CONFORMATIONALLY CONSTRAINED AND TRITIUM-LABELLED ANALOGUES OF ENDOMORPHINS

PhD Thesis

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I dedicate this thesis

to my mother, Elizabeth Hanzely, to whom I owe so much,

and to my wife, Andrea Hajnal, for all her understanding patience.

LIST OF PUBLICATIONS RELATED TO THE THESIS

[I] **Cs. Tömböly**, R. Dixit, I. Lengyel, A. Borsodi and G. Tóth "Preparation of Specifically Tritiated Endomorphins." *J. Label. Compds. Radiopharm.* **44**, 355-363 (2001)

[II] **Cs. Tömböly**, M. Spetea, A. Borsodi and G. Tóth "Synthesis of Tritium Labelled Endomorphin-2 and its Stability in the Radioreceptor Assay." *Czech. J. Phys.* **48/S1**, 893-896 (1998)

[III] A. Péter, G. Tóth, **Cs. Tömböly**, G. Laus and D. Torwé "Liquid Chromatographic Study of the Enzymatic Degradation of Endomorphins, with Identification by Electrospray Ionization Mass Spectrometry." *J. Chromatogr. A* **846**, 39-48 (1999)

[IV] M. Spetea, K. Monory, **Cs. Tömböly**, G. Tóth, E. Tzavara, S. Benyhe, J. Hanoune and A. Borsodi "In vitro Binding and Signalling Profile of the Novel μ Opioid Receptor Agonist Endomorphin-2 in Rat Brain Membranes." *Biochem. Biophys. Res. Commun.* **250**, 720-725 (1998)

[V] K. Monory, M.C. Bourin, M. Spetea, **Cs. Tömböly**, G. Tóth, H.W. Matthes, B.L. Kieffer, J. Hanoune and A. Borsodi "Specific Activation of the μ Opioid Receptor (MOR) by Endomorphin 1 and Endomorphin 2." *Eur. J. Neurosci.* **12**, 577-584 (2000)

[VI] G. Tóth, **Cs. Tömböly**, A. Péter, D. Biyashev, A. Borsodi, A. Rónai and R. Przewłocki "New Endomorphin Analogues: Design, Synthesis and Biological Properties." 26th European Peptide Symposium, Montpellier, 10-15 September, 2000; proceeding in *Peptides 2000*, 759-760 (2001)

[VII] R. Przewłocki, D. Łabuz, J. Mika, B. Przewłocka, **Cs. Tömböly** and G. Tóth "Pain Inhibition by Endomorphins." *Ann. N. Y. Acad. Sci.* **897**, 154-164 (1999)

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LIST OF ABBREVIATIONS

ACE	angiotensin converting enzyme, EC 3.4.15.1
ACN	acetonitrile
AcOH	acetic acid
BIT	2-(*p*-ethoxybenzyl)-1-diethylaminoethyl-5-isothiocyanobenzimidazole isothiocyanate
Boc	*tert*-butyloxycarbonyl
*t*Bu	*tert*-butyl
BuOH	1-butanol
Bzl	benzyl
CNS	central nervous system
CTOP	D-Phe-c[Cys-Tyr-D-Trp-Orn-Thr-Pen]-Thr-NH_2
DALDA	Tyr-D-Arg-Phe-Lys-NH_2
DAMGO	Tyr-D-Ala-N-MePhe-Gly-ol
DCC	N,N'-dicyclohexylcarbodiimide
DCM	dichloromethane
DIDII	Tyr-D-Ala-Phe-Glu-Ile-Ile-Gly-NH_2
DIEA	N,N-diisopropylethylamine
Dit	3',5'-diiodotyrosine
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DPDPE	Tyr-c[D-Pen-Gly-Phe-D-Pen]-OH
DPP IV	dipeptidyl-peptidase IV, EC 3.4.14.5
DTT	1,4-dithio-DL-threitol
EM1	endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2)
EM2	endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2)
EtOAc	ethyl acetate
EtOH	ethanol
ESI	electrospray ionization

Fmoc	9-fluorenylmethoxycarbonyl
GPCR	G-protein-coupled receptor
GPI	guinea pig ileum
HF	hydrogen fluoride
HOBt	N-hydroxybenzotriazole
HPLC	high-performance liquid chromatography
IC_{50}	half-maximal inhibitory concentration
JOM-13	Tyr-c[D-Cys-Phe-D-Pen]-OH
k'	capacity factor
M	mol dm^{-3}
MeOH	methanol
MERGL	Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu-OH
MS	mass spectrometry
MVD	mouse vas deferens
NEP	neutral endopeptidase, "enkephalinase", EC 3.4.24.11
NMR	nuclear magnetic resonance (spectroscopy)
NOE	nuclear Overhauser effect (spectroscopy)
PLO17	Tyr-Pro-N-MePhe-D-Pro-NH_2
PMSF	phenylmethanesulfonyl fluoride
R_f	retention factor
ROE	rotating frame Overhauser effect (spectroscopy)
SPPS	solid phase peptide synthesis
TAPP	Tyr-D-Ala-Phe-Phe-NH_2
TAPS	Tyr-D-Arg-Phe-Ser-OH
TFA	trifluoroacetic acid
TLC	thin-layer chromatography
Tris.HCl	tris(hydroxymethyl)aminomethane chloride
Tyr-W-MIF-1	Tyr-Pro-Trp-Gly-NH_2

1. INTRODUCTION

1.1. Opioid receptors

Opium has long been associated with human history. Sumerian ideograms, Egyptian medical papyri and Persian physicians already mentioned the medical use of opium. Morphine, the pharmacologically active component of opium, is one of the 40 opiate* alkaloids it contains. When the large-scale production of pure morphine became possible, and the hypodermic syringe and hollow needle were invented, the use of morphine for medical purposes began. Nearly two centuries after its introduction, the opiate morphine remains a clinically important drug with notable applications for analgesia. Major drawbacks to the clinical use of morphine, however, are that it causes physical dependence, tolerance and reward-seeking behaviour, it depresses respiration and it can induce hypotension and bradycardia (*Table 1*). The cause of these drawbacks is that exogenously administered opiates act at the same receptor as do endogenous opioids*; thus, they also produce such side-effects.

For centuries, scientists have sought to understand the pharmacology of the active constituents of opium, and other compounds with opiate activity. With the advent of tritiated ligands with high specific radioactivity, which could be used at low concentrations, the existence of stereospecific opiate binding sites (the opioid receptors) was demonstrated in the central nervous system [1,2]. Otherwise, agonists and antagonists have been shown to bind to the same receptor, but their binding can be differentiated [3]. From neurophysiological and behavioural observations on chronically spinalized dogs, Martin and his colleagues postulated that there are at least three different types of opioid receptors: μ (for morphine), κ (for ketocyclazocine) and σ (for SKF 10,047 or N-allylnormetazocine) [4,5]. However, the σ receptor is now not generally considered to be an opioid receptor [6]. Further investigations have proved the existence of an additional type of opioid receptor, the δ receptor [7]. Since these pioneering studies, other receptor types, such as the ε receptor [8] and the ζ receptor [9], have been postulated. Opioid receptors have characteristic ligand selectivity profiles [10,11], allowing their pharmacological differentiation and their further division into several subtypes. Pharmacologically, subtypes have been defined or confirmed on the basis of their ability to bind ligands selectively [12,13]; on their *in vivo* ability to affect certain physiological parameters but not others, and on the selective antagonism of these actions [14,15].

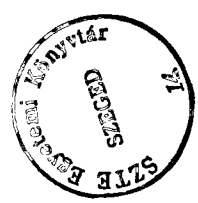
The existence of the pharmacologically established three major types, μ, δ and κ, of opioid receptor have also been confirmed by molecular biological methods. The δ opioid receptor was

TABLE 1. Main pharmacological effects mediated by opioid receptors

μ-opioid receptor	δ-opioid receptor	κ-opioid receptor
analgesia (supraspinal)	analgesia (spinal and supraspinal)	analgesia (spinal)
respiratory depression	respiratory depression	
sedation	sedation	sedation
euphoria		dysphoria
obstipation	obstipation	diuresis
bradycardia		
miosis		

cloned independently by two groups [16,17], and this was quickly followed by the cloning of μ and κ opioid receptors and by replications of the cloning of the δ receptor [18-27]. Further attempts to clone additional opioid receptor types and/or subtypes led several laboratories to isolate a cDNA encoding ORL-1 (opioid receptor-like) receptor [28-30]. This receptor, in contrast with μ, δ and κ receptors, mediates antiopioid effects. Further important results were derived from the investigation of transgenic animals lacking one of the opioid receptors. Thus, the significant role of μ opioid receptors in normal nociceptive processing was proved in homologous recombinant μ opioid receptor knockout animals [31,32]. The cloning of opiate receptors has also allowed the use of molecular biological techniques to localize them. For example, *in situ* hybridization and immunocytochemistry offer better resolution than do autoradiographic studies of binding sites, permitting the examination of single cells and the localization of receptors within the cell.

Opioid receptors are assigned to the large superfamily of rhodopsin-like G-protein-coupled receptors (GPCRs). This superfamily of GPCRs consist of integral membrane proteins that transduce optical and chemical signals across the cellular membrane. They share a common structure with a central core domain composed of seven transmembrane helices connected by three extracellular and intracellular loops [33]. The transmembrane regions of the receptors are responsible for the selective binding of extracellular ligands, while the cytoplasmic loops are involved in the selective coupling to G-proteins. Effector systems activated or blocked upon opioid receptor - G-protein interaction are adenyl cyclase, Ca^{2+} channels, K^+ channels or the phosphoinositol turnover [34]. Unfortunately, the high-resolution three-dimensional structures of these receptors remain elusive because X-ray diffraction and



NMR spectroscopic analysis cannot be applied. However, theoretical analysis has led to approximate receptor models [35], and the structure of rhodopsin is available with a resolution of 2.8 Å [36]; this is of interest for ligand docking studies and for the testing of hypotheses relating to ligand design.

Since the cloning of the opioid receptors, the individual pharmacological and biochemical profiles of the μ, δ and κ opioid receptors have been better defined, but a major problem still remains. The pharmacology of opioid receptors predicts a greater number of receptor subtypes than revealed by opioid receptor cloning [37]. Hetero-oligomerization interaction between the receptor types is a possible explanation. Oligomers of the μ and δ receptors [38], and the κ and δ receptors [39] allow the generation of a greater diversity of opioid signalling receptor units than predicted by the three cloned opioid receptor genes and provide greater physiological significance for the overlapping distribution of the closely related opioid receptor subtypes.

Opioid receptors are distributed more or less ubiquitously over the mammalian organism, indicating considerable functional significance [40]. A variety of functional roles, such as immunological importance [41-43], control of gastrointestinal functions [44], CNS functions [45] and control of reproductive mechanisms [46], have been proposed for these opioidergic systems. The peripheral presence of opioid receptors has been exploited to provide functional models of opioid action. Thus, various preparations of the isolated ileum of the guinea pig (GPI) and of the vas deferens from mouse (MVD), rat, rabbit and hamster have been used for more than 30 years in pharmacological assays to assess the agonist/antagonist properties of opioids. These compounds are able to inhibit neurotransmitter release (normally triggered by electrical field stimulation), giving rise to muscle contractions of GPI or MVD preparations [47,48].

1.2. Endogenous opioid peptides

The discovery of opioid receptors was the first example of the use of binding assays to demonstrate the presence of the receptors a neurotransmitter, and it took place before the endogenous ligands were identified. The view that an endogenous ligand acting at these sites exists was strongly supported by the observation that the antinociceptive effect caused by electrical stimulation of the periaqueductal grey of the midbrain was reversed by naloxone [49]. This phenomenon was best explained by the assumption that the electrical stimulation released an endogenous substance with morphine-like action, the effect of which was reversed by the

TABLE 2. Endogenous opioid peptides

Precursor protein	Opioid peptide	Sequence	Receptor selectivity
Proenkephalin	Met-enkephalin	YGGFM	$\mu \sim \delta >> \kappa$
	Leu-enkephalin	YGGFL	$\delta > \mu >> \kappa$
	Met-enkephalin-Arg^6-Phe^7	YGGFMRF	κ_2
	Met-enkephalin-Arg^6-Gly^7-Leu^8	YGGFMRGL	κ
	Peptide E	YGGFMRRVERPEWWMDYQKRYGGFL	κ
	Metorphamide	YGGFMRRV-NH_2	μ
Pro-opiomelanocortin	α-Endorphin	YGGFMTSEKSQTPLVT	
	β-Endorphin (1-26)	YGGFMTSEKSQTPLVTLFKNAIIKNA	$\mu > \delta >> \kappa$
	β-Endorphin (1-27)	YGGFMTSEKSQTPLVTLFKNAIIKNAY	
	β-Endorphin	YGGFMTSEKSQTPLVTLFKNAIIKNAYKKGE	
	γ-Endorphin	YGGFMTSEKSQTPLVTL	
Prodynorphin	Dynorphin A (1-8)	YGGFLRRI	$\kappa > \delta \sim \mu$
	Dynorphin A (1-13)	YGGFLRRIRPKLK	$\kappa > \delta \sim \mu$
	Dynorphin A	YGGFLRRIRPKLKWDNQ	κ
	Dynorphin B	YGGFLRRQFKVVT	κ
	Leumorphin	YGGFLRRQFKVVTRSQENPNAYYEELFDV	
	α-Neoendorphin	YGGFLRKYPK	
	β-Neoendorphin	YGGFLRKYP	
Others	Tyr-W-MIF-1	YPWG-NH_2	μ
ref. [77]	Dermorphin	YaFGYPS-NH_2	μ
ref. [77]	Dermenkephalin	YmFHLMD-NH_2	δ
ref. [77]	Deltorphin I	YaFDVVG-NH_2	δ
ref. [77]	Deltorphin II	YaFEVVG-NH_2	δ
β-Casein	β-Casomorphin (1-8)	YPFPGPIP	μ
Pronociceptin	Nociceptin	FGGFTGARKSARKLANQ	ORL_1
Unknown	Endomorphin-1	YPWF-NH_2	μ
	Endomorphin-2	YPFF-NH_2	μ

A: Ala, D: Asp, E: Glu, F: Phe, G: Gly, I: Ile, K: Lys, L: Leu, M: Met, N: Asn, P: Pro, Q: Gln, R: Arg, S: Ser, T: Thr, V: Val, W: Trp, Y: Tyr, a: D-Ala, m: D-Met

antagonist naloxone. The enkephalins, two closely related pentapeptides, were the first endogenous opioids (*Table 2*) isolated from brain extracts [50]. Screening of pituitary extracts for further endogenous ligands resulted in the identification of endorphins [51], which are pituitary peptides with morphine-like pharmacological action. Several other opioid peptides have been isolated, including κ opioid agonist dynorphins, which contain the Leu-enkephalin sequence [52-54]. Although most of these peptides bind to more than one type of opioid receptor, certain selectivities are obvious, e.g. selectivities of dynorphins for κ receptors, of enkephalins for δ receptors, and of endorphins not only for μ or δ, but also for ε receptors [55]. After isolation of the ORL-1 receptor, two groups independently reported the identification of its endogenous ligand, nociceptin [56,57].

