Dehydro-enkephalins. Part 7.[1] A Potent Dehydroleucine-enkephalin Resistant to Carboxypeptidase

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To preserve the enkephalin-like properties together with resistance to enzymatic degradation, we have synthesized unsaturated analogues of leucine enkephalin, containing dehydroleucine (ΔLeu[5]) ‡ in the *Z*-configuration (isopropyl and C=O, *trans*) at the *C*-terminus. The ΔLeu unit was introduced by the *N*-chlorination/dehydrochlorination method. The peptides [D-Ala[2],ΔLeu[5]]- and [ΔLeu[5]]-enkephalins were completely resistant to carboxypeptidase Y. In the radioligand binding assays, [D-Ala,[2]ΔLeu[5]]enkephalin displayed a greater affinity for the δ-enkephalin receptor binding sites. In particular, this dehydro-enkephalin was almost four times more active than its saturated [D-Ala[2],D-Leu[2]]enkephalin in the assay using [^{3}H]etorphine as tracer. It is suggested that the high δ-selectivity of [D-Ala[2],ΔLeu[5]]enkephalin may be responsible for a moderate *in vivo* analgesia effect.

An important key to the lack of *in vivo* biological activity after systemic administration of the enkephalins and their analogues may be their susceptibility to enzymatic degradation and poor passage through the blood-brain barrier.[2] It has been reported [3] that enzymatic cleavage is possible at all the peptide linkages in the enkephalin sequence, H-Tyr[1]-Gly[2]-Gly[3]-Phe[4]-Leu[5] (or Met[5])-OH. Preventing such degradation from occurring before the enkephalin reaches its receptors by chemical modifications such as replacement of L-amino-acids by D-amino-acids or *N*-methylation, however, usually causes a loss of δ-enkephalin *versus* μ-morphine receptor selectivity;[4] *i.e.* these modified peptides interact with the μ receptors as well as δ receptors. Our current studies of the incorporation of dehydroamino-acid residues into the enkephalin molecule have provided a unique kind of clarification of their structure–activity relationships.[5] We have synthesized ΔAla[2]-enkephalins, and showed that enhanced lipophilicity at position 2 facilitates the interaction with the μ receptors.[5,6] We also suggested, employing ΔAla[3]- and Ser[3]-enkephalins, that specific combinations of amino-acids at positions 2 and 3 are important to receptor preference, and especially that position 3 may have very specific interactions with the δ receptor but not with the μ receptor binding sites.[1] After synthesizing ΔPhe[4]-enkephalins, we found that the phenyl ring of the ΔPhe[4] residue oriented in the *Z*-configuration (phenyl ring and C=O, *trans*) enforced a sterically favourable interaction with the δ sites.[7,8] As a result of these studies, it has been shown that dehydrogenation of the enkephalin molecule can afford a peptide with full receptor activity having unchanged δ-selectivity.[5,7,8]

The special stability of dehydropeptides to enzymatic hydrolysis has been reported,[9] and recently we have reported the stability of some dehydrophenylalanine peptides to thermolysin and chymotrypsin.[10,11] One of the main routes of enzymatic degradation of the enkephalins is initiated from the *C*-terminus by carboxypeptidases.[3,12,13] In the past, this mode of degradation has been protected against by conversion

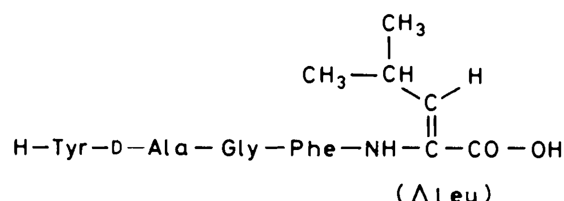

Figure 1. Structure of [D-Ala[2],ΔLeu[5]]enkephalin (8a) in the *Z*-configuration

of the carboxy-group to the corresponding amide, ester or alcohol.[12] However, these modifications invariably cause a change in receptor selectivity and at least partially eliminate the 'enkephalin-like' characteristics of the peptide,[2,4] since the presence of a *C*-terminal carboxy-group is the most important determining factor for δ-specific activity.[2,7] In the present study, we report the synthesis of ΔLeu[5]-enkephalins, their enzyme stability to carboxypeptidase Y, their receptor binding activities, and *in vivo* tail-flick analgesic activity.

