Evaluation of the Dmt−Tic Pharmacophore: Conversion of a Potent δ-Opioid Receptor Antagonist into a Potent δ Agonist and Ligands with Mixed Properties

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Analogues of the 2′,6′-dimethyl-L-tyrosine (Dmt)−1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) pharmacophore were prepared to test the hypothesis that a "spacer" and a third aromatic center in opioid peptides are required to convert a δ-antagonist into ligands with δ-agonist or with mixed δ-antagonist/μ-agonist properties. Potent δ-agonists and bifunctional compounds with high δ- and μ-opioid receptor affinities were obtained by varying the spacer length [none, NH−CH_2, NH−CH_2−CH_2, Gly−NH−CH_2] and C-terminal aromatic nucleus [1H-benzimidazole-2-yl, phenyl (Ph) and benzyl groups]. C-terminal modification primarily affected μ-opioid receptor affinities, which increased maximally 1700-fold relative to the prototype δ-antagonist H−Dmt−Tic−NH_2 and differentially modified bioactivity. In the absence of a spacer (**1**), the analogue exhibited dual δ-agonism (pEC_{50}, 7.28) and δ-antagonism (pA_2, 7.90). H−Dmt−Tic−NH−CH_2−1H-benzimidazol-2-yl (Bid) (**2**) became a highly potent δ-agonist (pEC_{50}, 9.90), slightly greater than deltorphin C (pEC_{50}, 9.56), with μ-agonism (pE_{50}, 7.57), while H−Dmt−Tic−Gly−NH−CH_2−Bid (**4**) retained potent δ-antagonism (pA_2, 9.0) but with an order of magnitude less μ-agonism. Similarly, H−Dmt−Tic−Gly−NH−Ph (**5**) had nearly equivalent high δ-agonism (pEC_{50}, 8.52) and μ-agonism (pEC_{50}, 8.59), while H−Dmt−Tic−Gly−NH−CH_2−Ph (**6**) whose spacer was longer by a single methylene group exhibited potent δ-antagonism (pA_2, 9.25) and very high μ-agonism (pEC_{50}, 8.57). These data confirm that the distance between the Dmt−Tic pharmacophore and a third aromatic nucleus is an important criterion in converting Dmt−Tic from a highly potent δ-antagonist into a potent δ-agonist or into ligands with mixed δ- and μ-opioid properties.

Introduction

The introduction of 2′,6′-dimethyl-L-tyrosine (Dmt)[1,2] into Tyr−1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic)-containing δ-opioid antagonists[3−9] enhanced both affinity and functional bioactivity by orders of magnitude.[2] This remarkable alteration in the properties of diverse opioid peptides underscores its application in the formation of ligands with new pharmacological properties,[10] including inverse agonism.[11] For example, the influence of Dmt can be appreciated in the alterations in the activity of [Dmt[1]]deltorphin B, an analogue of the potent δ_2-agonist deltorphin B,[12] which became a high affinity δ-/μ-agonist,[13] as well as the acquisition of higher μ-affinity and activity in [Dmt[1]]DALDA,[14] enkephalin analogues, which gained extraordinary high δ-affinity,[15] similar to the high μ-affinity observed with bis-Dmt-pyrazinone derivatives (Y. Okada, personal communications) and [Dmt[1]]endomorphins-1 and -2 (G. Toth, personal communications). Thus, Dmt facilitates ligand recognition toward both δ- and μ-opioid receptors.[16] Although the Tic residue appeared to be responsible for δ-antagonism[17] since the substitution of the Dmt−Tic pharmacophore at the N terminus of unrelated opioid agonists transformed them into δ-antagonists, such as enkephalin (δ),[18] dermorphin (μ),[18] and dynorphin A (1−11) NH_2 (κ),[19] some Dmt−Tic analogues have a distinct spectrum of mixed δ-/μ-antagonist/agonist activities.[20]

Building on these observations, recent attention focused on the transmutation of the Dmt−Tic pharmacophore into bifunctional or heterofunctional opioid compounds.[8,9,20−22] A variety of modifications to the Tic residue differentially modified receptor selectivity, including alterations in its electronic configuration[20,21] and a change in chirality[2] as well as its replacement by heteroaliphatic/heteroaromatic nuclei[17] or D-Phe.[23] Changes wrought by altering the distance to a third aromatic center at the C terminus[9] by an interposed sequence—a spacer—consisting of one or more amino acid residues[24,25] or selected C-terminal hydrophobic substituents,[8a] which was confirmed and extensively expanded upon by Pagé et al.,[8b] induced profound changes in the affinity, selectivity, and bioactivity of a

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ligand. For instance, modification at the *N* substituent of naltrindole, a non peptide δ-opiate antagonist,[26] converted it into either a μ-agonist[27] or a δ-agonist.[28] Similarly, the acquisition of high μ-receptor activity in other nonpeptide substances, such as 3-amino-3-phenylpropionamide,[29] methylmorphan compounds,[30] and diazatricyclodecane derivatives,[31] required phenethyl amide groups or a hexane chain for optimum activity. These data fully support the concept that spatially oriented hydrophobic or aromatic groups enhanced interaction between the ligand and the specific side chains within disparate receptors to elicit new or opposite opioid bioactivity.[8,17]

Rationale

"Nature teaches receptors to know their ligands."[32] We must decipher that logic by designing new ligands that conform to the dimensions of the binding site. The evolution of a potent δ-antagonist into a ligand, which elicits mixed antagonist/agonist properties for the δ- and μ-opioid receptors was initially associated with subtle as well as major alterations in structure: a change in chirality of the second residue[2,3,23] coupled with an absence of a negative charge shifted the selectivity toward the μ-receptor.[10] However, opioid dipeptides modified by C-terminal substituents[8,9,23] containing hydrophobic or aromatic centers[8,9] supported the concept that extended ligand topographies are potential requirements for μ-opioid activity.[23,33] Furthermore, several Dmt-pseudopeptides containing heteroaromatic or heteroaliphatic nuclei revealed a wide spectrum of mixed δ-/μ-opioid receptor properties: e.g., δ-antagonists exhibited μ-agonism[8,17] and weak μ-antagonism[17,20] as well as weak δ-/μ-agonism with weak μ-antagonism.[17] Additionally, a benzyloxy-methylene group in lieu of the carboxyl function in Dmt−Tic permitted the retention of high δ-affinity and δ-antagonism with the appearance of μ-agonism.[20] Interestingly, deletion of the carboxyl function in TIP, a δ-selective antagonist,[5] retained δ-affinity but possessed δ-agonism.[9] Therefore, the aromatic/hydrophobic substituent and the distance between aromatic centers may hold clues in the conversion of an antagonist into an agonist or a bifunctional opioid ligand. Evidence with nonpeptide opiates similarly demonstrated this shift in bioactivity: the replacement of *N*-cyclopropylmethyl by *N*-cyclohexylethyl, but not *N*-cyclohexylmethyl, transformed the high δ-affinity and δ-antagonism of naltrindole[26] into a potent μ-agonist,[27] whereas its substitution by a methyl group yielded a δ-agonist.[28] These data among other lines of evidence with opioid peptides[8] and nonpeptide opiates[29−31,34] exemplify this postulate.

