Evolution of the Dmt-Tic Pharmacophore: N-Terminal Methylated Derivatives with Extraordinary δ Opioid Antagonist Activity

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The δ opioid antagonist H-Dmt-Tic-OH (2′,6′-dimethyl-L-tyrosyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) exhibits extraordinary δ receptor binding characteristics [$K_i^{\delta} = 0.022$ nM; $K_i^{\mu}/K_i^{\delta} = 150\,000$] and δ antagonism ($pA_2 = 8.2$; $K_e = 5.7$ nM). A change in chirality of Dmt at Cα (**1, 2, 6, 8, 10, 13**) curtailed δ receptor parameters, while replacement of its α-amino function by a methyl group (**3**) led to inactivity; Tyr-Tic analogues **4** and **11** weakly interacted with δ receptors. *N*-Alkylation of H-Dmt-Tic-OH and H-Dmt-Tic-Ala-OH with methyl groups produced potent δ-opioid ligands with high δ receptor binding capabilities and enhanced δ antagonism: (i) *N*-Me-Dmt-Tic-OH **5** had high δ opioid binding ($K_i^{\delta} = 0.2$ nM), elevated δ antagonism on mouse vas deferens (MVD) ($pA_2 = 8.5$; $K_e = 2.8$ nM), and nondetectable μ activity with guinea pig ileum (GPI). (ii) *N*,*N*-Me_2-Dmt-Tic-OH (**12**) was equally efficacious in δ receptor binding ($K_i^{\delta} = 0.12$ nM; $K_i^{\mu}/K_i^{\delta} = 20\,000$), but δ antagonism rose considerably ($pA_2 = 9.4$; $K_e = 0.28$ nM) with weak μ antagonism ($pA_2 = 5.8$; $K_e = 1.58$ μM; GPI/MVD = 1:5640). *N*-Me-(**9**) and *N*,*N*-Me_2-Dmt-Tic-Ala-OH (**15**) also augmented δ opioid receptor binding, such that **15** demonstrated high affinity ($K_i^{\delta} = 0.0755$ nM) and selectivity ($K_i^{\mu}/K_i^{\delta} = 20\,132$) with exceptional antagonist activity on MVD ($pA_2 = 9.6$; $K_e = 0.22$ nM) and weak antagonism on GPI ($pA_2 = 5.8$; $K_e = 1.58$ μM; GPI/MVD = 1:7180). Although the amidated dimethylated dipeptide analogue **14** had high K_i^{δ} (0.31 nM) and excellent antagonist activity ($pA_2 = 9.9$; $K_e = 0.12$ nM), the increased activity toward μ receptors in the absence of a free acid function at the C-terminus revealed modest δ selectivity ($K_i^{\mu}/K_i^{\delta} = 1\,655$) and somewhat comparable bioactivity (GPI/MVD = 4500). Thus, the data demonstrate that *N*,*N*-$(Me)_2$-Dmt-Tic-OH (**12**) and *N*,*N*-Me_2-Dmt-Tic-Ala-OH (**15**) retained high δ receptor affinities and δ selectivities and acquired enhanced potency in pharmacological bioassays on MVD greater than that of other peptide or non-peptide δ antagonists.

Introduction[1]

Opioid peptides and their receptors represent a significant component in the physiological well-being of all animals.[2] Of the known opioids, two families of heptapeptide agonists, the dermorphins (μ selective) and the deltorphins (δ specific) obtained from skin of the Amazonian frogs *Phyllomedusa sauvagei* and *Phyllomedusa bicolor*, exhibited the highest degree of selectivity for either μ or δ mammalian receptors with subnanomolar affinities and with considerably higher potency than the endogenous enkephalins and endorphins.[2] Extensive structure−activity studies on their opioid properties resolved the function of each amino acid residue and the minimal sequence required for receptor binding and agonist bioactivity.[3−7] Those cumulative observations eventually led to the synthesis of di-, tri-, and tetrapeptide analogues containing the unnatural amino acid Tic in the second position; an interesting consequence of these opioid compounds was the acquisition of δ selectivity and behavior as δ antagonists.[8−11]

Even though Tic can be considered as a constrained analogue of Phe, the weak interaction of H-Tyr-Tic-OH and H-Tyr-Tic-Ala-OH with δ receptors indicated that these were the first opioids devoid of a phenyl side chain residues at positions 3 or 4,[9] a position considered crucial for binding to opioid receptors.[12] Although substitution of Tyr[1] by Dmt in a Tic[2]-containing tetrapeptide (DIPP) resulted in a compound with modest δ selectivity, *ca.* 500,[11] it was reasoned that replacement by the more hydrophobic residue Dmt[13,14] in the dipeptide might nonetheless be quite beneficial. As a result, the peptide retained *in vitro* δ opioid antagonism and exhibited exceptional δ receptor affinity ($K_i^{\delta} = 0.022$ nM) and extraordinarily high selectivity ($K_i^{\mu}/K_i^{\delta} = 150\,000$). Whereas the TIP(P) and Dmt-Tic di- and tripeptides are active *in vivo* when injected icv,[15,16] only H-Dmt-Tic-OH reversed the antinociception of δ opioid agonists by systemic administration.[17]

