Development, synthesis and tritium labelling of delta-opioid peptide antagonists

Ph.D. Thesis

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ABBREVIATIONS

AA	amino acid
ACN	acetonitrile
Aic	2-aminoindane-2-carboxylic acid
At	aminotetralin
Atc	2-aminotetralin-2-carboxylic acid
B_{max}	maximum number of binding sites
BBB	blood-brain barrier
Boc	N-*tert*-butoxycarbonyl
Boc_2O	di-*tert*-butyl dicarbonate
Bop	benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate
Bzl	benzyl
Cha	cyclohexylalanine
Chloramine-T	*N*-chloro-*p*-toluenesulfonamide
DADLE	H-Tyr-*D*-Ala-Gly-Phe-*D*-Leu-OH
DAMGO	H-Tyr-*D*-Ala-Gly- N(Me)Phe-Gly-ol
DCC	dicyclohexylcarbodiimide
DCM	dichloromethane
DCU	dicyclohexylurea
Deltorphin I	H-Tyr-*D*-Ala-Phe-Asp-Val-Val-Gly-NH_2
Deltorphin II	H-Tyr-*D*-Ala-Phe-Glu-Val-Val-Gly-NH_2
DIEA	N,N-diisopropylethylamine
DIPCDI	diisopropylcarbodiimide
DIPP	H-Dmt-Tic-Phe-Phe-OH
DIPP-NH_2	H-Dmt-Tic-Phe-Phe-NH_2
DIPP[Ψ]-NH_2	H-Dmt-Tic[CH_2-NH]-Phe-Phe-NH_2
Dit	3',5'-diiodotyrosine
DMF	N,N-dimethylformamide
DMS	dimethyl sulfide
DMSO	dimethyl sulfoxide
Dmt	2'6'-dimethyltyrosine
DNA	deoxyribonucleic acid
DPDPE	H-Tyr-c[*D*-Pen-Gly-Phe-*D*-Pen]-OH
DSLET	H-Tyr-*D*-Ser-Gly-Phe-Leu-Thr-OH
DTLET	H-Tyr-*D*-Thr-Gly-Phe-Leu-Thr-OH
EDT	1,2-ethanedithiol
EKC	ethylketocyclazocine
EtOAc	ethyl acetate
FDAA	Marfey's reagent ≡ 1-fluoro-2,4-dinitrophenyl-5-*L*-alanine amide
Fmoc	9-fluorenylmethoxycarbonyl
GITC	2,3,4,6-tetra-O-acetyl-β-*D*-glucopyranosyl isothiocyanate
GPI	guinea pig ileum
GTPγS	guanosine-5'-O-(3-thio)triphosphate
Hat	6-hydroxy-2-aminotetralin-2-carboxylic acid
H-αDmt-OH	2-methylamino-3-(2'6'-dimethyl-4-hydroxyphenyl)propionic acid
H-βTic	1,2,3,4-tetrahydroisoquinoline-3-yl acetic acid

HBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
Hfe	homophenylalanine
Hmt	2'-hydroxy-6'-methyltyrosine
HOBt	1-hydroxybenzotriazole
RP-HPLC	reversed-phase high-performance liquid chromatography
IC_{50}	the ligand concentration that inhibits specific radioligand binding by 50%
k'	capacity factor
K_d	equilibrium dissociation constant
K_e	the molar concentration required to double the agonist concentration to achieve the original response
K_i	equilibrium inhibitory constant
LSC	liquid scintillation counter
MBHA resin	4-methylbenzhydrylamine resin
β-MePhe	β-methyl-phenylalanine
MeTic	*L*-3-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid
MVD	mouse vas deferens
2-Nal	3-(2'-naphthyl)alanine
Nva	norvaline
Orn	ornithine
Ph	phenyl
Phe(*p*NO_2)	*para*-nitrophenylalanine
PyBOP	benzotriazol-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate
PyBrOP	bromo-tris(pyrrolidino)phosphonium hexafluorophosphate
RT	room temperature
SPPS	solid-phase peptide synthesis
t-Bu	*tert*-butyl
TBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TEA	triethylamine
TFA	trifluoroacetic acid
TFMSA	trifluoromethanesulfonic acid
Tia	1,2,3,4-tetrahydroisoquinoline-3-amine
TIA	thioanisole
Tic	1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid
TICP	H-Tyr-Tic-Cha-Phe-OH
TICP[Ψ]	H-Tyr-Tic[CH_2-NH]-Cha-Phe-OH
TIP	H-Tyr-Tic-Phe-OH
TIP[Ψ]	H-Tyr-Tic[CH_2-NH]-Phe-OH
TIPP	H-Tyr-Tic-Phe-Phe-OH
TIPP-NH_2	H-Tyr-Tic-Phe-Phe-NH_2
TIPP[Ψ]	H-Tyr-Tic[CH_2-NH]-Phe-Phe-OH
TNBS	2,4,6-trinitrobenzenesulfonic acid
TLC	thin-layer chromatography
U-69593	(5α,7α,8β)-N-methyl-[7-(1-pyrrolidinyl)-1-oxaspiro-[4,5]dec-8-yl]-benzeneacetamide
Z	benzyloxycarbonyl

LIST OF PUBLICATIONS RELATED TO THE THESIS

[I] **I. Kertész,** G. Balboni, S. Salvadori, L. H. Lazarus G. Tóth, Synthesis of 2',6'-dimethyltyrosine containing tritiated delta opioid-receptor selective antagonist dipeptide ligands with extraordinary affinity, *J. Lab. Comp. and Radiopharm.* 41 (1998) p.1083-1091

[II] D. Tourwe, E. Mannekens, T.D. Nguyen, P. Verheyden, H. Jaspers, G. Tóth, A. Péter, **I. Kertész**, G. Török, N.N. Chung, P.W. Schiller, Sidechain methyl substitution in the delta opioid-receptor antagonist TIPP has an important effect on the activity profile, *J. Med. Chem.* 41 (1998) p. 5167-5176

[III] **I. Kertész,** G. Tóth, G. Balboni, R. Guerrini, S. Salvadori, Tritiation of delta opioid-receptor selective antagonist dipeptide ligands with extraordinary affinity containing 2',6'-dimethyltyrosine, *Czech. J. Phys.* 49 (1999) p. 887-892

[IV] I. Szatmári, G. Tóth, , **I. Kertész**, P.W. Schiller, A. Borsodi, Synthesis and binding characteristics of [^{3}H]H-Tyr-TicΨ[CH2-NH]-Cha-Phe-OH, a highly specific and stable δ-opioid antagonist, *Peptides* 20 (1999) p. 1079-1083

[V] K. Monory, S.D. Bryant, **I. Kertész**, G. Balboni, R. Guerrini, G.Tóth, S. Salvadori, L.H. Lazarus, A. Borsodi, Characterization of N,N(Me)$_2$-Dmt-Tic-OH, a delta selective opioid dipeptide antagonist, *Neuroreport* 10 (2000) p. 2083-2086.

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1. INTRODUCTION

1.1. The opioid receptor and opioid peptides

1.1.1. The opioid receptor

Pain is a highly subjective, unique sensation. Opium has been extracted from the poppy plant and used to treat pain for thousands of years but with little understanding of its mechanism of action. Besides its analgesic effect, opium has several side-effects, among others mood changes, which are the basis of its non-medical use and of its abuse. The major active ingredient of opium is the alkaloid morphine, which was isolated from it by Friedrich Sertürner in 1805. Unfortunately, it proved to be as addictive as opium, also causing respiratory depression, which is the main limit to the clinical use of morphine. Despite the major advances in our understanding of the actions of opiates and the synthesis of hundreds of analogues, morphine remains one of the most widely used analgesics. The various discoveries have prompted biologists to find the mechanism of action of morphine in the human body, and chemists to develop more efficacious and safe drugs.

During World War II, Beecher observed that wounded soldiers required far less drug, despite the greater severity of their wounds, as compared with civilians, implying that they felt less pain [1]. He concluded that situational factors, such as the stress of combat, can modify the perception of pain. This study illustrated the lack of a clear correlation between the nociceptive input and the perception of pain. Recent studies have identified a pain modulatory system within the brain that may explain these observations. The fact that the analgesia is reversible by the specific opioid antagonist naloxone [2] implied the presence of an endogenous opioid system within the brain containing morphine-like factors. Opiates mimic the actions of these naturally occurring peptides.

Opiate receptors were first hypothesized in 1954 [3], almost 20 years before they were demonstrated experimentally in 1973 [4-6]. Opiate receptors consist seven transmembrane segments and interact with heterotrimeric G-proteins. Ligands acting on the receptors are named 'agonists' while compounds that inhibit the actions of agonists are called 'antagonists'. The first indication of multiple types came from studies of the interactions of morphine and nalorphine [7,8], which led Martin to propose the concept of "receptor dualism" [9]. Subsequently, he extended his classification and, on the basis of behavioral and neurophysiological observations on the chronic spinal dog, three different opioid receptors were distinguished [10] and named after the drugs used in the test: mu (μ, from morphine), kappa (κ, from ketocyclazocine) and sigma (σ, from SKF 10047 or N-allylnormetazocine).

Later, the σ-receptor proved to be non-opioid, and another type of opioid receptor, the delta (δ-receptor) was described by Lord et al. [11] on the basis of results of bioassays carried out on peripheral tissue preparations. The development of a potent antagonist for any peptide hormone or neurotransmitter is of great importance for studies aimed at elucidating its physiological role. In a situation of receptor heterogeneity, antagonists with selectivity for each receptor type are valuable pharmacological tools and may also have potential as therapeutic agents. Later, μ_1- and μ_2-receptor subtypes were suggested to explain the binding property of the μ-receptor [12]. Computer modelling programmes soon confirmed the presence of two morphine receptor subtypes [13]. Rothman came to a similar conclusion with his mu-delta complex, which corresponds to the μ_1-receptor [14]. While μ_1- and μ_2-receptors bind morphine quite well, their overall selectivities for other classes of opioids and opioid peptides differ significantly. Although their general distributions within the brain are somewhat similar, significant differences exist [15,16]. In the early 90s, the heterogeneity of the δ-receptor was suggested on the basis of pharmacological investigations. These studies showed that the effects of different δ-agonists are blocked unevenly by different, highly selective δ-antagonist ligands [17-20]. Two subtypes, the δ_1- and δ_2-receptors were distinguished. 7-Benzylidenenaltrexone [21] is a selective antagonist of the δ_1 subtype. Non-equlibrium antagonists of the δ_2-receptor are naltrindole-5'-isothiocyanate [22] and naltribene [17]. DPDPE is a selevtive δ_1-agonist, while deltorphin II is a selective agonist of the δ_2-receptor. However, these pharmacologically defined subtypes have not been identified at a molecular level to date. Devi et al. [23] provided biochemical and pharmacological evidence for the heterodimerization of two fully functional opioid receptors, κ and δ. This results in a new receptor that exhibits ligand binding and functional properties that are distinct from those of either receptor.

Molecular biology approaches have also been applied to demonstrate the existence of opioid receptors. In the early 90s, cloning of the opioid receptor cDNAs was reported by different groups [24-27]. The binding and functional properties of the cloned receptors are identical with those described by pharmacological experiments for the μ-, δ- and κ-receptors. Recently, mice lacking the μ-opioid receptor were generated by two independent laboratories [28,29]. Experiments performed on these animals proved that all the beneficial and non-desired effects of morphine are mediated *in vivo* exclusively by the μ-receptors. Therefore, drugs targeting this receptor are not likely to provide the ideal analgesic drug. κ-Opioid agonists have been shown to be potent analgesics, but, unfortunately, they also cause psychotomimetic and dysphoric effects. Since μ- and κ-opiates produce many of their side-effects via interaction with central opioid receptors, the current focus is on developing them as peripherally rather than centrally acting analgesics. Thus, ligands acting on the δ-receptors are of great interest.

There is a functional interaction between μ- and δ-receptors [30]. The μ "knockout" mice provided a means of investigating whether analgesia mediated by δ-receptors is maintained in the absence of μ-receptors. These investigations led to an ambiguous conclusion. Spinal and supraspinal analgesia evoked by deltorphin II [31] and DPDPE [31-33] was reported to be slightly less [31], unchanged [32], or drastically reduced [33] in the μ-receptor-deficient mice. Biochemical pathways activated by δ-agonists, G-protein activation and the inhibition of adenylate cyclase were retained in the μ-knockout mice. These data emphasize that antinociception mediated by δ-receptors is at least in part independent of the μ-receptors. This confirms that highly potent δ-agonists could gain clinical importance.

1.1.2. Opioid peptides

The opioid peptides are formed in the brain, the pituitary gland and the adrenal medulla by the proteolytic cleavage of three protein precursors. In mammals, three such precursors are known: proenkephalin A [34], which gives rise to enkephalins and related peptides; prodynorphine [35], from which dynorphins and neoendorphins are formed; and proopiomelanocortin [36] from which, amongst other hormonally active peptides, β-endorphin is formed. The primary structures of these precursors, each of which has approximately 260 amino acid residues, have been determined by the use of recombinant DNA techniques [37,38]. Enkephalins, the first discovered endogenous opioid ligands which act on the δ-receptors, were described in the mid-70s [39]. The dynorphins are generally acknowledged to be the endogenous ligands for the κ-receptors [40]. β-Endorphin was regarded as the possible endogenous ligand of the μ-receptor before the discovery of endomorphins [41]. The discovery of the endomorphin family, the endogenous ligands of the μ-receptors, was in the late-90s. The precursor molecule of these peptides is still unknown. (Table 1.)

Table 1. Mammalian endogenous opioid ligands

Precursor	Endogeneous Peptide	Amino acid sequence[b]
Proopiomelanocortin	β-Endorphin	YGGFMTSEKSQTPLVTLFKNA-IIKNAYKKGE
Proenkephalin	[Met]-Enkephalin [Leu]-Enkephalin Metorphamide	YGGFM YGGFL YGGFMRRV-NH_2
Prodynorphin	Dynorphin A Dynorphin B α-Neoendorphin β-Neoendorphin	YGGFLRRIRPKLKWDNQ YGGFLRRQFKVVT YGGFLRKYPK YGGFLRKYP
Proendomorphin[a]	Endomorphin-1 Endomorphin-2	YPWF-NH_2 YPFF-NH_2

[a] presumed to exist, awaiting discovery
[b] using a single letter code

Amongst many other biologically active compounds, frog skin contains opioid peptides: dermorphin [42] and the deltorphins [43,44]. Dermorphin is a highly potent and selective μ-ligand, while the deltorphins, and especially deltorphin I and II, are selective δ-ligands with subnanomolar K_i values in the binding assay. Deltorphin I and II are the most δ-selective natural opioids, with selectivity values of 21 000 and 3 450, respectively.

New δ-peptide agonists were developed in order to improve the affinity and rather poor selectivity of the natural enkephalins. DADLE [45], DSLET [46] and DTLET [47] are potent analogues, but their δ-selectivity is relatively low. Therefore, conformational restriction [48-51] of the enkephalin molecule was carried out by cyclization [49,50], resulting in a set of peptides, including DPDPE [52], which was both stable and highly selective for the δ-opioid receptor. Further chemical efforts have led to the replacement of the naturally occurring amino acids by conformationally constrained ones in the peptide sequence. Indeed, conformationally constrained DPDPE analogues were synthesized in order to enhance the selectivity of the parent peptide [53-55]. The development of a potent antagonist for any peptide hormone or neurotransmitter is of great importance for studies aimed at elucidating its physiological role.

1.1.2.1. TIPP family

The Tyr moiety is one of the key pharmacophores of opioid peptides. The discovery that opioid peptide analogues containing a Tic residue at position 2 are potent and highly selective δ-opioid antagonists led to the new class of TIP(P) peptides in 1992 [56]. Unexpectedly, the result of a receptor binding study with [^{125}I]TIPP indicated that substitution of an iodine atom at position 3' of Tyr in TIPP turned the δ-antagonist into a δ-agonist [57]. The corresponding iodination of the Tyr residue in TIPP[Ψ], TICP and TIP did not result in agonism, suggesting that the astonishing conversion observed with TIPP may be due to an overall conformational effect rather than to a direct local effect of the iodine substitution. It is interesting that the substitution of a bromine or a chlorine at position 3' of Tyr in TIPP produced partial agonists with intrinsic efficacies [58]. The Tyr(3'-F) analogue was again a pure δ-antagonist. Thus, systematic substitution of the halogen atoms, beginning with iodine and in the sequence of the periodic table, produced a progressive decrease in intrinsic activity and a concomitant increase in affinity at the δ-receptor. These findings indicate that the phenolic hydroxy group of Tyr contributes to the effective interaction with the opioid receptor, but is not an absolute requirement for opioid activity.