Results and Discussion

The synthetic scheme is illustrated in Figure 2. The ΔLeu moiety was prepared by the *N*-chlorination/dehydrochlorination sequence.[14] The free base H-Leu-OBzl was *N*-chlorinated by t-butyl hypochlorite, and the product was treated with 1,8-diazabicyclo[4.3.0]undec-7-ene (DBU). The resulting imine was allowed to rearrange to the enamine form (ΔLeu-OBzl·HCl) by treatment with hydrogen chloride at low temperature. Utilizing the water-soluble carbodi-imide–1-hydroxybenzotriazole (EDC–HOBt) method, the enamine was coupled with Boc-Phe-OH to afford a dipeptide (1). Compound (1) was obtained, surprisingly, in good yield (47%) after column chromatography, even though the nucleophilicity of the enamino-function is much less than that of a saturated amino-acid ester and such coupling methods as the mixed anhydride, acid chloride, and DCC are said to give poor yields.[15] The Boc group was removed by hydrogen chloride in ethyl acetate, and the resulting salt (2)·HCl was coupled with the dipeptide Boc-D-Ala (or Gly)-Gly-OH (4) by the EDC–HOBt method. The fully protected pentapeptides (7a) and (7b) were also prepared by the EDC–HOBt method. It was found that the ΔLeu residue was quite stable during these deblocking and coupling reactions.

Dehydro-enkephalins, [D-Ala[2],ΔLeu[5]]- (8a) and [ΔLeu[5]]-

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‡ Abbreviations according to IUPAC–IUB Commission, *Biochemistry*, 1972, **11**, 1726, are used throughout. Additional abbreviations: Δ, dehydro (α,β-unsaturated); Boc, t-butoxycarbonyl; DMF, *N,N'*-dimethylformamide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide; HOBt, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid; DADLE, [D-Ala[2],D-Leu[5]]enkephalin; ^{3}H-DHM, [^{3}H]dihydromorphine; ^{3}H-ETP, [^{3}H]etorphine; ^{3}H-NAL, [^{3}H]-naloxone.

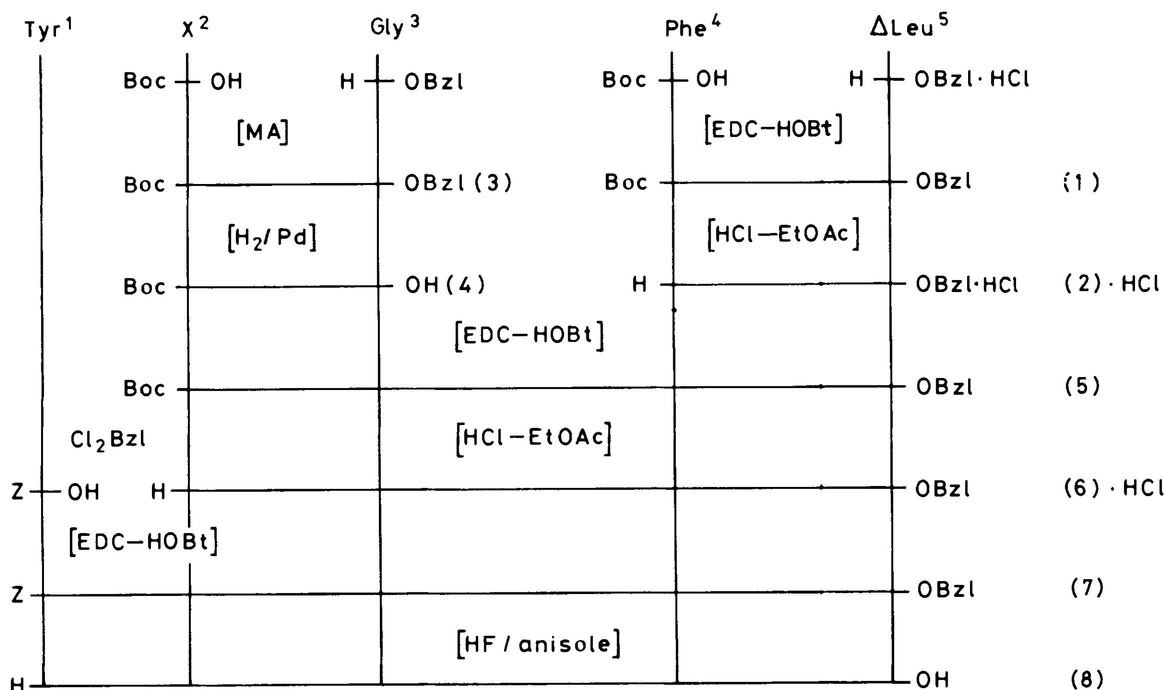

Figure 2. Synthetic scheme of ΔLeu[5]-enkephalins: [D-Ala[2],ΔLeu[5]] (8a, X = D-Ala) and [ΔLeu[5]]- (8b; X = Gly) enkephalins

Table 1. Enzyme stability of enkephalins to carboxypeptidase Y-catalysed hydrolysis

Enkephalins	% Hydrolysis [a] Phe[4]	Leu[5]
Tyr-Gly-Gly-Phe-Leu	100 (94) [b]	100 (100) [b]
Tyr-D-Ala-Gly-Phe-Leu	35	98
Tyr-D-Ala-Gly-Phe-ΔLeu	0	*c*
Tyr-D-Ala-Gly-ΔPhe-Leu	*c*	9.5

[a] The molar recovery of liberated amino-acids (Leu[5] followed Phe[4]) upon amino-acid analyses after incubation for 3 h at 25 °C. [b] The % molar recovery after 30 min incubation. [c] No amino-acids were detectable.

