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Synthesis & Conformations of Stereochemically Restricted Analogs of Leu^5-Enkephalinamide: Substitution of α-Aminoisobutyric Acid & 1-Aminocyclopentane-1-carboxylic Acid at Positions 2 & 3[a]

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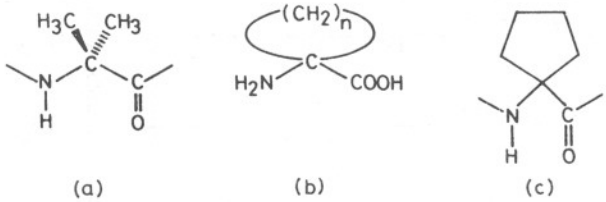
Six analogs of Leu-enkephalinamide containing α-aminoisobutyric acid (Aib) or 1-amino-cyclopentane-1-carboxylic acid (Acc^5) at positions 2 and 3 have been synthesized. The peptides Tyr-Aib-Gly-Phe-Leu-NH_2 (**1**), Tyr-Gly-Aib-Phe-Leu-NH_2 (**2**), Tyr-Aib-Aib-Phe-Leu-NH_2 (**3**), Tyr-Acc^5-Gly-Phe-Leu-NH_2 (**4**), Tyr-Gly-Acc^5-Phe-Leu-NH_2 (**5**) and Tyr-Acc^5-Acc^5-Phe-Leu-NH_2 (**6**) have been characterized by 270 MHz ^{1}H NMR and reverse phase HPLC. Intramolecular hydrogen bonding patterns have been established from NMR studies in $(CD_3)_2SO$ solution. Peptides **1** and **4** adopt a β-turn structure centred at positions 2 and 3, while **2** and **5** favour β-turns at positions 3 and 4. The analogs **3** and **6** favour consecutive β-turn conformations.

Small, biologically active peptides generally adopt a wide range of conformations in solution. The structural flexibility of the peptide backbone, in acyclic sequences, renders difficult the search for 'biologically active conformations', which are recognized by physiological receptors[1]. The introduction of backbone stereochemical constraints offers a means of simplifying the problem. If, structurally defined, conformationally rigid analogs are shown to be biologically active, then the range of structures recognizable by the receptor are necessarily limited. One of the simplest ways of reducing peptide backbone flexibility is by introduction of alkyl groups at the C^α carbon atom[2]. α-Aminoisobutyryl (Aib) analogs (Fig. 1) have been used in several cases[3]. Earlier studies from this laboratory have demonstrated the utility of Aib substitution to generate appropriately folded analogs of Met^5-enkephalinamide[4-7] and chemotactic tripeptides[8]. There have been few reports on the use of 1-aminocycloalkane-1-carboxylic acids[3,9-11] (Acc^n; where n is the cycloalkane ring size, Fig. 1), in generating stereochemically constrained analogs of biologically active peptides. No systematic study of the conformations of peptides containing these residues has been reported, so far. In this paper we describe the synthesis of Aib and 1-aminocyclopentane-1-carboxylic acid (Acc^5, cycloleucine)[b] analogs of Leu^5-enkephalinamide and present PMR spectral evidence for the presence of tightly folded backbone conformations in these peptides.



(a) (b) (c)

Fig. 1—Structures of (a) Aib residue (b) cycloalkane amino acid residue (c) Acc^5 residue