Endomorphin-1 (EM1) and endomorphin-2 (EM2), previously synthesized as a morphiceptin analogue [58], were isolated from bovine [59] and later from human [60] brain. The EMs are now considered to be the endogenous ligands of the μ opioid receptor. A dense distribution of the EMs has been shown throughout the CNS [59,61-64] and the peripheral tissues [65]. Versatile physiological actions of this new opioid peptides have been demonstrated in detail [66-69]. Despite the quite similar chemical structures of the EMs, they display some functional differences. EM1 and EM2 possess distinct analgesic features [70-74] and they also appear to differ in their actions on autonomic functions [75,76]. It has been proposed that EM1 and EM2 may act through distinct μ_1 and μ_2 opioid receptor subtypes, respectively [69]. Histological studies have revealed that their distributional patterns in the CNS differ too: EM1-like immunoreactivity is more widely and densely distributed in the brain, whereas that of EM2 is more prominent in the spinal cord [63].

All typical opioid peptides have the same N-terminal amino acid sequence, Tyr-Gly-Gly-Phe. In mammals, these endogenous compounds are encoded by three genes. Each of these genes encodes multiple bioactive peptides, and each contains segments that appear to be direct duplications of each other. The precursor proteins are proenkephalin (yields enkephalins and related peptides [78-80]), proopiomelanocortin (gives rise to β-endorphin [81]) and prodynorphin (processed into dynorphins and neoendorphins [82]). Expression of the opioid peptide precursor genes occurs in the CNS and in the peripheral nervous system, in the immune system, and in the endocrine system; however, additional tissues have been found to contain precursor gene transcripts or precursor protein cleavage products. The atypical opioid peptides originate from a variety of precursor proteins and carry various amino acid sequences

at their N-terminal regions - just the N-terminal Tyr residue is conserved (except nociceptin [56,57]). For example, hemorphins representing the degradation products of hemoglobin [83], cytochrophins derived from mitochondrial cytochrome B [84], and different natural peptide agonists and antagonists have been found among milk protein fragments [85]. The gene(s) encoding the precursor protein(s) of the EMs has(have) not yet been identified, but this information is crucial for an endogenous peptide.

1.3. Structure - activity relationships of μ-opioid receptor specific ligands

The μ opioid receptor has a special place within the opioid receptor family. It mediates the actions of morphine and most clinical analgesic agents, and also drugs of abuse such as heroin [86]. Efforts to explain the molecular basis for the opioid ligand - receptor interaction focus on structural and conformational features of the ligand, the molecular biology of the opioid system, and molecular modelling techniques.

New ligands specific for, rather than selective for a receptor subtype may cover the binding spectrum more completely and indicate whether ligands are binding to slightly different positions on the same receptor, thereby giving rise to different inhibition profiles. The inability to determine specific ligands for the receptor subtypes may be an indication that the differences observed *in vivo* are due to postbinding events (signalling). A number of synthetic peptides have been made which not only are less sensitive to enzymatic degradation, but additionally display selectivity for the receptor types [87]. The agonists with possibly the highest selectivity for the μ receptor have been discovered through structural modification of the enkephalins. DAMGO has become the most commonly used selective μ opioid receptor agonist [88]. The dermorphins, the naturally occurring amphibian heptapeptides, and their amides have high affinity and selectivity for the μ receptors [77]. Among the various analogues of the N-terminal tetrapeptide segment of dermorphin, the compounds TAPS [89], TAPP and DALDA [90] have been reported to be potent and selective μ agonists. Substitution of Dmt for Tyr^1 in DALDA resulted in a compound with excellent μ agonist potency and μ selectivity [91]. PLO17, synthesized on the basis of morphiceptin, is a μ agonist that has proved useful for studies of peripheral μ receptor-related functions [92,93]. The systematic investigation of conformational profiles and electronic properties of a series of morphiceptin analogues indicates that hydrogen bonding, but not electrostatic interactions, occurs between the C-terminal region and the μ

receptor binding site; furthermore, a candidate conformer for high-affinity binding at the μ opioid receptor has been identified as one with a β_{II}-like turn [94].

The putative endogenous μ opioid receptor ligands EM1 and EM2 are also potent and selective μ agonists [59]. The EMs are in the same class of opioid peptides as morphiceptin [95], PLO17 [92], and Tyr-W-MIF-1 [96,97], in which a Pro at the second position confers high selectivity on the μ-opioid receptor. The affinity of these peptides greatly depends on the nature of the amino acid at the fourth position. For example, the affinity increases 5-fold upon substitution of Gly in Tyr-W-MIF-1 with a hydrophobic residue and more than 50 times if Phe replaces Gly, as in EM1 [59]. Even though the presence of the C-terminal L-Phe in EM2 appears to generate the optimum μ receptor binding activity, the D-Phe^4 and the des-Phe^4 derivatives provide somewhat lower, but similar affinity. The negative impact of the N-terminal D-Tyr on μ affinity was minimal. Moreover, the Pro^2 and Phe^3 residues are quite essential, because their enantiomers reduce the μ affinity of the peptide diastereomers [98]. A similar tendency was observed in the case of EM1, where the [D-Pro^2]- and [D-Trp^3]EM1s possess lower μ affinity than that of the parent peptide, while N- and C-terminal amino acid inversion causes a moderate alteration [99]. Replacement of the amino acids with their β-isomers resulted in β-(R)-Pro^2-EM1, which was found to be more potent than endogenous EM1 [100].

The alkaloid morphine has an approximately 50-fold higher affinity for μ than for δ opioid receptors [101]. Among the non-peptide drugs, the piperidine derivative sufentanyl is a potent opioid agonist with high affinity and selectivity for the μ receptor [102,103]. The most potent and selective agonist at μ opioid receptors is the benzimidazole opioid etonitazene [101]. The only irreversible opioid agonist described is chloroxymorphamine, the 6β-N,N-bis(2-chloroethyl) derivative of oxymorphone [104]. Among steroids, 17α-acetoxy-6-dimethylaminoethyl-21-fluoro-3-ethoxypregna-3,5-dien-20-one (SC17599) is a selective, full agonist at the μ opioid receptor with *in vivo* antinociceptive potency and without affinity for the glucocorticoid receptor [105]. Although SC17599 contains a tertiary nitrogen, it lacks both the critical aromatic feature and a phenolic hydroxyl group corresponding to that typically found in opioid peptides and morphine-like opioids.

Naloxone was the first pharmacologically pure opioid antagonist identified. It is considered to be a universal opioid antagonist, and the action of an agonist is characterized as opioid receptor-mediated only if its effects are naloxone-reversible [106]. Naloxone has higher affinity for the μ opioid receptors than for the other opioid receptors [102]. β-Funaltrexamine

was the first agent characterized to antagonize selectively μ receptors [107,108]. Its ability to alkylate only μ opioid receptors demonstrates the subtle but important structural differences that exist between the different opioid receptors [109,110]. Using an amino-substituted analogue of the highly potent μ agonist etonitazene, Rice and his colleagues prepared a highly reactive and selective acylator of μ-opioid receptors, BIT [111]. Another compound with apparent selective, long-lasting μ receptor antagonist properties is naloxonazine, a C-6 azine-bridged dimer of naloxone [112]. It has been characterized as a selective antagonist at μ_1, a putative μ opioid receptor subtype [113]. Cyprodim [114] and somatostatin-related cyclic peptides, such as CTOP, are potent and selective μ opioid antagonism too [115,116].

The screening of combinatorial libraries has led to the identification of compounds structurally different from classical μ opioid peptide and alkaloid ligands (*Table 3*) [117]. Novel peptide antagonists with N-terminal basic amino acids (Arg, Lys) were isolated from the library of N-acetylated peptides (acetalins) and peptides containing all D-amino acids (e.g. Ac-Arg-Phe-Met-Trp-Met-Lys-NH_2 and Ac-D-Arg-D-Phe-D-Trp-D-Ile-D-Asn-D-Lys-NH_2). The *in vivo* potency of the agonist Ac-rfwink-NH_2 was found to be approximately twice that obtained for morphine [118]. Upon completion of the iterative deconvolution process, two potent μ opioid peptides, D-Ile-D-Phe-D-Thr-D-Trp-D-Tyr-D-Arg-NH_2 and D-Ile-D-Met-D-Ser-D-Trp-D-Trp-D-Gly-NH_2, were isolated from the non-acylated library made up entirely of D-amino acid hexapeptides. These peptides are another class of opioid peptides, in that they do not have the typical N-terminal free Tyr of endogenous ligands, nor do they have the N-terminal Arg of the acetalins. A tetrapeptide with a C-terminal basic amino acid was also identified (Trp-Trp-Pro-Arg-NH_2, IC_{50} = 10 nM) [119]. Combinatorial libraries of tetrapeptide amides containing non-proteinogenic amino acids resulted in the identification of Tyr-D-Nve-Gly-L-Nal-NH_2. According to these latest data, the general motif of active peptides identified at the μ-opioid receptor was Tyr-(D-amino acid)-(L-amino acid with small side-chain)-(L-aromatic amino acid)-NH_2 [120]. Screening of libraries of different peptidomimetics for compounds with μ opioid activity resulted, for example, in CHIR 4531, TPI418-1 and TPI632-4 (*Table 3*).

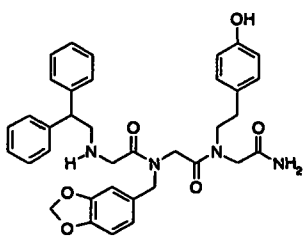
New ligands with widely varying structures are not so readily identified for other GPCRs. The plasticity of the μ opioid receptor may account for the high number of different, new and potent ligands identified.

Before the introduction of molecular biological methods, knowledge of the functions of the opioid receptors related directly to the available selective agents. Cloning of the opioid

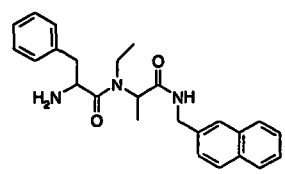
TABLE 3. μ-Opioid ligands identified from combinatorial libraries

Library	Ligand	Activity (nM)	Agonist/Antagonist	Ref.
OXXX-NH_2	YmFA-NH_2	IC_{50}=2	agonist	[121]
	YmFF-NH_2	IC_{50}=6	agonist	[121]
	Y(DNve)G(L-Nal)-NH_2	K_i=0.4	agonist	[120]
	YrAW-NH_2	K_i=1.8	agonist	[120]
OOXXXX-NH_2	YPFGFR-NH_2	IC_{50}=13	agonist	[119]
	YGGFMA-NH_2	IC_{50}=28	agonist	[122]
	WWPKHG-NH_2	IC_{50}=9	agonist	[119]
Ac-OOXXXX-NH_2	Ac-RFMWMK-NH_2	IC_{50}=5	antagonist	[123]
	Ac-RWIGWR-NH_2	IC_{50}=5	antagonist	[119]
	Ac-FRWWYM-NH_2	IC_{50}=33	agonist	[119]
ooxxxx-NH_2	iftwyr-NH_2	IC_{50}=5	#	[117]
	imswwg-NH_2	IC_{50}=10	#	[117]
Ac-ooxxxx-NH_2	Ac-rfwink-NH_2	IC_{50}=18	agonist	[118]
Trimer peptoid	CHIR 4531*	K_i=6	#	[124]
N-Alkylated dipeptide	TPI418-1*	K_i=6	agonist	[124]
Urea	TPI632-4*	IC_{50}=100	#	[117]

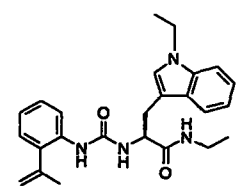
One-letter symbols: see below *Table 2*, Nve: norvaline, Nal: naphthylalanine, # no data
* Structures are:



CHIR 4531



TPI418-1



TPI632-4

receptors opened the way for structure - activity studies at the receptor level through the performance of site-directed mutagenesis, deletions or the construction of receptor chimeras.

The importance of the charged residues in transmembrane regions II, III and IV for the receptor function, and the involvement of extensive portions of N- and C-terminal receptor domains were underscored by molecular biological methods, deletions and site-directed mutagenesis [125]. Specific amino acid residues interacting directly with agonist and antagonist molecules were also identified by these methods [125-129]. Deletion of 64 N-terminal amino acids produced little effect on the function of the μ opioid receptor [27], but

the affinities of the alkaloid agonists and EMs for the truncated receptor relative to the wild-type μ opioid receptor were reduced by 20- to 60-fold [130]. In the case of the C-terminal truncated μ opioid receptor, morphine remained an effective agonist, while DAMGO lost much of its potency [125]. With δ/μ chimeric receptors, the region specifying the μ selective alkaloid binding was shown to be different from the determinant for binding of the μ selective enkephalin-related peptides [131,132]. Although the ligand-binding pocket consists mainly of residues from the transmembrane α-bundle, the extracellular loops of opioid receptors have also been shown to be important for interactions with many ligands [126,131-134]. Moreover, small non-peptide ligands with agonist properties, such as sufentanyl or morphine, bind to regions of the μ opioid receptor that are partially distinct from those bound by peptide agonists such as DAMGO [126,131]. These differences have also been described for the binding of the selective ligands at the δ and κ opioid receptors [135,136].

The three-dimensional structures of the transmembrane, seven α-helical domains and extracellular loops of the μ, δ and κ opioid receptors were determined by molecular modelling. According to these calculations, many of the opioid ligands considered have a similar arrangement in the receptors, with the tyramine moiety of alkaloids or Tyr^1 of opioid peptides interacting with conserved residues in the binding pocket and the tyramine N^+ and OH groups forming ionic interactions or H-bonds with a conserved Asp from helix III and a conserved His from helix VI, respectively [35,137,138].

1.4. Structural modification of peptides

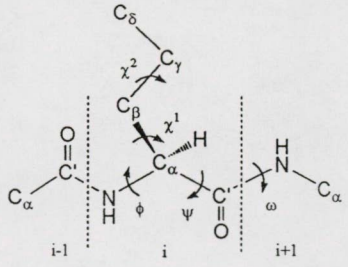
Native peptides are limited to use in clinical applications by their rapid enzymatic degradation, by the problem of passage through the blood-brain-barrier for centrally acting peptides, and eventually by their flexibility, which can lead to lesser specificity and thus undesirable side-effects. A major challenge in opioid peptide chemistry and pharmacology is the possibility to develop novel analgesics mimicking the endogenous opioid ligands instead of morphine. The general features of representative peptidomimetic strategies include the cyclization of peptides, incorporation of unnatural amino acids, linkages between consecutive residues, and the replacement of peptide bonds with amide isosteres [139]. The transformation of a peptide structure to a completely non-peptidic molecule (retaining pharmacophores and the required three-dimensional array) is also an attractive approach to the development of therapeutic

agents from native peptides. These types of drug candidates can incorporate metabolic stability and oral bioavailability, and can provide desirable selectivities.

