Synthesis and Antibiotic Activity of a Gramicidin S Analogue containing Bicyclic β-Turn Dipeptides

Kazuki Sato and Ukon Nagai *
Mitsubishi-Kasei Institute of Life Sciences, 11, Minamiooya, Machida-shi, Tokyo 194, Japan

In order to demonstrate the usefulness of a bicyclic dipeptide derivative designed to simulate the backbone conformation of a β-turn (type II'), the dipeptide (3*S*, 6*S*, 9*R*)-2-oxo-3-amino-7-thia-1-azabicyclo[4.3.0]nonane-9-carboxylic acid has been incorporated into gramicidin S (GS) at the β-turns in place of the D-Phe-Pro sequences.† The GS analogue exhibited equal antibacterial activity, and a closely similar c.d. spectrum, to that of GS, indicating that there are two β-turns in the biologically active conformation of GS. The method seems applicable to more flexible peptides of biological interest.

Peptide analogues of restricted conformational freedom are useful tools in elucidating the conformation of biologically active peptides in the environment in which they function. The high, and selective, activities of some cyclic analogues of biologically active peptides such as somatostatin,[1] enkephalin,[2-5] and α-melanocyte-stimulating hormone[6] are successful examples of such an approach. Another way of exploring conformational restriction is by the introduction of amino acid or dipeptide derivatives of low conformational flexibility. There have been several papers describing this approach.[7-9] Recently, we reported the synthesis of a bicyclic dipeptide derivative, (3*S*, 6*S*, 9*R*)-2-oxo-3-phthalimido-7-thia-1-azabicyclo[4.3.0]nonane-9-carboxylic acid (**1**) (Figure 1),[10] the most significant feature of which is the backbone conformation, which was designed to simulate the central part of a β-turn (type II' turn). The skeleton of (**1**) was named bicyclic β-*t*urn dipeptide (BTD). According to allowed nomenclature, compound (**1**) can be expressed as Pht<BTD–OH. Since BTD is a type of dipeptide, it can be inserted into a peptide chain at any point and, owing to the semi-rigid nature of the bicyclic skeleton, powerfully induce the peptide chain to take on a β-turn conformation at that point. In order to demonstrate the usefulness of the BTD unit, we attempted the synthesis of a gramicidin S (GS) analogue containing BTD units.

GS is a cyclic decapeptide antibiotic with the primary structure *cyclo*-(Val$^{1,1'}$-Orn$^{2,2'}$-Leu$^{3,3'}$-D-Phe$^{4,4'}$-Pro$^{5,5'}$-)$_2$. In solution, GS has β-sheet conformation with two β-turns at the sequences D-Phe-Pro, as illustrated in Figure 2.[11-13] Replacement of the two D-Phe-Pro sequences with BTD would fix the β-turns irreversibly. Thus, synthesis and testing of the antibiotic activity of the analogue is expected to show unambiguously the active conformation of GS.

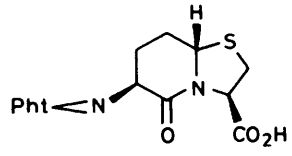

Figure 1. Structure of Pht BTD-OH (**1**)

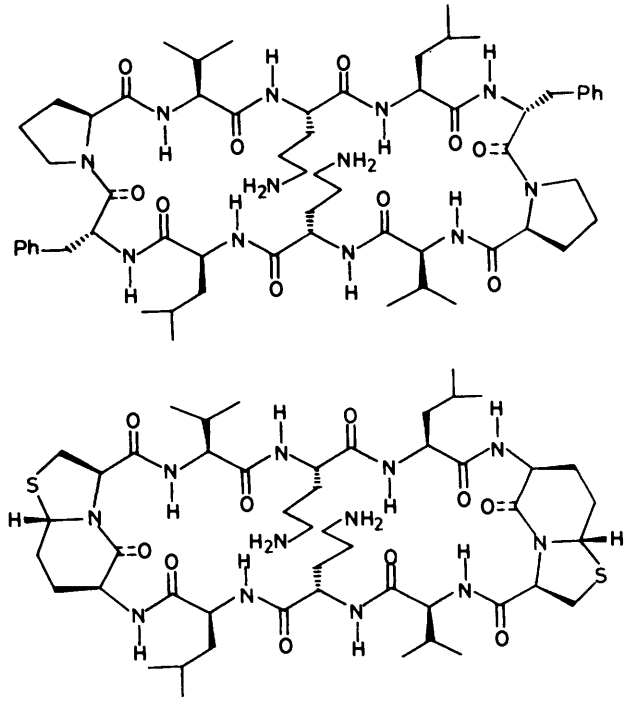

Figure 2. Schematic drawings of GS (upper) and [BTD$^{4-5,4'-5'}$]-GS (lower)