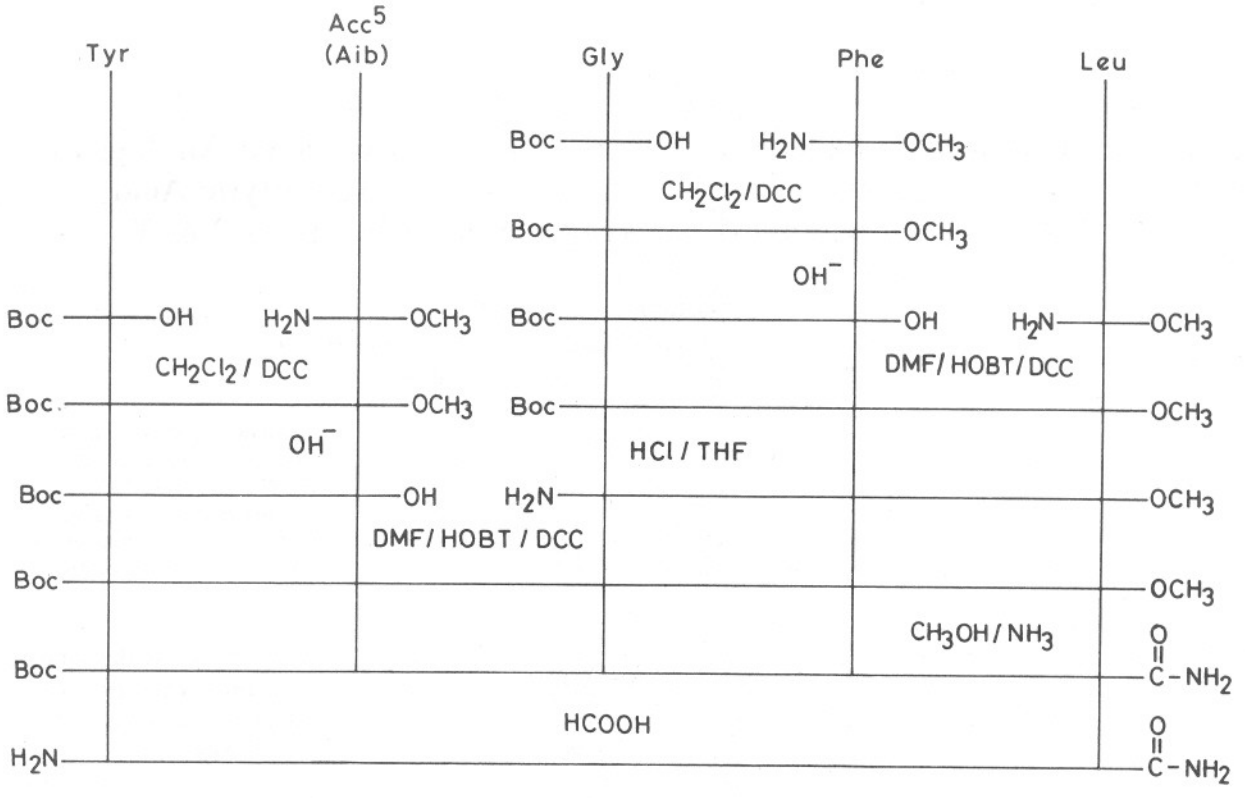
The Gly residues at positions 2 and 3 of Leu-enkephalin were individually and simultaneously replaced by Aib or Acc^5 residues. Peptides (**1-6**) were synthesized adopting 2+3 or 3+2 strategies, summarized in Schemes 1-4. The identity of the peptides was unambiguously established by 270 MHz PMR. Representative spectra for peptides **3** and **6** are illustrated in Fig. 2. The purity of the peptides was established by reverse phase HPLC and the chromatogram obtained for the Acc^5 analogs (**4-6**) are shown in Fig. 3 (see also Table 1). The synthesis of the analogs containing the sterically hindered Acc^5 residue proceed in good yields by N,N′-dicyclohexylcarbodiimide (DCC)-1-hydroxybenzotriazole (HOBT) mediated couplings.

PMR studies and assignment of resonances

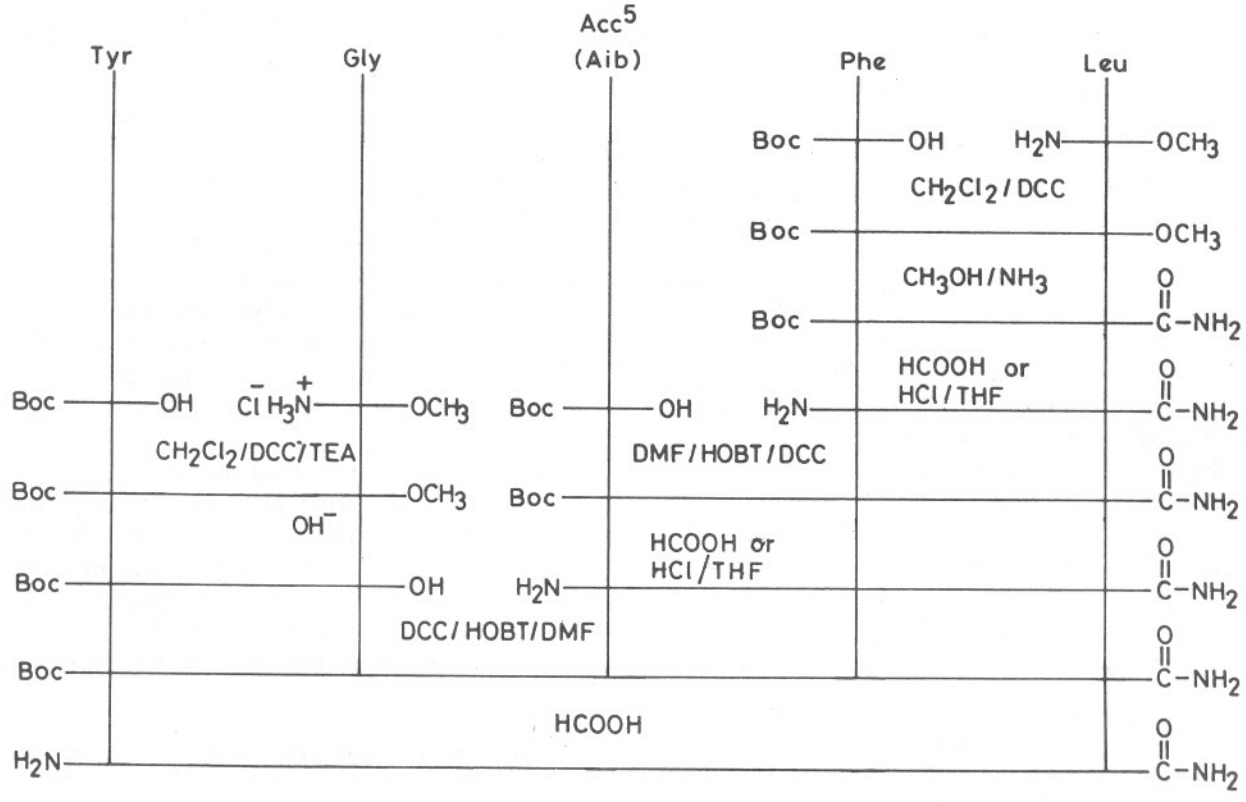
All six enkephalin analogs yield extremely well-resolved 270 MHz PMR spectra in $(CD_3)_2SO$. The amide NH resonances were assigned by a combination of decoupling and high temperature experiments. The doublet NH resonances due to the Phe and Leu residues could be assigned in the cases where there was

