Pergamon

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Tetrahedron Letters 44 (2003) 7369–7372

TETRAHEDRON LETTERS

Formation of β-hairpins in L-Pro-Gly containing peptides facilitated by 3-amino benzoic acid

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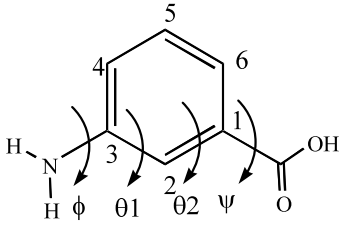
Received 22 May 2003; revised 11 July 2003; accepted 25 July 2003

Dedicated to Professor C. L. Khetrapal on the occasion of his 65th birthday

Abstract—Designed β-hairpin peptides tolerate insertion of 3-amino benzoic acid (3-Aba) and also permit accommodation of both enantiomers of Pro-Gly turn motifs.

Designing β-hairpins has been an active area of research.[1] In these designs the β-sheets invariably contain hydrophobic residues, while Pro-Gly is the most often used tight turn. It has been shown that for the two enantiomers of Pro-Gly, β-sheets with a natural twist,[2] accommodate only the loop containing the D-Pro-Gly and provide stable β-haipins.[3] These β-hairpins, have also been shown to tolerate insertion of β- and δ-amino amino acids without disrupting the designed fold.[4] In the present communication we demonstrate that insertion of 3-amino benzoic acid (3-Aba), **1**, a γ-amino acid, with a rigid extended framework, adds to the stability of the hairpins and also permits accommodation of both the enantiomers of Pro-Gly in the loop.

In 3-Aba, two intervening dihedral angles θ_1 (C2–C3) and θ_2 (C1–C2), between φ (N–C3) and ψ (C1–CO) (Fig. 1), with a fixed value of about 180°, are likely to promote a β-sheet like structure.[5] 3-Aba was first introduced in cyclic peptides behaving as artificial receptors for anions and more recently it has been incorporated into other peptides.[6] Substituted 3-Aba has also been used as a constrained residue in hetero duplexes and nanocavities of tunable sizes.[7] 3-Aba has the capability of having hydrogen bond donor as well as acceptor sites facing the same side of an extended sheet, which are likely to participate in H-bonds simultaneously.



3-Aba

1

Figure 1.

Among the various turn inducing dipeptide motifs, we chose D-Pro-Gly and L-Pro-Gly, for generating β-hairpins.[1,8] The peptides Boc-Ile-Val-(3-Aba)-^{L}Pro-Gly-(3-Aba)-Ile-Val-CO_2Me **2**, Boc-Ile-Val-(3-Aba)-^{D}Pro-Gly-(3-Aba)-Ile-Val-CO_2Me **3**, Boc-Ala-Gly-(3-Aba)-^{L}Pro-Gly-(3-Aba)-Val-Val-CO_2Me **4**, Boc-Ala-Gly-(3-Aba)-^{D}Pro-Gly-(3-Aba)-Val-Val-CO_2Me **5** were synthesized by standard methods of peptide coupling using DCC/HOBt and by the anhydride method using ethyl chloroformate.[9,10]

The results of structural studies carried out by NMR measurements in about 2 mM solutions in $CDCl_3$ at 30°C and supported by molecular dynamics (MD) calculations are reported below. In order to improve the resolution for **4** and **5**, 4% of DMSO-d_6 was added to the NMR solution. The NMR spectra clearly demonstrate wide dispersion of backbone amide and CαH chemical shifts,[11] supporting a well-defined organized structure for these peptides in solution.

For peptide **2** the solvent titration studies[12] show that 3-Aba(3) NH, 3-Aba(6) NH and Val(8) NH are hydrogen bonded as their chemical shifts change by only

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doi:10.1016/j.tetlet.2003.07.003

0.41, 0.18 and 0.42 ppm, respectively. This is further supported by appreciable down field shifts of their resonances in $CDCl_3$.

Strong ROESY cross peaks 3-Aba(3) H^6/Pro(4) H^δ and 3-Aba(6) H^6/Ile(7) H^N indicate that 3-Aba(3) and 3-Aba(6) adopt an extended structure. Figure 2(a) shows some of the important NOEs from the ROESY spectrum.

