Changes in Conformation and Antimicrobial Properties Caused by Replacement of D-Amino Acids with α-Aminoisobutyric Acid in the Gramicidin Backbone: Synthesis and Circular Dichroic Studies†

Masood Jelokhani-Niaraki,[a] Katsumi Yoshioka,[a] Hiroki Takahashi,[a] Fumio Kato[b] and Michio Kondo*[,a]

[a] *Department of Chemistry, Faculty of Science and Engineering, Saga University, Saga 840, Japan*
[b] *Department of Applied Biological Sciences, Faculty of Agriculture, Saga University, Saga 840, Japan*

In an attempt to mimic the stable helical structures of proteins with possible pore-forming ability in membranes, the linear gramicidin backbone has been changed by inserting achiral α-aminoisobutyric acids (Aib) in place of all of the alternatively sequenced D-amino acids. The conformation and biological activity of the synthetic gramicidin A and B analogues have been studied. CD measurements have been used to determine the conformation in solution. The original conformation of gramicidin clearly changes and its antimicrobial activity is reduced in Aib analogues. Although α-helical motifs can be clearly distinguished in analogues, the CD spectra show inherent complexities. The possibility of superposition of different conformations is considered. The potential pore-forming ability of analogues is briefly discussed.

Linear gramicidin (G), a well-known prototype for modelling membrane-spanning proteins and their ion-permeation mechanisms, is a hydrophobic pentadecapeptide antibiotic isolated as a mixture of three main components from *Bacillus brevis*,[2] HCO-Val(Ile)^{1}-Gly-Ala-D-Leu-Ala^{5}-D-Val-Val-D-Val-Trp-D-Leu^{10}-Xxx-D-Leu-Trp-D-Leu-Trp^{15}-Glyol [Xxx = Trp (GA), Phe (GB), Tyr (GC)].† Gramicidin A (GA) is the major component in this mixture (> 80%).

The alternative presence of D-amino acids in the peptide backbone gives a special helical sense to the gramicidin secondary structure. This conformation (known as a β-helix) can be adopted by regularly L,D-sequenced copolypeptides.[3,4]

In accordance with an original model,[5,6] the secondary structure of gramicidin in phospholipid membranes is a left-handed, single-stranded head-to-head β ^{6,3}-helical dimer with an approximate length of 25–30 Å, enabling it to act as an ion-transferer, selective for monovalent cations (channel structure). Gramicidin in organic solvents behaves differently and adopts double-helical conformations (pore structure) with comparable dimensions to that of the aforementioned structure in lipid bilayers.[7] CD studies in phospholipid bilayers and organic solutions also confirm this structural difference.[8] Crystal structures of the uncomplexed gramicidin, grown from alcoholic solutions, show double-stranded, antiparallel, left-handed β ^{5,6}-helices.[9,10] Interestingly, the same conformation was attributed to a caesium complex of gramicidin with 6.4 residues per turn.[11] Gramicidin pores and channels were reviewed recently.[12]

α-Aminoisobutyric acid (Aib)—widely found in pore-forming peptaibol antibiotics such as alamethicin, suzukacillin, zervamicin and emerimicin—constrains the conformation of peptides to certain areas of the Ramachandran plot,[13] most suitable for 3_{10}- and α-helical motifs.[14–18] For example, alamethicin adopts a largely right-handed α-helical structure in the crystal form[19] and retains this structure in organic solvents[20,21] and phospholipid vesicles.[21] Several models have been suggested to interpret the pore-forming ability of alamethicin in membranes.[19,21] These models were based on the ability of alamethicins to aggregate inside the hydrophobic area of membranes and form voltage-gated pores not as ion-selective as gramicidin channels.

Lately, α,α-dialkylated amino acids in general and Aib in particular have been studied, extensively, in an attempt to generalize their role in inducing certain ordered secondary structures.[22–25] In the case of Aib-containing peptides, the borderline between the two families of helical structures, *i.e.* 3_{10}- and α-helices, is very narrow. The energy difference between these two structures is small and they are interconvertible under minor environmental changes. Dominance of either structure in Aib-containing peptides depends on peptide length, milieu, size and distribution of amino acid side chains and the position of Aib residues in peptide backbone, although the latter was not emphasized much. In shorter peptides the 3_{10}-helix is dominant, whereas in longer peptides the α-helix is preferred. In solution, peptides longer than seven or eight residues favour α-helices.[22,23] Concomitantly, in crystals of one and the same molecule, different conformers of 3_{10}-, α- or mixed 3_{10}/α-helical structures were observed.[22,23,26–28]

3_{10}- and α-helices are the two common helical secondary structures manifested in proteins[29] and they contribute to the formation of the super-secondary structures responsible for various biological functions, such as ion-transfer through membranes or lipid-protein interactions. Peptides like gramicidin, as well as alamethicin and related peptides, though different in their helical conformation and mechanism of interaction with membranes, have long served to model these phenomena. As small peptides in comparison to proteins, they show more structural flexibility. Alamethicin gives more stable structures than gramicidin in different organic solvents and in being transferred from these solvents to aqueous solvents and finally to phospholipid bilayers.[21] This relatively more stable structure is due to the presence of Aib in the alamethicin

† Aib, α-aminoisobutyric acid (2-methylalanine); Boc, *tert*-butoxycarbonyl; *p*BrBz, *p*-bromobenzoyl; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; DMF, dimethylformamide; EA, ethyl acetate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; GAA, [Aib^{4,6,8,10,12,14}]gramicidin A; GBA, [Aib^{4,6,8,10,12,14}]gramicidin B; Glyol, 2-aminoethanol; HCO-ONSu, *N*-formyloxysuccinimide; HOBt, 1-hydroxybenzotriazole; IBCF, isobutylchloroformate; MA, mixed anhydride; NMM, *N*-methylmorpholine; LP, light petroleum (b.p. 30–60 °C); TEA, triethylamine; TFA, trifluoroacetic acid; TFE, trifluoroethanol; THF, tetrahydrofuran; Z, benzyloxycarbonyl.

The abbreviations in relation to amino acids and their derivatives are in accordance with the IUPAC-IUB rules: *Eur. J. Biochem.*, 1984, **138**, 9. All of the optically active amino acids are L-enantiomers, unless specified otherwise.

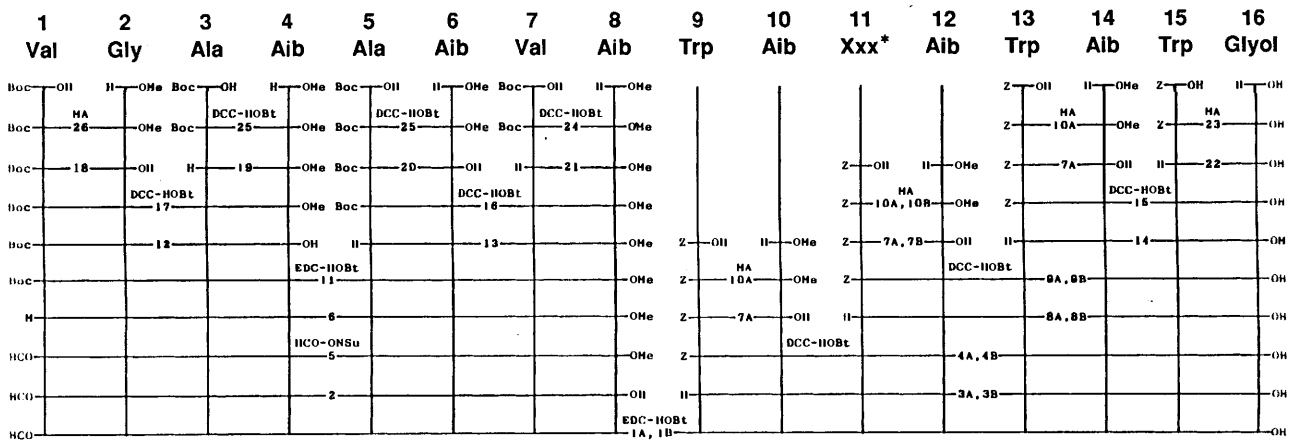

Fig. 1 Scheme for the syntheses of gramicidin A and B Aib analogues, GAA and GBA (* Xxx = Trp for A, and Phe for B)

backbone (40%), which gives the peptide less choice in conformational adaptability to environmental forces.