Both TIPP and TIP are stable in an aqueous buffer solution of pH 7.7, but they have been found to be unstable in organic solvents and in a lipophilic membrane environment, due to spontaneous diketopiperazine formation and concomitant cleavage of the Tic^2-Phe^3 peptide

bond [59]. Pseudopeptide analogues containing a reduced peptide bond between the latter two amino acid residues have been prepared [60]. The resulting analogue TIPP[Ψ] retained high δ-opioid antagonist potency, showed further improved δ-receptor affinity and displayed unprecedented δ-receptor selectivity. Similarly, TIP[Ψ] was also a more potent and more δ-selective antagonist than its parent TIP. Both TIPP[Ψ] and TIP[Ψ] proved to be very stable against chemical and enzymatic degradation. Since TIPP and TIPP[Ψ] have very similar conformational properties, they served as parent peptides for the design of analogues. Systematic substitution at every position is useful for the determination of structure-activity relationships.

Replacement of Tyr^1 with Dmt in TIPP produced a very significant enhancement of the δ-antagonist potency, but also a slight decrease in δ-receptor selectivity [58]. Similarly, the Dmt^1 analogue of the δ-agonist DPDPE showed increased δ-agonist potency and diminished δ-selectivity [61]. These observations indicate that substitution of Dmt for Tyr^1 in opioid peptides produces in general a substantial increase in δ-receptor affinity and an even more pronounced μ-receptor affinity enhancement. Interestingly, replacement of Tyr^1 in TIPP by Hmt also increased the δ-receptor affinity, but had no effect on the μ-receptor affinity [62].

Structural modifications were performed at the Tic^2 residue by replacement of the α-carbon of Tic with a nitrogen. Such substitutions resulted in TIP and TIPP analogues in which the Tic residue was replaced by AzaTic [63]. The tripeptide retained δ-antagonist affinity in the MVD assay, but was significantly less potent than the parent compound. The tetrapeptide turned out to be a quite potent μ-agonist in the GPI assay, and a weak δ-agonist in the MVD assay, in contrast with TIPP, which has very weak μ-receptor affinity. A molecular mechanics study of the modified tripeptide indicated that this peptide has conformational preferences similar to those of the parent TIP [64], but the heterocyclic ring in AzaTic is slightly different from that in Tic. The presence of the additional nitrogen in AzaTic creates the capacity for hydrogen-bond formation. Numerous substitutions with both natural and artificial amino acids have been performed at position 3 of the TIPP peptide sequence. The Trp^3 analogues of both TIPP and TIP showed somewhat higher δ-antagonist potency and δ-selectivity than their parent peptides [65]. Elongation of the side-chain at position 3, achieved by substitution of Hfe, resulted in a new analogue of TIPP, which showed subnanomolar δ-antagonist potency and very high δ-selectivity [58]. Substitution of a halogen atom in the *para* position of Phe^3 led to compounds with slightly higher δ-antagonist potency and higher δ-selectivity than those of TIPP [66]. Replacement at the same position with 2-Nal had little effect on the potency, but increased the δ-selectivity [58]. The aromatic residues at positions 3 and 4 are not essential for high δ-antagonism since they can be replaced by liophilic residues [65,67] or even omitted

[68]. It has been shown that TIPP analogues in which the Phe^3 residue has been replaced by Leu, Ile or Nva have only slightly lower δ-antagonist potencies than that of the parent peptide, and similar δ-selectivity [65]. The replacement of Phe^3 in TIPP with a Cha residue resulted in an analogue TICP, with a 10-fold increased δ-antagonist potency and a 4-fold improved δ-selectivity [65]. The corresponding pseudopeptide made from TICP was an even more potent and selective δ-antagonist with the adequate stability [65]. TICP[Ψ] is a stable δ-antagonist with both extraordinary potency and extraordinary δ-selectivity, being about 200 times more δ-selective than naltrindole and 3 times more potent.

Structural modifications have been made at the Phe^4 residue through incorporation of various substituents in the *para* position of the aromatic ring in TIPP. Halogenation produced four compounds that all showed a slight increase in antagonist potency and δ-selectivity. Phe(pNO_2) incorporation in TIPP resulted in a new compound which displayed higher δ-receptor affinity and selectivity than those of TIPP [69].

Determination of the hydrophobicity parameters by HPLC revealed that these modified peptides were significantly more lipophilic than TIPP [66]. To enhance the lipophilicity of TIPP and TIPP[Ψ], analogues containing various alkyl and aralkyl substituents were prepared [66]. The substituents were introduced on the N-terminal amino group of TIPP or on the secondary amino group of TIPP[Ψ]. Substitution of a n-hexyl group or of two ethyl groups on the N-terminal amino function of TIPP resulted in compounds with antagonist potency comparable to that of the TIPP peptide. In the receptor binding assays, all N-terminally substitued TIPP analogues showed high δ-receptor affinities and excellent δ-receptor selectivities. Introduction of a methyl substituent on the secondary amino group of the reduced peptide bond produced a compound with unchanged δ-antagonist potency, high δ-receptor affinity, and extraordinary δ-receptor selectivity. Analogous substitutions of TIPP[Ψ] with an ethyl, n-hexyl, or phenethyl group led to compounds that displayed only slightly reduced δ-antagonist potency as compared with TIPP[Ψ] [66]. These substitued pseudopeptides all had very weak affinity for μ-receptors, and therefore still showed high receptor selectivity. Measurement of the hydrophobicity constants indicated that all alkyl- or aralkyl-substituted TIPP or TIPP[Ψ] analogues had a markedly increased lipophilic character. These various substitutions alone, or in combination can be expected to improve the ability of these δ-antagonists to cross the BBB via passive diffusion.

1.1.2.2. The Tyr-Tic pharmacophore and the effect of charge

As mentioned above, deletion of the C-terminal Phe residue from TIPP resulted in a tripeptide analogue TIP, which was 4 times less potent δ-antagonist than the parent

tetrapeptide and retained considerable δ selectivity. Further, removal of the Phe residue in TIP furnished Tyr-Tic, which was the first opioid peptide without Phe that had δ-opioid selectivity and antagonist bioactivity [68]. Substitution of the Tyr in Tyr-Tic by Dmt afforded the dipeptide antagonist Dmt-Tic, which had exceptionally high δ-affinity, ultraselectivity, and *in vitro* δ-antagonist activity [70] and acted systemically *in vivo* to reverse antinociception by a δ-agonist [71]. Enhancement of the hydrophobic properties of Dmt-Tic through N-alkylation by methyl groups not only retained the high δ-affinity and δ-receptor selectivity, but also substantially enhanced the bioactivity [72,73]; other N-alkylating agents affected $K_{i\delta}$ and decreased the δ-selectivity [74]. Further studies on the Dmt-Tic pharmacophore demonstrated, that replacement of the N-terminal amide through alkylation by piperidin-1-yl, pyrrolidin-1-yl or pyrrol-1-yl was detrimental for all bioactivity measurements on MVD [75]. N-Alkylation by methyl groups to form secondary or tertiary amines was the only N-terminal substitution tolerated [74]. These observations revealed that the hydrophobicity imparted by the 2'6'-dimethylation of Tyr and N-alkylation was an important factor that influenced the interaction between the Dmt-Tic pharmacophore and the δ-opioid receptor ligand-binding domain in a tissue preparation. Amidation of TIP(P) [57,58,60], Tyr-Tic [68] and Dmt-Tic [70,72] allowed an exploration of the effect of charge on δ-receptor affinity. Suppression of the anionic function led to elevated μ-receptor binding, a phenomenon also observed with the deltorphins [76,77]. Dmt-Tic-X (X= -NH-NH_2, -NH-CH_3, -NH-tBu or -NH-1-adamantyl) had high δ-affinities with variable μ-affinities, to yield non-selective or weakly μ-selective analogues. Similarly, the elimination of the negative charge in δ-agonists or antagonists by reduction of the acid to an alcohol also augmented binding to μ-receptors [64]. N,N-$(Me)_2$Dmt-Tic-NH-1-adamantane exhibited dual δ- and μ-receptor affinities and potent δ-antagonism with μ-agonism [75]. Moreover, C-terminal amidation with a change of configuration to D-Tic produced weakly μ-selective compounds [58,70]. Other modification, such as the building-in of a methylene bridge between C_α of Tic and the carboxylate function to prevent diketopiperazine formation yielded H-Dmt-βHTic-OH, a biostable peptide with high δ-affinity and antagonism without μ-bioactivity [75]. The same chemical approach employing a methylene group between the amino group and C_α of Dmt was more detrimental [75].

Elimination of the C-terminal carboxyl function in the δ-antagonist H-Tyr-Tic-Atc-OH led to a compound H-Tyr-Tic-2-S-At, which turned out to be a full δ-agonist [78]. Removal of the C-terminal carboxyl group in the δ-antagonist TIP also produced a compound with δ-agonist properties, H-Tyr-Tic-NH-$(CH_2)_2$-Ph [79]. Interestingly, shortening or lengthening of the C-terminal phenylethyl substituent by one methylene group converted this parent compound to a δ-antagonist. Analogues of H-Tyr-Tic-NH-$(CH_2)_2$-Ph containing a fluoro- or

chloro substituent in the *para* position of the aromatic ring of the C-terminal phenylethyl substituent also proved to be δ-antagonists, whereas an analogue of the same compound with a fluoro substituent at the *ortho* position of the phenyl ring retained δ-agonist potency [80]. In comparison with the parent peptide, the analogue H-Tyr-Tic-NH-$(CH_2)_2$-Ph(*o*-Cl) exhibited increased δ-agonist potency in the MVD assay, and higher δ-receptor selectivity. Introduction of a second phenyl group on a β-carbon of the phenylethyl moiety resulted in a compound, H-Tyr-Tic-NH-CH_2-CH-$(Ph)_2$, which was 20 times more potent as a δ-agonist, with a 2-fold improved δ-selectivity. The corresponding analogue with a methylated N-terminal amino group, Tyr(NMe)-Tic-NH-CH_2-CH-$(Ph)_2$, displayed subnanomolar δ-agonist potency and still considerable δ-receptor selectivity. The most potent and selective agonist of this class of compounds was one of the isomers of H-Tyr-Tic-NH-CH_2-CH(Ph)COOEt, which had high δ-agonist potency and excellent δ-receptor selectivity. These results indicate that these ligands form a new class of potent and selective δ-opioid agonists and, because of their highly lipophilic character and low molecular weight, they are able to cross the BBB. Saturation of the aromatic ring of the phenylethyl moiety in H-Tyr-Tic-NH-$(CH_2)_2$-Ph again led to the compound H-Tyr-Tic-NH-$(CH_2)_2$-Ch with δ-antagonist properties [80]. Replacement of the phenyl substituent with a Py moiety also brought about a conversion from δ-agonism to δ-antagonism. Finally, reversal of the amide bond between the Tic residue and the phenylethyl substituent led to a compound, H-Tyr-Tia-CO-$(CH_2)_2$-Ph, that also turned out to be a δ-antagonist [80]. These results suggest that discriminative modifications at the C-terminus might result in opioids with new properties.

1.1.2.3. Conformational analysis of the TIPP family

The conformational behaviour of the tripeptide δ-agonist TIP was examined by theoretical conformational analysis based on molecular mechanics calculations and molecular dynamics simulations [81]. To determine possible receptor-bound conformations of TIP, a conformational comparison of its low-energy conformers with the structurally rigid non-peptide δ-antagonist naltrindole was performed. On the basis of this study, two different models were considered initially, one based on a low-energy conformer identified through superposition of the centroids of the Tyr^1 and Phe^3 aromatic rings and the terminal amino group with the centroids of the corresponding aromatic rings and the nitrogen atom in naltrindole, and the other based on a low-energy conformer showing good spatial overlap of the centroids of the Tyr^1 and Tic^2 aromatic rings and the N-terminal amino group with the corresponding moieties in naltrindole. The latter model was selected as the more plausible one because of the demonstrated importance of the Tic^2 aromatic ring for δ-antagonism [69], and

in view of the observation that the dipeptide H-Tyr-Tic-NH_2 and the tripeptide H-Tyr-Tic-Ala-OH were also δ-antagonists, albeit with much lower potency than that of TIP. This model is characterized by all-*trans* peptide bonds.

An alternative model of the receptor-bound conformation of δ-opioid peptide antagonists containing the N-terminal dipeptide segment H-Tyr-Tic- has been devised [68]. This theory is also based on spatial overlap of the Tyr^1 and Tic^2 aromatic rings and N-terminal amino group with the corresponding aromatic rings and nitrogen group in the structurally rigid non-peptide δ-antagonist naltrindole. In contrast with the model with all-*trans* peptide bonds [81], the peptide bond between the Tyr^1 and Tic^2 residues in the latter model is *cis* [68]. Furthermore, the Tyr^1 aromatic ring in this model is tilted about 60° relative to the phenol ring in naltrindole. In a subsequent comparative assessment of these two models, a theoretical conformational analysis was performed on six δ-opioid peptide antagonists of this class: H-Tyr-Tic-NH_2, H-Tyr-Tic-Ala-OH, TIP, TIP[Ψ], TIPP and TIPP[Ψ] [82]. Low-energy conformers consistent with both models were identified for all six compounds. The results of this study revealed that in all cases the conformers corresponding to the all-*trans* peptide bond model were lower in energy than the conformers corresponding to the model with the *cis* peptide bond. The conformers corresponding to the model with all-*trans* peptide bonds exhibited better coplanarity of their aromatic rings with the corresponding aromatic rings in naltrindole than did the conformers corresponding to the *cis* peptide bond-containing model. However, both models remained candidate structures for the receptor-bond conformation of the TIPP class.

Both models differ from the crystal structure of TIPP, which is stabilized by numerous intermolecular hydrophobic contacts and does not represent the receptor-bond conformation [83]. To distinguish between these two models, two pseudopeptides were prepared, H-TyrΨ[CH_2NH]Tic-Phe-Phe-OH and H-TyrΨ[CH_2NH]MeTic-Phe-Phe-OH, in which the reduced peptide bond between the Tyr and Tic (MeTic) residues cannot assume the *cis* configuration for steric reasons [64]. Both compounds retained δ-opioid antagonist activity. For both of them, the results of a molecular mechanics study indicated a low-energy conformation in which the torsion angle (ω_1) of the reduced peptide bond is 180° (*trans* configuration), in good agreement with the proposed model with all-*trans* peptide bonds. Furthermore, this conformational analysis revealed that neither of these peptides could exist in low-energy conformations in which ω_1 assumed a value of 0° (*cis* configuration). In the case of H-TyrΨ[CH_2NH]MeTic-Phe-Phe-OH, some conformations with the reduced peptide bond in the *gauche*(-) position (ω_1 has a value of -60°) were identified. However, these conformers were higher in energy than the lowest-energy conformer and displayed no spatial overlap of the

three pharmacophore groups with the corresponding moieties in naltrindole. These results confirmed that the receptor-bound conformation of δ-peptide antagonists containing an N-terminal H-Tyr-Tic dipeptide segment must have all-*trans* peptide bonding. The conformational behaviour of TIPP[Ψ] analogues with an alkyl substituent attached to the nitrogen of the reduced bond was also examined by theoretical conformational analysis [66]. At physiological pH, the tertiary amino group of the N-alkylated reduced peptide bond in these compounds may assume either the *R* or the *S* configuration. Molecular mechanics and molecular dynamics studies were performed on both diastereomers of H-Tyr-TicΨ[$CH_2N^+HCH_3$]-Phe-Phe-OH and of H-Tyr-TicΨ[$CH_2N^+HCH_2CH_2Ph$]-Phe-Phe-OH, and in both cases resulted in low-energy conformers that showed a good spatial overlap between the Tyr^1 and Tic^2 aromatic rings and the N-terminal amino group of the pseudopeptides with the corresponding aromatic rings and nitrogen atom of naltrindole. These low-energy conformers closely resembled the all-*trans* peptide bond model of the δ-receptor-bond conformation of the parent peptide TIPP[Ψ] [82]. This finding provides an explanation for the fact that N-alkylation of the reduced bond in TIPP[Ψ] resulted in compounds that retained high δ-antagonist potency [66]. Molecular dynamics simulations with the previous pseudopeptides containing the N-alkylated reduced peptide bond in protonated form revealed that the torsion angle ω_2 around the CH_2N^+HR bond predominantly assumes a value of -150° when the configuration is *R*, and a value of 150° in the case of the *S* configuration. These values are close to the value for the regular *trans* peptide bond. Fluctuations to ω_2 values of -60° and 60° (*R* and *S* configurations) were seen along the dynamics trajectory, but they were less frequent than for TIPP[Ψ] in the protonated state [60], indicating that the non-alkylated reduced peptide bond is structurally more flexible than the N-alkylated reduced peptide bonds. The intramolecular distances between the Tyr^1 and Tic^2 aromatic rings in TIP(P) peptides were determined and the experiments led to the conclusion that a close hydrophobic interaction between the Tyr^1 and Tic^2 aromatic rings is prevented by the intercalation of the Phe^3 aromatic ring in the tripeptide and of the Tic^2-Phe^3 peptide bond in the tetrapeptide. The Phe^3 and Phe^4 aromatic rings are then quite exposed and could engage in receptor interactions, which might explain the higher antagonist potency of the tetrapeptides as compared with that of the tripeptides. It is clear that in such small peptides the amino acid side-chains exhibit considerable conformational flexibility, and establishment of an exact three-dimensional arrangement of the structural moieties constituting the δ-antagonist pharmacophore of TIP(P) remains a challenging problem. The use of β-methylated amino acid to constrain the conformational mobility of the side-chain by biasing the population of the χ_1 torsion angle rotamers was pioneered by Hruby et al. [84,85].

