PARTIALLY MODIFIED RETRO-INVERSO ENKEPHALIN ANALOGUES CONTAINING GLY$^3\Psi$(NH—CO)PHE^4 MODIFICATION, CONFIGURATIONAL LABILITY*

Michael CHOREV[a], Noemy GILON[a], Eli ROUBINI[a], Elizabeth ACKERMAN[b], Dina LEVIAN-TEITELBAUM[c] and Marta ROSIN[d]

[a] *Department of Pharmaceutical Chemistry, School of Pharmacy, The Hebrew University, P.O. Box 12065, Jerusalem 91120, Israel*
[b] *Department of Anaesthesiology, Hadassah Medical Center, Jerusalem, Israel*
[c] *Department of Organic Chemistry, The Hebrew University of Jerusalem, Israel and*
[d] *Department of Pharmacology, School of Pharmacy, The Hebrew University of Jerusalem, Israel*

Received February 15th, 1988
Accepted May 10th, 1988

Dedicated to the memory of Dr Karel Bláha.

Two partially modified retro-inverso analogues of Leu-enkephalin in which the peptide bond between Gly^3-Phe^4 was reversed resulted from the incorporation of the Gly$^3\psi$(NH CO)Phe^4 unit. The presence of racemic 2-benzylmalonyl residue led to diastereomeric mixtures of analogues: H-Tyr-D-Ala-*g*Gly-(RS)-*m*Phe-Leu-NH_2 (*I*) and H-Tyr-Gly-*g*Gly-(RS)-*m*Phe-Leu-NH_2 (*II*). Analogue *I* was more potent than analogue *II* in the binding assay, induced guinea pig ileum assay as well as in antinociceptive tests. Both activities were naloxone reversible. No analgesia was observed after subcutaneous (SC) administration of 100 mg kg of either *I* or *II*. The problems related to configurational lability of the 2-alkylmalonyl residues in partially modified retro-inverso analogues is addressed.

Backbone modifications of the amide bond of biologically active peptides are designed either to introduce metabolic stability or to probe the role of

* Abbreviations used are according to IUPAC–IUB Joint Commission on Biochemical Nomenclature (1983) published in Int. J. Pept. Protein Res. *24*, 1 (1984). The following special abbreviations for partially modified retro-inverso peptides are used. The standard three-letter notation for amino acid residues preceded by the prefix *g* represents the gem-diaminoalkyl residue derived from the specified amino acid. The prefix *m* represents the malonic acid residue related to the amino acid residue specified by the three letter notation. The prefix *r* represents an amino acid residue bound in the reversed sense of direction. Configurational designation of the retro-inverso residues follows those of the amino acids. Further abbreviations: THF, tetrahydrofuran; HMPA, hexamethylphosphoramide; DMF, N,N-dimethylformamide; NMM, N-methylmorpholine; IBCF, isobutylchloroformate; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; HOBt, 1-hydroxybenzotriazole; EtOAc, ethyl acetate.

a particular peptide bond on the conformation and biological activity (see review by A. F. Spatola[1]). Detailed structure–activity relationship studies performed on the endogenous opioid peptides, the enkephalins, resulted in a better understanding of the structural moieties essential for potent biological activities[2]. Studies of the degradation patterns[3], by nonspecific exopeptidases as well as by the more specific dipeptidylcarboxypeptidase also known as "enkephalinase", had paved the way for a rational design of metabolically stable enkephalin analogues[4] and potent specific inhibitors of enkephalinase[5]. Since the enkephalins are small and relatively simple peptides they offered an attractive ground for the testing of novel peptide bond surrogates such as ψ[CH_2 - CH_2][6], ψ[CH_2 NH][6], ψ[CH_2 S][7], ψ[CH_2 O][8], ψ[(*E*)-or (*Z*) CH=CH][9,10], ψ[NH CO][11,12], ψ[CS NH][13] and ψ[CO CH_2][14].

The partial retro-inverso modification, which was introduced by some of us[15], was found to be in many cases an effective mode of backbone modification which resulted in potent analogues with protracted activity[11,12,16–18]. It offers a substantial topological resemblance to that of the parent peptide in the

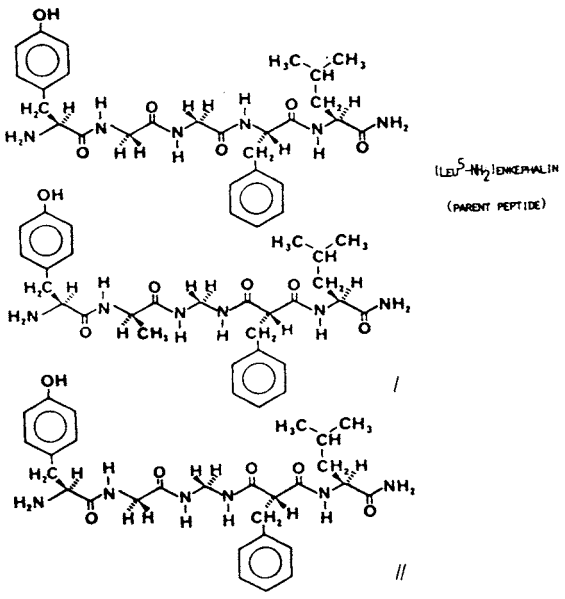


FIG. 1

Schematic representation of Leu-enkephalinamide (the parent peptide) and the two related partially modified retro-inverso analogues: [D-Ala^2, *g*Gly^3, (RS)-*m*Phe^4]- and [*g*Gly^3, (RS)-*m*Phe^4]-Leu-enkephaliamides *I* and *II* respectively, in their fully extended conformations

extended conformation and largely retains the spatial, electronic and hydrogen bond characteristics of the original peptide bond.

