# IBTM-Containing Gramicidin S Analogues: Evidence for IBTM as a Suitable Type II′ β-Turn Mimetic[1,2]

**David Andreu,*,[3a] Sergi Ruiz,[3a] Cristina Carreño,[3a] Jordi Alsina,[3a] Fernando Albericio,[3a] María Ángeles Jiménez,[3b] Natalia de la Figuera,[3c] Rosario Herranz,[3c] María Teresa García-López,[3c] and Rosario González-Muñiz[3c]**

*Contribution from the Department of Organic Chemistry, University of Barcelona, E-08028 Barcelona, Spain, and Institutes of Structure of Matter and Medicinal Chemistry, CSIC, E-28006 Madrid, Spain*

*Received February 24, 1997. Revised Manuscript Received August 6, 1997*[⊗]

**Abstract:** The 2-amino-3-oxohexahydroindolizino[8,7-*b*]indole-5-carboxylate system (IBTM) has been proposed as a dipeptide surrogate of type II′ β-turns. To evaluate which of the 11b*R* and 11b*S* diastereomers of IBTM best reproduces the conformational properties of type II′ β-turns, gramicidin S (GS), a cyclic antibiotic peptide that contains two such units, has been chosen as a test compound and the effect of either diastereomer on both conformation and activity of the resulting peptide analogues has been determined. A conventional approach to the cyclic peptide structure based on solution cyclization of a partially protected precursor was only practicable for the (*S*)-IBTM diastereomer. As an alternative, a solid phase mediated cyclization approach has been devised and applied successfully to both gramicidin S and its Lys$^{2,2'}$ analogue, then extended to the (*R*)-IBTM-containing analogues. NMR conformational analysis has clearly shown that only the (*R*) diastereomer of IBTM is a suitable mimic of the type II′ β-turn conformation typical of GS. Differences in antibacterial activity between the (*S*)- and (*R*)-IBTM-containing GS analogues confirm the conformational results.

The β-turn motif, a segment of four amino acid residues that reverse the direction of peptide chains, is an important structural feature of proteins. The surface localization of turns in proteins, and the predominance within them of residues containing potentially critical pharmacophoric information, has led to the hypothesis that turns play relevant roles in diverse recognition events.[4] There is substantial evidence to suggest that smaller peptides also possess β-turns in their bioactive conformations, and the resulting compact structures have clustered side chains available for interaction with receptors.[5] A general approach to confirm these suggestions involves the use of non-peptide building blocks which, when inserted into a peptide chain, enforce or stabilize a particular type of β-turn. In this respect, a variety of conformationally restricted compounds, mostly lactams, have been proposed as dipeptide mimetic replacements for the $i + 1$ and $i + 2$ residues of β-turns.[6,7] Incorporation of such β-turn surrogates into model peptides containing well-established β-turns produces analogues whose structural analysis indicates how well the replacements match the original β-turn conformation. A useful candidate for this type of studies is the antibiotic gramicidin S[8] [GS, *cyclo*(Val$^1$-Orn$^2$-Leu$^3$-D-Phe$^4$-Pro$^5$)$_2$, **1**], a cyclic decapeptide[9] that exists in a very stable solution conformation with two symmetrical type II′ β-turns

⊗ Abstract published in *Advance ACS Abstracts*, October 1, 1997.

(1) Abbreviations: AAA, amino acid analysis; Al, allyl; Boc, *tert*-butoxycarbonyl; ATCC, American Type Culture Collection; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; COSY, homonuclear correlated spectroscopy; DCM, dichloromethane; DIEA, *N,N*-diisopropylethylamine; DIPCDI, *N,N*′-diisopropylcarbodiimide; DMAP, *N,N*-dimethylaminopyridine; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; DSC, disuccinimidyl carbonate; Fmoc, 9-fluorenylmethoxycarbonyl; GS, gramicidin S; HATU, *N*-[(dimethylamino)(1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-yl)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HOAc, acetic acid; HOAt, 7-azahydroxybenzotriazole; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; IBTM, 2(*S*)-amino-3-oxohexahydroindolizino[8,7-*b*]indole-5(*S*)-carboxylic acid; MALDI-TOF MS, matrix-assisted laser desorption ionization, time-of-flight mass spectroscopy; MBHA, *p*-methylbenzhydrylamine (resin); MeCN, acetonitrile; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; ODS, octadecylsilica; Pam, 4-phenylacetamidomethyl (resin); PyAOP, (7-azabenzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate; Su, succinimidyl; TBTU, *N*-[(1*H*-benzotriazol-1-yl)dimethylaminomethylene)-*N*-methylmethanaminium tetrafluoroborate *N*-oxide; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TOCSY, total correlation spectroscopy; TSP, sodium 3-trimethylsilyl-[2,2,3,3-$^2H_4$]propionate.

(2) Parts of this work have been previously reported in preliminary form: (a) De la Figuera, N.; Jiménez, M. A.; Biacs, M.; García-López, M. T.; González-Muñiz, R.; Andreu, D. In *Peptides 1994: Proceedings of the Twenty-Third European Peptide Symposium*; Maia, H. L. S., Ed.; ESCOM Science Publishers: Leiden, The Netherlands, 1994; pp 702−703. (b) Andreu, D.; Ruiz, S.; Carreño, C.; Alsina, J.; Albericio, F.; Jiménez, M. A.; De la Figuera, N.; Herranz, R.; García-López, M. T.; González-Muñiz, R. In *Peptides 1996: Proceedings of the Twenty-Fourth European Peptide Symposium*; Ramage, R., Epton, R., Eds.; Mayflower Worldwide Ltd.: Kingswinford, England, 1997; in press.

(3) (a) University of Barcelona. (b) Institute of Structure of Matter. (c) Institute of Medicinal Chemistry.

(4) Rose, G. D.; Gierasch, L. M.; Smith, J. A. *Adv. Protein Chem.* **1985**, *37*, 1−109.

(5) (a) Dyson, H. J.; Cross, K. J.; Houghten, R. A.; Wilson, I. A.; Wright, P. E.; Lerner, R. A. *Nature* **1985**, *318*, 480−483. (b) Oka, M.; Montelione, G. T.; Scheraga, H. A. *J. Am. Chem. Soc.* **1984**, *106*, 7959−7969. (c) Freidinger, R. M.; Veber, D. F.; Perlow, D. S.; Brooks, J. R.; Saperstein, R. *Science* **1980**, *210*, 656−658.

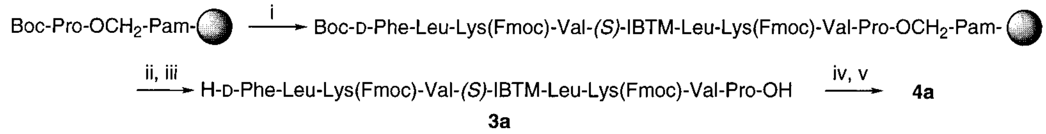
(6) For recent reviews on peptide conformation mimetics see: (a) Holzemann, G. *Kontakte* **1991**, 3−12, 55−63. (b) Kahn, M. *Synlett* **1993**, 821−826. (c) Olson, G. L.; Bolin, D. R.; Bonner, M. P.; Bös, M.; Cook, C. M.; Fry, D. C.; Graves, B. J.; Hatada, M.; Hill, D. E.; Kahn, M.; Madison, V. S.; Rusiecki, V. K.; Sarabu, R.; Sepinwall, J.; Vincent, G. P.; Voss, M. E. *J. Med. Chem.* **1993**, *36*, 3039−3049. (d) Liskamp, R. M. J. *Recl. Trav. Chim. Pays-Bas* **1994**, *113*, 1−19.

(7) Other representative examples of β-turn mimetics include: (a) Genin, M. J.; Johnson, R. L. *J. Am. Chem. Soc.* **1992**, *114*, 8778−8783. (b) Genin, M. J.; Gleason, W. B.; Johnson, R. L. *J. Org. Chem.* **1993**, *58*, 860−866. (c) Lombart, H.-G.; Lubell, W. D. *J. Org. Chem.* **1994**, *59*, 6147−6149. (d) Kemp, D. S.; Li, Z. Q. *Tetrahedron Lett.* **1995**, *36*, 4175−4178. (e) Hanessian, S.; Ronan, B.; Laoui, A. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1397−1400. (f) Robl, J. A. *Tetrahedron Lett.* **1994**, *35*, 393−396. (g) Mueller, R.; Revesz, L. *Tetrahedron Lett.* **1994**, *35*, 4091−4092. (h) Hanessian, S.; McNaughton-Smith, G. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1567−1572. (i) Slomczynska, U.; Chalmer, D. K.; Cornille, F.; Smythe, M. L.; Beusen, D. D.; Moeller, K. D.; Marshall, G. R. *J. Org. Chem.* **1996**, *61*, 1198−1204.

(8) Consden, R. J.; Gordon, A. H.; Martin, A. J. P.; Synge, R. D. M. *Biochem. J.* **1947**, *28*, 596−602.

