Brief Articles

Restriction of a Peptide Turn Conformation and Conformational Analysis of Guanidino Group Using Arginine-Proline Fused Amino Acids: Application to Mini Atrial Natriuretic Peptide on Binding to the Receptor

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Compounds (2*S*,4*S*)- and (2*S*,4*R*)-4-(2′-guanidinoethyl)proline have been synthesized as a conformationally restricted arginine. Their backbones fit the $i + 1$ position in a turn, and the side chains are restricted compared to that of arginine. These analogues were incorporated into mini atrial natriuretic polypeptide, which has an important turnlike conformation at Gly[6]-*Arg*[7]-Met[8]-Asp[9]. Structural analysis revealed that the size of the conformational space of Arg[7] on binding to the receptor was approximately one-third of the entire conformational space.

Introduction

Turn conformations play an important role in peptides and proteins; they have been implicated in molecular recognition and in protein folding.[1] Proline substitution has been widely used to search for turns[2] because proline is frequently found at the $i + 1$ position of a β-turn.[3] Proline can be easily incorporated into peptides via solid-phase synthesis, but functional side chains may be lost by proline substitution. To alleviate the problem, several proline analogues have been synthesized that retain the side chain moieties of α-amino acids.[4] However, detailed structural analysis of the side chain moieties has not been reported yet, despite of the importance of their direction on biological activity.

We have synthesized (2*S*,4*S*)- and (2*S*,4*R*)-4-(2′-guanidinoethyl)proline (4*S*-GEPro (**1**) and 4*R*-GEPro (**2**)) as arginine analogues, aiming at restricting the side chains as well as the backbone (Scheme 1). These analogues were used to determine the conformational space of the guanidino group of mini atrial natriuretic polypeptide (miniANP) on binding to the receptor.

MiniANP (**18**) (MCHFGGRMDRISCYR−NH_2), optimized by monovalent phage display, is the smallest analogue of ANP-related peptides.[5] MiniANP binds selectively to natriuretic peptide receptor A (NPR-A), which is a transmembrane protein composed of approximately 1060 residues,[6] causing cGMP synthesis. Our previous study by proline-scanning mutagenesis revealed that the receptor-bound conformation of miniANP had a turnlike conformation at residues 6−9.[7] In the study, proline substitution for Arg[7], corresponding to the $i + 1$ position of the turn, resulted in the most potent activity of all proline-substituted analogues. Influence of the absence of the side chain was estimated by comparing the activity of [Pro[7]]miniANP to that of [Ala[7]]miniANP. However, the activity of [Pro[7]]miniANP was lower than that of miniANP (**18**). Since the side chain of Arg[7] is also important for the activity, we have focused on the role of the side chain of Arg[7] and have investigated the conformational space of the guanidino group of Arg[7] on binding. [4*S*-GEPro[7]]miniANP (**19**) and [4*R*-GEPro[7]]miniANP (**20**) were synthesized, and their conformations were analyzed by NMR and systematic conformational search. The structural analysis together with the biological activity indicated that the position of the side chain of Arg[7] is limited to one-third of the entire conformationally accessible space on binding to the receptor.

Results and Discussion

Synthesis of N$^{\omega}$-Pbf-Fmoc-GEPro-OH (3,4) for the Solid-Phase Peptide Synthesis. The synthesis of cis-4-substituted proline analogue **3** started with *trans*-4-hydroxy-L-proline (**5**) as illustrated in Scheme 1. Compound **5** was protected by the general procedures to afford compound **6**. Improved Mitsunobu type reaction[8] of **6** with phenylsulfonylacetonitrile and cyanomethylenetributylphoshorane in toluene at 100 °C stereospecifically afforded cis-4-substituted proline derivative **7**. Removal of the phenylsulfonyl group[9] of **7** gave nitrile **8**. Reduction of **8** followed by guanylation[10] gave guanidine **9**. Conversion of **9** to Fmoc-amino acid **3** was accomplished by general procedures,[11] and free amino acid **1** was also obtained by deprotection of **10**. Compounds **2** and **4** were synthesized from cis alcohol **12**, which was easily prepared from trans alcohol **6**, by the same procedure as described for the synthesis of **1** and **3** from **6** as illustrated in Scheme 1.