One liability of small peptides is cyclization to a diketopiperazine[18] during the acidic steps involved in peptide synthesis.[19,20] This phenomenon occurs not only with peptides containing Tic or other constrained residues at the C-terminus,[20] but also with other amino acids.[19] Nonetheless, the presence of diketopiperazines in various biological fluids and tissues was suggested to occur by enzymatic degradation of larger molecules[21] (or even as products of thermal decomposition during isolation procedures[22]) since they elicit diverse and

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Table 1. Membrane Receptor Binding of Dmt-Tic and Tyr-Tic Analogues[a]

no.	peptide	K_i^δ (nM)	K_i^μ (nM)	K_i^μ/K_i^δ
	H-Dmt-Tic-OH	0.022 ± 0.0015 (6)	3320 ± 435 (7)	150800[b]
	H-Dmt-Tic-NH_2	1.22 ± 0.09 (6)	277 ± 27 (3)	227[b]
	H-Dmt-Tic-Ala-OH	0.285 ± 0.03 (6)	5810 ± 675 (4)	20400[b]
	H-Tyr-Tic-OH	191.7 ± 47.8 (7)	28411 ± 2941 (3)	148[b]
	TIPP[ψ]	2.37 ± 0.40 (6)	4995 ± 146 (3)	2107
1	H-D-Dmt-Tic-OH	123.9 ± 29.8 (3)	10962 ± 1612 (4)	88
2	H-D-Dmt-Tic-Ala-OH	12.97± 1.01 (3)	2555 ± 163 (3)	197
3	[des-NH_2-α-Me-Dmt]-Tic-OH	1304 ± 206 (3)	71373 ± 11498 (4)	55
4	H-*N*-Me-Tyr-Tic-OH	74.7 ± 6.9 (3)	12386 ± 1448 (3)	166
5	H-*N*-Me-Dmt-Tic-OH	0.203 ± 0.0367 (4)	379.4 ± 34.4 (3)	1869
6	H-*N*-Me-D-Dmt-Tic-OH	3.19 ± 0.54 (3)	2003 ± 332 (3)	628
7	H-*N*-Me-Dmt-Tic-NH_2	0.383 ± 0.0741 (4)	954.9 ± 164 (4)	2493
8	H-*N*-Me-D-Dmt-Tic-NH_2	11.2 ± 0.68 (3)	1329 ± 64.3 (4)	119
9	H-*N*-Me-Dmt-Tic-Ala-OH	0.136 ± 0.0299 (4)	5853 ± 591 (3)	43037
10	H-*N*-Me-D-Dmt-Tic-Ala-OH	106.1 ± 24.7 (4)	19210 ± 1915 (3)	181
11	*N*,*N*-$(Me)_2$-Tyr-Tic-OH	43.5 ± 6.27 (4)	11523 ± 1606 (4)	265
12	*N*,*N*-$(Me)_2$-Dmt-Tic-OH	0.118 ± 0.0219 (3)	2435 ± 462 (3)	20636
13	*N*,*N*-$(Me)_2$-D-Dmt-Tic-OH	5.74 ± 2.38 (4)	13089 ± 836 (3)	2280
14	*N*,*N*-$(Me)_2$-Dmt-Tic-NH_2	0.309 ± 0.049 (4)	511.4 ± 74.6 (4)	1655
15	*N*,*N*-$(Me)_2$-Dmt-Tic-Ala-OH	0.0755 ± 0.016 (4)	1520 ± 179 (3)	20013
16	*N*,*N*-$(Me)_2$-D-Dmt-Tic-Ala-OH	107.8 ± 35.8 (3)	18956 ± 484 (3)	176

[a] Receptor binding data are presented as the mean ± SEM with the number of repetitions from independent assays (*n*) listed in parentheses. The ratio of the affinities for δ and μ receptors (K_i^μ/K_i^δ) shows δ selectivity. [b] Data taken from ref 8.

distinct biological responses.[21,23] Diketopiperazine formation from Dmt-Tic peptides appeared to be essentially negligible under neutral conditions;[8,20] however, even when present, *cyclo*(Dmt-Tic) exhibited δ opioid receptor affinity (K_i^δ = 9.84 nM).[24] This activity was attributed to the enhanced hydrophobicity imparted by Dmt and a low-energy conformation deduced by Monte Carlo analysis in comparison to the inactive *cyclo*(Tyr-Tic) cognate.[25]

One means to stabilize peptides against diketopiperazine formation would be suppression of the ability of the protonated nitrogen at the N-terminus to form an intramolecular peptide bond with a carbonyl or the C-terminal carboxyl group that occurs through the inclusion of the CH_2-NH (ψ) bond in a tetrapeptide.[10] Previous modification of the N-terminal amine group of deltorphin C heptapeptide analogues[26] or residue alteration in cyclic opioid tetra-[27,28] and pentapeptides[29] resulted in a negative effect relative to δ affinity and selectivity. On the other hand, *N*-alkylation of Tyr^1 in other opioid peptides produced changes in their intrinsic activity depending on the type of alkyl group: e.g., *N*-alkylation of the Tyr^1 amino function in dynorphin A-(1−11) gave analogues with antagonist activity[30] and elevated κ selectivity,[31] whereas the trimethylammonium analogues of enkephalin exhibited minimal effect on its pharmacological action *in vitro* while suppressing receptor binding approximately 60-fold.[32]