Results and Discussion

[BTD$^{4-5,4'-5'}$]-GS was synthesized according to the scheme shown in Figure 3. Boc-Val-Orn(Z)-Leu-N_3[14] was condensed with H–BTD–OH (**2**) derived from Pht<BTD–OH (**1**) to yield Boc-Val-Orn(Z)-Leu-BTD-OH (**3**). A half portion of this pentapeptide equivalent was converted into its active ester Boc-Val-Orn(Z)-Leu-BTD-OSu (**4**) by treatment with HOSu and water-soluble carbodi-imide. The other half was treated with hydrogen chloride in formic acid to afford H-Val-Orn(Z)-Leu-BTD-OH (**5**). These two pentapeptide derivatives were coupled to yield Boc-[Val-Orn(Z)-Leu-BTD]$_2$-OH (**6**). The active ester of the linear decapeptide, Boc-[Val-Orn(Z)-Leu-BTD]$_2$-OSu (**7**), synthesized as for the ester (**4**) (above), was treated with hydrogen chloride in formic acid to give H-[Val-Orn(Z)-Leu-BTD]$_2$-OSu (**8**), which was then added dropwise to

* Abbreviations according to IUPAC-IUB, *Eur. J. Biochem.*, 1984, **138**, 9, are used throughout. Additional abbreviations: DMF, *N*,*N*-dimethylformamide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide; TEA, triethylamine; GS, gramicidin S; BTD, bicyclic β-turn dipeptide. Amino acids are of L-configuration unless otherwise stated.

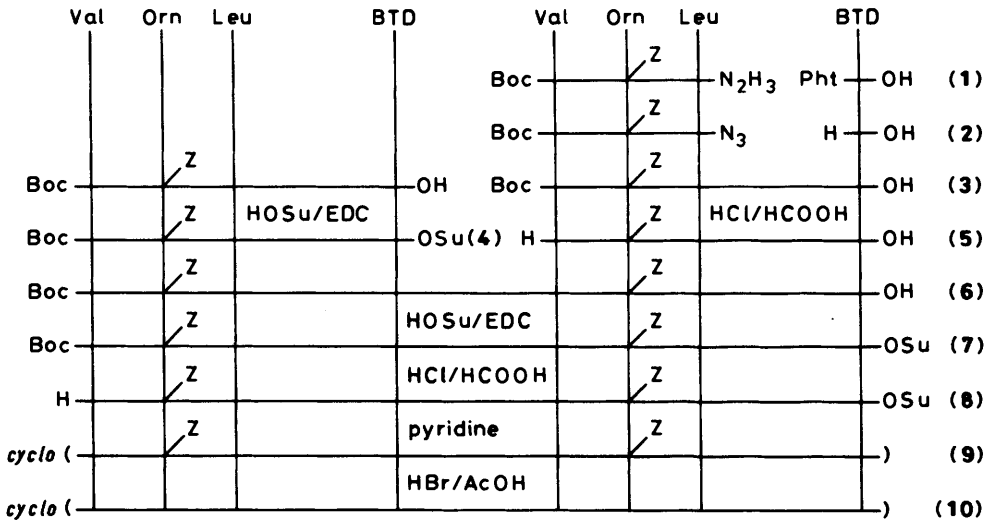

Figure 3. Synthetic scheme of [BTD$^{4-5,4'-5'}$]-GS (**10**)

