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X-ray diffraction studies of enkephalins

Crystal structure of [(4′-bromo)Phe⁴,Leu⁵]enkephalin

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In order to investigate the structure–activity relationship of [Leu⁵]- and [Met⁵]enkephalins, [(4′-bromo)Phe⁴,Leu⁵]-, [(4′-bromo)Phe⁴,Met⁵]- and [Met⁵]enkephalins were synthesized and crystallized. The crystal structure of [(4′-bromo)Phe⁴,Leu⁵]-enkephalin was determined by X-ray diffraction method using the heavy atom method and refined to $R = 0.092$ by the least-squares method. The molecule in this crystal took essentially the same type I′ β-turn conformation found in [Leu⁵]enkephalin [Smith & Griffin (1978) *Science* **199**, 1214–1216). On the other hand, the preliminary three-dimensional Patterson analyses showed that the most probable conformations of [(4′-bromo)Phe⁴,Met⁵]- and [Met⁵]enkephalins are both the dimeric extended forms. Based on these insights, the biologically active conformation of enkephalin was discussed in relation to the μ- and δ-receptors.

The discovery of two naturally occurring pentapeptides (enkephalins) with morphine-like action (Hughes *et al.*, 1975*a*) has induced numerous physicochemical studies in order to find the possible structural similarity between morphine and enkephalins. The primary structures of these peptides are H-L-Tyr-Gly-Gly-L-Phe-L-Met-OH ([Met⁵]enkephalin) and H-L-Tyr-Gly-Gly-L-Phe-L-Leu-OH ([Leu⁵]enkephalin). Since the enkephalins compete with morphine for binding to receptor, but these bindings are inhibited by morphine antagonists such as naloxone, it is likely that the enkephalins may bind to the same stereospecific receptor as does morphine or the other opiates (Hughes *et al.*, 1975*b*; Simantov & Snyder, 1976; Bradbury *et al.*, 1976; Horn & Rodgers, 1976). The comparison of the biologically active structures between opiates and enkephalins is therefore of especial importance to elucidate the mechanism of action at the opiate receptor site.

A great number of enkephalin analogues have been synthesized (Terenius *et al.*, 1976; Morgan *et al.*, 1976; Chang *et al.*, 1976; Ling & Guillemin, 1976; Pert *et al.*, 1976; Roemer *et al.*, 1977) and the establishment of structure–activity relationships has become important and essential for studies of biological activity. Thus, in the enkephalin sequence, the L-Tyr¹, Gly³ and L-Phe⁴ residues cannot be changed without significant loss of activity, while Gly² could be replaced by a D-amino acid, and the nature of the *C*-terminal residue is less critical for biological activity.

On the other hand, the preferential conformations of enkephalins have been investigated by n.m.r. (Roques *et al.*, 1976; Garbay-Jaureguiberry *et al.*, 1977; Bleich *et al.*, 1977; Combrisson *et al.*, 1976; Jones *et al.*, 1977; Stimson *et al.*, 1979), c.d. (Khaled *et al.*, 1977; Sudha & Balaram, 1981), Raman (Han *et al.*, 1980) and X-ray diffraction (Smith & Griffin, 1978) spectroscopies and by conformational energy calculations (DeCoen *et al.*, 1977; Isogai *et al.*, 1977; Momany, 1977; Balodis *et al.*, 1978; Loew & Burt, 1978). In general, these studies indicate that enkephalin takes a folded β-turn conformation not only in solution but also in crystal. However, there are some diversities in the nature of the conformation, especially in the location and type of bend in the backbone or in the extent of motional freedom of the side chain.

Moreover, recent n.m.r. studies (Zetta & Cabassi, 1982; Higashijima *et al.*, 1979; Fischman *et al.*, 1978; Bundi & Wüthrich, 1979; Garbay-Jaureguiberry *et al.*, 1980; Miyazawa & Higashi-

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jima, 1981) gave evidence that the flexible enkephalin molecule may exist as a dynamic equilibrium of nearly iso-energetical folded and extended conformers. A further complexity of the active conformation of enkephalin has recently been introduced by the findings that two or three different receptors (designated as μ, κ and δ) or multiple forms of the same receptor are involved in interaction with the enkephalins (Martin *et al.*, 1976; Lord *et al.*, 1977; Lemaire *et al.*, 1978; Chang & Cuatrecasas, 1979; Chang *et al.*, 1980). Binding studies of radiolabelled opiates and enkephalins to brain synaptic membranes or to the neuroblastoma glioma cell line suggest that the morphine binding site is not identical with that of the endogenous opiate ligands.

In view of these complicated circumstances it seems particularly important to perform the detailed structural analyses of enkephalins in the solid state. Even though the conformation obtained by X-ray analysis may not be the biologically active form, it certainly provides a foundation for further developing the structure–function correlation by various methods.

Preliminary X-ray analysis of [Leu^5]enkephalin was already reported by Smith & Griffin (1978). It is of interest for us to investigate whether the slightly modified [Leu^5]enkephalin takes the same conformation as the native one or not, because of the flexible conformation of this linear peptide as already stated. Therefore we synthesized the enkephalin and its analogues, and in this paper, we report the detailed X-ray result of [(4′-bromo)Phe^4,Leu^5]enkaphalin together with the preliminary X-ray crystal data of [Met^5]- and [(4′-bromo)Phe^4,Met^5]enkephalins.

Materials and methods

Synthetic methods

4′-Bromo-L-phenylalanine was obtained by the reaction of L-phenylalanine with bromine followed by fractionation on a column of Sephadex LH-20 [4cm × 100cm; elution with methanol/water (1 : 1, v/v)] according to the previous report (Faulstich *et al.*, 1973). The purity was verified by optical rotation ($[\alpha]_{D,H_2O}^{20} = -19°$) and ^{1}H-n.m.r. spectroscopy. [(4′-Bromo)Phe^4,Leu^5]enkephalin was synthesized by standard liquid phase methods according to the general scheme shown in Fig. 1. [Met^5]- and [(4′-bromo)Phe^4,Met^5]enkephalins were also synthesized by the same strategy. All deprotected pentapeptides were purified by gel filtration. Approx. 200mg of crude product dissolved in water was applied to a Sephadex LH-20 column (2cm × 100cm) and elution (50ml/h) was performed with the system methanol/acetic acid/water (4:1:5, by vol.). Purity was checked by

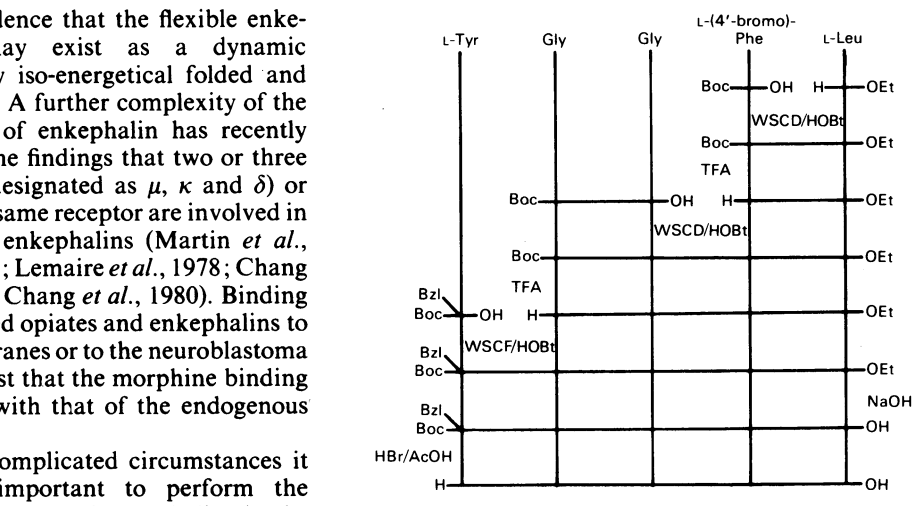

Fig. 1. *Synthetic strategy of [(4′-bromo)Phe^4,Leu^5]-enkephalin*

Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; WSCD, 1-ethyl-3-(3-dimethylaminopropyl)-carbodi-imide; HOBt, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid; AcOH, acetic acid.

h.p.l.c. on a Lichrosorb-NH_2 column [Merck; 0.4cm × 25cm; solvent acetonitrile/water (3:1, v/v)].