[a]Dedicated to Dr Nitya Anand on his 60th birth anniversary.

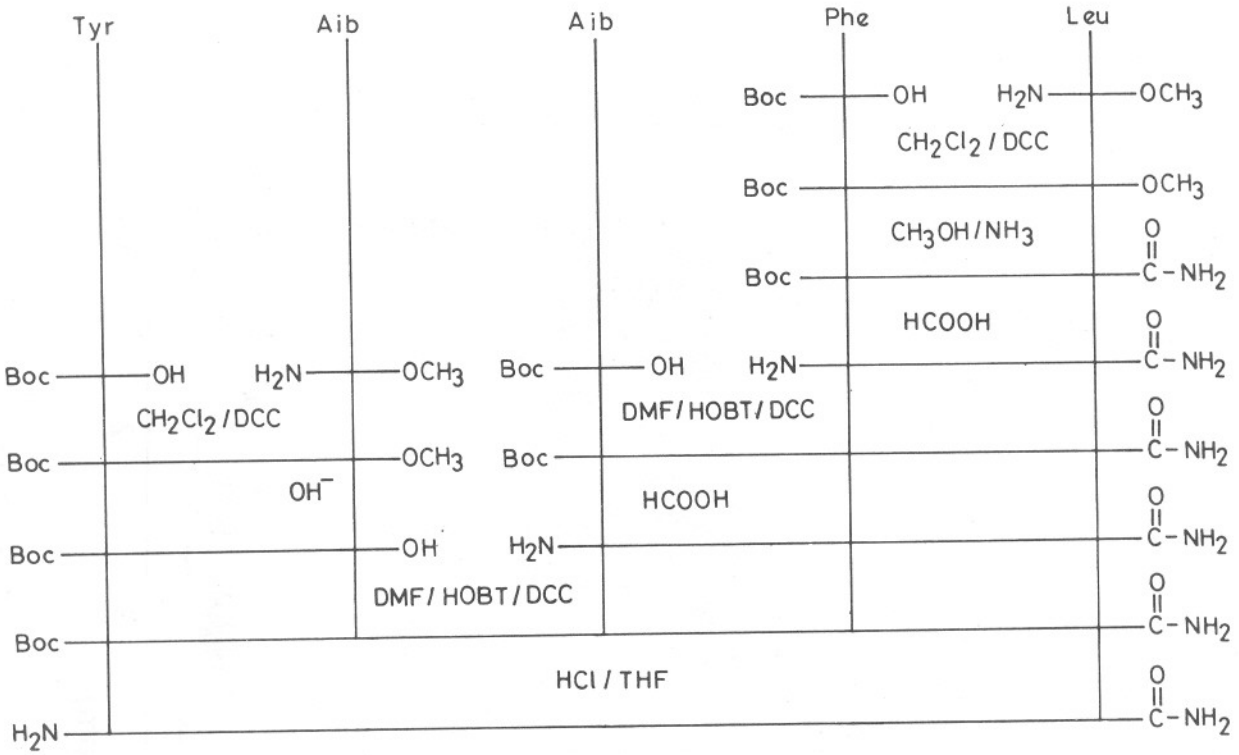
[b]1-amino-cyclopentane-1-carboxylic acid has been given the trivial name, cycloleucine and abbreviated as Cyl in the literature. We suggest the Acc^n nomenclature, in general, for 1,1-cycloalkane amino acids. This nomenclature was proposed by Prof. Claudio Toniolo, University of Padova, Italy.