Our systematic analysis presented herein extends and verifies the hypothesis on the conversion of a potent δ-opioid peptide antagonist into a ligand with extraordinary δ-agonist activity and analogues exhibiting mixed antagonist/agonist properties that depend on the length of the interposing spacer. We based our conclusions on the analysis of six analogues of the Dmt−Tic pharmacophore containing a free N terminus using the H−Dmt−Tic−R series of peptides (Figure 1).

Chemistry

All pseudopeptides were prepared in a stepwise procedure by standard solution peptide synthesis methods. Mixed carbonic anhydride coupling of *tert*-butyloxycarbonyl (Boc)−Gly−OH or Boc−βAla−OH or Boc−Gly−Gly−OH with *o*-phenylendiamine gave the corresponding crude intermediate monoamides, which were converted without purification to the desired heteroaromatic derivatives by cyclization and dehydration in acetic acid (AcOH), as outlined in Scheme 1 for Boc−Gly−OH. After N^{α} deprotection with trifluoroacetic acid (TFA), each derivative was condensed with Boc−Tic−OH and then with Boc−Dmt−OH via 1-ethyl-3-[3′-dimethyl)aminopropyl]carbodiimide (WSC)/1-hydroxy-1,2,3-benzotriazole (HOBt). Tripeptides containing C terminal benzyl amide or phenyl (Ph) amide (anilide) were obtained in a similar manner by condensation of Boc−Gly−OH with benzylamine or aniline via WSC/HOBt, respectively (Scheme 2).

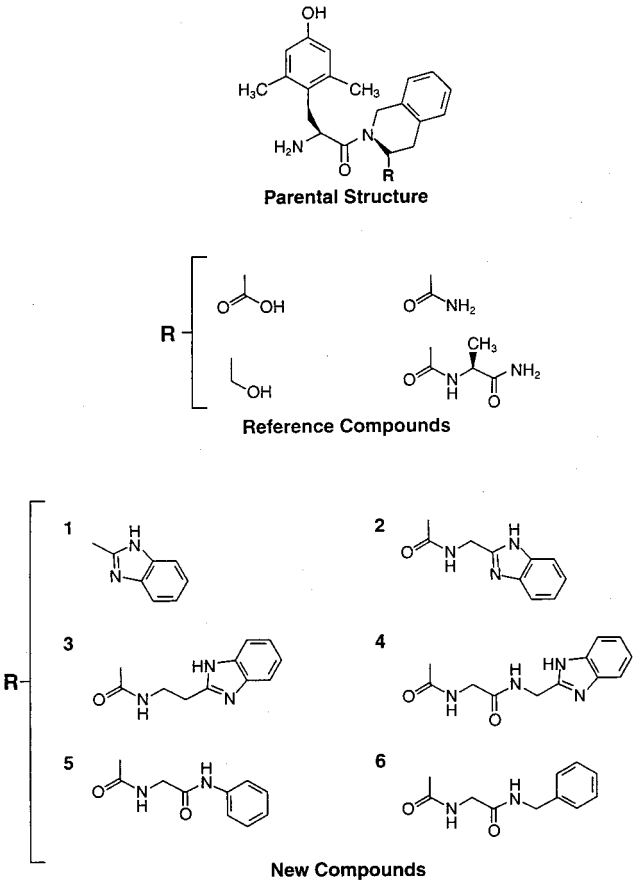


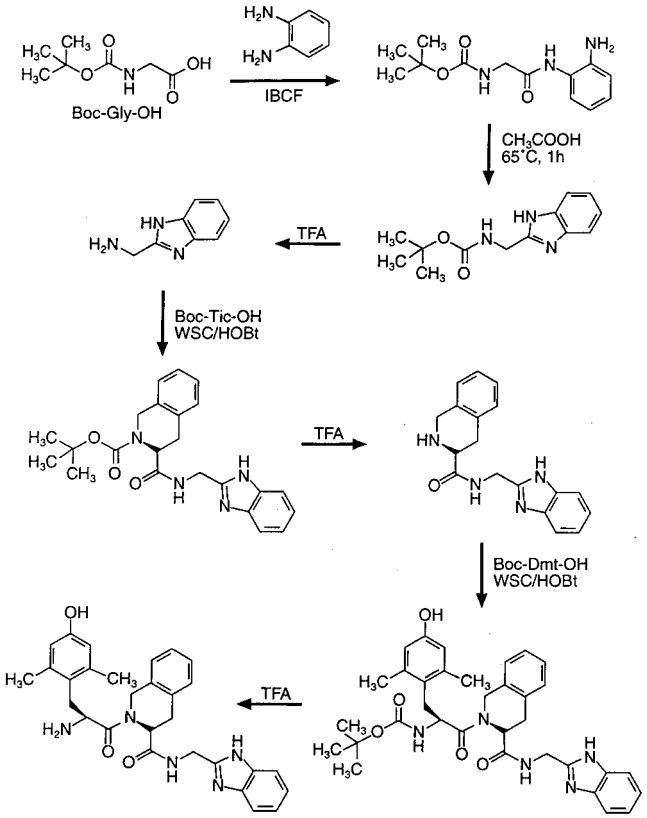
Figure 1. General structure of H−Dmt−Tic−R with the R for the parental structure and reference compounds, and R = **1**−**6** substituents for the new compounds.