1.1.2.4. Mixed μ-agonist / δ-antagonist members of the TIPP family

Recent data on the knock-out of the μ-opioid receptor in mice confirmed that the μ-receptor is primarily involved in the appearance of analgesia induced by morphine [28]. Chronic co-administration of morphine and naltrindole has been reported to attenuate the development of tolerance and the severity of the precipitated withdrawal syndrome in mice [86]. In agreement with these results, rats chronically treated with TIPP[Ψ] concurrently with chronic morphine administration over a 6-day period indicated no morphine tolerance and a significally reduced morphine dependence [87]. Recently, it was confirmed that δ-receptor occupation with naltrindole in rats chronically treated with morphine significantly attenuated the development of a physical dependence and antinociceptive tolerance, while not preventing the development of a tolerance to morphine-induced respiratory depression [88]. Another interesting observation was that chronic morphine treatment resulted in an upregulation of the δ-binding sites in rats [89]. The development of a morphine tolerance and dependence following 3 days of chronic morphine administration was blocked by an antisense oligodeoxynucleotide to the δ-opioid receptor [90]. Finally, morphine was shown to retain its μ-mediated analgesic activity in δ-receptor knock-out mice without producing analgesic tolerance upon chronic administration [91]. These various observations clearly indicate that the δ-opioid receptors play a key role in the development of morphine tolerance and dependence. These findings also suggest that δ-opioid antagonists may be useful therapeutic agents in analgesia in combination with μ-agonists such as morphine. Alternatively, the development of a single opioid compound acting as an agonist at the μ-receptor and as an antagonist at the δ-receptor might be of benefit in the management of chronic pain. Such a mixed μ-agonist/δ-antagonist would be an analgesic with a low propensity to produce analgesic tolerance and physical dependence. However, it should be noted that δ-receptor agonists act as non-addicting analgesic drugs [92], which produce an antinociceptive response without the appearance of cross-tolerance to μ-, or δ-opioid receptor agonists [93], and can lead to antinociception in the absence of the μ-receptor [32]. The first known compound with mixed μ-agonist / δ-antagonist properties was the TIPP-NH_2 [56]. It was a moderately potent μ-agonist in the GPI assay and a potent δ-antagonist in the MVD assay. In the receptor binding assay, it was quite δ-receptor-selective and showed no affinity for κ-receptors at concentrations up to 10 μM. Substitution of Dmt for Tyr^1 in TIPP-NH_2 resulted in a new compound, DIPP-NH_2, which exhibited a major potency increase in the GPI assay and also, an enhancement in δ-antagonist activity in the MVD assay [94]. The binding data revealed that this compound is still somewhat δ-receptor-selective. Reduction of the peptide bond between

Tic[2] and Phe[3] led to an analogue, DIPP[Ψ]-NH_2 [95], which displayed a further increased μ-agonist property and retained very high δ-antagonist activity. This ligand showed subnanomolar binding affinities for both μ- and δ-receptors and was the first example of a balanced mixed μ-agonist / δ-antagonist. DIPP[Ψ]-NH_2 did not display δ-agonist activity in the MVD assay, even at very high concentrations, and was stable against chemical and enzymatic degradation. The conformational behaviour of the potent μ-agonist / δ-antagonist DIPP[Ψ]-NH_2 was examined by theoretical conformational analysis based on molecular mechanics calculations. One low-energy conformer showed a good spatial overlap of its tyramine moiety and Phe[3] aromatic ring with the tyramine moiety of Hat[1] and the aromatic ring of Aic[3] of the cyclic μ-agonist peptide Hat-c[*D*-Orn-Aic-Glu] in a low-energy conformation. Another low-energy conformer is almost identical with the proposed model of the receptor-bond conformation of the δ-antagonist TIPP [64], showing spatial overlap with the corresponding moieties of naltrindole, as discussed above. These findings might explain the mixed μ-agonist / δ-antagonist profile of DIPP[Ψ]-NH_2.

1.1.2.5. Other features of δ-antagonists

Moderately δ-selective alkaloid antagonists have found special applivations in clinical trials; for example, amelioration of the effect of alcoholism [96], autism [97] and Tourette's syndrome [98]. In animal models, the δ-opiate antagonist naltrindole [99] inhibited the reinforcing properties of cocaine [100], moderated the behavioral effects of amphetamines [101], and brought about immunosuppression [102] suitable for organ transplantation. These transplantation effects were also seen when another non-peptide compound was used: 7-(benzylspiroindanyl)naltrexone [103].

1.2. Synthesis of amino acids [104]

There is growing interest in the synthesis, pharmacology and conformational properties of non-proteinogenic amino acids. In the opioid peptide field, numerous conformationally constrained peptide analogues have been synthesized by the incorporation of unnatural amino acids. Racemic and enantiomerically pure substituted amino acids have been prepared by a number of different routes:

- Strecker-Zelinsky synthesis
- amination of α-halogen acids
- amination via molecular rearrangement
- condensation of an aldehyde with an active methylene group
- Erlenmeyer condensation

- by condensation with N-substitued aminomalonic esters
- oxidation of amino alcohols
- Pictet-Spengler reaction [105]

Since the starting material for these reactions is an aldehyde or a ketone, these procedures obviously result in racemic products and a suitable resolution procedure is required before peptide synthesis or, in the case of incorporation in racemic form, the resulting diastereomeric peptides can be separated by HPLC during purification.

1.3. Separation of enantiomeric amino acids

The synthesis of amino acids often leads to a racemic mixture. Enantiomers have identical chemical and physical properties in a symmetrical environment, but in a chiral environment such as the human body they act as two different molecules with quite different biological activities. Thus, separation of the enantiomers and determination of the enantiomeric purity are of high importance. Separation procedures can be divided into two classes, depending on the scale.

1.3.1. Resolution of amino acids on a preparative scale

Preferential crystallization by providing seed crystals of one antipode in a supersaturated solution of a racemate was employed in the resolution of a few amino acids. This method often fails to accomplish total separation of the enantiomers.

Diastereomeric salts can be formed by the use of enantiomerically pure chiral ion-pair forming reagents. Due to the different solubilities of the resulting diastereomeric salts, they can be separated via differential crystallization from an appropriate solvent. Successful one-step resolution of a racemate presumes that the resolving agent forms a relatively tightly bound salt with at least one element of the racemate and that this salt is cleanly and quantitatively precipitable from that of the other isomer. Complete purification of each salt usually requires multiple crystallization and subsequent control of the enantiomeric purity.

The advantage of using biological procedures to resolve amino acid racemates is that their action towards amino acids of unknown optical structure can be predicted. The drawback of these procedures is that they are not applicable when both antipodes are needed. Several N-protected amino acids have been resolved by means of proteases, using bases such as aniline [106] or *p*-toluidine [107], with papain as enzyme. Asymmetric enzymatic hydrolysis takes place in solution and analytical methods are available for monitoring the progress of the reaction. The method applies *L*-directed enzymes. The enzymes most extensively used for the resolution of α-amino acids are acylases and amidases. *L*-Antipodes of N-acetyl, chloroacetyl

or trifluoroacetyl amino acids as substrates, except Pro, are readily hydrolyzed by acylases or carboxypeptidases.

1.3.2. Resolution of amino acids on an analytical scale

Among the analytical methods applied for the separation of enantiomers of amino acids, chromatography, including GC, HPLC and TLC, is the most widely used. Polarimetry is also useable, but a knowledge of the specific rotation of the pure enantiomer is required.

For direct separation, appropriate chiral selectors can be chiral ligands with metal ions [108], proteins [109], cyclodextrins [110], crown-ethers [111] and chiral counter ions [112]. These compounds, bound to a solid support, can also serve as a chiral stationary phase. This technique is a flexible and versatile one. Chiral derivatizing reagents applied for indirect enantiomeric resolution must fulfil many requirements. The reagent must be enantiomerically pure and provide a quantitative reaction without racemization or metabolism. It is advantageous to have both enantiomeric forms. The most widely used derivatizing agents for amino acids are Marfey's reagent [113] and the GITC reagent [114]. Lower limits of detection can be achieved by using fluorescent derivatizing agents such as *ortho*-phthalaldehyde together with chiral thiols [115].

Chiral TLC also provides a direct method for the resolution and analytical control of enantiomeric purity. This method is sensitive, and easy to carry out. Ligand exchange, ion exchange (optically pure chiral selector) and molecular inclusion complexation (cellulose [116] and cyclodextrin [117]) constitute the basis of these TLC separations. Quantification can be carried out by densitometry. The advantage of TLC over HPLC is that several analyses can be carried out on a single TLC plate while with GC or HPLC techniques all the analyses have to be performed sequentially. TLC additionally allows the easy control of synthetic processes.

1.4. Solid-phase peptide synthesis

Solid-phase peptide synthesis [118] is based on the sequential addition of α-amino or side-chain-protected amino acids to an insoluble polymeric support. The growing peptide chain is attached to the support via a linker through its C-terminus. The synthesis yields a peptide acid or amide, depending on the linker. Peptide synthesis can be carried out in a batchwise or continuous flow manner. Only the latter strategy is fully compatible with the continuous flow method. The acid-labile Boc or the base-labile Fmoc group is used for N-α-protection. After removal of the protecting group, the next protected amino acid is coupled by using either a coupling reagent or a preactivated protected amino acid derivative. Side-chain protecting groups are chosen so as to be cleaved simultaneously with detachment of the peptide from the

resin. In principle, Boc chemistry applies benzyl- and cyclohexyl-type protecting groups, while in Fmoc chemistry mainly *tert*-butyl- and trityl-type protecting groups are used. Cleavage of the Boc protecting group is achieved with TFA, and that of the Fmoc protecting group with piperidine. DCM and DMF are the solvents primarily used for resin deprotection, coupling and washing. The most widely used quantitative test for the presence or absence of free amino groups is the Kaiser test [119]. It should be noted that some unprotected amino acids do not show the expected colour (Ser, Asn and Asp) or, being secondary amino acids do not yield a positive reaction (Pro and Tic). In these cases, other methods, such as picric acid monitoring [120], the TNBS test [121] and for Pro the chloranil test [122] are recommended. When the Fmoc continuous flow method is used, the progress of each deprotection can be followed by monitoring the release of the cleaved Fmoc group at 300-320 nm.

Efficient peptide-bond formation requires chemical activation of the carboxyl component of N-α-protected amino acids. The activating group must be chosen carefully so as to achieve a very high coupling efficacy but to avoid side-reactions. There are four major types of these techniques: the carbodiimide, symmetrical anhydride, active ester and *in situ* activation methods. Carbodiimides are the most popular *in situ* activating agents in peptide synthesis. Reactions with DCC [123] are carried out at room temperature in DCM for up to 12 hours. The principal limitation to the use of carbodiimides is the dehydration of Asn or Gln. To prevent this side-reaction, HOBt should be used, which also has an accelerating effect on the coupling [124]. An important drawback to the use of DCC is the DCM-insoluble DCU during activation. Other carbodiimides used in SPPS, such as DIPCDI [125], form urea, which is more soluble in DCM than DCU. *In situ* activating reagents have recently become very popular because of the fast reactions and the lack of undesired side-reactions. They are mainly phosphonium and uronium salts and react in the presence of a tertiary base. The most important active ester is the HOBt ester [126]. This compound is formed *in situ* when HOBt, BOP [127], PyBOP [128], TBTU [129] or HBTU [130] is used, and is not isolated. BOP should be handled with care as the by-product formed during the activation is carcinogenic. Pentafluorophenyl esters [131] are also efficient, but react somewhat more slowly. PyBroP [132] allows fast and enantiomerization-free couplings, providing a significant improvement over existing reagents. Symmetrical anhydrides are formed *in situ* when two equivalents of the N-protected amino acid and one equivalent of DCC are used. This method is quite wasteful, and the anhydrides have to be prepared freshly prior to use.

Final cleavage of the peptidyl-resin and side-chain deprotection is achieved through the application of HF or TFMSA for Boc chemistry and TFA for Fmoc chemistry. Standard conditions for HF cleavage are 0-5 °C for a period of 30-60 min. The scavenger most widely

used to avoid side-reactions is anisole in combination with dimethyl sulfide. *p*-Thiocresol in combination with the previous scavenger can prevent the alkylation of Met and Cys residues. Unfortunately, HF is highly toxic and reactive, and special Teflon apparatus is required. As an alternative to HF, other strong acids, such as TFMSA, or for amide C-terminus peptides, 30% HBr/AcOH, can be applied in normal laboratory glassware. In Fmoc chemistry, cleavage of the peptide-resin bond and simultaneous deprotection is carried out with TFA. Met, Cys and Trp are extremely susceptible to alkylation by cations produced during the cleavage process. If scavengers such as EDT or thioanisole are added to the cleavage mixture, these side-reactions can largely be suppressed. A shorter process can be achieved if HBF_4 or trimethylsilylbromide is used to remove less acid-sensitive protecting groups as an alternative to TFA cleavage.

In most cases, the post-cleavage work-up consists in precipitation of the crude peptide from the cleavage mixture with diethyl ether. The peptide is then washed with diethyl ether to remove scavengers and is lyophilized from AcOH or from buffer solutions. In order to achieve high peptide purity, purification by RP-HPLC is necessary. Purified peptides can be stored as lyophilizates at -20 °C.

1.5. Tritium labelling of peptides

Tritium occurs naturally in the environment. Reactions between cosmic radiation and gases in the upper atmosphere produce most of the world's natural tritium:

$$^{14}_{7}N+^{1}_{0}n\rightarrow^{3}_{1}H+^{12}_{6}C$$

Tritium is a pure β-emitter with an E_{max} of 18 keV. It has a half-life of 12.4 years. A β^- particle is identical with an electron. One milliatom of tritium represents a radioactivity of 28.6 Ci (1.080 TBq).

In the opioid field, tritiated peptide ligands have proved to be essential for the performance of binding experiments, autoradiographic localization and distribution studies of the receptors, and to confirm that the cloned receptors are identical with those characterised in native tissues. Tritium incorporation can involve non-selective labelling, such as isotope exchange reactions [133], or selective labelling, such as the chemical or enzymatic synthesis of peptides from labelled amino acids, derivatization of the neuropeptides with [^{3}H]methyl iodide [134] or reductive methylation with tritiated metal hydrides [135] and the synthesis of [^{3}H]neuropeptides by using precursor peptides (Figure 1). The precursor can be obtained by peptide synthesis using amino acid derivatives containing halogens, or double or triple bonds. Tritium labelling can be performed on precursor peptides gained by direct synthesis or by post-synthetic modifications [136,137].

Tyr and His residues can be iodinated with I_2. For this purpose, I_2 can be used directly in a MeOH solution, formed from I^- by oxidation with chloramine-T or iodogen; by electrolysis, etc. Mono- and diiodinated analogues can be prepared, depending on the iodine/substrate ratio, the temperature and the reaction time, but the reaction mixture usually contains a mixture of iodinated and non-iodinated peptides. The diiodinated analogue can be separated by HPLC purification. The resulting substitutions are situated at positions 3' and 5' in the aromatic rings in Tyr or at positions 2' and 4' in the His residue. Peptides containing Trp, Met or Cys may be damaged in the case of direct halogenation. To prevent such obstacles, fragment condensation is an appropriate approach [138]. Tritiated neuropeptides can also be prepared by using precursor peptides obtained by peptide synthesis. Boc and Fmoc chemistry can equally be used, but in the event of double bonds Fmoc chemistry is preferred. The most popular amino acids are Dit or 3',5'-dibromotyrosine. 2',6'-Dibromotyrosine can be used to place the labels in more stable positions [139]. The use of *p*-iodophenylalanine results in lower specific activity in the tritiated peptide, but the label itself will be more stable [140]. Other frequently used precursor amino acids are 3,4-dehydroproline [141], 4,5-dehydroleucine [142] and propargyl- [143] or allylglycine.