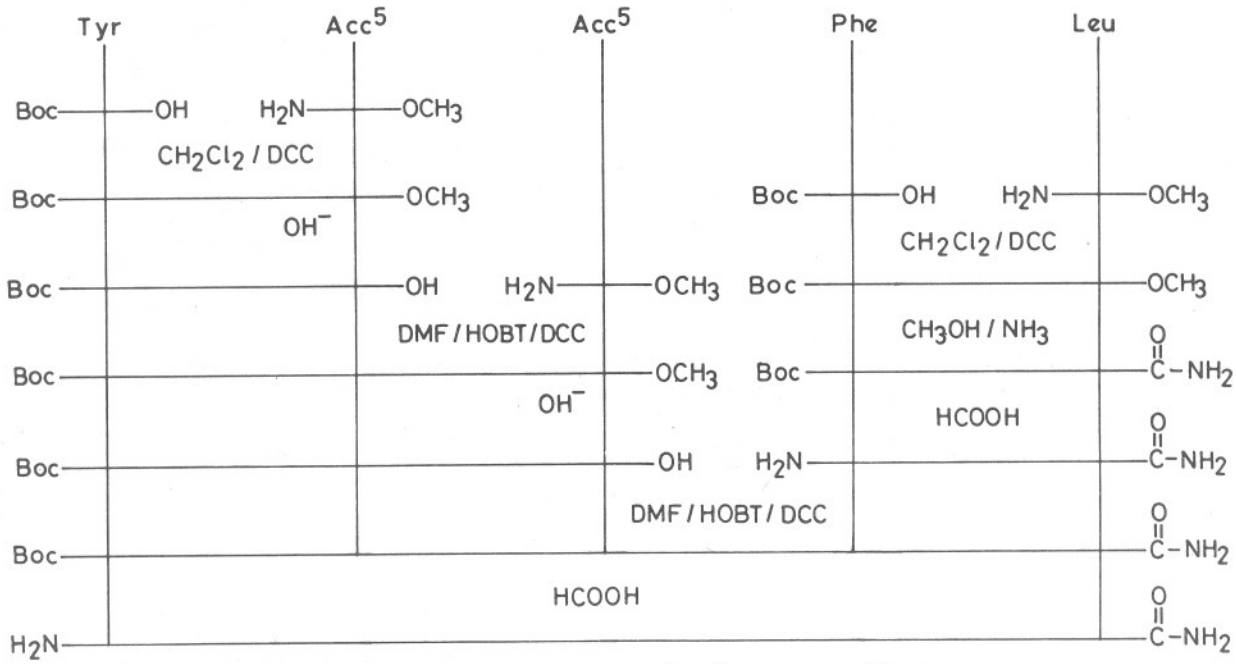
Scheme 1—Synthesis of Tyr-Aib-Gly-Phe-Leu-NH_2 (**1**) and Tyr-Acc^5-Gly-Phe-Leu-NH_2 (**4**)



Scheme 2—Synthesis of Tyr-Gly-Aib-Phe-Leu-NH_2 (**2**) and Tyr-Gly-Acc^5-Phe-Leu-NH_2 (**5**)



Scheme 3—Synthesis of Tyr-Aib-Aib-Phe-Leu-NH_2 (3)



Scheme 4—Synthesis of Tyr-Acc^5-Acc^5-Phe-Leu-NH_2 (6)

no overlap of C^{α}-H resonances. The Phe ($\sim 2.0\,\delta$) and Leu ($\sim 1.55\,\delta$) $C^{\beta}-H_2$ resonances could be unambiguously identified and decoupling experiments permitted the $C^{\beta}-H_2-C^{\alpha}-NH$ connectivity to be established. The terminal primary amide resonances (S_1 and S_2) were assigned to the singlets, which broaden and merge on heating; a characteristic feature of primary amides, due to speeding up of rotation about the C – N bond. The remaining singlet could be assigned to the Aib or Acc^5 residue at position-3. The Gly NH resonance was unequivocally recognized by its triplet nature.