In order to mimic a protein helical structure with the potential for inducing pore-forming structures in lipid bilayers and yet more stable than gramicidin in different milieu, Aib analogues of gramicidin were designed. In this study, D-amino acids present in gramicidin backbone were replaced with Aib. As a result, new hydrophobic pentadecapeptides were generated with an alternative sequence of L- and achiral amino acids: HCO-Val[1]-Gly-Ala-Aib-Ala[5]-Aib-Val-Aib-Trp-Aib[10]-Xxx-Aib-Trp-Aib-Trp[15]-Glyol [Xxx = Trp(GAA), Phe(GBA), Tyr(GCA)]. In these analogues, the first three amino acids of the *N*-terminus half of the gramicidin were retained. The Trp and other aromatic residues of the *C*-terminus half of the original pentadecapeptide were also preserved. The latter part of the molecule is believed to be responsible for the gramicidin incorporation into the membranes, although the mechanism of this phenomenon is a subject of controversy.[30]

As will be discussed later, the helical sense of the natural gramicidin is changed in these analogues from an ion-permeable, left-handed β-helix to right-handed helices with smaller diameters and less flexible structures that may not allow ions to permeate directly through. Conformational studies are based on CD spectra and therefore cannot be as specific as other structure-determining techniques, such as X-ray crystallography. In these solution studies GBA is used in all CD comparisons with gramicidin.

Results and Discussion

Synthesis.—We have already studied the structural effects of Aib in an Aib-containing synthetic analogue of the cyclic peptide gramicidin S.[31] In this study, the syntheses of GAA and GBA were carried out as depicted in Fig. 1. Non-protected tryptophan residues were used in these syntheses, although N^i-formyltryptophans were also used in a separate synthesis of GAA (unpublished study). The tryptophan's indole damage is considerably less in the present study. The solution-phase synthesis of gramicidin A[32] was considered as a guideline in the synthesis of the *C*-terminus heptapeptide. To complete this study, synthesis of the last and more hydrophilic analogue, GCA, is being undertaken at present in our laboratory.

CD Spectra.—CD measurements were used to compare the structural changes of gramicidin and its Aib analogues, GAA and GBA, in solvents of different polarity and different hydrogen-bonding properties. In organic solvents gramicidin was considered as a mixture of four double-helical dimers in equilibrium with monomers.[7] The net CD spectra of this peptide in alcoholic solvents and dioxane are believed to be a superposition of the spectrum of each individual species. However, in trifluoroethanol gramicidin is a monomer and its conformation is not well-defined.[12] The flexible nature of gramicidin in solvents of different polarity is shown in Fig. 2(*a*) and was investigated before.[33] In an aqueous solution (70% methanol) the gramicidin spectrum show similar patterns to that of TFE; however, the minimum is transferred to the positive area of the mean residue ellipticity. The conformational mixture of gramicidin in dioxane and methanol show two characteristic negative Cotton effects around 210 and 230 nm, which are also present in the spectra of some of the individual dimers.[7]

CD spectra of one of the Aib analogues of gramicidin, GBA, were measured under similar conditions employed for gramicidin [Fig. 2(*b*)]. GBA was not very soluble in dioxane and a saturated solution was used for CD measurements. Solution conformation of GBA shows different patterns from those of gramicidin. A distinguished helical structure can be observed in all of the four solvent systems. In methanol and dioxane, negative Cotton effects, characteristic of α-helix (208 and 222 nm), can be observed. The minimum at 222 nm appears as a small shoulder which then extends to a broader one at shorter wavelengths. CD spectra in methanol, TFE and 70% methanol show a positive Cotton effect with a maximum at 192 nm, another special feature of helical structures. The minimum at 208 nm is more negative than the one at 222 nm. This phenomenon has been observed both in the CD spectra of Aib-containing peptides[20] and 3_{10}-helical motifs.[34,35] In the case of the TFE spectrum, the double minimum is somehow flattened and the broad shoulder between 210 and 220 nm is changed and becomes less distinct; instead a broad minimum shows itself around 210 nm. Other features of the GBA spectra are the presence of a minimum around 230 nm and another minimum around 216 nm. These aspects can be readily detected in the spectra in methanol, dioxane and TFE.

Comparison of the two sets of CD spectra in Figs. 2(*a*) and (*b*), shows less solvent-dependent behaviour. *i.e.* a more stable conformation, for GBA. The content of ordered secondary structures present in GBA changes in different environments, but in general at least a helical motif is preserved throughout the whole range of solvents. In contrast, gramicidin shows different conformations in the same set of solvents.

Fig. 3 shows superimposed spectra of the two analogues, GAA and GBA, in methanol. The two spectra almost cover each other.

The presence of a variety of negative Cotton effects in

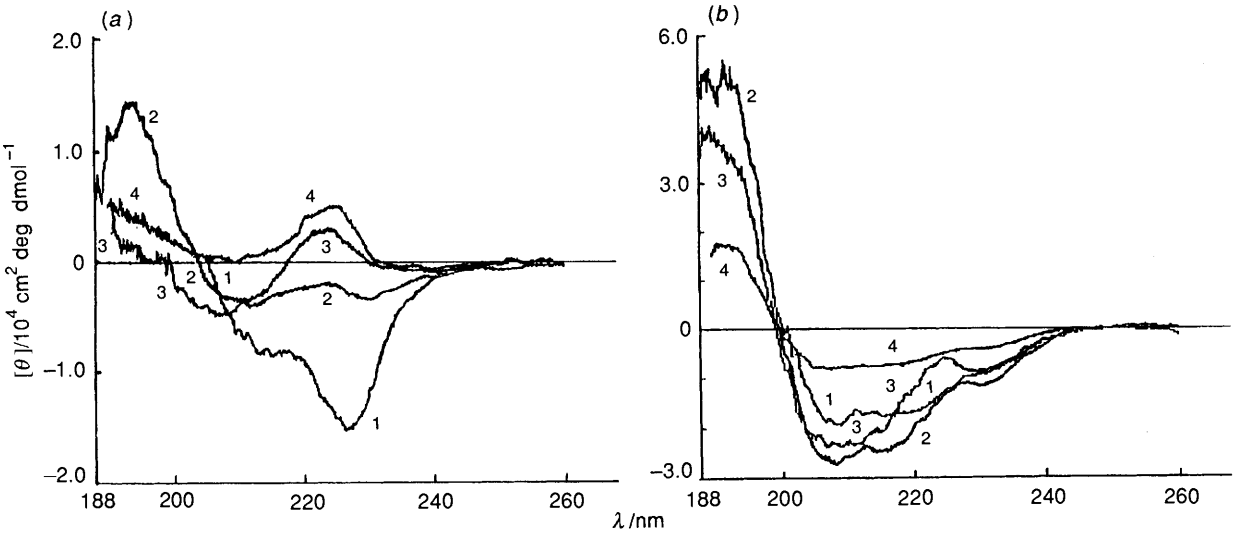

Fig. 2 CD spectra of (*a*) the naturally occurring gramicidin and (*b*) the synthetic gramicidin B Aib analogue (GBA) in solvents of different polarity: dioxane (1), methanol (2), TFE (3) and aqueous 70% methanol (4). (All concentrations are 1 mg cm^{-3} except that of GBA in dioxane, a saturated solution, which is less than 1 mg cm^{-3}).