$PdO/BaSO_4$ is the most widely applied catalyst for the tritiation of halogen-containing peptides [144], but Pd/C and carrier-free Pd catalyst have also been applied for the labelling of opioid peptides [145,146].

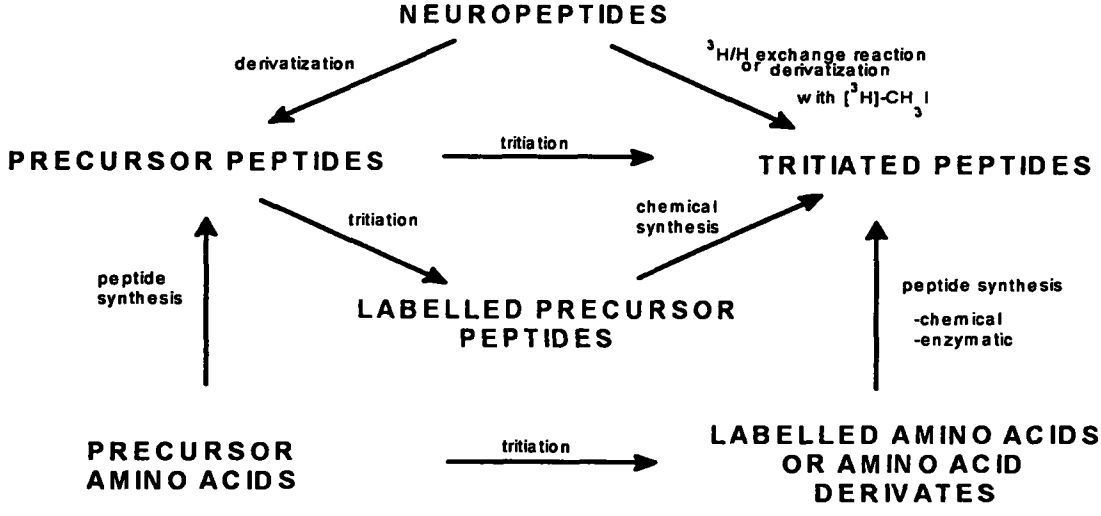

Figure 1. Reaction routes rot the synthesis of tritiated peptides

2. AIMS AND SCOPE

The development of antagonists, which selectively block the action of the agonist, presumably by binding to the receptors, has been of spectacular help in elucidating the function of those ligands for which antagonists have been developed. We set out to synthesize several peptides from the TIPP peptide family in order to study their biological properties. The systematic substitution of every position with β-methylated amino acids in the δ-antagonist TIPP and the mixed μ-agonist / δ-antagonist TIPP-NH_2 is useful for the determination of structure-activity relationships. In the case of δ-antagonist TIPP, we wished to develop new, highly potent and selective δ-opioid antagonist peptide ligands. Substitution of Dmt for Tyr^1 in opioid peptides in general produces a substantial increase in δ-receptor affinity and the exchange improves the ability of these δ-antagonists to cross the BBB. Accordingly we set out to devise a new method for synthesis of the optically pure *L*-Dmt, and to use this amino acid in our new, Dmt-substitued analogues. In the case of the mixed μ-agonist / δ-antagonist TIPP-NH_2, it was hoped that the β-methyl substitution might result in more balanced μ- and δ-affinities, thereby leading to new analgesics with a diminished propensity to produce tolerance and dependence.

Tritium-labelled biologically active peptides are valuable tools for the biological characterization of receptors and binding sites. The metabolic pathway of tritium-labelled compounds is also easily traceable, facilitating the study of biological properties. After biological characterization of our new, structure-activity-tested analogues we choose some of the promising ligands and prepare them in tritium-labelled form. We decided to synthesize labelled forms of some other δ-antagonists reported to be the best in their field too, because data from indirect membrane receptor binding assays sometimes resulted in ambiguous findings. To determine the correct values, we synthesize two new, highly potent and selective ligands with sufficient stability, TICP[Ψ] and N,N-$(Me)_2$Dmt-Tic-OH in radiolabelled form from their corresponding halogen-containing precursor peptides.The tritiated ligands of high specific radioactivity could help to decide the question and might be useful tools for the biological characterization of other new δ-antagonists.

3. MATERIALS AND METHODS

Protected and unprotected amino acids (except β-methyl amino acids and Dmt), resins, coupling reagents, GITC and Carboxypeptidase A, Type II from bovine pancreas (5 000 U / 5.1 cm^3) were purchased from Sigma-Aldrich Ltd., from Bachem Feinchemicalien AG or from Fluka. All other reagents and solvents were of analytical or reagent grade and were used without further purification.

TLC analyses were performed on precoated plates (Merck, Kieselgel 60 F_{254}) using the following solvent systems: (1) hexane-ethyl acetate (9:1)

(2) hexane-ethyl acetate (1:1)

(3) methanol-chloroform-ammonium hydroxide (5:5:1)

(4) n-butanol-acetic acid-water (4:1:1)

(5) acetonitrile-methanol-water (4:1:1)

(6) n-butanol-acetic acid-pyridine-water (38:6:24:20)

(7) ethyl acetate-acetic acid-pyridine-water (60:6:20:11)

UV light, I_2 and ninhydrin were applied to detect the compounds. Radioactive traces were checked with a Berthold Radichromatogram Tracemaster.

RP-HPLC analyses of the synthesis of Dmt were performed on a Spectra-Physics SpectraSystem P 4000, UV 2000 and DataJet Integrator. The column was a Vydac 218TP54. The detection was at 275 nm.

Other analyses were carried out on a Merck-Hitachi chromatograph, applying a Vydac 218TP54 column for analytical and a Vydac 218TP1010 column for preparative purposes. UV detection at 215 nm was applied for detection. Final products were obtained as lyophilizates. For radiochemical purification, we used a Jasco PU 980 HPLC equipped with Merck 50943 LiChroCart 125-4 LiChrospher 100 RP-18 or Vydac 218TP54 columns. Detection was performed with a Jasco UV-975 detector at 215 nm and a Packard Flow-one/β A-500 radiodetector. Radioactivity was counted with a Searle-Delta-300 liquid scintillation counter in a toluene-Triton X-100 scintillation cocktail.

Analytical characterizations were performed by using a linear gradient of ACN-0.1% TFA (eluent B) from 0 to 80% over 30 min. Water-0.1% TFA was eluent A

H-[^{3}H]Dmt-Tic-OH was purified to homogeneity with a gradient programme: 0 min, 15% B; 10 min, 20% B; 15 min, 32% B and 25 min, 50% B. For N,N(Me_2)-[^{3}H]Dmt-Tic-OH, the following programme was applied: 0 min, 20% B; 10 min, 30% B; 15 min, 40% B.

[^{3}H]TICP[Ψ] and [^{3}H]-N(Me)Phe^{3}TIPP[Ψ] were assessed by using a linear gradient from 25% to 50% ACN in 0.1% TFA over 30 min.

[^{3}H]-(*L*)-*threo*-Phe^3-TIPP, [^{3}H]-(*D*)-*threo*-Phe^3-TIPP and [^{3}H]-(*L*)-*threo*-Phe^3-DIPP were purified by using a linear gradient from 25 to 60% ACN in 0.1% TFA over 30 min.

The molar masses of the peptides were determined by electrospray ionization mass spectrometry (Finnigan TSQ 7000) and by fast atom bombardment ionization mass spectrometry (AEI/902S) using xenon gas. The GC-MS was equipped with a Chirasil-Val 50 m column.

^{1}H NMR measurements were performed on a Bruker AC 250-P 250 MHz spectrometer.

Melting points were determined on a Büchi 510 apparatus and were uncorrected.

The catalyst for tritiation, $PdO/BaSO_4$ (10% Pd), was from Merck. A Pd/C catalyst (10% type 90, Johnson Matthey) was applied for the hydrogenation of methyl butenoate.

Synthesis of L-2′,6′-dimethyltyrosine

O-Carbethoxy-3,5-dimethylphenol

60 cm^3 of pyridine was added to a solution of 74 g (0.6 mol) of 3,5-dimethylphenol in 500 cm^3 of benzene, and 75 cm^3 (0.74 mol) of ethyl chloroformate was added dropwise under stirring. The reaction mixture was stirred for 1 hour at RT. The reaction mixture was filtered and the filtrate was evaporated to an oily residue. The oil was distilled under reduced pressure in a one-piece distillation flask equipped with a Vigreux column. The fraction with b.p. 62-64 °C / 13.3 Pa was collected to give 76.7 g (65.2%) of O-carbethoxy-3,5-dimethylphenol. TLC was performed to follow the reaction. The R_f(1) values of 3,5-dimethylphenol and the product were 0.28 and 0.58, respectively.

O-Carbethoxy-3,5-dimethyl-4-chloromethylphenol

Anhydrous hydrogen chloride was introduced at a flow rate of 70-80 cm^3/min into a stirred mixture of 12.8 g (66 mmol) of O-carbethoxy-3,5-dimethylphenol, 51 cm^3 of 37% aqueous formaldehyde (0.678 mol) and 25 cm^3 of 32% hydrochloric acid and the temperature was maintained at 40 °C for 6.5 hours. The product was formed as a white solid. The crystalline product was filtered off, washed twice with water, and then extracted with ethyl acetate. The organic solution was dried over anhydrous sodium sulfate and evaporated in vacuum. The procedure resulted in 15.2 g of solid material (95%). This solid was distilled under reduced pressure (0.66 Pa and at 116-118 °C) in a one-piece flask equipped with a Vigreux column, to give 6.84 g (45%) of a mixture of O-carbethoxy-3,5-dimethyl-2-chloromethylphenol and O-carbethoxy-3,5-dimethyl-4-chloromethylphenol. The ratio of the isomers was investigated by GC-MS: 11.9% of O-carbethoxy-3,5-dimethyl-2-

chloromethylphenol, 85.9% of O-carbethoxy-3,5-dimethyl-4-chloromethylphenol and 2.2% impurity was found. TLC was applied and the $R_f(1)$ values of O-carbethoxy-3,5-dimethylphenol and the mixture of O-carbethoxy-3,5-dimethyl-2-chloromethylphenol and O-carbethoxy-3,5-dimethyl-4-chloromethylphenol proved to be 0.58 and 0.43, respectively.

Diethyl acetamido-(2,6-dimethyl-4-hydroxybenzyl)malonate

Sodium ethoxide was prepared by adding 1.1 g of Na (47.6 mmol) to 90 cm^3 of abs. ethanol. 10.2 g of diethyl acetamidomalonate (47 mmol) and 9.4 g of a mixture of O-carbethoxy-3,5-dimethyl-2-chloromethylphenol and O-carbethoxy-3,5-dimethyl-4-chloromethylphenol (39 mmol) were added to this solution. The mixture was refluxed for 2 hours, cooled and filtered; filtrate was then evaporated to give a foam. This was dissolved in about 120 cm^3 of ethyl acetate and washed twice with about 60 cm^3 of water. When the organic phase was dried over anhydrous sodium sulfate and evaporated again, it started to crystallize. The collected crystals were recrystallized from benzene. The procedure resulted in 7.2 g of diethyl acetamido-(2,6-dimethyl-4-hydroxybenzyl)malonate. TLC was performed and the $R_f(2)$ values of the starting compound and the product were found to be 0.9 and 0.33, respectively. The melting point was 154-155 °C. To verify the purity and to determine the ratio of *ortho* and *para* isomers, RP-HPLC analysis was performed; the t_R value of diethyl acetamido-(2,6-dimethyl-4-hydroxybenzyl)malonate was 18.8 min, while that of diethyl acetamido-(4,6-dimethyl-2-hydroxybenzyl)malonate was 18.5 min.

(D,L)-N-Acetyl-2′,6′-dimethyltyrosine

20.4 g of diethyl acetamido-(2,6-dimethyl-4-hydroxybenzyl)malonate (58 mmol) was hydrolysed by refluxing in 75 cm^3 of 2 M sodium hydroxide solution for 40 min. After cooling, 3 M HCl solution was added dropwise to the mixture under stirring until pH = 2 was attained. The separated acetylamino acid was filtered off, and washed with cold acidified water (pH = 2) and acetonitrile. 12.7 g of (*D,L*)-N-acetyl-2,6-dimethyltyrosine (87%) was formed. HPLC gave t_R = 11.5 min. The $R_f(3)$ value was 0.83.

(D,L)-2′,6′-Dimethyltyrosine

10 g of (*D,L*)-N-acetyl-2′,6′-dimethyltyrosine (40 mmol) was refluxed with 80 cm^3 of 6 M HCl for 6.5 hours. The solution was concentrated in vacuum to give (*D,L*)-2′,6′-dimethyltyrosine hydrochloride. It was then dissolved in about 50 cm^3 of warm water, and the solution was neutralized to pH = 6.5 with aqueous sodium carbonate, and cooled. The crystals of Dmt were collected and washed with acetone or diethyl ether to give 6.05 g of product (72.6%). HPLC gave t_R = 9.6 min. MS (ESI): 210 (MH^+). The m.p. was 230-231 °C, with decomposition.

(D,L)-N-Trifluoroacetyl-2′,6′-dimethyltyrosine

3 g of Dmt (12 mmol) was dissolved in 24 cm^3 of trifluoroacetic acid, the flask was placed in an ice-bath, and 7 cm^3 of trifluoroacetic anhydride was added dropwise. The mixture was stirred and after stirring for 15 min, the temperature was allowed to return to RT. The reaction time was 3 hours. After evaporation, the yield was 4.3 g (98%). MS (ESI): 306 (MH^+). HPLC gave t_R = 16 min.

L-2′,6′-Dimethyltyrosine

(*D,L*)-N-Trifluoroacetyl-2′,6′-dimethyltyrosine (4.3 g) was suspended in 45 cm^3 of 0.1 M K_2HPO_4 buffer (pH = 7.5), the pH was adjusted to 6.5-7.5 with 2 M NaOH, 120 µl of a suspension of Carboxypeptidase A, Type II was added and the reaction mixture was shaken for 6 hours at 37 °C. From time to time, the pH was adjusted to 6.5-7.5. The solution was then evaporated, the solid residue was dissolved in water, the pH was adjusted to 2-3 with dilute HCl and the solution was extracted 3 times with EtOAc. The aqueous phase was next neutralized with 2 M NaOH and evaporated to about half volume, and the crystals were filtered off. 0.98 g (65.3% for the resolution) product was obtained. ^1H NMR (DMSO-d_6/10% D_2O): δ 2.16 (6H,s,Ar-CH_3), 2.63-2.72 (1H,dd,H^β J = 8.3 and 14.6 Hz), 3-3.12 (1H,dd,H^β J = 14.6 and 6.3 Hz), 3.24-3.29 (1H,dd,H^α J = 8.3 and 6.3 Hz), 6.38 (2H,s,Ar-H). HPLC gave t_R = 9.6 min. MS (ESI): 210 (MH^+). M.p. 230-231 °C with decomposition. To determine the enantiomer excess, GC-MS assay was performed after derivatization; this gave a value of 91 %.

Synthesis of erythro-β-methyltyrosine

2-Phenyl-4-[α-E/Z-p-methoxyphenylethylidene]-5(4H)-oxazolone

A mixture of 15 g hippuric acid (0.08 mol) and 23 cm^3 of acetic anhydride was cooled to about 10 °C and 9 cm^3 of H_2SO_4 was added dropwise under stirring. To this solution, 12.5 g of methoxyacetophenone (0.09 mol) was introduced and the mixture was allowed to reach RT. After heating at 60 °C for 15 min, the mixture was left under stirring overnight at RT. The resulting brown oil was poured into 150 cm^3 of ice-cold water, when the product precipitated. It was washed 3 times with cold water and 100 cm^3 of hot water was added to the solid during stirring. The residue was filtered off, dried over P_2O_5, and crystallized from 350 cm^3 of hot ethanol to give 20.4 g (83%) of product (*E/Z* 70:30). Repeated crystallization from ethanol resulted in a mixture of oxazolone (*E/Z* 98:2) with a yield of 68%. ^1H NMR ($CDCl_3$): δ 2.64 (3H,s,CH_3), 3.85 (3H,s,OCH_3), 6.93 (2H,d,arom. J = 8.4 Hz), 7.5 (5H,arom.), 8.08 (2H,d,arom. J = 8.4 Hz). MS (FAB): 294 (MH^+). M.p. 123-127 °C, TLC: $R_f(2)$ = 0.77.