Rationale

Increased hydrophobicity and alteration of peptide conformation represent consequences of *N*-alkylation that might therefore minimally disrupt the physical parameters of the peptide. Peptide alkylation by N^α- or C^α-methylation, however, is known to affect conformation by induction of 3_{10}-, α-helical,[33] or β-turn conformations[34] and provided conformational constraints used to study structure−activity relationships.[35,36] For example, the incorporation of N^α- or C^α-Me-Phe^3 residues in deltorphin C, which reduced receptor binding and bioactivity, was accompanied by an observable change in its molecular dynamics conformation;[37] *cyclo*[Dmt-(N^α-Me-Phe)] abolished δ opioid receptor binding;[24] and an N^α-pentamethylated analogue of enkephalin was similarly devoid of activity.[32] Comparable substitutions of N^α-Me-Phe as well as other methylated residues in conformationally constrained and linear opioid analogues[27,28,37,38] affected receptor binding and bioactivity related to specific changes in dihedral angles that modified an assumed bioactive conformation.[35,39] On the other hand, methylation also alters peptide hydrophobicity[37,40] and heightens stability against proteolysis,[35] which could account for enhanced biological activity in some cases,[28,34] although curtailment of bioactivity was a more prevalent attribute of peptide methylation.[28,32,33,35,38]

Thus, we sought to determine whether *N*-alkylation could modify the δ opioid receptor−ligand interaction of Dmt-Tic di- and tripeptides, since selective *N*-alkylation with methyl groups create secondary and tertiary amines. Furthermore, these peptides might serve as probes in order to deduce the requirement for hydrophobic factors in coupling an opioid peptide within the δ receptor pocket[41] as suggested by site-directed mutagenesis[42] and chimeric receptor molecules,[43] as well as other detailed structure−activity studies.[3−6,12,24−29,37,39,44]

Results

***δ* Opioid Receptor Properties.** *N*-Alkylated analogues of Dmt-Tic di- and tripeptides exhibited high δ opioid binding properties, comparable to the title peptides even though a 5−10-fold increase in K_i^δ was observed among some analogues (**5, 7, 9, 12, 14**) and most were better than TIPP[ψ] (Table 1). Change in chirality from L- to D-Dmt at Cα in the title compounds was detrimental for binding (**1** and **2**) (*infra vide*), while replacement of the N-terminal amine by a methyl group, des-NH_2-α-Me-Dmt^1 (**3**) (Figure 1), reduced δ affinity over 5 orders of magnitude to yield an essentially inactive peptide. *N*-Alkylation of the Tyr-Tic cognates (**4** and **11**) generally elevated δ and μ affinities without affecting selectivity; nevertheless, their overall binding remained substantially less than the comparable Dmt-Tic analogues. Relative to the H-Dmt-Tic-NH_2 title peptide, the *N*-Me-dipeptide (**7**) increased δ affinity and selectivity *ca.* 3- and 10-fold, respectively, such that the receptor properties of analogues **5** and **7** were similar.

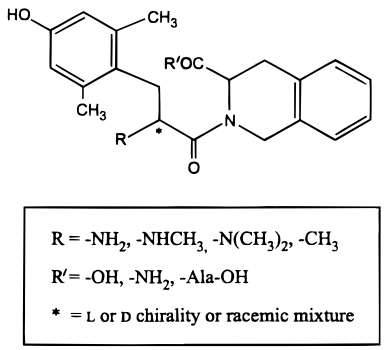

Figure 1. Schematic diagram of R-Dmt-Tic-R′. The prototypic high δ affinity and ultraselective opioid peptide antagonist is when R = NH_2 and R′ = OH.[8]

Binding affinity of the *N*-Me-tripeptide **9** to δ receptors doubled in comparison to the title compound (H-Dmt-Tic-Ala-OH) without altering μ affinity.

N,*N*-Dimethylation differentially affected the δ receptor binding properties of Dmt-containing di- and tripeptides. The δ affinity of peptide **12** moderately declined (5-fold) in comparison to H-Dmt-Tic-OH; however, a δ affinity of *ca*. 0.1 nM and selectivity of 20 000 would certainly qualify a peptide to be considered an exceptional opioid ligand.[10,44] The δ affinity and selectivity of dipeptide **14** was similar to analogue **7**, and both exhibited binding properties 10-fold greater than amidated H-Dmt-Tic-NH_2, while *N*,*N*-dimethylation of the tripeptide **15** enhanced δ affinity 4-fold without affecting δ selectivity.