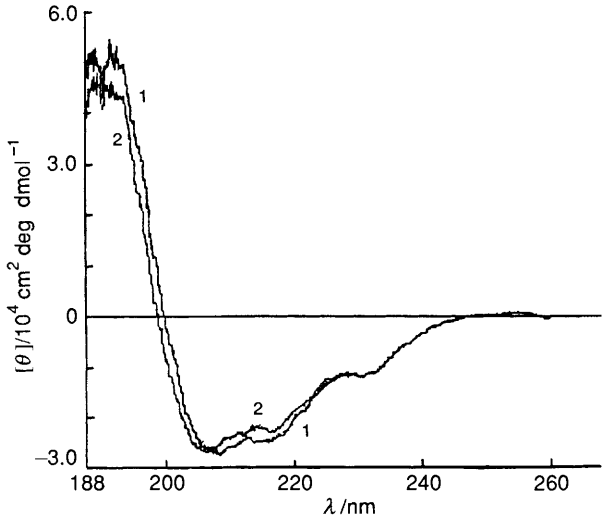

Fig. 3 CD spectra of GAA (2) and GBA (1) in methanol. (Concentrations are 1 mg cm^{-3}).

GAA and GBA spectra, characteristic of at least one ordered secondary structure, and the possibility of aromatic side chain interaction with the CD spectra[8,33] make any detailed conformational interpretation rather complex.

Figs. 4(*a*) and 4(*b*) show CD spectral comparisons between gramicidin and GBA in three solvents. Although in methanol and dioxane, gramicidin exists in dimeric conformations, the net spectra represents some characteristics of the individual conformers.[7] Thus in Fig. 4(*a*), two negative Cotton effects of gramicidin in methanol and dioxane can be compared to those of GBA in the same region. The same comparison can be made in methanol and TFE in Fig. 4(*b*). Interesting similarities, though not in intensity, can be distinguished in these spectra.

CD spectra of GBA, its *N*-terminus octapeptide and *C*-terminus heptapeptide fragments in methanol and TFE were compared previously.[1]

Results of CD studies are in agreement with the potent tendency of Aib to form common helical secondary structures[36] found in proteins.[29] In addition, other amino acids present in the backbone, with the exception of Gly, are not strong helix destabilizers.[36,37] Therefore, an α-helical conformation can be attributed to both of the Aib analogues studied. Crystal structures of hydrophobic Aib-containing peptides of different length (6–20 residues) were reviewed recently,[22] and 3_{10}-, α- or mixed 3_{10}/α-helical conformations were detected in their crystals. The crystals of alternatively sequenced *p*BrBz-(Aib-Ala)$_n$ deca- and dodecapeptides basically show α-helical structures with disruptions near their *C*-termini. These disruptions are due to the presence of C_{10} (β-bend) and C_{16} (π-bend) motifs.[38]

Finally, in the case of GAA and GBA, as far as CD analysis permits, one may deduce a possible superposition of an α-helical motif with other secondary structures such as 3_{10}-helix or random coil. The existence of other helical structures or different conformers in solution cannot be confirmed or rejected by the present CD based conformational analysis.

These less flexible, synthetic hydrophobic Aib analogues may show pore-forming abilities in phospholipid bilayers and experiments in this area are planned. Aib-containing amphiphilic peptides of the same length (15–16 residues), such as zervamicin, can exhibit pore-forming abilities.[39,40] As helical structures have unsatisfied hydrogen-bonding donors and acceptors at and near their termini, there is always the possibility of head-to-tail aggregation in crystals or less ordered structures such as phospholipid bilayers. In fact, such an aggregation in crystals has been reported for peptaibol antibiotic fragments and hydrophobic synthetic peptides.[22] This hydrogen-bonding ability may provide a tendency for hydrophobic peptides, shorter than the average bilayer thickness, to span the membrane to reach the more hydrophilic zones.[25]

Biological Activity.—Biological activities of the natural gramicidin, GAA and GBA, were tested against certain microbial organisms. The results, shown in Table 1, suggest considerable reduction in the antimicrobial inhibitory activity of the Aib analogues compared to that of gramicidin. The relative activities of GAA and GBA are quite comparable. It can be concluded that the change of structure induced by inclusion of an unusual amino acid such as Aib in the gramicidin backbone drastically reduces the biological activities of the natural product.

Experimental

Instrumental.—Circular dichroism was measured by a JASCO J-600 CD spectropolarimeter. To detect and determine the Trp content of the peptides synthesized a JASCO Ubest UV–VIS spectrophotometer was used. Optical rotations were

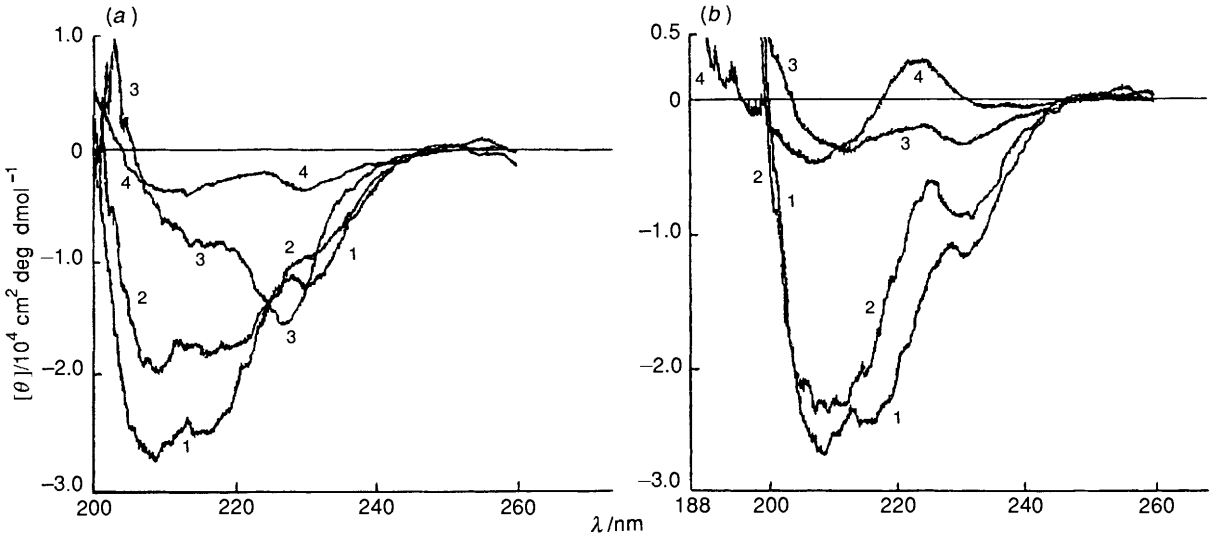

Fig. 4 CD spectra of (*a*) the naturally occurring gramicidin and the synthetic gramicidin B Aib analogue (GBA) in methanol (4 and 1, respectively) and dioxane (3 and 2, respectively), and (*b*) the naturally occurring gramicidin and the synthetic gramicidin B Aib analogue (GBA) in methanol (3 and 1, respectively) and TFE (4 and 2, respectively). (All concentrations are 1 mg cm^{-3} except that of GBA in dioxane, which is less than 1 mg cm^{-3}).