Isolation of 2-phenyl-4-[α-Z-p-methoxyphenylethylidene]-5(4H)-oxazolone

A solution of 0.3 g of the oxazolone (*E/Z* 70:30) in 15 cm^3 of pyridine was stirred for 10 min at RT. The mixture was poured into 0.24 M ice-cold HCl solution. The precipitated material was quickly filtered off, the residue was dissolved in 300 cm^3 of hot ethanol and the dicalite was filtered off. On addition of extra water to the solution, the oxazolone (*E/Z* 4:96) crystallized with a yield of 73% (0.22 g). ^1H NMR ($CDCl_3$): δ 2.77 (3H,s,CH_3), 3.877 (3H,s,OCH_3), 6.98 (2H,d,arom. J = 8.2 Hz), 7.5 (5H,arom.), 8.07 (2H,d,arom. J = 8.2 Hz). MS (FAB): 294 (MH^+). M.p. 95-98 °C. TLC: $R_f(2) = 0.77$.

E-2-Benzoylamino-3-p-methoxyphenyl-2-butenoic acid methyl ester

To a solution of 125 mg of Na (5.43 mmol) in 50 cm^3 of anhydrous methanol, 5 g of oxazolone (*E/Z* 98:2) (17.1 mmol) was added cautiously. This mixture was shaken at RT for 2 hours. The precipitated solid was filtered off and washed with methanol. The filtrate was neutralized with cc. HCl solution, and further precipitation started. The product was collected by filtration again. The combined solids were crystallized from ethanol/water and a yield of 4.77 g (86%) of the *E* isomer was obtained. ^1H NMR ($CDCl_3$): δ 2.16 (3H,s,CH_3), 3.5 (3H,s,OCH_3), 3.87 (3H,s,$COOCH_3$), 6.86 (2H,d,arom. J = 8.7 Hz), 7.16 (2H,d,arom. J = 8.7 Hz), 7.35 (3H,arom.), 7.86 (2H,arom.). MS (FAB): 326 (MH^+). M.p. 147-148 °C, TLC: $R_f(2) = 0.33$.

Z-2-Benzoylamino-3-p-methoxyphenyl-2-butenoic acid methyl ester

The synthesis started from *Z*-oxazolone (*E/Z* 4:96) and the procedure was the same as above. ^1H NMR ($CDCl_3$): δ 2.29 (3H,s,CH_3), 3.5 (3H,s,OCH_3), 3.8 (3H,s,$COOCH_3$), 6.86 (2H,d,arom. J = 8.7 Hz), 7.16 (2H,d,arom. J = 8.7 Hz), 7.35 (3H,arom.), 7.86 (2H,arom.). M.p. 138-140 °C, MS and TLC $R_f(2)$ identical with that of the *E* isomer.

erythro-N-Benzoyl-β-methyl-p-methoxyphenylalanine methyl ester

A mixture of 4.73 g of the *E* isomer of the foregoing butenoic acid methyl ester (14.5 mmol) in 190 cm^3 of methanol and 0.4 g of Pd/C catalyst was hydrogenated for 4 days at RT at a pressure of 345 kPa in a Parr hydrogenator. The catalyst was then filtered off over a layer of celite, and the filtrate was evaporated in vacuum. The resulting yellow oil was dissolved in hot ethanol and crystallization started after the addition of water. The yield was 3.77 g (76%). ^1H NMR ($CDCl_3$): δ 1.38 (3H,d,CH_3 J = 7.2 Hz), 3.46 (1H,m,H^β), 3.72 (3H,s,$COOCH_3$), 3.78 (3H,s,OCH_3), 5 (1H,dd,H^α J = 8.1 and 5.2 Hz), 6.28 (1H,d,NH J = 8.1 Hz), 6.86 (2H,d,arom.

J = 8.6 Hz), 7.12 (2H,d,arom. J = 8.6 Hz), 7.45 (3H,arom.), 7.67 (2H,d,arom. J = 8.7 Hz). MS (FAB): 328 (MH^+). M.p. 80-83 °C, TLC: $R_f(2) = 0.42$.

threo-N-Benzoyl-β-methyl-p-methoxyphenylalanine methyl ester

The synthesis started from butenoic acid methyl ester (*E/Z* 20:80) and the hydrogenation was the same as above (the spectrum contained additional signals of the *erythro* isomer). ^1H NMR ($CDCl_3$): δ 1.43 (3H,d,CH_3 J = 7.3 Hz), 3.46 (1H,m,H^β), 3.72 (3H,s,$COOCH_3$), 3.78 (3H,s,OCH_3), 4.96 (1H,d,H^α J = 5.2 Hz), 6.54 (1H,d,NH J = 8.2 Hz), 6.86 (2H,d,arom. J = 8.6 Hz), 7.12 (2H,d,arom. J = 8.6 Hz), 7.45 (3H,arom.), 7.67 (2H,d,arom. J = 8.7 Hz). M.p. 125-128 °C. MS and TLC $R_f(2)$ identical with that of the *erythro* isomer.

erythro-β-Methyltyrosine

To a solution of 5.2 cm^3 of 48% aqueous HBr and 10.4 cm^3 of acetic acid, 1 g (3 mmol) of *erythro*-N-benzoyl-β-methyl-*p*-methoxyphenylalanine methyl ester was added. The mixture was refluxed for 24 hours and evaporated. The resulting oil was dissolved in water and evaporated again. After repeated addition of 5 cm^3 of water, the flask was placed into a refrigerator. The precipitated crystals were filtered off and the filtrate was extracted with ethyl acetate. The aqueous phase was adjusted to pH = 6 with NH_4OH, and *erythro*-β-methyltyrosine precipitated. The crystals were filtered off and recrystallized from hot water; a yield of 361 mg (58%) was obtained. ^1H NMR (D_2O/TFA): δ 0.94 (3H,d,CH_3 J = 7.2 Hz), 3.07 (1H,m,H^β), 3.65 (1H,d,H^α J = 7.2 Hz), 6.43 (2H,d,arom. J = 8.6 Hz), 6.75 (2H,d,arom. J = 8.6 Hz), 13.6 (1H,s,COOH). MS (FAB): 196 (MH^+). M.p. 228-231 °C, TLC: $R_f(4)$ = was 0.47.

threo-β-Methyltyrosine

threo-N-Benzoyl-β-methyl-*p*-methoxyphenylalanine methyl ester was hydrolysed in 48% aqueous HBr and acetic acid as above. Any *erythro* isomer was removed by chromatography on a Lobar (Merck) LiChroprep RP8 (40-63) column, using 1% aqueous methanol as eluent. ^1H NMR (D_2O/TFA): δ 1.31 (3H,d,CH_3 J = 7 Hz), 3.37 (1H,m,H^β), 4.11 (1H,d,H^α J = 7.2 Hz), 6.43 (2H,d,arom. J = 8.6 Hz), 6.81 (2H,d,arom. J = 8.6 Hz), 13.6 (1H,s,COOH). MS (FAB): 196 (MH^+). M.p. 228-231 °C, TLC: $R_f(4) = 0.47$.

N-tert-Butyloxycarbonylamino acids [147]

Boc-AAs were prepared from the given AA in dioxane-water 2:1 at pH = 10 with 1.1 equivalents of Boc_2O. After stirring for 3 hours, an additional portion of Boc_2O (0.3

equivalents) was added and stirring was continued overnight at RT. After extraction with EtOAc, the resulting brownish oil was dissolved in EtOAc and precipitated with petroleum ether.

Solid-phase peptide syntheses

These were performed by the manual solid-phase technique, using 4-methylbenzhydrylamine resin for peptide amides or Merrifield resin for peptide acids. Coupling was performed by shaking the resin with two equivalents of Boc-amino acid, HOBt and DCC for 1-1.5 hours. The resin was then washed with DCM, EtOH and DCM again (3 times each). Coupling was monitored with the Kaiser test. Boc-deprotection was carried out by treating the peptidyl resin with 50% TFA/DCM containing 2% of anisole (5+20 min), and the resin was washed then with DCM, (3 times) neutralized with 10% DIEA/DCM (2 times) and washed with DCM again (3 times). *threo*-β-MePhe was incorporated as racemate into the TIPP analogue peptide sequences. After RP-HPLC purification, two diastereomeric peptides were obtained. After acid hydrolysis, [142] GITC derivatizations were performed and the resulting mixtures were compared with standards. Side-chain protection was with dichlorobenzyl for Tyr. Simultaneous side-chain deprotection and cleavage from the resin were accomplished by treatment with HF and anisole as scavenger (1 cm^3 of anisole and ~10 cm^3 of HF/g peptide resin) at 0 °C for 1 hour. After evaporation of the HF, the peptide resin mixture was washed with diethyl ether and the peptide was extracted with glacial acetic acid and lyophilized.

Postsynthetic modifications

Iodination of peptides wase performed as follows: The peptide was dissolved in ACN/water (1:1), and NaI (in ACN/water solution) and chloramine-T (in ACN/water solution) were added. After stirring at RT, the product, which contained a mixture of iodinated and diiodinated peptides, was separated by HPLC. Purified precursor peptides were characterized by HPLC and MS analyses.

Acid hydrolysis was performed as follows: To 1 mg of peptide, 1 cm^3 of 6 M HCl was added, and the mixture was incubated at 110 °C for 2 days in a sealed ampoule. The hydrolysate was analysed by RP-HPLC after GITC derivatization.

GITC derivatization was performed as follows: Equal volumes of amino acid solution (1 mg/cm^3, dissolved in 0.4% TEA/ACN:water (1:1)) and 0.2% GITC solution in acetonitrile were mixed. The mixture was allowed to stand at RT for 60-90 min, and was then injected directly into the HPLC. GITC-AA has an absorption maximum at 250 nm.

Tritium labelling of peptides

This was carried out in an in-house designed vacuum apparatus [148] in a fume cupboard. The purified precursor peptide was dissolved in DMF and the catalyst was suspended in the solution. The reaction vessel was then connected to the tritiation manifold and cooled with liquid nitrogen, and the air was removed by vacuum. Tritium gas was liberated from uranium tritide by heating above 300 °C, and it was expanded into the reaction vessel. The reaction mixture was agitated with a magnetic stirrer at RT. The reaction was terminated by freezing the solution and adsorbing the remaining tritium on pyrophoric uranium in another reservoir. The catalyst was removed by filtration through Whatman GF/C filters, and was washed several times with ethanol. Labile tritium was removed by repeated evaporation of an ethanol–water (1:1) solution. In most cases, further TLC or HPLC purification was necessary. The purified labelled peptide was dissolved in ethanol and stored under liquid nitrogen.

The specific activities of the labelled peptides were determined by dividing the measured activity by the amount of purified peptide. UV detection was performed on a Shimadzu-160 spectrophotometer. The quantity of the purified labelled material was determined from its UV absorption, or by HPLC, using a calibration curve.

Receptor binding assays were performed with 50 mM Tris-HCl buffer (pH 7.4) in a final volume of 1 cm^3 and at a protein concentration of 0.3-0.5 mg/cm^3. Incubation was started by addition of the rat brain membrane suspension, and terminated by rapid vacuum filtration through Whatman GF/C filters. The radioactivity was measured by LSC in a toluene-based scintillation cocktail. Non-specific binding was determined in the presence of 10 μM non-labelled naloxone. Specific binding is the difference between the total binding and the non-specific binding. Scatchard plots were used for the analysis of radioligand binding saturation curves. The displacement curves of [^{3}H]TICP[Ψ] were analyzed with the LIGAND program [149], using a non-linear least squares algorithm. K_i values were determined with GraphPad Prism 2.01. for [^{3}H]N,N(Me)$_2$-Dmt-Tic-OH.

G-proteins are activated by binding GTP. Hydrolysis of bound GTP to GDP terminates activation. Receptors activated by agonists catalyze the release of the inactive GDP as well as the binding of GTP, and thereby increase the relative level of G-protein activation. This effect can be inhibited by adding antagonists. [^{35}S]GTPγS binding was performed [150] on membranes of the δ-receptor-rich frontal cortex of rat brain. Different concentrations of N,N(Me)$_2$-Dmt-Tic-OH were incubated in the absence and presence of 1 μM Ile^{5,6}deltorphin II, a potent δ-agonist. Incubations were carried out for 60 min at 30 °C.

4. RESULTS AND DISCUSSION

4.1 Synthesis

The synthesis of Dmt was accomplished by literature methods [151] with some modifications.

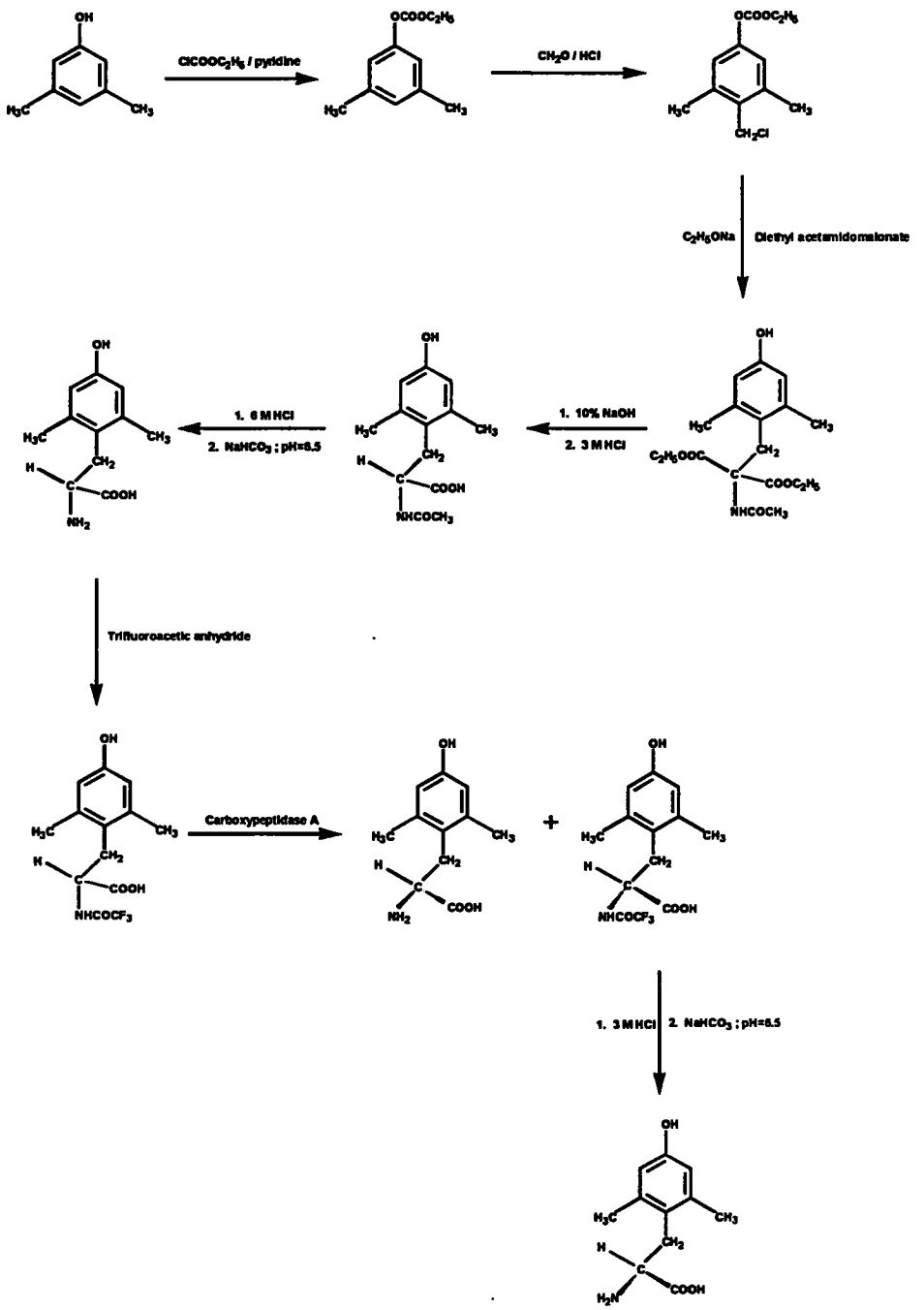

Figure 2. Synthesis of *L*-Dmt and *D*-Dmt

Racemic Dmt was synthesized from commercially available 3,5-dimethylphenol in a multistep reaction. The overall yield was 10.5%. Under our conditions, some problems were encountered. The first by-product formed on a larger scale was O-carbethoxy-3,5-dimethyl-2-chloromethylphenol during the Blank reaction. The ratio of the product and by-product depends on the HCl flow rate, and the temperature presumably too has an important role. It is possible, that the amount of formaldehyde also influences the ratio of the products. The only way to remove this impurity was the recrystallization of diethyl acetamido-(2,6-dimethyl-4-hydroxybenzyl)malonate from benzene. After decarboxylation to simplify the reaction route and the resolution of *L*-Dmt, acylase digestion was performed, but it was found that (*D,L*)-N-acetyl-2′,6′-dimethyltyrosine was resistant to acylase I digestion. We therefore developed a new enzymatic method.