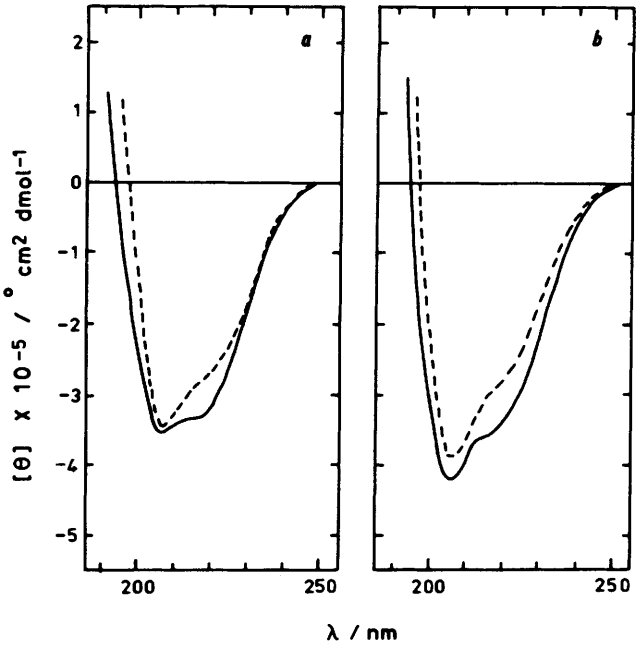

Figure 4. C.d. spectra of [BTD$^{4-5,4'-5'}$]-GS (— — —) and GS (——) in (*a*) H_2O and (*b*) MeOH solution.

Table. Antibacterial activity of GS and its analogue (minimum inhibitory concentration, μg/ml)

Strain	GS	[BTD$^{4-5,4'-5'}$]-GS
Staphylococcus aureus MCI-1380	3.13	3.13
Bacillus subtilis marfurg 168	3.13	3.13
Escherichia coli C-600	>100	>100

pyridine with stirring (final concentration = 3 mM). Work-up of the reaction product[15] gave *cyclo*-[Val-Orn(Z)-Leu-BTD-]$_2$ (**9**) [29% yield from (**8**)]. This side-chain protected cyclic decapeptide was treated with hydrogen bromide in acetic acid to afford the desired [BTD$^{4-5,4'-5'}$]-GS (**10**) as the dihydrobromide.

The antibiotic activity of the synthesized analogue was compared with that of GS. The minimum concentration of the compound necessary for the complete inhibition of the growth of several micro-organisms was determined by a dilution method using a nutrient agar. The results in the Table show that [BTD$^{4-5,4'-5'}$]-GS has much the same activity as natural GS indicating unambiguously that GS retains the conformation it has in solution (with two β-turns) in the environment in which it exhibits antibiotic activity. It is interesting to note that the analogue exhibits equal activity, despite the lack of aromatic side-chains at the turn positions. This may be explained by the more rigid backbone conformation of the analogue relative to GS.

The c.d. spectra of [BTD$^{4-5,4'-5'}$]-GS and GS were measured for solutions in water and MeOH (Figure 4). The close similarity of the c.d. spectrum of the analogue to that of GS indicates that both compounds have a similar conformation in solution. The decreased intensity at *ca.* 220 nm in the analogue seems to be caused by the absence of the aromatic chromophore, since other analogues of GS possessing aliphatic residues in place of D-Phe show only a weak shoulder at *ca.* 220 nm.[14] That [BTD$^{4-5,4'-5'}$]-GS and GS exhibited almost temperature independent c.d. spectra, suggests that they have quite stable conformations.

Although we chose a biologically active peptide with a fairly rigid conformation in solution as the first target molecule in our attempt to confirm the usefulness of BTD, a similar approach seems applicable to more flexible peptides. Any biologically active analogue so obtained will yield significant information about the active conformation of the parent peptide.

The use of a monocyclic lactam as a tool for restricting peptide conformational freedom as described above was pioneered by Freidinger *et al.*[7] who obtained a highly active analogue of LH-RH. A monocyclic lactam, however, can fix only a single dihedral angle and leaves the other important conformational features susceptible to perturbation by the environment. A bicyclic structure such as was introduced here, however, can fix the dihedral angles essential for the β-turn, and thus seems to have advantages over the monocyclic one.

Wyvratt *et al.*[16] synthesized a very similar structure in the course of their study on the inhibitors of angiotensin converting enzyme. The compound has an ε-lactam ring instead of the δ-lactam in our compound. Both compounds were synthesized by similar routes to that used in the famous synthesis of penicillin by Sheehan *et al.*[17] Although both compounds share a similar design concept in that a bicyclic skeleton is expected to give restricted conformation, their flexibility differs considerably,

judging from the inspection of molecular models; *i.e.* the δ-lactam has much less flexibility than the ε-lactam ring. Thus it seems that the ψ-value obtained by *X*-ray crystal analysis by Wyvratt *et al.* may not be valid in every environment owing to the flexibility of the seven-membered ring. Since our compound was designed to simulate a β-turn conformation from the start, the ε-lactam ring had been excluded from consideration. In our view, it is of importance that a fairly rigid dipeptide skeleton with a backbone β-turn conformation was obtained and has proved useful as a powerful β-turn inducing building block. No comment was given in the Wyvratt paper[16] on the possible use of their compound in fixing β-turns. Despite their apparent close similarity, the importance of the compound described in Wyvratt's study and that described here is quite different.