Crystallization and data collection

Details of crystallization, cell parameters and X-ray data collection are given in Table 1. The crystals were obtained by the slow evaporation of each solution (0.2M) in a temperature-controlled room (20 ± 1°C) over 1 week, respectively. The number of molecules of water of crystallization was determined by elemental and thermal analyses. A single crystal of each enkephalin was sealed in a glass capillary with some mother liquor to prevent the concomitant loss of resolved X-ray diffraction patterns. The crystal system and space group of each crystal were determined from the symmetries and systematic absences of X-ray diffraction peaks by means of oscillation and Weissenberg photographs. The lattice parameters were obtained from a least-squares fit of 2θ values of 35 high-angle reflections ($40° < 2\theta < 60°$) recorded on a Rigaku automatic four-circle diffractometer with graphite-monochromated CU K_α radiation [$\lambda = 1.5418$Å (0.15418nm)]. Intensities for the three compounds were collected with the same diffractometer at 50kV and 180mA (temperature 18 ± 2°C). The intensities of four standard reflections showed no deterioration of each crystal during the data collection. Corrections were made for Lorentz-polarization effects. Absorption corrections by the

Table 1. *Summary of crystallization and data collection*

	[(4′-Bromo)Phe⁴,Leu⁵]enkephalin	[Met⁵]enkephalin	[(4′-Bromo)Phe⁴,Met⁵]enkephalin
Molecular formula	$C_{28}H_{36}N_5O_7Br$	$C_{27}H_{35}N_5O_7S$	$C_{27}H_{34}N_5O_7SBr$
Molecular weight	634.5	573.7	652.6
Solvents for crystallization	Ethanol/water (1:1, v/v)	Water	Water
Crystal shape	Plate	Tabular	Tabular
Waters of crystallization	2.5	5	4
Crystal system	Monoclinic	Monoclinic	Triclinic
Space group	C2	$P2_1$	P1
a (Å)	31.577(16)	16.481(3)	11.553(2)
b (Å)	8.631(3)	17.892(2)	11.581(2)
c (Å)	12.576(4)	11.595(1)	12.944(2)
α (°)	90	90	93.77(1)
β (°)	97.74(4)	91.23(1)	95.96(1)
γ (°)	90	90	86.96(1)
V (Å^3)	3396.3(23)	3418.2(9)	1716.9(4)
Z	4	4	2
F (0 0 0)	1420	1416	756
ρ (calc.) (g/cm^3)	1.329	1.290	1.402
ρ (obs.) (g/cm^3) by flotation in CCl_4/C_6H_6 mixture	1.329(1)	1.285(2)	1.405(1)
Index range measured	± 37, 10, 14	19, 21, ± 13	± 13, 13, ± 15
Scan type	$\omega - 2\theta$	$\omega - 2\theta$	$\omega - 2\theta$
θ range (°)	1–65	1–65	1–65
Scan speed (2θ) (°/min)	2	2	2
Scan width (2θ) (°)	1.45	1.50	1.20
Background time(s) at both sides of peak	5	5	5
Standard reflections	4 every 50 reflections	4 every 50 reflections	4 every 50 reflections
Collected reflections	3113	6012	5908
Non-zero reflections	2787	4406	5349
Crystal size (mm)	$\sim 0.2 \times 0.5 \times 0.3$	$\sim 0.2 \times 0.2 \times 0.3$	$\sim 0.4 \times 0.4 \times 0.3$
Absorption coefficient (cm^{-1})	21.14	13.55	27.10

Furnas (1957) method were also applied for each crystal, because of the large linear absorption coefficient and the use of a glass capillary.

Structure determination and refinement of [(4′-bromo)Phe⁴,Leu⁵]enkephalin

Among the crystal data listed in Table 1, we first tried to determine the crystal structure of [(4′-bromo)Phe⁴,Leu⁵]enkephalin, because the space group and density of this crystal showed that there is one crystallographically independent molecule in the asymmetric unit. The almost identical lattice parameters with those of [Leu⁵]enkephalin [space group C2, $a = 31.871$, $b = 8.535$, $c = 12.467$ Å; $\beta = 96.53°$; $Z = 4$ (Smith & Griffin, 1978)] imply that the molecular packing modes are almost the same as each other. The bromine atom position was determined by a Patterson synthesis. Successive Fourier refinements using normalized structure factors as coefficients revealed the locations of the peptide backbone and the Phe⁴ and Leu⁵ side chains. At this stage, it was found that the phenol ring of the Tyr¹ residue was in disorder and occupied two discrete positions with nearly equal weight, similar to that of [Leu⁵]enkephalin. The occupancies for these disordered atoms were therefore taken as half, respectively. Difference Fourier synthesis was used to locate the two oxygen atoms out of the 2.5 water molecules. The structure was refined by the full-matrix least-squares method with isotropic temperature factors and then by block-diagonal least-squares with anisotropic factors. The disordered atoms were simply refined with isotropic ones. Some hydrogen atoms were not resolved in difference Fourier syntheses. Hence, their idealized positions were calculated and kept fixed during the refinement. The final R index $[\Sigma||F_o| - k|F_c||/\Sigma|F_o|]$ was 0.092 for all observed reflections and 0.085 for 2787 non-zero reflections, where k is the scale factor. The minimized function was $\Sigma w(|F_o| - k|F_c|)^2$ with $w = [\sigma^2(F_o) + 0.25709 \quad |F_o| + 0.00658|F_o|^2]^{-1}$, where $\sigma(F_o)$ is the standard deviation based on counting statistics. The atomic and anomalous scattering factors were taken from Cromer & Waber (1974). All the numerical calculations were carried out at the Crystallographic Research Center, Institute for Protein Research, and at the Computing Center of Osaka University, using the programs of 'The Universal Crystallographic Computing System – Osaka (1979)'. The observed and calculated structure factors, and anisotropic temperature factors for non-hydrogen atoms have been deposited as Supplementary Publication SUP 50124 (17 pages) with the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained as indicated in *Biochem. J.* (1984) **217**, 5.

The final positional and isotropic thermal parameters of non-hydrogen atoms are given in Table 2.

Results and discussion

[(4′-Bromo)Phe⁴,Leu⁵]enkephalin takes essentially the same β-bend conformation as found in [Leu⁵]enkephalin

Fig. 2 shows the molecular conformation of [(4′-bromo)Phe⁴,Leu⁵]enkephalin. The molecule takes the type-I′ β-turn structure with the Gly²-Gly³ residues at the corners of the bend. The torsion angles defining the backbone and side chain conformations are listed in Table 3, in which those of [Leu⁵]enkaphalin are also included for comparison. No noteworthy discrepancy is observed between their torsion angles: the maximum deviation is 14° for χ_2 of the Leu⁵ residue. Therefore it should be pointed out that the conformation observed here is essentially same as that of [Leu⁵]enkephalin.