The conformational flexibility of opioid peptides has hampered numerous attempts at determining the relationship between the solution conformation and activity by the use of spectroscopic and modelling methods. Insight into the conformational requirement of peptide binding has been obtained through the synthesis of analogues with a more rigid backbone scaffold [140], or by studies in media that promote structure, such as viscous solvents [141], lipids [142] or lyotropic liquid crystals [143].

Because of their flexibility, small peptides produce secondary structures only with low probability; these can be defined by the torsional angles ϕ, ψ and ω (*Figure 1*). Thus, an equally important area is the three-dimensional structure of the side-chain moieties, which can be characterized by the torsional angles χ^1, χ^2, etc., i.e. the chi space. The χ angles, in conjunction with the backbone angles, define the positions of side-chain functional groups in space and thus must be regarded as of key importance in an understanding of the mode of action of peptides [144]. Of the many tools required to aid investigations of the χ-space, an array of non-proteinogenic α-amino acids with well-defined complementary χ-characteristics are clearly of considerable importance.

The χ-constrained, conformationally restricted amino acids can be divided into three



ϕ=C'-N-C_α-C'

ψ=N-C_α-C'-N

ω=C_α-C'-N-C_α

χ^1=N-C_α-C_β-C_γ

χ^2=C_α-C_β-C_γ-C_δ

FIGURE 1. Definition of the dihedral angles of a peptide

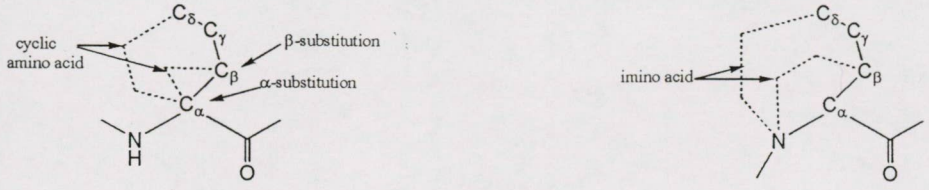
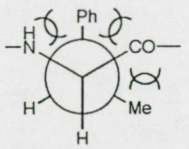
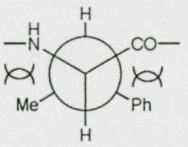


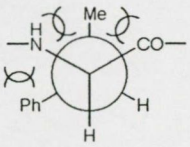
FIGURE 2. Classification of constrained α-amino acids



gauche (+)
($\chi^1 = +60°$)



trans
($\chi^1 = 180°$)



gauche (-)
($\chi^1 = -60°$)

Stereoisomers of β-MePhe	Preferred conformation
(2S, 3S)	*gauche (-)*
(2S, 3R)	*trans*
(2R, 3R)	*gauche (+)*
(2R, 3S)	*trans*

FIGURE 3. The effect of β-substitution on the population of χ^1 rotamers

classes (*Figure 2*). In contrast with α-substitution, which has little effect on χ^1, the introduction of a methyl group at the β position directly enhances the population of one χ^1 rotamer. The torsional angle χ^1 of the substituted amino acids is restricted by van der Waals interactions. As illustrated for (2S,3R)-β-MePhe (*Figure 3*), these interactions between vicinal substituents suggest that a *trans* side-chain conformation is preferred to the *gauche (-)* and *gauche (+)* conformations. The same analysis for each of the four stereoisomers of β-MePhe has led to the predictions summarized in *Figure 3*.

In consequence of the steric property of the β-methylated side-chain favouring one conformation of the torsional angle χ^1, the biochemical properties of the modified ligand change (e.g. when the stereoisomers of β-MePhe were incorporated as Phe^4 replacement in DPDPE [145] or as Phe^3 replacement in JOM-13, a δ-opioid agonist [146]). *Table 4* lists the μ and δ binding affinities of the four β-$MePhe^3$ analogues of JOM-13 and additionally provides

TABLE 4. Binding affinities and energy differences of X^3 side-chain rotamers of JOM-13 and β-$MePhe^3$-containing analogues [147]

Ligand	K_i (nM)		Relative energy (kcal/mol) of χ^1 rotamers		
	μ	δ	- 60°	180°	+ 60°
JOM-13	51.5	0.74	0.1	0.0	0.9
[(2S,3S)β-$MePhe^3$]JOM-13	259	1.52	0.0	2.2	1.5
[(2S,3R)β-$MePhe^3$]JOM-13	1000	12.3	0.2	0.0	2.0
[(2R,3R)β-$MePhe^3$]JOM-13	> 10000	4.8	6.3	6.1	0.0
[(2R,3S)β-$MePhe^3$]JOM-13	> 10000	237	4.7	0.0	1.2

JOM-13: Tyr-c[D-Cys-Phe-D-Pen]-OH

the relative energies of the residue 3 rotamers calculated for each analogue [147]. As can be seen from this Table, in [(2S,3S)β-MePhe^3]JOM-13 and [(2R,3R)β-MePhe^3]JOM-13, the analogues displaying the highest δ affinity, the same spatial orientation of the phenyl side-chain is strongly favoured: the χ^1 = -60° rotamer of the former is favoured by 1.5-2.2 kcal/mol, while the spatially equivalent (since the α stereochemistry is inverted) χ^1 = +60° rotamer of the latter is favoured by more than 6 kcal/mol. These results imply the presence of the χ^1 = -60° rotamer of Phe^3 in the binding conformer of JOM-13.

1.5. Preparation of neuropeptide radioligands

1.5.1. Tritium labelling of peptides

Special experimental tools are needed for the examination of receptor macromolecules, in order to ensure the accuracy and reproducibility of quantitative measurements at low concentration, in consequence of their physical properties. The analogues of biologically active compounds used in these studies can contain radioactive, fluorescent, chemiluminescent and/or affinity labelling probes. In opioid research, tritium-labelled ligands have proved to be essential for the *in vitro* characterization, autoradiographic localization and distribution studies of the receptors.

Tritium is a soft β-emitting (18.6 keV) radionuclide without gamma emission, and has a medium half-life (12.35 years). Furthermore, its decay product, ^{3}He, is innocuous. Thus, this hydrogen isotope is the safest radioactive nuclide in the life sciences. According to its half-life, one tritium atom incorporated per molecule represents a specific radioactivity of 1.06 TBq/mmol (28.6 Ci/mmol). This value is appropriate for the study of neuropeptides in the nano- and subnanomolar ranges. On the other hand, tritium-labelled compounds are ideal for high-resolution autoradiographic studies, because the range of β-particles is inversely proportional to the density of the medium (photoemulsion, ρ = 3.5 g/cm^3).

The main methods for the tritium labelling of neuropeptides [148] include β-radiation-induced isotope exchange reactions [149], catalytic isotope exchange [150], chemical or enzymatic synthesis from precursor peptides or labelled amino acids, methylation of peptides with [^{3}H]methyl iodide [151] or reductive methylation using tritiated metal hydrides [152]. Tritiation of the appropriate precursor peptides, and the synthesis of tritiated peptides from available labelled amino acids, ensure the most varied and most specific labelling. With this method, tritium can be incorporated into a discretionary residue, resulting in radioligands with

high specific radioactivities. These specifically labelled peptides are of great importance in investigations of their catabolic processes.

The appropriate precursor peptides can be obtained by postsynthetic modifications or by direct synthesis. The most frequently used chemical modification is iodination of the aromatic side-chains of the peptides with iodine or its monochloride. Iodine can be used directly in MeOH solution or can be generated from iodides by oxidizing agents (e.g. chloramine-T), by electrolysis or enzymatically, through the use of peroxidases. Precursor peptides containing halogenated or unsaturated amino acid(s) can be prepared directly before the tritiation step. Such halogenated amino acids are Dit [153,154], 2',6'-dibromo-Tyr, which leads to more stable tritium incorporation [155], 4'-iodo-Phe [156], 2',5'-diiodo-His [157] and 5',7'-dibromo-Trp [158]. Among the unsaturated amino acids, 3,4-dehydro-Pro [159,160], 4,5-dehydro-Leu [161], 4,5-dehydro-Ile, 2,6-diamino-4-hexenoic acid [162], and propargyl- [163] or allyl-Gly have been reported. The most widely applied heterogeneous catalysts for the tritiation of peptides with tritium gas are $Pd/BaSO_4$, Pd/Al_2O_3, Pd/C, PdO, Pd and Rh [148].

Radio-HPLC is often the method of choice to separate, purify and quantify the activity levels of radiolabelled peptides. This is the technology applied for the continuous measurement of radioactivity in HPLC effluent streams. Flow scintillation homogeneous counting provides real-time analysis during the chromatographic run, and high counting efficiencies (up to 60%) for the low-energy β-emitter tritium [164]. When a liquid (homogeneous) flow cell is used, an effluent stream splitter is applied to divert a fraction of the HPLC effluent to a fraction collector in order to avoid the mixing of the tritiated compound with the fluor cocktail and to perform the purification of tritiated peptides.

1.5.2. Solid-phase peptide synthesis (SPPS)

The solid-phase peptide synthesis [165,166] proposed by Merrifield is based on the attachment of the (N-blocked) C-terminal residue to an insoluble polymeric support (resin), followed by deprotection and acylation of the exposed amino group (coupling) with the penultimate residue and continuation of the procedure by similar cycles of deprotection and coupling. The peptide is separated from the support only after the completion of chain building. Most polymeric supports applied in this technique are gels rather than real solids, and the reactions take place in the inside of the particles. The most common strategy in SPPS is $C \rightarrow N$ directed chain elongation. This approach requires that both the coupling reaction and the unmasking of α-

amino groups be carried to completion; otherwise, deletion sequences will contaminate the final product.

During the SPPS, three basic protection steps are needed: protection of the peptide C-terminal, temporary N^{α}-protection, and semipermanent blocking of the side-chains. The C-terminal amino acid is anchored to the solid support by the formation of either an ester, a thioether or an amide bond. The two major N^{α}-protection schemes frequently used today are the Boc/Bzl and Fmoc/*t*Bu approaches. In the former, the acid-labile *tert*-butyloxycarbonyl (Boc) group is used for N^{α}-protection, and the side-chains of the various amino acids are protected with benzyl- or cyclohexyl-type protecting groups. In the latter approach, the base-labile 9-fluorenylmethoxycarbonyl (Fmoc) group is used as N^{α}-protection, and the side-chains are protected with tertiary carbon-based protecting groups (*tert*-butyl or trityl). In order to convert carboxylic acids into strong acylating agents, they must be activated by an electron-withdrawing group to enhance the electrophilicity of the carbonyl group. The most important types of activating groups are azides, halogenides, anhydrides, active esters, phosphonium and uronium salts. For monitoring of the coupling or deprotection, the Kaiser [167] or chloranil [168] qualitative test is used. Final cleavage of the peptidyl-resin involves the application of $HF_{(l)}$ or trifluoromethanesulfonic acid for Boc chemistry and trifluoroacetic acid (TFA) for Fmoc chemistry. Side-reactions (e.g. alkylation) can be avoided by the use of appropriate scavengers, such as anisole, dimethyl sulfide or *p*-thiocresol. Peptides are extracted from the cleavage mixture with aqueous AcOH or buffer solution, and are then lyophilized.

The final step in the preparation of high-purity peptides is purification by HPLC. Reversed-phase HPLC is used for this purpose on C_{18}, C_8, or C_4 derivatized silica, depending on the hydrophobicity and the size of the peptide. If the peptide contains charged side-chain groups, ion-exchange chromatography can also be used for purification.

1.6. Neuropeptide catabolism

The metabolism of peptides, defined as the cleavage of a peptide bond, is an important area of study. This process produces the endogenous opioid peptides from their precursor proteins, and degrades these endogenous opioids too. Metabolic fragmentation may also lead to the inactivation of exogenously administered opioids. The establishment of inactivation pathways allows the design of enzyme inhibitors that may be of pharmacological interest or can help in the design of more stable and potent peptide-based compounds. A classical example is

"enkephalinase" (neutral endopeptidase, NEP, EC 3.4.24.11), which generates inactive metabolites from enkephalins, and its inhibitors, which are analgesic and substitute in opiate abstinence [169]. The metabolism may also generate products that maintain activity, but are less potent, or, more interestingly, molecules with different activity. An outstanding example is the ACE-inhibitors, which block the activity of angiotensin converting enzyme (ACE, EC 3.4.15.1).

In both nervous and peripheral tissues, peptides are degraded extracellularly, probably by a limited number of enzymes with relatively broad specificities. Most of these are ectoenzymes, i.e. integral membrane proteins that have their active sites facing the extracellular space, and many are Zn-metallopeptidases [169]. Despite their relatively broad specificities, a certain *in vivo* specificity seems to be achieved, governed by the distribution of a given peptidase and by that of its potential substrates. Several peptidases have been shown to be involved in the metabolism of opioid peptides. Thus, neprilysin (EC 3.4.24.11) cleaves the Gly-Phe bond in both Met-enkephalin and Leu-enkephalin [170,171]. Inhibitors of neprilysin, such as thiorphan, slow the degradation of enkephalins *in vivo*, possess analgesic actions themselves, and potentiate the analgesia induced by enkephalins [172-174]. In contrast, endopeptidase-24.15 (EC 3.4.24.15) rapidly converts dynorphin (1-8) and α- and β-neoendorphins into Leu-enkephalin, and converts Met-enkephalin-Arg^6-Gly^7-Leu^8 (MERGL) into Met-enkephalin [175,176]. Inhibitors of endopeptidase-24.15 produce naloxone-reversible analgesia, and enhance MERGL or dynorphin (1-8) analgesia [174,177]. Aminopeptidase M (EC 3.4.11.2) has a broad substrate specificity for the removal of N-terminal amino acids from peptides. When a Pro residue is preceded by a bulky hydrophobic residue, e.g. Leu, Tyr or Trp, secondary reactions can arise, such that the Xaa-Pro combination is released as an intact dipeptide [178]. In the cerebrospinal fluid, a significant role can be attributed to aminopeptidase M in the degradation of low molecular weight opioid peptides [179]. It has been shown that this enzyme hydrolyses the Tyr-Gly bond of Leu-enkephalin [180]. Dipeptidyl-peptidase IV (DPP IV, EC 3.4.14.5) is a highly specialized aminopeptidase that removes dipeptides only from peptides with an N-terminal penultimate Pro or Ala [181]. DPP IV has contact with neuropeptides mainly in the cerebrospinal fluid. A potent and selective inhibitor of DPP IV is Ala-pyrrolidonyl-2-nitrile, which elicits dose-dependent analgesia and potentiates the analgesic actions of EM2 [182]. There is other evidence to indicate that DPP IV is involved in the degradation of the EMs [183].

EM1 and EM2 have been reported to produce only a short-lasting analgesic effect after intrathecal administration, due to the rapid breakdown in the spinal cord [184]. In contrast with the initial report of the tail-flick test [59], where intracerebroventricularly administered EMs produced prolonged analgesia, the effects of intracerebroventricular EMs in a later study [72] appeared to be much more short-acting. In the formalin test, EM1 has been shown to produce prolonged analgesia in rats, after intrathecal injection in higher doses [185,186].