Experimental

All m.p.s were measured on a Yanagimoto melting point apparatus, and are uncorrected. T.l.c. was carried out on Silica Gel 60 F_{254} (E. Merck). R_F Values are reported for the following solvent systems: R_F^1, $CHCl_3$–MeOH (5:1); R_F^2, $CHCl_3$–MeOH–AcOH (95:5:1); R_F^3, BuOH–AcOH–pyridine–H_2O (4:1:1:2); R_F^4, BuOH–AcOH–H_2O (4:1:5, organic phase). Optical rotations were measured on a JASCO DIP-360 digital polarimeter. FAB mass spectra were measured on a JEOL HX-100 mass spectrometer.

H-BTD-OH (**2**).—To a solution of Pht<BTD–OH (**1**) (1.00 g, 2.9 mmol) in a mixture of EtOH (25 ml) and water (20 ml) was added a solution of NH_2NH_2·H_2O (0.70 ml, 14.5 mmol) in EtOH (25 ml). The solution was refluxed for 2 h and evaporated under reduced pressure. The residue was dissolved in water (30 ml) and the pH of the solution was adjusted to 1 with 4M-HCl. The solution was stored at 4 °C overnight after which the precipitate so formed was removed by centrifugation, and the supernatant applied to a column (2.2 × 17 cm) of Dowex 1 (OH^- form). The column was washed with water and the desired product eluted with 1M-AcOH; the solvent was then evaporated and the crude product recrystallized from H_2O–EtOH (543 mg, 87%), m.p. > 300 °C; $[\alpha]_D^{24}$ −276° (*c* 1.0, H_2O); R_F^3 0.25, R_F^4 0.07 (Found: C, 44.15; H, 5.4; N, 13.1. $C_8H_{12}N_2O_3S$ requires C, 44.43; H, 5.59; N, 12.95%).

Boc-Val-Orn(Z)-Leu-BTD-OH (**3**).—To a solution of Boc-Val-Orn(Z)-Leu-N_2H_3[14] (237 mg, 0.4 mmol) in DMF (5 ml) were added 2M-hydrogen chloride in EtOAc (0.6 ml) and isopentyl nitrite (0.062 ml, 0.44 mmol) at −60 °C. After being stirred at −20 °C for 10 min, the solution was cooled again to −60 °C and neutralized with TEA (0.17 ml, 1.2 mmol). To the mixture was added a chilled solution of (**2**) (86 mg, 0.4 mmol) and TEA (0.056 ml, 0.4 mmol) in water (3 ml). The reaction mixture was stirred at 5 °C for 2 days and then evaporated. Addition of 10% citric acid to the residue gave a white precipitate. The crude product (298 mg, 96%) dissolved in MeOH (2 ml) was applied to a column (3 × 170 cm) of Sephadex LH-20 and eluted with MeOH. The fractions (661—774 ml) containing the desired product (detected by u.v. absorption and t.l.c.) were collected and evaporated, and the residue was recrystallized from EtOAc–ether (165 mg, 53%), m.p. 135—137 °C; $[\alpha]_D^{24}$ −101° (*c* 0.5, MeOH); R_F^1 0.13, R_F^2 0.09, R_F^3 0.74, R_F^4 0.75 (Found: C, 56.35; H, 7.25; N, 10.7. $C_{37}H_{56}O_{10}N_6S$·0.5H_2O requires C, 56.54; H, 7.31; N, 10.69%).

Boc-Val-Orn(Z)-Leu-BTD-OSu (**4**).—To a chilled solution of (**3**) (78 mg, 0.1 mmol) and HOSu (23 mg, 0.2 mmol) in DMF (2 ml) was added a solution of EDC·HCl (38 mg, 0.2 mmol) in $CHCl_3$ (1 ml). After being stirred at 5 °C overnight, the solution was evaporated under reduced pressure. The precipitate formed by the addition of chilled water was filtered off and dried *in vacuo*. The product was used for the next reaction without further treatment (80 mg, 92%); R_F^1 0.75, R_F^2 0.21.