This β-turn conformation is mainly stabilized by two intramolecular hydrogen bondings formed between the Tyr¹ and Phe⁴ residues: N1(Tyr)–O4(Phe) = 2.80(1) Å and O1(Tyr)–N4(Phe) = 2.987(8) Å. Many further short contacts, less than 3.1 Å, are found between the intramolecular nitrogen and oxygen atoms. These are listed in Table 4. On the other hand, we previously reported the crystal structure of Boc-Gly-Gly-L-Phe-OEt (Ishida *et al.*, 1983), and it was confirmed that the molecule adopts the type-I′ β-turn conformation at Gly-Gly residues, i.e. similar to the conformation of the brominated and native [Leu⁵]enkephalin molecules studied here. However, this bend conformation in Boc-Gly-Gly-L-Phe-OEt did not involve any intramolecular hydrogen bonding. Therefore, it appears that the Gly-Gly-L-Phe sequence can favour the β-turn conformation intrinsically. The formation of the intramolecular hydrogen bonds and short contacts may stabilize this conformation in the crystalline state, although multiple low-energy conformers of the enkephalins exist in solution (Khaled *et al.*, 1977; Manavalan & Momany, 1981; Maigret & Premilat, 1982).

Similar to [Leu⁵]enkephalin, the two aromatic side chains were in the outside position of the peptide backbone chain. The tyrosine residue was disordered around the C^α–C^β bond. The dihedral angle between these disordered tyrosine rings is 38(1)°, and the separation distance between phenolic oxygen atoms is 2.53(2) Å. The planarity of the benzene ring of Phe⁴ is comparable with phenylalanine derivatives (r.m.s. = 0.013 Å) and the bromine atom lies on the plane with a fluctuation of 0.09(2) Å. This plane makes angles of 19(1) and 54(1)° with the disordered tyrosine planes respec-

Table 2. *Atomic co-ordinates of non-hydrogen atoms* ($\times 10^4$) *with estimated standard deviations in parentheses*

Atom	x	y	z	$B_{eq.}$*
N1	3749(2)	1004(8)	8679(5)	5.1
C1	3601(2)	298(10)	9622(5)	4.8
C11	3734(3)	1266(21)	10626(7)	9.0
C12†	4199(5)	2018(21)	10687(11)	5.3
C13	4357(8)	3421(23)	10322(20)	8.3
C14	4784(6)	3794(27)	10425(28)	10.1
C15	5079(5)	2757(24)	10949(12)	5.9
O15	5495(4)	3068(17)	11073(11)	6.8
C16	4953(6)	1474(32)	11331(19)	8.4
C17	4502(7)	1094(31)	11132(19)	8.3
C12′	4182(4)	842(22)	11085(11)	5.2
C13′	4420(5)	−561(22)	11088(13)	6.0
C14′	4844(5)	−709(23)	11465(13)	6.1
C15′	5070(4)	698(22)	11830(13)	5.5
O15′	5485(4)	650(17)	12207(11)	7.1
C16′	4841(6)	2055(26)	11888(18)	7.1
C17′	4427(6)	2149(21)	11488(13)	6.1
C1′	3119(2)	199(8)	9417(4)	4.1
O1	2911(2)	1381(6)	9174(3)	4.6
N2	2944(2)	−1196(7)	9492(4)	4.8
C2	2488(3)	−1314(11)	9331(6)	6.1
C2′	2268(2)	−759(9)	8251(5)	4.9
O2	1909(2)	−312(9)	8160(4)	6.4
N3	2507(2)	−838(7)	7442(4)	4.5
C3	2333(3)	−329(8)	6375(5)	5.1
C3′	2429(2)	1369(8)	6102(5)	4.0
O3	2322(2)	1819(6)	5177(3)	5.4
N4	2624(2)	2232(6)	6888(4)	4.1
C4	2688(2)	3910(7)	6758(5)	4.3
C41	2412(3)	4807(9)	7428(6)	5.5
C42	1948(3)	4693(10)	7021(6)	5.6
C43	1687(3)	3590(13)	7436(8)	7.0
C44	1262(3)	3521(17)	7062(9)	8.3
C45	1096(3)	4475(17)	6293(9)	8.1
Br	496(1)	4453(2)	5773(1)	9.0
C46	1341(4)	5549(17)	5815(12)	9.5
C47	1765(4)	5624(14)	6202(10)	8.2
C4′	3150(2)	4352(9)	7075(5)	4.7
O4	3366(2)	3753(10)	7865(6)	8.5
N5	3290(2)	5520(7)	6545(5)	4.9
C5	3713(2)	6152(10)	6787(6)	5.3
C51	3924(3)	6249(12)	5739(7)	6.5
C52	4032(4)	4633(15)	5337(8)	7.6
C53	4114(5)	4716(25)	4153(10)	11.0
C54	4388(6)	3946(28)	6048(13)	13.2
C5′	3723(3)	7770(12)	7328(7)	6.7
O51	3392(3)	8504(9)	7291(5)	7.9
O52	4068(3)	8199(15)	7778(10)	13.5
O1W‡	3394(3)	6027(11)	9992(7)	9.6
O2W	5693(5)	5928(15)	10363(11)	13.6

* $B_{eq.}$ values excluding the Tyr aromatic ring were calculated from anisotropic temperature factors (B_{ij}) by using $B_{eq.} = 4/3\ (B_{11}a^2 + B_{22}b^2 + B_{33}c^2 + acB_{13} \cdot \cos\beta)$.

† C12∼C17 and C12′∼C17′ atoms are the disordered moiety of the Tyr residue.

‡ W, water of crystallization.

tively. The side chain conformations of Tyr[1], Phe[4] and Leu[5] appear to be all stable forms because similar stable conformations of [Leu[5]]enkephalin have been observed in solution (Stimson *et al.*, 1979) and in related peptides in the crystal form (Sasisekharan & Ponnuswamy, 1971; Bhat *et al.*, 1979). All the peptide groups in the molecule are *trans* and nearly planar with 7.1(6)° as the