Enzymatic digestion of N-TFA-Dmt resulted in good resolution. According to TLC and RP-HPLC, Carboxypeptidase A [152] can digest only the *L* enantiomer of N-TFA-Dmt in 10 hours at 37 °C. According to the GC-MS assay after derivatization, the reaction yielded 66% of an enantiomerically pure product. Esterification was performed with 3 M HCl in isopropanol and followed by N-protection with trifluoroacetic anhydride. This reaction resulted in N-trifluoroacetyl-2,6-dimethyltyrosine isopropyl ester. The result revealed that the material contained 95.4% *L*-Dmt and 4.6% *D*-Dmt, and the enantiomer excess was 91%. Unfortunately, recovery of the *D*-Dmt was not really successful. The yield was much lower than before, the material contained 90.3% *D*-Dmt and 9.7% *L*-Dmt, and the enantiomer excess was 81%.

Since our study required all stereoisomers of β-MeTyr, a rapid synthetic route involving Erlenmeyer condensation between hippuric acid and 4-methoxyacetophenone [153-155] and resulting in racemates was chosen, rather than a stereoselective synthesis for each isomer. From the resulting 70/30 mixture of *E*- and *Z*-oxazolone, the *E* isomer was easily isolated by crystallization [156]. *E*-Oxazolone can be isomerized to the *Z* isomer in pyridine [153]. Further catalytic hydrogenation and subsequent deprotection steps afforded the *erythro*-β-MeTyr and *threo*-β-MeTyr racemates. After Boc-derivatization both the *erythro* and *threo* β-MeTyr isomers were incorporated into the TIPP analogues [**II**] as racemic pairs. Peptide synthesis was performed by SPPS, using either a Merrifield resin or a 4-methylbenzhydrylamine resin. The resulting epimeric peptides were characterized by RP-HPLC, TLC and MS analyses (Table 3). The absolute configuration at the α-carbon of the β-methyl-amino acid in each peptide was determined after acid hydrolysis and derivatization of the resulting amino acids with GITC or FDAA [157].

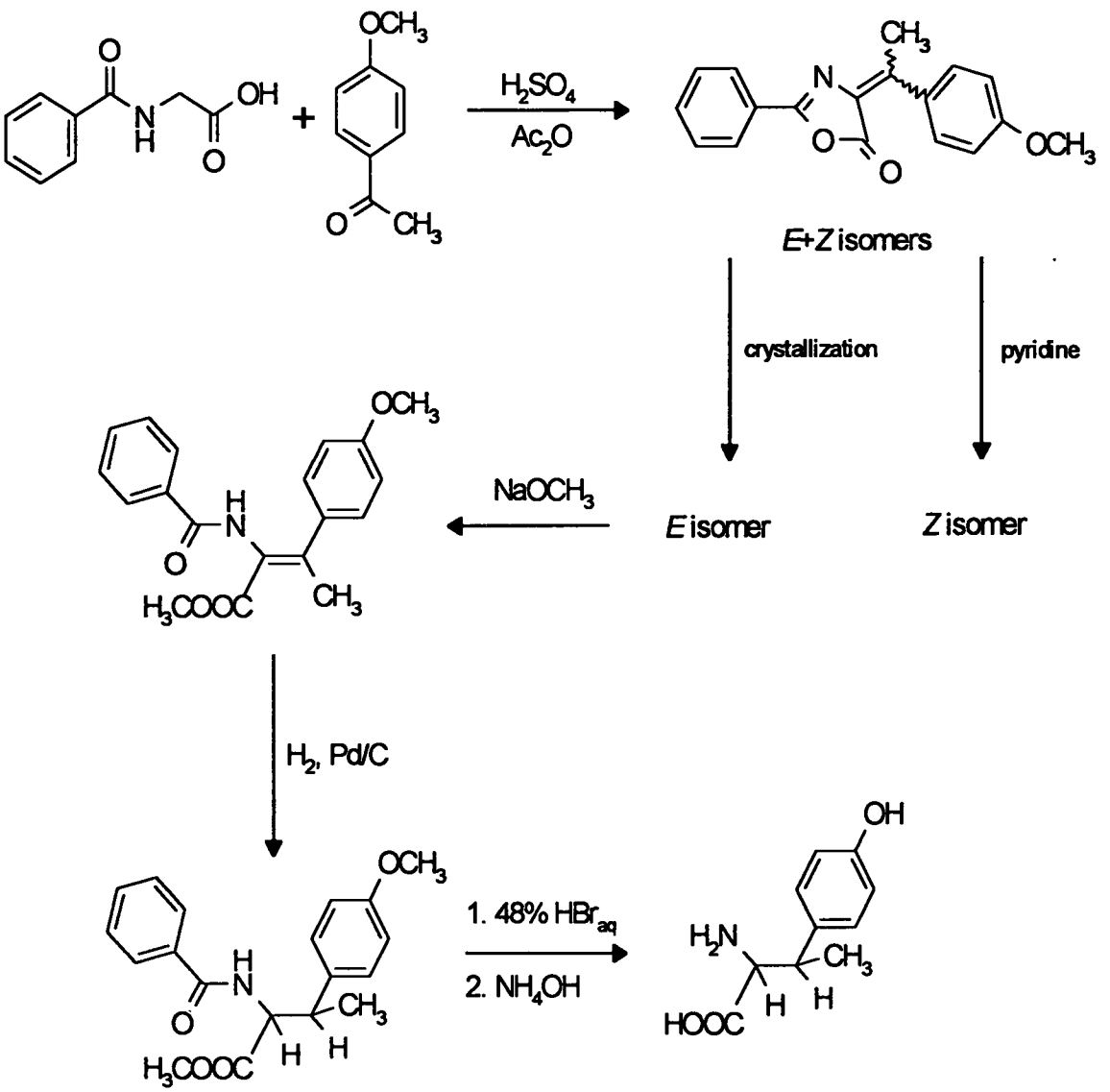

Figure 3. Synthesis of β-MeTyr isomers

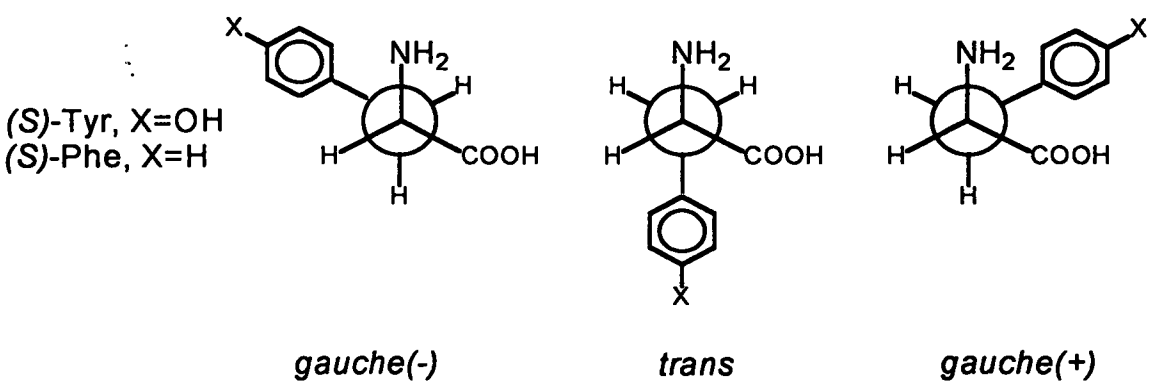

Figure 4. Possible side-chain conformations of *S*-Tyr and *S*-Phe

Table 2. Influence of the configuration of aromatic β-methyl amino-acids on the population of the side-chain

	(2*S*,3*S*)	(2*S*,3*R*)	(2*R*,3*R*)	(2*R*,3*S*)
gauche(-)	++	+	+	-
trans	-	+	-	+
gauche(+)	+	-	++	++

-, *strongly disfavoured;* +, *allowed;* ++, *favoured*

Table 3. Physicochemical data on TIPP analogues

Peptide	HPLC k'	TLC R_f			MS Mol. wt.
		(4)	(5)	(7)	
H-TIPP-OH	3.9	0.65	0.82	0.53	635
H-TIPP-NH_2	3.23	0.71	0.88	0.62	634
(*SS*)-β-MeTyr[1]-TIPP-NH_2	4.2	0.83	0.88	0.68	648
(*SR*)-β-MeTyr[1]-TIPP-NH_2	3.23	0.83	0.88	0.72	648
(*RR*)-β-MeTyr[1]-TIPP-NH_2	4.3	0.71	0.89	0.78	648
(*RS*)-β-MeTyr[1]-TIPP-NH_2	2.56	0.72	0.89	0.72	648
(*SS*)-β-MePhe[3]-TIPP-NH_2	3.83	0.78	0.81	0.74	648
(*SR*)-β-MePhe[3]-TIPP-NH_2	3.23	0.78	0.81	0.71	648
(*RR*)-β-MePhe[3]-TIPP-NH_2	16.66	0.78	0.75	0.62	648
(*RS*)-β-MePhe[3]-TIPP-NH_2	14.76	0.75	0.79	0.61	648
(*SR*)-β-MePhe[3]-TIPP-OH	4.24[a]	0.77	0.82	0.69	649
(*RS*)-β-MePhe[3]-TIPP-OH	14.68[a]	0.77	0.88	0.55	649
(*SS*)-β-MePhe[4]-TIPP-NH_2	4.43	0.77	0.79	0.7	648
(*SR*)-β-MePhe[4]-TIPP-NH_2	8.5	0.76	0.76	0.67	648
(*RR*)-β-MePhe[4]-TIPP-NH_2	8.7	0.76	0.75	0.67	648
(*RS*)-β-MePhe[4]-TIPP-NH_2	3	0.75	0.82	0.76	648
(*SR*)-β-MePhe[3]-DIPP-OH	4.78[b]	0.69	0.77	0.8	677[c]
(*RS*)-β-MePhe[3]-DIPP-OH	5.67[b]	0.67	0.74	0.78	677[c]
(*SR*)-β-MePhe[3]-I_2-DIPP-OH	7.31[b]	0.82	0.69	0.64	929[c]

HPLC k' on a Vydac 218TP54 column; flow rate 1 cm^3/min. Solvent system: 0.1% TFA in water, 0.1% TFA in ACN. Solvent front breakthrough at 3.0 min.
Isocratic 27% organic component
[a] *Isocratic 30% organic component*
[b] *Gradient of 25-60% organic component in 30 min;*
FAB ionization; [c] *ES ionization*

Table 4. Capacity factors of GITC-derivatized amino acids

GITC-AA	k'
Tyr	0.93
Tic	1.61
Phe	2.19
L-threo-β-MePhe	2.74
D-threo-β-MePhe	3.26

HPLC k' on a Vydac 218TP54 column; flow rate 1 cm^3/min. Solvent system: 0.1% TFA in water, 0.1% TFA in ACN. Solvent front breakthrough at 2.8 min.
Gradient of 35-45% organic component in 30 min;

Preparation of tritiated δ-opioid antagonists

Preparation of H-[$^3H_2$]-Tyr-Tic[CH_2-NH]-Cha-Phe-OH

The precursor was prepared by a standard SPPS method, using Dit, described in the literature [IV]. 2.5 μmol of the iodinated precursor peptide was dissolved in 1 cm^3 of DMF and 60 μl of 0.1 M TEA in DMF, 12 mg of PdO/$BaSO_4$ was added as catalyst, and tritium gas was introduced. The reaction time was 80 min. The total radioactivity of the labelled peptide was measured by liquid scintillation counting, and was found to be 4.4 GBq (118.96 mCi). The crude tritiated peptide was purified on reaction-zone type preparative TLC plates. It was found earlier that approximately 30-50 mCi of radioactivity can be conveniently purified in one run. The solvent system was acetonitrile-methanol-water (4:1:1). Radioactivity was detected by autoradiography of a photography emulsion, and the silica gel containing adsorbed radioactive material was then extracted with ethanol. This procedure resulted in pure material with a total activity of 1.44 GBq (39 mCi). The purity of the radiolabelled peptide was assessed by TLC, with detection of radioactivity by a TLC plate scanner in three different solvent systems, RP-HPLC and a liquid scintillation detector. It was found that the tritiated compounds were pure and identical with the non-labelled parent compounds. The radiochemical purity was found to be at least 95%. The specific radioactivity of the purified [^{3}H]TICP[Ψ] was 1.53 TBq/mmol (41.28 Ci/mmol). This specific radioactivity was high enough to perform biological tests. This was not the theoretical maximum, which could be a result of hydrogen impurity in the tritium gas or exchange reactions with labile protons of the reagents, the target compound, the solvent, etc. The tritiated compound was stored as a 37 MBq/cm^3 ethanolic solution under liquid nitrogen.

Preparation of H-[$^3H_2$]-Tyr-Tic[CH_2-NH]-N(Me)Phe-Phe-OH

The precursor was prepared by a standard SPPS method, using Dit. 2.5 μmol of the iodinated precursor peptide was dissolved in 1 cm^3 of DMF and 1 μl TEA, 10 mg of $PdO/BaSO_4$ was added as catalyst, and tritium gas was introduced. The reaction time was 70 min. The total radioactivity of the labelled peptide was measured by liquid scintillation counting, and was found to be 3.56 GBq (95.96 mCi) The crude tritiated peptide was purified on reaction-zone type preparative TLC plates using an acetonitrile-methanol-water (4:1:1) solvent system. The purity of the radiolabelled peptide was assessed by TLC, with detection of radioactivity by a TLC plate scanner in three different solvent systems, RP-HPLC and a liquid scintillation detector. The radiochemical purity was found to be at least 95%. The specific radioactivity of the purified [^{3}H]N(Me)Phe^{3}TIPP[Ψ] was 1.06 TBq/mmol (28.65 Ci/mmol). The tritiated compound was stored as a 37 MBq/cm^3 ethanolic solution under liquid nitrogen.

Preparation of H-[3',5'-^{3}H]Dmt-Tic-OH

The synthesis of H-[3',5'-I_2]-Dmt-Tic-OH has been described in the literature [I]. To a solution of 2.5 μmol of precursor in 1 cm^3 of DMF, 12 mg of $PdO/BaSO_4$ and 0.8 μl of TEA were added, and tritium gas was introduced. The reaction time was 80 min. The total radioactivity of the radiolabelled peptide was measured by liquid scintillation counting, and proved to be 3.96 GBq (107 mCi). The crude tritiated peptide was purified by RP-HPLC. During tritiation, diketopiperazine formation was observed, with k' = 12.7. The purity of the radiolabelled peptide was assessed by RP-HPLC, and was found to be at least 95%. After purification, 1.6% of diketopiperazine was found. During storage in methanol, this value increased to 18.9% at 24 hours. The amount of peptide was determined by UV spectrometry, using unlabelled TFA×H-Dmt-Tic-OH as a standard (ε = 29.74 dm^3/mmol×cm at 214 nm) [29 740 cm^2/mmol]. The specific activity of the purified H-[^{3}H]Dmt-Tic-OH was 1.65 TBq/mmol (44.67 Ci/mmol). The purified peptide was stored in a concentration of 18.5 MBq/cm^3 (0.5 mCi/cm^3) in methanol/water 1:4, with the pH adjusted to 9 with NH_4OH, and with the solution under liquid nitrogen.

Preparation of N,N(Me)$_2$-[3',5'-^{3}H]Dmt-Tic-OH

The synthesis of N,N(Me)$_2$[3',5'-I_2]-Dmt-Tic-OH has been described in the literature [I]. To a solution of 2.5 μmol of the precursor in 1 cm^3 of DMF, 12 mg of $PdO/BaSO_4$ as catalyst and 0.9 μl of TEA were added, and tritium gas was introduced. The reaction was completed in 80 min. The total radioactivity of the tritiated peptide was measured by liquid scintillation counting, and proved to be 2.96 GBq (80 mCi). The crude tritiated peptide was

purified by RP-HPLC. The amount of peptide was determined by UV spectrometry, using unlabelled TFA×N,N(Me)2-Dmt-Tic-OH as a standard (ε = 30.92 dm^3/mmol×cm at 214 nm) [30 920 cm^2/mmol]. The specific activity of the purified N,N(Me)$_2$-[^{3}H]Dmt-Tic-OH was 2.22 TBq/mmol (59.88 Ci/mmol). The purified peptide was stored in a concentration of 18.5 MBq/cm^3 (0.5 mCi/cm^3) in acetonitrile/water 1:4 under liquid nitrogen.