Affinities of the *N*-alkylated D-amino acid analogues (**6** and **13**) increased in comparison to their nonmethylated counterparts (**1** and **2**); however, the δ affinities of the D-Dmt peptides (**1, 2, 6, 8, 10, 13,** and **16**) declined 10−400-fold relative to the Dmt-Tic title compounds or Tyr-Tic cognates (**4** and **11**) and ablation of δ affinity (5600-fold loss) occurred in the nonalkylated D-isomer-containing dipeptide (**1**). On the other hand, comparison between the change in chirality and *N*-alkylation among pairs of peptides demonstrated that the negative effect of the D-isomer was partially attenuated by *N*-methylation; i.e., δ affinity decreased about an order of magnitude between peptide pair **5** and **6** and pair **7** and **8**, while an even greater loss of 750-fold was noted between **9** and **10**. Interestingly, *N*-methylation permitted retention of high δ binding affinities in the presence of C-terminal amidation (Table 1). Thus, *N*-alkylation of Dmt-Tic peptides yielded several peptide analogues with high δ affinities and selectivities, although somewhat less than the title dipeptide, but greater than TIPP[ψ], which is the biologically stable analogue of the TIP(P) pseudopeptides[10,11] and more active than other Tyr-Tic-containing peptides.[9]

Functional Bioactivity. Bioassay data on MVD and GPI are listed in Table 2. The pA_2 (Figure 2) (and derived K_e values) (Table 2) of the methylated peptides generally exhibited greater potency than the title compounds and TIPP[ψ], except for the D-isomers (**1**, **2**, **6**, and **8**), which ranged from minimal activity to inactivity on mouse vas deferens (MVD). Similarly, analogues **2** and **6** had reduced binding affinities (590- and 145-fold less, respectively, than H-Dmt-Tic-OH) and exhibited 20−25-fold losses in bioactivity. The biological potency of the *N*,*N*-dimethylated di- (**12, 13**) and tripeptides (**14, 15**) on MVD ranged from 2- to 16-fold higher than the title peptides and exhibited a 2−4-fold higher activity than the *N*-methylated peptides (**5**, **9**); compounds **12, 14**, and **15** exceeded that of naltrindole, which produced substantial antagonist activity with electrically contracted guinea pig ileum (GPI) (GPI/MVD = 76) (Table 2). The bioactivity ratio for compounds **12** and **15** were 5640 and 7180, respectively, while that for peptide **14** was somewhat comparable at 4500 due to weak μ antagonist activity on GPI (*supra vide*) (Table 2). Thus, our data revealed that *N*,*N*-dimethylation of H-Dmt-Tic-OH and H-Dmt-Tic-Ala-OH yielded peptide antagonists with considerably higher biological potency than the title compounds,[8] naltrindole and TIPP[ψ].[10,11]

Discussion

The *N*-alkylated derivative of H-Dmt-Tic-OH and H-Dmt-Tic-Ala-OH retained relatively high δ receptor affinity and selectivity, and in particular, *N*,*N*-dimethylation of analogues **12** and **15** augmented δ antagonist bioactivity *ca*. 20-fold. These results enable us to propose the following conclusions: (i) hydrophobic forces represent a critical factor in the discrimination of ligand binding within the δ opioid receptor binding pocket, compatible with the proposed 3-D model of the δ receptor[41−43,45] that was suggested by Balboni *et al*.[24] in the "three-contact point attachment" hypothesis, and (ii) a nitrogen atom is required at the N-terminus. Replacement of the N-terminal amine by a methyl group (**3**, Figure 1) eliminated potential interactions between a protonated nitrogen in the peptide with an anionic site in the receptor in which the nitrogen would coordinate a receptor binding interaction.[45] Although our data would appear to contrast with the maintenance of high μ receptor binding observed with [des-NH_2-Tyr[1]]dermorphin analogues (a μ agonist) or peptides containing a nonprotonated nitrogen,[24,46] it should be noted that those peptides exhibited weak bioactivities[24,47] and further supported the concept that a protonated nitrogen is required for the optimal expression of activity.

Alteration of the chirality at Cα of Dmt diminished both δ receptor affinities (Table 1). This contrasts to the simultaneously decreased δ and enhanced μ affinities upon changing the chirality of Tic in several Tic[2]-containing peptides in which selectivity was inverted ($\delta \rightarrow \mu$) upon C-terminal amidation.[8,10] Reversal of peptide selectivity was similarly observed with analogues of enkephalin and dermorphin containing Tic in the second position.[7] C-Terminal amidation of Dmt-Tic di- and tripeptides further amplified the interaction to μ receptors,[8] as observed with numerous deltorphin tetra-[3] and heptapeptide analogues[4] and the TIP(P) pseudopeptides,[10,11] and supports the concept that a free acid function in opioid peptides discriminates between δ and μ receptor binding sites by an electrostatic repulsion between the δ ligand and μ receptor.[3−6] In terms of the D-enantiomer of Dmt, *N*-methylation partially compensated for its detrimental effect relative to nonmethylated title peptides (Table 1) imposed by the change in chirality, implying that methylation might permit the peptide to conform to the physical constraints of the receptor pocket (*infra vide*).