Table 2, Receptor binding activities of unsaturated and saturated Leu[5]-enkephalins

	Enkephalins	IC_{50} (nN) ^{3}H-DADLE [a]	^{3}H-DHM [a]	^{3}H-ETP
(8a)	[D-Ala[2],ΔLeu[5]]	2.5 (76) [b]	8.7 (87) [b]	23 (370) [b]
(8b)	[Gly[2],ΔLeu[5]]	6.6 (29)	32 (24)	440 (20)
(9a)	[D-Ala[2],D-Leu[5]]	1.9 (100)	7.6 (100)	86 (100)
(9b)	[Gly[2],Leu[5]]	2.4 (79)	15 (51)	*c*

[a] Assayed in the presence of bacitracin (100 µg/ml, Sigma). [b] Relative activities (%) as compared with [D-Ala[2],D-Leu[5]]enkephalin (DADLE). [c] Not determined.

(8b) enkephalins were liberated with anhydrous hydrogen fluoride in the presence of anisole, and their homogeneity was determined by t.l.c., paper electrophoresis, and amino-acid analysis. Acid hydrolysis of a ΔLeu peptide converts this residue into ammonia and a ninhydrin negative α-keto-acid (2-oxo-4-methylpentanoic acid), whereas hydrogenation of (8a) and (8b) followed by acid hydrolysis of the product allowed detection of leucine generated upon amino-acid analysis.

The configuration of the ΔLeu moiety was determined as the *Z*-form (isopropyl and C=O, *trans*) by determining the chemical shift of the $C_{\gamma}H$ (2.38 p.p.m.) in its ^{1}H n.m.r. spectrum in $CDCl_3$.[16] The presence of an unsaturated unit in the peptides was clearly shown by u.v. difference spectroscopy (ε 5 000—7 000 at 220—230 nm), in which the u.v. spectrum of an unsaturated peptide was scanned with the saturated analogue as a reference.[17]

The profiles in the enzymatic hydrolyses of saturated and unsaturated (dehydro) enkephalins are shown in Table 1, employing carboxypeptidase Y [EC 3.4.16.1.] and quantitative analysis using an amino-acid analyzer. Leucine[5]-enkephalin was promptly degraded from the *C*-terminus, liberating Leu[5] and Phe[4] within 30 min, followed slowly by Gly.[3] In contrast, no phenylalanine was detected for ΔLeu[5]-enkephalins (8a) and (8b) under the same conditions within 3 h. This indicates that the *C*-terminal ΔLeu[5] residue makes the peptide completely resistant to carboxypeptidase Y. When ΔPhe[4]-enkephalins [5] were used as substrate, it was found that the rate of hydrolysis at the ΔPhe[4]-Leu[5] peptide bond was much slower (over 10-fold) than that of the saturated Phe[4]-Leu[5] bond (Table 1). In conclusion, it has been proved quantitatively, for the first time, that an α,β-dehydroamino-acid residue is very strongly resistant to carboxypeptidase at its carboxy-side, and 'completely' resistant at the amino-peptide linkage.

Multiple opiate receptors exist in brain,[18,19] and the δ-enkephalin and μ-morphine receptors have been well characterized by receptor binding [20,21] and pharmacological assay systems.[19,22] For the selective labelling and characterization of each receptor, specific ligands with a greater selectivity will be required. So-called 'enkephalin-like' peptides such as [D-Ala[2], D- or L-Leu[5]]enkephalin bind to the δ receptors about 5—8 times more strongly than to the μ receptors,[7,19,20] while the alkaloids dihydromorphine and naloxone bind to the μ receptors with much greater affinity (20—30-fold) than to the δ receptors.[4,19,20] On the other hand, etorphine binds both δ and μ receptors equally well.[4] Table 2 shows the binding affinities of the ΔLeu[5]-enkephalins, (8a) and (8b), and their

Table 3. Specific receptor binding activities and δ-selectivity of enkephalin analogues