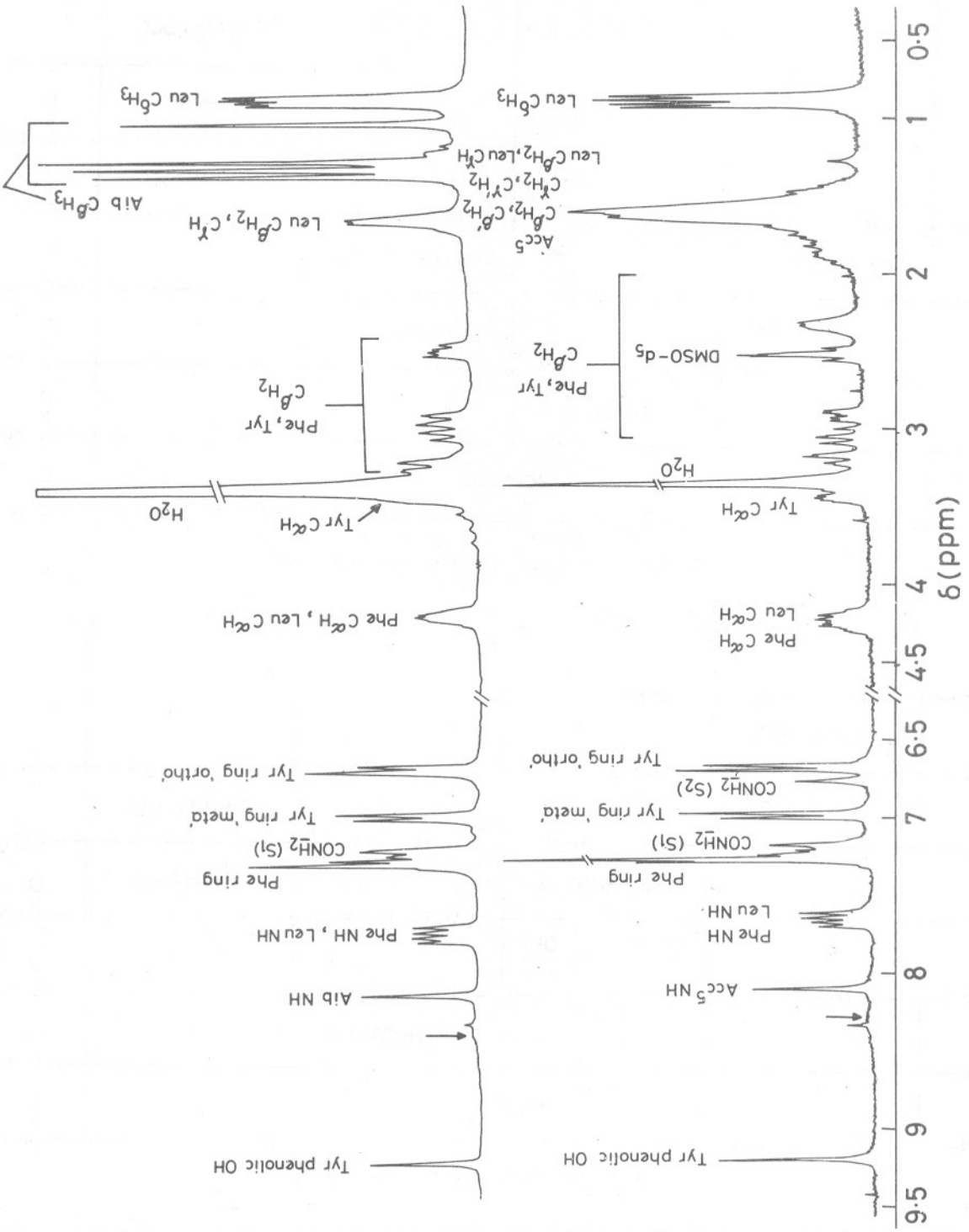


Fig. 2—270 MHz PMR spectra of Tyr-Aib-Aib-Phe-Leu-NH_2 (**3**) (top) and Tyr-Acc^5-Acc^5-Phe-Leu-NH_2 (**6**) (bottom) in $(CD_3)_2SO$

Interestingly, in all the six peptides one NH resonance appeared as a very broad peak, which was at times difficult to observe. A comparison of the spectra of the Aib-Gly, Gly-Aib, Acc^5-Gly and Gly-Acc^5 analogs revealed that the Gly NH appeared as a sharp triplet only when Gly was at position-3. It thus appears that the NH of residue-2 is broadened in all the peptides, suggesting that the N-terminal amino function may catalyze exchange of this proton with the water present in the solvent. This effect could, in principle, reflect a preference for conformations which place residue-2 NH proximate to the amino group. In the cases of peptides studies as free bases (**3-6**), the phenolic OH of Tyr was observed at $\sim 9.2\,\delta$, whereas in peptides (**1**, **2**), studied as acetate salts, this resonance was not seen due to rapid exchange with water.

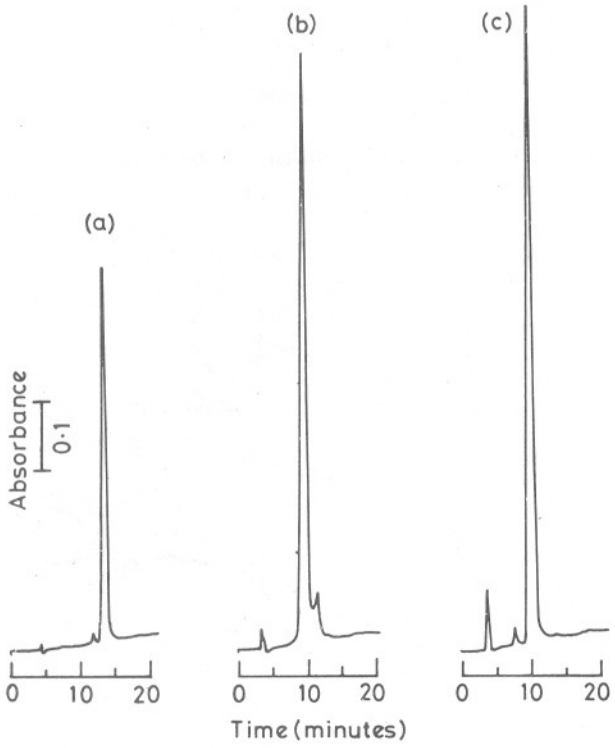


Fig. 3—HPLC of the Acc^5 analogs of Leu-enkephalinamide (a) -Acc^5-Gly-(**4**), (b) -Gly-Acc^5-(**5**) and (c) -Acc^5-Acc^5-(**6**) [Column Lichrosorb RP-18; gradient of 55-85% MeOH in H_2O containing 0.1% trifluoroacetic acid over 20 min; and flow rate 0.8 ml min^{-1}]

Delineation of intramolecularly hydrogen-bonded NH groups

The temperature dependence of NH chemical shifts in $(CD_3)_2SO$ was measured and the temperature coefficient ($d\delta/dT$) values are summarized in Table 2. The $d\delta/dT$ values can often be used as diagnostic for the involvement of NH groups in hydrogen bonds[12–14]. In general, low $d\delta/dT$ values (<0.003 ppm/K) are characteristic of solvent shielded and/or intramolecularly hydrogen-bonded NH groups. The $d\delta/dT$ values >0.0045 ppm/K are observed for free or solvent-exposed NH groups, in a polar, hydrogen bonding solvent like $(CD_3)_2SO$. $d\delta/dT$ values, which lie between 0.003 and 0.0045 ppm/K, have often been attributed to weak hydrogen bonds[15], although such an interpretation is fraught with uncertainty.