Results

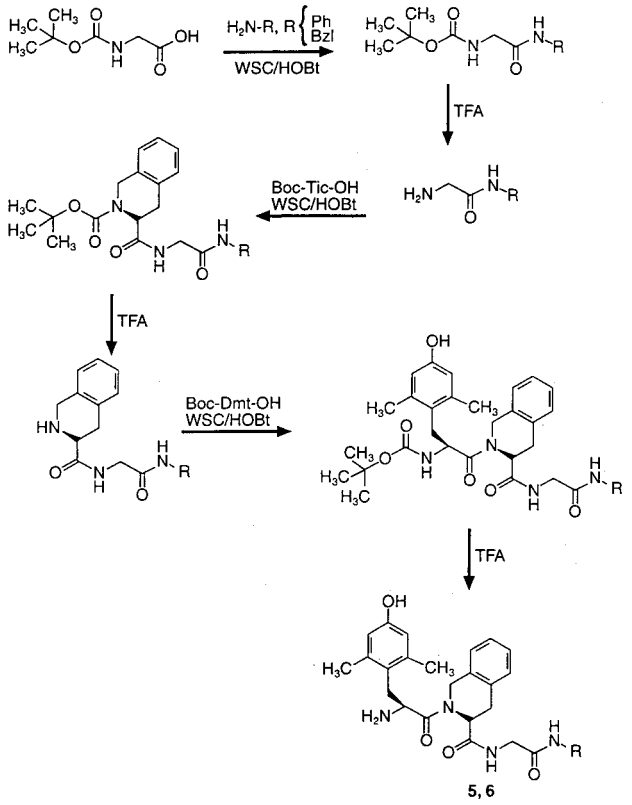
Peptide Interaction with Opioid Receptors. The appearance of high δ-affinity (Table 1) depends on the quality of the radioligand, either [^{3}H]DPDPE (*cyclic*[D-Pen^{2,5}]enkephalin), [^{3}H]DAGO ([D-Ala^2, *N*−Me−Phe^4, Gly ol^5] enkephalin), [^{3}H]*N*,*N*(CH$_3$)$_2$−Dmt−Tic−OH,[35,36] or [^{3}H]deltorphin B, which all yielded consistently comparable K_i values.[8] As long as the synaptosomal preparations are prepared from fresh rat brains and stored at −80 °C in glycerol,[37] stability and reliability of the radioligand remained the key factors.

The Dmt−Tic pharmacophore peptides (**1**−**6**) had exceptionally high δ-affinities, in which the K_i values

Scheme 1. Synthesis of Compound **2**



Scheme 2. General Synthesis of Compounds **5** and **6**



were 0.1 nM or less (Table 1). The C-terminal substituents containing a spacer and an aromatic ring substituent exerted essentially minimal influence on δ-binding parameters in these analogues; i.e., no linker (**1**) or containing NH−CH_2 (**2**), NH−CH_2−CH (**3**), Gly−NH (**5**), or Gly−NH−CH_2 (**4**, **6**) were nearly equivalent. The interaction between peptide ligand and μ-receptors, on the other hand, demonstrated remarkable variation (Table 1): (a) C terminal aromatic groups enhanced μ-receptor binding by several orders of magnitude with K_i values < 1 nM (**2**, **5**, **6**) while compounds **1**, **3**, and **4** had a $K_i\mu$ values of 5−20 nM; however, it should be noted that **3** and **4** were essentially nonselective. Ligand affinity for the μ-receptor increased over 1700-fold with **5** and **6** relative to H−Dmt−Tic−NH_2, a standard prototypic δ-opioid antagonist.[2] (b) The length of the spacer in the 1H-benzimidazol-2-yl (Bid)-containing series (**1**−**4**) greatly affected the acquisition of high μ-affinity (Table 1). (c) Ligand interaction with μ-receptors required a definitive distance between the aromatic nuclei of Tic and Bid. However, the chemical and physical nature of the C terminal substituent play a role in receptor recognition as seen with bulky 1-adamantane and *tert*-butyl derivatives[8] as well as numerous other hydrophobic and aromatic compounds.[8b]

Functional Bioactivity Studies. The in vitro pharmacological assays are summarized in Tables 1 and 2. H−Dmt−Tic−NH−CH_2−Bid (**2**) and H−Dmt−Tic−Gly−NH−Ph (**5**) exhibited potent δ-agonist activity; in particular, compound **2** (negative log of the molar concentration of an agonist to produce 50% of the maximum effect (pEC_{50}) = 9.90) was greater than that of deltorphin C; its weak μ-agonism was nearly two orders of magnitude less than dermorphin. To determine if the δ-agonist activities of **2** and **5** were mediated by the δ-receptor, the pA_2 (negative log of the molar concentration required to double the agonist concentration to achieve the original response) for naloxone using the δ-selective agonist deltorphin C was determined. The pA_2 values were comparable for all δ-agonists confirming that in the mouse vas deferens (MVD), agonist activity was mediated by the δ-receptor (Table 2). Similarly, *N*,*N*$(Me)_2$−Dmt−Tic−OH, a highly selective δ-antagonist,[7] yielded analogous results with deltorphin C and compound **2**, while compound **5** had 5-fold lower activity. Thus, the activity of **2** is mediated by δ-opioid receptors and δ-agonism is more than 2 orders of magnitude greater than its μ-agonist activity. Interestingly, while compounds **4** and **6** retained potent δ-antagonist activity, only **6** had remarkably high μ-agonism.

The functional pharmacological activity of compound **1** had an unusual spectrum of behavior that depended on peptide concentration. At low doses, it was possible to estimate δ-antagonist activity against deltorphin C, while at high concentration (up to 1 μM), electrical stimulation of the tissue was reduced by 83%.

Discussion

Nature is proving to be a formidable opponent and a difficult teacher. Nonetheless, our analogues subtly probed the requirements for developing a potent δ-opioid agonist on the scaffold of a potent δ-antagonist, in addition to the formulation of new compounds with mixed δ-antagonist/μ-agonist properties. In doing so, it brings to light structural features required within the receptor[38] and the ligand[9,10,16,17,20] that must be considered in the design of new opioid peptides.