S0002-7863(97)00575-1 CCC: $14.00 

**Scheme 1**




( *i*) 8 cycles of Boc solid phase synthesis. (ii) HF-anisole (9:1 v/v, 0 °C). (iii) reverse phase purification. (iv) Cyclization: peptide (50 μM), BOP (10 eq), DIEA (20 eq), DMF, 6 h. (v) Piperidine-DMF (1:4 v/v), 20 min; reverse phase purification.

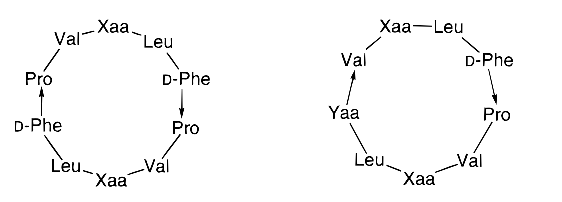
connected by a short antiparallel $\beta$-sheet.[10] The replacement of the D-Phe-Pro residues in GS by rigid mimetics has become a standard method for measuring their capacity to serve as surrogates of the $i + 1$ and $i + 2$ residues in type II′ $\beta$-turns.[11] Moreover, since the antibacterial activity of GS has been related to its particular conformation,[12] the cyclic decapeptide provides not only structural but also functional evidence to assess the adequacy of $\beta$-turn mimetic candidates.

In a recent paper we have proposed the 2-amino-3-oxo-hexahydroindolizino[8.7-*b*]indole-5-carboxylate system (H-IBTM-OH[1]) as a new type of $\beta$-turn surrogate.[13] Molecular dynamics studies on model structures Ac-*S*-IBTM-NHMe and Ac-*R*-IBTM-NHMe have revealed that, although both derivatives are able to adopt conformations close to an ideal type-II′ $\beta$-turn, the diastereoisomer with the 11b*R* configuration is a more effective replacement than the 11b*S* isomer for the $i + 1$ and $i + 2$ residues of this type of $\beta$-turn.[13] In a similar way, the temperature dependence of the N*H*-Me amide hydrogen chemical shift of the 11b*R* isomer in DMSO ($\Delta\delta/\Delta T = -2.8$ ppb/K) indicated that this proton is involved in an intramolecular hydrogen bond,[14] characteristic of $\beta$-turn conformations. The corresponding value for the *S* derivative (−3.9 ppb/K), however, was within a region where it is difficult to draw concrete conclusions with respect to hydrogen bonding.[9g]

In search of a more conclusive evaluation of the fitness of the IBTM system as a type II′ $\beta$-turn mimetic, we decided to replace one of the D-Phe[4]-Pro[5] dipeptide units in GS with either *S*- or *R*-IBTM surrogates. This in turn depended on convenient access routes to these IBTM-containing GS analogues. The present paper reports the synthesis of three such peptides, [(*S*)-IBTM[4,5]Lys[2,2′]]GS (**4a**), [(*R*)-IBTM[4,5],Lys[2,2′]]GS (**4b**), and [(*R*)-IBTM[4,5]]GS (**5b**), and the comparison of (*S*)- and (*R*)-IBTM-containing peptidomimetics to GS by NMR conformational analysis. Additional evidence on how well these dipeptide surrogates mimic the topology of D-Phe[4]-Pro[5] in GS has been provided by the activity of compounds **4a**, **4b**, and **5b** against several bacterial strains, in comparison with those of GS and its [Lys[2,2′]] analogue **2**.

## Results and Discussion

**Synthesis.** Our initial approach to IBTM-containing GS analogues chose a conventional synthetic strategy based on solution cyclization of a partially protected linear precursor (Scheme 1) assembled by solid-phase methods[15] on a Pam[16] resin. As in previous studies on GS analogues,[11c] the replacement of the two Orn residues in **1** by Lys was primarily dictated by synthetic reasons, *i.e.*, commercial availability of the orthogonally protected derivative Boc-Lys(Fmoc)-OH. Pro[4′] was chosen as *C*-terminal residue to minimize the risk of epimerization at the cyclization step, and the peptide was built from that residue by Boc-based chemistry[17] (Scheme 1). In the case of **4a**, the (*S*)-IBTM pseudodipeptide unit was incorporated into the sequence as the Boc derivative **6a**. HF acidolysis of the peptide-resin gave the linear bis-Fmoc protected precursor **3a**, which was purified on reverse phase, cyclized under high dilution conditions, and deprotected to give the target compound **4a**, satisfactorily characterized by AAA and MALDI-TOF MS. A parallel synthesis with the (*R*)-IBTM derivative **6b** readily provided the expected H-D-Phe-Leu-Lys(Fmoc)-Val-(*R*)-IBTM-Leu-Lys(Fmoc)-Val-Pro-OH intermediate. However, attempts to cyclize this precursor using a variety of activation reagents and conditions led invariably to very complex crudes in which no significant levels of **4b** could be detected after Fmoc




**1:** Xaa=Orn (gramicidin S)
**2:** Xaa=Lys

**4a** : Xaa=Lys; Yaa=*(S)*-IBTM
**4b** : Xaa=Lys; Yaa=*(R)*-IBTM
**5b** : Xaa=Orn; Yaa=*(R)*-IBTM

deprotection. In view of these difficulties, an alternative route, involving solid-phase-mediated cyclization as the key step, was explored. This type of cyclization has been successfully applied

(9) For recent reviews on cyclic peptides see: (a) Kopple, K. D. *J. Pharm. Sci.* **1972**, *61*, 1345−1356. (b) Blout, E. R. *Biopolymers* **1981**, *20*, 1901−1912. (c) Hruby, V. J. *Life Sci.* **1982**, *31*, 189−199. (d) Ovchinikov, Y. A.; Ivanov, V. T. In *The Proteins*; Neurath, H., Hill, R. L., Eds.; Academic Press: New York, 1982; pp 307−642. (e) Schmidt, U. *Pure Appl. Chem.* **1986**, *58*, 295−301. (f) Tonelli, A. E. In *Cyclic Polymers*; Semlyen, J. A., Ed.; Elsevier Applied Science: London, 1986; pp 261−284. For reviews on cyclic peptides as models for conformational studies see: (g) Kessler, H. *Angew. Chem., Int. Ed. Engl.* **1982**, *21*, 512−523. (h) Rizo, J.; Gierasch, L. M. *Annu. Rev. Biochem.* **1992**, *61*, 387−418.

(10) (a) Dygert, M.; Go, N.; Scheraga, H. A. *Macromolecules* **1975**, *8*, 750−761. (b) Huang, D.-H.; Walter, R.; Glickson, J. D.; Kirshna, N. R. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 672−675. (c) Krauss, E. M.; Chan, S. I. *J. Am. Chem. Soc.* **1982**, *104*, 6953−6961.

(11) (a) Sato, K.; Nagai, U. *J. Chem. Soc., Perkin Trans. 1* **1986**, 1231−1234. (b) Bach, A. C.; Markwalder, J. A.; Ripka, W. C. *Int. J. Peptide Protein Res.* **1991**, *38*, 314−323. (c) Ripka, W. C.; De Lucca, G. V.; Bach, A. C.; Pottorf, R. S.; Blaney, J. M. *Tetrahedron* **1993**, *49*, 3609−3628. (d) Lombart, H.-G.; Lubell, W. D. In *Peptides: Chemistry, Structure and Biology. Proceedings of the 14th American Peptide Symposium*; Kaumaya, P. T. P., Hodges, R. S., Eds.; Mayflower Scientific Ltd.: Kingswinford, England, 1996; pp 695−696.

(12) (a) Imazu, S.; Shimohigashi, Y.; Kodama, H.; Sakaguchi, K.; Waki, M.; Kato, T.; Izumiya, N. *Int. J. Peptide Protein Res.* **1988**, *32*, 298−306. (b) Shimohigashi, Y.; Sakamoto, H.; Yoshikomi, H.; Waki, M.; Kawano, K.; Ohno, M. In *Peptides, Chemistry and Biology, Proceedings of the 12th American Peptide Symposium*; Smith, J. A., Rivier, J. E., Eds.; Escom: Leiden, 1992; pp 271−272. (c) Tamaki, M.; Akabori, S.; Muramatsu, I. *Int. J. Peptide Protein Res.* **1996**, *47*, 369−375.

(13) De la Figuera, N.; Alkorta, I.; García-López, M. T.; Herranz, R.; González-Muñiz, R. *Tetrahedron* **1995**, *51*, 7841−7856.