Table 1 Relative inhibitory activities[a] of the naturally occurring gramicidin (GD) and its Aib analogues, GAA and GBA, against certain microorganisms at concentrations of 1 and 10 μg cm^{-3}

Microbial strain	GD		GAA		GBA	
	1	10	1	10	1	10
B. subtilis	100[b]	100	0	6.5	0.5	8
S. aureus	100	100	0	0	1	1
E. coli	0	22	4.5	9.5	1	11.5
M. morganii	0	0	0	0	0	0

[a] Inhibitory activities were evaluated in bouillon broth medium (pH 6.5) at 37 °C after 6 h, by fixed wavelength measurement at 660 nm (OD_{660}). [b] 100 represents maximum inhibitory activity (no microbial growth, hence no increase in absorption at OD_{660} comparing to that of the blank); 0 represents minimum inhibitory activity (complete microbial growth, hence increase in absorption at OD_{660} with the same rate as the blank).

determined by a JASCO DIP-370 polarimeter. Elemental analyses were performed at Kyushu and Nagasaki Universities.

TLC and Paper Chromatography.—Silica gel GF_{254} plates (Merck) and Toyo 50 papers (Toyo Roshi) were used, respectively. Solvent systems [represented by R_{fn} (n = 1–8) in the text]: (1) chloroform–methanol (5:1); (2) chloroform–methanol (9:1); (3) chloroform–methanol (20:1); (4) chloroform–methanol–acetic acid (8:1:1); (5) chloroform–methanol–acetic acid (50:10:2); (6) chloroform–methanol–acetic acid (95:5:1); (7) n-butanol–acetic acid–water (4:1:5); (8) n-butanol–acetic acid–pyridine–water (4:1:1:2). Spray reagents: 10% H_2SO_4; 0.5% ninhydrin in acetone (in the case of the *N*-protected peptides TLC plates were first sprayed with HCl (2 mol dm^{-3}) and then with the ninhydrin reagent); 1% *p*-dimethylaminobenzaldehyde in acetone–HCl (9:1) (Ehrlich reagent).

Ion Exchange Chromatography.—Dowex 1 × 4 (mesh 100–200) and Dowex 50[w] × 4 (mesh 100–200) ion exchange resins were used in their OH^- and H^+ cycles, respectively. The resins were equilibrated with methanol.

Natural Gramicidin.—Gramicidin D (Dubos) was obtained from P-L Biochemicals, Inc. (USA) and used in CD measurements without further purification.

Peptide Synthesis.—The peptides were synthesized by conventional solution-phase procedures. All of the reported melting points are uncorrected. Boc-Val-OH, Boc-Ala-OH, Z-Trp-OH and H-Glyol·HCl were obtained from the Peptide Institute, Inc. (Japan) and used as received. Z-Phe-OH was synthesized, under Schotten–Baumann conditions, by coupling the free amino acid to benzylchlorocarbonate. H-Gly-OMe· HCl, H-Ala-OMe·HCl and H-Aib-OMe·HCl were prepared by the esterification of the corresponding free amino acids through a thionyl chloride procedure in methanol.[41] Purities of the *N*-protected amino acid and methyl ester hydrochlorides were examined by TLC, paper chromatography and melting point determination.

Boc-Val-Gly-OMe (**26**). Boc-Val-OH was coupled to H-Gly-OMe·HCl *via* the MA method to give the methyl ester **26** (71%), m.p. 111–112 °C (from EA–LP) (lit.,[42] 110–112 °C); R_{f4} 0.75.

Boc-Val-Gly-OH (**18**). The methyl ester **26** was saponified with 2 mol dm^{-3} NaOH (2 equivalents) and the free acid **18** was used in a further step.

Boc-Ala-Aib-OMe (**25**). Boc-Ala-OH was coupled to H-Aib-OMe·HCl *via* the DCC-HOBt method to yield the oily methyl ester **25** (86%); R_{f4} 0.33, R_{f6} 0.47.

Boc-Ala-Aib-OH (**20**). The dipeptide **25** was saponified with 2 mol dm^{-3} NaOH (2 equivalents) in methanol to give the acid **20** (93%), m.p. 164–165 °C; $[\alpha]_D^{20}$ −30.0 ± 0.5° (*c* 0.1 in methanol); R_{f2} 0.10.

H-Ala-Aib-OMe·HCl (**19**). The dipeptide **25** (4.33 g, 15 mmol) was dissolved in 4 mol dm^{-3} HCl/dioxane (7.5 cm^3, 30 mmol) at 0 °C. After 5 h, the solvent was evaporated under reduced pressure and the residual oil was dried over KOH *in vacuo*. The oil (**19**) was used in a further step (3.40 g, 100%); R_{f1} 0.27; paper chromatography: R_{f7} 0.52, R_{f8} 0.66.

Boc-Val-Aib-OMe (**24**). Boc-Val-OH was coupled to H-Aib-OMe·HCl *via* the DCC-HOBt method to give the methyl ester **24** (70%), m.p. 114–115.5 °C (lit.,[43] 115–118 °C, lit.,[44] 140 °C); R_{f4} 0.77.

H-Val-Aib-OMe·HCl (**21**). The dipeptide **24** (3.16 g, 10 mmol) was dissolved in 4 mol dm^{-3} HCl/dioxane (25.0 cm^3, 100 mmol) at 0 °C. After 6 h, the solvent was evaporated under reduced pressure and the residue was treated as described for **19**. The oil (**21**) was used in a further step (2.50 g, 100%); R_{f2} 0.16, R_{f3} 0.10; paper chromatography: R_{f7} 0.42, R_{f8} 0.73.

Boc-Val-Gly-Ala-Aib-OMe (**17**). The dipeptide **19** (4.02 g, 18 mmol) was dissolved in DMF (50 cm^3), and TEA (2.52 cm^3, 18

mmol) was added when the solution had cooled. A solution of **18** (4.11 g, 15 mmol) in DMF (50 cm^3), was added. HOBt (2.43 g, 18 mmol), and after a few minutes, DCC (3.09 g, 15 mmol), were added successively. The reaction mixture was stirred at 0 °C for 2 h and stirring continued at room temperature for 70 h. The insoluble DCU was filtered off and the filtrate was concentrated to oil and dissolved in EA. The solution was washed, successively, with 5% $KHSO_4$, 0.5 mol dm^{-3} $NaHCO_3$ and saturated NaCl solution, then dried over Na_2SO_4. The solvent was removed, and the residue was crystallized from LP to yield the tetrapeptide methyl ester **17** (6.40 g, 96%), m.p. 78–80 °C (Found: C, 53.7; H, 8.2; N, 12.1. $C_{20}H_{36}N_4O_7$ requires C, 54.0; H, 8.2; N, 12.6%); $[\alpha]_D^{20}$ −29.0 ± 0.5° (*c* 0.1 in methanol); R_{f2} 0.45, R_{f4} 0.64.

Boc-Ala-Aib-Val-Aib-OMe (**16**). The dipeptide **21** (7.58 g, 30 mmol) was dissolved in DMF (100 cm^3), and TEA (4.20 cm^3, 30 mmol) was added when the solution had cooled. The dipeptide **20** (8.23 g, 30 mmol) was added together with HOBt (4.86 g, 36 mmol). After a few minutes, DCC (6.19 g, 30 mmol) was added. The reaction mixture was treated as described for **17**. The final residue was crystallized from LP to yield the tetrapeptide methyl ester **16** (9.78 g, 69%), m.p. 176 °C (Found: C, 56.0; H, 8.5; N, 11.65. $C_{22}H_{40}N_4O_7$ requires C, 56.0; H, 8.5; N, 11.9%); $[\alpha]_D^{20}$ −30.0 ± 0.4° (*c* 0.1 in methanol); R_{f1} 0.71, R_{f2} 0.51, R_{f5} 0.33.