	Enkephalins			IC_{50} (nM ± s.e.) [b]		
	Amino acid residues [a]					
	4	5	C-terminus	δ-assay ^{3}H-DADLE (NG108 15 cells)	μ-assay ^{3}H-NAL (rat brain)	δ-Selectivity ratio [c]
(8a):	Phe	ΔLeu	CO_2H	1.68 (±0.12)	11.7 (±1.64)	7.0
(10):	Phe	Leu	CO_2H	1.04 (±0.05)	6.76 (±1.09)	6.5
(11):	ΔPhe	Leu	CO_2H	1.45 (±0.10)	10.1 (±1.66)	7.0
(12):	Phe	Leu	$CONH_2$	1.41 (±0.10)	1.14 (±0.19)	0.8

[a] All the peptides have the same sequence of Tyr-D-Ala-Gly in residues one to three. [b] All the assays were performed in the presence of bacitracin (100 μg/ml) at 25 °C. [c] Calculated as the values of ratio of IC_{50} using ^{3}H-NAL *vs.* IC_{50} using ^{3}H-DADLE, showing the selectivity for the δ receptors.

saturated analogues in rat brain membrane preparations, using [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin (^{3}H-DADLE), [^{3}H]-dihydromorphine (^{3}H-DHM) and [^{3}H]etorphine (^{3}H-ETP) as tracers. [D-Ala^2,ΔLeu^5]Enkephalin (8a) is almost as active as the saturated analogue [D-Ala^2,D-Leu^5]enkephalin (9a; DADLE) in both ^{3}H-DADLE and ^{3}H-DHM assays, and almost twice as active as Leu^5-enkephalin in ^{3}H-DHM assay. On the other hand, [Gly^2, ΔLeu^5]enkephalin (8b) displays 40—50% of the activity of Leu^5-enkephalin (9b) and 20—30% that of DADLE. Since there is a large difference (3—4-fold) in affinity between D-Ala^2 and Gly^2, ΔLeu^5-enkephalins, and no such difference between the saturated analogues with D-Ala^2 and Gly^2 residues, it is difficult to explain the low affinity of compound (8b), unless possibly a conformational change (due to a β-turn) which includes positions 2 and 5 might account for this.[7,23]

In the δ,μ-combined ^{3}H-ETP assay, which did not contain bacitracin as enzymolysis inhibitor, compound (8b) has only 20% the activity of DADLE and shows a total loss of activity in the *in vivo* bioassay (Table 4). This may be due primarily to degradation of the Tyr^1-Gly^2 bond by amino-peptidases,[24,25] despite its resistance to carboxypeptidases. Compound (8a) having a D-Ala^2 residue, surprisingly, displayed almost four times greater potency than DADLE in this etorphine assay. This result could be due to resistance conferred by the D-Ala^2 and ΔLeu^5 residues to both amino- and carboxy-peptidases. Indeed, the lower potency of saturated DADLE as compared to (8a) is unexpected because the D-Leu^5 residue of DADLE should also cause resistance to degradation by carboxypeptidases.

Neuroblastma-glioma hybrid (NG108-15) cell lines contain only δ receptors.[26] Consequently, very low concentrations of ^{3}H-DADLE (0.10 nM) in HG108-15 cells and [^{3}H]naloxone (^{3}H-NAL, 0.15 nM) in rat brain membrane label almost selectively δ and μ receptors, respectively [7,21] (Table 3). When ^{3}H-DADLE was used as tracer in these cells, all D-Ala^2 enkephalins display very high affinity (IC_{50} = 1.0—1.7 nM) for the δ binding sites. In the μ assay using ^{3}H-NAL and rat brain, *C*-terminal free enkephalins (8a), (10), and (11) drastically lose their affinity for the μ-sites (7—12 nM), while the amidated analogue [D-Ala^2,Leu^5] enkephalin amide (12) sustains its high potency (IC_{50} = 1.14 nM). These results suggest either either that the *C*-terminal amidation of enkephalin causes a favourable interaction with the μ sites, or that the *C*-terminal carboxy-group is a predominant factor for δ receptor interaction.[2,7] ΔLeu^5-Enkephalin (8a) is almost as active as [D-Ala^2,L-Leu^5]enkephalin (10) and [D-Ala^2,ΔPhe^4,Leu^5]enkephalin (11), which are good δ agonists.

The δ-selectivity as an indicator of the descriminative ability of enkephalins for the δ receptor binding sites was calculated using the ratio of IC_{50}'s in ^{3}H-NAL μ assay *versus* ^{3}H-DADLE δ assay.[7,21] A non-selective peptide with the same potency in both assays will have a selectivity of 1.0; for example, [D-Ala^2,Leu^5]enkephalin amide (12) is non-selective (Table 3). All of the *C*-terminal free enkephalins show high δ-selectivity (ratio of 6.5—7.0), indicating that the incorporation of α,β-dehydroamino-acids (ΔLeu^5 or ΔPhe^4) does not affect its receptor preference. We are impressed by the fact that simple α,β-dehydrogenation of the Leu^5 residue in the enkephalin molecule causes full receptor activity to be sustained along with high δ-selectivity while showing complete resistance to enzymatic hydrolysis by carboxypeptidases. It is clear that the opiate receptors are insensitive to chirality of position 5, since [D- and L-Leu^5]-enkephalins have almost the same receptor binding affinities,[28] and ΔLeu^5-enkephalin (8a) retains its receptor affinity in spite of the achirality at position 5.