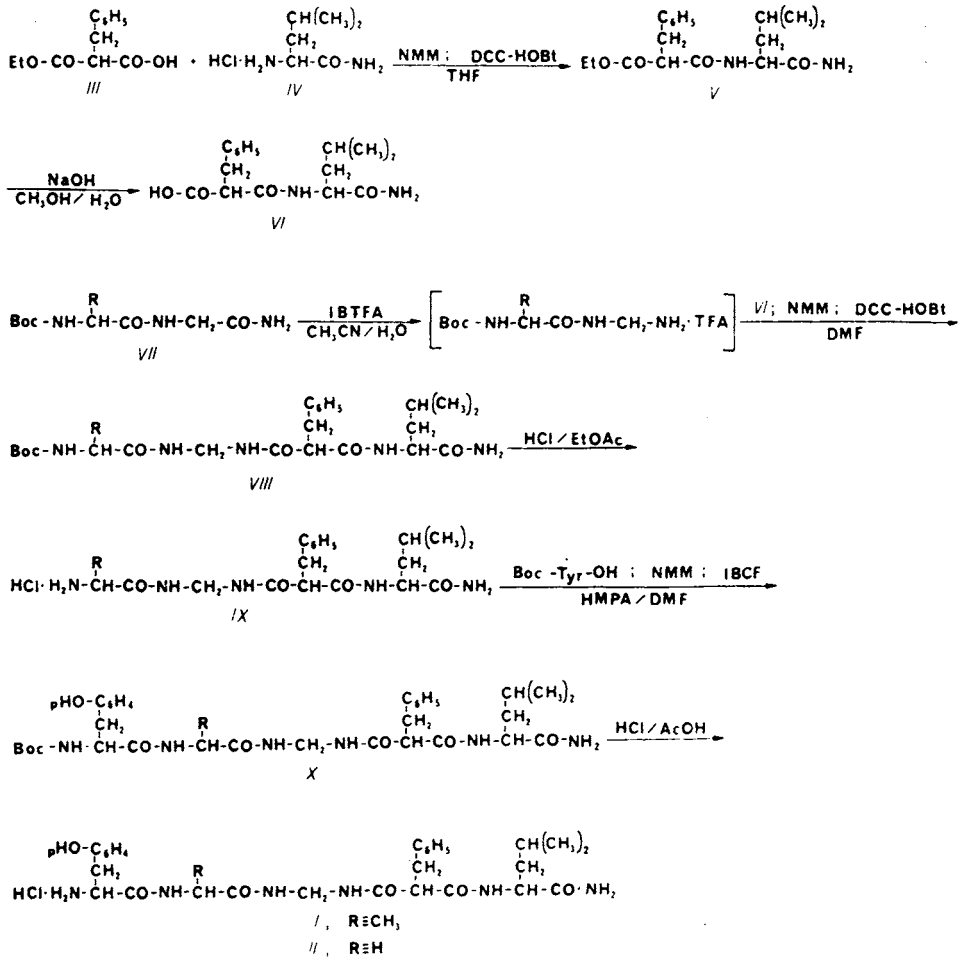
Partial retro-inverso modification of peptide bond Phe[4]-Leu[5] or Phe[4]-Met[5] in enkephalins resulted in highly potent analogues both in vitro and in vivo assays[11,12]. This peptide bond, which is proximate to the C-terminus was also found to tolerate other structural modifications such as N-methylation[19], methylenethio[7] and thioamide[13] replacements. On the other hand modifications at the peptide bond Gly[3]-Phe[4] by either N-methylation or its replacements by peptide bond surrogates such as trans C=C bond[10], ethylenic group[6], methyleneamino[6] and methylenethio[6] resulted in substantial loss of activities either in binding assay or in analgesia tests. According to Hudson's model, the carbonyl of this peptide bond aligned to a putative polar site, corresponds to the C-6 hydroxyl group of morphine and should play an important role in the interaction with the opiate receptor[6].

This paper describes the synthesis of two partially modified retro-inverso analogues of Leu-enkephalin which include the Gly[3]ψ[NH–CO]Phe[4] modification i.e. compounds *I* and *II* in Fig. 1. The issues addressed here include: (i) metabolic stability, (ii) the importance of this particular peptide bond to biological activity, and (iii) the configurational lability of the 2-alkylmalonyl residue in the ψ(NH–CO) modification.

RESULTS

Chemistry

Synthesis was designed in such a way that the gem-diaminoalkyl residue participating in the pseudopeptidic bond will be already monoacylated by either Ala[2] or Gly[2] residues prior to its in-situ formation from the corresponding t-butyloxycarbonyl-dipeptidyl amide *VIIa* and *VIIb*, respectively (see Scheme 1). Transformation of *VIIa* and *VIIb* to the corresponding TFA salt of monoacylated gem-diaminoalkyl derivative was accomplished by an oxidative rearrangement employing bis(trifluoroacetoxy)iodobenzene (IBTFA)[20,21]. Pallai and coworkers have shown that this route of rearrangement of a N-acyl aminoacylamide to the corresponding gem-diaminoalkyl derivative preserves the chiral integrity of the asymmetric center involved in the reaction[22]. The newly formed protonated monoacylated gem-diaminoalkyl residue was immediately coupled to the C-terminal fragment i.e. 2-benzylmalonylleucylamide (*VI*) employing DCC HOBt to avoid side-reactions observed in the case of the mixed-anhydride method, which was used otherwise in all the rest of the couplings. Moreover, avoiding the deprotection steps in which a urethane type protecting group on the nitrogen of gem-diaminoalkyl moiety is removed improves yields and reduces side-product formation[23,24].



In formulae *VII*–*X*: *a*, R = CH_3 *b*, R = H

SCHEME 1

Stereochemistry

Coupling of a racemic 2-alkylmalonyl residue such as ethyl hydrogen (RS)-2-benzylmalonate (*III*) with an optically active peptide or amino acid derivative as the hydrochloride of L-leucinamide (*IV*) gives rise to a diastereomeric mixtures. Reversed-phase high performance liquid chromatography (RP-HPLC) employing a C_{18}-column and a solvent system composed of

methanol–water could resolve diastereomeric mixtures of tetra and pentapeptides either in their protected or deprotected forms with base-line separation and selectivity constants better than 1.42 (see Table I).

In order to study the configurational lability of the diastereoisomers composing the mixtures and its implications on biological activity, they were resolved into their fast and slow moving components (F and S, respectively) by RP-HPLC, pooled and stored at 0°C. Fast isomerization was observed at 40°C

TABLE I
RP-HPLC characterization of the diastereomeric mixtures on Whatman 10-ODS-3 (250 × 4.6 ID mm) column, detection at 210 nm

Compound	Solvent system	k'(F) [a]	k'(S) [b]	α
I	A	3.0	5.17	1.72
II	A	3.0	5.6	1.87
VIIIa	W[c]	19.66	29.0	1.47
VIIIb	W	11.25	17.75	1.56
IXa	A[d]	2.0	5.0	1.5
IXb	A	1.75	4.25	2.43
Xa	W	6.0	8.5	1.42
Xb	W	5.0	8.14	1.63

[a] k'(F) is k' value of the fast moving component (diastereoisomer); [b] k'(S) is k' value of the slow moving component; [c] methanol–water (40:60), flow rate 2 ml/min; [d] methanol–0.05M-$AcONH_4$, pH 6.0, flow rate 1 ml/min.

TABLE II
Summary of the isomerization characteristics of the partially modified retro-inverso enkephalin analogues *I* and *II*

Isolated isomer[a]	$t_{1/2}$, h [b]	Composition of equilibrium mixture
I(S)	6.2	*I*(S)/*I*(F); 64.5 : 35.5
I(F)	8.9	
II(S)	4.8	*II*(S)/*II*(F); 60 : 40
II(F)	4.0	

[a] Isomerization initiated with 100% of the diastereomer at $t = 0$; [b] time interval required for the formation of 50 % of the equilibrium concentration of the newly formed diastereoisomer, measured at 40 °C.

leading to equilibrium mixtures. Same equilibrium mixtures were independently reached starting from either the F or S isomer. Kinetic parameters of these isomerizations are summarized in Table II. The isomerization profiles of the isolated F and S diastereoisomers of *II* are depicted in Fig. 2.

Fast isomerization was also observed for an isolated diastereoisomer of the protected pseudopentapeptide *Xb* in methanol–water at 30°C. In this case the final equilibrium was reached after about half an hour.