The *N*-Me-Dmt-Tic-Ala-OH analogue (**9**) exhibited a doubling in δ opioid receptor selectivity relative to the parental compound (Table 1); a similar enhancement of the selectivity of [*N*-alkylated-Tyr[1]]dynorphin A-(1−11) for κ receptors was also observed.[31] In the *N*,*N*-

Table 2. Functional Bioactivity of Dmt-Tic Analogues and Other Compounds[a]

		MVD (nM)		GPI (μM)			
no.	cmpd	pA_2	K_e	pA_2	K_e	IC_{50}	GPI/MVD
	naltrindole	9.2 (8.5−9.7)	0.7	7.3 (6.7−7.7)	0.053	−[b]	76
	TIPP[ψ]	8.1 (8.0−8.2)	6.4	−	−	>10	>1560
	H-Dmt-Tic-OH	8.2 (7.5−8.9)	5.7	−	−	>10	>1750
	H-Dmt-Tic-Ala-OH	8.4 (8.0−8.8)	4.0	−	−	>10	>2500
1	H-D-Dmt-Tic-OH	−	−	−	>10	−	
2	H-D-Dmt-Tic-Ala-OH	6.9 (5.5−8.3)	114	−	−	>10	>88
5	H-*N*-Me-Dmt-Tic-OH	8.5 (8.4−8.6)	2.8	−	−	>10	>3570
6	H-*N*-Me-D-Dmt-Tic-OH	6.8 (6.3−7.6)	141	−	−	>10	>71
7	H-*N*-Me-Dmt-Tic-NH_2	8.6 (7.1−10.2)	2.2	−	−	>10	>4550
8	H-*N*-Me-D-Dmt-Tic-NH_2	−	−	−	>10	−	
9	H-*N*-Me-Dmt-Tic-Ala-OH	8.8 (8.7−8.9)	1.41	−	−	>10	>7100
12	*N*,*N*-$(Me)_2$-Dmt-Tic-OH	9.4 (8.6−10.3)	0.28	5.8 (5.2−6.6)	1.58	−	5640
13	*N*,*N*-$(Me)_2$-D-Dmt-Tic-OH	8.6 (7.7−10.0)	2.38	−	−	>10	>4200
14	*N*,*N*-$(Me)_2$-Dmt-Tic-NH_2	9.9 (8.8−11.0)	0.12	6.3 (5.5−7.0)	0.54	−	4500
15	*N*,*N*-$(Me)_2$-Dmt-Tic-Ala-OH	9.6 (7.9−10.8)	0.22	5.8 (4.9−6.5)	1.58	−	7180

[a] Antagonist activity is defined by pA_2 (with the range in values given in parentheses) and derived from a dose−response curve detailed in the legend to Figure 2. [b] Inactive throughout the table is denoted by a dash (−). The GPI/MVD ratio is based on K_e and IC_{50} values.

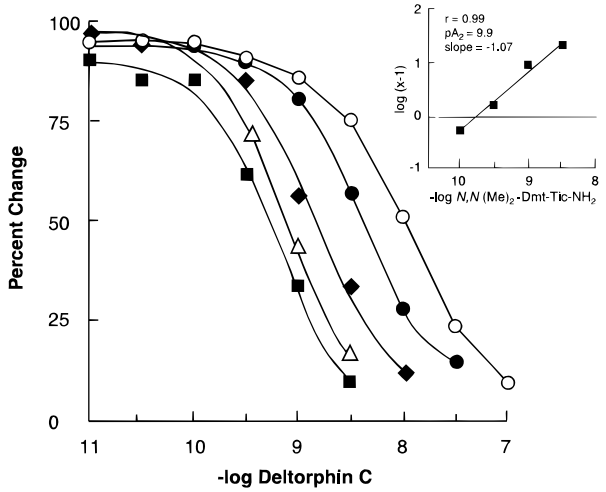

Figure 2. Concentration−response displacement curves of deltorphin C by *N*,*N*-$(Me)_2$-Dmt-Tic-NH_2 (**14**). Each point represents the average of five or six individual experiments and the SEM was less than 10% in each case. Symbols are as follows: control (■); compound **14,** 1×10^{-10} M (△); 3×10^{-10} M (◆); 1×10^{-9} M (●) and 3×10^{-9} M (○). The Schild plot[60] is shown in the insert [r =0.99, pA_2 = 9.9 and slope (■) = −1.07].

methylated peptides (**12** and **15**) bioactivity increased 14−18-fold (Table 2), even though δ receptor selectivity of analogue **12** was less than that of its title compound (Table 1), it nevertheless represented a marked achievement for *N*,*N*-alkylated peptides. One key inference of these results is that the bulky *N*,*N*-methyl groups of the di- (**12**) and tripeptide (**15**) contributes to the physical interaction of the peptide within the binding cleft of δ opioid receptors.[41] Hydrophobic (nonhydrogen bonded) interactions involving *N*-methylation[48] might influence receptivity within the ligand-binding domain through increased van der Waals forces with aliphatic or aromatic residues in the receptor binding site or improve alignment of the phenol side chain to permit efficient H-bonding.[41,42,49] Furthermore, the hydrophobic methyl groups might provide enhanced attachment points with the side chains responsible for the principal $\pi-\pi$ bond interactions between aromatic residues.[42,49]

Another aspect that should be considered, however, is the overall shape of the molecule which would offer the ligand a better opportunity to be inserted within the receptor cavity. This was recently demonstrated by the incorporation into DNA of the nonpolar difluorotoluene nucleoside which has an identical shape to thymidine.[50] A further important consequence of *N*,*N*-alkylation to form a tertiary amine might be the prevention of cyclization to a diketopiperazine, since small peptides containing Tic or other constrained residues cyclize spontaneously under nonphysiological (acidic) conditions.[18−20] In the case of H-Dmt-Tic-OH, the diketopiperazine *cyclo*(Dmt-Tic) exhibited a K_i^{δ} of 9.84 nM, but was nearly inactive on MVD.[24]