The data in Table 2 clearly indicate that the residue-3 NH group is solvent exposed ($d\delta/dT$ >0.0045 ppm/K) in all the peptides studied. The Phe NH is hydrogen-bonded in the Aib-Gly (**1**) and Acc^5-Gly (**4**) analogs, whereas Leu NH is hydrogen-bonded in the Gly-Aib (**2**) and Gly-Acc^5 (**5**) analogs ($d\delta/dT$

Table 1—Characterization of Leu-Enkephalinamide Analogs Tyr-X-Y-Phe-Leu-NH_2

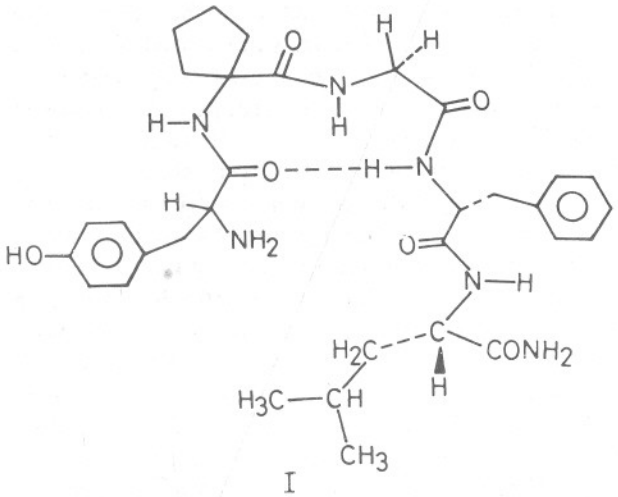
X – Y	m.p. (°C)	HPLC retention time (min)[a]	$[\alpha]_D^b$	Chemical shifts (δ, ppm)[c]
Aib-Gly	157	7.9	10.5	8.24, (*t*, 1H, Gly NH), 7.94 (*d*, 1H, Phe NH), 7.76 (*d*, 1H, Leu NH), 7.06 [*s*, 1H, $CONH_2$ (S_1)], 6.92 [*s*, 1H, $CONH_2$ (S_2)]
Gly-Aib	103	7.8	13.5	8.42 (*s*, 1H, Aib NH), 7.78 (*d*, 1H, Phe NH), 7.62 (*d*, 1H, Leu NH), 7.08 [*s*, 1H, $CONH_2$ (S_1)], 6.92 [*s*, 1H, $CONH_2$ (S_2)]
Aib-Aib	109	8.8	−10.5	9.23 (*s*, 1H, Tyr OH), 8.13 (*s*, 1H, Aib NH), 7.78 (*d*), 7.71 (*dd*, 2H, Phe and Leu NH), 7.2 [*s*, 1H, $CONH_2$ (S_1)]
Acc^5-Gly	126	9.2	1.5	9.22 (*s*, 1H, Tyr OH), 8.12 (*t*, 1H, Gly NH), 7.85 (*d*, 1H, Leu NH), 7.80 (*d*, 1H, Phe NH), 7.08 [*s*, 1H, $CONH_2$ (S_1)], 6.96 [*s*, 1H, $CONH_2$ (S_2)]
Gly-Acc^5	108	9.1	−1.5	9.25 (*s*, 1H, Tyr OH), 8.44 (*s*, 1H, Acc^5 NH), 7.78 (*d*, 1H, Phe NH), 7.57 (*d*, 1H, Leu NH), 7.07 [*s*, 1H, $CONH_2$ (S_1)], 6.96 [*s*, 1H, $CONH_2$ (S_2)]
Acc^5-Acc^5	82	13.2	27	9.19 (*s*, 1H, Tyr OH), 8.09 (*s*, 1H, Acc^5 NH), 7.67 (*d*, 1H, Phe NH), 7.61 (*d*, 1H, Leu NH), 7.15 [*s*, 1H, $CONH_2$ (S_1)], 6.74 [*s*, 1H, $CONH_2$ (S_2)]

(a) HPLC conditions are given in legend to Fig. 7.
(b) $[\alpha]_D$ measured at ambient temperature (~ 25°C); $C=0.33$ (MeOH).
(c) Only NH and OH chemical shifts are listed.