A considerable body of evidence reveals that the Dmt−Tic pharmacophore is responsible for high

Table 1. In Vitro Opioid Activity of Dmt-Tic Analogues[a]

compd	receptor affinity (nM)			functional bioactivity		
	$K_i\delta$	$K_i\mu$	μ/δ	MVD (pEC_{50})	MVD (pA_2)	GPI (pEC_{50})
			Ref Compds			
H−Dmt−Tic−OH	0.022	3320	150 780		−8.2	*b*
H−Dmt−Tic−NH_2	1.22	277	227		−7.2	*b*
H−Dmt−Tic−ol	0.44	151	344		−7.0	*b*
H−Dmt−Tic−Ala−NH_2	0.22	47	195		−8.0	*b*
deltorphin C	0.21	387	1840[c]	9.56 (0.30)		
dermorphin	82.5	0.28	0.0034[d]			9.15 (0.10)
			New Compds			
1	0.13 ± 0.04 (4)	7.22 ± 0.96 (4)	56[e]	7.28 (0.45)	7.90 (0.30)	
2	0.035 ± 0.006 (3)	0.50 ± 0.054 (3)	14	9.90 (0.32)		7.57 (0.14)
3	0.067 ± 0.015 (4)	5.49 ± 0.93 (3)	82		8.32 (0.25)	6.97 (0.18)
4	0.058 ± 0.005 (3)	20.5 ± 2.4 (3)	353		9.00 (0.29)	6.45 (0.20)
5	0.042 ± 0.007 (3)	0.16 ± 0.003 (3)	3.6	8.52 (0.99)		8.59 (0.48)
6	0.031 ± 0.002 (3)	0.16 ± 0.018 (3)	5.3		9.25 (0.3)	8.57 (0.14)

[a] The K_i values (nM) were determined by Cheng and Prusoff[53] using a rat brain receptor (P_2 synaptosome) assay (see Experimental Section); the mean ± SE with *n* repetitions in parentheses was determined using PrismJ (GraphPad). Each repetition was an independent bioassay conducted in duplicate using 5−8 graded dosages of peptide; confidence levels are in parentheses. pA_2 is the negative log of the molar concentration required to double the agonist concentration to achieve the original response. pEC_{50} is the negative log of the molar concentration of an agonist to produce 50% of the maximum effect. [b] Salvadori et al.[2] [c] Lazarus et al.[51] [d] Tomatis et al.[54] [e] Balboni et al.[17]

Table 2. Pharmacological Activity of Selected δ-Antagonists/μ-Agonists[a]

compd	MVD pEC_{50}	MVD, antagonist activity (pA_2)	
		naloxone	*N*,*N*(CH_3)$_2$−Dmt−Tic−OH
deltorphin C	9.56	7.5	9.76
2	9.90	7.45	9.70
5	8.52	7.47	9.04

[a] Data are the means of at least four independent experiments. $pA_2 = \log[C - 1]/[\text{antagonist}]$, where C = concentration.

δ-affinity[2,7−10,17,39] and whose activity toward the μ-receptor is modified by alterations at the C terminus,[2,3,8,20] but for the most part, the δ-receptor maintained high ligand affinities.[7,8] Alteration in the C terminus of the Dmt−Tic pharmacophore appeared to mimic changes observed with the amphibian μ-opioid heptapeptides dermorphin[40] and deltorphin.[12] Similarly, the coupling of bulky anionic or hydrophobic fluorescent dyes to [Lys^7]dermorphin, deltorphin C, and H−Tyr−Tic−Phe−(Phe)−OH (TIPP), which were initially modified through the C-terminal addition of Cys, substantially reduced receptor affinity and selectivity.[41] This adds further weight to our postulate that C-terminal groups restrict the activity of an opioid peptide toward their specific receptor,[8a] and this was substantiated recently in considerable detail by Pagé et al.[8b] It is known that the carboxylic acid function of Dmt−Tic (Figure 1, R = −COOH) determines the δ-opioid receptor selectivity by excluding interaction with μ receptors (Table 1) and that this compound essentially fails to interact with κ receptors.[2]

The results presented herein (Tables 1 and 2) demonstrate that C-terminal modification of the Dmt−Tic pharmacophore converted a δ-antagonist into pseudopeptides with remarkable new properties. For example, some analogues demonstrated an improvement in μ-opioid affinity and μ-agonist activity with guinea pig ileum (GPI) assay as noted previously.[17] In an effort to discover different approaches to replace or modify Tic, we reported the introduction of benzoimidazole, pyridoindole, and spiroinden derivatives of Dmt, which acquired interesting properties.[20] Among the benzoimidazole derivatives, 2-amino-1-[3-(1H-benzoimidazole-2-yl)-3,4-dihydro-1H-isoquinoline-2-yl]-3-(4-hydroxyl-2,6-dimethyl-Ph)-propane-1-one (Figure 1, **1**) showed high δ-binding affinity, being 10-fold higher than H−Dmt−Tic−NH_2 and 3-fold greater than H−Dmt−Tic−ol. Compound **1** also increased affinity toward the μ-receptor nearly 40-fold as compared to H−Dmt−Tic−NH_2; published data indicated δ-antagonist (pA_2, 7.67) and μ-agonist activities (IC_{50}, 30 nM).[17] This observation was substantiated with the functional bioassays of newly synthesized preparations of **1** that revealed δ-antagonist (pA_2, 7.90) and δ-agonist activities (pEC_{50}, 7.28) (Table 1). These results are impressive in light of the fact that all hitherto for described Dmt−Tic analogues are all δ-antagonists,[10,16] although hydrophobic and aromatic substituents at the C terminus provided evidence that they substantially modified the properties of the Dmt−Tic pharmacophore.[8]

Our results clearly demonstrate that a third pharmacophore, namely, 1-H-benzimidazol-2-yl (Bid), in lieu of the carboxylic acid function of H−Dmt−Tic−OH or in analogues containing a linker in the general formula H−Dmt−Tic−X−Bid, are generally better than *N*-Ph amide to elicit δ-agonist or antagonist activities (compare **3** or **4** to **6**). Interestingly, H−Dmt−Tic−Gly−NH−Ph (**5**) had essentially equivalent activity as both a δ-agonist and a μ-agonist. However, replacing the *N*-Ph amide (**5**) with an *N*-benzyl amide (**6**) function not only increased the distance between aromatic nuclei but also converted the analogue into a potent δ antagonist with excellent μ-agonist activity, although about one-fourth of that for dermorphin. The pharmacological profile of that analogue might be expected to have analgesic properties with lower liability to tolerance and dependence.