Discrepancies between receptor binding and pharmacological bioassays *in vitro* of one or more orders of magnitude were consistently recognized by many investigators.[5,24,29,32,36,44, 47,51,52] In particular, Vaughn *et al*.[51] reported that a cyclic opioid peptide with a modified Phe residue, cyclic[D-Pen^{2,5},*p*Cl-Phe^4]enkephalin, exhibited a disparity between receptor binding and functional bioassays with MVD. If it appears that Phe (or a closely related structural homologue)[37,51] is responsible for eliciting biological activity[12,44] (since substitutions lead to marked deviations between measurements for δ opioid activity[5,9,37,51]), then it may be reasonable to conclude that di- and tripeptides (Table 2) lacking this amino acid would demonstrate even greater variation and subsequently differentiate them from the group of TIP(P) pseudopeptides.[10,11] Furthermore, the advent of cloning the genes for opioid receptors revealed that alternative splicing mechanisms for mRNA[53] or alternate transcription start sites[54] in some peripheral tissues produce receptor variants. The formation of "transcriptionally derived mutant" receptors may also explain the observed discrepancies between the apparent activity in central nervous system membranes versus peripheral receptors in tissue preparations.[5,37,51,52] Moreover, splice variants yielding receptor isoforms, a concept that was also applied to the evolutionary diversity found in naturally occurring peptide variants,[2] may resolve the issue on the existence of pharmacologically defined receptor subtypes.[55] Nonetheless, other factors in addition to receptor sequence and peptide conformation[5,24,37] and receptivity may affect binding: e.g., modification of the bioactive conformation of the receptor by cations in the extracellular milieu, changes associated with intracellular receptor-coupled G-proteins, and the availability of receptors due to differential rates of receptor turnover and internalization.

Conclusions

Our data extend the observations that Dmt-Tic peptides represent a unique class of high-affinity δ opioid

antagonists with extraordinary selectivity.[8] The fact that *N*- and *N,N*-alkylation greatly increased δ antagonist activity implied that bulky methyl groups enabled the peptide to better fit within the ligand-binding domain of the δ receptor and supports site-directed or chimeric mutagenesis studies on opioid receptors. On the other hand, acylation[24] or the N-terminal replacement of an amine with a methyl group drastically reduced binding and bioactivity. Retention of high δ opioid antagonist activity with the tertiary amine (**12** and **15**) indicates that *N,N*-dimethylation of H-Dmt-Tic-OH or H-Dmt-Tic-Ala-OH provides a scaffold upon which to build novel peptides with enhanced antagonist activity and, in contrast to non-peptide and other opioid peptide antagonists, alkylation of the amino function would not require specific allyl or cyclopropylmethyl groups.

Experimental Section

Materials. H-L-Dmt-OH was a generous gift from and synthesized by Dygos *et al.*[13] The racemic mixture of H-L-/D-Dmt-OH was prepared by Commonwealth Biotechnologies (Richmond, VA). Boc-*N*-Me-Tyr-OH, H-Tic-OH, and H-D-Tic-OH were obtained from Bachem Feinchemikalien AG. [^{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).

Peptide Synthesis. All peptides were prepared by standard solution methods.[8] *N*-Monomethylated di- and tripeptides were obtained by condensation of Boc-*N*-Me-L-Dmt(Bzl)-OH or Boc-*N*-Me-Tyr-OH with H-Tic-O*t*Bu or H-Tic-Ala-O*t*Bu via WSC/HOBt. Final products were treated with TFA or hydrogenolysis in the presence of C/Pd (10%) followed TFA treatment. *N*-Dimethylated di- and tripeptides were obtained by exhaustive methylation of the corresponding deprotected linear di- and tripeptides with aqueous formaldehyde and $NaBH_3CN$ in acetonitrile.[56] L-Dmt-containing peptides were prepared from the optically pure L-Dmt amino acid. The D-Dmt peptides were synthesized from the L-/D-Dmt racemic mixture, and the distereoisomers of di- and tripeptides were separated by HPLC (*infra vide*) and identified by comparing the retention time of the enantiomeric L-Dmt-containing peptides. The racemic phenylpropionic acid derivative employed in the synthesis of compound **3** was obtained in accordance with the method of Abrash *et al.*[57] using methylmalonate diethyl ester instead of acetamidomalonate diethyl ester.