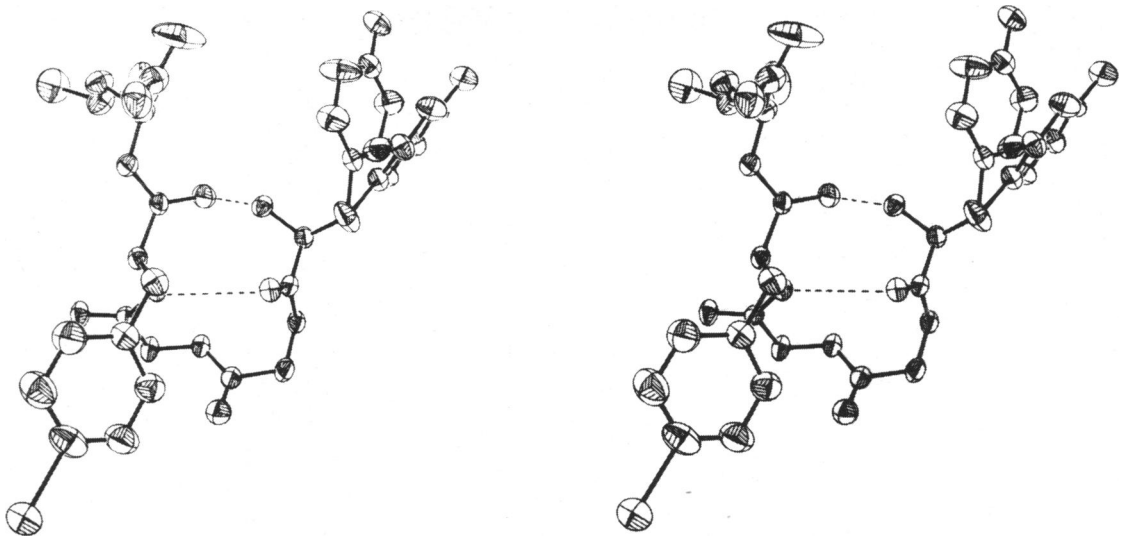

Fig. 2. *Stereoscopic view of the observed conformation of* [(*4'-bromo*)*Phe*^{4},*Leu*^{5}]*enkephalin showing type-I' β-turn structure* The broken lines represent possible intramolecular hydrogen bonds.

Table 3. *Comparison of conformational angles* (°) *between* [(*4-bromo*)*Phe*^{4},*Leu*^{5}]- *and* [*Leu*^{5}]*enkephalins* The notation of torsion angles for respective amino acid residues are in accordance with the IUPAC–IUB Commission on Biochemical Nomenclature (1971).

	[(4-Bromo)Phe^4,Leu^5]enkephalin	[Leu^5]enkephalin
Tyr^1		
ψ	123.9(7)	126
ω	178.2(7)	173
χ_1	−38(2), −84(1)	−44, −86
χ_2	−89(2), −31(3)	−89, −30
Gly^2		
ϕ	59(1)	59
ψ	27(1)	25
ω	−179.5(7)	179
Gly^3		
ϕ	93.6(8)	97
ψ	−6(1)	−7
ω	−172.9(6)	−174
Phe^4		
ϕ	−127.8(7)	−136
ψ	147.9(6)	145
ω	176.1(7)	180
χ_1	−69.4(8)	−62
χ_2	95(1)	90
Leu^5		
ϕ	−108.0(9)	−105
ψ	−16(1)	−4
χ_1	−70(1)	−69
χ_2	164(1)	178

maximum value of $\Delta\omega$ at the Gly-Phe peptide bond.

The molecule exists as a zwitterion form with tyrosine NH_3^+ group and a terminal CO_2^- carboxylate group. These polar atoms participated in many intermolecular hydrogen bond formations with the neighbouring polar atoms of enkephalin and water molecules (discussed later).

Table 4. *Intramolecular hydrogen bonds and short contacts*

Hydrogen bond (D-H—A)

Atom (D)	Atom (A)	Distance (Å) D—H	Distance (Å) H—A	Angle (°) D-H—A
N1	O4	2.795(11)	1.95(16)	175(16)
N4	O1	2.987(8)	2.05(14)	160(12)

Short contact

Atom	Atom	Distance (Å)
N1	O1	2.821(8)
N3	O1	3.049(8)
O1	O4	3.100(10)
N3	N2	2.773(9)
N4	N3	2.777(8)
N4	O4	2.819(10)
N5	O51	2.745(11)

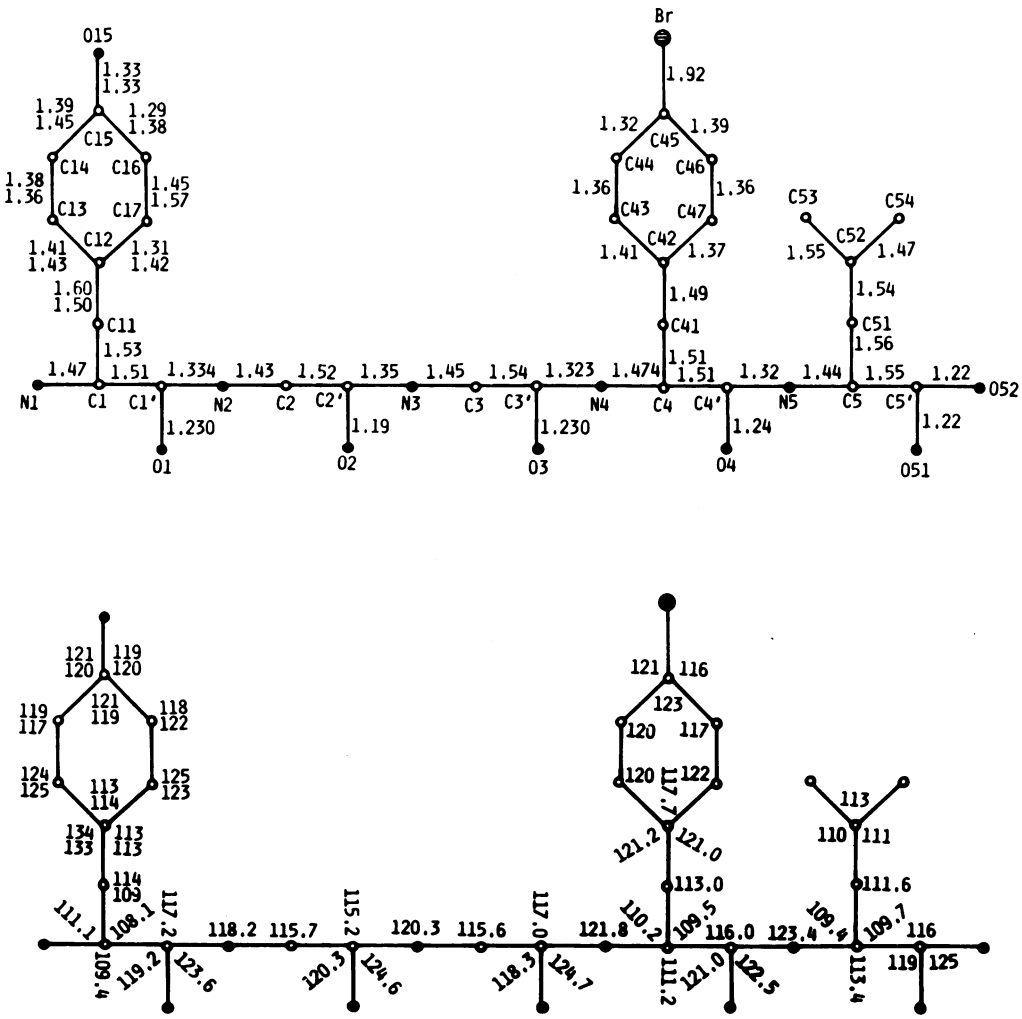

Fig. 3. *Bond lengths and angles of* [(*4'-bromo*)*Phe*^{4},*Leu*^{5}]*enkephalin*
The estimated standard deviations range from 0.008 to 0.03 Å (Tyr^1 moiety: 0.03–0.04 Å) for bond lengths and from 0.6 to 1.5° (Tyr^1 moiety: 1.1–2.4°) for bond angles, respectively.