Table 4. *In vivo* tail flick analgesic activity of enkephalin analogues

Enkephalins	ED_{50} (mg/kg)
(8a)	22
(8b)	>50
Met^5-enkephalin	>50
FK33 824	2.2
Morphine	0.5

Table 4 shows the potency of these peptides in the *in vivo* mouse tail flick analgesia test by intravenous injection. [Gly^2,ΔLeu^5]- (8b) and [Gly^3,Met^5]-enkephalins have no activity in doses of up to 50 mg/kg. Compound (8a) showed a weak analgesic activity, which was almost 40-fold less than morphine and 10-fold less than δ,μ-ligand FK33 824. Fluorescence [29] and differential autoradiographical [30] studies have suggested that δ and μ receptors have different locations and functions in the brain, and that the analgesic effect is mediated through the μ receptors.[2,31] The poor analgesic potency of (8a) in the present study may be due to its high δ receptor selectivity, as shown in the binding studies.

The results of the present study clearly indicate the availability and the usefulness of the incorporation of α,β-dehydroamino-acids into peptide hormones, in regard to potency and enzyme stability. Such studies should be very useful in the further clarification of the structure–activity relationships of other peptide hormones.

Experimental

All m.p.s were measured on a 6427-H10 Thomas Hoover Melting Point Apparatus, and are uncorrected. Elemental analyses were determined by Atlantic Microlab, Inc. (Atlanta, Georgia). Amino-acid analyses were performed on a Beckman Model 119Cl Amino Acid Analyzer under standard conditions after hydrolysis of the peptide with 6M-HCl in

sealed, deaerated tubes for 24 h at 110 °C. Electrophoresis was carried out on Whatman 3 MM chromatography paper at pH 1.9 in a solvent mixture of HCO_2H–MeOH–AcOH–H_2O (1 : 3 : 6 : 10, v/v). Migration values are reported with respect to lysine as R_{Lys}. Silica gel columns were packed with Silica Gel 60 (230—400 mesh, Merck).

T.l.c. was carried out on Silica Gel K6GF (Whatman) with detection of the components by u.v. light or heating after spraying with 10% H_2SO_4 or ninhydrin. R_F Values are reported for the following solvent systems: R_F^1, $CHCl_3$–MeOH–AcOH (95 : 5 : 1); R_F^2, $CHCl_3$–MeOH (5 : 1); R_F^3, $CHCl_3$–EtOAc (1 : 1); R_F^4, $CHCl_3$–EtOAc (3 : 1); R_F^5, Bu^nOH–AcOH–pyridine–H_2O (4 : 1 : 1 : 2); R_F^6, Bu^nOH–AcOH–H_2O (4 : 1 : 5, organic); R_F^7, 0.1% AcOH–Bu^nOH–pyridine (11 : 5 : 3, organic).

The ^1H n.m.r. spectra were recorded on a Varian EM-390 90 MHz NMR Spectrometer with tetramethylsilane as internal standard. The u.v. spectra were measured on a Varian Cary 219 and a Beckman UV 5260 Spectrophotometer. Optical rotations were measured with a Perkin-Elmer Model 141 Polarimeter.