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. Preparative reversed-phase HPLC was conducted with a Waters Delta Prep 3000 Å (30 × 3 cm; 15 μm) column. Peptides were eluted with a gradient of 0−60% B in 25 min at a flow rate of 30 mL/min using the following mobile phases: solvent A (10%, v/v, acetonitrile in 0.1% TFA) and solvent B (60%, v/v, acetonitrile in 0.1% TFA). Analytical HPLC analyses were carried out with a Bruker liquid chromatography LC 21-C instrument using a Vydac 218 TP 5415 C18 column (250 × 4.6 mm, 5 μm particle size) and equipped with a Bruker LC 313 W variable-wavelength detector. Recording and quantification were accomplished with a chromatographic data processor coupled to an Epson computer system (QX10). Analytical determination of the capacity factor (K') was determined using HPLC conditions in the above solvent systems programmed at a flow rate of 1 mL/min in a linear gradient from 0 to 100% B in 25 min. All analogues showed less than 1% impurities when monitored at 220 nm. TLC was performed on precoated plates of silica gel F254 (Merck, Darmstadt, Germany) using the following solvent systems: (A) 1-butanol/HOAc/H_2O (3:1:1), (B) $CHCl_3$/benzene/methanol (17:1:2), and (C) EtOAc/hexane (1:1). Ninhydrin (1%, Merck), fluorescamine (Hoffman-La Roche), and chlorine reagent were used as sprays. Open column chromatography (2 × 70 cm, 0.7−1 g material) was run on silica gel 60 (70−230 mesh, Merck) using the same eluent systems. The analytical properties of the peptides are listed in Table 3.

Boc-L-/D-Dmt-OH. To a solution of H-L-/D-Dmt-OH·HCl (0.9 g, 3.66 mmol) in *t*BuOH/H_2O (2:1, 15 mL) were added 1 N NaOH (7.3 mL, 7.3 mmol) and di-*tert*-butyl dicarbonate (0.9 g, 4 mmol) at 0 °C. The reaction mixture was stirred for 3 h at 0 °C and 24 h at room temperature. Then H_2O (20 mL) and solid citric acid (5 g) were added. The product was extracted with AcOEt (100 mL), dried (Na_2SO_4), and evaporated to dryness. The residue was crystallized from petroleum ether: yield 0.85 g (75%); R_f (B) 0.2; HPLC $K' = 7.57$; mp 175−177 °C; MH^+ 310; ^{1}H-NMR (DMSO) $\delta = 1.32$ (s, 9H), 2.17 (s, 6H), 2.85 (dd, 2H), 3.96 (m, 1H), 6.37 (s, 2H), 7.13 (δ, 1H), 8.95 (s, 1H), 12.5 (bs, 1H). Boc-L-Dmt-OH was prepared in the same manner[12a,b] and had an optical rotation, $[\alpha]^{20}_D$, of +20.0.

Z-Tic-OH. To a solution containing H-Tic-OH (5.31 g, 30 mmol) in DMF (20 mL) and 1 N NaOH (30 mL), was added Z-OSu (6.73 g, 27 mmol) at 0 °C. The reaction mixture was stirred for 3 h at 0 °C and 24 h at room temperature. After evaporation of DMF, the residue was washed in AcOEt with a solution of citric acid (10%) and brine. The organic solution was then dried (Na_2SO_4) and evaporated to dryness. The residue was crystallized from Et_2O: yield 6.28 g (74.7%); R_f (A) 0.78; HPLC $K' = 7.85$; mp 144−145 °C; $[\alpha]^{20}_D$ +22.6; MH^+ 312; ^{1}H-NMR (DMSO) $\delta = 3.1$ (m, 2H), 4.51 (m, 3H), 5.18 (s, 2H), 7.19 (m, 5H), 7.37 (m, 4H).

Z-Tic-O*t*Bu. To a solution of Z-Tic-OH (3.49 g, 11.21 mmol) in CH_2Cl_2 (25 mL) at −70 °C was added concentrated H_2SO_4 (0.11 mL), and then isobutylene (11.21 mL) was bubbled into the solution. After 15 days at room temperature, the organic phase was washed with citric acid (10%), $NaHCO_3$ (5%), and brine and then dried and evaporated to dryness: yield 3.25 g (79%); R_f (A) 0.92; HPLC $K' = 10.28$; oil; $[\alpha]^{20}_D$ +5.7; MH^+ 368; ^{1}H-NMR (DMSO) $\delta = 1.16$ (s, 9H), 3.1 (m, 2H), 4.51 (m, 3H), 5.18 (s, 2H), 7.19 (m, 5H), 7.37 (m, 4H).

H-Tic-O*t*Bu. To a solution of Z-Tic-O*t*Bu (3.25 g, 8.85 mmol) in MeOH (30 mL) was added C/Pd (5%, 0.2 g) and H_2 was bubbled through for 2 h at room temperature. After filtration, the solution was evaporated to dryness: yield 1.62 g (78%); R_f (B) 0.69; HPLC $K' = 6.92$; oil; $[\alpha]^{20}_D$ −88.93; MH^+ 233; ^{1}H-NMR ($CDCl_3$) $\delta = 1.49$ (s, 9H), 2.13 (s, 1H), 3.0 (m, 2H), 3.61 (dd, 2H), 4.1 (s, 2H), 7.13 (m, 4H).