Preparation of H-[$^3H_2$]-Tyr-Tic-(L)-threo-β-MePhe-Phe-OH

The precursor was prepared by a standard SPPS method, using Dit. 1.7 μmol of the iodinated precursor peptide was dissolved in 1 cm^3 of DMF, 5.5 μl of 10% TEA in DMF and 10.5 mg of PdO/$BaSO_4$ as catalyst were added and tritium gas was introduced. The reaction time was 85 min. The total radioactivity of the labelled peptide was measured by liquid scintillation counting, and was found to be 3.92 GBq (106 mCi). The crude tritiated peptide was purified by RP-HPLC. The purity of the radiolabelled peptide was assessed by RP-HPLC and a liquid scintillation detector. The radiochemical purity was found to be at least 95%. The quantity of the purified labelled material was determined by HPLC, using a calibration curve. The specific radioactivity of the purified [^{3}H]-(*L*)-*threo*-β-MePhe^3-TIPP was 1.99 TBq/mmol (53.7 Ci/mmol). The tritiated compound was stored as a 37 MBq/cm^3 ethanolic solution under liquid nitrogen.

Preparation of H-[$^3H_2$]-Tyr-Tic-(D)-threo-β-MePhe-Phe-OH

The precursor was prepared by a standard SPPS method, using Dit. 1.65 μmol of the iodinated precursor peptide was dissolved in 1 cm^3 of DMF, 5.5 μl of 10% TEA in DMF and 12.3 mg of PdO/$BaSO_4$ as catalyst were added and tritium gas was introduced. The reaction time was 100 min. The total radioactivity of the labelled peptide was measured by liquid scintillation counting, and was found to be 3.17 GBq (85.75 mCi). The crude tritiated peptide was purified by RP-HPLC. The purity of the radiolabelled peptide was assessed by RP-HPLC and a liquid scintillation detector. The radiochemical purity was found to be at least 95%. The quantity of the purified labelled material was determined by HPLC, using a calibration curve. The specific radioactivity of the purified [^{3}H]-(*D*)-*threo*-β-MePhe^3-TIPP was 2.09 TBq/mmol (56.5 Ci/mmol). The tritiated compound was stored as a 37 MBq/cm^3 ethanolic solution under liquid nitrogen.

Iodination of H-Dmt-Tic-(L)-threo-β-MePhe-*Phe-OH*

2.5 μmol of peptide was dissolved in 100 μl of ACN/water (1:1) and 4 equivalents of NaI (10 mg/cm^3 ACN/water solution) and 4 equivalents of chloramine-T (10 mg/cm^3

ACN/water solution) were added. After stirring for 300 s at RT, the products were separated by HPLC to give the desired diiodinated peptides in 70-75% yield. Purified I_2-Dmt-containing precursor peptides were characterized by HPLC and MS analyses.

Preparation of H-[$^3H_2$]-Dmt-Tic-(L)-threo-β-MePhe-*Phe-OH*

0.62 μmol of the iodinated precursor peptide was dissolved in 1 cm^3 of DMF and 3 μl of 10% TEA in DMF, 10 mg of $Pd/BaSO_4$ was added as catalyst and tritium gas was introduced. The reaction time was 80 min. The total radioactivity of the labelled peptide was measured by liquid scintillation counting, and was found to be 0.86 GBq (23.28 mCi). The crude tritiated peptide was purified by RP-HPLC. The purity of the radiolabelled peptide was assessed by RP-HPLC and a liquid scintillation detector. The radiochemical purity was found to be at least 95%. The quantity of the purified labelled material was determined by HPLC, using a calibration curve. The specific radioactivity of the purified [^{3}H]-(*L*)-*threo*-β-MePhe^3-DIPP was 2.15 TBq/mmol (58.06 Ci/mmol). The tritiated compound was stored as a 18.5 MBq/cm^3 ethanolic solution under liquid nitrogen.

Table 5. Physicochemical data and specific radioactivities of labelled peptides

Peptide	HPLC k'	TLC R_f			TBq/mmol Specific radioactivity
		(4)	(5)	(6)	
[^{3}H]TICP[Ψ]	12	0.62	0.49	0.66	1.53
[^{3}H]-N(Me)Phe^3-TIPP[Ψ]	8.1	0.63	0.55	0.68	1.06
H-[^{3}H]Dmt-Tic-OH	10.2[b]	–	–	–	1.65
N,N(Me)$_2$-[^{3}H]Dmt-Tic-OH	8.1[c]	0.5	0.29	0.53	2.22
[^{3}H]-(*L*)-*threo*-β-MePhe^3-TIPP	5.6[d]	0.77	0.82	0.75	1.99
[^{3}H]-(*D*)-*threo*-β-MePhe^3-TIPP	6.51[d]	0.77	0.88	0.72	2.09
[^{3}H]-(*L*)-*threo*-β-MePhe^3-DIPP	5.94[d]	0.69	0.77	0.76	2.15

HPLC k' on a Merck 50943 LiChroCart 125-4 LiChrospher 100 RP-18 column; flow rate 1 cm^3/min. Solvent system : 0.1% TFA in water, 0.1% TFA in ACN. Solvent front breakthrough at 1.2 min.
Gradient of 25-50% organic component in 30 min
[b]*Gradient program: at 0 min 15%, at 10 min 20%, at 15 min 32% and at 25 min 50% organic component*
[c]*Gradient program: at 0 min 20%, at 10 min 30% and at 15 min 40% organic component*
[d]*Vydac 218TP54 column; Solvent front breakthrough at 2.85 min.*
Gradient of 15-70% organic component in 30 min

Under the TLC conditions, the short piperazine formation made it impossible to determine the R_f of H-[^{3}H]Dmt-Tic-OH.

4.2. Biological data

4.2.1. β-Methyl amino acid substituted TIPP analogues

The new TIPP analogues were examined with regard to their binding properties to rat brain opioid receptors and their *in vitro* bioactivities, via their abilities to inhibit an electrically induced contraction of the MVD or GPI. δ-Antagonist potencies were determined against agonist DPDPE.

For the characterization of β-methyl amino acid substituted TIPP analogues, [^{3}H]DPDPE was used as δ-, [^{3}H]sufentanil as μ- and [^{3}H]U-69593 as κ-radioligand, respectively. The binding affinities of these ligands are summarized in Table 6.

δ-Opioid antagonists may be useful therapeutic agents in analgesia in combination with μ-agonists such as morphine, or the development of a single opioid compound acting as an agonist at the μ-receptor and as an antagonist at the δ-receptor, might be of benefit in the management of chronic pain. The modification of TIPP-NH_2, a mixed μ-agonist/δ-antagonist, served to find an analgesic with a low propensity to produce analgesic tolerance and physical dependence.

Table 6. Receptor binding affinities of TIPP analogues containing β-methyl amino acids

Peptides	K_i (nM) ± SEM		
H-TIPP-OH	>10 000	0.48±0.07	>10 000
H-TIPP-NH_2	178±26	0.83±0.11	838±116
(*SS*)-β-MeTyr^1-TIPP-NH_2	352±100	2.88±0.25	>10 000
(*SR*)-β-MeTyr^1-TIPP-NH_2	284±63.5	1.21±0.06	>10 000
(*RR*)-β-MeTyr^1-TIPP-NH_2	1 596±412	28±2	1 180±63.5
(*RS*)-β-MeTyr^1-TIPP-NH_2	>10 000	113±14	2 509±300
(*SS*)-β-MePhe^3-TIPP-NH_2	973±309	2.91±0.4	3 396±655
(*SR*)-β-MePhe^3-TIPP-NH_2	149±31	0.66±0.07	4324±1978
(*RR*)-β-MePhe^3-TIPP-NH_2	>10 000	74±5.1	442±52
(*RS*)-β-MePhe^3-TIPP-NH_2	84±21	0.5±0.07	1 190±452
(*SR*)-β-MePhe^3-TIPP-OH	>10 000	0.38±0.01	>10 000
(*RS*)-β-MePhe^3-TIPP-OH	810±185	0.76±0.10	>10 000
(*SS*)-β-MePhe^4-TIPP-NH_2	47±12.4	1.6±0.18	1 282±87
(*SR*)-β-MePhe^4-TIPP-NH_2	2 021±202	1.68±0.15	253±10
(*RR*)-β-MePhe^4-TIPP-NH_2	498±150	4.1±0.3	766±18
(*RS*)-β-MePhe^4-TIPP-NH_2	>10 000	11±1.01	2 844±519

μ [a]: [^{3}H]sufentanil (rat forebrain), δ [b]: [^{3}H]DPDPE (NxG 108 CC 15 cells), κ [c]: [^{3}H]U-69593 (guinea pig cerebellum)

The *L*-β-methyl amino acid containing TIPP analogues [II] displayed only slight changes in δ-receptor affinity as compared with the parent peptides TIPP or TIPP-NH_2. The effect of change of the configuration at the β-carbon on the δ-receptor binding was found to be small. The μ-receptor affinity was influenced to a larger extent, particularly as concerns the residue at position 4.

In the *D*-β-methyl amino acid containing TIPP analogues, the effects were more dramatic. The replacement of Tyr^1 resulted in new compounds, the (2*R*,3*R*) conformer proving more potent than the (2*R*,3*S*). This was in contrast with β-$MePhe^3$-TIPP, where the (2*R*,3*R*)-β-$MePhe^3$ analogue displayed a 10 times lower δ-affinity than its corresponding parent peptide and no μ-receptor affinity, and the epimeric (2*R*,3*S*) compound exhibited subnanomolar δ-receptor affinity. It was expected, that replacement of the C-terminal carboxamide function in (2*R*,3*S*)-β-$MePhe^3$-TIPP-NH_2 by a carboxylic acid group would lead to a new compound with enhanced δ-receptor selectivity. The β-$MePhe^4$ substitution resulted in the still quite potent and δ-receptor selective ligands. The κ-receptor binding remained low in all cases.

In the MVD and GPI assays [II], all compounds of the *L*-β-methyl amino acid containing series retained δ-antagonist properties with the exception of the partial agonist (2*S*,3*R*)-β-$MeTyr^1$-TIPP-NH_2. The results are summarized in Table 7.The δ-antagonist properties in general correlated with the values obtained in experiments on the δ-receptor affinity. The analogue (2*S*,3*R*)-β-$MePhe^3$-TIPP had a very high δ-antagonist potency, with no μ-antagonist and μ-agonist properties. The β-$MePhe^4$-TIPP-NH_2 analogues were mixed μ-agonist/δ-antagonists, with nearly the same properties as those of TIPP-NH_2. (2*S*,3*S*)-β-$MePhe^4$-TIPP-NH_2 was a better μ-agonist; it had a smaller K_e value than the parent peptide, and it might be a promising ligand for the treatment of pain.

In the *D*-β-methyl amino acid containing TIPP analogue series, β-$MeTyr^1$ replacement resulted in weakly potent δ-antagonists, in agreement with their low δ-receptor affinities. Interestingly, the analogue (2*R*,3*R*)-β-$MePhe^3$-TIPP-NH_2 was a moderately potent, but quite selective δ-antagonist, while the epimer (2*R*,3*S*)-β-$MePhe^3$-TIPP-NH_2 was a very potent full δ-agonist with a moderately potent μ-agonist potency. The corresponding peptide with a carboxy terminal had partial agonist properties at both receptors. The β-$MePhe^4$ analogues maintained moderate δ-antagonist potencies, whereas the (2*R*,3*R*)-β-$MePhe^4$ analogue was a partial μ-agonist, and (2*R*,3*S*)-β-$MePhe^4$-TIPP-NH_2 was a very weak, but full μ-agonist

Table 7. Activities in MVD and GPI bioassays of TIPP analogues containing β-methyl amino acids

Peptides	Bioassay	
	GPI (IC_{50}) ± SEM agonist [nM]	MVD (K_e DPDPE) ± SEM antagonist [nM]
H-TIPP-OH	>10 000	4.8±0.2
H-TIPP-NH_2	1 700±220	18±2.2
(*SS*)-β-MeTyr^1-TIPP-NH_2	>10 000	102±6
(*SR*)-β-MeTyr^1-TIPP-NH_2	1 210±260	IC_{25} = 1030±120 part. agon. max. 50%
(*RR*)-β-MeTyr^1-TIPP-NH_2	>10 000	1020±150
(*RS*)-β-MeTyr^1-TIPP-NH_2	>10 000	317±62
(*SS*)-β-MePhe^3-TIPP-NH_2	5 520±640	18.2±3.4
(*SR*)-β-MePhe^3-TIPP-NH_2	5 080±700	7.24±0.11
(*RR*)-β-MePhe^3-TIPP-NH_2	>10 000	54.4±5.8
(*RS*)-β-MePhe^3-TIPP-NH_2	127±19	IC_{50} = 1.78±0.25 agonist
(*SR*)-β-MePhe^3-TIPP-OH	>10 000	0.192±0.025
(*RS*)-β-MePhe^3-TIPP-OH	IC_{35} = 519±62 part. agon. max. 70%	IC_{30} = 0.623±0.12 part. agon. max. 60%
(*SS*)-β-MePhe^4-TIPP-NH_2	636±62	5.36±1.05
(*SR*)-β-MePhe^4-TIPP-NH_2	2 749±360	5.37±0.84
(*RR*)-β-MePhe^4-TIPP-NH_2	IC_{35} = 1 710±270 part. agon. max. 70%	20.5±0.7
(*RS*)-β-MePhe^4-TIPP-NH_2	4 510±750	27.7±5.5

The intramolecular distance between the aromatic rings of Tyr^1 and Tic^2 is an important factor determining the δ-antagonism in TIP(P). The aromatic rings of Phe^3 and Phe^4 may prevent the hydrophobic collapse of the Tyr-Tic rings and can engage in additional receptor interactions. The introduction of a β-methyl group on each of the residues may therefore have effects on the receptor affinity, on the selectivity and on the agonist/antagonist character, by biasing the side-chain topology. In general, it was clear that the introduction of *L*-β-methyl amino acids in TIPP [**II**] had an unimportant effect on the δ-receptor affinity and on the δ-antagonist potency. Both of the β-MeTyr^1 isomers allowed a *gauche*(-) conformation, which was not consistent with the theoretical models of the Tyr-Tic dipeptide analogue [158]. For β-MePhe^3, the *threo* isomer-containing peptide was more potent than its *erythro* analogue. This indicated that a *trans* χ_1 was preferred, and a *gauche*(+) conformation was out of the question as a bioactive conformation. In the lowest-energy conformation of TIPP[Ψ], the Phe^3 assumed the *trans* orientation, which made it more exposed and accessible for the interaction with the δ-receptor [79]. The receptor selectivity was further increased in (2S,3R)-β-MePhe^3-TIPP-OH, and the high potency confirmed the previous conclusion. This compound had a K_e value of

0.192 nM, which was comparable to those of the most potent δ-antagonists, such as TICP[Ψ], DIPP and N,N-(Me)$_2$Dmt-Tic-OH. In the β-MePhe^4-TIPP-NH$_2$ analogues, the configuration of the β-carbon had little effect on the δ-receptor affinity and potency. This can be explained by the fact that both of the isomers can exist in the *gauche*(-) instead of the *trans* conformation. The difference between the isomers was more stressed in the case of the μ-affinity; this resulted in the higher δ-selectivity of the (2*S*,3*R*)-β-MePhe^4-TIPP-NH$_2$ ligand. Some important effects can be observed in the *D*-β-methyl amino acid containing series. Since the *D*-Tyr^1 residue is not tolerated in opioid peptides, the low potency was not surprising in these analogues. An interesting observation was made in the case of the *D*-β-MePhe^3 peptides. While the (2*R*,3*R*)-β-MePhe^3-TIPP-NH$_2$ analogue retained an antagonist character, the epimer with the (2*R*,3*S*) configuration proved to be a very potent δ-agonist and a moderately potent μ-agonist. It was supposed that the χ_1-rotamer, which was highly disfavoured in the (2*R*,3*R*) isomer, but existed in the other isomer, can cause this effect. Change of the C-terminal amide to the carboxylic acid in (2*R*,3*S*)-β-MePhe^3-TIPP-NH$_2$ resulted in a new compound with partial δ-agonism. The effect of *D*-β-MePhe^4 substitution was similar to that, in the case of the *L*-amino acid. Both compounds retained moderate δ-antagonism, but in (2*R*,3*R*)-β-MePhe^4-TIPP-NH$_2$ partial μ-agonism was observed.

4.2.2. Binding characteristics of [^{3}H]TICP[Ψ] and [^{3}H]N,N(Me)$_2$-Dmt-Tic-OH

This work was carried out by the Receptor Research Group of BRC.

The results of the binding kinetics experiments indicated [**IV**], that a steady state was reached in 60 min. with [^{3}H]TICP[Ψ] (0.5 nM) at 25 °C (Figure 5). The determined association rate constant was 1.2 10^6 sec^{-1}M^{-1} and the dissociation rate constant was 5.6 10^{-4} sec^{-1}. From these rate constants, a binding K_d of 0.47 nM was calculated.