Boc-Val-Gly-Ala-Aib-OH (**12**). The tetrapeptide **17** (3.56 g, 8 mmol), in methanol (35 cm^3), was saponified with 2 mol dm^{-3} NaOH (8 cm^3, 16 mmol) at 0 °C. After 45 min, the reaction was continued at room temperature for 5 h. The solvent was evaporated under reduced pressure and the residue was dissolved in water, washed with ether and acidified to pH 3 with citric acid when the mixture had cooled. The separated oil was extracted with chloroform, washed with saturated NaCl solution and dried over Na_2SO_4. Chloroform was removed by evaporation and the residue was crystallized from ether to yield the acid **12** (2.82 g, 82%), m.p. 168–169 °C (Found: C, 52.9; H, 7.9; N, 12.9. $C_{19}H_{34}N_4O_7$ requires C, 53.0; H, 8.0; N, 13.0%); $[\alpha]_D^{20}$ −32.5 ± 0.5° (*c* 0.1 in methanol); R_{f1} 0.12.

H-Ala-Aib-Val-Aib-OMe·TFA (**13**). The tetrapeptide **16** (4.73 g, 10 mmol) was dissolved in TFA (20 cm^3) at 0 °C and kept at this temperature for 30 min. After removal of the solvent under reduced pressure, the residue was solidified by the addition of ether, and dried over KOH *in vacuo*. The salt **13** was used later without further purification (4.86 g, 100%).

Boc-Val-Gly-Ala-Aib-Ala-Aib-Val-Aib-OMe (**11**). The tetrapeptide salt **13** (2.92 g, 6 mmol), TEA (0.84 cm^3, 6 mmol) and the tetrapeptide **12** (2.58 g, 6 mmol) were dissolved in DMF (35 cm^3). HOBt (0.81 g, 6 mmol), and after a few minutes, EDC (1.27 g, 6.6 mmol), were added. The reaction mixture was stirred at 0 °C for 2 h and then at room temperature for 50 h. The solution was evaporated to oil under reduced pressure and the oil was dissolved in EA. The EA solution was washed and dried as described for **17**. After removal of the solvent by evaporation, the residue was crystallized from LP to yield the octapeptide methyl ester **11** (2.80 g, 60%), m.p. 115–116 °C (Found: C, 54.2; H, 8.1; N, 13.7. $C_{36}H_{64}N_8O_{11}\cdot H_2O$ requires C, 53.85; H, 8.3; N, 14.0%); $[\alpha]_D^{20}$ −8.0 ± 0.3° (*c* 0.1 in methanol); R_{f1} 0.66, R_{f2} 0.40, R_{f5} 0.74, R_{f6} 0.34.

H-Val-Gly-Ala-Aib-Ala-Aib-Val-Aib-OMe·HCl (**6**). The octapeptide **11** (2.35 g, 3 mmol) was dissolved in TFA (10 cm^3) at 0 °C, and the solution was left at this temperature for 30 min. TFA was evaporated under reduced pressure, the residue was dissolved in 4 mol dm^{-3} HCl/dioxane (10 cm^3) and kept at room temperature for 1 h. After evaporation of the solvent, the residue was crystallized by addition of ether and dried over KOH *in vacuo*. The salt **6** was used later without further purification (2.13 g, 98%), m.p. 141–143 °C; R_{f1} 0.43, R_{f5} 0.18.

HCO-Val-Gly-Ala-Aib-Ala-Aib-Val-Aib-OMe (**5**). (*a*) HCO-ONSu was prepared by the reaction of *N*-hydroxysuccinimide (1.15 g, 10 mmol), formic acid (0.38 cm^3, 10 mmol) and DCC (2.06 g, 10 mmol) in THF (15 cm^3) at 0 °C. The reaction mixture was stirred for 2 h at this temperature, then the reaction was continued at room temperature for another 3 h. DCU was filtered off, the filtrate was evaporated, and the residue was crystallized from LP. The hygroscopic compound was dried over P_2O_5 *in vacuo* and used in the next reaction (1.24 g, 86%); R_{f6} 0.30.

(*b*) The octapeptide salt **6** (1.59 g, 2.2 mmol) was dissolved in DMF (15 cm^3) and kept at 0 °C. TEA (0.39 cm^3, 2.75 mmol), and after a few minutes, the freshly prepared HCO-ONSu (0.40 g, 2.75 mmol), were added. The reaction mixture was stirred for 20 min at 0 °C, and then at room temperature for another 3 h. The solvent was evaporated under reduced pressure, the residue was dissolved in water–ethanol (9:1), and the formylated octapeptide was extracted with chloroform from the aqueous solution. The chloroform phase was then washed with water, dried over Na_2SO_4 and evaporated. The residue was crystallized from LP–ether to yield the formyloctapeptide methyl ester **5** (0.92 g, 59%), m.p. 219–221 °C (decomp.) (Found: C, 53.2; H, 7.9; N, 15.5. $C_{32}H_{56}N_8O_{10}\cdot 0.5H_2O$ requires C, 53.2; H, 8.0; N, 15.5%); $[\alpha]_D^{20}$ −14.2 ± 0.3° (*c* 0.1 in methanol); R_{f1} 0.55, R_{f2} 0.37, R_{f5} 0.53, R_{f6} 0.38.

HCO-Val-Gly-Ala-Aib-Ala-Aib-Val-Aib-OH (**2**). The formyloctapeptide **5** (0.71 g, 1 mmol), in methanol (15 cm^3), was saponified with 2 mol dm^{-3} NaOH (2 cm^3, 4 mmol) at 0 °C over 10 min, then the reaction was continued at room temperature for 30 h. The solvent was evaporated under reduced pressure, and the residue was dissolved in water, washed with chloroform and acidified to pH 3 with 10% H_2SO_4. The water was evaporated under reduced pressure and the residue was extracted with methanol. After being centrifuged, the supernatant was evaporated. The residue was triturated in ether–EA and recrystallized from methanol–EA to yield the formyloctapeptide acid **2** (0.60 g, 85%), m.p. 184–186 °C (decomp.) (Found: C, 52.1; H, 7.75; N, 15.5. $C_{31}H_{54}N_8O_{10}\cdot H_2O$ requires C, 52.0; H, 7.9; N, 15.6%); $[\alpha]_D^{20}$ −15.0 ± 0.5° (*c* 0.1 in methanol); R_{f5} 0.42, R_{f8} 0.81.

Z-Trp-Glyol (**23**). Z-Trp-OH (3.38 g, 10 mmol), in THF (40 cm^3), was cooled to −20 °C. NMM (1.10 cm^3, 10 mmol) was added, and after 30 min IBCF (1.32 cm^3, 10 mmol) was added while the reaction mixture was stirred. NMM·HCl salt began to precipitate. After 5 min, a cold solution of H-Glyol·HCl (1.02 g, 10.5 mmol) and TEA (1.47 cm^3, 10.5 mmol) in chloroform (20 cm^3) was added at −20 °C. The reaction mixture was kept for 2 h between −10 and −20 °C and then the reaction was allowed to continue at room temperature for another 20 h. The white precipitate was filtered off and the filtrate evaporated under reduced pressure. The residue was dissolved in EA and washed, successively, with 5% $KHSO_4$, 0.5 mol dm^{-3} $NaHCO_3$ and water, then dried over Na_2SO_4. After evaporation of the solvent, the residue was recrystallized twice from EA–LP to give **23** (2.80 g, 73%), m.p. 102.5–104 °C (lit.,[32] 124–125 °C); $[\alpha]_D^{20}$ −5.0 ± 0.4° (*c* 0.1 in methanol) [lit.,[32] −4.6 ± 1.5° (*c* 0.86 in methanol)]; R_{f1} 0.65, R_{f2} 0.46, R_{f3} 0.19, R_{f6} 0.35.