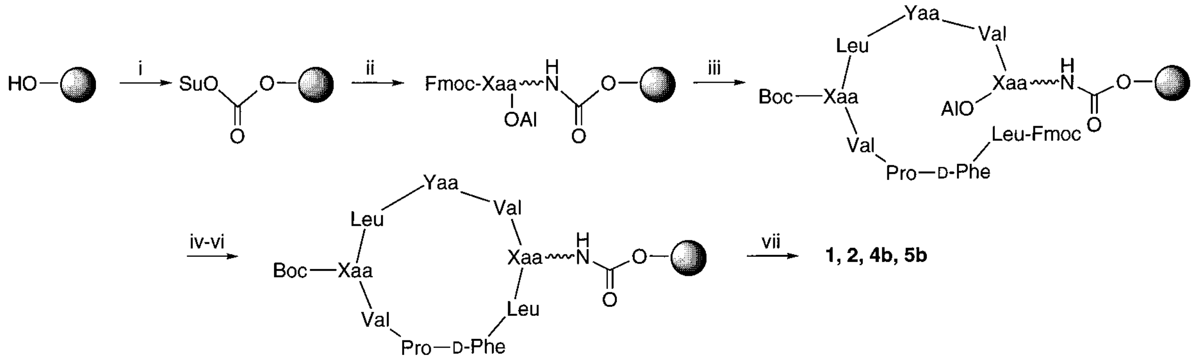
(14) De la Figuera, N. Unpublished results.

(15) (a) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149−2154. (b) Merrifield, R. B. In *Peptides. Synthesis, Structures and Applications*; Gutte, E., Ed.; Academic Press: San Diego, 1995; pp 93−169.

(16) Mitchell, A. R.; Kent, S. B. H.; Engelhard, M.; Merrifield, R. B. *J. Org. Chem.* **1978**, *43*, 2845−2852.

(17) Barany, G.; Merrifield, R. B. In *The Peptides. Analysis, Synthesis, Biology*; Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1979; Vol. 2, pp 1−284.

**Scheme 2**




(i) $(SuO)_2CO$-DMAP (10:1 eq), DMF. (ii) Fmoc-Xaa-OAl trifluoroacetate (10 eq), (Xaa= Orn, Lys), DIEA (20 eq), DMF. (iii) Fmoc solid phase synthesis; Yaa= D-Phe-Pro for **1** and **2**, *(R)*-IBTM for **4b** and **5b**. (iv) $Pd^0(PPh_3)_4$ in DMSO-THF-0.5 N aqueous HCl-morpholine (20:20:10:1 v/v), 2.5 h, 25°C. (v) Piperidine-DMF (1:4 v/v), 20 min. (vi) PyAOP-HOAt-DIEA (10 eq each), DMF, 2 h, rt. (vii) TFA-$H_2O$ (19:1), 2 h, 25°C.

to the synthesis of cyclic peptides in recent years.[18] The original approach used carboxyl (Asp, Glu)- or carboxamide (Asn, Gln)-containing residues for side chain anchoring to the polymer, with the α-COOH of those residues orthogonally protected with respect to the chemistry used for chain elongation. Selective removal of both *N*- and *C*-terminal protections allows cyclization to proceed, ideally under the pseudodilution conditions provided by the polymeric matrix.[19] One of our laboratories has developed a variation of this strategy that allows anchoring through the side chain amino groups of Lys or Orn, via a carbamate-functionalized resin.[20] The procedure requires Fmoc chemistry for chain elongation and uses orthogonal allyl protection of the α-COOH of the Lys or Orn residue. We decided to apply this method to prepare the (*R*)-IBTM-containing analogues of GS that were not accessible by conventional solution cyclization.

Trial syntheses of GS (**1**) and [Lys$^{2,2'}$]GS (**2**) were first performed (Scheme 2). The starting resin, 3-[4-(hydroxymethyl)phenoxy]propionyl-Ala-MBHA[21] (with Ala used as the internal reference amino acid[22]), was activated with DSC/DMAP to give a Su-carbonate resin, to which the trifluoroacetate salt of Fmoc-Xaa-OAl (Xaa = Orn, Lys) was anchored in the presence of DIEA. From this point, chain assembly was continued by Fmoc chemistry, with Boc protection for the side chains of Orn or Lys. Deprotection of the *C*-terminal allyl ester, followed by Fmoc removal at the *N*-terminus, allowed cyclization on the solid phase, with activation of the α-carboxyl of Orn/Lys by means of PyAOP/HOAt/DIEA.[23] Finally, acidolytic deprotection and cleavage furnished **1** and **2** in good purity (Figure 1), although in moderate yields (*ca.* 10% after purification, based on initial resin substitution).

Extension of this approach to the (*R*)-IBTM-containing mimetics **4b** and **5b** was essentially straightforward with use of the Fmoc derivative of (*R*)-IBTM, **7b**, as replacement for positions 4,5. Its incorporation to the peptide resin was facilitated by the strong activating agent HATU.[23] A deprotection and cleavage sequence similar to the ones used for **1** and **2** above provided the target compounds, albeit in much lower yields, presumably due to the enhanced hydrophobicity of IBTM, which substantially decreased recoveries at the purification steps. The cyclic pseudopeptides were satisfactorily characterized by AAA and MALDI-TOF MS.

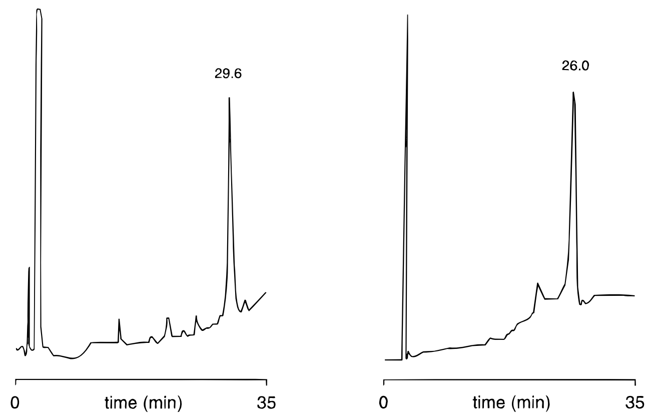



**Figure 1.** HPLC analysis of crude **1** (left panel) and **2** (right panel) after acidolytic cleavage from resin. ODS column (0.4 × 25 cm, 5 $\mu$m). Elution by a linear 5−95% gradient of MeCN (+0.036% TFA) into $H_2O$ (+0.045% TFA) over 30 min, followed by 5 min isocratic at 95%. UV detection at 220 nm. Retention times are indicated above the peaks.

**NMR Conformational Analysis.** Since type II′ β-turns present negative $\phi_3$ angles, and since only an equatorial disposition of the $C_5H$ proton in [(*S*)-IBTM$^{4,5}$,Lys$^{2,2'}$]GS (**4a**) and [(*R*)-IBTM$^{4,5}$,Lys$^{2,2'}$]GS (**4b**) is compatible with negative $\phi_3$ angles (Figure 2), NMR analysis was started by investigating the equatorial or axial disposition of that particular proton. A $^3J_{5,6}$ of ∼0 Hz is expected for the downfield $C_6H$ proton of IBTM in an equatorial disposition, while a large value would be expected for an axial disposition. The experimental $^3J_{5,6}$ value observed for compounds **4a** and **4b** in aqueous solution at pH 3.0 and in DMSO is ∼0 Hz, which confirms the equatorial disposition of the $C_5H$ proton in both **4a** and **4b**. Thus, both (*S*)- and (*R*)-IBTM skeletons prefer conformations with negative $\phi_3$ angles, as required for the formation of a type II′ β-turn.

The NMR parameters of [(*S*)-IBTM$^{4,5}$,Lys$^{2,2'}$]GS (**4a**) under both experimental conditions investigated are very different from those previously reported for native GS.[10c] Differences in the

(18) For recent examples of solid phase mediated cyclizations, see: (a) Kates, S. A.; Solé, N. A.; Albericio, F.; Barany, G. In *Peptides: Design, Synthesis and Biological Activity*; Basava, C., Anantharamaiah, G. M., Eds.; Birkhäuser: Boston, 1994; pp 39−58 and references cited therein. (b) Valero, M. L.; Camarero, J. A.; Adeva, A.; Verdaguer, N.; Fita, I.; Mateu, M. G.; Domingo, E.; Giralt, E.; Andreu, D. *Biomed. Pept. Proteins Nucleic Acids* **1995**, *1*, 133−140. (c) Valero, M. L.; Giralt, E.; Andreu, D. *Tetrahedron Lett.* **1996**, *37*, 4229−4232.

(19) Mazur, S.; Jayalekshmy, P. *J. Am. Chem. Soc.* **1979**, *101*, 677−683.

(20) Alsina, J.; Rabanal, F.; Giralt, E.; Albericio, F. *Tetrahedron Lett.* **1994**, *35*, 9633−9636.

(21) Albericio, F.; Barany, G. *Int. J. Peptide Protein Res.* **1985**, *26*, 92−97.

(22) Matsueda, G. R.; Haber, E. *Anal. Biochem.* **1980**, *104*, 215−227.

(23) Carpino, L. A.; El-Faham, A.; Minor, C. A.; Albericio, F. *J. Chem. Soc., Chem. Commun.* **1994**, *201*, 203.