Bond distances and angles

The bond distances and angles of [(4′-bromo)-Phe^4, Leu^5]enkephalin are given in Fig. 3. As far as the main backbone chain is concerned, the bond lengths and angles are quite close to those found in other peptides (Marsh & Donohue, 1967), and are all within the accepted values (Kennard, 1968). The following are averaged values in the present case: Cα–C′ = 1.53, C′–O = 1.22, C′–N = 1.33, N–Cα = 1.45 Å; Cα–C′–O = 120.0, Cα–C′–N = 116.3, O–C′–N = 123.9, C′–N–Cα = 120.9°. The bond lengths and angles in the Tyr side chain are not precisely determined because of disordering. Since the thermal motions of the Phe^4 and Leu^5 side chain atoms are relatively high compared with those of main chain atoms, the bond lengths and angles of these hydrophobic chains have rather high standard deviations, but the quoted values appear to be normal within their estimated standard deviations. In the CO_2^- carboxylate group, both the C–O bonds are nearly the same as each other, implying the delocalization of the π-electron on the group.

[(4′-Bromo)Phe^4,Leu^5]enkephalins form a rigid dimeric column by intermolecular hydrogen bond formation

Fig. 4 shows a stereodrawing of the crystal packing viewed along the *b*-axis. There is no large cavity in the unit cell, and the aromatic rings of the Tyr^1 and Phe^4 residues exposed to the outside of the β-turn backbone chain are nearly on the (2 0 1) plane. The molecules are stabilized by extensive hydrogen bonds and short contacts with the neighbouring enkephalin and water molecules. The details of hydrogen bonds are given in Table 5, in which the hydrogen bonds that the disordered O15 and O15′ and water oxygen atoms participate in, as donor, were inferred from the distances between donor (D) and acceptor (A) atoms and the angles of C (Carbon)-D—A and/or D—A-C, because of the lack of clear-cut appearance of hydrogen atoms attached to the respective donor atoms. Fig. 5 shows the possible hydrogen bonding modes formed in the crystal.

As shown in Fig. 5, the essential feature of the molecular packing is that the enkephalins related

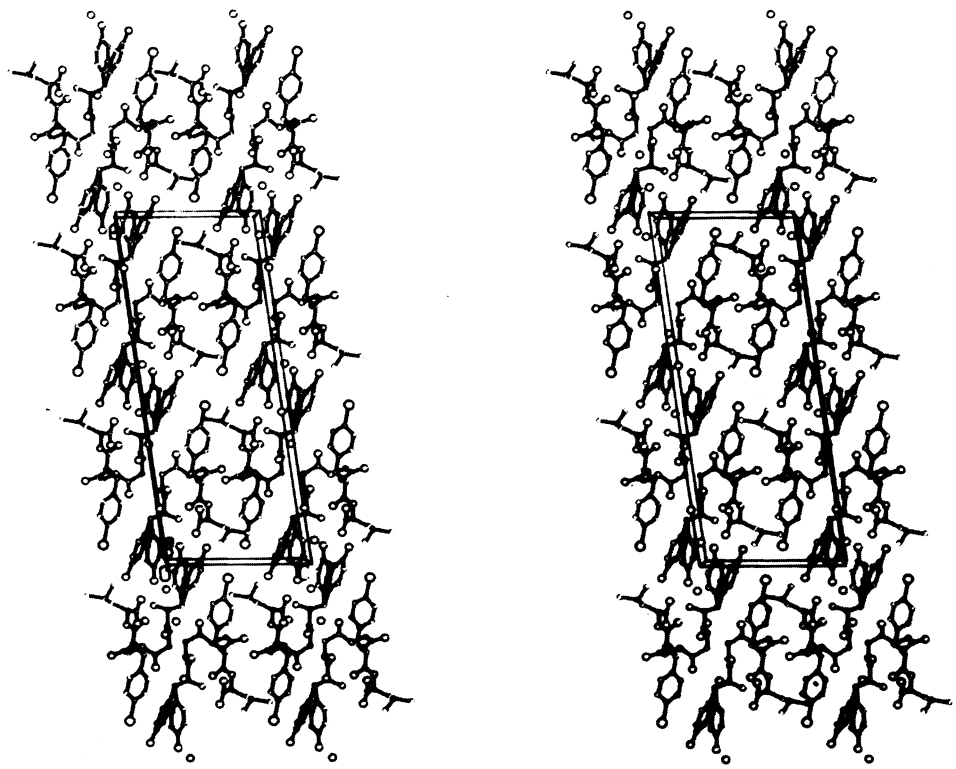

Fig. 4. *Stereoscopic view of [(4′-bromo)Phe^4,Leu^5]enkephalin crystal packing, viewed along the b-axis (vertical, a-axis; horizontal, c-axis)*