Incubation with brain homogenate [187,188], rat brain extract [189], intact pieces of brain [190], brain slices [191,192] or immunocytochemically pure cell cultures [180] can be used for an *in vitro* evaluation of the possible fate of neuropeptides in the CNS. In contrast with brain homogenates, the cellular structures are conserved in brain slices, and the levels of cytosolic and soluble peptidases are limited [193]. The *in vivo* metabolism of neuropeptides can be studied after microinjection into brain structures. Dissection of the brain areas is followed by analysis of the degradation products [194]. Microdialysis combined with mass spectrometry allows the continuous and sensitive monitoring of biochemical events in the cerebral extracellular tissue space *in vivo* [195]. The incubation or the digestion step is generally followed by a separation step, in order to separate the metabolites and the examined neuropeptide before the quantification. Separation can be achieved most commonly with chromatographic methods such as HPLC [180,188,189,192,195] and size-exclusion chromatography [194] or by capillary electrophoresis [191].

2. AIMS AND SCOPE

Since the endogenous mammalian opioid ligands are not particularly selective towards the different opioid receptor classes, one important goal of the present work was to develop new, highly selective and potent μ opioid peptides. The putative endogenous μ opioid ligands EM1 (Tyr-Pro-Trp-Phe-NH_2) and EM2 (Tyr-Pro-Phe-Phe-NH_2) were selected as basic compounds. As aromatic groups on a peptide ligand often play a central role in the interaction with the receptor, modification of the Phe moieties was expected to influence the binding characteristics of the EMs. In order to reduce the conformational freedom of the side-chains of these residues, β-MePhe isomers were incorporated into EM1 and EM2 in place of the Phe^3 and Phe^4 residues.

Following the biological characterization of the new EM analogues and the results of NMR experiments, both carried out by collaborating groups, structure - activity relationships can be established, the conformational requirements of the amino acid side-chains for bioactivity can be examined and the characteristics of the μ opioid pharmacophore can be further clarified.

Further important goal of this study was to prepare specifically tritium-labelled EMs as appropriate tools for radioligand binding assays, and for examination of the *in vitro* metabolism of EM1 and EM2 in detail. This study of the metabolism had the aim of establishing whether the metabolism involves the deactivation or a modification of the original effects of EMs.

3. EXPERIMENTAL PROTOCOLS

3.1. General methods

TLC was performed on silica gel 60 F_{254}-precoated glass plates (Merck), and the separation of optically active compounds on glass plates coated with C_{18}-RP silica gel and impregnated with a chiral selector and copper(II) ions (Chiralplate, Macherey-Nagel). The following solvent systems were used: (1) ACN-MeOH-water 4:1:1, (2) *n*-BuOH-AcOH-water 4:1:1, (3) ACN-MeOH-water 4:1:1 on Chiralplate, (4) ACN-$CHCl_3$-AcOH 8:1:1, (5) $CHCl_3$-MeOH-AcOH 90:8:2, (6) EtOAc-pyridine-AcOH-water 60:20:6:11. Chiralplate layers were activated at 100°C for 15 min before use. TLC spots were detected under UV light, in I_2 vapour and with ninhydrin spray. Radio-TLC was performed similarly. Radioactive spots were detected with a Berthold LB 2821 position-sensitive windowless proportional counter (filling gas: 9.9% methane/Ar) connected to a Berthold LB 511 data-processing unit.

HPLC analyses were carried out on a Merck-Hitachi chromatographic system, a Vydac 218TP54 column being applied for analytical purposes and a Vydac 218TP1010 semipreparative column for preparative purposes. UV detection was carried out at 216 nm. After the preparative HPLC process, pure peptides were obtained as lyophilizates. A Shimadzu SIL-6B auto-injector combined with an SCL-6B system controller was used to inject samples for degradation studies. The mobile phases for linear gradient elutions were (A) water, (B) ACN, (C) 0.1% (v/v) TFA in water, and (D) 0.08% (v/v) TFA in ACN. Radio-HPLC analyses were performed on a Jasco instrument with a Vydac 218TP54 column. Radioactivity in effluents was detected continuously with a Radiomatic 505TR Flow Radiochromatography Detector (Canberra Packard) with the Ultima-Flo M scintillation cocktail. Chemiluminescence-corrected data collected by time-resolved counting (TR-LSC) were processed by FLO-ONE software (Canberra Packard).

Tritiation was carried out in a self-designed vacuum manifold [148]. $^3H_2$ gas was purchased from Technobexport, Russia, and contained at least 98% tritium. The radioactivity of tritiated compounds was measured with a Searle Delta 300 liquid scintillation counter in a toluene-Triton X-100 cocktail.

3.2. Synthetic methods

3.2.1. Diethyl (1-phenylethyl)acetamidomalonate

67.1 g (0.31 mol) of diethyl acetamidomalonate was dissolved in a NaOEt solution in EtOH (7.2 g (0.31 mol) of Na was dissolved in 320 mL of absolute EtOH), 68.6 g (0.37 mol) of (1-bromoethyl)benzene was added, and the mixture was stirred at 30-35°C for 20 h. The precipitated NaBr was filtered off and washed with hot EtOH. The combined EtOH solution was concentrated *in vacuo*, and the product was crystallized from EtOH:water 1:1. 82.2 g (0.26 mol), 83% yield, mp.: 124-125°C.

3.2.2. N-Acetyl-DL-β-methylphenylalanine

82.2 g (0.25 mol) of diethyl (1-phenylethyl)acetamidomalonate was hydrolysed with 380 mL of 10% NaOH solution for 45 min, and the solution was then carefully acidified with conc. HCl solution to pH=2. The resulting suspension was allowed to stand in a refrigerator overnight, after which the solid was filtered off and used for the next reaction without further purification; yield 46.3 g (84%).

3.2.3. DL-β-Methylphenylalanine

46.3 g (0.21 mol) of N-acetyl-DL-β-methylphenylalanine was refluxed with 300 mL of 6 M HCl solution for 12 h, and the solution was then evaporated to dryness. The residue was diluted with water and evaporated again. The diastereomeric amino acids were separated by fractional crystallization of their hydrochloride salts, and the free amino acids were liberated with aqueous NH_3 solution. In the crystal phase, the *erythro* ((2S,3S) and (2R,3R)) enantiomers were more abundant, while in the mother liquor, the *threo* ((2S,3R) and (2R,3S)) enantiomers were more abundant. The ratio of the diastereomeric amino acids was checked by HPLC. Diastereomers with >98% purity was used for peptide synthesis.

7.2 g *e*-β-MePhe (38%); mp.: 225-227°C. ^{1}H-NMR (D_2O) δ 1.36 (d, 3H, J=7.2 Hz, CH_3), 3.24 (t, 1H, J=7.3 Hz, H_β), 3.75 (d, 1H, J=7.7 Hz, H_α), 7.35 (m, 5H, ArH). TLC $R_f(1)$=0.42, $R_f(2)$=0.44, $R_f(3)$=0.39 and 0.57; HPLC k'=5.34 (0-2% B/A/30 min).

3.4 g *t*-β-MePhe (18%); mp.: 222-224°C. ^{1}H-NMR (D_2O) δ 1.34 (d, 3H, J=7.3 Hz, CH_3), 3.48 (t, 1H, J=7.2 Hz, H_β), 3.88 (d, 1H, J=5.0 Hz, H_α), 7.35 (m, 5H, ArH). TLC $R_f(1)$=0.42, $R_f(2)$=0.44, $R_f(3)$=0.46 and 0.57; HPLC k'=6.06 (0-2% B/A/30 min).

3.2.4. Boc-e- and t-DL-β-methylphenylalanine

1.5 g (8.3 mmol) of *e*- or *t*-β-MePhe was dissolved in a mixture of 26 mL of peroxide-free dioxane and 13 mL of water, and the solution was basified with 4 M NaOH solution to pH=9 and cooled in an ice bath. Next, 1.85 g Boc_2O (8.5 mmol) was added and the mixture was stirred at room temperature. The pH of the solution was kept at 9. After overnight stirring, the dioxane was removed under reduced pressure and the mixture was extracted with EtOAc. The combined organic solutions were extracted with 10% citric acid solution and then with brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated and the resulting oil was crystallized from EtOAc/petroleum ether.

1.2 g Boc-*e*-DL-β-MePhe (52%); mp.: 108-110°C. ^{1}H-NMR (DMSO-d_6) δ 1.21 (s, 3H, βCH_3), 1.33 (s, 9H, $BocCH_3$), 3.17 (s, 1H, H_β), 4.14 (s, 1H, H_α), 7.22 (m, 5H, ArH). TLC $R_f(4)=0.75$, $R_f(5)=0.56$; HPLC k'=5.33 (20-90% D/C/30min).

1.3 g Boc-*t*-DL-β-MePhe (56%); mp.: 96-98°C. ^{1}H-NMR (DMSO-d_6) δ 1.19 (d, 3H, J=5.5 Hz, βCH_3), 1.26 (s, 9H, $BocCH_3$), 3.05 (s, 1H, H_β), 4.08 (s, 1H, H_α), 7.23 (m, 5H, ArH). TLC $R_f(4)=0.72$, $R_f(5)=0.57$; HPLC k'=5.17 (20-90% D/C/30 min).

3.2.5. Synthesis of peptides

Peptides were synthesized by manual solid-phase techniques. Peptide amides were prepared on 4-methylbenzhydrylamine resin and peptide acids on chloromethylated resin. The Boc-amino acid resin esters were prepared by the method of Gisin [196]: 1 g of chloromethylated resin was carefully stirred with 1 mmol of Boc-amino acid and 1 mmol of $CsHCO_3$ dissolved in DMF at 40°C. N^α-*t*-Boc chemistry with HOBt and DCC as coupling agents was employed for peptide elongation. The following steps were performed in each cycle: ① addition of Boc-amino acid in DCM (2 equiv.); ② addition of HOBt (2 equiv.); ③ addition of DCC (2 equiv) and mixing for 3 h; ④ washing with DCM; ⑤ monitoring with the ninhydrin test; ⑥ Boc-deprotection with 50% (v/v) TFA in DCM containing 0.5% DTT (20 min); ⑦ washing with DCM; ⑧ neutralization with 10% (v/v) DIEA in DCM; and ⑨ washing with DCM and EtOH. Peptides were cleaved from the resin by treatment with anhydrous HF (10 mL/g resin) in the presence of anisole (1 mL/g resin) and dimethyl sulfide (1 mL/g resin) for 60 min at 0°C. After evaporation of the HF, the resin was extracted with Et_2O to remove the scavengers, and subsequently with 30% AcOH. Crude peptides were obtained in solid form through lyophilization of the diluted AcOH extract (yields 60-70%). Purification was performed by

TABLE 5. Analytical parameters of endomorphin analogues and fragments

Peptide	M_r	MS	HPLC[a]	TLC[a]		
		$[M+H]^+$	k'	$R_f(1)$	$R_f(2)$	$R_f(6)$
H-Tyr-Pro-Trp-Phe-NH_2	610.7	611.6	3.33[b]	0.45	0.56	0.34
H-Tyr-Pro-Trp-(2S,3S)β-MePhe-NH_2	624.7	625.4	3.80[b]	0.43	0.57	0.35
H-Tyr-Pro-Trp-(2R,3R)β-MePhe-NH_2	624.7	625.4	4.37[b]	0.36	0.53	0.31
H-Tyr-Pro-Trp-(2S,3R)β-MePhe-NH_2	624.7	625.4	3.69[b]	0.44	0.57	0.36
H-Tyr-Pro-Trp-(2R,3S)β-MePhe-NH_2	624.7	625.4	4.41[b]	0.37	0.55	0.34
H-Tyr-Pro-Phe-Phe-NH_2	571.7	572.7	2.83[b]	0.43	0.55	0.37
H-Tyr-Pro-(2S,3S)β-MePhe-Phe-NH_2	585.7	586.4	3.67[b]	0.43	0.55	0.42
H-Tyr-Pro-(2R,3R)β-MePhe-Phe-NH_2	585.7	586.5	5.55[b]	0.37	0.56	0.36
H-Tyr-Pro-(2S,3R)β-MePhe-Phe-NH_2	585.7	586.5	3.68[b]	0.47	0.53	0.37
H-Tyr-Pro-(2R,3S)β-MePhe-Phe-NH_2	585.7	586.5	5.56[b]	0.39	0.56	0.38
H-Tyr-Pro-Phe-(2S,3S)β-MePhe-NH_2	585.7	586.6	3.28[b]	0.42	0.56	0.38
H-Tyr-Pro-Phe-(2R,3R)β-MePhe-NH_2	585.7	586.7	4.39[b]	0.39	0.53	0.36
H-Tyr-Pro-Phe-(2S,3R)β-MePhe-NH_2	585.7	586.5	3.23[b]	0.44	0.57	0.40
H-Tyr-Pro-Phe-(2R,3S)β-MePhe-NH_2	585.7	586.4	4.47[b]	0.41	0.55	0.38
H-Tyr-Pro-Trp-Phe-OH	611.7	612.4	5.52[c]	0.53	0.53	0.27
H-Tyr-Pro-Phe-Phe-OH	572.7	573.6	5.30[c]	0.53	0.54	0.29
H-Tyr-Pro-OH	278.3	279.4	1.55[c]	0.41	0.56	0.26
H-Tyr-Pro-Trp-OH	464.5	465.4	4.08[c]	0.45	0.47	0.21
H-Tyr-Pro-Phe-OH	425.5	426.4	3.90[c]	0.44	0.47	0.24
H-Pro-Trp-Phe-NH_2	447.5	448.5	4.37[c]	0.26	0.42	0.26
H-Pro-Phe-Phe-NH_2	408.5	409.5	4.04[c]	0.26	0.44	0.28
H-Pro-Trp-Phe-OH	448.5	449.5	5.01[c]	0.29	0.41	0.16
H-Pro-Phe-Phe-OH	409.5	410.4	4.61[c]	0.29	0.41	0.17
H-Pro-Trp-OH	301.3	302.4	2.61[c]	0.21	0.33	0.10
H-Pro-Phe-OH	262.3	263.4	2.33[c]	0.22	0.31	0.12
H-Trp-Phe-NH_2	350.4	351.4	3.92[c]	0.56	0.57	0.43
H-Phe-Phe-NH_2	311.4	312.3	3.35[c]	0.60	0.56	0.48
H-Trp-Phe-OH	351.4	352.4	4.58[c]	0.56	0.57	0.30
H-Phe-Phe-OH	312.3	313.3	4.14[c]	0.57	0.55	0.32
H-Phe-NH_2	164.2	165.2	1.45[c]	0.36	0.43	0.27
H-Dit-Pro-Trp-Phe-NH_2	862.5	863.0	4.83[b]	0.63	0.46	0.42
H-Tyr-ΔPro-Trp-Phe-NH_2	608.7	609.3	2.70[b]	0.52	0.50	0.37
H-Dit-Pro-Phe-Phe-NH_2	823.5	824.1	4.59[b]	0.60	0.46	0.45
H-Tyr-ΔPro-Phe-Phe-NH_2	569.7	570.2	2.17[b]	0.51	0.50	0.38
H-Tyr-Pro-Phe(4'-I)-Phe-NH_2	697.6	698.0	4.30[b]	0.50	0.36	0.37

[a] Concentration of peptides: 0.1 mg/mL; t_0 = 2.6 min (HPLC)
[b] 20-40% D/C/20 min
[c] 5-50% D/C/20 min

HPLC with D/C linear gradients. Each peptide was at least 98% pure, as assessed by TLC and analytical HPLC. The molecular weights of the peptides were confirmed by ESI-MS (*Table 5*).