The presence of the cross peaks Gly(5) H^N/3-Aba(6) H^N as well as much stronger Pro(4) H^α/Gly(5) H^N compared to Gly(5) H^α/3-Aba(6) H^N clearly show the existence of a type-II β-turn involving Pro(5)/Gly(6) residues. The unequivocal evidence for a stable β-hairpin comes from interstrand ROESY cross peaks between 3-Aba(3) H^2/3-Aba(6) H^2, Val(2) H^α/Ile(7) H^α, Ile(1) H^N/Val(8) H^N, Val(2) H^α/Ile(7) CH_3^γ and Boc/OCH_3 (Fig. 3) as well as participation of Ile(1) NH, 3-Aba(3) NH, 3-Aba (6) NH and Val (8) NH in interstrand hydrogen bonds. The large value of $^3J(H^N–H^\alpha)$ (8.4/Ile(1), 9.0/Val(2), 8.9/Ile(7) and 8.2/Val(8), Hz) in addition to the down field appearance of H^α protons (4.91/Val(2), 5.02/Ile(7) and 4.55/Val(8), ppm) as well as the wide dispersion of strong inter-residue H^α–H^N and weak intra-residue H^N-H^α cross peaks, strongly support backbone dihedral angles for the two strands to be in the β region of the Ramachandran plot. Interestingly the β-hairpin does not seem to fray much in the termini.

For peptide **3**, the structure is very similar to that for the diastereomer **2**. The turn is the 'mirror image' type-II′ β-turn. The picture is very similar for peptides **4** and **5**, where amide protons at residues 3, 6 and 8 are hydrogen bonded and most of the NOE cross peaks observed in **2** and **3** are present.

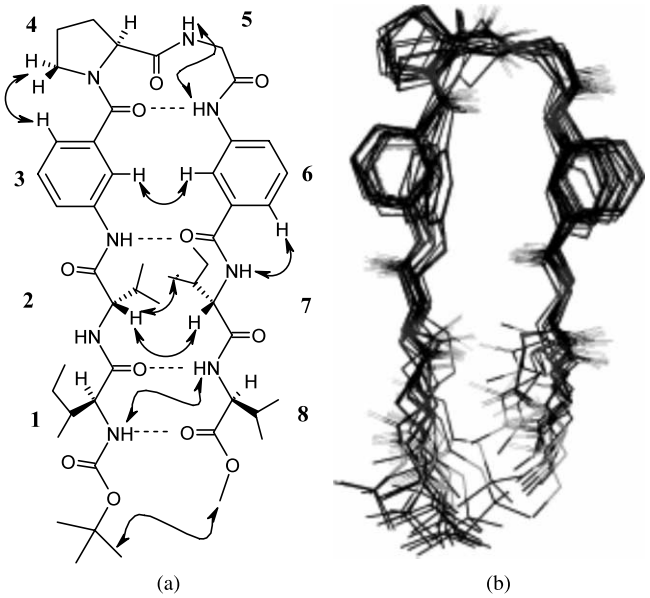

Figure 2. (a) Diagnostic NOEs and the H-bonds in **2**. (b) Superimposed 20 minimum energy structures from the MD simulation of **2**.

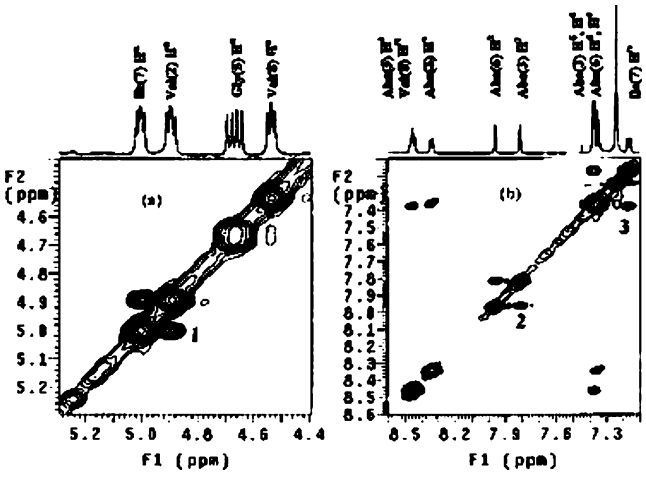

Figure 3. Expanded regions of the ROESY spectrum of **2**. (a) Val(2) H^α/Ile(7) H^α (1) and (b) 3-Aba(3) H^2/3-Aba(6) H^2 (2) and Ile(7) H^N/3-Aba(6) H^6 (3).

The β-turns for **3**, the D-Pro containing peptides, are similar to those in designed β-hairpins with all α-amino acid residues.[3] As against this, insertion of β amino acids results in hairpins with both type-I′β and type-II′β turns.[4] The variation in the presentation of the functional groups in the strands may have a role in deciding the type of the turn.