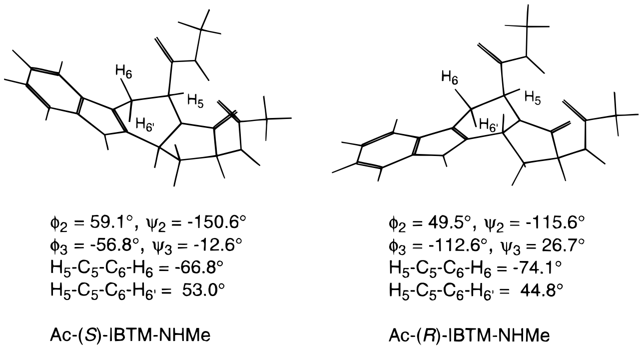



**Figure 2.** Significant dihedral angles of the $\beta$-turn-like conformations of Ac-(*S*)- and Ac-(*R*)-IBTM-NHMe model compounds.

**Table 1.** Chemical Shifts of Amide and Pro C$\delta$H protons ($\delta$, ppm from TSP)

| | GS (**1**) | | (*S*)-IBTM-GS (**4a**) | | (*R*)-IBTM-GS (**4b**) | |
|---|---|---|---|---|---|---|
| residue no. | H$_2$O[a] | DMSO[a] | H$_2$O[b] | DMSO[c] | H$_2$O[b] | DMSO[c] |
| Val 1 | 7.62 | 7.22 | 8.44 | 8.14 | 7.53 | 7.23 |
| Xaa 2[d] | 8.61 | 8.68 | 8.57 | 8.12 | 8.64 | 8.52 |
| Leu 3 | 8.81 | 8.34 | 7.78 | 7.44 | 8.27 | 8.10 |
| Yaa 4[d] | 8.99 | 9.11 | 7.80 | 7.91 | 9.39 | 9.46 |
| Val 1′ | 7.62 | 7.22 | 8.20 | 7.90 | 8.44 | 8.14 |
| Xaa 2′[d] | 8.61 | 8.68 | 8.19 | 7.81 | 8.56 | 8.53 |
| Leu 3′ | 8.81 | 8.34 | 8.18 | 7.92 | 8.72 | 8.46 |
| D-Phe 4′ | 8.99 | 9.11 | 8.39 | 8.35 | 9.01 | 8.82 |
| Pro 5′[e] | 3.68 | 3.60 | 3.67 | 3.58 | 3.66 | 3.58 |
| | 2.59 | 2.48 | 3.29 | 3.30 | 2.54 | 2.49 |

[a] Values from ref 10c. [b] DMSO, 20 °C. [c] H$_2$O, pH 3.0, 5 °C. [d] Xaa is Orn in GS and Lys in compounds **4a** and **4b**; Yaa is D-Phe-Pro in GS, and IBTM in compounds **4a** and **4b**. [e] Values corresponding to C$\delta$H and C$\delta'$H protons.

**Table 2.** Temperature Coefficients for Amide Protons ($\Delta\delta/\Delta T$, ppm 10$^3$/K)

| | GS (**1**) | (*S*)-IBTM-GS (**4a**) | | (*R*)-IBTM-GS (**4b**) | |
|---|---|---|---|---|---|
| residue no. | DMSO[a] | H$_2$O[b] | DMSO[c] | H$_2$O[b] | DMSO[c] |
| Val 1 | −1.6 | −9.0 | −5.2 | −3.0 | −1.6 |
| Xaa 2[d] | −5.0 | −8.5 | −2.5 | −9.0 | −5.3 |
| Leu 3 | −2.8 | −1.5 | −3.3 | −2.5 | −1.6 |
| Yaa 4[e] | −7.4 | −1.0 | −0.6 | −8.5 | −4.7 |
| Val 1′ | −1.6 | −5.5 | −4.9 | +0.5 | −0.4 |
| Xaa 2′[d] | −5.0 | −9.0 | −4.0 | −8.5 | −5.1 |
| Leu 3′ | −2.8 | −9.0 | −6.4 | −3.0 | −2.4 |
| D-Phe 4′ | −7.4 | −10.5 | −5.4 | −12.0 | −7.6 |

[a] Values from ref 11b. [b] pH 3.0, temperature range 5−25 °C. [c] Temperature range 20−50 °C. [d] Xaa is Orn in GS and Lys in compounds **4a** and **4b**. [e] D-Phe-Pro in GS, and IBTM in compounds **4a** and **4b**.

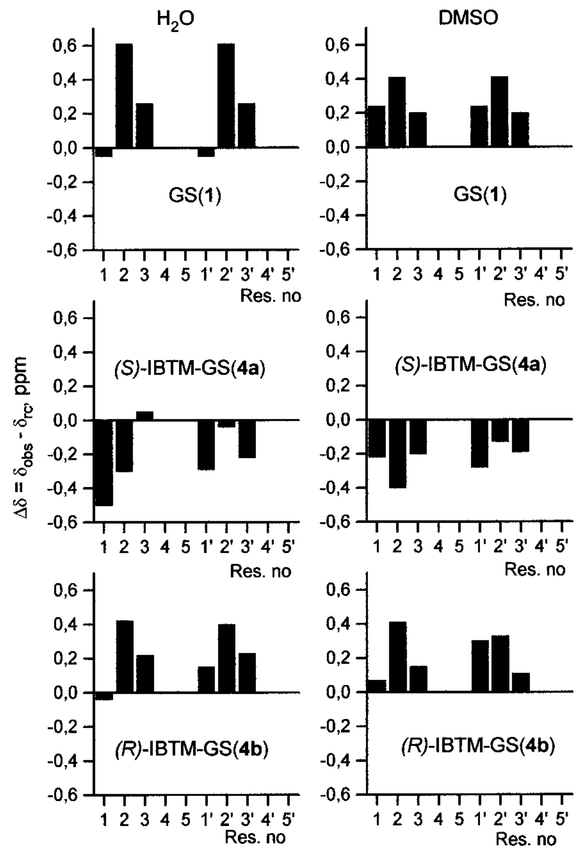



**Figure 3.** Conformational C$\alpha$H $\Delta\delta$ shifts ($\Delta\delta = \delta_{observed} - \delta_{random\ coil}$, ppm) as a function of sequence obtained for GS ($\Delta\delta$ was obtained from the $\delta$-values reported by Krauss and Chan[10c]) for compound **4a** and compound **4b** in aqueous solution and in DMSO. IBTM and D-Phe residues are not included as no random coil values have been reported for them.

$\delta$-values of most NH and C$\alpha$H protons of [(*S*)-IBTM$^{4,5}$, Lys$^{2,2'}$]GS (**4a**) with respect to GS (**1**) are large (Table 1). The C$\alpha$H protons of residues involved in an antiparallel $\beta$-sheet are expected to be downfield shifted[24] with respect to the random coil values.[25] As shown in Figure 3, this is the case for the C$\alpha$H protons of Val$^{1,1'}$, Orn$^{2,2'}$, and Leu$^{3,3'}$ in GS (**1**), but not for the same C$\alpha$H protons in [(*S*)-IBTM$^{4,5}$,Lys$^{2,2'}$]GS (**4a**), which are upfield shifted. A small absolute value for the temperature coefficient of an amide proton indicates its protection from solvent exchange by either involvement in an intramolecular hydrogen bond or inaccessibility to solvent. The temperature coefficients measured for the amide protons of [(*S*)-IBTM$^{4,5}$, Lys$^{2,2'}$]GS (**4a**) do not fit with the hydrogen-bonding pattern expected for a GS-like structure (Table 2). If **4a** were to adopt a GS-like structure such as shown in Figure 4, one should expect temperature coefficients for Val$^{1,1'}$ and Leu$^{3,3'}$ to be small (in absolute value), as previously reported for GS (**1**);[10c] however, Val$^{1,1'}$ and Leu$^{3'}$ have large coefficients, indicative of their accessibility to bulk solvent. The pattern of $^3J_{C\alpha H-NH}$ coupling constants of **4a** is also very different from that of GS (Table 3). Small values for Val$^1$ and Lys$^2$ may suggest the presence of reverse turns involving these residues. The observation of C$\alpha$H(Val$^1$)−NH(Leu$^3$), NH(Val$^1$)−NH(Lys$^2$), and NH(Lys$^2$)−NH(Leu$^3$) NOE connectivities is also compatible with the presence of a type I $\beta$-turn centered at Val$^1$-Lys$^2$. In addition to those NOEs, several long-range NOEs involving the IBTM nucleus and Val$^1$, Leu$^{3'}$, and D-Phe$^{4'}$ residues were found for **4a** in both aqueous solution and DMSO (Table 4 and Figure 5). These nonsequential NOEs could be explained by the formation of antiparallel dimers that bring the indole ring and the Val$^1$, Leu$^{3'}$, and D-Phe$^{4'}$ residues into close proximity, or by monomeric species with a hydrophobic cluster in which the IBTM skeleton and the hydrophobic side chains of Leu$^{3'}$ and D-Phe$^{4'}$ interact. In any event, the $\beta$-turn involving the D-Phe$^{4'}$-Pro$^{5'}$ in GS residues is not detected in **4a**.