H-Val-Orn(Z)-Leu-BTD-OH·HCl (**5**)·HCl.—Compound (**3**) (78 mg, 0.1 mmol) was dissolved in 0.1M-hydrogen chloride in formic acid (2 ml). After 30 min at room temperature the solution was evaporated to leave an oil which crystallized upon addition of ether. The product (69 mg, 96%) was used for the next reaction without further treatment; R_F^1 0.06, R_F^3 0.63, R_F^4 0.43.

Boc-[Val-Orn(Z)-Leu-BTD]$_2$-OH (**6**).—To a chilled solution of (**5**)·HCl (69 mg, 0.096 mmol) and TEA (0.027 ml, 0.192 mmol) in DMF (2 ml) was added (**4**) (80 mg, 0.092 mmol), and the solution was stirred at room temperature overnight. It was then evaporated and 10% citric acid added to the residue to give a white precipitate. The crude product (118 mg, 89%) dissolved in MeOH (2 ml) was applied to a column (3 × 170 cm) of Sephadex LH-20 and eluted with MeOH. The fractions (461—540 ml) containing the desired product were collected and evaporated to leave crystals which were then recrystallized from MeOH (77 mg, 58%), m.p. 242—245 °C; $[\alpha]_D^{24}$ −182° (*c* 0.1, MeOH); R_F^1 0.22, R_F^2 0.13, R_F^3 0.76, R_F^4 0.79 (Found: C, 57.15; H, 7.25; N, 11.6. $C_{69}H_{102}N_{12}O_{17}S_2$·$H_2O$ required C, 57.01; H, 7.21; N, 11.56%).

Boc-[Val-Orn(Z)-Leu-BTD]$_2$-OSu (**7**).—To a chilled solution of (**6**) (72 mg, 0.05 mmol) and HOSu (12 mg, 0.1 mmol) in DMF (2 ml) was added a solution of EDC·HCl (19 mg, 0.1 mmol) in $CHCl_3$ (1 ml). The reaction mixture was treated as described for (**4**); (72 mg, 94%), R_F^1 0.80, R_F^2 0.18.

H-[Val-Orn(Z)-Leu-BTD]$_2$-OSu·HCl (**8**)·HCl.—Compound (**7**) (72 mg, 0.047 mmol) was treated with 0.1M hydrogen chloride in formic acid (0.94 ml) as described for (**5**)·HCl; (66 mg, 96%), R_F^1 0.33.

cyclo-[Val-Orn(Z)-Leu-BTD-]$_2$ (**9**).—A solution of (**8**)·HCl (66 mg, 0.045 mmol) in DMF (2 ml) was added dropwise to pyridine (13 ml) at room temperature to a final concentration of 3 mM. The reaction mixture was stirred overnight and evaporated, and the residue was dissolved in MeOH–H_2O (20 ml; 3:1), and applied to columns (1.6 × 10 cm each) of Dowex 50 (H^+ form) and Dowex 1 (OH^- form). The columns were washed with the same solvent (100 ml) and the combined effluent was evaporated to leave a white solid, which was collected with the aid of H_2O. The crude product (28 mg, 47%) dissolved in MeOH (1 ml) was applied to a column (3 × 170 cm) of Sephadex LH-20 and eluted with MeOH. The fractions (633—690 ml) containing the desired product were collected and evaporated. The residue was further purified by silica gel column chromatography (1.7 × 25 cm) using a mixture of $CHCl_3$–MeOH–AcOH (95:5:1) as eluant. The product was recrystallized from MeOH–EtOAc–ether (17 mg, 29%), m.p. 193—195 °C; $[\alpha]_D^{24}$ −235° (*c* 1.0, MeOH); R_F^1 0.73, R_F^2 0.29, R_F^3 0.91, R_F^4 0.93 (Found: C, 57.65; H, 7.1; N, 12.5. $C_{64}H_{92}O_{14}N_{12}S_2$·$H_2O$ requires C, 57.55; H, 7.09; N, 12.58%).

cyclo-(Val-Orn-Leu-BTD-)$_2$·2HBr ([$BTD^{4-5.4'-5'}$]-GS·2HBr) (**10**)·2HBr.—Compound (**9**) (13 mg, 0.01 mmol) was dissolved in a mixture of ethyl methyl sulphide (0.05 ml) and 25% hydrogen bromide in acetic acid (0.5 ml), and the solution was kept at room temperature for 1 h. Addition of ether to the solution gave white precipitate which was recrystallized from MeOH–ether (9 mg, 74%), m.p. > 300 °C; $[\alpha]_D^{24}$ −212° (*c* 0.5, MeOH); R_F^3 0.52, R_F^4 0.22 (Found: C, 46.7; H, 6.7, N, 13.55%;

M^+, 1 048. $C_{48}H_{80}O_{10}N_{12}S_2 \cdot 2HBr \cdot H_2O$ requires C, 46.90; H, 6.89; N, 13.67%; *M*, 1 048).