3.2.6. Determination of the configuration of β-MePhe in peptides

Two diastereomeric peptides containing *e*-β-MePhe isomers and two diastereomeric peptides containing *t*-β-MePhe isomers were obtained after HPLC purification (see capacity factors in *Table 5*). 1 mg of each peptide was hydrolysed separately with 1 mL of 6 M HCl under Ar in a glass ampoule for 24 h at 110°C. The solvent was removed by evaporation, and the mixture of amino acids was analysed by chiral TLC. These R_f values were compared with literature data [145].

3.2.7. Preparation of tritiated endomorphins

2 μmol of precursor peptide was dissolved in 1 mL of DMF and 1.5 μl of TEA (in the case of halogenated precursors) and 10 mg of $PdO/BaSO_4$ was added. The solution was frozen with liquid N_2 and evacuated. Tritium gas was liberated from uranium tritide by heating, and 555 GBq (15 Ci) of it was introduced into the reaction vessel. The reaction mixture was stirred at room temperature for 80 min. Tritiation was controlled by following the pressure with a manometer. After the reaction had been completed, the reaction mixture was frozen with liquid N_2, and unreacted tritium gas was adsorbed on pyrophoric uranium. The catalyst was filtered off through a Whatman GF/C filter and washed with EtOH:water (1:1). The mother liquor was evaporated and labile tritium was removed by repeated evaporation from EtOH:water (1:1). The total radioactivity (A_{tot}) of the labelled peptides was measured by liquid scintillation counting, and was found as given in *Table 6*. Crude products were purified by HPLC to give a radiochemical purity of >95%, checked by both TLC and HPLC (*Table 7*). Specific radioactivity (a) was determined by using calibration curves of the EMs. Purified tritiated peptides dissolved in spectroscopic EtOH were stored in a concentration of 37 MBq/mL (1 mCi/mL) under liquid N_2.

3.2.8. Tritium distribution in labelled endomorphins

0.74 MBq (20 μCi) of each tritiated EM and 0.06 mg of unlabelled EM were hydrolysed with 1 mL of 6 M HCl under Ar pressure in a closed ampoule for 24 h at 110°C. The solvent was removed by evaporation, and the samples were then dissolved in 1 mL of 0.2 M borate buffer (pH=7.7). 0.2 mL of 9-fluorenylmethyl chloroformate in acetone (15 mM) was added to 0.2 mL of aqueous sample solution. After 45 sec, the mixture was extracted with pentane, and the aqueous phase was analysed by HPLC with a linear gradient of 30-80% D/C/20 min.

TABLE 6. Synthesis of tritiated endomorphins

Precursor peptide	Labelled peptide	A_{tot} (GBq)
H-Dit-Pro-Trp-Phe-NH_2	H-[^{3}H]Tyr-Pro-Trp-Phe-NH_2	4.11
H-Tyr-ΔPro-Trp-Phe-NH_2	H-Tyr-[^{3}H]Pro-Trp-Phe-NH_2	3.67
H-Dit-Pro-Phe-Phe-NH_2	H-[^{3}H]Tyr-Pro-Phe-Phe-NH_2	3.25
H-Tyr-ΔPro-Phe-Phe-NH_2	H-Tyr-[^{3}H]Pro-Phe-Phe-NH_2	3.18
H-Tyr-Pro-Phe(4'-I)-Phe-NH_2	H-Tyr-Pro-[^{3}H]Phe-Phe-NH_2	1.40

TABLE 7. Radioanalytical data on tritiated endomorphins

Labelled peptide	Radio-HPLC	Radio-TLC			Spec. activity
	k'[a]	R_f(1)	R_f(2)	R_f(6)	(TBq/mmol)
H-[^{3}H]Tyr-Pro-Trp-Phe-NH_2	3.31	0.46	0.55	0.34	1.53
H-Tyr-[^{3}H]Pro-Trp-Phe-NH_2	3.36	0.44	0.57	0.35	2.35
H-[^{3}H]Tyr-Pro-Phe-Phe-NH_2	2.81	0.42	0.55	0.39	1.97
H-Tyr-[^{3}H]Pro-Phe-Phe-NH_2	2.79	0.42	0.54	0.37	1.88
H-Tyr-Pro-[^{3}H]Phe-Phe-NH_2	2.85	0.43	0.56	0.37	0.77

[a] 20-40% D/C/20 min

3.3. Methods for investigation of the metabolism of endomorphins

3.3.1. Preparation of matrices from rat brain

Brains of Wistar rats were homogenized in 50 mM Tris.HCl buffer (pH=7.4) to give a final protein content of 5.4 mg/mL. Centrifugation (22000×g, 30 min, 4°C) of the rat brain homogenate resulted in a supernatant (2.3 mg/mL protein) and a pellet (3.4 mg/mL protein) fraction. Protein concentrations were determined by the method of Bradford [197]. The crude membrane fraction used in receptor binding assays was prepared from the rat brain homogenate [198].

3.3.2. Digestion of peptides with matrices derived from rat brain

20 μL of 1 mM peptide stock solution in 50 mM Tris.HCl buffer (pH=7.4) was added to 180 μL of the matrix and the mixture was incubated at 37°C. For preincubation, inhibitors were added 10 min before the peptide addition to yield the concentrations [180] given in *Table 12.* Aliquots were withdrawn from the incubation mixtures and immediately acidified with 0.1 M HCl solution. 10 μL of supernatant obtained after centrifugation (11340×g, 5 min, 25 °C) of the samples was analysed by HPLC. Kinetic parameters of the EMs were calculated by assuming pseudo-first-order degradation.

3.3.3. Digestion of tritiated endomorphins with rat brain homogenate

The minimum detectable radioactivity was measured as 370 Bq at an HPLC effluent rate of 1 mL/min. Thus, the basic amount of radioactivity for measurement with flow-through radioactivity detection was chosen as 3700 Bq, which was multiplied by the ratio of the specific activities of the [^{3}H]EMs, resulting in the same chemical concentration of peptides in the incubation mixtures (*Table 8*). After preincubation of the rat brain homogenate, 192 nM [^{3}H]EM was incubated with it in 50 mM Tris.HCl buffer (pH=7.4) at 37°C in a final volume of 200 µL. 50 µL aliquots were withdrawn after incubation for 1, 5 and 10 min, and immediately acidified with 50 µL of 0.1 M HCl solution. Following centrifugation (11340×g, 5 min) of the samples, 50 µl of supernatant was analysed by radio-HPLC at a gradient of 5-50% D/C/20 min.

3.3.4. Preparation of solutions from purified enzymes

Carboxypeptidase A (EC 3.4.17.1, type II, aqueous suspension with toluene added, from bovine pancreases), carboxypeptidase Y (EC 3.4.16.1, lyophilized powder, from baker's yeast), aminopeptidase M (EC 3.4.11.2, type IV-S, suspension in 3.5 M $(NH_4)_2SO_4$ solution, pH=7.7, containing 10 mM $MgCl_2$, from porcine kidney microsomes) and proteinase A (EC 3.4.23.6, lyophilized powder, from baker's yeast) were purchased from Sigma. The homogenity of each of these commercially available enzymes was checked by sodium dodecylsulfate-polyacrylamide gel electrophoresis. Carboxypeptidase A and aminopeptidase M were used in their original concentrations, 400 units/mL and 1080 units/mL, respectively. Carboxypeptidase Y solution (0.5 mg/mL, 9 units/mL) was made by dissolving the powder in 50 mM Tris.HCl buffer (pH=7.4). Proteinase A was dissolved in 50 mM Tris.HCl buffer (pH=7.4) containing 10% glycerol to give a 0.5 mg/mL (9.5 units/mL) solution.

TABLE 8. Radioactivity concentrations in the incubation mixtures

Labelled peptide	Radioactivity concentration (Bq/µL)
H-[^{3}H]Tyr-Pro-Trp-Phe-NH_2	291
H-Tyr-[^{3}H]Pro-Trp-Phe-NH_2	450
H-[^{3}H]Tyr-Pro-Phe-Phe-NH_2	376
H-Tyr-[^{3}H]Pro-Phe-Phe-NH_2	359
H-Tyr-Pro-[^{3}H]Phe-Phe-NH_2	148

3.3.5. Enzymatic digestion of peptides

A solution of enzymes (5 μL of carboxypeptidase A, 30 μL of carboxypeptidase Y, 10 μL of aminopeptidase M, 30 μL of proteinase A) was diluted with 50 mM Tris.HCl buffer (pH=7.4) to 1 mL, and was then preincubated for 15 min at 37°C. 0.2 mL of a 1 mM solution of peptide in 50 mM Tris.HCl buffer (pH=7.4) was added to the solution of enzymes. The digestion mixture was kept at 37°C. At designated intervals, 20 μL aliquots were removed and acidified with 20 μL of 0.1 M HCl solution. The times of sampling were chosen so that a kinetic curve could be constructed. 10 μL of acidified sample was analysed by HPLC with a linear gradient of 5-50% D/C/20 min. Chromatograms were corrected by means of blanks prepared from peptide-free samples. Kinetic curves were analysed by assuming pseudo-first-order degradation. Half-lives ($t_{1/2}$) were calculated from the rate constants (k) as (ln2)/k. The sequence of metabolites was verified in part by HPLC-MS and by the use of synthetic EM fragments.

4. RESULTS AND DISCUSSION

4.1. Synthesis and structure determination of β-MePhe-endomorphins

The preparation of EM analogues substituted with β-methylphenylalanine (β-MePhe) isomers required the synthesis of this amino acid, which was achieved by malonester synthesis starting from racemic (1-bromoethyl)benzene [199] (*Figure 4*). The *erythro* and *threo* isomers can be separated by fractional crystallization of their benzoyl derivatives, and the optical resolution of the respective isomers can be attained by treating the benzyloxycarbonyl derivatives with quinine or quinidine [199]. Moreover, the pure *e*-β-MePhe hydrochloride salt can be obtained by fractional crystallization from water, while *t*-β-MePhe can be obtained from the mother liquor by repeated crystallization from water [145].

Fractional crystallization of the hydrochloride salts resulted in *e*- and *t*-β-MePhe with purities of >98% which was satisfactory for peptide synthesis. The purities of the amino acids were controlled by HPLC. Boc-protected racemic *e*- and *t*-β-MePhe were prepared by reaction with di-*tert*-butyl dicarbonate in the presence of NaOH. The Boc derivatives of e- and t-β-MePhe were incorporated into the peptides in racemic form.

EM analogues were synthesized manually by SPPS using Boc chemistry. The 4-methylbenzhydrylamine resin was chosen as support for the synthesis, because EMs are peptide

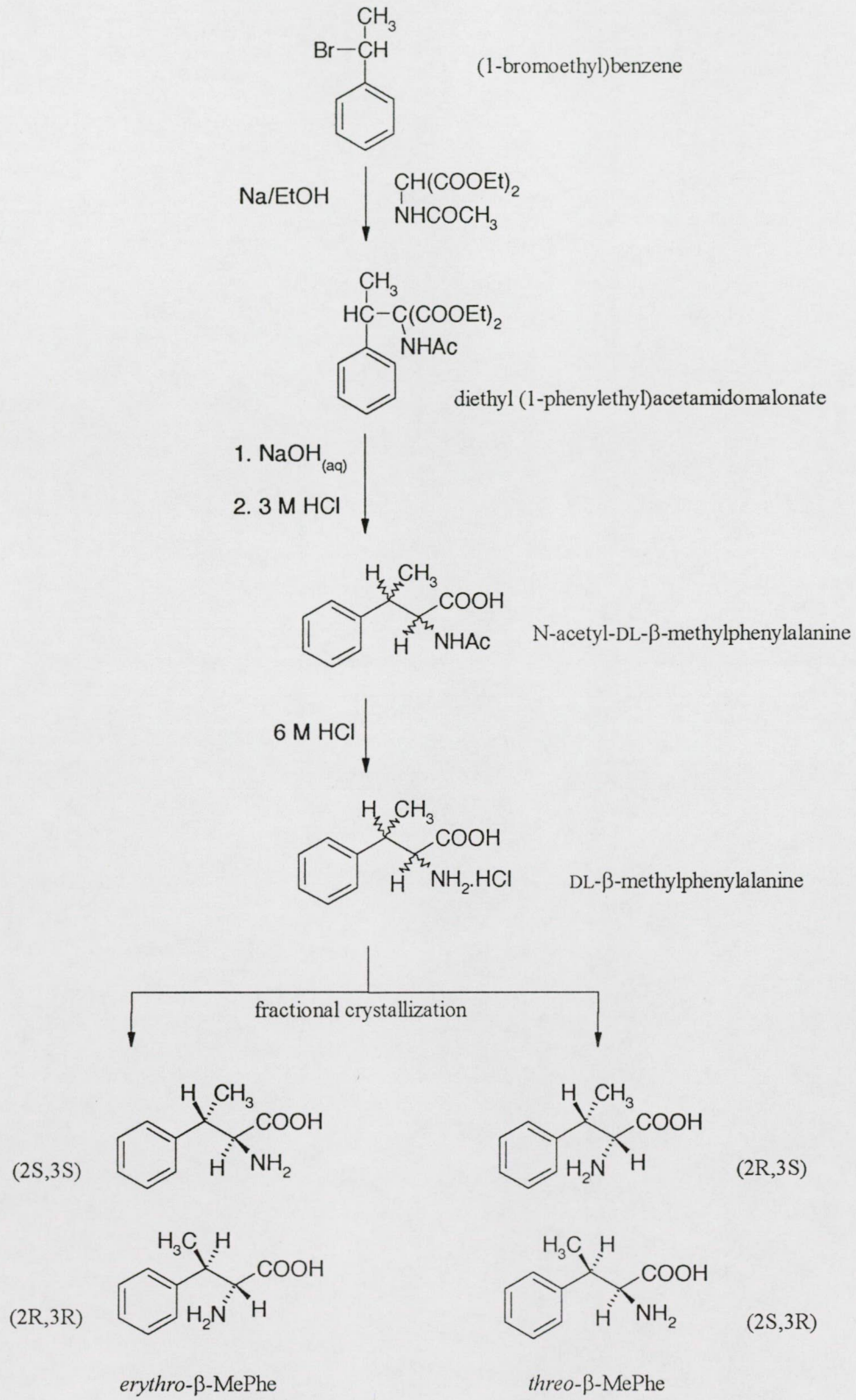


FIGURE 4. Synthesis of *erythro*- and *threo*-β-methylphenylalanine

amides. HOBt and DCC were used as coupling agents, and 50% TFA in DCM, which contained 0.5% DTT as scavenger, was used for deprotection of the Boc-peptidyl resin. Coupling and deprotection of Boc-β-MePhe isomers could be accomplished by the standard method. HPLC analysis of the crude peptides indicated that the ratio of the diastereomeric peptides is nearly 1:1. Thus, the peptide bonds are formed with the different Boc-β-MePhe isomers at the same reaction velocity. Peptides were cleaved from the polymeric support with liquid HF in the presence of anisole and dimethyl sulfide as scavengers. After cleavage, the scavengers were removed by washing the mixture with diethyl ether, and the peptides were extracted with aqueous acetic acid and finally lyophilized. Crude peptides were generally obtained in 60-70% yield (referring to the amount of NH_2 groups on the resin) and were purified by reversed-phase HPLC on a semipreparative C_{18} column. A binary eluent of ACN as organic modifier and water, containing 0.08% and 0.1% TFA as ion-pairing agent, respectively, afforded good separation for the diastereomeric peptides (differences in k' were 0.57-1.88, *Table 5*). To determine the absolute configurations of β-MePhe isomers incorporated into EMs, peptides were hydrolysed under acidic conditions, and the mixture of amino acids was then analysed by TLC. β-MePhe isomers could be separated on the chiral stationary phase of Macherey-Nagel, and were identified via their retention factors [145]. Comparison of the TLC data with the HPLC chromatograms clearly reveals that the compound eluting first from the reversed-phase HPLC column contains (2S)β-MePhe isomers.