Peptide Syntesis. MiniANP (**18**), [4*S*-GEPro[7]]-miniANP (**19**), [4*R*-GEPro[7]]miniANP (**20**), [Pro[7]]miniANP (**21**), and [Ala[7]]miniANP (**22**) were synthesized using

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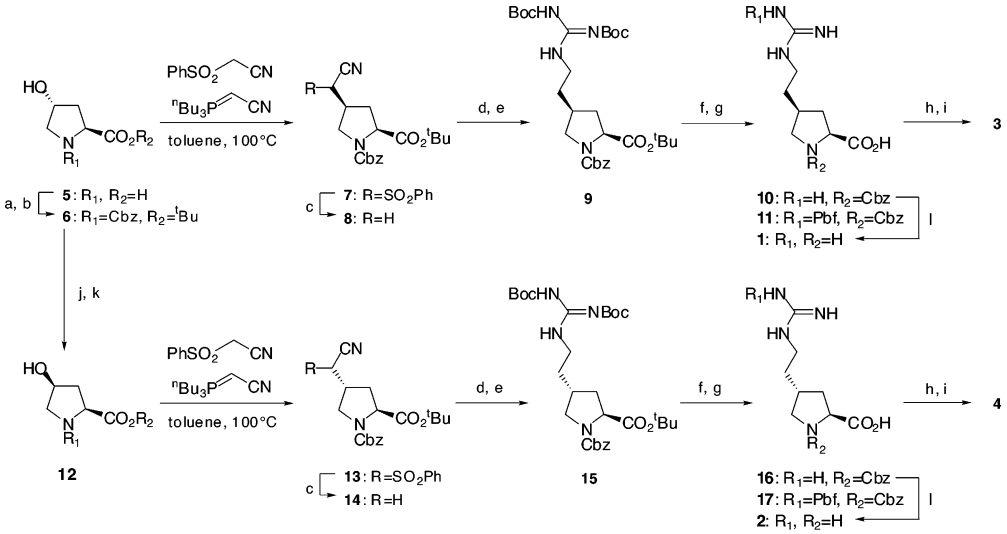
Scheme 1. Synthesis of Protected Amino Acids **3** and **4**[a]



[a] Conditions: (a) CbzCl, 2 N NaOH (aq), room temp, 20 h; (b) $Me_2NCH(O^tBu)_2$, 80 °C, 6 h, 66% from **5**; (c) Mg, $HgCl_2$ (1 mol %), MeOH, THF, room temp, 2 h, 75% **8** (from **6**), 62% **14** (from **12**); (d) $NaBH_4$, $CoCl_2$, MeOH, −20 °C, 2 h; (e) *N*,*N*′-bis(*tert*-butoxycarbonyl)-1*H*-pyrazole-1-carboxamidine, THF, room temp, 12 h, 61% **9** (from **8**), 71% **15** (from **14**); (f) TFA, CH_2Cl_2, 0 °C, 10 h; (g) PbfCl, NaOH (aq), THF, 0 °C, 10 h, 56% **11** (from **9**), 61% **17** (from **15**); (h) H_2, 10% Pd/C, MeOH, room temp, 2 h; (i) FmocOSu, 10% Na_2CO_3 solution, DME, room temp, 12 h, 56% **3** (from **11**), 58% **4** (from **17**); (j) PDC, MS4A, CH_2Cl_2, room temp, 2 h, 84%; (k) LiAlH(O^tBu), THF, −78 °C, 2 h, 98%, 99% ee; (l) TMSOTf, PhSMe, TFA, 0 °C, 2 h, 49% **1** (from **9**), 49% **2** (from **15**).

Table 1. Biological Activity of MiniANP and Analogues

peptide	biological activity[a] EC_{50} (nmol)	relative biological activity EC_{50}(analogue)/ EC_{50}(miniANP)
miniANP (**18**)	3.1 ± 0.5	1.0
[4*S*-GEPro[7]]miniANP (**19**)	4.6 ± 0.4	1.5
[4*R*-GEPro[7]]miniANP (**20**)	1.3 ± 0.6	0.4
[Pro[7]]miniANP(**21**)	58.1 ± 17.2	18.7
[Ala[7]]miniANP(**22**)	137.3 ± 11.3	44.3

[a] The mean values of three independent experiments.

FastMoc chemistry by a solid-phase peptide synthesizer (model 433A PE Biosystems, Tokyo, Japan). After the formation of a disulfide bond by DMSO/HCl, they were purified to homogeneity by reverse-phase HPLC.