The MD calculations on **2** and **3** show that the extended geometry of 3-Aba facilitates the accommodation of two additional carbons in the backbone without disrupting the hairpin. For **2**, twenty superimposed minimum energy structures obtained from a 600 ps MD run are shown in Figure 2(b). Since 3-Aba is achiral, ϕ and ψ take both signs with the same propensity. The magnitudes of ϕ and ψ for 3-Aba(3) and 3-Aba(6) are 132±4°, 132±9°, 140±5° and 150±6°, respectively, which are in the range required for β-antiparallel sheets. The hairpin is stabilized by four cross-strand hydrogen bonds. There is significant fraying at the termini as shown by the large variation for ϕ and ψ of the terminal residues. The angle between the 3-Aba(3) and 3-Aba(6) aromatic rings is around 13–80°. The hydrophobic effects involving side chain–side chain interactions are critical determinants of antiparallel β-sheet stability.[13] Interactions between the 3-Aba rings, like those observed between the aromatic rings in proteins, might contribute to additional stabilization of the hairpins.[14]

The CD spectra of 100 μM solutions of **2** and **3** in methanol are given in Figure 4. They show a broad extrema at about 230–240 nm with **2** having a maximum at 238 nm and **3** a minimum at about 230 nm. These spectra are anomalous and differ considerably from those reported for conventional β-hairpin peptides which show a strong negative CD band at about 220 nm. The CD spectra for **2** and **3** reflect a strong influence of the aromatic group in 3-Aba[15]

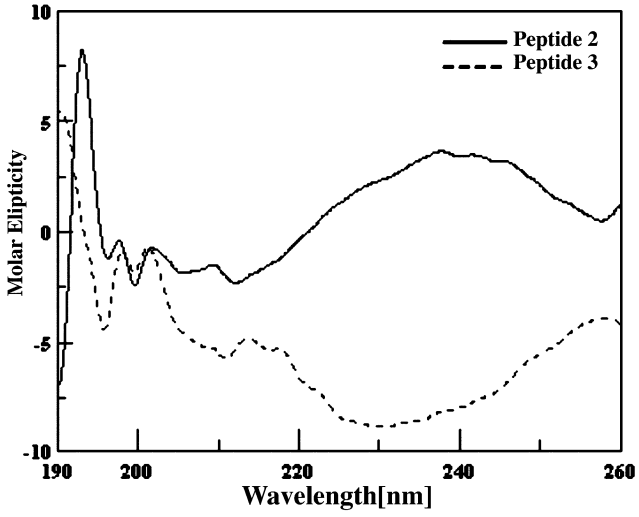

Figure 4. CD Spectra of **2** and **3** in MeOH.

The peptides **2** and **3** fold into β-hairpin structures irrespective of the chirality of Pro at the β-turn. The relationship between the β-turn and the β-hairpin promotion arises from the right handed twist preference of the β-sheets, which must be matched in the loop.[2] L-Pro-Gly leads to left-handed type-I/type-II β-turns, which are not compatible with the twist of the strand for L-residues. It is thus very likely that the β-hairpin stability in these molecules is due to the absence of any perceptible twist of the strands, which enables accommodation of both enantiomeric turns. The presence of NOEs across the diagonally placed residues has been attributed to the curvature/twist of the sheets.[16] We only observed NOE interactions between residues placed directly across from each other and not between the diagonal ones, further supporting the absence of curvature. Additional support for the absence of the twist of the strands comes from a recent report where a peptide containing an L-Pro-Ala turn motif has been shown to exist as a three strand mono-layer film of β-hairpins extended parallel to the plane of the air–water interface.[17] In vinylogous peptides Schreiber et al.[18] have also shown the ability of the L-Pro-Gly motif to lead to 'minimal' antiparallel sheet conformations with a tight turn.

In conclusion we have shown that the incorporation of 3-Aba, a γ-amino acid, in designed hairpins is very well tolerated. The observation of a β-hairpin with an L-Pro-Gly turn inducing motif is novel and unusual. We are presently studying the incorporation of these amino acids in designs containing multiple antiparallel β-strands.

Acknowledgements

We thank Dr. T. K. Chakraborty for several helpful discussions. M.H.V.R.R. and S.K.K. thank CSIR, New Delhi for fellowships.