In order to find molecular determinants for high μ opioid affinity and selectivity, the structures of the native EMs and their diastereomeric analogues were investigated by NMR spectroscopy, molecular modelling, circular dichroism and opioid receptor binding studies [98,99,200].

NMR experiments on β-MePhe-substitited EMs were performed with standard one- and two-dimensional homo- and heteronuclear techniques in DMSO-d_6 (27°C). The one-dimensional ^1H spectra of β-MePhe-EMs showing two sets of signals indicate the presence of a conformational exchange slow on the NMR time scale between the *cis*- and *trans*-isomers of the investigated peptides. The relative integrated intensities of the signals corresponding to the different isomers indicate that the β-MePhe-EMs reside in a population ratio of 1:2 in the *cis*- and *trans*-isomers, respectively, with respect to the Pro-omega bond. The NMR assignments of the isomers were based on the characteristic sequential NOEs observed between the Tyr^1 and Pro^2 residues, and were supported by the characteristic ^{13}C chemical shifts of Pro^2. A

significant difference of *ca.* 0.8 ppm was observed between the proton chemical shifts of Pro^2-H_α in the *cis*- and *trans*-isomers. A similar isomeric ratio was reported for EM1, which exists in the *cis*- and *trans*-configurations in approximately 25% and 75% populations, respectively. The significance of the *trans*-isomer as concerns peptide binding and selectivity was investigated through structural comparisons with other μ selective peptides [200]. The upfield shift of the Pro^2-H_α signals of the *cis*-isomers indicates the spatial proximity of the aromatic side-chain to the Pro ring. Only a few non-sequential ROESY cross-peaks were observed for the investigated peptides, which is an indication of the existence of random extended conformations in DMSO solution. However, the sequential NH_i - NH_{i+1} NOEs observed between Phe^3 and β-$MePhe^4$, and the small temperature coefficient of β-$MePhe^3$-NHs (-3 ppb/K), suggest that some folded conformers may also exist in conformational equilibrium with the extended ones. The side-chain conformations of the Tyr^1, β-$MePhe^3$ and β-$MePhe^4$ residues were deduced from the three-bond homo- and heteronuclear coupling constants and were corroborated by the ROE patterns observed between the backbone and side-chain protons. Tyr^1 prefers the *trans*-conformation (up to 45-50% of the population) in the *cis*-peptides, but in the *trans*-isomers all three staggered conformers are almost equally populated. It is noteworthy that the *trans*-conformation of the Tyr^1 side-chain in the *cis*-isomers allows the spatial proximity of the aromatic and Pro rings. In the *trans*-isomers of β-$MePhe^3$-EM2 analogues the β-MePhe side-chain prefers *gauche (-)* and *gauche (+)* conformations (up to *ca.* 45-45%), while in the *cis*-isomers the *gauche (-)* conformation is favoured (60%) in the rotamer equilibrium. The β-$MePhe^4$ side-chain prefers the *gauche (-)* conformation in both isomers, while the *trans* arrangement is found to be the least favoured in both cases.

4.2. Tritium labelling

Radioisotopomers of both EM1 and EM2 were prepared to examine the enzymatic degradation of the EMs *in vitro*, and to prepare satisfactory experimental tools for the biological characterization of EMs. The radioactive nuclides most widely used for peptide labelling are ^3H, ^{14}C, ^{35}S and ^{125}I. The incorporation of ^{125}I provides high specific radioactivity, but evidently changes the physicochemical properties of the molecule (e.g. the size of the molecule, and the hydrophobicity and acidity of the phenolic OH of Tyr), which in turn results in different biological characteristics. The target of the iodine labelling is an activated aromatic side-chain ring of the peptide, such as Tyr. However, the Tyr moiety of opioid ligands is a key

pharmacophore, and it is therefore highly recommended to keep this moiety intact. In contrast with this logic, Goldberg and his colleagues reported that radioiodination did not appreciably affect the affinity of the EMs in binding assays [201]. Tritium labelling is a better method with which to obtain a suitable biological tool, because the incorporation of tritium atom(s) instead of hydrogen atom(s) does not result in significant changes in the molecular properties. The incorporation of one tritium atom into one molecule results in a specific radioactivity of 1.06 TBq/mmol (28.6 Ci/mmol), which is sufficient not only for radioligand binding assays, but also for autoradiography.

There are several methods to prepare tritiated peptides [148]. A chemical synthetic method was used to prepare the specifically labelled EMs [**I, II**]. The specificity of the labelling in this case means that only one amino acid residue of the peptide should contain a tritium atom(s). This is a necessary condition for qualitative investigation of the degradation of the EMs to provide an unambiguous assignment of the degradation products. Specifically tritiated EMs were prepared through their halogenated or unsaturated precursor peptides. These precursor peptides were synthesized manually on a solid phase, incorporating the commercially available Dit, 3,4-dehydro-Pro or 4'-iodo-Phe into the EM sequences. The precursor peptides were deiodinated or saturated with tritium gas in a tritiating apparatus manufactured in our laboratory [148]. The reactions were carried out in DMF solution, and triethylamine was added to the deiodination reactions in order to neutralize the HI formed. 10% Pd (oxidized form) on $BaSO_4$ was applied as catalyst. Separation of the tritiated EMs from the corresponding precursor peptides was accomplished by HPLC (see *Table 5* vs. *Table 7*). Purity control of the resulting compounds was performed by both TLC and HPLC, with radioactivity detection with a position-sensitive proportional counter and a flow-through liquid scintillation detector, respectively. The tritiated peptides were >95% pure and identical with the non-labelled parent compounds. Specific radioactivity (a) was calculated from the amount of substance and its radioactivity (*Table 7*). To determine the chemical amount from the known radioactivity of [^{3}H]EM1 and [^{3}H]EM2 isotopomers, calibration curves of non-labelled EM1 and EM2 prepared by HPLC (peak area vs. amount of substance) were used. Tritiated compounds were stored as 37 MBq/mL ethanolic solutions under liquid nitrogen and were found to be stable for more than a year. Radiolysis resulted in about 5% impurities yearly in the peptide solutions.

Incorporation of tritium was attained with a good yield in each labelled peptide: >70% of the theoretical maximum (*Table 9*). The difference could be a result of the hydrogen impurity

TABLE 9. Incorporation of tritium into endomorphins

Tritiated peptide	a (TBq/mmol)	a/a_{max}	^3H atoms/molecule
H-[^{3}H]Tyr-Pro-Trp-Phe-NH_2	1.53	72%	1.4
H-Tyr-[^{3}H]Pro-Trp-Phe-NH_2	2.35	109%	2.2
H-[^{3}H]Tyr-Pro-Phe-Phe-NH_2	1.97	93%	1.9
H-Tyr-[^{3}H]Pro-Phe-Phe-NH_2	1.88	89%	1.8
H-Tyr-Pro-[^{3}H]Phe-Phe-NH_2	0.77	73%	0.7

in the tritium gas, or exchange reactions with labile protons of the reagents, target peptides, solvent, etc. Examination of the tritium distribution at an amino acid residue level within the peptide molecules and control of the specificity of the tritium incorporation were crucial aspects of the work before the tritiated peptides were used for metabolism investigations. For this purpose, labelled peptides were hydrolysed in the presence of unlabelled EMs under acidic conditions, and the mixtures of amino acids were then analysed through their Fmoc derivatives by HPLC. Fmoc derivatization decreases the UV detection limit of the amino acids, and ensures appropriate retention and separation for analysis of the hydrolysates [202]. The distribution of tritium within the EMs was calculated from the molar ratio of tritiated Fmoc-amino acids (*Table 10*). For peptides tritiated on the Pro^2 residue ([^{3}H]Pro^2-EM1 and [^{3}H]Pro^2-EM2), some tritium was incorporated into the aromatic amino acids Tyr and Phe. Aromatic amino acid residues could be tritiated in catalytic exchange reactions during the longer reaction time applied for the tritiation of ΔPro^2-EM1 and ΔPro^2-EM2. Application of a reaction time longer than in the case of other precursors was necessary to minimize the amount of the precursor peptide in the crude product, because the HPLC resolution of unsaturated and saturated EMs was low, and appreciable labelled EMs would have been lost during the purification step. However, the longer reaction time resulted in slight exchange reactions and therefore in a higher specific activity with a decreased specificity of labelling. On the other

TABLE 10. Distribution of tritium within labelled endomorphin molecules

Tritiated peptide	Fmoc-[^{3}H]Tyr	Fmoc-[^{3}H]Pro	Fmoc-[^{3}H]Trp	Fmoc-[^{3}H]Phe
H-[^{3}H]Tyr-Pro-Trp-Phe-NH_2	99%	-	[a]	-
H-Tyr-[^{3}H]Pro-Trp-Phe-NH_2	7%	83%	[a]	10%
H-[^{3}H]Tyr-Pro-Phe-Phe-NH_2	99%	-	--	-
H-Tyr-[^{3}H]Pro-Phe-Phe-NH_2	1%	91%	--	8%
H-Tyr-Pro-[^{3}H]Phe-Phe-NH_2	2%	-	--	98%

[a] Trp decomposes during acidic hydrolyses

hand, the tritium content of the Tyr and Phe residues is the reason why [^{3}H]Pro^2-EM1 had a specific activity higher than the theoretical value (a/a_{max}=109%). For the other tritiated EMs, the labelling was practically specific, resulting in satisfactory radioligands for metabolic studies and radioligand-binding experiments [I].

4.3. Metabolism

The degradation of neuropeptides may influence the *in vivo* physiological effects and *in vitro* receptor binding properties through their peptide fragments, and produces physiological effects that decrease in time. For instance, the analgesia produced by EM1 and EM2 was shorter in duration than that with morphine [72]. The EMs are relatively stable as compared with other opioid peptides, but a metabolite of EM1, the tripeptide Pro-Trp-Phe-NH_2, has been isolated in parallel to EM1 from the human brain [60]. It has also been reported that dipeptidyl peptidase IV [182] and other actinonin-sensitive proteolytic enzymes [183] play a role in the inactivation of EMs.

To characterize the metabolic process of EM1 and EM2 *in vitro*, peptides (100 μM) were digested with rat brain homogenate (5.4 mg/mL protein), and the digestion mixtures were analysed by HPLC. Quantitative data were obtained by measuring the change in peak area of the parent peptides, and this alteration in time was analysed from the aspect of the kinetics. Various enzyme inhibitors were used to determine the nature of the proteolytic enzymes involved in the degradation of the EMs. Quantitative degradation maps were constructed by the digestion of specifically tritium-labelled radioisotopomers of EM1 and EM2, and the effects of some known peptidases on the EMs were investigated.

4.3.1. Quantitative details of the metabolism of endomorphins

Peptide fragments of the EMs (*Table 5*) were synthesized as authentic standards in order to find chromatographic conditions suitable for the separation of all metabolites and the EMs. A linear gradient of ACN (D) as organic modifier in water (C), both containing TFA as ion-pairing agent, satisfied this separation requirement, and was applied to study the *in vitro* metabolism of the EMs. Peptides were examined at a concentration (100 μM) much higher than those of the endogenous EMs (2-150 pmol/g brain [60]). Accordingly, this *in vitro* approach was satisfactory only for study of the kinetics of degradation of the synthetic EMs, and this saturating concentration of the neuropeptides resulted in the proteolytic enzymes not

TABLE 11. Kinetics of degradation of endomorphins

Medium	Inhibitor	EM1		EM2	
		k [min^{-1}]	$t_{1/2}$ [min]	k [min^{-1}]	$t_{1/2}$ [min]
Brain hom.	none	0.1415 ± 0.0055	4.94 ± 0.19	0.1841 ± 0.0090	3.81 ± 0.19
Brain hom.	PMSF	0.0995 ± 0.0020	6.97 ± 0.14	0.1245 ± 0.0055	5.60 ± 0.24
Brain hom.	1,10-phen	0.0247 ± 0.0015	28.26 ± 1.68	0.0316 ± 0.0016	22.04 ± 1.20
Brain hom.	bestatin	0.0932 ± 0.0019	7.45 ± 0.15	0.1283 ± 0.0034	5.42 ± 0.14
Brain hom.	captopril	0.0792 ± 0.0017	8.76 ± 0.19	0.1225 ± 0.0052	5.69 ± 0.25
Brain hom.	thiorphan	0.1050 ± 0.0063	6.64 ± 0.39	0.1648 ± 0.0078	4.22 ± 0.19
Brain hom.	actinonin	0.0737 ± 0.0020	9.41 ± 0.26	0.0596 ± 0.0012	11.30 ± 0.19
Membrane	none	0.0247 ± 0.0018	28.35 ± 2.05	0.0255 ± 0.0005	27.27 ± 0.59
Cytosol	none	0.1649 ± 0.0018	4.20 ± 0.04	0.2114 ± 0.0092	3.29 ± 0.14

Data are arithmetic means of 3-6 measurements ± S.E.M. Protein contents were 5.4 mg/mL in the brain homogenate, 3.4 mg/mL in the membrane fraction and 2.3 mg/mL in the cytosol. The EM content was 100 μM. 1,10-phen: 1,10-phenanthroline.