Biological Activity. The production of cGMP in Chinese hamster ovary cells expressing NPR-A in response to peptides **18**−**22** was measured. Table 1 shows that the biological activity of [Pro[7]]miniANP (**21**) is approximately 19 times lower than that of miniANP (**18**), but [4*S*-GEPro[7]]miniANP (**19**) and [4*R*-GEPro[7]]-miniANP (**20**) are as potent as miniANP (**18**) despite the chiral difference of the C^4 atom. Therefore, it appears important that residue 7 supports a β-turn structure and presents a guanidino group to achieve full activity.

Solution Structure. Although the overall conformation of the analogues **19** and **20** could not be determined by NMR, in each case the distance constraints were consistent with and converged well onto a type I β-turn; this structure was not so clear in the case of miniANP itself (Figure 1). Heavy atom root-mean-square deviations (rmsd) of the turn region was 0.69 Å for [4*S*-GEPro[7]]miniANP (**19**) and 0.44 Å for [4*R*-GEPro[7]]-miniANP (**20**). The stability difference would be due to van der Waals interaction between the guanidinoethyl group and the carbonyl group (van der Waals interaction reaches ∼15 Å, while NOE reaches ∼6 Å); namely, the guanidinoethyl group and the carbonyl group were

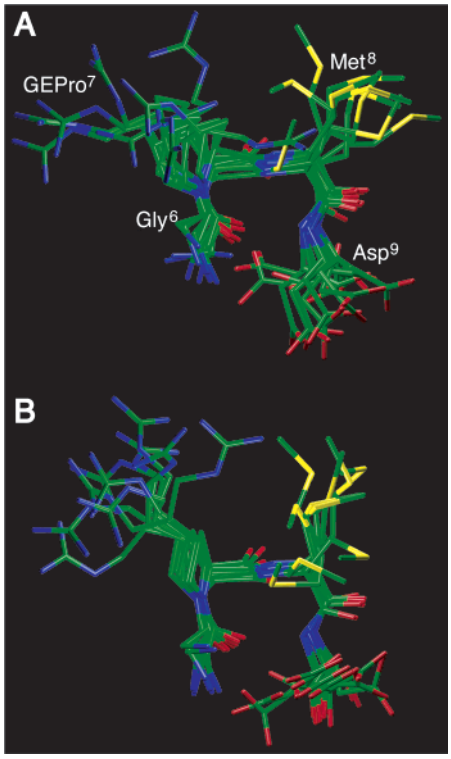


Figure 1. Turn conformation of [4*S*-GEPro[7]]miniANP (A) and [4*R*-GEPro[7]]miniANP (B) on their mean coordinates. Backbone heavy atoms in residues 6−9 were used for alignment. Hydrogens are omitted for clarity.

located on the same side of the proline ring for 4*S*-GEPro (**1**), while they were on the opposite side for 4*R*-GEPro (**2**).

The averaged turn of the analogues **19** and **20** overlapped well (rmsd = 0.23 Å). Moreover, their H^α and H^N chemical shifts including miniANP (**18**) were in good agreement with each other within 0.04 ppm except for residues 5−9. Therefore, the analogues **19** and **20**

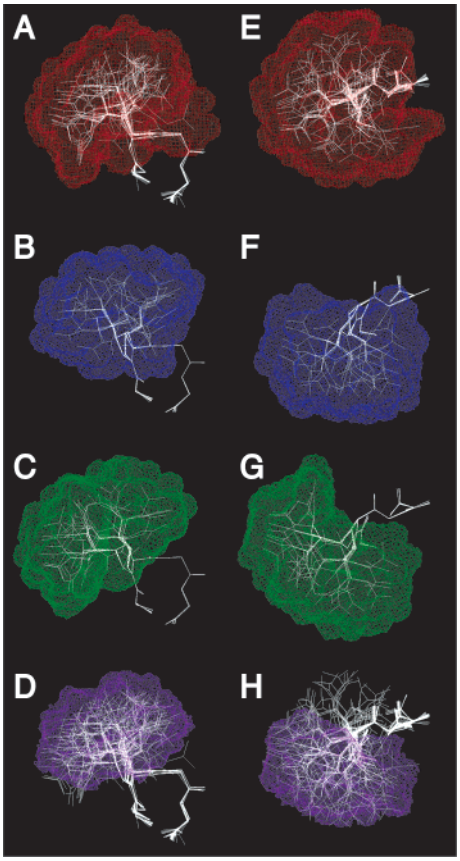


Figure 2. Conformations accepted by the systematic conformational search and molecular volumes of the guanidino groups of Arg (A and E), 4*S*-GEPro (B and F), and 4*R*-GEPro (C and G) and the intersection region of each volume (D and H). Parts D and H contain all conformations. (A−D) Front view of the turn. (E−H) Top view. The red, blue, green, and magenta areas represent Arg, 4*S*-GEPro, 4*R*-GEPro, and the intersection region, respectively. The conformations are aligned using backbone atoms. The side chains of Met and Asp are omitted for clarity.

should possess almost the same conformational properties as for the backbone.