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10. **Standard amide coupling using DCC and HOBt**: Solid 1-hydroxybenzotriazole (HOBt, 1 equiv.) and dicyclohexylcarbodiimide (DCC, 1 equiv.) were added sequentially at 0°C to a stirred solution of *N*-protected amino acid/peptide in dry CH_2Cl_2 or in a mixture of dry DMF and CH_2Cl_2 in cases where the solubility was poor in CH_2Cl_2, under N_2. After a period of $\sim$0.25 h, the reaction mixture was mixed with amino acid methyl ester/amine-free peptide methyl ester in dry CH_2Cl_2. The combined mixture was stirred at room temperature for 6 h, the precipitate dicylohexyl urea filtered and the residue washed with CH_2Cl_2 and the combined filtrates were

washed sequentially with cold 0.5 N HCl, saturated $NaHCO_3$ and NaCl solutions. The organic extract was dried over Na_2SO_4 and evaporated in vacuo. The residue was purified by column chromatography using EtOAc/hexane as eluent over 60–120 silica gel.

General procedure for peptide coupling using ethyl chloroformate: *N*-Methyl morpholine (NMM, 1 equiv.) and ethyl chloroformate (1 equiv.) were added sequentially at −20°C to a stirred solution of *N*-protected amino acid/peptide in dry CH_2Cl_2 or in a mixture of dry DMF and CH_2Cl_2 in cases where the solubility was poor in CH_2Cl_2, under N_2. After a period of ~0.25 h, the reaction mixture was mixed with amino acid methyl ester/amine free peptide methyl ester (freshly generated from its hydrochloride salt/TFA salt, using triethylamine) in dry CH_2Cl_2. The combined mixture was stirred at room temperature for 6 h, the residue washed with CH_2Cl_2 and the combined filtrates were worked up as above. The organic extract was dried over Na_2SO_4 and evaporated in vacuo. The residue was purified by column chromatography using EtOAc/hexane as eluent over 60–120 silica gel.

11. **Spectral data of selected compounds 2**: ^{1}H NMR ($CDCl_3$, 500 MHz): δ 10.30 (s, 1H, 3-Aba3-N*H*), 9.35 (s, 1H, 3-Aba6-N*H*), 8.48(d, $J=7.4$ Hz, 1H, 3-Aba6-C4*H*), 8.47 (d, $J=8.2$ Hz, 1H, Val8-N*H*), 8.35 (d, $J=7.5$ Hz, 1H, 3-Aba3-C4*H*), 8.00 (s, 1H, 3-Aba6-C2*H*), 7.84 (s, 1H, 3-Aba3-C2*H*), 7.41 (m, 1H, 3-Aba6-C5*H*), 7.39 (m, 1H, 3-Aba3-C6*H*), 7.38 (m, 1H, 3-Aba6-C6*H*), 7.37 (m, 1H, 3-Aba3-C5*H*), 7.18 (d, $J=8.9$ Hz, 1H, Ile7-N*H*), 6.97 (d, $J=9.0$ Hz, 1H, Val2-N*H*), 6.42 (dd, $J=4.0$, 9.1 Hz, 1H, Gly5-N*H*), 5.41 (d, $J=8.4$ Hz, 1H, Ile1-N*H*), 5.02 (dd, $J=5.5$, 8.9 Hz, 1H, Ile7-Cα*H*), 4.91 (dd, $J=6.2$, 9.0 Hz, 1H, Val2-Cα*H*), 4.68 (dd, $J=9.1$, 17.4 Hz, 1H, Gly5-Cα*H*), 4.55 (dd, $J=5.6$, 8.2 Hz, 1H, Val8-Cα*H*), 4.30 (dd, $J=3.8$, 8.1 Hz, 1H, Pro4-Cα*H*), 4.16 (m, 1H, Ile1-Cα*H*), 3.84 (m, 2H, Pro4-Cδ*H* & Cδ*H′*), 3.75 (s, 3H, OCH_3), 3.73 (dd, $J=4.0$, 17.3 Hz, 1H, Gly5-Cα*H′*), 2.35 (m, 1H, Pro4-Cγ*H*), 2.22 (m, 1H, Pro4-Cβ*H*), 2.20 (m, 1H, Val8-Cβ*H*), 2.17 (m, 1H, Pro4-Cβ*H′*), 2.07 (m, 1H, Val2-Cβ*H*), 2.00 (m, 1H, Pro4-Cγ*H′*),1.97 (m, 1H, Ile1-Cβ*H*), 1.73 (m, 1H, Ile7-Cβ*H*), 1.50 (m, 1H, Ile1-Cγ*H*), 1.44 (s, 9H, *Boc*), 1.42 (m, 1H, Ile1-Cγ*H*), 1.16 (m, 1H, Ile1-Cγ*H′*), 0.97 (d, 3H, Val8-CγCH_3), 0.97 (d, 3H, Val8-CγCH'_3), 0.96 (m, 1H, Ile8-Cγ*H′*), 0.95 (d, 3H, Val2-CγCH_3), 0.95 (d, 3H, Val2-CγCH'_3), 0.82 (d, $J=6.9$ Hz, 3H, Ile7-CγCH_3), 0.72 (t, $J=7.4$ Hz, 3H, Ile7-CδC*H*3); FABMS (*m*/*z* %): M+Na (971, 14), M^+ (949, 12); IR (KBr, cm^{-1}): 3287, 2966, 2927, 1746, 1718, 1637, 1588, 1545, 1490, 1442, 1387, 1312, 1249, 1206, 1166, 1020.