H-Trp-Glyol (**22**). The peptide **23** (1.91 g, 5 mmol) was dissolved in methanol–acetic acid (1:1) (8 cm^3) and a Pd black catalyst (*ca.* 0.3 g) was added. The reaction was continued under an H_2 atmosphere for 2 h. The catalyst was filtered off and the filtrate was evaporated under reduced pressure. The residue was eluted with methanol through a Dowex 1 column (OH^- form, equilibrated with methanol). The eluent was evaporated and the residue was recrystallized from methanol–ether when cooled (the crystallization process was slow) to yield **22** (1.10 g, 89%), m.p. 144 °C (lit.,[32] 144 °C); $[\alpha]_D^{20}$ +19.0 ± 0.7° (*c* 0.1 in methanol) [lit.,[32] +18.2 ± 1° (*c* 1 in methanol)]; R_{f1} 0.37, R_f[2] 0.21, R_{f5} 0.14.

Z-Xxx-Aib-OMe [Xxx = Trp (**10A**), Phe (**10B**)]. Z-Xxx-OH [Xxx = Trp (1.69 g), Phe (1.50 g), 5 mmol], in THF (25 cm^3), was neutralized with NMM (0.55 cm^3, 5 mmol) under the conditions stated for peptide **23**. IBCF (0.66 cm^3, 5 mmol) was added, and after 5 min a cold suspension of H-Aib-OMe·HCl (0.81 g, 5.25 mmol) and TEA (0.735 cm^3, 5.25 mmol) in chloroform (15 cm^3) were also added. The reaction was worked up as described for **23** to give the dipeptide methyl ester **10A** (1.94 g, 89%) or **10B** (1.91 g, 96%) [attempts at recrystallization failed in the case of **10A** (foam-like, triturated in LP and a few drops of EA) and **10B** was crystallized from ether]; m.p. **10A**: 62–65 °C (Found: C, 65.4; H, 6.2; N, 9.5. $C_{24}H_{27}N_3O_5$ requires C, 65.9; H, 6.2; N, 9.5%), **10B**: 88–90 °C (Found: C, 66.5; H, 6.6; N, 7.0. $C_{22}H_{26}N_2O_5$ requires C, 66.3; H, 6.6; N, 7.0%); $[\alpha]_D^{20}$ −12.5 ± 0.3° (**10A**), −8.5 ± 0.1° (**10B**) (*c* 0.1 in methanol); **10A**: R_{f2} 0.53, R_{f6} 0.37, **10B**: R_{f2} 0.63, R_{f5} 0.88.

Z-Xxx-Aib-OH [Xxx = Trp (**7A**), Phe (**7B**)]. Dipeptide **10A** (1.31 g, 3 mmol) [or **10B** (1.20 g, 3 mmol)] was saponified in methanol (10 cm^3) using 2 mol dm^{-3} NaOH (1.8 cm^3, 3.6 mmol) at 0 °C over 1 h. The reaction was continued at room temperature for a further 5.5 h. Methanol was evaporated under reduced pressure and the residue was dissolved in water, washed with ether, and acidified with 2 mol dm^{-3} HCl to pH 3. The acidic dipeptide was extracted with EA and dried over Na_2SO_4. EA was removed by evaporation to yield **7A** (1.19 g, 94%; a pure foam-like substance used without further purification) [or **7B** (crude 0.89 g, 77%, recrystallised from EA–LP: 0.74 g, 65%)], m.p. **7A**: 86–88 °C (Found: C, 64.7; H, 6.1; N, 9.7. $C_{23}H_{25}N_3O_5$ requires C, 65.2; H, 5.95; N, 9.9%), **7B**: 161 °C (Found: C, 65.5; H, 6.2; N, 7.3. $C_{21}H_{24}N_2O_3$ requires C, 65.6; H, 6.3; N, 7.3%); $[\alpha]_D^{20}$ −15.5 ± 0.3° (**7A**), −9.5 ± 0.5° (**7B**) (*c* 0.1 in methanol); **7A**: R_{f5} 0.62, **7B**: R_{f5} 0.60.

Z-Trp-Aib-Trp-Glyol (**15**). The dipeptide **7A** (1.27 g, 3 mmol), the peptide **22** (0.74 g, 3 mmol) and HOBt (0.49 g, 3.6 mmol) were dissolved in DMF (7 cm^3), and kept at 0 °C. A cooled solution of DCC (0.65 g, 3.15 mmol) in DMF (3 cm^3) was added. The reaction mixture was stirred for 1 h, the reaction was continued at room temperature for another 22 h, then DCU was filtered off and solvent removed under reduced pressure. The residue was dissolved in EA, washed, successively, with 5% $KHSO_4$, 0.5 mol dm^{-3} $NaHCO_3$ and water, then dried over Na_2SO_4. EA was evaporated under reduced pressure and the foam-like residue was triturated in LP to give the tripeptide **15** (1.83 g, 94%), m.p. 117–120 °C (Found: C, 64.9; H, 6.4; N, 12.1. $C_{36}H_{40}N_6O_6 \cdot H_2O$ requires C, 64.5; H, 6.3; N, 12.5%); $[\alpha]_D^{20}$ −15.0 ± 0.6° (*c* 0.1 in methanol); R_{f2} 0.42, R_{f5} 0.54.

H-Trp-Aib-Trp-Glyol (**14**). The tripeptide **15** (1.96 g, 3 mmol) was dissolved in methanol–acetic acid (5:4) (5.4 cm^3), and Pd black (*ca.* 0.3 g) was added. The reaction mixture was stirred under an H_2 atmosphere for 3 h. The catalyst was filtered off and the evaporated filtrate was passed through Dowex 1 (OH^- cycle) as described for **22**. The methanolic eluent was evaporated under reduced pressure and the foam-like residue was triturated in LP to yield the *N*-terminus free tripeptide **14** (1.39 g, 89%), m.p. 120–123 °C (Found: C, 63.45; H, 6.9; N, 15.25. $C_{28}H_{34}N_6O_4 \cdot 0.5H_2O$ requires C, 63.7; H, 6.7; N, 15.9%); $[\alpha]_D^{20}$ −20.0 ± 0.3° (*c* 0.1 in methanol); R_{f2} 0.36, R_{f5} 0.33.

