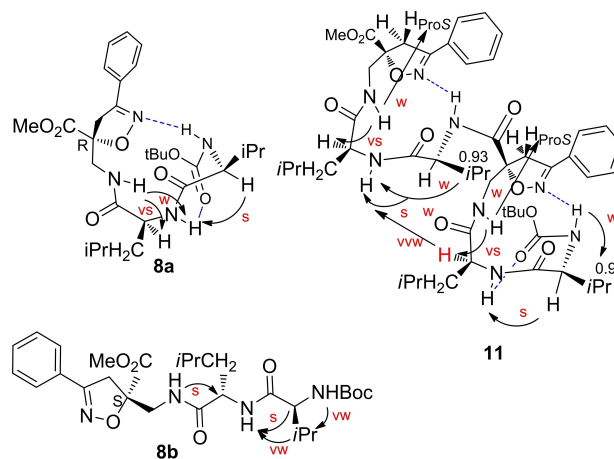


Figure 6. Conformations of the peptides **8a**, **8b** and **11**; NOEs (arrows), H-bonds (blue dotted lines)

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- $\beta^{2,2}$ AA in short tri- and hexapeptide models, we observed an unexpected  $\alpha$ -turn-like motif, thanks to an unprecedented strong H-bond between  $\text{NH}_{\text{Val}}$  and the C=N of isoxazoline side chain of our  $\beta^{2,2}$ AA.

## Experimental Section

**General information.** The NMR spectroscopic experiments were carried out either on a Varian OXFORD 300 MHz (300 and 75 MHz for  $^1\text{H}$  and  $^{13}\text{C}$ , respectively), or Bruker Avance Bruker Avance I 500 MHz spectrometers (500 and 125 MHz for  $^1\text{H}$  and  $^{13}\text{C}$ , respectively). Chemical shifts  $\delta$  are given in ppm relative to the  $\text{CHCl}_3$  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 Finnigan 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**.<sup>[54]</sup> 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/ $\text{H}_2\text{O}$  (3:1, 12 mL).  $\text{NaN}_3$  (450 mg, 6.72 mmol) was added to the solution and the mixture was stirred for 20 minutes. The reaction mixture was diluted with  $\text{CH}_2\text{Cl}_2$  (20 mL), washed with  $\text{H}_2\text{O}$  and s.s. NaCl and dried over  $\text{Na}_2\text{SO}_4$ . The solvent was removed under reduced pressure, affording a colorless oil. (485.2 mg, 98 %).<sup>[27]</sup>

**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  $\text{H}_2\text{O}$  (15 mL) and finally dried over  $\text{Na}_2\text{SO}_4$ . 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 0.1 M solution. Afterwards,  $\text{H}_2\text{O}$  (0.37 mL, 20.72 mmol, 7 eq) and a 0.1 M solution of  $\text{PMe}_3$  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-Isox $\beta^{2,2}$ AA-OME (6).** In a round bottom flask equipped with magnetic stirrer, **5** (693 mg, 2.96 mmol) was suspended in dry  $\text{CH}_2\text{Cl}_2$  (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  $\text{KHSO}_4$  (5%, 20 mL),  $\text{NaHCO}_3$  (20 mL), brine (25 mL), then the organic layer was dried over  $\text{Na}_2\text{SO}_4$ . 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.

***NH*<sub>2</sub>-Leu-Isox $\beta^{2,2}$ AA-OME (7).** In a round bottom flask equipped with magnetic stirrer, **6** (850 mg, 1.91 mmol) was suspended in  $\text{CH}_2\text{Cl}_2$  (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  $\text{NaHCO}_3$  and the organic layer was extracted with  $\text{CH}_2\text{Cl}_2$  (10 mL) and dried over  $\text{Na}_2\text{SO}_4$ . 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 ( $\text{CH}_2\text{Cl}_2$ /MeOH 10:1 + 0.1% TEA) afforded **7a** (266 mg) and **7b** (251 mg) as white solids.

***N*-Boc-Val-Leu-Isox $\beta^{2,2}$ AA-OME (8).** In a round bottom flask equipped with magnetic stirrer, *NH*-Boc-(*L*)-Valine (176 mg, 0.811 mmol) was suspended in dry  $\text{CH}_2\text{Cl}_2$  (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  $\text{KHSO}_4$  (5%, 20 mL),  $\text{NaHCO}_3$  (20 mL), brine (25 mL), then the organic layer was dried over  $\text{Na}_2\text{SO}_4$ . 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*)-Isox $\beta^{2,2}$ AA-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  $\text{KHSO}_4$  (5%, 20 mL) and the organic

layer was extracted with CH<sub>2</sub>Cl<sub>2</sub> (10 mL), then dried over Na<sub>2</sub>SO<sub>4</sub>. The solvent was removed under reduced pressure, affording **9a** as white solid with quantitative yield.