*t-Butoxycarbonyl-*L*-phenylalanyl-(*Z*)-α,β-dehydroleucine Benzyl Ester* (1).—A solution of H-Leu-OBzl (2.21 g, 10 mmol) in anhydrous ether (10 ml) was treated with t-butyl hypochlorite (1.2 ml, 10 mmol) for 30 min at 0 °C with the exclusion of light. After evaporation *in vacuo* the oily residue was dissolved in $CHCl_3$ and the solution was washed with 2% HCl (10 ml) and water (10 ml), dried (Na_2SO_4) at 0 °C, and evaporated to yield crude *N*-chloroleucine benzyl ester. To a solution of this *N*-chloro-derivative in ether (40 ml) was added a solution of DBU (1.5 ml, 10 mmol) in ether (10 ml) at room temperature. After 30 min the resulting DBU·HCl was filtered *in vacuo* and 3.0M HCl–EtOAc (5 ml) was added to the filtrate at −70 °C. The precipitated H-ΔLeu-OBzl·HCl was collected and dried, wt. 2.33 g (91%), and used in the next step without further purification. To a solution of Boc-Phe-OH (2.41 g, 9.1 mmol), H-ΔLeu-OBzl·HCl (2.33 g, 9.1 mmol), and Et_3N (1.27 ml, 9.1 mmol) in $CHCl_3$ (60 ml) were added HOBt (1.68 g, 11 mmol) and EDC·HCl (2.11 g, 11 mmol) at −10 °C. The reaction mixture was stirred for 2 h at 0 °C and overnight at room temperature. After evaporation, the residual oil was dissolved in EtOAc and the solution was washed successively with 4% $NaHCO_3$, 10% citric acid, and water, and dried (Na_2SO_4) and evaporated. Purification was carried out on a silica gel column (2.2 × 50 cm) eluted with $CHCl_3$–EtOAc (9 : 1). The fractions containing a pure product were pooled, evaporated, and the residual oil was crystallized from ether–light petroleum to yield compound (1) (2.00 g, 47%), m.p. 140—141 °C; $[\alpha]_D^{25}$ −21.2° (*c* 1.0, $CHCl_3$); R_F^1 0.86, R_F^3 0.92; u.v. by difference spectroscopy, ε 7 400 (λ 222 nm, MeOH), ε 6 100 (λ 227 nm, CH_3CN); δ ([$^2H_6$]Me_2NCHO): 0.96 (d, *J* 6.6 Hz, 6 H, ΔLeu 2 CH_3), 1.33 (s, 9 H, Boc), 2.5 (m, 1 H, ΔLeu $C_\gamma H$), 2.95 (d, *J* 5.4 Hz, Phe $C_\beta H_2$), 4.2—4.5 (m, 1 H, Phe $C_\alpha H$), 5.16 (s, 2 H, OCH_2Ph), 6.40 (d, *J* 10.8 Hz, 1 H, ΔLeu $C_\beta H$), 6.8 (d, *J* 9 Hz, 1 H, Phe NH), 7.32 (s, 5 H, ArH), 7.43 (s, 5 H, ArH), and 9.31 (s, 1 H, ΔLeu NH) (Found: C, 69.5; H, 7.35; N, 6.0. $C_{27}H_{34}N_2O_5$ requires C, 69.34; H, 7.58; N, 5.98%).

*t-Butoxycarbonyl-*D*-alanyl-glycine Benzyl Ester* (3a).—To a chilled solution of Boc-D-Ala-OH (2.27 g, 12 mmol) and Et_3N (1.68 ml, 12 mmol) in tetrahydrofuran (20 ml) was added isobutyl chloroformate (1.58 ml, 12 mmol) at −10 °C. After 10 min a chilled solution of H-Gly-OBzl·TosOH (4.05 g, 12 mmol) and Et_3N (1.68 ml) in $CHCl_3$ was added. The reaction mixture, treated as described for compound (1), yielded (3a) (3.50 g, 87%), m.p. 84—85 °C, $[\alpha]_D^{25}$ 25.3° (*c* 1.0, $CHCl_3$); R_F^1 0.62, R_F^2 0.87, R_F^4 0.46 (Found: C, 60.6; H, 7.2; N, 8.3. $C_{17}H_{24}N_2O_5$ requires C, 60.70; H, 7.19; N, 8.33%).

t-Butoxycarbonyl-glycyl-glycine Benzyl Ester (3b).—Compounds (3b)—(8b) were prepared in the same manner as described for (3a)—(8a), respectively. Therefore, the analytical data are described for the b (Gly^2) series without a detailed description; yield of (3b) was 91%, m.p. 82—84 °C; R_F^1 0.57, R_F^2 0.91, R_F^4 0.19 (Found: C, 59.6; H, 6.9; N, 8.7. $C_{16}H_{22}N_2O_5$ requires C, 59.76; H, 6.92; N, 8.67%).

*t-Butoxycarbonyl-*D*-alanyl-glycine* (4a).—Compound (3a) (2.69 g, 8 mmol) was dissolved in MeOH (20 ml) and treated with hydrogen in the presence of 10% palladium on activated carbon. The filtrate from the catalyst was evaporated, and the resulting solid was recrystallized from ether–light petroleum to give (4a) (1.61 g, 82%), m.p. 126—127 °C, $[\alpha]_D^{25}$ 4.1° (*c* 1.0, $CHCl_3$), R_F^1 0.42 (Found: C, 48.8; H, 7.4; N, 11.35. $C_{10}H_{18}N_2O_5$ requires C, 48.77; H, 7.36; N, 11.38%).

t-Butoxycarbonyl-glycyl-glycine (4b), yield (86%, m.p. 133—134 °C, R_F^1 0.25 (Found: C, 46.5; H, 6.95; N, 12.05. $C_9H_{16}N_2O_5$ requires C, 46.54; H, 6.95; N, 12.04%).