Table 2—Temperature Coefficients ($d\delta/dT$, ppm/K) of NH Resonances[a] in Enkephalin Analogs Tyr-X-Y-Phe-Leu-NH_2 in $(CD_3)_2SO$

Analog		Residue: 3	Phe	Leu	$-CONH_2$	
					S_1	S_2
-Aib-Gly-	(1)	0.0045	0.0019	0.0039	0.0051	0.0027
-Gly-Aib-	(2)	0.0059	0.0035	0.0018	0.0052	0.0027
-Aib-Aib-	(3)	0.0063	0.0020[b]	0.0023[b]	0.0066	c
-Acc^5-Gly-	(4)	0.0045	0.0018	0.0049	0.0059	0.0036
-Gly-Acc^5-	(5)	0.0059	0.0037	0.0019	0.0054	0.0025
-Acc^5-Acc^5-	(6)	0.0062	0.0019	0.0021	0.0075	0.0016

(a) In all peptides the residue-2 NH was very broad and not easily observed (See text).
(b) The C^a-H resonances overlap precluding specific NH assignments. However, the observation of similar, low, $d\delta/dT$ values suggests that both Phe and Leu NH groups are hydrogen-bonded.
(c) Not observed.



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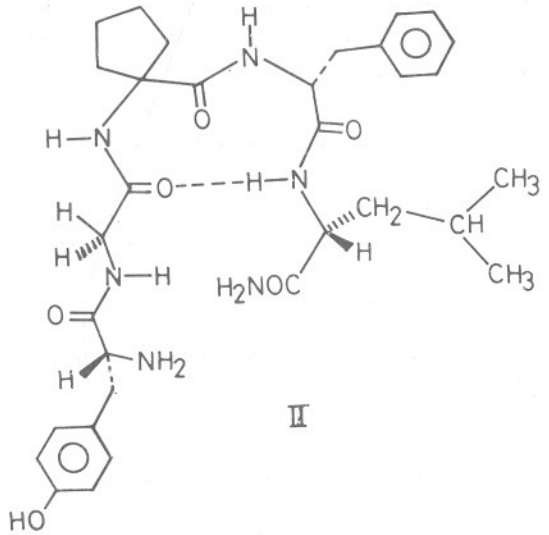

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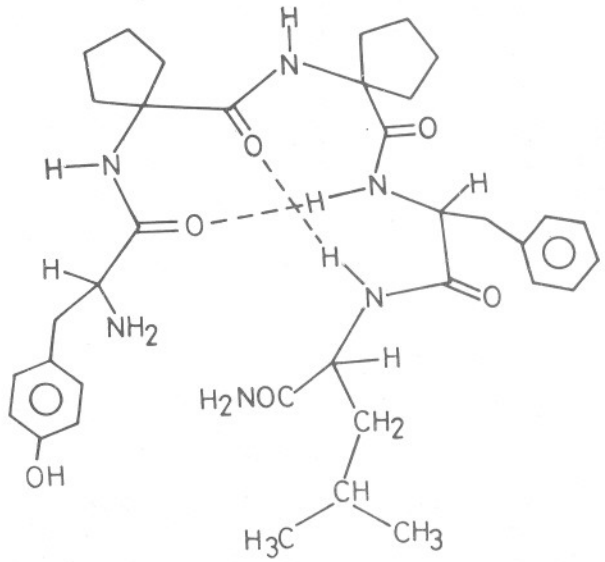

III

<0.003 ppm/K). Similarly both Phe and Leu NH are hydrogen-bonded in the Aib-Aib (**3**) and Acc^5-Acc^5 (**6**) analogs. The low $d\delta/dT$ value for one of the C-terminal primary amide resonances is not interpreted as indicative of hydrogen bonding. This is because exchange effects (rotation about the C–N bond) tend to shift this proton to low field, while increasing temperature would normally result in upfield shift of a solvent-exposed proton. The two opposing effects may result in a low $d\delta/dT$ value. The PMR results suggest that there is one intramolecular hydrogen bond in peptides **1**, **2**, **4** and **5** and two hydrogen bonds in **3** and **6**.

Aib residues have been shown to strongly promote β-turn formation[16-18], stabilized by $4\rightarrow1$ (C_{10}) hydrogen bonding in Aib-X sequences. A similar tendency may be expected for Acc^5 residues by considering molecular models and theoretical energy calculations (unpublished data). The similarity of the PMR data for the Aib and Acc^5 analogs supports this conclusion. The conformations consistent with the known stereochemical preferences of Aib residues and the observed PMR data are illustrated in structures (I-III); proposed β-turn conformation of peptides (**1** and **4**) is represented by structure(I) and that of **2** and **5** by structure(II). The consecutive β-turn (incipient 3_{10}-

helical) conformation[19] shown in III has, in fact, been observed for the protected tetrapeptide Boc-Aib-Aib-Phe-Met-NH_2, in the crystalline state by X-ray diffraction[20]. Similar folded conformations have been suggested from PMR studies of Aib analogs of Met-enkephalinamide[7]. Of the three crystal structures reported thus far for Leu-enkephalin[21,22] and a p-bromo-Phe derivative[23], in two cases a Type-I′ β-turn between residues 2 and 3 has been observed[22,23]. In the third structure, an extended peptide conformation is observed in the crystal[21]. The conformation of analogs **1** and **4** resemble the β-turn conformation of Leu-enkephalin in the crystalline state[22,23]. The possibility that enkephalins can adopt distinctly different conformational states is relevant in view of the existence of multiple opiate receptor sites, which appear to have different affinities for certain analogs[21,23]. The generation of various types of folded analogs using Aib or Acc^5 residues should allow studies of the strucutral requirements for interaction with specific types of receptor sites.