Conclusions

We can draw the following conclusions based on the data in Tables 1 and 2. (i) A third aromatic nucleus determines δ-opioid agonist activity when it is located at a prescribed distance by use of a spacer from the Dmt−Tic pharmacophore. This in turn implies that the μ-receptor binding site not only accommodates but also requires a physically larger and bulkier peptide ligand

in order to bind and trigger an agonist response. However, the differences in activity between ligands containing a NH−CH_2 or Gly−NH−CH_2 linker indicate that enhanced flexibility of the latter spacer fails to permit the proper positioning of the peptide in the region of the receptor that elicits agonism. Thus, the spatial orientation of the C-terminal portion of the peptide, the "address domain", in contrast to the N-terminal "message domain", putatively defines receptor selectivity.[24] We can further surmise that these changes reflect inherent differences in the structural domains of δ- and μ-opioid receptors in the binding pocket; normally, the changes are associated primarily in the extracellular loop regions,[42] although subtle differences exist within the α-helical transmembrane helices,[43−45] which would comprise portions of the binding region for Dmt.

(ii) A Bid substituent is a better pharmacophore than a *N*-Ph amide function in determining the agonist activation of the δ receptor (compare **2** to **5**), whereas the opposite is true in terms of δ antagonism (compare **4** to **6** and **3**). However, the 1H-benzimadazol-2-yl pharmacophore was responsible for agonist activity, whether covalently bonded to Tic (**1**) or via a NH−CH_2 linker (**2**).

(iii) All modifications at the C terminus of the H−Dmt−Tic−OH or H−Dmt−Tic−Xaa−OH pharmacophores drastically increased μ-opioid receptor affinity and μ-agonist activity (Table 1) as observed previously with hydrophobic substituents.[8] Interestingly, based on its pharmacological profile, compound **1** appears to be intermediate between H−Dmt−Tic−NH_2, a pure δ-antagonist,[11] and H−Dmt−Tic−NH−CH_2−Bid (**2**), a δ-agonist (Table 2). On the other hand, this bulky aromatic moiety in H−Dmt−Tic−Gly−NH−CH_2−Bid (**4**) with a slightly long linker only yields potent δ-antagonist activity.

(iv) Analogue **6**, which has mixed δ-antagonist/μ-agonist activity, is an ideal candidate peptide to ascertain analgesia with a lower liability toward tolerance and dependence.

In general, these data will assist in the future development of more selective opioid ligands and will clarify the optimum binding requirements for the δ- and μ-opioid receptors to permit the differentiation between agonist and antagonist responses. In fact, highly selective opioids,[10] analogous to opioid knock-out mice,[46] afford a window of opportunity into the mechanism of action by a specific receptor relative to a physiological response. The multifaceted transformation of a single pharmacophore seems to echo the words of Byron, "Tis strange—but true; for truth is always strange; stranger than fiction."

Experimental Section

Materials. H−L−Dmt−OH was synthesized as reported and compared to a sample generously supplied by J. H. Dygos et al.[47] Boc−Tic−OH was obtained from Bachem (Heidelberg, Germany). [^{3}H]DPDPE (32.0 Ci/mmol) was a product of NEN-DuPont (Bilirica, MA), and [^{3}H]DAGO (58.0 Ci/mmol) was obtained from Amersham (Arlington Heights, IL).

General Methods. Crude peptides were purified by preparative reversed-phase high-performance liquid chromatography (HPLC) using a Waters Delta Prep 4000 system with Waters PrepLC 40 mm Assembly column C_{18} (30 cm × 4 cm, 300 D, 15 μm particle size column). The column was perfused at a flow rate of 50 mL/min with mobile phase solvent A (10% acetonitrile in 0.1% TFA, v/v), and a linear gradient from 0 to 50% solvent B (60% acetonitrile in 0.1% TFA, v/v) in 25 min was adopted for the elution of the products. Analytical HPLC analyses were performed with a Beckman System Gold with a Beckman ultrasphere ODS column (5 μm; 4.6 mm × 250 mm). Analytical determinations and capacity factor (K') of the products were determined using HPLC conditions in the above solvent systems (solvents A and B) programmed at a flow rate of 1 mL/min using the following linear gradients: (a) from 0 to 100% B in 25 min and (b) from 10 to 70% B in 25 min. All analogues showed less than 1% impurities when monitored at 220 and 254 nm.

Thin-layer chromatography (TLC) was performed on precoated plates of silica gel F254 (Merck, Darmstadt, Germany) using the following solvent systems: (A) 1-butanol/AcOH/H_2O (3:1:1, v/v/v) and (B) CH_2Cl_2/toluene/methanol (MeOH, 17:1:2, v/v/v). Ninhydrin (1%, Merck), fluorescamine (Hoffman-La Roche), and chlorine reagents were used as sprays. Open column chromatography (2 cm × 70 cm, 0.7−1 g of material) was run on silica gel 60 (70−230 mesh, Merck) using the same eluent systems.

Melting points were determined on a Kofler apparatus and are uncorrected. Optical rotations were determined at 10 mg/mL in MeOH with a Perkin-Elmer 241 polarimeter with a 10 cm water-jacketed cell. All ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a Bruker 200 MHz spectrometer. MALDI-TOF analyses (matrix assisted laser desorption ionization time-of-flight) mass spectrometry of peptides were conducted using a Hewlett-Packard G 2025 A LD-TOF system. The samples were analyzed in the linear mode with 28 kV accelerating voltage, mixing them with a saturated solution of α-cyano-4-hydroxycinnamic acid matrix.

(1H-Benzimidazol-2-yl-methyl)carbamic Acid *tert*-Butyl Ester (Boc−NH−CH_2−Bid). A solution of Boc−Gly−OH (1.7 g, 9.6 mmol) and 4-methyl morpholine (NMM, 1 mL, 9.6 mmol) in *N*,*N*-dimethylforamide (DMF, 10 mL) was treated at −20 °C with isobutyl chloroformate (1.2 mL, 9.6 mmol). After 10 min at −20 °C, *o*-phenylendiamine (1 g, 9.6 mmol) was added. The reaction mixture was allowed to stir while slowly warming to room temperature (1 h) and was then stirred for 3 h.