The binding of [^{3}H]N,N(Me)$_2$-Dmt-Tic-OH [**V**] was rapid, a steady state being reached after 25 min of incubation at 25 °C.

For estimation of the total and non-specific binding, increasing concentrations of [^{3}H]TICP[Ψ] and [^{3}H]N,N(Me)$_2$-Dmt-Tic-OH were incubated with membranes in the absence and presence of 10 μM naloxone.

Saturation binding experiments on [^{3}H]TICP[Ψ] [**IV**] were performed with the radiolabelled ligand by varying the concentration over the range from 0.02 nM to 3 nM. The specific binding was found to be saturable. The Scatchard plot (Figure 6) was used to evaluate the data, and this indicated the existence of a single binding site with a K_d of 0.35±0.03 nM, a value in reasonable agreement with the K_d obtained from the binding kinetics experiments. The maximum number of binding sites was 112.1±9.6 fmol/mg protein.

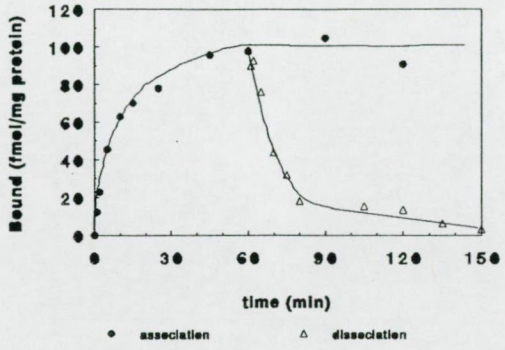

Figure 5. Association and dissociation curves of [^{3}H]TICP[Ψ] binding to rat brain membranes at 25 °C

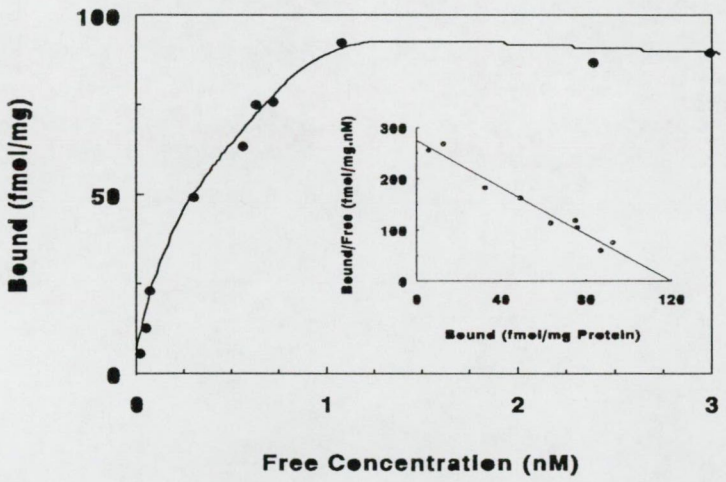

Figure 6. Saturation binding curve and Scatchard plot (insert) of [^{3}H]TICP[Ψ] binding to rat brain membranes. Plots on graph represent specific binding.

The specific binding of [^{3}H]N,N(Me)$_2$-Dmt-Tic-OH [**V**] was also saturable and Scatchard analysis (Figure 7) of the saturation binding data was best fitted with a single binding site model. K_d and the maximum number of binding sites were determined and found to be 0.42 ± 0.03 nM and 63.12 ± 1.22 fmol/mg protein, respectively.

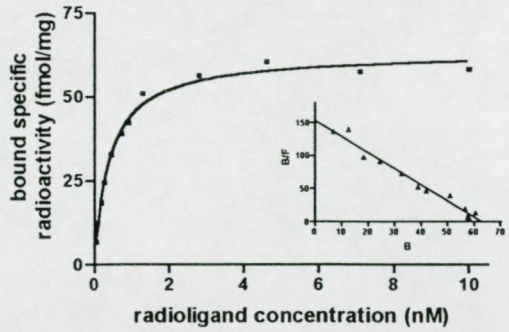

Figure 7. Saturation binding curve and Scatchard plot (insert) of [^{3}H]N,N(Me)$_2$-Dmt-Tic-OH binding to rat brain membranes. Plots on graph represent specific binding.

Equilibrium competition experiments were performed with the use of various unlabelled ligands selective for μ-, δ- or κ-receptors to displace bound [^{3}H]TICP[Ψ]. The rank order of potency within the series of compounds tested was δ>>κ>μ (Table 8). The determined K_i values indicate that TICP[Ψ] has a higher affinity for δ-receptors than all the used unlabelled ligands. In similar experiments, the inhibition of the binding of [^{3}H]N,N(Me)$_2$-Dmt-Tic-OH by various unlabelled specific opioid ligands was studied (Table 8). The rank order of potencies was found to be δ >> μ ≈ κ. The reported K_i values indicated that naltribene and naltrindole had the highest affinities for the δ-receptor. The results also confirmed that N,N(Me)$_2$-Dmt-Tic-OH bound to the δ-receptor with a high affinity comparable with that of naltrindole, and more tightly than TIPP[Ψ].

For both radioreceptor binding and localization studies, there were a number of requirements that a labelled compound had to fulfil: most importantly a low amount of non-specific binding, reaching of the steady state in a feasible time, a high specific radioactivity and a high selectivity. The high amount of non-specific binding was the problem, for example, with H-Tyr-Tic[CH$_2$-NH]-N(Me)Phe-Phe-OH. [^{3}H]TICP[Ψ] and [^{3}H]N,N(Me)$_2$-Dmt-Tic-OH fulfil all of these criteria. Both of our new tritiated ligands had high specific radioactivities. The ratio of non-specific binding was about 45-50% for [^{3}H]TICP[Ψ] (Table 9), and the specific binding reached a steady state in approximately 60 min. The conclusion from this work is that [^{3}H]TICP[Ψ] is a radioligand with high δ-receptor affinity and unprecedented δ-receptor selectivity. TICP[Ψ] proves to be a very convenient ligand for investigation and identification of the δ-opioid receptor. The non-specific binding of [^{3}H]N,N(Me)$_2$-Dmt-Tic-OH was around 10-15%, while the specific binding reached a steady state in approximately 25 min. As for specificity, [^{3}H]N,N(Me)$_2$-Dmt-Tic-OH is an exceptionally δ-specific ligand. The differences between the affinities of the subtypes of the δ-receptor resulted in differences in the B_{max} values of these ligands. Measurement of the K_i values of [^{3}H]N,N(Me)$_2$-Dmt-Tic-OH against a

number of δ-specific opioid ligands in radioreceptor binding assays revealed that the inhibition constants were in the subnanomolar or the low nanomolar range and the lowest K_i value was measured against naltrindole; this was even lower than that against $N,N(Me)_2$-Dmt-Tic-OH itself. This extraordinarily low K_i value might be due to the conformational similarities between this opioid dipeptide and naltrindole. TIPP[Ψ] gave a K_i value close to 1 nM. Interestingly, despite the high sequence similarities, its parent compound, TIPP, gave the highest K_i values among all the δ-compounds. We can conclude that the dipeptide [^{3}H]$N,N(Me)_2$-Dmt-Tic-OH is one of the most specific and potent δ-radioligands available for characterization of the δ-opioid receptor in *in vitro* and *in vivo* studies.

Table 8. Inhibition of [^{3}H]TICP[Ψ] and [^{3}H]$N,N(Me)_2$-Dmt-Tic-OH binding by various opioid receptor-selective ligands

Unlabelled ligand	Type	K_i±SEM (nM)[a]	K_i± SEM (nM)[b]
Ile^{5,6} Deltorphin II	δ	1.54±0.85	3.39±0.47
Deltorphin II	δ	2.48±0.22	0.28±0.07
Naltrindole	δ	0.61±0.19	0.14±0.001
DSLET	δ	3.35±0.4	2.10±0.15
DPDPE	δ	10.47±2.51	1.91±0.55
TIPP	δ	4.62±3.2	16.23±3.76
TIPP[ψ]	δ	2.49±0.9	1.31±0.12
Naltribene	δ	–	0.02±0.01
TICP[Ψ]	δ	0.35±0.03	–
$N,N(Me)_2$-Dmt-Tic-OH	δ	–	0.44±0.13
Dermorphin	μ	–	569.67±289.2
DAMGO	μ	416.5±16	1 053.2±216.8
Dihydromorphine	μ	126.9±20.8	–
Levorphanol	μ	5.45±0.75	8.10±0.69
Dextrorphan	μ	> 10 μM	> 10 μM
Endomorphin 1	μ	–	20 046.67±12 894.7
Endomorphin 2	μ	–	2 580.33±1 137.9
U69593	κ	331.29±194.58	5 964.00±609
Nor-binaltorphimine	κ	46.3±7.6	–
EKC	κ	–	660.19±117.66
Dynorphin A (1-11)	κ	–	269.07±65.81

[a], *[^{3}H]TICP[Ψ]*, [b], *[^{3}H]N,N(Me)$_2$-Dmt-Tic-OH*

Table 9. Changes in the non-specific binding values of tritiated ligands

	TIPP	TIPP[Ψ]	TICP[Ψ]	N,N(Me)$_2$-Dmt-Tic-OH
Non-specific binding	35%	30%	45-50%	10-15%

It was further concluded that both of our new tritiated ligands were about 10 times more potent than TIPP or TIPP[Ψ] (Table 10). Moreover, it was important that TICP[Ψ] and N,N(Me)$_2$-Dmt-Tic-OH were very stable against chemical degradation under storage conditions, and against enzymatic degradation under binding conditions.

Table 10. K_d and B_{max} values of tritiated ligands based on Scatchard analysis and K_e values

Analogues	TIPP	TIPP[Ψ]	TICP[Ψ]	N,N(Me)$_2$-Dmt-Tic-OH
B_{max} fmol/mg	82.4±20.4	105.5±49	112.4±7.5	63.12 ± 1.22
K_d (nM)	0.64±0.20	0.98±0.12	0.35±0.03	0.42 ± 0.03
K_e (nM)	4.8[a]	2.89[a]	0.219[a]	0.28[b]

[a] *ref. 159,* [b] *ref. 75*

The antagonist property of the peptide, shown previously in MVD tests, was further confirmed here by a biochemical approach, [^{35}S]GTPγS binding. As shown in Figure 8, N,N(Me)$_2$-Dmt-Tic-OH potently and effectively reversed the activation of GTP-binding proteins by a specific δ-agonist peptide, Ile^{5,6}deltorphin II, as measured by the binding of the non-hydrolysable GTP analogue, GTPγS. The ED_{50} value of this effect was in the nanomolar range (8.1 nM), which emphasizes the potential usefulness of this molecule.

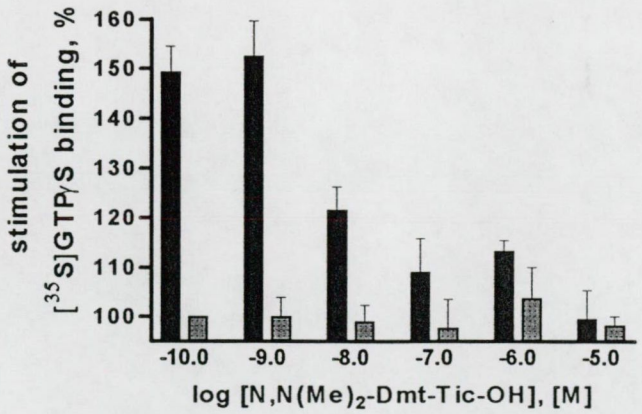

Figure 8. Inhibition of agonist-stimulated [^{35}S]GTPγS binding by N,N(Me)$_2$-Dmt-Tic-OH in membranes of rat frontal cortex. Grey bars mean the absence and black bars mean the presence of 1 μM Ile^{5,6}deltorphin II. Non-specific binding was 61%; non-stimulated [^{35}S]GTPγS binding was 67.3 ± 11.6 fmol/mg protein. Points are means ± S.E.M. of data from three independent experiments carried out in triplicate.

5. SUMMARY

Our aim was the develolopment of a new δ-antagonist or a mixed μ-agonist/δ-antagonist from the TIPP family. The idea was based on the peptide library: a slightly constrained, β-methyl amino acid substitution on each position of TIPP or TIPP-NH_2. To reach our aims, we achieved the following results:

- We performed the Erlenmeyer condensation between hippuric acid and 4-methoxyacetophenone to gain β-MeTyr.
- Both the *erythro* and *threo* β-MeTyr isomers were incorporated into the TIPP analogues and the resulting epimeric peptides were characterized by RP-HPLC, TLC and MS, separated and purified by RP-HPLC. The absolute configuration at the α-carbon of the β-methyl-amino acid in each peptide was determined after acid hydrolysis and derivatization of the resulting amino acids with GITC.
- We performed the racemic synthesis of Dmt and developed a new enzymatic resolution method.

We found that there were crucial effects on the receptor selectivity and on the agonist/antagonist character following the introduction of β-methyl amino acids. Tyr-Tic was previously found to be the first opioid peptide without Phe that had δ-opioid selectivity and antagonist bioactivity. The presented work confirmed that substitution at Phe^3 and Phe^4 can strongly influence the profile of biological properties. Despite the fact that a single β-methyl substituent is reported not to constrain the side-chain position of aromatic amino acids extensively, it was revealed that these topographically constrained amino acids resulted in differences in biological activity in the TIPP series. These results were hard to explain in terms of the topographical requirements of the receptor as concerns the side-chain conformations. New, larger substituents with higher energy differences between the rotamers may provide additional information about this question.

We have obtained some promising ligands, such as (2*S*,3*R*)-β-$MePhe^3$-TIPP-OH, which exhibited subnanomolar δ-receptor affinity and the K_e value of which was comparable to those of TICP[Ψ] and N,N$(Me)_2$-Dmt-Tic-OH. Another result was the development of (2*S*,3*S*)-β-$MePhe^4$-TIPP-NH_2, which had a better μ-agonist property and was a more potent δ-antagonist than the parent TIPP-NH_2, and might be a candidate ligand for the treatment of pain or to serve as a basis for further development.

We synthesized the tritiated ligands for radioreceptor binding. With this work, we wished to develop a series of δ-antagonist radioligands suitable for any receptor binding conditions. It had been reported previously that TICP[Ψ] and N,N$(Me)_2$-Dmt-Tic-OH were

good δ-antagonist candidates for tritium labelling. We chose some promising δ-antagonist ligands from our former work and made them in radiolabelled form. Unfortunately, the biological characterization of these TIPP analogues has not yet been completed. We have prepared the following tritiated analogues:

- [^{3}H]TICP[Ψ]
- [^{3}H]-N(Me)Phe^3-TIPP[Ψ]
- H-[^{3}H]Dmt-Tic-OH
- N,N(Me)$_2$-[^{3}H]Dmt-Tic-OH
- [^{3}H]-(*L*)-*threo*-β-MePhe^3-TIPP
- [^{3}H]-(*D*)-*threo*-β-MePhe^3-TIPP
- [^{3}H]-(*L*)-*threo*-β-MePhe^3-DIPP

The radioreceptor binding studies revealed that both [^{3}H]TICP[Ψ] and N,N(Me)$_2$-[^{3}H]Dmt-Tic-OH were suitable for δ-receptor characterization. They had high specific radioactivity, they were very stable against chemical and enzymatic degradation under storage and binding conditions, they had low ratio of non-specific binding (even N,N(Me)$_2$-[^{3}H]Dmt-Tic-OH), both had a very high δ-receptor affinity and unprecedented δ-receptor selectivity, and the specific binding reached a steady state in a feasible period of time. The most important conclusion was that both of our new ligands were 10 times more potent δ-antagonists than TIPP[Ψ]. All these parameters made them very convenient ligands for investigation and identification of the δ-opioid receptor. It is important to emphasize that the H-Dmt-Tic-OH sequence represents the minimum sequence of the δ-antagonism to date, and this statement gives peculiar importance to N,N(Me)$_2$-[^{3}H]Dmt-Tic-OH radioligand.

[^{3}H]-(*L*)-*threo*-β-MePhe^3-TIPP is also a very potent δ-antagonist with a subnanomolar K_e value, and it was known from earlier results that [^{3}H]TIPP has a lower non-specific binding than [^{3}H]TICP[Ψ]. Replacement of Tyr^1 with Dmt in (*L*)-*threo*-β-MePhe^3-TIPP may produce further enhancement of the δ-antagonist potency, but also a slight decrease in δ-receptor selectivity. The resulting labelled peptide [^{3}H]-(*L*)-*threo*-β-MePhe^3-DIPP may be the most potent δ-receptor antagonist. We hope that these new ligands can fulfil all criteria of radioligands and can prove to be useful research tools.

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