Experimental Procedures

Synthesis of peptides

All peptides were prepared by conventional solution phase procedures as outlined in Scheme 1-4. Representative procedures for preparation of Tyr-Acc^5-Acc^5-Phe-Leu-NH_2 are given below.

Boc-Tyr-Acc^5-OH

Boc-Tyr-OH (1.7 g, 6.05 mmol) in CH_2Cl_2 (20 ml) was cooled to 0°C and H-Acc^5-OMe (0.9 g, 6.3 mmol) was added, followed by DCC (1.4 g). The reaction mixture was stirred for 4 hr at 0°C and overnight at room temperature. The precipitated dicyclohexylurea (DCU) was filtered off and EtOAc (150 ml) added to the filtrate. The solution was washed successively with 2*N* HCl, 1*M* $NaHCO_3$ and H_2O. Drying and evaporation of the organic layer yielded Boc-Tyr-Acc^5-OMe as a white solid; yield 2.0 g (81%); m.p. 58°.

The above ester (2 g) was dissolved in MeOH (10 ml) and 2*N* NaOH (5.5 ml) added to it. After saponification was complete, as monitored by TLC, MeOH was evaporated and H_2O (60 ml) added. The aqueous solution was washed with ether and acidified with 2*N* HCl. Extraction with EtOAc followed by drying and evaporation of the organic layer gave Boc-Tyr-Acc^5-OH as a white solid; yield 1.4 g (72%); m.p. 167°.

Boc-Tyr-Acc^5-Acc^5-OH

Boc-Tyr-Acc^5-OH (2 g, 5.1 mmol) dissolved in dimethylformamide (DMF, 15 ml) was cooled to 0°C and to this was added H-Acc^5-OMe (0.7 g, 5 mmol) followed by HOBT (0.68 g) and DCC (1.1 g). The reaction mixture was stirred for 6 hr at 0°C and for 20 hr at room temperature. The DCU was filtered, DMF evaporated *in vacuo*, the residue taken up in EtOAc and washed successively with 2*N* HCl, 1*M* $NaHCO_3$ and H_2O. Drying and evaporation yielded 1.1 g (42%) of Boc-Tyr-Acc^5-Acc^5-OMe as a solid; yield 1.1 g (42%); m.p. 160°.

The tripeptide ester was saponified as described for the dipeptide to obtain the tripeptide acid as a solid; yield 0.9 g (84%); m.p. 99°.

Boc-Tyr-Acc^5-Acc^5-Phe-Leu-NH_2

Boc-Tyr-Acc^5-Acc^5-OH (0.9 g, 1.7 mmol), dissolved in DMF (5 ml), was cooled to 0°C and to this were added in succession H-Phe-Leu-NH_2[24] (0.5 g, 1.8 mmol), HOBT (0.28 g) and DCC (0.5 g). The mixture was stirred for 6 hr at 0°C, for 48 hr at room temperature and worked-up as described for the tripeptide ester. The crude product was chromatographed on a silica gel column using $CHCl_3$ – MeOH as the eluant to give the pentapeptide; yield 0.6 g (44%); m.p. 122°.

Tyr-Acc^5-Acc^5-Phe-Leu-NH_2 (**6**)

Boc-Tyr-Acc^5-Acc^5-Phe-Leu-NH_2 (0.5 g, 0.66 mmol) was dissolved in 98-100% formic acid (5 ml). After complete removal of the Boc group, as monitored by TLC, formic acid was evaporated and the residual oil dissolved in H_2O (40 ml). The aqueous solution was washed with ether and made alkaline with Na_2CO_3. Extraction with EtOAc (4 × 50 ml) followed by drying and evaporation gave **6** as a crude solid. This was purified by column chromatography (silica gel, 3% $CH_3OH - CHCl_3$) to give the pure peptide; yield 0.23 g (53%); m.p. 82°.

The reaction conditions and protocol used for the synthesis of peptides **1-5** were essentially similar to those described above. In all the cases intermediates were characterized by 60 MHz PMR and shown to have satisfactory purity by TLC on silica gel. The Aib-Aib (**3**), Acc^5-Gly (**4**) and Gly-Acc^5 (**5**) analogs were purified by silica gel chromatography. The Aib-Gly (**1**) and Gly-Aib (**2**) analogs were purified by gel filtration on a Sephadex G-10 column using 10% acetic acid as eluant. The peptides **1** and **2** were obtained as acetate salts after lyophilization of the column fractions.

Characterization of peptides

All six enkephalin analogs were characterized by 270 MHz PMR. Spectra were recorded on a Bruker WH-270 FT-NMR spectrometer, at the Sophisticated Instruments Facility, Bangalore. HPLC analyses were performed on an LKB-HPLC system using a Lichrosorb RP-18 column and absorbance detection at 226 nm.

Acknowledgement

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