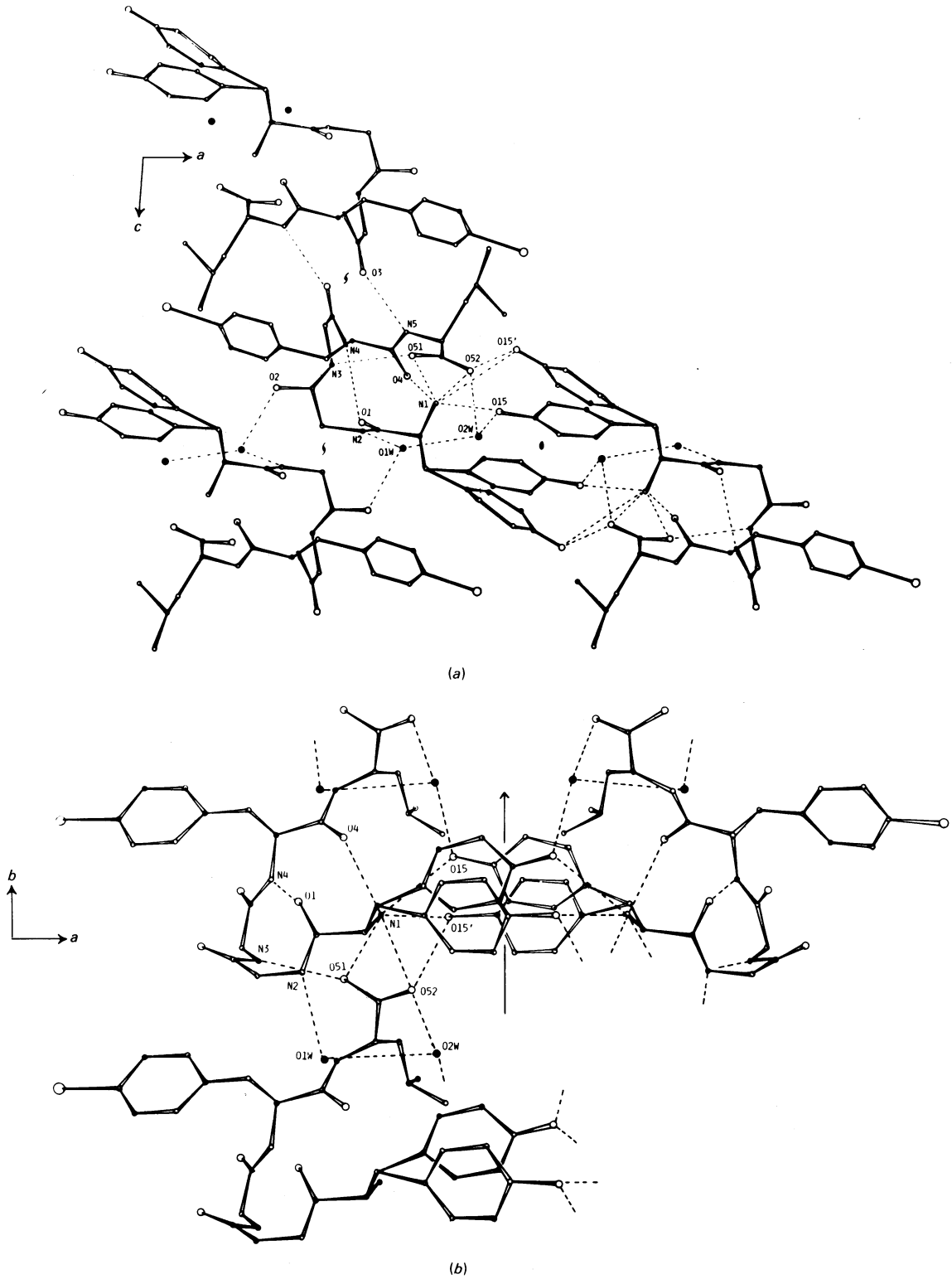

Fig. 5. *Hydrogen bonding networks formed among the neighbouring molecules*
The filled circles represent water molecules. The possible hydrogen bonds are shown by the broken lines. (*a*) Viewed along *b*-axis; (*b*) viewed along *c*-axis.

by twofold symmetry form a dimer by the hydrogen bonds of N1(Tyr)–O15(Tyr) [2.96(2)Å] and of N1(Tyr)–O15′(Tyr) [2.81(2)Å]. The N1 atom is further hydrogen-bonded to the O51 and O52 atoms of the *C*-terminal group translated by one unit cell along the *b*-axis, consequently forming an infinite dimeric column along the diad axis. This structure is further stabilized by the O15′–O52 and O15–O2W–O1W–N2 hydrogen bond formations. These dimeric columns are stabilized by N5–O3 and O1W–O2 hydrogen bonds and many short contacts along the *c*- and *a*-directions.

The probable conformation of [Met⁵]enkephalin is the extended form

Existence of two independent molecules per asymmetric unit in [Met⁵]enkephalin accelerated the difficulty of the structure determination by usual methods. Various attempts to solve the structure by direct methods or three-dimensional Patterson analyses have failed. But its Patterson map showed the characteristic peak distributions. The major peaks lie on the (U 0 W) and its Harker section (U 1/2 W), but no relatively high peaks on (U V W) planes. The peaks on the (U 0 W) and (U 1/2 W) planes shown in Fig. 6 roughly form a line parallel to the (2 0 $\bar{2}$) directions with a mean separation distance of 4.8Å, which correspond to the vectors between the dimeric *trans*-zigzag extended peptides linked by N-H—O hydrogen bonds. Therefore it would be easily conceivable from the Patterson map that two molecules associate to form the antiparallel extended sheet as shown in Fig. 7. This extended conformation of [Met⁵]enkephalin in solution was already proposed by means of various spectroscopic (Khaled *et al.*, 1977; Zetta & Cabassi, 1982; Kobayashi *et al.*, 1980; Higashijima *et al.*, 1979) and theoretical (DeCoen *et al.*, 1977) studies.

In order to investigate whether this extended structure also exists in the modified [(4′-bromo)-Phe⁴,Met⁵]enkephalin crystal, its Patterson function was calculated, and two independent rows of the peaks parallel to the ($\bar{2}$ 0 2) direction of the (U 0 W) section with a separation distance of approx. 4.8 Å appeared as similar as those of [Met⁵]enkephalin. These results imply that [Met⁵]enkephalin probably prefers the dimeric extended structure. Structural analyses for these two crystals are now in progress.

Concerning the biologically active conformation of enkephalin

Pharmacological studies on various types of opiates and opioid peptides have led to the concept of opiate receptor heterogeneity. The existence of at least three different receptors, μ, κ and δ, is now widely accepted. While morphine-related opiates interact with the μ-receptor selectively, the natural enkephalins show a binding preference for the δ

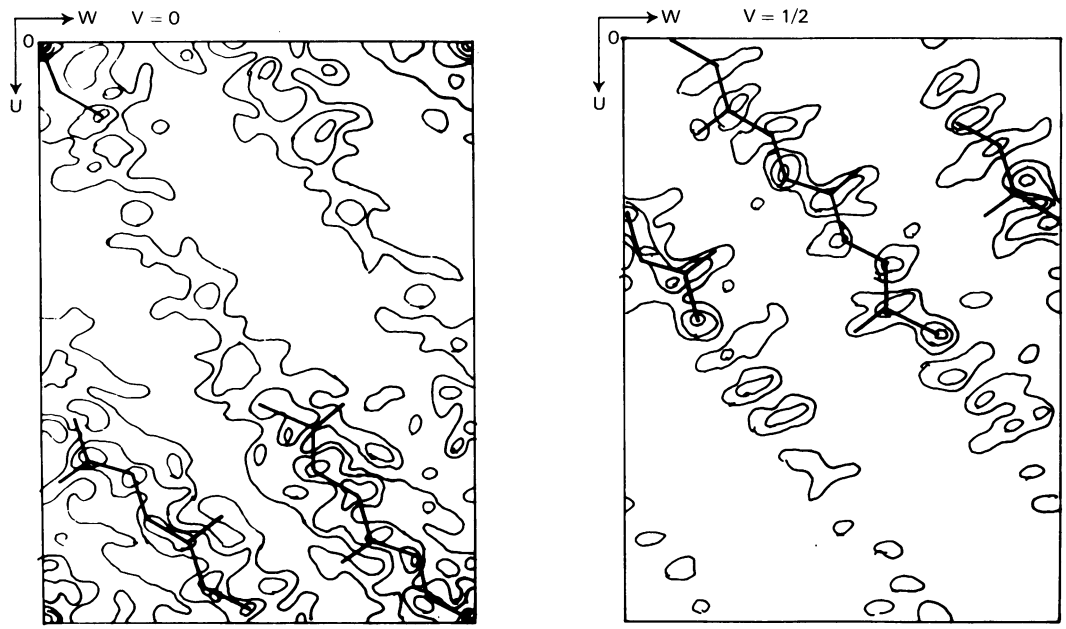

Fig. 6. *Patterson maps of [Met⁵]enkephalin crystal at (U 0 W) and (U 1/2 W) sections*
Possible backbone chain arrangements of the molecule are indicated by the stick bonds.

Table 5. *Intermolecular hydrogen bonds [D-H (at x, y, z)—A (at symmetry operation)]*

Atom (D)	Atom (A)	Symmetry operation	Distance (Å) D—A	Distance (Å) H—A	Angle (°) D-H—A
O15	N1	$1-x,y,2-z$	2.962(16)		
O15′	O52	$1-x,-1+y,2-z$	2.543(19)		
O15′	N1	$1-x,y,2-z$	2.813(16)		
N1	O51	$x,-1+y,z$	2.906(11)	2.0(1)	176(13)
N1	O52	$x,-1+y,z$	2.908(15)	2.1(2)	173(15)
N2	O1W	$x,-1+y,z$	2.815(11)	1.9(1)	171(14)
N3	O51	$x,-1+y,z$	2.884(10)	2.1(1)	153(13)
N5	O3	$0.5-x,0.5+y,1-z$	2.926(8)	2.1(2)	174(16)
O1W	O2	$0.5-x,0.5+y,2-z$	2.873(12)		
O1W	O2W	$1-x,y,2-z$	2.977(26)		
O2W	O15	x,y,z	2.727(26)		
O2W	O52	$1-x,y,2-z$	3.067(25)		