Systematic Conformational Search. The NMR results above suggest that peptides **18**−**20** share a similar backbone structure. Since the analogues **19** and **20** have similar biological activity as well, it seems likely that their guanidino groups interact with the ANP receptor in a similar conformational space. The conformational space accessible to the guanidino groups of 4*S*- and 4R-GEPro (**1**, **2**) and arginine were mapped out systematically and compared. It should be noted that the receptor-bound conformation of residues 6−9 could be different from the typical type I β-turn used in the conformational search. However, our previous study indicated that miniANP (**18**) has a turnlike conformation on binding[12] and proline has a strong propensity to form a β-turn as shown by the solution structures. Therefore, the difference, if any, would be only to a slight degree.

Figure 2 shows the conformations accepted by the search together with molecular volumes. Since the potential energy of the conformations in the binding site could not be estimated, all conformations were used in the analysis. The volume of arginine can encompass almost the total volumes of 4*S*- and 4*R*-GEPro (**1**, **2**). In the case of shorter or longer side chains, such as 4-guanidinomethylproline or 4-(3′-guanidino-*n*-propyl)-proline, their molecular volumes did not overlap well with that of arginine (data not shown).

The size of the intersection region of all molecular volumes (295 Å^3) was approximately one-third of that of arginine (969 Å^3). For arginine, 36 conformations were included inside the intersection region while 97 conformations were searched. At least one of those conformations would bind to the receptor. The intersection region contains mainly the equatorial configuration of the guanidinoethyl groups in 4*S*- and 4*R*-GEPro (**1**, **2**) and arginine with an extended side chain. Since those conformations were calculated to be relatively stable, the guanidino group in each peptide (**18**−**20**) takes the same arrangement easily.

Conclusion

This report demonstrates the use of the arginine-proline fused amino acids to determine the conformational space where the guanidino group interacts with the receptor. The backbones of the arginine analogues fit the turn because of the bent backbone of proline. The systematic conformational search showed that the side chains of 4*R*- and 4*S*-GEPro are restricted compared to that of arginine, and the conformational spaces accessible to the guanidino group differed depending on the chirality of the C^4 atom as shown in Figure 2. Since the biological activity of the analogues (**19**, **20**) was as potent as that of miniANP (**18**), it can be concluded that the receptor-bound conformation of the guanidinoethyl group would be one of the conformations included in the intersection region of each conformationally accessible space.

Analogous proline analogues can also be designed and synthesized readily. Therefore, the method we proposed here can be used widely and may shed light on the structural analysis of ligands containing a turn.

Experimental Section

NMR Measurements and Structure Calculations. 2D DQF-COSY, TOCSY, and NOESY experiments were carried out on 2.0 mM samples dissolved in 90% H_2O/10% D_2O using a Bruker DMX 750 spectrometer. Proton signals were assigned by the conventional assignment strategy. NOE volumes were converted into upper-bound distance restraints classified as strong, medium, weak, or very weak, corresponding to 2.7, 3.7, 5.0, or 6.0 Å, respectively. The lower bound distance restraints were set to 1.8 Å. $^3J_{HN,Ha}$ coupling constants were measured from a 1D spectrum and the DQF-COSY spectrum and were converted into dihedral angle restraints. An amide proton temperature coefficient (ds_{HN}/dT), whose value greater than −4.6 ppb/K is a good indicator of a hydrogen bond, was analyzed by measuring 1D NMR spectra at 15, 25, 35, 45, and 55 °C. Structure calculations were carried out using torsion-angle molecular dynamics with the program XPLOR-NIH. A total of 100 structures were generated, and the 10 lowest energy structures were submitted to subsequent analysis.