TABLE 12. Effects of protease inhibitors on endomorphin degradation

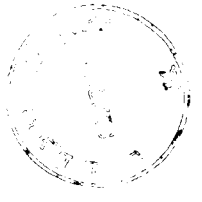
Inhibitor	Conc. (μM) [180]	% Inhibition[a] of degradation of	
		EM1	EM2
PMSF	1000	41	47
1,10-Phenanthroline	1000	472	478
Bestatin	100	51	42
Captopril	20	77	49
Thiorphan	20	34	10
Actinonin	100	90	196

[a] Ratio of half-lives with and without inhibitors

competing for substrate utilization [192]. Furthermore, at high substrate concentrations many enzyme-catalysed reactions display approximately first-order kinetics (zero-order with respect to substrate). Thus, degradation half-lives ($t_{1/2}$) were obtained by a least square linear regression analysis of plots of logarithmic EM peak areas ($\ln(A_t/A_0)$) vs. time (*Table 11*), using a minimum of five points. The breakdown of the EMs took place relatively slowly in the brain homogenate ($t_{1/2}$(EM1) = 4.94 min, $t_{1/2}$(EM2) = 3.81 min). In contrast, Leu-enkephalin was completely destroyed within 1 min under similar conditions [188]. A similar tendency was observed in the newborn rat spinal cord [183]. Different rates of degradation of EM1 and of EM2 by the rat brain homogenate was observed. EM1 was 1.3 times more resistant than EM2 to peptidases.

To identify specific peptidases involved in the metabolism of the EMs, peptides (100 μM) were incubated in rat brain homogenate in the presence of various general and specific enzyme inhibitors (*Tables 11* and *12*). PMSF is a general inhibitor of serine proteases and caused moderate inhibition of the EMs to the same extent (41 and 47%), supporting a role for serine-type protease(s) in the metabolism of EMs. 1,10-Phenanthroline, a general metalloprotease inhibitor, resulted in a high degree of inhibition. Incubation with bestatin, a general aminopeptidase inhibitor, produced a 50% inhibition of EM1, but inhibited EM2 degradation less. Since bestatin is a non-specific inhibitor of aminopeptidases [203], at the concentration used its low efficiency on the degradation of EMs could result from the activity of specific peptidases observable only when the degradation by aminopeptidases is already retarded. Such a phenomenon has already been suggested for Met-enkephalin degradation because inhibition of aminopeptidases by bestatin unmasks the formation of Tyr-Gly-Gly and Phe-Met by NEP and/or ACE in intact rat brain slices [192]. Captopril (20 μM), a selective inhibitor of ACE, produced a significant inhibition of EM1, but inhibited EM2 degradation to a smaller extent. Thiorphan (20 μM), a selective inhibitor of NEP, produced the least inhibition of EM1, and had practically no effect on EM2 degradation. Actinonin, an inhibitor of multiple enkephalin-degrading enzymes, including aminopeptidase M, resulted in a substantial inhibition of both EM1 and EM2, and reversed the stability of EM1 and EM2 against proteolysis. The robust effects of 1,10-phenanthroline and actinonin, together with the inhibitory effect of bestatin, suggest that the aminopeptidase activity is the predominant pathway for the degradation of EM1 and EM2.

These data must be considered critically, as the brain homogenate contains many cytosolic peptidases to which EMs may not be exposed during their action and CNS pharmacological studies, and additionally the distributions of the different poteolytic enzymes in the brain are non-uniform. The latter point can be overcome, because a lot of enzyme distribution data are available [e.g. 192,204]. Since the opioid receptors are membrane-bound, their ligands compete for binding in the extracellular space and are exposed to both membrane-bound proteases and enzymes dissolved in extracellular liquid, e.g. in the cerebrospinal fluid. The cytosolic and membrane fractions of the brain homogenate were therefore separated by centrifugation, and the peptidase activities associated with them were examined separately. The membrane fraction and the cytosolic fraction were examined for proteolytic activity. In the presence of the membrane fraction, EM1 and EM2 have 6- and 7-fold longer half-lives,



respectively, than in the total homogenate (*Table 11*) i.e. the cytosolic fraction contains the bulk of the enzyme activity of the brain homogenate.

The metabolic stabilities of all EM fragments were also measured in the rat brain membrane fraction (3 mg/mL protein) (*Table 13*). There were few fragments which had relatively long half- lives compared to those of the parent peptides. These are the peptide acid analogues, the N-terminal di- and tripeptide fragments of EM1 and EM2, and eventually the C-terminal tripeptide fragment of EM1. If these fragments are present in the incubation mixtures of labelled EMs, they represent *in vivo* metabolites, and it is worthwhile to examine their biological activities. If they are not present during the degradation of radiolabelled peptides, they represent relatively stable candidates in the search for the shortest sequence responsible for the opioid activity of the EMs.

There is another factor which initiated the examination of the metabolic stability of the EMs in brain membrane preparations. Radioligand binding experiments are important and simple methods for the investigation of new ligands of a receptor *in vitro*. These methods use special membrane preparations [198] to examine the ligands of the membrane-bound opioid

TABLE 13. Metabolic stabilities of endomorphins and their fragments in membrane preparation

Peptides	Half-lives[a] (min)
H-Tyr-Pro-Trp-Phe-NH_2	24.5 ± 0.5
H-Tyr-Pro-Trp-Phe-OH	17.4 ± 0.3
H-Tyr-Pro-Trp-OH	16.0 ± 0.8
H-Tyr-Pro-OH	18.9 ± 0.1
H-Pro-Trp-Phe-NH_2	10.8 ± 0.6
H-Pro-Trp-Phe-OH	2.2 ± 0.1
H-Pro-Trp-OH	1.5 ± 0.2
H-Trp-Phe-NH_2	1.3 ± 0.1
H-Trp-Phe-OH	4.4 ± 0.1
H-Tyr-Pro-Phe-Phe-NH_2	19.3 ± 3.5
H-Tyr-Pro-Phe-Phe-OH	11.4 ± 0.2
H-Tyr-Pro-Phe-OH	15.9 ± 0.1
H-Pro-Phe-Phe-NH_2	2.7 ± 0.1
H-Pro-Phe-Phe-OH	4.6 ± 0.2
H-Pro-Phe-OH	1.4 ± 0.1
H-Phe-Phe-NH_2	1.1 ± 0.1
H-Phe-Phe-OH	1.3 ± 0.1

[a] Means of three measurements. The protein content was 3 mg/mL.

receptors. Before the experiment, it is advisable to scrutinize the stability of the ligand in question under the conditions of the binding study. EM1 and EM2 exhibit long half-lives in the presence of the rat brain membrane preparation (0.3 mg/mL protein): 295 min and 230 min, respectively [I]. Since it takes only 45 min to incubate the ligands with the membrane before the measurement, therefore, virtually an inconsiderable degree of degradation occurs, and binding studies of the EMs result in correct data.

4.3.2. Determination of the metabolic pathway of endomorphins

The metabolism of the EMs has also been studied in the rat brain homogenate *in vitro* by the digestion of specifically labelled peptide radioisotopomers. An advantage of the use of tritium-labelled peptides is that it allows measurements at low concentrations without the disturbing effect of the self-proteolytic products of the brain. The basic principle of this approach is that all the radioactive metabolites of the tritiated peptides, but only these can be detected. The specificity of the labelling and the radiochemical purity of the compounds were therefore crucial points in these studies, and were proved to be satisfactory [I]. The minimum radioactivity detected by radio-HPLC was determined to be 370 Bq. The use of 3700 Bq of radioactivity in the samples therefore led to the possibility of identification of metabolites present at a level of 10%. In accordance with this consideration, the degradation of tritiated peptides was measured at a concentration of 192 nM, which is in the range of concentration of the endogenous EMs [60]. Radiochromatograms obtained from the digestion mixtures of the radioisotopomers contain the peaks of metabolites involving the corresponding tritiated amino acid. Representative radiochromatograms of EM1 and EM2 radioisotopomers incubated with rat brain homogenate are shown in *Figure 5.* The main peaks in the radiochromatograms of the corresponding radioisotopomers denoted [^{3}H]Tyr (t_R = 4.6 min), [^{3}H]Pro (t_R = 2.6 min) and [^{3}H]Phe (t_R = 7.0 min) as end-products of the peptide catabolism. Furthermore, a common metabolite with moderate stability at a retention time of 7.3 min could be seen in the radiochromatograms of [^{3}H]Tyr-EM1, [^{3}H]Pro-EM1, [^{3}H]Tyr-EM2 and [^{3}H]Pro-EM2. The only interpretation of this feature is that this metabolite should be the dipeptide Tyr-Pro, because only this compound contains both Tyr and Pro, and only this sequence is present in both EM1 and EM2. In addition, a fine difference could be observed between the degradations of EM1 and EM2. A minor metabolite was identified in the radiochromatogram of [^{3}H]Pro-EM1. Since this metabolite was observed only in this digestion mixture and not in the case of

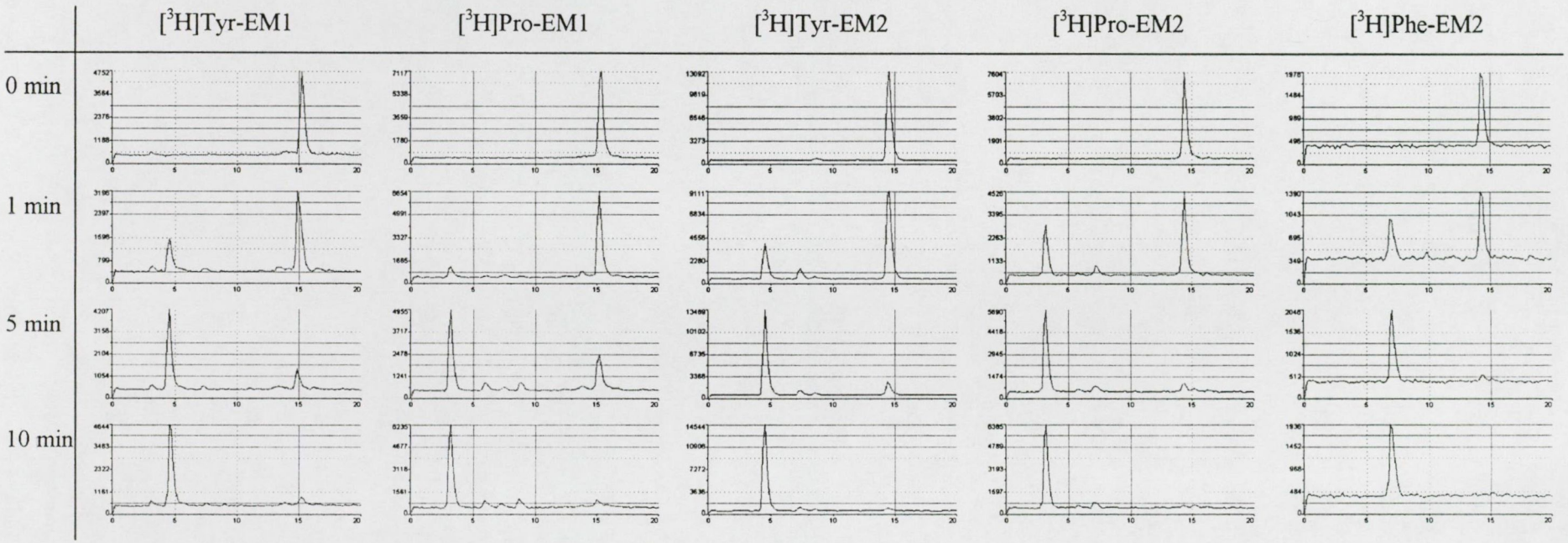


FIGURE 5. Degradation of [^{3}H]endomorphins in rat brain homogenate

Degradation was studied at 37°C in rat brain homogenate (protein content: 5.4 mg/mL). The chemical concentration of EM radioisotopomers was 192 nM (291 Bq/µL of [^{3}H]Tyr-EM1, 450 Bq/µL of [^{3}H]Pro-EM1, 376 Bq/µL of [^{3}H]Tyr-EM2, 359 Bq/µL of [^{3}H]Pro-EM2 and 148 Bq/µL of [^{3}H]Phe-EM2). Analyses of samples were performed by radio-HPLC: 5-50% D/C/20 min.

[^{3}H]Tyr-EM1, it contains Pro and not Tyr. Therefore, it was a tripeptide fragment Pro-Trp-Phe-NH_2, which was confirmed by comparing its retention time of 14.0 min with that of the synthetic tripeptide.

Consequently, EM1 and EM2 are degraded by similar pathways, but the degradation of EM1 contains an additive minor route. The first step in the catabolism of EM1 and EM2 is the cleavage of Pro^2-Trp^3 and Pro^2-Phe^3 peptide bonds, respectively. The dipeptides formed are then hydrolysed into amino acids. In contrast with the dipeptide Tyr-Pro, the dipeptides Trp-Phe-NH_2 and Phe-Phe-NH_2 have short half-lives (*Table 13*). Therefore, only [^{3}H]Phe (t_R = 7.0 min) can be seen in the radiochromatogram of digested [^{3}H]Phe-EM2, but not the dipeptide [^{3}H]Phe-Phe-NH_2. Furthermore, the metabolic pattern observed in the presence of only the membrane fraction of the brain homogenate was the same.

4.3.3. Investigation of the effects of proteolytic enzymes on endomorphin degradation

Four different enzymes, carboxypeptidase A (EC 3.4.17.1), carboxypeptidase Y (EC 3.4.16.1), proteinase A (EC 3.4.23.6) and aminopeptidases M (EC 3.4.11.2), were chosen to investigate their effects on the stability of the EMs [III].

Carboxypeptidase A is a metalloprotease which prefers peptide and protein substrates with an aromatic or branched-chained C-terminal amino acid. As amidated peptides, EM1 and EM2 exhibited high stability against digestion with this enzyme. No sign of degradation could be observed after digestion for 24 h at 37°C.

Carboxypeptidase Y can catalyse the hydrolysis of both peptide amides and peptide acids [205]. The degradation of the EMs by this enzyme took place in two steps. Hydrolysis of the tetrapeptide amides was followed by cleavage of the C-terminal Phe. N-Terminal tripeptides are the end-products of the degradation: even after 20 h of hydrolysis, no other product could be observed. This pattern was evidenced by HPLC-MS measurements too. EM1 proved to be more stable than EM2 against carboxypeptidase Y, and the degradation of EM1 and EM2

TABLE 14. Stability of endomorphins against proteolytic enzymes

Enzyme	Activity (U/mL)	$t_{1/2}$ (min)	
		EM-1	EM-2
Carboxypeptidase A	9.00	> 24 h	> 24 h
Carboxypeptidase Y	0.23	6.5	0.8
Aminopeptidase M	0.33	6.7	20.0
Proteinase A	0.24	210	102

could be described with half-lives of 6.5 and 0.8 min, respectively (*Table 14*). Proteinase A, an endopeptidase which also catalyses the hydrolysis of amidated peptides [206], degraded EMs similarly as did carboxypeptidase Y. In the first step, the EMs were hydrolysed to peptide acids, and the C-terminal Phe was then released.

Aminopeptidase M removes N-terminal amino acids, but EM1 and EM2 were split between Pro^2-Trp^3 and Pro^2-Phe^3. This finding can be explained in terms of the broad specificity of this enzyme, i.e. Xaa-Pro dipeptides are cleaved when a Pro residue is preceded by a bulky hydrophobic residue [178]. EM1 and EM2 were degraded with half-lives of 6.7 and 20.0 min, respectively. The resulting C-terminal dipeptide fragments, Trp-Phe-NH_2 and Phe-Phe-NH_2, were hydrolysed further to amino acids. The N-terminal fragment, Tyr-Pro, was relatively stable, because Tyr appeared in the chromatogram after 5 h.