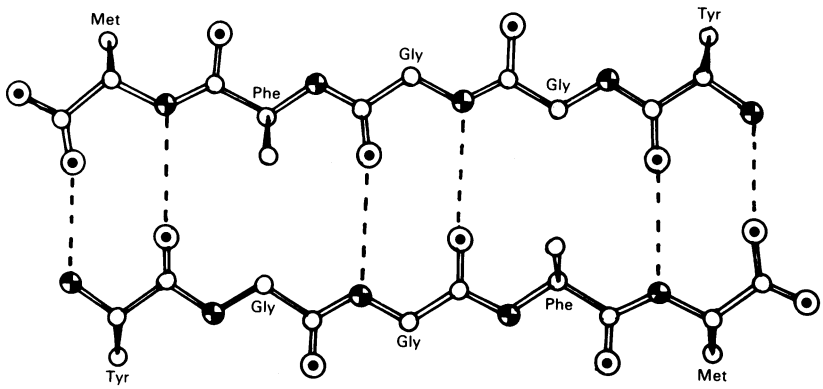

Fig. 7. *Proposed backbone chain conformation of [Met⁵]enkephalin as it exists in the crystal* The molecules take a dimer form showing an extended antiparallel sheet structure.

receptor. The interaction at δ and μ sites may be mediated through different conformations of the peptides. Cyclic [Leu⁵]enkephalin analogues replaced at Gly² and the *C*-terminus by a $-[CH_2]_nNH-$ chain ($n = 1$–4) were reported to indicate selective binding to the μ receptor (DiMaio *et al.*, 1982). This cyclization makes the [Leu⁵]enkephalin a folded form. Therefore it seems to be reasonable to compare the β-turn conformation observed in the [(4′-bromo)Phe⁴,Leu⁵]-enkephalin crystal with a morphine structure having a high μ-character.

The chemical formula of morphine is shown in Fig. 8, together with its atomic numbering. Several models for the opiate pharmacophore have been proposed (Portorghese, 1965; Feinberg *et al.*, 1976; Horn & Rodgers, 1977; Gorin & Marshall, 1977; Clarke *et al.*, 1978; Gorin *et al.*, 1978; Childers *et al.*, 1979). Opiate activity essentially requires the correct spatial orientation of the phenolic A-ring, the ammonium group of the D-ring, and additional

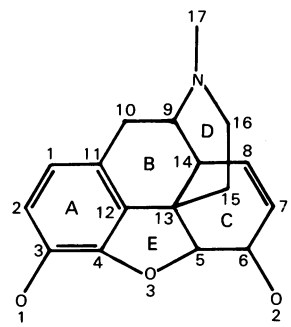

Fig. 8. *Structure and atomic numbering of morphine*

atoms of the C-ring including the C6–O2 bond. By considering these spatial requirements for μ-receptor activity, the conformational comparison of the enkephalin with morphine (Gylbert, 1973) led us to elucidate the following conclusions. (1)

The Tyr[1] hydroxy group of enkephalin corresponds to the phenyl hydroxy group of the morphine A-ring. (2) CPK model building suggests that the disposition of the Tyr residue permits relatively large rotation around the Cl–C11 bond without any accompanying steric hindrance. (3) When the Tyr[1] residue takes the torsion angle χ_1 of 76°, the relative spatial arrangements among O15, N1 and O51 are nearly the same as those among the O1, N and O2 atoms of morphine, respectively, i.e., the C5–O51 carbonyl bond of the *C*-terminal is equivalent to the C6–O2 bond of the morphine C-ring, and the N1(Tyr[1]) amino group is equivalent to the ammonium group of the D-ring (N1–O15 = 6.5, N1–O51 = 6.8, O15–O51 = 4.6 Å for enkephalin and N–O1 = 7.1, N–O2 = 6.5, O1–O2 = 4.6 Å for morphine). Similar relationship among these atoms were already proposed by Smith & Griffin (1978), but the proposed conformations are different. Fig. 9 illustrates the comparison of the morphine and the proposed enkephalin conformations. In the conformation of enkephalin, the disposition of the C2′–N3–C3–C3′–N4–C4–C4′–N5–C5 backbone chain to the Tyr[1] residue is similar to that of the C- and D-rings of morphine to the phenolic A-ring.

On the other hand, the most probable structure of [Met[5]]enkephalin would be the dimeric form consisting of the two antiparallel extended sheets, which is in contrast to that of [Leu[5]]enkephalin. This dimer formation, although it is at present not yet determined, seems to be stable and rigid because of six intermolecular hydrogen bond formations (see Fig. 7). The conformational difference between [Met[5]]- and [Leu[5]]enkephalins in crystals would in part reflect the binding selectivities for the δ- and μ-opiate receptors. We wish here to postulate that the δ-receptor can interact with the dimeric extended form of enkephalins, while the μ-receptor may interact selectively with the β-turn form. The dimeric state could also be observed in the present [Leu[5]]enkephalin crystal (see Fig. 5). This would imply a possible explanation of why the folded conformation of [Leu[5]]enkephalin also interacts with the δ-receptor. The dimerization of the enkephalin was already shown to exist also in solution (Khaled *et al.*, 1977; Higashijima *et al.*, 1979).

References

Balodis, Y. Y., Nikiforovich, G. V., Grinsteine, I. V., Vegner, R. E. & Chipens, G. I. (1978) *FEBS Lett.* **86**, 239–242

Bhat, T. N., Sasisekharan, V. & Vijayan, M. (1979) *Int. J. Pept. Protein Res.* **13**, 170–184

Bleich, H. E., Day, A. R., Freer, R. J. & Glasel, J. A. (1977) *Biochem. Biophys. Res. Commun.* **74**, 592–598

Bradbury, A. F., Smyth, D. G. & Snell, C. R. (1976) *Nature (London)* **260**, 165–166

Bundi, A. & Wüthrich, K. (1979) *Biopolymers*, **18**, 285–297

Chang, K. J. & Cuatrecasas, P. (1979) *J. Biol. Chem.* **254**, 2610–2618

Chang, K. J., Fong, B. T. W., Pert, A. & Pert, C. B. (1976) *Life Sci.* **18**, 1473–1482

Chang, K. J., Hazum, E. & Cuatrecasas, P. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4469–4473

Childers, S. R., Creese, I., Snouman, A. M. & Snyder, S. (1979) *Eur. J. Pharmacol.* **55**, 11–18

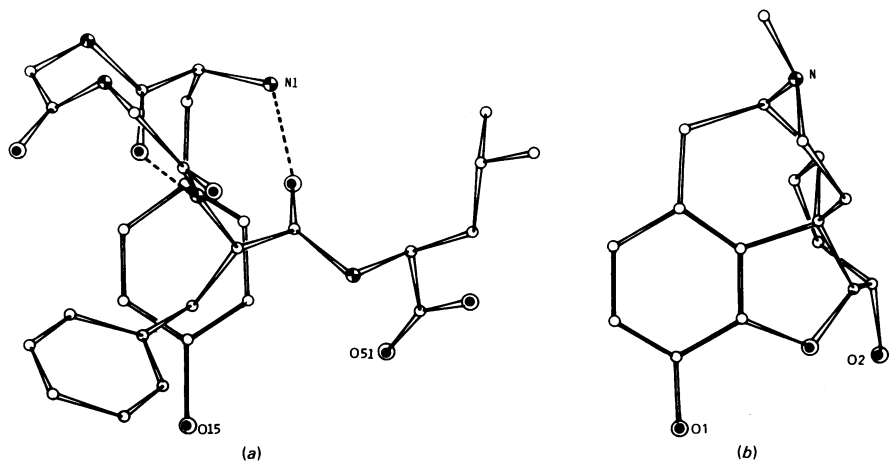

Fig. 9. *Conformational comparision between the crucial groups necessary for the μ-opiate activity in [Leu[5]]enkephalin and morphine*

(*a*) [Leu[5]]enkephalin built from the present X-ray data [χ_1(Tyr[1]) = 76°]. The broken lines represent the intramolecular hydrogen bonds. (*b*) Morphine.