In contrast to the previous data, the NMR parameters found for [(*R*)-IBTM$^{4,5}$,Lys$^{2,2'}$]GS (**4b**) in both aqueous solution, pH 3.0, and DMSO clearly indicate that it adopts a GS-like structure. The $\delta$ values of NH and C$\alpha$H protons in both solvents are very close to those reported for GS (**1**), while substantially different from those of [(*S*)-IBTM$^{4,5}$,Lys$^{2,2'}$]GS (**4a**) (Table 1). As expected for residues in a $\beta$-strand[24] and observed in GS,[10c] $\delta$ values of the C$\alpha$H protons of Val$^{1,1'}$, Lys$^{2,2'}$, and Leu$^{3,3'}$ are

(24) (a) Wishart, D. S.; Sykes, B. D. *Methods Enzymol*. **1994**, *239*, 363−392. (b) Case, D. A.; Dyson, H. J.; Wright, P. E. *Methods Enzymol*. **1994**, *239*, 392−416.

(25) Wütrich, K. *NMR of Proteins and Nucleic Acids*; Wiley: New York, 1986.

**Table 3.** Coupling Constants of Amide Protons ($^3J_{NH-C\alpha H}$, Hz)

| | GS (**1**) | (*S*)-IBTM-GS (**4a**) | | (*R*)-IBTM-GS (**4b**) | |
|---|---|---|---|---|---|
| residue no. | DMSO[a] | $H_2O$[b] | DMSO[c] | $H_2O$[b] | DMSO[c] |
| Val 1 | 9.7 | 5.5 | 9.5 | 8.9 | 12.0 |
| Xaa 2[d] | 8.9 | 2.8 | 7.5 | 8.9 | 10.3 |
| Leu 3 | 9.2 | 9.6 | 8.1 | 8.0 | 9.1 |
| Yaa 4[e] | 2.6 | 7.0 | 8.2 | 6.4 | 4.3 |
| Val 1′ | 9.7 | 9.0 | 12.5 | 9.3 | 12.3 |
| Xaa 2′ | 8.9 | 8.8 | 8.4 | 8.4 | 9.7 |
| Leu 3′ | 9.2 | 5.5 | 7.3 | 8.0 | 10.3 |
| D-Phe 4′ | 2.6 | 8.6 | 7.9 | 3.8 | 2.6 |

[a] 25 °C; values from ref 11b. [b] 5 °C, pH 3.0. [c] 50 °C. [d,e] As in Table 2.

**Table 4.** Long-Range NOEs observed for (*S*)-IBTM-GS (**4a**, 5 mM)

| | | NOE intensity in | |
|---|---|---|---|
| residue i | residue j | $H_2O$, pH 3.0, 5 °C | DMSO, 20 °C |
| NH Val 1 | $N_{11}H$ IBTM | weak | not detected |
| CαH Val 1 | $N_{11}H$ IBTM | strong | *a* |
| $C\gamma H_3$ Val 1 | $N_{11}H$ IBTM | medium | medium |
| $C\gamma H_3$ Val 1 | $C_{10}H$ IBTM | medium | strong |
| CαH Leu 3′ | $N_{11}H$ IBTM | strong | *b* |
| $C_\delta H_3$ Leu 3′ | $N_{11}H$ IBTM | medium | medium[c] |
| $C_{\delta'}H_3$ Leu 3′ | $C_{10}H$ IBTM | weak | strong[c] |
| $C_\beta H$ D-Phe 4′ | $C_{11b}H$ IBTM | strong | not detected |
| $C_{\beta'}H$ D-Phe 4′ | $C_{11b}H$ IBTM | medium | not detected |

[a] NOE connectivity could not be observed due to closeness to diagonal. [b] NOE connectivity could not be observed due to overlapping with other NOE crosspeaks. [c] $C_\delta H_3$ and $C_{\delta'}H_3$ protons of Leu 3′ have the same δ value.

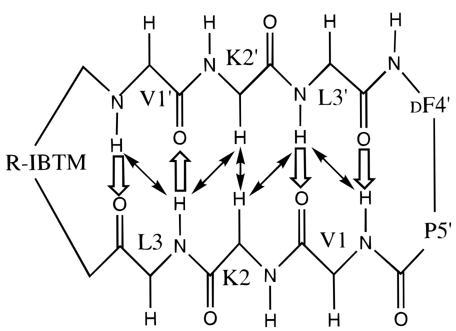



**Figure 4.** Schematic representation of the GS-like backbone conformation. Hydrogen bonds are indicated by arrows and the expected NOEs by black lines.

downfield shifted (Figure 3) with respect to random coil values.[25] The amide protons of residues $Val^{1,1'}$ and $Leu^{3,3'}$ are hydrogen bonded, as in the GS structure (Figure 4). Their temperature coefficients, which are small in absolute value in both solvents (Table 2), as expected for hydrogen-bonded amide protons, confirm that [(*R*)-$IBTM^{4,5}$,$Lys^{2,2'}$]GS (**4b**) adopts a GS-like structure. The strongest evidence for the existence of such structure comes from the analysis of NOE connectivities. The CαH($Lys^2$)−CαH($Lys^{2'}$), CαH($Lys^2$)−NH($Leu^{3'}$), and NH($Leu^3$)−CαH($Lys^{2'}$) NOE crosspeaks, as well as some non-sequential $i,i+2$ NOEs involving at least one side chain proton, fully support the proposed GS-like structure (Figure 6 and Table 5). Further confirmation is obtained from the $^3J_{C\alpha H-NH}$ coupling constants of **4b** (Table 3).

**Antibacterial Activity.** Minimal inhibitory concentrations of mimetics **4a**, **4b**, and **5b**, GS (**1**), and its [$Lys^{2,2'}$] analogue **2** were determined against two gram-positive and two gram-negative organisms (Table 6). The results showed again a clear difference between the (*R*)- and (*S*)-IBTM GS analogues. As is the case with GS, none of the IBTM-containing peptides showed significant activity toward gram-negative microorganisms. However, (*R*)-IBTM derivatives **4b** and **5b** were quite comparable in antibiotic activity to the reference GS against gram-positive strains, while the (*S*)-IBTM analogue **4a** was practically inert against these organisms. The Lys for Orn replacement in **2** did not produce any significant difference in antibiotic potency relative to GS.

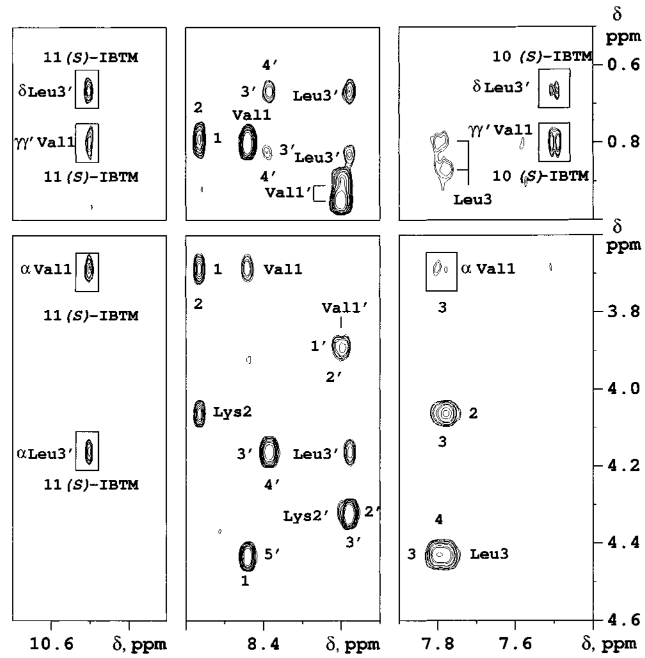



**Figure 5.** Selected regions of the NOESY spectrum of [(*S*)-$IBTM^{4,5}$,$Lys^{2,2'}$]GS (**4a**) in $H_2O-D_2O$ (9:1 v/v), pH 3.0 at 5 °C. Nonsequential NOEs are boxed and labeled. The sequential assignment for most residues can also be followed through CαH-NH($i$,$i$+1) NOEs (lower regions) and through CγH−NH($i$,$i$+1) and CδH−NH($i$,$i$+1) NOEs (upper regions).

**Table 5.** Nonsequential NOEs Observed for (*R*)-IBTM-GS (**4b**, 5 mM)

| | | NOE intensity in | |
|---|---|---|---|
| residue i | residue j | $H_2O$, pH 3.0, 5 °C | DMSO, 20 °C |
| NH Val 1 | NH Leu3′ | weak | weak |
| CαH Lys 2 | CαH Lys 2′ | *a* | strong |
| CαH Lys 2 | NH Leu 3′ | *b* | medium |
| NH Leu 3 | NH Val 1′ | weak | *a* |
| NH Leu 3 | CαH Lys 2′ | *b* | medium |
| Cγγ′H Val 1 | NH Leu 3 | not detected | weak |
| CβH Val 1 | NH Leu 3′ | not detected | weak |
| NH Val 1′ | CγH Leu 3 | weak | not detected |
| CβH Val 1′ | NH Leu 3 | not detected | weak |
| Cγγ′H Val 1′ | NH Leu 3 | weak | medium |
| Cγγ′H Val 1′ | Cγββ′H Leu 3 | medium | medium |
| CβH Val 1 | NH Leu 3′ | weak | weak |

[a] NOE connectivity could not be observed due to closeness to the diagonal. [b] NOE connectivity could not be observed due to overlapping with other NOEs.