3: ^{1}H NMR ($CDCl_3$, 500 MHz): δ 10.16 (s, 1H, 3-Aba3-N*H*), 9.35 (s, 1H, 3-Aba6-N*H*), 8.46 (d, $J=9.1$ Hz, 1H, Val8-N*H*), 8.43(d, $J=7.4$ Hz, 1H, 3-Aba6-C4*H*), 8.22 (dt, $J=1.6$, 1.9, 7.2, Hz, 1H, 3-Aba3-C4*H*), 7.84 (t, $J=1.6$, 1H, 3-Aba6-C2*H*), 7.81 (t, $J=1.6$ Hz, 1H, 3-Aba3-C2*H*), 7.34 (m, 1H, 3-Aba3-C6*H*), 7.34 (m, 1H, 3-Aba6-C6*H*) 7.35 (m, 1H, 3-Aba3-C5*H*), 7.33 (d, $J=8.5$ Hz, 1H, Ile7-N*H*), 7.23 (dt, $J=1.6$, 7.4 Hz, 1H, 3-Aba6-C5*H*), 6.74 (dd, $J=3.5$, 9.3 Hz, 1H, Gly5-N*H*), 6.42 (d, $J=6.9$ z, 1H, Val2-N*H*), 5.68 (d, $J=8.4$ Hz, 1H, Ile1-N*H*), 4.80 (t, $J=8.5$ Hz, 1H, Ile7-Cα*H*) 4.72 (dd, $J=9.3$, 17.4 Hz, 1H, Gly5-Cα*H*), 4.55 (dd, $J=5.6$, 9.1 Hz, 1H, Val8-Cα*H*),), 4.40 (dd, $J=6.9$, 7.5 Hz, 1H, Val2-Cα*H*), 4.36 (dd, $J=3.9$, 8.1 Hz, 1H, Pro4-Cα*H*), 4.03 (t, $J=8.4$, 1H, Ile1-Cα*H*), 3.85 (m, 2H, Pro4-Cδ*H* and Cδ*H′*), 3.72 (s, 3H, OCH_3), 3.69 (dd, $J=3.5$, 17.4 Hz, 1H, Gly5-Cα*H′*), 2.32 (m, 1H, Pro4-Cγ*H*), 2.21 (m, 1H, Pro4-Cβ*H*), 2.07 (m, 1H, Val2-Cβ*H*), 2.05 (m, 1H, Val8-Cβ*H*), 2.13 (m, 1H, Pro4-Cβ*H′*), 1.98 (m, 1H, Pro4-Cγ*H′*), 1.89 (m, 1H, Ile1-Cβ*H*), 1.88 (m, 1H, Ile7-Cβ*H*), 1.65 (m, 1H, Ile7-Cγ*H*), 1.59 (m, 1H, Ile1-Cγ*H*), 1.45 (s, 9H, Boc), 1.22 (m, 1H, Ile1-Cγ*H′*), 1.01 (d, $J=6.9$ Hz, 3H, Val2-CγCH_3), 1.01 (d, $J=6.9$ Hz, 3H, Val2-CγCH'_3), 0.99 (m, 1H, Ile7-Cγ*H′*), 0.96 (d, $J=7.5$ Hz, 3H, Ile7-CγCH_3), 0.88 (t, $J=7.5$ Hz, 3H, Ile7-CδC*H*3), 0.72 (d, $J=7.1$ Hz, 3H, Val8-CγCH_3), 0.72 (d, $J=7.1$ Hz, 3H, Val8-CγCH'_3), 0.71 (d, $J=7.1$ Hz, 3H, Val8-CγCH'_3); ESI-MS (*m*/*z*.%): M^++Na (972,15), M+2H (950, 8); IR (KBr, cm^{-1}): 3314, 2966, 2905, 1764, 1654, 1596, 1546, 1441, 1405, 1396, 1346, 1263, 1225, 1164, 1050.

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