Systematic Conformational Search. Conformational spaces accessible to the guanidinoethyl group at a turn were searched for systematically with the program Search Compare (Accelrys, San Diego, CA). To simplify the search, only a type I β-turn (Gly-*Pro*-Met-Asp) was used. First, (4*S*)- and (4*R*)-methylproline were constructed and the proline ring conformations were searched, during which the N^1−C^2 bond was cleaved, allowing other bonds to rotate freely. While C^3−C^4 and C^4−C^5 bonds were rotated 360° every 10°, C^2−C^3 and C^5−N^1 bonds were automatically rotated by the algorithm. The

conformations were energy-minimized with hydrogen bond restraints to keep the turn. After removal of identical conformations, 4*S*- and 4*R*-GEPro (**1**, **2**) were built for the resulting conformations. The $C^4-C^{1'}$, $C^{1'}-C^{2'}$, and $C^{2'}-N^{3'}$ bonds were rotated 360° every 10° in the second search. The conformations were energy-minimized, and identical conformations were removed. The same procedure was applied to arginine (Gly-*Arg*-Met-Asp), corresponding to miniANP (**18**).

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Supporting Information Available: Spectral data and synthesis procedures for compounds **3** and **4** and methods for measurement of intracellular cGMP. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Rose, G. D.; Gierasch L. M.; Smith, J. A. Turns in peptides and proteins. *Adv. Protein Chem.* **1985**, *37*, 1−109.

(2) (a) Morimoto, A.; Irie, K.; Murakami, K.; Ohigashi, H.; Shindo, M.; Nagao, M.; Shimizu, T.; Shirasawa, T. Aggregation and neurotoxicity of mutant amyloid *β* (A*β*) peptides with proline replacement: importance of turn formation at positions 22 and 23. *Biochem. Biophys. Res. Commun.* **2002**, *295*, 306−311. (b) Situ, H.; Balasubramanian, S. V.; Bobek, L. A. Role of α-helical conformation of histatin-5 in candidacidal activity examined by proline variants. *Biochim. Biophys. Acta* **2000**, *1475*, 377−382.

(3) Guruprasad, K.; Rajkumar, S. *β*- and *γ*-turns in proteins revisited: a new set of amino acid turn-type dependent positional preferences and potentials. *J. Biosci.* **2000**, *25*, 143−156.

(4) (a) Flamant-Robin, C.; Wang, Q.; Chiaroni, A.; Sasaki, N. A. An efficient method for the stereoselective synthesis of *cis*-3-substituted prolines: conformationally constrained-α amino acids. *Tetrahedron* **2002**, *58*, 10475−10484. (b) Damour, D.; Pulicani, J.-P.; Vuilhorgne, M.; Mignani, S. *cis*- and *trans*-(3-Indolyl)proline derivatives as conformationally restricted analogues of tryptophan. *Synlett* **1999**, *6*, 786−788.

(5) Bing, L.; Jeff, Y. K. T.; David, O.; Randy, Y.; Wayne, J. F.; James, A. W.; Brian, C. C. Minimization of a polypeptide hormone. *Science* **1995**, *270*, 1657−1660.

(6) Koller, K. J.; Goeddel, D. V. Molecular biology of the natriuretic peptides and their receptors. *Circulation* **1992**, *86*, 1081−1088.

(7) Sugase, K.; Oyama, Y.; Kitano, K.; Akutsu, H.; Ishiguro, M. Structure−activity relationships for mini atrial natriuretic peptide by proline-scanning mutagenesis and shortening of peptide backbone. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1245−1247.

(8) Tunoda, T.; Nagaku, M.; Nagino, C.; Kawamura, Y.; Ozaki, F.; Hioki, H.; Ito, S. Carbon−carbon bond formation with new Mitsunobu reagents. *Tetrahedron Lett.* **1995**, *36*, 2531−2534.

(9) Lai, J.-Y.; Yu, J.; Hawkins, R. D.; Falck, J. R. Two-carbon elongation/annulation of alcohols to nitriles. *Tetrahedron Lett.* **1995**, *36*, 5691−5694.

(10) Bernatowicz, M. S.; Wu, Y.; Matsueda, G. R. Urethane protected derivatives of 1-guanylpyrazole for the mild and efficient preparation of guanidines. *Tetrahedron Lett.* **1993**, *34*, 3389−3392.

(11) Ramage, R.; Green, J.; Blake, A. J. An acid labile arginine derivative for peptide synthesis: N^{γ}-2,2,5,7,8-pentamethylchroman-6-sulphonyl-L-arginine. *Tetrahedron* **1991**, *47*, 6353−6370.

(12) Sugase, K.;Oyama, Y.; Kitano, K.; Iwashita, T.; Fujiwara, T.; Akutsu, H.; Ishiguro, M. Designing analogues of mini atrial natriuretic peptide based on structural analysis by NMR and restrained molecular dynamics. *J. Med. Chem.* **2002**, *45*, 881−887.

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