4.4. Receptor binding and pharmacology

The EMs are suggested to be the endogenous ligands of the μ opioid receptor, but their precursor protein(s) and gene(s) encoding are as yet unidentified. To resolve or to decrease this contradiction, much indirect evidence has been collected (see section 1.2.). Tritiated EMs are useful research tools for this purpose. The receptor binding properties of EM1 and EM2 were examined in rat brain membranes with the help of [^{3}H]Tyr-EM2. This radioligand labelled a receptor population of 114.8 fmol/mg protein with a K_d value of 1.12 nM **[IV]**. However, in μ receptor-deficient mutant mice, no binding was detected at any of the radioligand concentrations used**[V]**. These observations show that EM2 specifically labels μ opioid receptor sites and hence directly demonstrate the high specificity of this compound for the μ receptor, as suggested by Zadina and his colleagues [59].

[^{3}H]Tyr-EM2 was also successfully applied in the characterization of EM analogues containing β-MePhe **[VI]**. The new EM analogues were examined with regard to their binding properties to rat brain opioid receptors. For the characterization of β-MePhe-containing EM analogues, [^{3}H]DAMGO was used as another μ radioligand, and [^{3}H]DIDII and [^{3}H]dynorphin A as δ and κ radioligands, respectively. EM1 and EM2 were also characterized for comparison. K_i values characteristic of the binding affinities of the ligands are listed in *Table 15*. EMs substituted with L-β-MePhe isomers (2S,3S) or (2S,3R) have higher μ affinities than those of their epimeric peptides. (2S,3S)β-$MePhe^4$-EMs were 4-5 times more active than the parent peptides. (2S,3S)β-$MePhe^4$-EM2 was the most selective analogue for the μ opioid

TABLE 15. Opioid receptor binding affinities and characteristics of opioid action of β-MePhe-containing endomorphins in isolated organs

PEPTIDE	$K_{i\mu}$ (nM)		$K_{i\delta}$ (nM)	$K_{i\kappa}$ (nM)[a]	IC_{50} (nM)	
	[^{3}H]EM2	[^{3}H]DAMGO	[^{3}H]DIDII	[^{3}H]Dynorphin A	GPI	MVD
Tyr-Pro-Trp-Phe-NH_2	1.62 ± 0.11	4.21 ± 0.31	6390 ± 5390	52.7 ± 22.7	10.1 ± 1.22	31.9 ± 10.3
Tyr-Pro-Trp-(2S,3S)β-MePhe-NH_2	0.47 ± 0.08	0.80 ± 0.09	567 ± 222	33.7 ± 17.5	-	-
Tyr-Pro-Trp-(2R,3R)β-MePhe-NH_2	43.6 ± 11.3	45.3 ± 7.75	1460 ± 1170	467 ± 226	-	-
Tyr-Pro-Trp-(2S,3R)β-MePhe-NH_2	23.4 ± 9.7	26.3 ± 5.82	4630 ± 3690	65.4 ± 39.7	-	-
Tyr-Pro-Trp-(2R,3S)β-MePhe-NH_2	47.0 ± 15.30	107.0 ± 32.8	745 ± 431	316.0 ± 92.0	-	-
Tyr-Pro-Phe-Phe-NH_2	4.00 ± 1.22	9.53 ± 2.19	2650 ± 1750	10.6 ± 6.9	9.22 ± 0.96	22.1 ± 6.5
Tyr-Pro-(2S,3S)β-MePhe-Phe-NH_2	73.1 ± 7.1	45.3 ± 13.8	179 ± 147	83.0 ± 56.7	-	-
Tyr-Pro-(2R,3R)β-MePhe-Phe-NH_2	6980 ± 4070	7090 ± 1310	6760 ± 1250	4470 ± 3400	-	-
Tyr-Pro-(2S,3R)β-MePhe-Phe-NH_2	35.0 ± 11.4	106.0 ± 9.3	> 10000	55.7 ± 3.5	-	-
Tyr-Pro-(2R,3S)β-MePhe-Phe-NH_2	-	4910 ± 328	> 10000	990 ± 930	-	-
Tyr-Pro-Phe-(2S,3S)β-MePhe-NH_2	0.97 ± 0.30	1.67 ± 0.47	6360 ± 2380	11.9 ± 2.4	3.46 ± 0.55	9.15 ± 2.9
Tyr-Pro-Phe-(2R,3R)β-MePhe-NH_2	127.0 ± 35.5	250.0 ± 65.5	5180 ± 4380	181.0 ± 95.5	816.7 ± 84.0	> 10000
Tyr-Pro-Phe-(2S,3R)β-MePhe-NH_2	23.4 ± 11.5	69.50 ± 6.61	4900 ±3990	28.1 ± 18.7	89.9 ± 16.1	235.6 ± 47.8
Tyr-Pro-Phe-(2R,3S)β-MePhe-NH_2	47.7 ± 22.6	104.0 ± 15.3	> 10000	94.3 ± 30.4	589.2 ± 88.7	> 10000

Arithmetic means of 3-5 measurements ± S.E.M. [a] [^{3}H]Dynorphin A is not a κ selective opioid receptor ligand: it also binds to μ receptors; therefore, the K_i values are relatively low.

receptor ($K_{i\delta}/K_{i\mu}$(DAMGO) = 3808, $K_{i\kappa}/K_{i\mu}$(DAMGO) = 7). The corresponding analogue of EM1, (2S,3S)β-MePhe^4-EM1, exhibited an 11-fold increased δ and a 1.5-fold increased κ receptor binding affinity; thus, its selectivity was lower than that of the parent EM1. β-MePhe^3 substitution in EM2 resulted in compounds that were less active than both the β-MePhe^4 analogues and the parent EM2.

The bioactivities of the most active and selective analogues were further investigated *in vitro* via their abilities to inhibit an electrically evoked neurotransmitter release (and the resulting muscle contraction) in the MVD and the GPI preparations. In terms of biological activity, β-MePhe-substituted analogues of EM2 exhibited similar structure - activity trends in GPI and MVD bioactivity assays than in radioligand binding assays. Differencies are due to the nature of these preparations: the GPI preparation contains predominantly μ opioid receptors, but also κ receptors, while in the MVD preparation δ receptors are predominant, but contains μ and κ receptors too [47]. The above experiments indicated that the β-MePhe-EMs were agonists. Eventually, the most hopeful (2S,3S)β-MePhe^4-containing compounds were studied *in vivo* on animal models of different types of pain [**VII**]. (2S,3S)β-MePhe^4-EM1 increased the tail-flick latency dose-dependently. Its analgesic effect was comparable with that exerted by the parent peptide. The increase in paw pressure latency was only slightly stronger and lasted longer than that evoked by the parent peptide, EM1. (2S,3S)β-MePhe^4-EM2 had a much more marked antinociceptive activity than that of EM2. It increased the tail-flick latency to radiant heat dose-dependently. The maximum effect was observed at 15 and 30 min after injection, but the effect was significant up to 120 min after an intrathecal dose of 1 μg/mice. The paw pressure latency was slightly increased at 15 min after doses of 0.1 and 0.5 μg, but it was very pronounced and lasted up to 120 min only after a dose of 1 μg.

Tritiated EM isotopomers were compared to examine the effects of the difference in the position of the incorporated tritium. For this purpose, the radioligands were investigated in radioligand binding assays. The similarity of the biochemical data demonstrated that the radioisotopomers of [^{3}H]EM1 and [^{3}H]EM2 were not significantly different in the radioreceptor assay; accordingly, the position of the labelling does not significantly influence the potency of the ligand [**I**].

Peptide fragments of EM1 and EM2 were investigated in displacement studies. Only three of the fragments caused any inhibition (*Table 16*). Tyr-Pro-Trp-Phe-OH abolished [^{3}H]EM1 binding at 10 μM concentration. Tyr-Pro-Trp-OH displaced almost all of the [^{3}H]EM1

TABLE 16. Displacement of [^{3}H]endomorphin binding by their fragments

Fragments of EM1	Displacement of [^{3}H]EM1 (%)	K_d (nM) [^{3}H]EM1	Fragments of EM2	Displacement of [^{3}H]EM2 (%)	K_d (nM) [^{3}H]EM2
Tyr-Pro-Trp-Phe-NH_2	100	0.4 ± 0.07	Tyr-Pro-Phe-Phe-NH_2	100	4.00 ± 1.22
Pro-Trp-Phe-NH_2	47.8 ± 0.1	7876 ± 346	Pro-Phe-Phe-NH_2	35.1 ± 13.4	-
Trp-Phe-NH_2	17.5 ± 0.3	-	Phe-Phe-NH_2	34.5 ± 10.6	-
Tyr-Pro-Trp-Phe-OH	100 ± 2.7	139 ± 11	Tyr-Pro-Phe-Phe-OH	80.9 ± 2.3	420.5 ± 173.1
Pro-Trp-Phe-OH	27.5 ± 2.1	-	Pro-Phe-Phe-OH	33.7 ± 9.1	-
Trp-Phe-OH	15.8 ± 1.8	-	Phe-Phe-OH	36.3 ± 7.8	-
Tyr-Pro-Trp-OH	87.8 ± 4.3	706 ± 66	Tyr-Pro-Phe-OH	92.7 ± 1.7	69.6 ± 24.4
Tyr-Pro-OH	4.8 ± 2.6	-	Tyr-Pro-OH	37.6 ± 7.0	-

Displacement of 0.4 nM [^{3}H]EM1 and [^{3}H]EM2 values are given as percentages of the specific binding. Rat brain membranes were incubated with the tritiated ligands in the presence of the peptide fragments in concentrations in the range 0.01-10000 nM.

binding, while Pro-Trp-Phe-NH_2 displaced about 50% of it. The tripeptide Tyr-Pro-Trp-OH, as a Tyr-W-MIF-1 fragment, was earlier characterized in binding assays (inhibition of [^{3}H]DAMGO), and had a K_i value of 0.405 μM for μ opioid receptors [207]. The other peptides displaced less than 30% of the tritiated ligand binding. Furthermore, none of the degradation products exerted an analgesic effect in the tail-flick tests. EM2-related peptides exhibited a similar tendency, but only two fragments, Tyr-Pro-Phe-Phe-OH and Tyr-Pro-Phe-OH, displaced [^{3}H]EM2 binding to an extent of 80-90%. It was already reported that Tyr-Pro-Phe-OH binds to the μ opioid receptor with a K_i value of 46.3 nM [98]. These findings suggest that the remaining portion of the tripeptide contained key factors for binding within the receptor pocket. On the other hand, the degradation of EMs led to total deactivation, because their peptide fragments are not able to modify the physiological effects of the parent peptides.

5. SUMMARY

Conformationally restricted amino acids were incorporated into the EMs, in order to examine the conformational requirements of the amino acid side-chains for bioactivity, and in the hope of obtaining more selective and potent μ opioid ligands. The incorporation of each stereoisomer of β-methylated derivatives of Phe resulted in the same backbone conformation, but produced different topographies of the peptides, thus leading to significant differencies in potency and selectivity.

The conformationally constrained Phe analogues were prepared in racemic syntheses by literature methods. Peptides were synthesized on solid phase, and the constrained building blocks were incorporated in racemic form. The resulting diastereomeric peptides were purified and separated by reversed-phase HPLC. To determine the absolute configuration of the β-MePhe in the peptides, they were hydrolysed under acidic conditions and the resulting mixture of amino acids was analysed by chiral TLC.

The new ligands were subjected to biological characterization by collaborating groups. Two peptides, Tyr-Pro-Trp-(2S,3S)β-MePhe-NH_2 and Tyr-Pro-Phe-(2S,3S)β-MePhe-NH_2, exhibited affinities 4 times higher than the parent EMs towards the μ opioid receptor [**VI**]. Furthermore, Tyr-Pro-Phe-(2S,3S)β-MePhe-NH_2 exhibited a 10 times higher μ vs. δ and μ vs. κ selectivity as compared to the parent EM2. *In vitro* bioassays on GPI and MVD demonstrated that the new ligands, just like the parent compounds, are full agonists. (2S,3S)β-MePhe^4-substituted EMs exhibited potencies higher than those of the endogenous EMs in *in vivo* analgesic tests too [**VII**]. Substitution of the Phe^3 moiety of EM2 resulted in a lower μ affinity, similarly to the substitution of the C-terminal position of the EMs with the other β-MePhe isomers.

In accordance with the NMR experiments and the literature data [145,146], (2S,3S)β-MePhe prefers the *gauche (-)* side-chain conformation, which can mean a favourable arrangement for μ opioid receptor binding. Moreover, the proposed bioactive form of the EMs, the *trans*-Pro^2-omega bond isomer [200], predominates over the *cis*-isomer in the (2S,3S)β-MePhe^4-EMs.

Anatomical, pharmacological and functional investigations of receptors require potent and preferably highly selective labelled ligands. EMs containing a regioselective radioactive label were prepared by chemical synthetic methods [**I, II**]. These new ligands were characterized in rat brain membrane preparations and were found to be potent and selective radioligands.

Therefore, they are excellent tools for investigations of the opioid receptor system. The corresponding radioisotopomers of EM1 and EM2 proved to be similar in affinity and receptor labelling. Thus, the position of the tritium label does not influence the action of these radioligands.

The *in vitro* metabolism of the EMs was examined in a rat brain homogenate, in its membrane and cytosolic fractions, and in the presence of different proteolytic enzymes. First, a rapid, highly reproducible chromatographic system was developed which separates EMs and related fragments. The kinetics of the metabolism of the EMs was characterized by analysing the digestion mixtures with HPLC. The velocity constants and half-lives were then calculated on the basis of pseudo-first-order kinetics. In contrast with the enkephalins, the EMs were degraded relatively slowly in the rat brain homogenate ($t_{1/2}$(EM1) = 4.94 min, $t_{1/2}$(EM2) = 3.81 min). On the other hand, no appreciable degradation of EM1 and EM2 occurred in the presence of the rat brain membrane preparation in the radioligand binding assays: $t_{1/2}$ = 295 min and 230 min, respectively [**I**].

Some general and specific enzyme inhibitors were applied to identify the nature and specificity of the enzyme system responsible for the catabolism of EM1 and EM2. The inhibition of metalloproteases and aminopeptidases stabilized the EMs to the greatest extent. The catabolism of the EMs was examined by the digestion of tritiated EM isotopomers. These studies established that only the aminopeptidase pathways are essential for inactivation of the exogenous EMs, and that the tetrapeptides were degraded by cleavage of the Pro^2-Trp^3/Phe^3 bond. The end-products of the catabolism are amino acids; the fragments Tyr-Pro-OH and Pro-Trp-Phe-NH_2 were present as intermediates. Metabolites produced by brain carboxypeptidases were not detected, because the EMs are peptide amides and the C-terminal amidation largely protects against degradation by carboxypeptidases.

Biological examination revealed that all fragments of EM1 and EM2 had low or zero affinity for μ opioid receptors. The catabolism of the EMs therefore results in their deactivation.

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