*t-Butoxycarbonyl-*D*-alanyl-glycyl-*L*-phenylalanyl-(*Z*)-α,β-dehydroleucine Benzyl Ester* (5a).—Compound (1) (0.42 g, 0.9 mmol) was dissolved in 2.8M HCl–EtOAc (3.2 ml). After 2 h at room temperature the solvent was evaporated under reduced pressure and the evaporation was repeated after the addition of EtOAc; yield of oily H-Phe-ΔLeu-OBzl·HCl [(2)·HCl] 0.36 g (100%). Compound (5a) was prepared immediately from (4a) (0.22 g, 0.9 mmol), (2)·HCl (0.36 g, 0.9 mmol), Et_3N (0.13 ml, 0.9 mmol), HOBt (0.17 g, 1.1 mmol), and EDC·HCl (0.19 g, 1.0 mmol) as described for compound (1); yield of (5a) 0.46 g (85%), m.p. 149—151 °C; $[\alpha]_D^{25}$ −24.9° (*c* 1.0, $CHCl_3$); R_F^1 0.42, R_F^1 0.65; u.v. (MeOH) by difference spectroscopy, λ 221 nm (ε 7 400); δ ([$^2H_6$]Me_2NCHO) 6.40 [d, *J* 10.5 Hz, 1 H, ΔLeu C=C*H*·CH(CH_3)$_2$] and 9.39 (s, 1 H, ΔLeu NH) (Found: C, 64.45; H, 7.2; N, 9.35. $C_{32}H_{42}N_4O_7$ requires C, 64.62; H, 7.12; N, 9.42%).

*t-Butoxycarbonyl-glycyl-glycyl-*L*-phenylalanyl-(*Z*)-α,β-dehydroleucine Benzyl Ester* (5b).—This tetrapeptide was prepared by the procedure used for (5a) and the crude product was purified by silica gel chromatography using a column (2.8 × 17 cm) and elution with $CHCl_3$–EtOAc (3 : 1) to remove an impurity followed by EtOAc–acetone (3 : 1) for the elution of pure (5b) (89%), m.p. 143—145 °C, $[\alpha]_D^{25}$ −18.1° (*c* 1.0, $CHCl_3$), R_F^1 0.48, R_F^2 0.83, R_F^3 0.14; δ ([$^2H_6$]Me_2NCHO): 6.38 [d, *J* 10.8 Hz, 1 H, ΔLeu C=C*H*·CH(CH_3)$_2$], and 9.40 (s, 1 H, ΔLeu NH) (Found: C, 63.1; H, 7.05; N, 9.45. $C_{31}H_{40}N_4O_7\cdot\frac{1}{2}H_2O$ requires C, 63.14; H, 7.01; N, 9.50%).

*Benzyloxycarbonyl-O-2,6-dichlorobenzyl-*L*-tyrocyl-*D*-alanyl-glycyl-*L*-phenylalanyl-(*Z*)-α,β-dehydroleucine Benzyl Ester* (7a).—Compound (5a) (387 mg, 0.65 mmol) was dissolved in 2.8M HCl–EtOAc (2.3 ml). After 2 h at room temperature, the solution was evaporated to give oily H-D-Ala-Gly-Phe-ΔLeu-OBzl·HCl [(6)·HCl] (344 mg, 100%). Compound (7a) was prepared from Z-Tyr(Cl_2Bzl)-OH[8] (308 mg, 0.65 mmol), (6)·HCl (344 mg, 0.65 mmol), Et_3N (0.09 ml, 0.65 mmol), HOBt (119 mg, 0.78 mmol), and EDC·HCl (138 mg, 0.72 mmol) as described for compound (1). The crude product was purified by recrystallization twice from DMF–ether; yield 463 mg (75%), m.p. 184—186 °C, $[\alpha]_D^{25}$ −17.8° (*c* 0.5, DMF), R_F^1 0.27, R_F^2 0.77, δ ([$^2H_6$]Me_2NCHO) 6.38 [d, *J* 10.8 Hz, 1 H, ΔLeu C=C*H*·CH(CH_3)$_2$], and 9.39 (s, 1 H, ΔLeu NH) (Found: C, 64.35; H, 5.65; N, 7.4. $C_{51}H_{53}Cl_2N_5O_9$ requires C, 64.42; H, 5.62; N, 7.37%).