Paper Electrophoresis.—Electrophoresis was carried out using Whatman No. 1 paper and a solvent system of formic acid–AcOH–MeOH–H_2O (1:3:6:10, pH 1.8) for 2 h at 500 V/30 cm. [$BTD^{4-5,4'-5'}$]-GS showed a single spot, and the mobility was as follows: [$BTD^{4-5,4'-5'}$]-GS, 7.3 cm; GS, 7.1 cm.

C.D. Measurement.—C.d. spectra were recorded on a JASCO J-40 spectropolarimeter in 0.1 mM solutions using a cell of 1 mm path length at 25 °C.

Microbiological Assays.—The minimum concentration of the compounds necessary for the complete inhibition of growth of several micro-organisms was determined by a dilution method using a nutrient agar, 'Eiken' Sensitivity Test Agar E-MC10.

Acknowledgements

We thank Dr. Hideshi Nakamura for measurement of FAB-MS spectra, Mrs. Rika Nakamura for assistance in the preparation of H-BTD-OH (**2**), and Miss Rika Kato for assistance in microbiological assays.

References

1 D. F. Veber, F. W. Holly, R. F. Nutt, S. Bergstrand, S. F. Brady, R. Hirschman, M. S. Glitzer, and R. Saperstein, *Nature* (*London*), 1979, **280**, 512.
2 J. Dimaio and P. W. Schiller, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 7162.
3 P. W. Schiller, B. Eggiman, J. Dimaio, C. Lemieux, and T. M. D. Nguyen, *Biochem. Biophys. Res. Commun.*, 1981, **101**, 337.
4 P. W. Schiller and J. Dimaio, *Nature* (*London*), 1982, **297**, 74.
5 H. I. Mosberg, R. Hurt, V. J. Hruby, J. J. Galligan, T. F. Burks, K. Glee, and H. I. Yamamura, *Biochem. Biophys. Res. Commun.*, 1982, **106**, 506.
6 T. K. Sawyer, V. J. Hruby, P. S. Darman, and M. E. Hadley, *Proc. Natl. Acad. Sci. USA*, 1982, **79**, 1751.
7 R. M. Freidinger, D. F. Veber, D. S. Perlow, J. R. Brooks, and R. Saperstein, *Science*, 1980, **210**, 656.
8 J. L. Krestenansky, R. L. Baranowski, and B. L. Currie, *Biochem. Biophys. Res. Commun.*, 1982, **109**, 1368.
9 D. S. Kemp and E. T. Su, *Tetrahedron Lett.*, 1982, **23**, 3759.
10 U. Nagai and K. Sato, *Tetrahedron Lett.*, 1985, **26**, 647.
11 D. C. Hodgkin and B. M. Oughton, *Biochem. J.*, 1957, **65**, 752.
12 A. Stern, W. A. Gibbons, and R. C. Craig, *Proc. Natl. Acad. Sci. USA*, 1968, **61**, 734.
13 M. Ohnishi and D. W. Urry, *Biochem. Biophys. Res. Commun.*, 1969, **36**, 194.
14 M. Kawai and U. Nagai, *Biopolymers*, 1978, **17**, 1549.
15 K. Sato and U. Nagai, *Bull. Chem. Soc. Jpn.*, 1983, **56**, 3329.
16 M. J. Wyvratt, M. H. Tischler, J. P. Springer, E. W. Tristram, and A. A. Patchett, in 'Peptides—Structure and Function, Proceedings of the Eighth American Peptide Symposium,' eds. V. J. Hruby and D. H. Rich, Pierce Chemical Company, Rockford, 1983, p. 551.
17 J. C. Shehan and K. R. Henry-Logan, *J. Am. Chem. Soc.*, 1959, **81**, 3089.

Received 14*th October* 1985; *Paper* 5/1767