**NH<sub>2</sub>-Val-Leu-(R)-Isoxβ<sup>2,2</sup>AA-OME (10a)**. In a round bottom flask equipped with magnetic stirrer, **8a** (350 mg, 0.64 mmol) was suspended in CH<sub>2</sub>Cl<sub>2</sub> (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)-Isoxβ<sup>2,2</sup>AA-Val-Leu-(R)-Isoxβ<sup>2,2</sup>AA-OME (11)**. In a round bottom flask equipped with magnetic stirrer, **9a** (120 mg, 0.26 mmol) was suspended in dry CH<sub>2</sub>Cl<sub>2</sub> (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 KHSO<sub>4</sub> (5 %, 10 mL), NaHCO<sub>3</sub> (10 mL), brine (10 mL). The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub>. 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 Kα X-rays (λ=0.71073 Å) with a nominal source power of 50 kV×30 mA. **7a** crystallizes in the monoclinic acentric polar P2<sub>1</sub> 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, β = 104.470(2) deg, V = 925.06(8) Å<sup>3</sup>. 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.<sup>[55]</sup> The overall agreement factor was R(F) = 0.0415 for 3682 reflections with F<sub>o</sub> > 4σ(F<sub>o</sub>), with goodness-of-fit 0.950 and largest Fourier residuals as low as ±0.13 e·Å<sup>-3</sup>. The absolute structure was secured by the Parson's method<sup>[56]</sup> 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**.

Deposition Number 2172521 (for **7a**) contains the supplementary crystallographic data for this paper. These data are provided free of charge by the joint Cambridge Crystallographic Data Centre and Fachinformationszentrum Karlsruhe Access Structures service www.ccdc.cam.ac.uk/structures.

### Theoretical calculations

Peptide **8a** was initially constructed using the MOE software.<sup>[57]</sup> A conformational search (CS) was then performed with the

same software. The Low Mode Molecular Dynamics CS method was applied,<sup>[58]</sup> using the Amber10EHT forcefield.<sup>[59,60]</sup> The Reaction-Field implicit solvation model,<sup>[61]</sup> 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.<sup>[62]</sup> The density functional theory (DFT) was applied in combination with the MPW1B95 functional and the 6-31G(d) basis set.<sup>[63]</sup> This functional was chosen based on our experience with conformational analyses of non-natural peptides.<sup>[50]</sup> 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<sup>[64]</sup> and GD3 empirical correction to dispersive interactions.<sup>[65]</sup> 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,<sup>[66]</sup> except the maximum number of Newton iterations and the step size that were 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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### Conflict of Interest

The authors declare no conflict of interest.

### Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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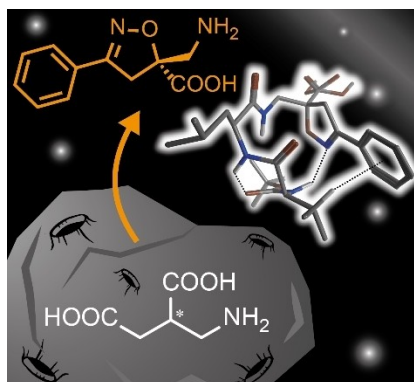


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## RESEARCH ARTICLE

New peptidomimetics containing a  $\beta^{2,2}$ -isoxazoline amino acid, 5-(aminomethyl)-3-phenyl-4,5-dihydroisoxazole-5-carboxylic acid (Isox- $\beta^{2,2}$ AA), were prepared and studied by NMR and theoretical calculations. Interestingly, inserting *R*-Isox- $\beta^{2,2}$ AA in short tri- and hexapeptide models, an unexpected  $\alpha$ -turn-like motif was observed, thanks to an unprecedented strong H-bond involving C=N of isoxazoline side chain of  $\beta^{2,2}$ AA.



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**A Non-coded  $\beta^{2,2}$ -Amino Acid with Isoxazoline Core Able to Stabilize Peptides Folding through an Unprecedented Hydrogen Bond**



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