*Benzyloxycarbonyl-*O*-2,6-dichlorobenzyl-*L*-tyrocyl-glycyl-glycyl-*L*-phenylalanyl-*(Z)*-α,β-dehydroleucine Benzyl Ester* (7b).—This peptide was prepared by the procedure used for (7a) and the crude product was purified by gel filtration on a Sephadex LH-20 column (1.9 × 75 cm) using DMF for elution; yield 96%, m.p. 175—177 °C, $[\alpha]_D^{25}$ −15.4° (*c* 0.5, DMF), R_F^1 0.44, R_F^2 0.89; δ ([$^2H_6$]Me_2NCHO) 6.38 [d, *J* 10.2 Hz, 1 H, ΔLeu C=C*H*·CH(CH_3)$_2$] and 9.43 (s, 1 H, ΔLeu NH) (Found: C, 64.05; H, 5.5; N, 7.45. $C_{50}H_{51}Cl_2N_5O_9$ requires C, 64.10; H, 5.49; N, 7.48%).

L*-Tyrocyl-*D*-alanyl-glycyl-*L*-phenylalanyl-*(Z)*-α,β-dehydroleucine* ([D-Ala^2,ΔLeu^5]*Enkephalin*) (8a).—Compound (7a) (285 mg, 0.3 mmol) was treated with anhydrous liquid HF (5 ml) and anisole (0.2 ml) for 1 h at 0 °C. The solution was evaporated and the oily product was dissolved in 1M-AcOH. The aqueous solution was washed with ether, evaporated to a small volume under reduced pressure and subjected to gel filtration on a column (1.9 × 91 cm) of Bio-Gel P-2 (200—400 mesh) in 1M-AcOH. The fractions (140—220 ml) were pooled, lyophilized, and the lyophilization was repeated by the addition of water; yield 126 mg (85%), m.p. 164 °C (decomp.), $[\alpha]_D^{25}$ 31.2° (*c* 0.5, 1M-AcOH); R_F^5 0.83, R_F^6 0.59, R_F^7 0.84; R_{Lys} 0.53. Amino-acid ratios in acid hydrolysate: Tyr, 1.03; Ala, 0.98; Gly, 1.05; Phe, 1.00; NH_3, 1.15.

An analytical amount of (8a) was hydrogenated in 1M-AcOH in the presence of 10% palladium on activated carbon, and hydrolysed in 6M-HCl for 24 h at 110 °C. The amino-acid ratio in this hydrolysate was as follows: Tyr, 0.98; Ala, 1.02; Gly, 1.04; Phe, 1.00; Leu, 0.94.

L*-Tyrocyl-glycyl-glycyl-*L*-phenylalanyl-*(Z)*-α,β-dehydroleucine* ([ΔLeu^5]*enkephalin*) (8b).—This compound was prepared from (7b) as above; yield 77%, m.p. 165 °C (decomp.), $[\alpha]_D^{25}$ 13.0° (*c* 0.5, 1M-AcOH), R_F^5 0.74, R_F^6 0.70, R_F^7 0.84; R_{Lys} 0.55. The amino-acid ratios in the acid hydrolysate of (8b): Tyr, 0.99; Gly, 2.08; Phe, 1.00; NH_3, 1.16, and of hydrogenated (8b): Tyr, 0.98; Gly, 2.06; Phe, 1.00; Leu, 0.92.

Enzyme Assay.—The peptides (0.5—0.6 mM) were incubated with carboxypeptidase Y (EC 3.4.16.1., 2.7 μM; Worthington, NJ) in 0.1M-pyridine acetate buffer (pH 5.5) at 25 °C as described by Hayashi.[32] At certain intervals, 100 μl aliquots of the reaction mixture (1 ml) were lyophilized after immediate freezing, and the residues were dissolved in citrate buffer (pH 2.2) and injected into an amino-acid analyzer. After subtraction of control analyses (enzyme alone), the free amino-acid values were used for the calculation of the rate of hydrolysis. The results are summarized in Table 1.

Biological Assays.—Receptor binding assays using a rat brain membrane preparation were carried out essentially as described by Pert and Snyder.[33] The δ receptor assay using neuroblastma-glioma hybrid (NG108-15) cell lines was performed according to the procedure of Chang *et al.*[26,34] Tritiated opioids were purchased from New England Nuclear (Boston, MA), and the saturated enkephalin analogues from Peninsula Laboratories Inc. (San Carlos, CA). All binding curves were repeated in at least duplicate experiments. Results were analyzed by the computer program 'ALLFIT' to construct the least-squares estimates of the logistic curves relating binding of labelled ligand to concentrations of unlabelled ligand.[35] Doses which produce a 50% inhibition of binding (IC_{50}) were obtained together with their standard error. The results are summarized in Tables 2 and 3.

In vivo analgesic activity was measured using male CF-1 derived mice (18—22 g) as follows. The test peptides in 0.01M-AcOH were injected intravenously and 2 min later the mouse was tested for analgesia by the tail flick method.[36] When a significant number of responders (5/6—6/6) was noted, additional doses of 0.03 log intervals lower were tested until 0/6—1/6 responders were noted, starting at a dose of 50 mg/kg. These data were used to calculate ED_{50}'s by the procedure of Finney.[37]

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