## Conclusions

The NMR studies reported here clearly show that (*R*)-IBTM, when incorporated into [$Lys^{2,2'}$]GS, does not disrupt the conformation of this peptide. Moreover, [(*R*)-$IBTM^{4,5}$]GS and [(*R*)-$IBTM^{4,5}$,$Lys^{2,2'}$]GS retain the biological activity of the parent peptides. These results conclusively demonstrate that (*R*)-IBTM is an effective type II′ β-turn mimetic. However, similar studies on the (*S*)-IBTM-containing peptide analogue do not support this type of conformation.

The versatility of (*R*)-IBTM as a suitable dipeptide surrogate is evidenced by its incorporation into peptide sequences by solid-

**Table 6.** Minimal Inhibitory Concentrations (μg/mL) of GS and Analogues

| | gram-positive | | gram-negative | |
|---|---|---|---|---|
| peptide | *Bacillus subtilis* | *Staphylococcus aureus* | *Escherichia coli* | *Pseudomonas aeruginosa* |
| GS (**1**) | 4 | 4 | 128 | 256 |
| [Lys2,2′]GS (**2**) | 4 | 8 | 256 | 256 |
| [(*S*)-IBTM$^{4,5}$,Lys$^{2,2'}$]GS (**4a**) | 128 | 128 | 128 | >512 |
| [(*R*)-IBTM$^{4,5}$,Lys$^{2,2'}$]GS (**4b**) | 4 | 16 | >64 | >64 |
| [(*R*)-IBTM$^{4,5}$]GS (**5b**) | 2 | 8 | >128 | >128 |

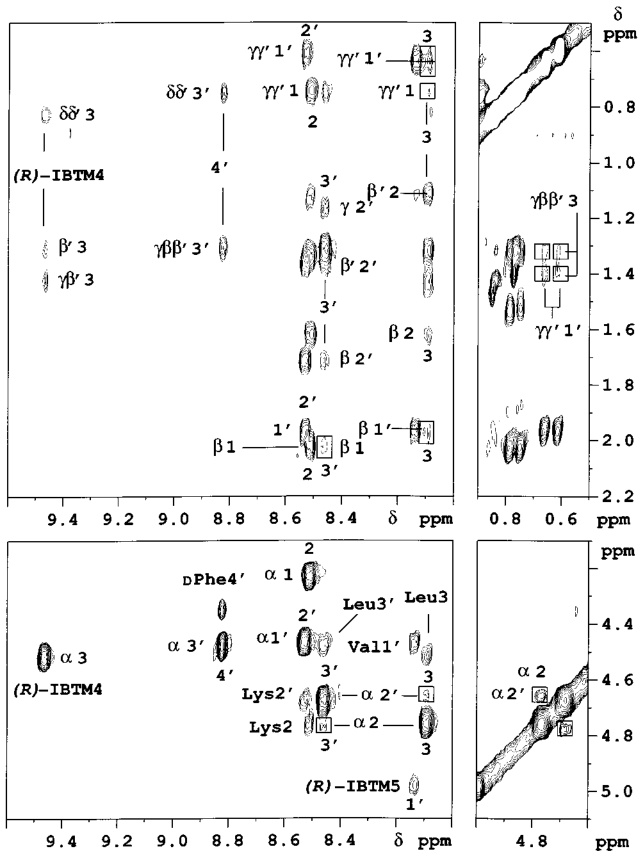
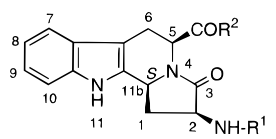



**Figure 6.** Selected regions of NOESY spectra of [(*R*)-IBTM$^{4,5}$Lys$^{2,2'}$]-GS (**4b**) in DMSO at 20 °C. Nonsequential NOEs are boxed and labeled. The sequential assignment for most residues can also be followed through CαH−NH(*i*,*i*+1) NOEs (lower regions) and through C*β*H−NH(*i*,*i*+1), CγH−NH(*i*,*i*+1), and CδH−NH(*i*,*i*+1) NOEs (upper regions). Intraresidue crosspeaks at the upper regions are not labeled.

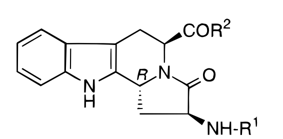
phase synthesis, using both Boc and Fmoc chemistries. From a structural point of view, it is interesting to note the presence of an indole moiety in (*R*)-IBTM which can be considered as the side chain of the *i* + 2 residue of the *β*-turn mimetic. This fact could represent an advantage for mimicking *β*-turns with aromatic or hydrophobic amino acids at this position, since most of the dipeptide mimetics reported so far lack substituents for the corner residue side chains. Finally, one can easily envisage the extension of the solid-phase-mediated cyclization scheme used in this work to other cyclic peptidomimetics.

## Experimental Section

**General.** Full details on the synthesis and characterization of (*R*)- and (*S*)-IBTM amino acids and their Boc derivatives **6a,b** have appeared previously.[13] Conversion of the zwitterion H-(*R*)-IBTM-OH into its Fmoc (**7b**) derivative is described below. Boc-protected amino acids, BOP, TBTU, and MBHA resin were purchased from Novabiochem (Läufelfingen, Switzerland). Boc-Pro-OCH$_2$-Pam resin (0.73 mmol/g) was from Applied Biosystems (Foster City, CA). Fmoc-protected amino acids and HATU, HOAt, and PyAOP coupling reagents were from PerSeptive Biosystems (Framingham, MA). All other chemicals were of the highest purity commercially available. Solvents for peptide synthesis (DMF and DCM) and HPLC supplies (MeCN and ODS columns, 4 × 250 mm, 5 μm) were from Scharlau (Barcelona, Spain). Analytical HPLC runs used linear gradients (usually 5−95%) of MeCN (+0.036% TFA) into $H_2O$ (+0.045% TFA) over 30 min at 1 mL/min, with UV detection at 220 nm. Amino acid analysis of peptide hydrolysates (6 N HCl, 150 °C, 4 h) were run in a Beckman 6300 autoanalyzer. Mass spectra of peptides were acquired by the MALDI-TOF ionization technique in a Kompact I instrument (Kratos Analytical, Manchester, UK) with an α-cyano-4-hydroxycinnamic acid matrix. Electrospray mass spectra were obtained in a VG Quattro instrument (Fisons Instruments, Wythenshawe, UK) working in the positive mode, using nitrogen as nebulizing and drying gas (10 and 450 L/h, respectively), a source temperature of 80 °C, a capillary voltage of 3.5 kV, and a focusing voltage of 60 V. The same instrument, fitted with a Cs gun, was used to acquire FAB spectra.




**6a**: Boc-*(S)*-IBTM-OH; R$^1$= Boc




**6b:** Boc-*(R)*-IBTM-OH; R$^1$= Boc
**7b:** Fmoc-*(R)*-IBTM-OH; R$^1$= Fmoc

**Solid-Phase Synthesis and Purification of [(*S*)-IBTM$^{4,5}$,Lys$^{2,2'}$]-GS (4a).** Boc-Pro-OCH$_2$-Pam resin (300 mg, 0.22 mmol) was submitted to a cycle of Boc solid-phase synthesis for each residue of the sequence, as follows: (i) deprotection with TFA-DCM (2:3 v/v; 1 + 20 min); (ii) washes with DCM (4 × 0.5 min) and DMF (2 × 1 min); (iii) neutralization with DIEA-DCM (1:19 v/v; 2 × 1 min); and (iv) coupling with Boc-amino acid (0.66 mmol), TBTU (0.66 mmol), and DIEA (1.32 mmol) in dry DMF for 60 min. All couplings were complete after this time by the Kaiser test[26] except for the (*S*)-IBTM residue, which required a second coupling with **6a** (0.33 mmol), HATU (0.33 mmol), and DIEA (0.66 mmol) during 60 min for complete acylation. The fully protected linear sequence (50 μmol, 150 mg of resin) was cleaved from the resin and Boc-deprotected at the N$^{\alpha}$ function by acidolysis in anhydrous HF−anisole (9:1 v/v) for 1 h at 0 °C. After evaporation, the residue was triturated with anhydrous ethyl ether, dissolved in glacial HOAc, and lyophilized to give 37 μmol (74% yield) of the bis-Fmoc protected linear precursor **3a**. Prior to cyclization, 18 μmol of this peptide was lyophilized several times from $H_2O$ to remove traces of HOAc and dried under vacuum over $P_2O_5$ and KOH overnight. The dried, HOAc-free peptide was dissolved in dry DMF (45 μM final concentration) under nitrogen, then treated with DIEA (0.36 mmol, 20 equiv) and BOP (0.18 mmol, 10 equiv) under mild stirring at 25 °C. Once HPLC analysis showed the cyclization to be complete (*ca.* 6 h) the reaction mixture was concentrated to 20 mL and treated with 100 mL of piperidine−DMF (1:4 v/v) for 2 h at 25 °C. Evaporation gave an oil that was redissolved in glacial HOAc, lyophilized, and purified by reverse-phase chromatography (ODS, 2 × 25 cm, 15−20 μm) with use of a 10−70% linear gradient of MeCN into $H_2O$ over 4 h at 3 mL/min. Fractions judged to be homogeneous by HPLC were pooled to give **4a** (4 μmol; cyclization and purification yield 22%), with the expected amino acid composition and molecular mass (MALDI-TOF; calculated average molecular mass for $C_{63}H_{93}N_{13}O_{10}$ 1192.52; positive spectrum, *m*/*z* 1194.4 [MH$^+$]).