High Resolution ^{1}H NMR

An essential part of the characterization of synthetic intermediates and final enkephalin analogues was based on 300 MHz ^{1}H NMR analysis in hexadeuterodimethylsulfoxide. In this way direct information on the non-amino acid residues incorporated into different pseudopeptides could be obtained. This information is not available through amino acid analysis since the gem-diaminoalkyl residue decomposes upon acid hydrolysis and the malonyl residue does not react with ninhydrin. As we reported previously the gem-diaminoalkyl and malonyl residues have very characteristic chemical shifts which are markedly distinguished from those of the corresponding amino acid residues[25,26]. The C^{α}-protons of the gem-diaminoalkyl and the malonyl residues

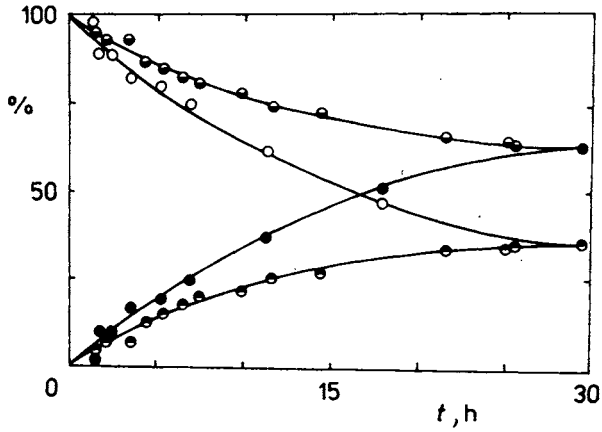


FIG. 2

Time course for the isomerization of an isolated slow and fast components of [D-Ala^2, gGly^3, (RS)-*m*Phe^4]-Leu-enkephalinamide *I*(S)(○) and *I*(F)(◒), respectively. Formation of the fast component *I*(F)(◓) and slow component *I*(S)(●) from the isolated slow and fast diastereoisomers, respectively; was followed, until equilibrium mixtures were reached, by RP-HPLC at 210 nm as described in the Experimental

are shifted relative to the C^{α}-protons of amino acid residues downfield and upfield, respectively. In Table III we have summarized the detailed ^1H NMR data for all the pseudopeptides described in this work.

Biological Activity

Opioid activity was determined by in vitro and in vivo assays. In vitro assays included a binding assay to a rat brain membrane preparation which measured displacement of [^{3}H]-naloxone and inhibition of electrically induced contraction of guinea pig ileum (GPI). In vitro results are summarized in Table IV. In the in vivo tests, analogues were administered intrathecally to rats. Tail pressure test was performed in order to assess analgesic potency of each of the compounds; results are summarized in Table V. The analgesic effect observed following administration of compounds *I* and *II* could be reversed by subcutaneous (SC) administration of 0.1 mg/kg of naloxone. High doses of partial retro-inverso analogues, included in this study, induced severe catatonia and loss of righting reflex which lasted for several hours. Subcutaneous administration of analogues *I* and *II* to mice, up to 100 mg/kg did not cause any observable analgesic effect as tested by the hot plate method. On the other hand, in an identical test, SC administration of 6 mg/kg of morphine was sufficient to cause a three-fold increase in response latency.

DISCUSSION

The somewhat higher biological potency of analogue *I*, which includes both D-Ala^2 and Gly$^3\psi$(NHCO)Phe^4 modifications, as compared to that of analogue *II*, which contains only the latter modification, may reflect differences in proteolytic resistance towards degradation by aminopeptidases. Binding studies reveal analogue *I* to have higher affinity for the opiate receptor than analogue *II*, which correlate well with the results obtained in the GPI. This is in line with the enhanced affinities of D-Ala^2 enkephalins and their amide analogues as compared to the native forms of enkephalin[27,28]. It is obvious that the partial retro-inverso modification diminished the affinities of analogues *I* and *II* to the opiate receptor as compared with [D-Ala^2, Met^5-NH$_2$]enkephalin. Linear or cyclic analogues containing a reversed peptide bond at Phe^4-Leu^5 were reported to have binding affinities of the same order of magnitude as their corresponding peptide analogues[29]. Thus the higher potency and much longer duration of analgesic activity, following intrathecal administration of analogues *I* and *II*, should be attributed to improved metabolic resistance to proteolytic degradation. Cleavage of the Tyr^1-Gly^2 peptide bond was postulated[30,31] and subsequently proven[32,33] to be a major non-specific deactivation pathway in

TABLE III
High resolution (300 MHz) ^{1}H NMR of peptide and pseudopeptide intermediates and the partially modified retro-inverso enkephalin analogs in hexadeuteriodimethylsulfoxide

Peptide	NH					α-CH						β-CH				γ-OH Leu	δ-OH Leu	OH Tyr	Arom.		NH_2	Boc	Misc.
	Tyr	D-Ala	Gly	*q*Gly	Leu	Tyr	D-Ala	Gly	*q*Gly	*m*Phe	Leu	Tyr	D-Ala	*m*Phe	Leu				Tyr	*m*Phe			
I					8.202					3.759	4.167			3.022	1.282	1.142	0.657				7.049		CH_3 1.103
																							CH_3–CH_3
															1.425		0.732				6.920		4.029
																							COOH
VI					8.135					3.668	4.126			2.991	1.276	1.081	0.647		7.211 –	7.054			12.625
																	0.725		7.170	6.930			
VIIa		7.106	8.024				3.920	3.559					1.172								7.157	1.374	
																					7.106		
VIIb			7.054[a]					3.555[a]													7.219	1.371	
			7.957[b]					3.626[b]													7.073		
VIIIa		6.844		8.460	7.962		3.949		4.390	3.476	4.148		1.139	3.000	1.331	1.236	0.751		7.243 –	7.146	1.368		
				8.374											1.433		0.678		7.164	7.003			
VIIIb			6.898	8.479	7.990			3.461	4.407	3.512	4.148			3.006	1.441	1.250	0.757		7.248 –	7.148	1.379		
				8.388													0.679		7.173	7.003			
IXa				8.988	8.218		3.768		4.450	3.577	4.135		1.320	2.988	1.454	1.234	0.754		7.254 –	7.143			H_3O^+ 8.175
				8.719											1.333		0.679		7.150	7.052			
IXb				8.964	8.204			3.496	4.455	3.573	4.140			3.001	1.446	1.250	0.762		7.254 –	7.146			H_3O^+ 8.175
				8.743	9.066										1.366				7.155	7.105			
Xa	6.858	7.989		8.512	7.935	4.075	4.240		4.387	3.484	4.156	2.801	1.096	2.955	1.430	1.236	0.760	9.142	7.017	7.240 –	7.143	1.320	
				8.463								2.629			1.349		0.681		6.635	7.143	7.000		
Xb	6.882		8.128	8.500	7.995	4.123		3.700	4.409	3.479	4.188	2.888		3.001	1.435	1.241	0.768	9.150	7.040	7.224 –	7.151	1.309	
				8.482	7.929								2.621			1.340		0.681		7.173	7.027		
I		8.021		8.630	7.990	3.360[c]	4.223		4.381	3.485	4.145	2.460[d]	1.102	2.998	1.430	1.239	0.765	9.177	6.658	7.243 –	7.146		AcO 1.907
				8.490	7.926							2.892			1.326		0.684		6.690	7.159	7.035		
II			8.131	8.566	7.995	3.380[c]		3.687	4.406	3.474	4.175	2.848		2.998	1.430	1.239	0.771	9.185	7.003	7.251 –	7.149		AcO 1.907
			8.503	7.929								2.450			1.330		0.676		6.667	7.089	7.057		

[a] Chemical shifts related to Gly^1 residue; [b] chemical shifts related to Gly^2 residue; [c] peak is located under the water peak (3.335 ppm); [d] peak is located under the DMSO peak (2.510 ppm).

vivo. Non-specific aminopeptidase activity either in blood[34] or in brain[35] was shown to be responsible for fast degradation of the endogenous enkephalins. Replacement of Gly^2 by D-Ala in [Met^5]enkephalin resulted in elevated potency and longer lasting in vivo analgesic activity following intracisternal administration of the enkephalin analogue[36], an effect of which was even more pronounced in the case of [D-Ala^2, Met^5-NH$_2$]enkephalin[37]. The higher potency and protracted activity of analogue *I* as compared to that of analogue *II* is probably due to the additional stability towards aminopeptidases which was absent in analogue *II*.