The solvent was evaporated, and the residue was partitioned between ethyl acetate (EtOAc) and H_2O. The EtOAc layer was washed with 5% $NaHCO_3$ and brine and dried over Na_2SO_4. The solution was filtered, the solvent was evaporated, and the residual solid was dissolved in glacial AcOH (10 mL). The solution was heated at 65 °C for 1 h. After the solvent was evaporated, the residue was crystallized from diethyl ether (Et_2O)/petroleum ether (Pe) (1:9, v/v); yield 2.11 g (89%); R_f (B) 0.43; HPLC $K' = 3.95$; mp 131−133 °C; MH^+ 248. ^{1}H NMR (dimethyl sufoxide (DMSO)): δ 1.40 (s, 9H), 4.22−4.40 (m, 2H), 7.26−7.70 (m, 5H), 7.98 (s, 1H).

2TFA·2-Aminomethylbenzimidazole (H_2N−CH_2−Bid).[48] Boc−NH−CH_2−Bid (1.00 g, 4.05 mmol) was treated with TFA (1 mL) for 0.5 h at room temperature. Et_2O/Pe (1:1, v/v) was added to the solution until the product precipitated; yield 1.49 g (98%); R_f (A) 0.38; HPLC $K' = 1.50$; mp 220−222 °C; MH^+ 148.

Boc−Tic−NH−CH_2−Bid. To a solution of Boc−Tic−OH (0.3 g, 1.08 mmol) and 2 TFA·H−NH−CH_2−Bid (0.41 g, 1.08 mmol) in DMF (10 mL) at 0 °C were added NMM (0.24 mL, 2.16 mmol), HOBt (0.18 g, 1.19 mmol), and WSC (0.23 g, 1.19 mmol). The reaction mixture was stirred for 3 h at 0 °C and 24 h at room temperature. After DMF was evaporated, the residue was solubilized in EtOAc and washed with $NaHCO_3$ (5%) and brine. The organic phase was dried and evaporated to dryness. The residue was crystallized from Et_2O/Pe (1:9, v/v); yield 0.36 g (82%); R_f (B) 0.68; HPLC $K' = 8.12$; mp 142−144 °C; $[\alpha]^{20}_D$ −12.1; MH^+ 407. ^{1}H NMR (DMSO): δ 1.38−1.42 (d, 9H), 3.08−3.15 (m, 2H), 4.22−4.53 (m, 5H), 7.05−7.71 (m, 9H), 7.97 (s, 1H).

2TFA·H−Tic−NH−CH_2−Bid. Boc−Tic−NH−CH_2−Bid (0.36 g, 0.89 mmol) was treated with TFA (1 mL) for 0.5 h at

room temperature. Et_2O/Pe (1:1, v/v) was added to the solution until the product precipitated; yield 0.46 g (97%); R_f (A) 0.43; HPLC K' = 4,11; mp 180−182 °C; $[\alpha]^{20}_D$ −14.8; MH^+ 307.

Boc−Dmt−Tic-NH−CH_2−Bid. To a solution of Boc−Dmt−OH (0.25 g, 0.82 mmol) and 2TFA·H−Tic−NH−CH_2−Bid (0.44 g, 0.82 mmol) in DMF (10 mL) at 0 °C were added NMM (0.18 mL, 1.64 mmol), HOBt (0.14 g, 0.90 mmol), and WSC (0.17 g, 0.90 mmol). The reaction mixture was stirred for 3 h at 0 °C and for 24 h at room temperature. After DMF was evaporated, the residue was solubilized in EtOAc and washed with $NaHCO_3$ (5%) and brine. The organic phase was dried and evaporated to dryness. The residue was crystallized from Et_2O/Pe (1:9, v/v); yield 0.39 g (80%); R_f (B) 0.68; HPLC K' = 8.98; mp 142−144 °C; $[\alpha]^{20}_D$ −22.5; MH^+ 598. ^1H NMR (DMSO): δ = 1.32−1.39 (d, 9H), 2.17 (s, 6H), 2.85−3.10 (m, 4H), 4.22−4.65 (m, 6H), 6.37 (s, 2H), 7.05−7.71 (m, 10H), 7.97 (s, 1H).

2TFA·H−Dmt−Tic−NH−CH_2−Bid (2). Boc−Dmt−Tic−NH−CH_2−Bid (0.1 g, 0.17 mmol) was treated with TFA (1 mL) for 0.5 h at room temperature. Et_2O/Pe (1:1, v/v) was added to the solution until the product precipitated; yield 0.12 g (97%); R_f (A) 0.31; HPLC K' = 5.40; mp 152−154 °C; $[\alpha]^{20}_D$ −35.2; MH^+ 499.

(1H-Benzimidazol-2-ylethyl)carbamic Acid *tert*-Butyl Ester (Boc−NH−CH_2−CH_2−Bid). This product was obtained from Boc−βAla−OH and *o*-phenylenediamine as reported for Boc−NH−CH_2−Bid; yield 0.8 g (85%); R_f (B) 0.52; HPLC K' = 4.25; mp 142−144 °C; MH^+ 262. ^1H NMR (DMSO): δ = 1.38 (s, 9H), 2.81−3.29 (m, 4H), 7.25−7.68 (m, 5H), 7.95 (s, 1H).

2TFA·2-(Bid)ethylamine (H_2N−CH_2−CH_2−Bid).[48] Boc−NH−CH_2−CH_2−Bid was treated with TFA as reported for 2TFA·H_2N−CH_2−Bid; yield 0.52 g (96%); R_f (A) 0.43; HPLC K' = 1.78; mp 229−231 °C; MH^+ 162.

Boc−Tic−NH−CH_2−CH_2−Bid. This substance was obtained by condensation of Boc−Tic−OH with 2TFA·H_2N−CH_2−CH_2−Bid via WSC/HOBt as reported for Boc−Tic−NH−CH_2−Bid; yield 0.36 g (88%); R_f (B) 0.72; HPLC K' = 8.38; mp 147−149 °C; $[\alpha]^{20}_D$ −10.7; MH^+ 421. ^1H NMR (DMSO): δ = 1.39−1.41 (d, 9H), 2.80−3.31 (m, 6H), 4.21−4.55 (m, 3H), 7.07−7.69 (m, 9H), 7.99 (s, 1H).