(26) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595−598.

**Fmoc-(*R*)-IBTM-OH (7b).** To a suspension of the zwitterion H-(*R*)-IBTM-OH (400 mg, 1.4 mmol) in 10% $NaHCO_3$−dioxane−DMSO (20:5:1 v/v/v, 26 mL) was added Fmoc-Cl (541 mg, 2.1 mmol) in three portions over a 30-min period, and the mixture was stirred for another 3.5 h at 25 °C. The pH was maintained at 9 throughout this period by addition of 10% $NaHCO_3$ as required. The reaction mixture was chilled in an ice bath for 20 min and filtered, and the filtrate was extracted with hexane (3 × 150 mL) and acidified to pH 3 with 1 M $KHSO_4$, whereupon **7b** separated as an off-white solid (370 mg, yield 52%) that was not recrystallized, given its insolubility in most solvents except DMSO and DMF. The purity of the material was judged to be adequate for peptide synthesis (>95%) by analytical HPLC (see above) with use of a 0−100% MeCN (+0.036% TFA) into $H_2O$ (+0.045% TFA) gradient over 30 min at 1 mL/min (retention time 23.5 min). $^{13}C$ NMR (50 MHz, $CD_3SOCD_3$) $\delta$ 172.4 (COOH), 171.8 (CON), 141.0 ($C_{ar}$), 136.5 ($C_{ar}$), 132.8 ($C_{ar}$), 127.6 ($C_{ar}$-H), 127.0 ($C_{ar}$-H), 126.6 ($C_{ar}$-H), 125.0 ($C_{ar}$-H), 121.3 ($C_{ar}$-H), 119.9 ($C_{ar}$-H), 118.8 ($C_{ar}$-H), 117.8 ($C_{ar}$-H), 111.0 ($C_{ar}$-H), 104.6 ($C_{ar}$), 66.2 ($CH_2$), 52.0 (CH), 50.6 (CH), 50.3 (CH), 46.8 (CH), 32.4 ($CH_2$), 23.5 ($CH_2$). Mass spectroscopy (FAB, "magic bullet", dithioerythritol−dithiothreitol, 3:2 v/v): calculated monoisotopic mass of $C_{30}H_{26}N_3O_5$ 507.86, positive spectrum, *m/z* 508.1 [$MH^+$].

**Fmoc-Orn-OAl, Trifluoroacetate Salt.** A solution of Fmoc-Orn(Boc)-OH (4 g, 8.8 mmol) in MeCN (15 mL) was treated with allyl bromide (20 mL, 231 mmol) and DIEA (2.9 mL, 17 mmol) for 5 h at 40 °C, with magnetic stirring. The reaction mixture was diluted with ethyl acetate (200 mL) and washed with 0.1 N HCl (4 × 125 mL), 10% $NaHCO_3$ (4 × 125 mL), and saturated NaCl (4 × 125 mL), and the organic phase was dried over $MgSO_4$ and evaporated to give Fmoc-Orn(Boc)-OAl as a white solid in quantitative yield. To this intermediate was added dropwise 100 mL of TFA-DCM (1:1 v/v), and the solution was stirred for 2 h at 25 °C. Solvent removal followed by repeated evaporations from ethyl ether provided Fmoc-Orn-OAl as a clear oil (yield 4.1 g, 92%; $R_f$ 0.56, $CHCl_3$/MeOH/HOAc, 75:25:2), which was used without further purification. $^1H$ NMR (200 MHz, $CD_3SOCD_3$) $\delta$ 7.28−7.92 (m, 9H), 5.85 (m, 1H), 5.29 (dd, $J_1$ = 1.3 Hz, $J_2$ = 17.4 Hz, 1H), 5.19 (dd, $J_1$ = 1.3 Hz, $J_2$ = 10.4 Hz, 1H), 4.58 (d, $J$ = 5.1 Hz, 2H), 4.29 (t, $J$ = 15.1 Hz, 1H), 4.28 (d, $J$ = 15.1 Hz, 2H), 4.06 (m, 1H), 2.79 (m, 2H), 1.50−1.80 (m, 4H). Mass spectroscopy (electrospray): calculated monoisotopic mass of $C_{23}H_{26}N_2O_4$ 394.19, positive spectrum, *m/z* 395.1 [$MH^+$].

**Fmoc-Lys-OAl, Trifluoroacetate salt.** Fmoc-Lys(Boc)-OH (1 g, 2.2 mmol) was treated with allyl bromide (7 mL, 89 mmol) and DIEA (0.77 mL, 4.4 mmol), then deprotected with TFA as described above to give the title compound as a clear oil (yield 0.99 g, 89%; $R_f$ 0.25, $CHCl_3$/MeOH/HOAc, 75:25:2), which was used without further purification. $^1H$ NMR (200 MHz, $CD_3SOCD_3$) $\delta$ 7.28−7.93 (m, 9H), 5.87 (m, 1H), 5.29 (dd, $J_1$ = 1.7 Hz, $J_2$ = 17.2 Hz, 1 H), 5.19 (dd, $J_1$ = 1.7 Hz, $J_2$ = 10.2 Hz, 1H), 4.57 (d, $J$ = 5.2 Hz, 2H), 4.29 (t, $J$ = 14.7 Hz, 1H), 4.28 (d, $J$ = 14.7 Hz, 2H), 4.03 (m, 1H), 2.75 (m, 2H), 1.30−1.85 (m, 6H). Mass spectroscopy (electrospray): calculated monoisotopic mass of $C_{24}H_{28}N_2O_4$ 408.19, positive spectrum, *m/z* 409.1 [$MH^+$].

**Side Chain Anchoring of Fmoc-Orn-OAl and Fmoc-Lys-OAl.**[20] MBHA resin (0.5 g, 0.56 mmol/g) was reacted with Boc-Ala-OH (265 mg, 1.4 mmol) in the presence of equimolar amounts of DIPCDI and HOBt in DMF. The Boc-Ala-resin (Ala serving as internal reference amino acid[22]) was deprotected with TFA-DCM (2:3 v/v), neutralized with DIEA-DCM (1:19 v/v), and loaded with the bifunctional spacer 3-(4-(hydroxymethyl)phenoxy)propionic acid (165 mg, 0.84 mmol) in the presence of equimolar amounts of DIPCDI and HOBt in DMF. The resulting polymer was placed under an Ar stream, reacted with DSC (2.8 mmol, 10 equiv) and DMAP (0.28 mmol, 1 equiv) in dry DMF, and stirred mechanically for 2 h, with periodic Ar bubbling through the suspension. After filtration and DMF washes (5 × 0.5 min), the polymer was reacted with the trifluoroacetate salt of Fmoc-Orn-OAl or Fmoc-Lys-OAl (2.8 mmol, 10 equiv) and DIEA (5.5 mmol, 20 equiv) in dry DMF for 4 h at 25 °C, with periodic Ar bubbling. Amino acid analysis of the polymers after 12 N HCl−propionic acid (1:1) hydrolysis (4 h, 150 °C) showed incorporation of Orn and Lys to be 71% and 74%, respectively, corresponding to actual substitutions of *ca*. 0.45 mmol/g in both resins.