It is interesting to point out that in our previous study we reported a potent partially modified retro-inverso analogue of substance P related peptide i.e.

TABLE IV
Potencies of enkephalin analogues in the guinea pig ileum (GPI) assay and binding to rat brain membranes (RBM)

Compound	GPI		RBM	
	IC_{50} mol l^{-1}	relative potency[a]	IC_{50} mol l^{-1}	relative potency[a]
[D-Ala^2, *g*Gly^3, (*RS*)-*m*Phe^4, Leu^5-NH$_2$]enkephalin (*I*)	$2{\cdot}5\pm0{\cdot}2 \cdot 10^{-6}$	0·01	$6\pm0{\cdot}6 \cdot 10^{-6}$	0·015
[*g*Gly^3, (*RS*)-*m*Phe^4, Leu^5-NH$_2$]enkephalin (*II*)	$3{\cdot}0\pm0{\cdot}2 \cdot 10^{-5}$	0·001	$5\pm0{\cdot}5 \cdot 10^{-5}$	0·001 8
[D-Ala^2, Met^5-NH$_2$]enkephalin	$2{\cdot}9\pm0{\cdot}2 \cdot 10^{-8}$	1	$9\pm0{\cdot}9 \cdot 10^{-8}$	1
Morphine	$1{\cdot}4\pm0{\cdot}2 \cdot 10^{-7}$	0·21	–	–
Naloxone	–	–	$7\pm0{\cdot}8 \cdot 10^{-9}$	12·8

[a] Potency relative to [D-Ala^2, Met^5-NH$_2$]enkephalin (= 1).

TABLE V
Analgesic activity in rats after intrathecal administrations of partially modified retroinverso enkephalin analogues

Compound	Analgesic dose $ED_{50} \cdot 10^{-7}$, mol l^{-1}	Duration of analgesia, h	Onset time min
I	0·25	2–3	1–2
II	3·2	0·25	1–2
morphine	3·5	2·5–3	5

[pGlu^6, *g*Phe^8, *m*Gly^9]SP$_{6-11}$ which was found to be highly resistant to a broad spectrum of proteolytic enzymes as well as in sliced tissue preparations[38]. In that report a single retro-inverso modification confers resistance to proteolysis not only on the reversed peptide bond and the two adjacent peptide bonds, formed by non-amino acid residues, but also on amide bonds which are further removed from site of structural modification. Such a long-range stabilizing effect must involve either changes in the overall conformation to prevent effective interactions with the active sites of endopeptidases participating in degradation, or the elimination of an essential binding site in the substrate. Evidently, this is not the case with [*g*Gly^3, (RS)-*m*Phe^4, Leu^5-NH$_2$]enkephalin (*II*) in which Tyr^1-Gly^2, is separated by only one peptide bond from the reversed (NH—CO) bond and which remains susceptible to proteolysis. We conclude therefore that the extent of proteolytic resistance induced by a single retro-inverso modification on peptide bonds removed from the site of modification is probably sequence dependent.

The lack of analgesic activity following SC administration of analogues *I* and *II* even in large doses (100 mg/kg) may reflect the failure of these analogues to pass the blood-brain barrier.

Testing a mixture of separable (see Table I) diastereoisomers in different bio-assays to establish structure–activity relationship deserves a due justification. In light of the high configurational lability of the chiral center in the 2-benzylmalonyl residue incorporated at position 4 in the partially modified retro-inverso enkephalins *I* and *II*, established through studies of the kinetics of the isomerization of the separated diastereoisomers (see Table II), testing of the isolated diastereoisomers has not been attempted. The enhanced metabolic stability of analogues *I* and *II* which results in protracted in vivo activity may also allow the isomerization of separated diastereoisomers into equilibrium mixtures typical to the microenvironment in which it will take place. Fast rate of isomerization (half life time for the formation of the steady-state equilibrium from an isolated diastereoisomer is in the range of few hours) was also observed by us in the case of partially modified retro-inverso analogue of substance P related peptide i.e. [pGlu^6, *g*Phe^7, (RS)-*m*Phe^8]SP$_{6-11}$ (ref.[39]). Pallai et al. reported that H$_2$N-(S)-*m*Phe-Val-OH isomerized only to 1.7% after 1 hour and to 22.7% after 21 hours, which they considered as a slow enough process to be ignored in most of the biological assays[24]. On the other hand the same authors reported a very fast isomerization for the separated diasteroisomers of [Ala^4, *g*Phe^7, D-*r*Trp^8, D-*r*Thr^{10}, (RS)-*m*Phe^{11}]somatostatin upon incubation at 34°C in ammonium acetate buffer (40% of the initial isomer had been transformed into the other isomer within 1 hour)[40]. All the above mentioned cases contain the same malonyl residue namely *m*Phe, however they differ in the sequence in which this residue is located. Isomerization can take place in neutral as well as

charged sequences, and in water–methanol or buffered solutions. Thus, we conclude that the isomerization results via a general acid-base catalysis and is sequence dependent and probably residue dependent. A tentative mechanism in which water molecules play the role of general acid-base catalysis was suggested by us recently[39].

We feel that the configurational lability of a malonyl residue in a given sequence has to be established for each newly prepared partially modified retro-inverso peptide analogue in order to evaluate the need for separation of diasteroisomers and characterization of their biological activities.

In light of the configurational lability of the malonyl residues in pseudopeptides containing them, it seems impractical, as proposed by Pallai and coworkers, to separate a diastereomeric mixture of pseudopeptidic intermediates containing malonyl residue, assign their configuration and, to complete the synthesis with the separated diastereoisomers[41].

The retention of substantial biological potencies in [D-Ala^2, *g*Gly^3, (RS)-*m*Phe^4, Leu^5-NH$_2$]enkephalin (*I*) as compared to the relatively low activities observed for other types of peptide bond surrogates at the same location[6,7,10], implies that this partial retro-inverso modification retains to a large extent the characteristics necessary for an efficient interaction with the opioid receptor. Interestingly, reversal of the Gly^3-Phe^4 amide bond should interfere with the anticipated alignment of the corresponding carbonyl to a polar site at the receptor, which is considered by Hudson et al. to be essential for an effective interaction[6].

The results of this study suggest that introduction of a partial retro-inverso modification in a single peptide bond is a rewarding effort. Replacement of the amide bond Gly^3-Phe^4 by a reversed amide bond led to a potent opiate agonist with protracted in vivo activity. Since the retro-inverso modification decreases the affinity for opiate receptors, as assigned through binding studies, the major consequences of this modification are probably the enhancement of metabolic stability towards proteases. The intriguing implications of configurational lability as a potential mechanism for achieving receptor–ligand complementarity are very exciting. Ongoing work in our laboratories addresses itself to the introduction of partial retro-inverso modification to other bioactive peptides and aims to delineate the relationship between the nature of the malonyl residue and its location within sequence to the degree of configurational lability.

EXPERIMENTAL

Melting points were taken on a Thomas Hoover capillary melting point instrument and are uncorrected. Optical rotations were measured on a Perkin Elmer 141 polarimeter in a 10 cm water-jacketed cell. HPLC analysis was performed on a Tracor 950 liquid chromatograph equipped with Tracor 970A variable-wavelength detector and Tracor 980A solvent programmer. During

HPLC analysis peptides were monitored either at 210 or at 254 nm. A Whatman 10-ODS-3 column (0.46 × 25 cm) was used in all analytical separations. TLC were performed on 0.25 mm Polygram silica gel plates UV 254 (Macherey–Nagel), using the following solvent systems: (A) $CHCl_3$–MeOH (4 : 1), (B) $CHCl_3$–MeOH (9 : 1), (C) BuOH–AcOH–H_2O (4 : 1 : 5, upper phase). The plates were developped with spray reagents: 0.1% ninhydrin (Merck) or fluorescamine (Fluram, Hoffman La Roche) and/or with iodine vapors. For chlorination test a modified Reindel–Hoppe procedure was used[42] on precoated TLC aluminium plates.