Z-Xxx-Aib-Trp-Aib-Trp-Glyol [Xxx = Trp (**9A**), Phe (**9B**)]. The dipeptide Z-Xxx-Aib-OH [**7A** (0.85 g), **7B** (0.77 g), 2 mmol], the tripeptide **14** (1.04 g, 2 mmol) and HOBt (0.325 g, 2.4 mmol) were dissolved in DMF (7 cm^3) and kept at 0 °C. A cooled solution of DCC (0.435 g, 2.1 mmol) in DMF (3 cm^3) was added. The reaction mixture was stirred for 2 h, the reaction was continued at room temperature for a further 21 h, then the reaction mixture was treated as described for **15**. The pentapeptide was recrystallized from EA–LP to yield **9A** (1.44 g, 78%) or **9B** (1.41 g, 80%), m.p. **91**: 149–151 °C (Found: C, 65.4; H, 6.4; N, 13.3. $C_{51}H_{57}N_9O_8 \cdot H_2O$ requires C, 65.0; H, 6.3; N, 13.4%), **9B**: 154–155 °C (Found: C, 65.9; H, 6.4; N, 12.5. $C_{49}H_{56}N_8O_8 \cdot 0.5H_2O$ requires C, 65.8; H, 6.4; N, 12.5%); $[\alpha]_D^{20}$ −23.5 ± 0.3° (**9A**), −24.5 ± 0.4° (**9B**) (*c* 0.1 in methanol); **9A**: R_{f1} 0.53, R_{f5} 0.70, **9B**: R_{f2} 0.47, R_{f5} 0.73, R_{f6} 0.35.

H-Xxx-Aib-Trp-Aib-Trp-Glyol [Xxx = Trp (**8A**), Phe (**8B**)]. The pentapeptide **9A** (1.11 g, 1.2 mmol) [or **9B** (1.06 g, 1.2 mmol)] was hydrogenated using Pd black catalyst (*ca.* 0.2 g), in methanol–acetic acid (2:1) (2.4 cm^3) under an H_2 atmosphere (reaction time: 2 h). The reaction mixture was worked up further as described for **22**. After being passed through Dowex 1 (OH^- form), the hydrogenated pentapeptide was recrystallized from EA–LP to yield **8A** (0.87 g, 92%) [or **8B** (0.78 g, 86%)], m.p. **8A**: 125–129 °C (Found: C, 63.6; H, 6.8; N, 14.7. $C_{43}H_{51}N_9O_6 \cdot H_2O$ requires C, 63.9; H, 6.6; N, 15.6%), **8B**: 126–128 °C (Found: C, 64.3; H, 6.9; N, 14.3. $C_{41}H_{50}N_8O_6 \cdot H_2O$ requires C, 64.0; H, 6.8; N, 14.6%); $[\alpha]_D^{20}$ −31.0 ± 0.7° (**8A**), −43.0 ± 0.2° (**8B**) (*c* 0.1 in methanol); **8A**: R_{f2} 0.33, **8B**: R_{f2} 0.40, R_{f6} 0.04, R_{f7} 0.82, R_{f8} 0.89.

Z-Trp-Aib-Xxx-Aib-Trp-Aib-Trp-Glyol [Xxx = Trp (**4A**), Phe (**4B**)]. The pentapeptide **8A** (798 mg, 1 mmol) [or **8B** (751 mg, 1 mmol)], the dipeptide **7A** (423 mg, 1 mmol) and HOBt (162 mg, 1.2 mmol) were dissolved in DMF (3 cm^3) and kept at 0 °C. A cooled solution of DCC (227 mg, 1.1 mmol), in DMF (2 cm^3), was added. After 1 h, the reaction was continued at room temperature for another 21 h. The reaction mixture was then treated as described for **15** to give the heptapeptide methyl ester **4A** (1.052 g, 88%) (crystallized from ether) [or **4B** (0.952 g, 80%) (crystallized from LP)], m.p. **4A**: 154–158 °C (Found: C, 64.8; H, 6.7; N, 13.3. $C_{66}H_{74}N_{12}O_{10} \cdot 2H_2O$ requires C, 64.4; H, 6.4; N, 13.65%), **4B**: 154–155 °C (Found: C, 65.4; H, 6.4; N, 13.0. $C_{64}H_{73}N_{11}O_{10} \cdot H_2O$ requires C, 65.5; H, 6.4; N, 13.1%); $[\alpha]_D^{20}$ −10.7 ± 0.4° (**A**), −13.3 ± 0.4° (**4B**) (*c* 0.1 in methanol); **4A**: R_{f1} 0.36, R_{f5} 0.56, **4B**: R_{f2} 0.45, R_{f5} 0.65, R_{f6} 0.16.

H-Trp-Aib-Xxx-Aib-Trp-Aib-Trp-Glyol [Xxx = Trp (**3A**), Phe (**3B**)]. The heptapeptide **4A** (837 mg, 0.7 mmol) [or **4B** (809 mg, 0.7 mmol)] was hydrogenated by Pd black catalyst (*ca.* 0.2 g), in methanol–acetic acid (5:1) (6 cm^3), under an H_2 atmosphere (reaction time: 2 h). The reaction mixture was worked up further as described for **22**. After being eluted from a Dowex 1 (OH^- form) column with methanol, the hydrogenated heptapeptide was crystallized from ether to yield **3A** (705 mg, 95%) or **3B** (665 mg, 93%), m.p. **3A**: 155–158 °C (Found: C, 63.4; H, 7.0; N, 14.3. $C_{58}H_{68}N_{12}O_8 \cdot 2H_2O$ requires C, 63.5; H, 6.6; N, 15.3%), **3B**: 149–152 °C (Found: C, 64.3; H, 6.8; N, 14.2. $C_{56}H_{67}N_{11}O_8 \cdot H_2O$ requires C, 64.7; H, 6.7; N, 14.8%); $[\alpha]_D^{20}$ −25.3 ± 0.8° (**3A**), −23.3 ± 0.5° (**3B**) (*c* 0.1 in methanol); **3A**: R_{f5} 0.49, **3B**: R_{f2} 0.23, R_{f5} 0.50.

HCO-Val-Gly-Ala-Aib-Ala-Aib-Val-Aib-Trp-Aib-Xxx-Aib-Trp-Aib-Trp-Glyol [Xxx = Trp (**1A**), Phe (**1B**)]. The heptapeptide **3A** (531 mg, 0.5 mmol) [or the heptapeptide **3B** (511 mg, 0.5 mmol)], the formyloctapeptide **2** (367 mg, 0.525 mmol) and HOBt (112 mg, 0.825 mmol) were dissolved in DMF (8 cm^3) and kept at 0 °C. A cooled suspension of EDC (144 mg, 0.75 mmol) in DMF (2 cm^3) was added. The reaction mixture was stirred for 2 h, the reaction was continued at room temperature for a further 70 h, then the precipitate was filtered off and the filtrate evaporated under reduced pressure to an oil. The residue was purified by being eluted, with methanol, through Dowex 50 (H^+ cycle) and Dowex 1 (OH^- cycle) columns, respectively. The methanolic eluent was collected in four main fractions. The first fraction consisted of a chromatographically pure peptide but the second fraction showed traces of impurity. The third fraction was also rich in the desired peptide, but was more impure than the second fraction. The fourth fraction showed major impurities. The chromatographically pure peptide was recrystallized from methanol–ether to yield the pentadecapeptide **1A** (crude: 425 mg, 48%; chromatographically pure from fractions one and two: 201 mg, 23%) [or **1B** (crude: 412 mg, 48%;

chromatographically pure from fractions one and two: 238 mg, 28%)], m.p. **1A**: 215–219 °C (Found: C, 60.85; H, 7.4; N, 16.1. $C_{89}H_{120}N_{20}O_{17}\cdot H_2O$ requires C, 60.7; H, 7.8; N, 15.9%), **1B**: 245–247 °C (Found: C, 60.7; H, 7.3; N, 15.3. $C_{87}H_{119}N_{19}O_{17}\cdot H_2O$ requires C, 60.7; H, 7.1; N, 15.5%); $[\alpha]_D^{20}$ −10.0 ± 0.3° (**1A**), −8.0 ± 0.4° (**1B**) (*c* 0.1 in methanol) and −9.0 ± 0.5° (**1A**), −6.5 ± 0.4° (**1B**) (*c* 0.1 in DMF); **1A**: R_{f1} 0.45, R_{f5} 0.32, **1B**: R_{f1} 0.52, R_{f5} 0.41.