2TFA·H−Tic−NH−CH_2−CH_2−Bid. Boc−Tic−NH−CH_2−CH_2−Bid was treated with TFA as reported for 2TFA·H−Tic−NH−CH_2−Bid; yield 0.35 g (98%); R_f (A) 0.48; HPLC K' = 4.18; 176−178 °C; $[\alpha]^{20}_D$ −12.3; MH^+ 321.

Boc−Dmt−Tic−NH−CH_2−CH_2−Bid. This compound was obtained by condensation of Boc−Dmt−OH with 2TFA·H−Tic−NH−CH_2−CH_2−Bid via WSC/HOBt as reported for Boc−Dmt−Tic−NH−CH_2−Bid; yield 0.29 g (83%); R_f (B) 0.72; HPLC K' = 9.03; mp 151−153 °C; $[\alpha]^{20}_D$ −19.9; MH^+ 612. ^1H NMR (DMSO): δ = 1.33−1.41 (d, 9H), 2.16 (s, 6H), 2.81−3.31 (m, 4H), 4.22−4.64 (m, 4H), 6.38 (s, 2H), 7.04−7.69 (m, 10H), 7.98 (s, 1H).

2TFA·H−Dmt−Tic−NH−CH_2−CH_2−Bid (3). Boc−Dmt−Tic−NH−CH_2−CH_2−Bid was treated with TFA as reported for 2TFA·H−Dmt−Tic−NH−CH_2−Bid; yield 0.052 g (87%); R_f (A) 0.34; HPLC K' = 4.88; mp 156−158 °C; $[\alpha]^{20}_D$ −30.7; MH^+ 513.

Boc−Gly−NH−CH_2−Bid. This compound was obtained from Boc−Gly−Gly−OH and *o*-phenylenediamine as reported for Boc−NH−CH_2−Bid; yield 0.73 g (82%); R_f (B) 0.55; HPLC K' = 4.53; mp 148−150 °C; MH^+ 305. ^1H NMR (DMSO): δ = 1.40−1.42 (d, 9H), 3.85−4.46 (m, 4H), 7.25−7.71 (m, 6H), 7.94 (s, 1H).

2TFA·H−Gly−NH−CH_2−Bid. Boc−Gly−NH−CH_2−Bid was treated with TFA as reported for 2TFA·H_2N−CH_2−Bid; yield 0.49 g (97%); R_f (A) 0.45; HPLC K' = 1.88; mp 187−189 °C; MH^+ 205.

Boc−Tic−Gly−NH−CH_2−Bid. This substance was obtained by condensation of Boc−Tic−OH with 2TFA·H−Gly−NH−CH_2−Bid via WSC/HOBt as reported for Boc−Tic−NH−CH_2−Bid; yield 0.38 g (84%); R_f (B) 0.73; HPLC K' = 8.16; mp 146−148 °C; $[\alpha]^{20}_D$ −10.4; MH^+ 464. ^1H NMR (DMSO): δ = 1.40−1.42 (d, 9H), 3.08−3.15 (m, 2H), 3.85−4.55 (m, 7H), 7.03−7.70 (m, 10H), 7.95 (s, 1H).

2TFA·H−Tic−Gly−NH−CH_2−Bid. Boc−Tic−Gly−NH−CH_2−Bid was treated with TFA as reported for 2TFA·H−Tic−NH−CH_2−Bid; yield 0.32 g. (95%); R_f (A) 0.47; HPLC K' = 4.35; mp 163−165 °C; $[\alpha]^{20}_D$ −10.1; MH^+ 364.

Boc−Dmt−Tic−Gly−NH−CH_2−Bid. This compound was obtained by condensation of Boc−Dmt−OH with 2TFA·H−Tic−Gly−NH−CH_2−Bid via WSC/HOBt as reported for Boc−Dmt−NH−CH_2−Bid; yield 0.28 g (85%); R_f (B) 0.73; HPLC K' = 9.03; mp 146−148 °C; $[\alpha]^{20}_D$ −19.3; MH^+ 655. ^1H NMR (DMSO): δ = 1.35−1.40 (d, 9H), 2.17 (s, 6H), 2.85−3.12 (m, 4H), 4.20−4.72 (m, 8H), 6.37 (s, 2H), 7.05−7.75 (m, 11H), 7.99 (s, 1H).

2TFA·H−Dmt−Tic−Gly−NH−CH_2−Bid (4). Boc−Dmt−Tic−Gly−NH−CH_2−Bid was treated with TFA as reported for 2TFA·H−Dmt−Tic−NH−CH_2−Bid; yield 0.068 g (96%); R_f (A) 0.35; HPLC K' = 4.95; mp 155−157 °C; $[\alpha]^{20}_D$ −30.8; MH^+ 557.

Boc−Tic−Gly−NH−Ph. This compound was obtained by condensation of Boc−Tic−OH with TFA·H−Gly−NH−Ph[49] as reported for Boc−Tic−NH−CH_2−Bid; yield 0.24 g (85%); R_f (B) 0.67; HPLC K' = 10.73; mp 131−133 °C; $[\alpha]^{20}_D$ −36.8; MH^+ 410. ^1H NMR (DMSO): δ = 1.39−1.41 (d, 9H), 3.08−3.15 (m, 2H), 3.61 (d, 2H), 4.22−4.92 (m, 3H), 6.91−7.31 (m, 10H), 8.96 (bs, 1H).

TFA·H−Tic−Gly−NH−Ph. Boc−Tic−Gly−NH−Ph was treated with TFA as reported for 2TFA·H−Tic−NH−CH_2−Bid; yield 0.18 g (96%); R_f (A) 0.35; HPLC K' = 6.07; mp 165−167 °C; $[\alpha]^{20}_D$ −32.5; MH^+ 310.

Boc−Dmt−Tic−Gly−NH−Ph. This compound was obtained by condensation of Boc−Dmt−OH with TFA·H−Tic−Gly−NH−Ph as reported for Boc−Dmt−Tic−NH−CH_2−Bid; yield 0.14 g (84%); R_f (B) 0.64; HPLC K' = 9.9; mp 144−146 °C; $[\alpha]^{20}_D$ −19.7; MH^+ 601. ^1H NMR (DMSO): δ = 1.40−1.42 (d, 9H), 2.17 (s, 6H), 3.08−3.15 (m, 4H), 3.61 (d, 2H), 4.22−4.92 (m, 3H), 6.37 (s, 2H), 6.91−7.31 (m, 11H), 8.94 (bs, 1H).