Amino acid analysis was performed on LKB-4400 amino acid analyzer equipped with a Spectra-Physics SP-4100 printer-plotter computing integrator using a 4-component sodium buffer system and a standard 70 min programme. Potassium-cationized field-desorption (FD) mass spectroscopy was performed on a Varian CH5-DF mass spectrometer equipped with an EI, FI, and FD ion sources. Fast atom bombardment mass spectrometry (FAB-MS) was taken by Prof. H. Schwartz at the Technical University in Berlin. ^1H NMR spectra were measured on a Bruker 300 MHz spectrometer, in $(CD_3)_2SO$ (99.9% D), and chemical shifts are reported relative to tetramethylsilane as internal standard.

Preparative column chromatography was used extensively to purify crude products. Open column chromatography was run on silica gel 60 (70 – 230 mesh, Merck). High performance low pressure liquid chromatography (HPLPLC), at 1 300 – 2 000 Pa, was run on silica gel 60 (230 – 400 mesh, Merck). The effluent was monitored at 254 nm by an ISCO-UA5 multiwavelength absorbance monitor.

Solvents were purified in the following way: $CHCl_3$, DMF and HMPA were distilled from P_2O_5. DMF was then redistilled from ninhydrin. Glacial acetic acid was distilled from $B(OAc)_3$. THF, toluene and methanol were distilled from CaH_2.

Peptides were prepared by the liquid-phase methods using Boc and amides or ethyl esters as amino and carboxy protecting groups, respectively. Coupling methods employed were the following: *A*) DCC with HOBt. *B*) the excess mixed anhydride method using IBCF and NMM. The general procedures for both methods have been described in detail in a previous report[17]. Boc protected compounds were treated with either *a*) dry HCl in EtOAc or *b*) HCl in glacial acetic acid either in presence or absence of 1% thioanisole[17].

Ethyl Hydrogen (RS)-2-Benzylmalonate (*III*)

To a stirred solution of diethyl 2-benzylmalonate (20 g, 80 mmol) in ethanol (160 ml) at 0 °C was added dropwise 4 M-NaOH (18 ml) during 12 h. The residue obtained after removal of ethanol under reduced pressure was taken in water (75 ml) and extracted with EtOAc (30 ml). The aqueous phase was acidified with HCl (c) to pH 2 and extracted with EtOAc (4 × 100 ml). The combined EtOAc washings were extracted with brine and dried over $MgSO_4$. Removal of EtOAc under reduced pressure gave the oily monoethyl ester *III*: yield 15 g (84.4%); TLC R_f 0.42 (A); MS (*m z*): 222 $(M)^+$, 176 $(M - CO_2H)^+$, 148 $(M - CO_2Et)^+$, 131 $(M - C_6H_5CH_2)^+$, 91 $(C_7H_7)^+$. For $C_{12}H_{14}O_4$ (222.44) calculated: 64.85% C, 6.35% H; found: 65.14% C, 6.85% H.

EtO-(RS)-*m*Phe-Leu-NH_2 (*V*)

The carboxyl component *III* (4.82 g, 21.7 mmol) and the leucinamide hydrochloride (*IV*) (3.0 g, 18.07 mmol) were dissolved in dry THF (90 ml). To this solution were added NMM (2.0 ml, 18.1 mmol) and HOBt (3.66 g, 27.1 mmol) followed by DCC (4.1 g, 20.1 mmol). Reaction mixture was stirred at room temperature for 24 h, after which it was worked up in a standard fashion. The organic extracts were dried over $MgSO_4$, concentrated under reduced pressure, and stored overnight at 0 °C. The formed precipitate was filtered off. Addition of petroleum ether to the filtrate formed

a precipitate which was collected and dried under vacuum over P_2O_5. Yield 5.6 g (92.8%); m.p. 110 °C; TLC R_f 0.59 (A), 0.56 (B); MS (*m/z*): 334 (M)$^+$, 317 (M − NH_3)$^+$, 290 (M − $CONH_2$)$^+$, 261 (M − CO_2Et)$^+$. For $C_{18}H_{26}N_2O_4$ (334.42) calculated: 64.65% C, 7.84% H, 8.37% N; found: 64.91% C, 7.86% H, 8.71% N.

HO-(RS)-*m*Phe-Leu-NH_2 (*VI*)

To a solution of compound *V* (2.9 g, 8.7 mmol), a solution of 0.3 M-NaOH (30 ml) was added dropwise during 20 min. The reaction mixture was stirred at room temperature for 30 min. The solution was concentrated in vacuo, diluted with water (100 ml), extracted with EtOAc (3 × 50 ml) and acidified to pH 2 with 1 M-HCl. The product was collected from the ice cold mixture, washed thoroughly with cold distilled water, dried under vacuum over P_2O_5. Yield 2.5 g (95%); m.p. 157 °C; TLC R_f 0.47 (A), 0.21 (B); MS (*m/z*): 262 (M − CO_2)$^+$, 246 (262 − NH_2)$^+$, 218 (246 − CO)$^+$, 91 (C_7H_7)$^+$. For $C_{16}H_{22}N_2O_4.1\,2\,H_2O$ (315.37) calculated: 60.94% C, 7.35% H, 8.88% N; found: 60.40% C, 7.48% H, 9.36% N.

Boc-D-Ala-Gly-OEt

To a solution of Boc-D-Ala-OH (8.25 g, 45 mmol) in DMF (50 ml) were added consecutively NMM (4.6 ml, 42 mmol) and IBCF (5.5 ml, 42 mmol). To this mixture was added solution of HCl.GlyOEt (8.4 g, 45 mmol) and NMM (4.95 ml, 45 mmol) in DMF (50 ml). Coupling proceeded at −25 °C for 2 h after which it was worked up in a usual manner. The protected dipeptide was obtained as an oil. Yield 14.4 g (98.8%); $[\alpha]_D$ +25.6 (*c* 1.0, AcOH); TLC R_f 0.59 (A), 0.57 (B); MS (*m/z*): 274 (M)$^+$, 201 (M − CO_2Et)$^+$, 173 (M − Boc)$^+$. For $C_{12}H_{22}N_2O_5$ (274.32) calculated: 52.54% C, 8.08% H, 10.21% N; found: 52.57% C, 8.07% H, 10.80% N.

Boc-D-Ala-Gly-NH_2 (*VIIa*)

Dipeptide ester Boc-D-Ala-Gly-OEt (7.1 g, 25.5 mmol) was treated with saturated methanolic ammonia (200 ml) for 12 h. Removal of the solvent under reduced pressure gave white crystals which were dried under vacuum over P_2O_5 and H_2SO_4 (c) to yield *VIIa*. Yield 6 g (95%); m.p. 133 °C; $[\alpha]_D$ 11.9 (*c* 1.0, AcOH); TLC R_f 0.19 (B); MS (*m/z*): 246 (M)$^+$, 186 (M − tBu)$^+$, 173 (M − tBuO)$^+$, 145 (M − Boc)$^+$. For $C_{10}H_{19}N_3O_4$ (245.28) calculated: 48.97% C, 7.81% H, 17.31% N; found: 48.71% C, 7.57% H, 17.57% N.