Analytical Procedures in Relation to the Final Compound.— Each of the components of the eluent (in fractions three and four, three spots were detected by TLC) were examined by UV spectroscopy and amino acid analysis, and only one component (the one with the lowest R_f and present in the first fraction as a pure compound) was, both qualitatively and quantitatively, consistent with the desired pentadecapeptide analogues. UV spectra of the purified peptides showed the main characteristics of a Trp spectrum, with maxima at 280 and 288 nm. The Trp content of peptides **1A** and **1B** was roughly estimated by comparing their UV spectra to that of the free Trp in methanol: 81% (**1A**); 97% (**1B**). In this estimate the interaction of the aromatic side chains, the solvent effect, and more complicated conformational interactions considered in determining the Trp in proteins[45] were neglected.

Acknowledgements

The authors wish to show their gratitude to Dr. Setsuko Ando (Fukuoka University), Dr. Ken-ichi Sato and Mr. Teruo Yasunaga (Saga University) for their technical advice and kind assistance.

References

1 Part of this work was presented at the 29th Symposium on Peptide Chemistry, Tokyo, 1991: M. Jelokhani-Niaraki, H. Takahashi, F. Kato and M. Kondo, *Peptide Chemistry*, 1991, in the press.
2 (*a*) R. J. Dubos, *J. Exp. Med.*, 1939, **70**, 1; (*b*) R. D. Hotchkiss and R. J. Dubos, *J. Biol. Chem.*, 1940, **132**, 791.
3 E. Benedetti, B. Di Blasio and C. Pedone, *Nature*, 1979, **282**, 630.
4 P. De Santis, S. Morosetti and R. Rizzo, *Macromolecules*, 1974, **7**, 52.
5 D. W. Urry, *Proc. Natl. Acad. Sci. USA*, 1971, **68**, 672.
6 D. W. Urry, M. C. Goodall, J. D. Glickson and D. F. Mayers, *Proc. Natl. Acad. Sci. USA*, 1971, **68**, 1907.
7 W. R. Veatch, E. T. Fossel and E. R. Blout, *Biochemistry*, 1974, **13**, 5249.
8 B. A. Wallace, W. R. Veatch and E. R. Blout, *Biochemistry*, 1981, **20**, 5754.
9 D. A. Langs, *Science*, 1988, **241**, 188.
10 D. A. Langs, G. D. Smith, C. Courseille, G. Precigoux and M. Hospital, *Proc. Natl. Acad. Sci. USA*, 1991, **88**, 5345.
11 B. A. Wallace and K. Ravikumar, *Science*, 1988, **241**, 182.
12 B. A. Wallace, *Annu. Rev. Biophys. Biophys. Chem.*, 1990, **19**, 127.
13 G. N. Ramachandran and V. Sasisekharan, *Adv. Protein Chem.*, 1968, **23**, 283.
14 A. W. Burgess and S. J. Leach, *Biopolymers*, 1973, **12**, 2599.
15 Y. Paterson, S. M. Rumsey, E. Benedetti, G. Nemethy and H. A. Scheraga, *J. Am. Chem. Soc.*, 1981, **103**, 2947.
16 C. Toniolo, *CRC Crit. Rev. Biochem.*, 1980, October, 1.
17 G. D. Rose, L. M. Gierasch and J. A. Smith, *Adv. Protein Chem.*, 1985, **37**, 1.
18 W. F. De Grado, *Adv. Protein Chem.*, 1988, **39**, 51.
19 R. O. Fox and F. M. Richards, *Nature*, 1982, **300**, 325.
20 G. Jung, N. Dubischar and D. Leibfritz, *Eur. J. Biochem.*, 1975, **54**, 395.
21 M. Casico and B. A. Wallace, *PROTEINS: Structure. Function, and Genetics*, 1988, **4**, 89.
22 I. L. Karle and P. Balaram, *Biochemistry*, 1990, **29**, 6747.
23 G. R. Marshall, E. E. Hodgkin, D. A. Langs, G. D. Smith, J. Zagrocki and M. T. Leplawy, *Proc. Natl. Acad. Sci. USA*, 1990, **87**, 487.
24 C. Toniolo and E. Benedetti, *Macromolecules*, 1991, **24**, 4004.
25 E. E. Hodgkin, J. D. Clark, K. R. Miller and G. R. Marshall, *Biopolymers*, 1990, **30**, 533.
26 R. Bosch, G. Jung, H. Schmitt, G. M. Sheldrick and W. Winter, *Angew. Chem., Int. Ed. Engl.*, 1984, **23**, 450.
27 I. L. Karle, J. L. Flippen-Anderson, K. Uma, H. Balaram and P. Balaram, *Proc. Natl. Acad. Sci. USA*, 1989, **86**, 765.
28 I. L. Karle, J. L. Flippen-Anderson, M. Sukumar and P. Balaram, *Proc. Natl. Acad. Sci. USA*, 1987, **84**, 5087.
29 D. J. Barlow and J. M. Thornton, *J. Mol. Biol.*, 1988, **201**, 601.
30 A. M. O'Connell, R. E. Koepper II, O. S. Andersen, *Science*, 1990, **250**, 1256.
31 M. Kondo, M. Kimura, K. Sato and H. Horimoto, *Bull. Chem. Soc. Jpn.*, 1987, **60**, 1391.
32 R. Sarges and B. Witkop, *J. Am. Chem. Soc.*, 1965, **87**, 2020.
33 D. W. Urry, J. D. Glickson, D. F. Mayers and J. Haider, *Biochemistry*, 1972, **11**, 487.
34 R. W. Woody, in *The Peptides*, ed. V. J. Hruby, Academic Press, New York, 1985, **vol. 8**, p. 33.
35 M. C. Manning and R. W. Woody, *Biopolymers*, 1991, **31**, 569.
36 K. T. O'Neil and W. F. De Grado, *Science*, 1990, **250**, 646.
37 P. Y. Chou and G. D. Fasman, *Annu. Rev. Biochem.*, 1978, **47**, 251.
38 E. Benedetti, B. Di Blasio, V. Pavone, C. Pedone, A. Santini, A. Bavoso, C. Toniolo, M. Crisma and L. Sartore, *J. Chem. Soc., Perkin Trans. 2*, 1990, 1829.
39 K. L. Rinehart, Jr., L. A. Gaudioso, M. L. Moore, R. C. Padneyand and J. Carter Cook, Jr., *J. Am. Chem. Soc.*, 1981, **103**, 6517.
40 I. I. Karle, J. L. Flippen-Anderson, S. Agarwalla and P. Balaram, *Proc. Natl. Acad. Sci. USA*, 1991, **88**, 5307.
41 (*a*) M. Brenner and W. Huber, *Helv. Chim. Acta*, 1953, **36**, 1109; (*b*) R. A. Boissonas, St. Guttmann, P. A. Jacquenoud and J. P. Waller, *Helv. Chim. Acta*, 1955, **38**, 1491.
42 D. W. Urry, W. D. Cunningham and T. Ohnishi, *Biochemistry*, 1974, **13**, 609.
43 R. Nagaraj, N. Shamala and P. Balaram, *J. Am. Chem. Soc.*, 1979, **101**, 16.
44 H. Schmitt and G. Jung, *Liebigs Ann. Chem.*, 1985, 321.
45 H. Edelhoch, *Biochemistry*, 1967, **6**, 1948.

Paper 2/00338D
Received 21*st January* 1992
Accepted 23*rd March* 1992