**Synthesis and Purification of Gramicidin S (1).** Polymer (0.5 g) loaded with Fmoc-Orn-OAl (*ca*. 0.22 mmol Orn) was submitted to one cycle of Fmoc solid-phase synthesis for each residue of the sequence, as follows: (i) deprotection with piperidine−DMF (1:4 v/v, 2 × 1 min + 1 × 10 min); (ii) DMF washes (5 × 0.5 min); (iii) coupling of Fmoc amino acid (4 equiv) in the presence of TBTU (4 equiv) and DIEA (8 equiv) in DMF for 60 min; and (iv) DMF washes (5 × 0.5 min). Couplings were monitored for completion by the Kaiser[26] or chloranil[27] tests; all residues were satisfactorily incorporated after the first coupling. Once the target sequence, Fmoc-Leu-D-Phe-Pro-Val-Orn(Boc)-Leu-D-Phe-Pro-Val-Orn(OAl)-polymer, was assembled, the peptide-resin (0.28 mmol/g, 160 mg, 44 $\mu$mol) was suspended on 4 mL of DMSO−THF−0.5 M HCl−morpholine (2:2:1:0.1 v/v) and treated with $Pd(PPh_3)_4$ (0.44 mmol, 10 equiv) for 2.5 h under Ar. The resin was then washed with THF (3 × 2 min), DMF (3 × 2 min), DCM (3 × 2 min), DIEA−DCM (1:19) (3 × 2 min), DCM (3 × 2 min), sodium diethyldithiocarbamate−DMF (5 g/L; 3 × 15 min), DMF (5 × 2 min), and DCM (3 × 2 min). Following *N*-terminal deprotection with piperidine−DMF (as above), cyclization was performed with PyAOP (229 mg, 0.44 mmol, 10 equiv), HOAt (60 mg, 10 equiv), and DIEA (153 $\mu$L, 20 equiv) in dry DMF for 2 h at 25 °C, after which ninhydrin analysis showed no remaining free amino groups. Acidolysis with TFA−$H_2O$ (19:1 v/v, 2 h, 25 °C) and diethyl ether precipitation furnished 11.9 mg of crude **1**, *ca*. 90% pure by HPLC. This material was dissolved in 3 mL of THF−$H_2O$ (1:4 v/v), loaded onto a reverse-phase column (ODS, 2 × 25 cm, 15−20 $\mu$m) and eluted with a linear 20−50% gradient of THF into $H_2O$ at 1.5 mL/min. Fractions judged to be homogeneous by HPLC were pooled to give 5.2 mg (4.55 $\mu$mol, purification yield 47%) of purified **1**, with the expected amino acid composition and molecular mass (MALDI-TOF, calculated average mass for $C_{60}H_{92}N_{12}O_{10}$ 1141.46; positive spectrum, *m/z* 1142.5 [$MH^+$]).

**Synthesis and Purification of [$Lys^{2,2'}$]Gramicidin S (2).** Starting from a resin loaded with Fmoc-Lys-OAl (*ca*. 0.21 mmol of Lys), the target sequence, Fmoc-Leu-D-Phe-Pro-Val-Lys(Boc)-Leu-D-Phe-Pro-Val-Lys(OAl)-polymer, was assembled, deprotected (*N*- and *C*-termini), and cyclized (40 $\mu$mol scale) by a procedure analogous to the one described above for GS (**1**). Acidolysis yielded 9.4 mg (8 $\mu$mol, 20% yield) of crude product that was purified as above to give 5.1 mg (yield 54%) of **2**, with the expected amino acid composition and molecular mass (MALDI-TOF, calculated average mass for $C_{62}H_{96}N_{12}O_{10}$ 1169.53; positive spectrum, *m/z* 1170.6 [$MH^+$]).

**Synthesis and Purification of [(*R*)-$IBTM^{4,5}$]GS (5b).** The same protocols described for **1** and **2** were employed to prepare the target sequence, Fmoc-Leu-D-Phe-Pro-Val-Orn(Boc)-Leu-(*R*)-IBTM-Val-Orn(OAl)-polymer. Fmoc-(*R*)-IBTM was activated with HATU (2 equiv) and DIEA (4 equiv) in DMF and coupled for 2 h at 25 °C. Deprotection and cyclization of a 40 $\mu$mol resin sample furnished 12.8 mg (11 $\mu$mol, yield 27.5%) of crude **5b** that was purified on reverse phase (ODS, 2 × 25 cm, 20 $\mu$m) by means of a 50−100% linear gradient of methanol into $H_2O$ to give 1.4 mg of pure **5** (11% yield), with the expected amino acid composition and molecular mass (MALDI-TOF, calculated average mass for $C_{61}H_{89}N_{13}O_{10}$ 1164.47; positive spectrum, *m/z* 1165.7 [$MH^+$]).

**Synthesis and Purification of [(*R*)-$IBTM^{4,5}$,$Lys^{2,2'}$]GS (4b).** The same protocol described for **5b** was employed to assemble the target sequence, Fmoc-Leu-D-Phe-Pro-Val-Lys(Boc)-Leu-(*R*)-IBTM-Val-Lys(OAl)-polymer. Deprotection and cyclization of a 35 $\mu$mol resin sample provided 13.9 mg (11.6 $\mu$mol, yield 32%) of crude that was purified as above to give 1.2 mg of pure **4b** (9% yield), with the expected AAA and mass (MALDI-TOF, calculated average mass for $C_{63}H_{93}N_{13}O_{10}$ 1192.52; positive spectrum, *m/z* 1192.4 [$MH^+$]).

**NMR Conformational Analysis.** NMR samples were about 5 mM in $H_2O/D_2O$ (9:1) or in DMSO. pH of the samples in aqueous solution was adjusted to 3.0 by addition of minute amounts of DCl or NaOD. pH measurements were not corrected for isotope effects. Sodium 3-trimethylsilyl(2,2,3,3-$^2H_4$)propionate (TSP) was used as internal reference. NMR experiments were performed on a Bruker AMX-600 spectrometer. All the two-dimensional spectra were acquired in the phase sensitive mode with the use of the time proportional phase

(27) Christensen, T. *Acta Chem. Scand*. **1979**, *33*, 760−762.

incrementation (TPPI) technique[28] with presaturation of the water signal. COSY[29] and NOESY[30] spectra were recorded by using standard phase-cycling sequences. Short mixing times (200 ms) were used in the NOESY experiments in order to avoid spin-diffusion effects. TOCSY[31] spectra were acquired with use of the standard MLEV17 spinlock sequence and a 80 ms mixing time. The size of the acquisition data matrix was 2048 $\times$ 512 words in $f_2$ and $f_1$, respectively, and prior to Fourier transformation the 2D data matrix was multiplied by a phase-shifted square-sine bell window function in both dimensions and zero-filled to 4096 $\times$ 1024 words. The phase shift was optimized for every spectrum.

Assignment of the $^1$H NMR resonances of [(*S*)-IBTM$^{4,5}$,Lys$^{2,2'}$]GS (**4a**) and [(*R*)-IBTM$^{4,5}$,Lys$^{2,2'}$]GS (**4b**) in both aqueous solution and DMSO was readily performed by using the sequential assignment method.[32,25] For the connection of spin systems through sequential NOE connectivities, the $C_2H$ and $C_5H$ IBTM protons were considered as CαH protons. Strong CαH−CδH($i$,$i$+1) and CαH−Cδ′H($i$,$i$+1) NOEs permitted the connection between D-Phe 4′ and Pro 5′ and indicated the *trans* conformation of the X-Pro bond in compounds **4a** and **4b**. The sequential assignment of [(*R*)-IBTM$^{4,5}$,Lys$^{2,2'}$]GS (**4b**) in DMSO can be followed in the NOESY spectral region shown in Figure 6. The δ values of the NH amide protons of compounds **4a** and **4b** in both aqueous solution and in DMSO are given in Table 1 and those for the other protons are available as Supporting Information.

**Antibiotic Activity.** Minimal inhibitory concentrations of the peptides were determined against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538P, *Pseudomonas aeruginosa* ATCC 9027, and *Escherichia coli* ATCC 10799, using a 100 μL inoculum of a 2 $\times$ 10$^5$ CFU/mL log phase bacterial culture. Serial dilutions of the peptides covering a 256−0.01 μg/mL range were prepared in 10 mM sodium phosphate buffer, pH 7.3. After incubation at 37 °C for 20 h, inhibition of bacterial growth was determined by turbidimetry measurement with a microplate reader at 650 nm.

**Acknowledgment.** We thank Dr. Irene Fernández (Mass Spectrometry Service, University of Barcelona) for the mass spectra. Work at the University of Barcelona was supported by Fondo de Investigaciones Sanitarias (94/0007) and by Centre de Referència de Biotecnologia (Generalitat de Catalunya, Spain). J.A. is a predoctoral fellow from the Ministerio de Investigación y Ciencia, Spain. Work at the Institute of Medicinal Chemistry was supported by CICYT (SAF94-0705). Work at the Institute of Structure of Matter was supported by DGICYT (PB93-0189).

**Supporting Information Available:** Tables of $^1$H chemical shifts of CαH and side chain protons of **4a** and **4b** (2 pages). See any current masthead page for ordering and Internet access instructions.

JA9705755

(28) Marion, D.; Wüthrich, K. *Biochim. Biophys. Res. Commun.* **1983**, *113*, 967−974.

(29) Aue, W. P.; Bartholdi, E.; Ernst, R. R. *J. Chem. Phys.* **1976**, *64*, 2229−2246.

(30) Kumar, A.; Ernst, R. R.; Wüthrich, K. *Biochem. Biophys. Res. Commun.* **1980**, *95*, 1−6.

(31) Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *65*, 355−360.

(32) Wüthrich, K.; Billeter, M.; Braun, W. *J. Mol. Biol.*, **1984**, *180*, 715−740.