Boc-D-Ala-*g*Gly-(RS)-*m*Phe-Leu-NH_2 (*VIIIa*)

To a stirred suspension of amide *VIIa* (1.27 g, 5.2 mmol) in a mixture of acetonitrile–water (64 ml, 5:3 v/v) was added iodobenzen-bistrifluoroacetate (IBTFA)[43] (2.52 g, 5.5 mmol). The mixture was stirred at room temperature until complete disappearance of starting material *VIIa*, as monitored by TLC (about 6 h). The solvent was removed under reduced pressure and the oily residue obtained was kept overnight under vacuum over P_2O_5. The yellow sticky solid was triturated twice with dry ether. The residue left from the trituration was dried under vacuum to yield the crude TFA salt of the acylated *g*Gly residue, as a white amorphous solid which was used without further purification in the following coupling step. The TFA salt was dissolved in DMF (23 ml) followed by the addition of the carboxy component *VI* (1.6 g, 5.2 mmol), NMM (0.57 ml, 5.2 mmol), DCC (1.06 g, 5.2 mmol) and HOBt (0.70 g, 5.2 mmol). Coupling proceeded at room temperature for 24 h after which it was worked up in a usual manner. The crude product was purified on HPLPLC silica gel column (2.5

× 40 cm) and eluted by a gradient of MeOH in $CHCl_3$ (0 to 10%). The fraction that eluted around 7% MeOH in $CHCl_3$ was pooled and rechromatographed on the same column using an identical solvent gradient. The pure pseudotetrapeptide *VIIIa* was obtained as white solid. Yield 1.4 g (53.2%); TLC R_F 0.58 (A), 0.36 (C); FD-MS (*m z*): 545 (MH + K)$^+$, 506 (MH)$^+$. For $C_{25}H_{39}N_5O_6$ (505.62) calculated 59.39% C, 7.77% H, 13.85% N; found 59.17% C, 7.66% H, 13.80% N.

HCl.H-D-Ala-*g*Gly-(RS)-*m*Phe-Leu-NH_2 (*IXa*)

Deprotection of the pseudotetrapeptide *VIIIa* was carried out with HCl in EtOAc. The hydrochloride pseudotetrapeptide *IXa*: yield 1.0 g (81.7%); m.p. 226°C; TLC R_f 0.34 (C); FAB-MS (*m/z*): 406 (MH, free base)$^+$, 389 (406 − NH_3)$^+$, 361 (406 − $CONH_2$)$^+$, 318 (406 − H-Ala-NH)$^+$, 306 (406 − H-Ala-$NHCH_2$)$^+$, 289 (406 − H-Ala-*g*Gly)$^+$, 261 (289 - CO)$^+$, 131 (H_2-Leu-NH_2)$^+$, 101 (H-Ala-NH-CH_2)$^+$. For $C_{20}H_{32}ClN_5O_4$ (441.96) calculated 54.32% C, 7.29% H; found 54.16% C, 7.16% H.

Boc-Tyr-D-Ala-*g*Gly-(RS)-*m*Phe-Leu-NH_2 (*Xa*)

To a solution of Boc-Tyr-OH (0.31 g, 1.0 mmol) in DMF (2 ml) were added NMM (120 µl, 1.08 mmol) and IBCF (142 µl, 1.08 mmol), followed by the deprotected pseudotetrapeptide *IXa* (0.48 g, 1.08 mmol) in HMPT–DMF (6 ml, 1:2 v/v). Coupling proceeded for 2 h at −25 C followed by the usual work up. The crude product (0.70 g) was chromatographed on HPLPLC silica gel column (2.5 × 40 cm), employing a solvent gradient of MeOH (0 to 10%) in $CHCl_3$. The fraction obtained around 5% MeOH in $CHCl_3$ was rechromatographed on a LH-20 column (1.5 × 150 cm) and eluted with 45% MeOH in water. The major peak was pooled and lyophilized to give the pure protected pseudopentapeptide *Xa*. Yield 0.31 g (51%); TLC R_F 0.62 (A), 0.28(B); FAB-MS (*m z*): 691 (M + Na)$^+$, 669 (MH)$^+$, 569 (MH − Boc)$^+$, 406 (569 − Tyr)$^+$, 335 (406 − Ala)$^+$, 306 (335 − CH_2NH)$^+$. For $C_{34}H_{48}N_6O_8$ (668.8) calculated 61.07% C, 7.23% H; found: 59.52% C, 7.47% H.

AcOH.H-Tyr-D-Ala-*g*Gly-(RS)-*m*Phe-Leu-NH_2 (*I*)

Protected pseudopentapeptide *Xa* (65 mg, 0.097 mmol) was deprotected in 1 M-HCl in AcOH. The crude pseudopentapeptide *I* (55 mg, 94.3%) was purified by partition chromatography on Sephadex G-25 column (2.5 × 100 cm) employing a two phase solvent system which consisted of BuOH–AcOH–H_2O (4:1:5, v/v). The main fraction was lyophilized. The enkephalin analogue *I* was obtained as a fine powder. Yield 28.8 mg (49.5%); TLC R_f 0.55 (C); FAB-MS (*m z*): 569 (MH acetate free)$^+$, 406 (569 − Tyr)$^+$. Amino acid analysis: Tyr 1.02, Ala 1.00, Leu 1.02.

Boc-Gly-Gly-OEt

Boc-Gly-OH (5.0 g, 28.5 mmol) in THF (70 ml) was treated with IBCF (3.5 ml, 26.5 mmol) in presence of NMM (2.92 ml, 26.5 mmol). A mixture of HCl.H-Gly-OEt (4.88 g, 35 mmol) and NMM (3.86 ml, 35 mmol) in DMF (70 ml) was added to the reaction mixture. Coupling proceeded for 2 h at −25 C and it was worked up in a standard fashion. Residue obtained after removal of EtOAc under reduced pressure yielded the protected dipeptide as an oily product. Yield 6.5 g (94.2%); TLC R_F 0.69 (A), 0.54 (B); MS (*m/z*): 260 (M)$^+$, 203 (M − tBu)$^+$, 187 (M − tBuO)$^+$, 158 (M − Boc)$^+$, 130 (158 − NH–CH_2)$^+$. For $C_{11}H_{20}N_2O_5$ (260.29) calculated: 50.76% C, 7.74% H, 10.76% N; found: 50.47% C, 7.90% H, 10.48% N.

Boc-Gly-Gly-NH_2 (*VIIb*)

Boc-Gly-Gly-OEt (6.2 g, 23.8 mmol) was treated with a saturated methanolic ammonia (200 ml) for 12 h. Removal of the solvent under reduced pressure gave the amide *VIIb* as white crystals which were dried under vacuum over P_2O_5 and H_2SO_4 (c). Yield 5.25 g (95.2%); m.p. 129 – 130 C; TLC R_f 0.37 (A), 0.26 (B); MS (*m z*): 231 (M)$^+$, 174 (M – tBu)$^+$, 130 (M – Boc)$^+$, 115 (130 – NH)$^+$, 101 (115 – CH_2)$^+$, 182 (M – CO-NH_2)$^+$. For $C_9H_{17}N_3O_4$ (231.26) calculated: 46.75% C, 7.41% H; found: 49.60% C, 7.57% H.