Clarke, F. H., Jaggi, H. & Lovell, R. A. (1978) *J. Med. Chem.* **21**, 600–606
Combrisson, S., Roques, B. P. & Oberlin, R. (1976) *Tetrahedron Lett.* 3455–3458
Cromer, D. T. & Waber, J. T. (1974) in *International Tables for X-ray Crystollography* (Ibers, J. A. & Hamilton, E. C., eds.), vol. 4, pp. 71–73, Kynoch Press, Birmingham
DeCoen, J. L., Humblet, C. & Koch, M. H. J. (1977) *FEBS Lett.* **73**, 38–42
DiMaio, J., Nguyen, T. M. D., Lemieux, C. & Schiller, P. W. (1982) *J. Med. Chem.* **25**, 1432–1438
Faulstich, H., Smith, H. O. & Zobeley, S. (1973) *Liebigs Ann. Chem.* 765–771
Feinberg, A. P., Creese, I. & Snyder, S. H. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 4215–4219
Fischman, A. J., Riemen, M. W. & Cowburn, D. (1978) *FEBS Lett.* **94**, 236–240
Furnas, T. C. (1957) *Single Crystal Orienter Instruction Manual*, General Electric Co., Milwaukee, WI
Garbay-Jaureguiberry, C., Roques, B. P., Oberlin, R., Anteunis, M., Combrisson, S. & Lallemand, J. Y. (1977) *FEBS Lett.* **76**, 93–98
Garbay-Jaureguiberry, C., Baudet, J., Florentin, D. & Roques, B. P. (1980) *FEBS Lett.* **115**, 315–318
Gorin, F. A. & Marshall, G. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5179–5183
Gorin, F. A., Balasubramanian, T. N., Barry, C. D. & Marshall, G. R. (1978) *J. Supramol. Struct.* **9**, 27–39
Gylbert, L. (1973) *Acta Crystallogr. Sect. B.* **29**, 1630–1635
Han, S. L., Stimson, E. R., Maxfield, F. R. & Scheraga, H. A. (1980) *Int. J. Pept. Protein Res.* **16**, 173–182
Higashijima, T., Kabayashi, J., Nagai, U. & Miyazawa, T. (1979) *Eur. J. Biochem.* **97**, 43–57
Horn, A. S. & Rodgers, J. R. (1976) *Nature (London)* **260**, 795–797
Horn, A. S. & Rodgers, J. R. (1977) *J. Pharm. Pharmacol.* **29**, 257–265
Hughes, J., Smith, T. W., Kosterlitz, H. W., Fothergill, L. A., Morgan, B. A. & Morris, H. R. (1975*a*) *Nature (London)* **258**, 577–579
Hughes, J., Smith, T. W., Morgan, B. A. & Fothergill, L. A. (1975*b*) *Life Sci.* **16**, 1753–1758
Ishida, T., Tanabe, N. & Inoue, M. (1983) *Acta Crystallogr. Sect. C.* **39**, 110–112
Isogai, Y., Némethy, G. & Scheraga, H. A. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 414–418
IUPAC-IUB Commission on Biochemical Nomenclature (1971) *Biochem. J.* **121**, 577–585
Jones, C. R., Garsky, V. & Gibbons, W. A. (1977) *Biochem. Biophys. Res. Commun.* **76**, 619–625
Kennard, O. (1968) *International Tables for X-ray Crystallography*, vol. 3, p. 276, Kynoch Press, Birmingham
Khaled, M. A., Long, M. M., Thompson, W. D., Bradley, R. J., Brown, G. B. & Urry, D. W. (1977) *Biochem. Biophys. Res. Commun.* **76**, 224–231
Kobayashi, J., Higashijima, T., Nagai, U. & Miyazawa, T. (1980) *Biochim. Biophys. Acta*, **621**, 190–203
Lemaire, S., Berube, A., Derome, G., Lemaire, I., Magnan, J., Regoli, D. & St-Pierre, S. (1978) *J. Med. Chem.* **21**, 1232–1235
Ling, N. & Guillemin, R. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3308–3310
Loew, G. H. & Burt, S. K. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 7–11
Lord, J. A. H., Waterfield, A. A., Hughes, J. S. & Kosterlitz, H. W. (1977) *Nature (London)* **267**, 495–499
Maigret, B. & Premilat, S. (1982) *Biochem. Biophys. Res. Commun.* **104**, 971–976
Manavalan, P. & Momany, F. A. (1981) *Int. J. Pept. Protein Res.* **18**, 256–275
Marsh, R. E. & Donohue, J. (1967) *Adv. Protein Chem.* **22**, 235–256
Martin, W. R., Eades, C. G., Thompson, J. A., Huppler, R. E. & Gilbert, P. E. (1976) *J. Pharmacol. Exp. Ther.* **197**, 517–532
Miyazawa, T. & Higashijima, T. (1981) *Biopolymers*, **20**, 1949–1958
Momany, F. A. (1977) *Biochem. Biophys. Res. Commun.* **75**, 1098–1103
Morgan, B. A., Smith, C. F. C., Waterfield, A. A., Hughes, J. & Kosterlitz, H. W. (1976) *J. Pharm. Pharmacol.* **28**, 660–661
Pert, C. B., Pert, A., Chang, J. K. & Fong, B. T. W. (1976) *Science* **194**, 330–332
Portoghese, P. S. (1965) *J. Med. Chem.* **8**, 609–616
Roemer, D., Buescher, H. H., Hill, R. C., Pless, J., Bauer, W., Cardinaux, F., Closse, A., Hauser, D. & Huguenin, R. (1977) *Nature (London)* **268**, 547–549
Roques, B. P., Barbay-Jaureguiberry, C., Oberlin, R., Anteunis, M. & Lala, A. K. (1976) *Nature (London)* **262**, 778–779
Sasisekharan, V. & Ponnuswamy, P. K. (1971) *Biopolymers* **10**, 583–592
Simantov, R. & Snyder, S. H. (1976) *Life Sci.* **18**, 781–788
Smith, G. D. & Griffin, J. F. (1978) *Science* **199**, 1214–1216
Stimson, E. R., Meinwald, Y. C. & Scheraga, H. A. (1979) *Biochemistry* **18**, 1661–1671
Sudha, T. S. & Balaram, P. (1981) *FEBS Lett.* **134**, 32–36
Terenius, L., Wahlström, A., Lindeberg, G., Karlsson, S. & Ragnarsson, U. (1976) *Biochem. Biophys. Res. Commun.* **71**, 175–179
The Universal Crystallographic Computing System-Osaka (1979) Library of Programs, Computing Center, Osaka University
Zetta, L. & Cabassi, F. (1982) *Eur. J. Biochem.* **122**, 215–222