TFA·H−Dmt−Tic−Gly−NH−Ph (5). Boc−Dmt−Tic−Gly−NH−Ph was treated with TFA as reported for 2 TFA·H−Dmt−Tic−NH−CH_2−Bid; yield 0.07 g (97%); R_f (A) 0.41; HPLC K' = 7.18; mp 155−7 °C; $[\alpha]^{20}_D$ −21.8; MH^+ 444.

Boc−Tic−Gly−NH−CH_2−Ph (Bzl). This compound was obtained by condensation of Boc−Tic−OH with TFA·H−Gly−NH−Bzl[50] as reported for Boc−Tic−NH−CH_2−Bid; yield 0.26 g (88%); R_f (B) 0.72; HPLC K' = 11.02; mp 123−125 °C; $[\alpha]^{20}_D$ −35.2; MH^+ 424. ^1H NMR (DMSO): δ = 1.39−1.41 (d, 9H), 3.08−3.15 (m, 2H), 3.61 (d, 2H), 4.22−4.92 (m, 5H), 6.91−7.31 (m, 10H), 8.25 (t, 1H).

TFA·H−Tic−Gly−NH−Bzl. Boc−Tic−Gly−NH−Bzl was treated with TFA as reported for 2 TFA·H−Tic−NH−CH_2−Bid; yield 0.20 g (97%); R_f (A) 0.38; HPLC K' = 6.14; mp 151−153 °C; $[\alpha]^{20}_D$ −30.8; MH^+ 324.

Boc−Dmt−Tic−Gly−NH−Bzl. This compound was obtained by condensation of Boc−Dmt−OH with TFA·H−Tic−Gly−NH−Bzl as reported fro Boc−Dmt−Tic−CH_2−Bzl; yield 0.15 g (81%); R_f (B) 0.68; HPLC K' = 10.4; mp 138−140 °C; $[\alpha]^{20}_D$ −18.9; MH^+ 615. ^1H NMR (DMSO): δ = 1.40−1.42 (d, 9H), 2.17 (s, 6H), 3.08−3.15 (m, 4H), 3.61 (d, 2H), 4.22−4.92 (m, 5H), 6.37 (s, 2H), 6.91−7.31 (m, 11H), 8.25 (t, 1H).

TFA·H−Dmt−Tic−Gly−NH−Bzl (6). Boc−Dmt−Tic−Gly−NH−Bzl was treated with TFA as reported for 2 TFA·H−Dmt−Tic−NH−CH_2−Bid; yield 0.07 g (98%); R_f (A) 0.44; HPLC K' = 7.39; 148−150 °C; $[\alpha]^{20}_D$ −20.1; MH^+ 516.

Radioreceptor Assays. The peptides were assayed in a competition assay under equilibrium conditions with a P_2 preparation of rat brain synaptosomes[37] using [^{3}H]DPDPE (32.0 Ci/mmol; NEN-DuPont, Bilirica, MA) for δ-receptors and [^{3}H]DAGO (58.0 Ci/mmol; Amersham, Arlington Heights, IL) for μ-receptors in 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid, pH 7.5, containing $MgCl_2$ and a cocktail of several protease inhibitors and glycerol.[51] After the peptides were incubated for 120 min at room temperature (23−24 °C), the samples were rapidly filtered through Whatman GF/C glass fiber filers and washed three times with 2 mL of ice cold 0.05 M Tris, pH 7.4−7.5, containing 0.1% bovine serum

albumin. (Presoaking filters in 0.1% polyethyeneimine is only required in assays with peptides carrying an overall positive charge, such as dynorphin or nociceptin.)[52] Nonspecific binding was determined using 2 μM unlabeled DPDPE or DAGO for δ- or μ-assays, respectively. The K_i data, determined according to Cheng and Prusoff,[53] represent the means ± standard error (SE) from three or more independent assays using at least three different synaptosomal preparations and testing the analogues with 5−8 graded dosages over 2−3 orders of magnitude in concentration.

Functional Pharmacological Bioassays. The in vitro pharmacological assays used a single MVD for δ receptors and a 2−3 cm segment of GPI for μ receptors with each suspended in 20 mL organ baths containing balanced salt solutions in a physiological buffer.[2] Peptide analogues were assayed for μ-agonist activity by the inhibition of electrically stimulated contractions in comparison to dermorphin; δ-antagonist activity used deltorphin C (δ_1-agonist).[12] Data were obtained from four independent assays and tissue samples. Agonism is expressed as the IC_{50} (nM) value and antagonism as the pA_2 value.[1]

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(1) In addition to the IUPAC−IUB Commission on Biochemical Nomenclature (*J. Biol. Chem*. **1985**, *260*, 14−42), this paper uses the following symbols and abbreviations: AcOH, acetic acid; Bid, 1H-benzimidazol-2-yl; Boc, *tert*-butyloxycarbonyl; Bzl, CH_2−Ph; DAGO, [D-Ala^2, *N*-Me−Phe^4, Gly ol^5] enkephalin; DMF, *N*,*N*-dimethylforamide; Dmt, 2′,6′-dimethyl-L-tyrosine; DPDPE, *cyclic*[D-Pen^{2,5}]enkephalin; Et_2O, diethyl ether; EtOAc, ethyl acetate; GPI, guinea pig ileum; HOBt, 1-hydroxy-1,2,3-benzotriazole; HPLC, high-performance liquid chromatography; MeOH, methanol; MVD, mouse vas deferens; NMM, 4-methyl morpholine; pA_2, negative log of the molar concentration required to double the agonist concentration to achieve the original response; Pe, petroleum ether; pEC_{50}, negative log of the molar concentration of an agonist to produce 50% of the maximum effect; Ph, phenyl; TEA, triethylamine; TFA, trifluoroacetic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TIPP, H−Tyr−Tic−Phe−(Phe)−OH; TLC, thin-layer chromatography; WSC, 1-ethyl-3-[3′-dimethyl)aminopropyl] carbodiimide.

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