Boc-Gly-*g*Gly-(RS)-*m*Phe-Leu-NH_2 (*VIIIb*)

To a stirred suspension of amide *VIIb* (1.4 g, 6.0 mmol) in a mixture of acetonitrile–water (75 ml, 3:2, v v) was added IBTFA (2.6 g, 6.0 mmol). The mixture was stirred at room temperature untill complete disappearance of starting *VIIb*, as monitored by TLC (about 6 h). Workup of reaction mixture followed the procedure outlined for compound *VIIIa*. The TFA salt which was obtained as an amorphous solid was used in the following coupling step without further purification. The TFA salt obtained in the preceeding step was dissolved in DMF (30 ml) followed by consecutive addition of the carboxyl-component *VI* (1.84 g, 16 mmol), NMM (0.67 ml, 6.0 mmol), HOBt (0.81 g, 6.0 mmol) and DCC (1.24 g, 6.0 mmol). Coupling proceeded for 24 h at room temperature. Residue obtained after removal of solvent under reduced pressure was redissolved in methanol and passed through an Amberlyst A-21 (OH-form), ion exchange resin. The crude residue obtained after removal of solvent under reduced pressure (2.8 g, 95%) was purified on HPLPLC silica gel column (2.5 × 40 cm) employing a solvent gradient (0 – 10%) MeOH in $CHCl_3$. Removal of solvents from the pooled fractions of the major peak which eluted at about 7% $CHCl_3$ yielded the pure protected pseudotetrapeptide *VIIIb*. Yield 1.77 g (60%); TLC R_f 0.74 (A), 0.42 (B); FAB-MS (*m/z*): 514 (MH + Na)$^+$, 492 (MH)$^+$, 435 (MH – tBu)$^+$, 419 (MH – tBuO)$^+$, 392 (MH – Boc)$^+$, 376 (M – Boc-NH)$^+$, 334 (M – Boc-Gly)$^+$, 319 (334 – NH)$^+$, 290 (419 – Leu-NH_2)$^+$, 262 (290 – CO)$^+$. Amino acid analysis: Gly 1.06, Leu 1.00. For $C_{24}H_{37}N_5O_6$ (491.59) calculated: 58.64% C, 7.59% H, 14.25% N; found: 58.44% C, 7.68% H, 14.01 % N.

HCl.H-Gly-*g*Gly-(RS)-*m*Phe-Leu-NH_2 (*IXb*)

Deprotection of the pseudotetrapeptide *VIIIb* (0.40 g, 0.81 mmol) was carried out with HCl in EtOAc. The hydrochloride salt *IXb* was obtained as a solid. Yield 0.34 g (79%); TLC R_f 0.36 (C); FAB-MS (*m z*): 392 (MH free base)$^+$, 347 (391 – $CONH_2$)$^+$, 318 (391 – H-Gly-NH)$^+$, 288 (391 – H-Gly-*g*GlyH)$^+$, 261 (391 – H-Gly-*g*Gly-CO)$^+$.

Boc-Tyr-Gly-*g*Gly-(RS)-*m*Phe-Leu-NH_2 (*Xb*)

To a solution of Boc-Tyr-OH (0.40 g, 1.42 mmol) in THF (4.5 ml) were added NMM (150 µl, 1.35 mmol) and IBCF (176 µl, 1.35 mmol), followed by the deprotected pseudotetrapeptide *IXb* (0.56 g, 1.31 mmol). Coupling proceeded for 2 h at –25 C after which it was worked up in a usual manner. The pure protected pseudopentapeptide *Xb* was obtained as a white solid. Yield 725 mg (83.5%); TLC R_f 0.51 (A), 0.72 (C); FAB-MS (*m z*): 677 (MNa)$^+$, 655 (MH)$^+$, 555 (MH – Boc)$^+$, 392 (MH – Boc-Tyr)$^+$, 319 (M – *g*Gly-Phe-Leu-NH_2)$^+$, 306 (MH – Boc-Tyr-Gly-NH-CH_2)$^+$, 262 (M – Gly-*g*Gly-Phe-Leu-NH_2)$^+$, 263 (MH – Boc-Tyr-Gly-*g*Gly-CO)$^+$. For $C_{33}H_{46}N_6O_8$ (654.77) calculated: 60.53% C, 7.08% H, 12.83% N; found: 60.50 % C, 7.31% H, 12.81% N.

AcOH.H-Gly-*g*Gly-(RS)-*m*Phe-Leu-NH_2 (*II*)

Protected pseudopentapeptide *Vb* (120 mg, 0.18 mmol) was deprotected with 1 M-HCl in AcOH. The crude pseudopentapeptide *II* (89 mg, 90.4%) was subjected to a purification procedure identical to the one described in the preparation of analogue *I*. The enkephalin analogue *II* was obtained as a fine powder. Yield 30 mg (30.5%). FAB-MS (*m z*): 555 (MH free base)$^+$, 392 (555 – Tyr)$^+$, 278 (555 – H-Phe-Leu-NH_2)$^+$, 131 (H_2-Leu-NH_2)$^+$. Amino acid analysis: Tyr 1.05, Gly 0.98, Leu 1.00.

Biological Assays

Inhibition of electrically induced contraction of the GPI: Briefly, male guinea pigs (300 to 400 g) were killed by cervical dislocation. The small intestine was dissected out and the lumen washed with 20 ml of Krebs solution. A segment of ileum (2–3 cm) 10 cm from the ileocecal junction was mounted under 0.5 g tension in a 15 ml organ bath containing thermostated (37 C) oxygenated (95% O_2, 5% CO_2) Krebs solution of the following composition, mmol l^{-1}: NaCl 118; $CaCl_2$ 2.54; KCl 4.75; KH_2PO_4 1.19; $MgCl_2$ 1.2; $NaHCO_3$ 25; glucose 11.2. A Grass apparatus stimulator delivered repetitive transmural stimulations, through platinum wire electrodes, consisting of rectangular pulses (40 V, 0.05 Hz, 0.5 msec duration). Contractions were recorded with a Hugo Sachs Mark isotonic transducer connected to a Hugo Sachs VH linear recorder. Stock solutions of the opiate agonist were prepared in DMSO and all dilutions were made with double distilled water. The concentration of DMSO in the organ bath did not exceed 0.04%. Determination in the reduction of the twitch height at various doses permitted the construction of log dose-response curves. Log dose-response curves were determined with [D-Ala^2, Met^5]enkephalinamide (DAMEA) and morphine as standards for the ileum preparation. IC_{50} values of the enkephalin analogues were determined and compared to those of morphine and DAMEA.

Binding studies: Rat brain (less *cerebellum*) preparations were carried out essentially as reported elsewhere[41], measuring the displacement of [^{3}H]naloxone, except that membranes were prepared without using discontinuous sucrose gradient purification step. Incubations were performed in sodium-free buffer (20 mM Tris HCl, pH 7.4) at 37 C for 25 min, the medium containing 15 µl of washed membranes, [^{3}H]naloxone (40 Ci mmol, 10 nmol) the inhibitory cold ligand and enough buffer to make a total volume of 100 µl. Specific [^{3}H]naloxone binding accounted for 60–65% of total [^{3}H]naloxone binding. Reported IC_{50} values represent the concentration of inhibitor that caused half-maximal inhibition of specific binding. All experiments were carried out in duplicates and repeated at least three times. The in vitro results are summarized in Table IV.

Analgesis tests: Rats (200–250 g males, Wistar) were prepared under pentobarbitone anesthesia, with polyethylene catheters (PE10) which were introduced into the subdural space above the lumber regions of the spinal cord and advanced 2 cm towards the lumber region. The catheter was attached to the exposed laminal with acrylic bone cement and the muscle and skin sutured. Two to three days later, the response of the animals to painful stimulus was assessed by the Randall-Selitto method, in which increasing degrees of pressure were applied to the tail. The pressure (in arbitrary units, AU) at which a struggle or squeak was obtained was taken as the "Pain threshold". The cutoff point of pressure was 30. Morphine and enkephalin analogues or saline were injected into the catheter in a total volume not exceeding 0.1 ml (including wash-in with saline). Reaction of the rat to pressure assessed every 5 min for 15 min and at 30 min, after injection. All doses were expressed as µg rat.

REFERENCES

1. Spatola A. in: *Chemistry and Biochemistry of Amino acids, Peptides and Proteins* (B. Weinstein, Ed.). Vol. 7, p. 267. Dekker, New York 1983.
2. Morley J. S.: Ann. Rev. Pharmacol. Toxicol. *20*, 81 (1980).
3. Malfroy B., Swerts J. P., Guyon A., Roques B. P., Schwartz J. C.: Nature *276*, 523 (1978).
4. Roemer D., Buescher H. H., Hill R. C., Pless J., Bauer W., Cardinaux F., Closse A., Hauser D., Huguenin R.: Nature *268*, 547 (1977).
5. Roques B. P., Fournier-Zaluski M. C., Soroca E., Lecomte J. M., Malfroy B., Llorens C., Schwartz J. C.: Nature *288*, 286 (1980).
6. Hudson D., Sharpe R., Szelke M.: Int. J. Pept. Protein Res. *15*, 122 (1980).
7. Spatola A. F., Saneii H., Edwards J. V., Bettag A. L., Anwer M. K., Rowell P., Browne B., Lahti R., von Voigtlander P.: Life Sci. *38*, 1243 (1986).
8. Rubini E., Gilon C., Levian-Teitelbaum D., Selinger Z., Weinstock-Rosin M., Chorev M. in: *Peptides 1984* (U. Ragnarsson, Ed.), p. 137. Almquist & Wiksell, Stockholm 1984.
9. Hann M. M., Sammes P. G., Kennewell P. D., Taylor J. B.: J. Chem. Soc., Perkin Trans. 1, *1982*, 307.
10. Cox M. T., Gromley J. J., Hayward C. F., Peter N. N.: J. Chem. Soc., Chem. Commun. *1980*, 800.
11. Chorev M., Shavitz R., Goodman M., Minick S., Guillemin R.: Science *204*, 1210 (1979).
12. Berman J. M., Jenkins N., Hassan M., Goodman M., Nguyen T. M. D., Schiller P. W. in: *Peptides: Structure and Function* (V. J. Hruby and D. H. Rich, Eds), p. 283. Pierce Chem. Corp., Rockford IL. 1983.
13. Clausen K., Anwer M. K., Bettag A. L., Benovitz D. E., Edwards J. V., Lawesson S. O., Spatola A. F. in: *Peptides: Structure and Function* (V. J. Hruby and D. H. Rich, Eds), p. 307. Pierce Chem. Corp., Rockford IL. 1983.
14. Almquist R. G., Olsen C. M., Uyeno E. T., Toll L.: J. Med. Chem. *27*, 115 (1984).
15. Goodman M., Chorev M.: Acc. Chem. Res. *12*, 1 (1979).
16. Goodman M., Chorev M. in: *Perspectives in Peptide Chemistry* (A. Eberle, R. Geiger and T. Wieland, Eds), p. 283. Karger, Basel 1981.
17. Chorev M., Rubini E., Gilon C., Wormser U., Selinger Z.: J. Med. Chem. *26*, 129 (1983).
18. Salvadori S., Balboni G., Marastoni M., Sarto G., Tomatis R. in: *Peptides 1984* (U. Ragnarsson, Ed.), p. 309. Almquist & Wiksell, Stockholm 1984.
19. Gesellchen P. D., Frederickson R. C. A., Tafur S., Smiley D. in: *Peptides: Synthesis, Structure, Function* (D. H. Rich and E. Gross, Eds), p. 621. Pierce Chem. Corp., Rockford IL. 1981.
20. Swaminathan K., Venkatasubranian N.: J. Chem. Soc., Perkin Trans. 2, *1975*, 1161.
21. Radhakrishna A. S., Pharham M. E., Riggs R. M., Loudon G. M.: J. Org. Chem. *44*, 1746 (1979).
22. Pallai P., Goodman M.: J. Chem. Soc., Chem. Commun. *1982*, 280.
23. Rubini E., Chorev M., Gilon C., Friedman Z. Y., Wormser U., Selinger Z. in: *Peptides: Synthesis, Structure, Function* (D. H. Rich and E. Gross, Eds), p. 593. Pierce Chem. Corp., Rockford IL. 1981.
24. Pallai P., Richman S., Struthers R. S., Goodman M.: Int. J. Pept. Protein Res. *21*, 84 (1983).
25. Ribiero A., Chorev M., Goodman M.: Biopolymers *22*, 1869 (1983).
26. Ribiero A., Jardetzky O., Gilon C., Teitelbaum D., Chorev M.: Biopolymers *22*, 247 (1983).
27. Coy D. H., Kastin A. J., Schally A. V., Morin O., Caron F., Walker J. M., Fertel R., Bernston G. G., Sandman C. A.: Biochem. Biophys. Res. Commun. *73*, 632 (1976).
28. Pert C. B., Bowie D. L., Fong B. T. W., Chang J. K. in: *Advances in Clinical Chemistry* (O. Bodanszky and A. L. Latner, Eds), Vol. 18, p. 79. Academic Press, New York 1976.

29. Berman J. M., Goodman M., Nguyen T. M. D., Schiller P. W.: Biochem. Biophys. Res. Commun. *115*, 864 (1983).
30. Pasternak G. W., Goodman R., Snyder S. H.: Life Sci. *16*, 765 (1975).
31. Hughes J.: Brain Res. *88*, 295 (1975).
32. Hambrook J. M., Morgan B. A., Rance J. M., Smith C. F. C.: Nature *262*, 782 (1976).
33. Meck J. L., Yang H. Y. Costa E.: Neuropharmacology *16*, 151 (1977).
34. Coletti-Previero M. A., Mattras H., Descamps B., Coletti-Previero A.: Biochim. Biophys. Acta *657*, 122 (1981).
35. Hersh L. B., McKelvy J. F.: J. Neurochem. *36*, 171 (1981).
36. Walker J. M., Bermston G. G., Sandman C. A., Coy D. H., Schally A. V., Kastin A. J.: Science *196*, 85 (1976).
37. Pert C. B., Pert A., Chang J. K., Fong B. T. W.: Science *194*, 330 (1976).
38. Chorev M., Rubini E., Hart Y., Gilon C., Wormser U., Selinger Z.: Eur. J. Pharmacol. *127*, 187 (1986).
39. Chorev M., Yaion M., Wormser U., Levian-Teitelbaum D., Gilon C., Selinger, Z.: Eur. J. Med. Chem., Chim. Ther. *21*, 96 (1986).
40. Pallai P., Richman S., Goodman M. in: *Peptides: Synthesis, Structure, Function* (D. H. Rich and E. Gross, Eds), p. 85. Pierce Chem. Corp., Rockford IL. 1981.
42. Von Arx E., Faupe M., Brugger M.: J. Chromatogr. *120*, 224 (1976).
43. Maletina I. I., Orda V. V., Yagupolskii L. M.: J. Org. Chem. (U.S.S.R.) *10*, 294 (1974).
44. Tolkovski A. M.: Mol. Pharmacol. *22*, 648 (1982).