Boc-L-/D-Dmt-Tic-O*t*Bu. To a solution of Boc-L-/D-Dmt-OH (0.31 g, 1 mmol) and H-Tic-O*t*Bu (0.23 g, 1 mmol) in DMF (10 mL) at 0 °C were added HOBt (0.18 g, 1.2 mmol), WSC (0.21 g, 1.2 mmol), and TEA (0.17 mL, 1.2 mmol). The reaction mixture was stirred for 3 h at 0 °C and 24 h at room temperature. After evaporation of DMF, the residue was solubilized in EtOAc and washed with citric acid (10%), $NaHCO_3$ (5%), and brine. The organic phase was dried and evaporated to dryness. The residue was crystallized from Et_2O/petroleum ether (1:1): yield 0.41 g (78%); R_f (B) 0.78; HPLC $K' = 10.87$; mp 103−105 °C; $[\alpha]^{20}_D$ −15.3; MH^+ 525; ^{1}H-NMR (DMSO/D_2O, 9:1, v/v) $\delta = 1.18$ (s, 9H), 1.32 (s, 9H), 2.17 (s, 6H), 2.23 (m, 1H), 2.85−3.1 (m, 3H), 3.96−4.15 (m, 2H), 4.52 (m, 1H), 4.97 (m, 1H), 6.35 (s, 2H), 7.35 (m, 4H).

TFA·H-L-/D-Dmt-Tic-OH. Boc-L-/D-Dmt-Tic-O*t*Bu (0.38 g, 0.72 mmol) was treated with TFA (2 mL) for 0.5 h at room temperature. Et_2O was added to the solution until the product precipitated: yield 0.18 g (79%); R_f (A) 0.67; 0.69; HPLC $K' =$ 3.76 and 4.06; mp 135−137 °C; $[\alpha]^{20}_D$ +23.7; MH^+ 369. Diastereoisomers were separated by preparative HPLC. The identity of TFA·H-Dmt-Tic-OH was confirmed by independent synthesis starting from H-L-Dmt-OH. TFA·H-Dmt-Tic-OH: yield 0.09 g (79%); R_f (A) 0.69; HPLC $K' = 4.06$; mp 136−138 °C; $[\alpha]^{20}_D$ +36.9; MH^+ 369.

TFA·H-D-Dmt-Tic-OH (1): Yield 0.08 g (76%); R_f (A) 0.67; HPLC $K' = 3.76$; mp 139−141 °C; $[\alpha]^{20}_D$ +12.1; MH^+ 369.

TFA·H-D-Dmt-Tic-Ala-OH (2) was obtained by condensation of Boc-L-/D-Dmt-OH with H-Tic-Ala-O*t*Bu as reported for the synthesis of **1**. The diastereoisomeric mixture was separated by preparative HPLC: yield (85%); R_f (A) 0.72; HPLC $K' = 4.11$; mp 157−159 °C; $[\alpha]^{20}_D$ −3.2; MH^+ 440.

2-(4-Hydroxy-2,6-dimethylbenzyl)-2-methylmalonic Acid Diethyl Ester. A solution of sodium ethoxide was prepared by adding sodium (1.4 g, 61 mmol) to absolute

ethanol (200 mL). To this solution were added 2-methylmalonic acid diethyl ester (10.5 g, 60 mmol) and, after 10 min, *O*-carbethoxy-3,5-dimethyl-4-(chloromethyl)phenol[56] (14.5 g, 60 mmol). The mixture was refluxed for 2 h, cooled, and filtered. The filtrate was evaporated *in vacuo* to a clear colorless oil which was purified by silica gel chromatography (mobile phase, diethyl ether/petroleum ether, 1:1): yield 8.3 g (45%); R_f (Et_2O/petroleum ether, 1:1) 0.48; HPLC $K' = 10.33$; oil; MH^+ 309; ^{1}H-NMR ($CDCl_3$) $\delta = 1.18-1.29$ (m, 9H), 2.19 (s, 6H), 3.44 (s, 2H), 4.07−4.23 (m, 4H), 5.35 (bs, 1H), 6.47 (s, 2H).

2-(4-Hydroxy-2,6-dimethylbenzyl)-2-methylmalonic Acid. The above compound (0.9 g, 3 mmol) suspended in 4 N NaOH (10 mL) was refluxed for 3 h. The solution was acidified with 1 N HCl and extracted with ethyl acetate, and the extract was dried and evaporated *in vacuo*. Title compound was crystallized from Et_2O/petroleum ether (1:1): yield 0.72 g (95%); R_f (B) 0.3; HPLC $K' = 4.27$; mp 160−165 °C; MH^+ 253; ^{1}H-NMR ($CDCl_3$, DMSO) $\delta = 1.13$ (s, 3H), 2.2 (s, 6H), 3.39 (s, 2H), 6.45 (s, 2H), 8.57 (s, 1H), 10.5−16 (bs, 2H).

3-(4-Hydroxy-2,6-dimethylphenyl)-2-methylpropionic Acid [Des-NH$_2$-α-Me-Dmt-OH]. The malonic acid derivative (0.4 g, 2 mmol) was suspended in xylene (20 mL) and refluxed for 3 h. After evaporation the crude product was crystallized with petroleum ether: yield 0.38 g (92%); R_f (B) 0.6; HPLC $K' = 4.83$; mp 138−141 °C; MH^+ 209; ^{1}H-NMR ($CDCl_3$) 1.11 (d, 3H), 2.28 (s, 6H), 2.65−2.74 (m, 2H), 2.96−3.05 (m, 1H), 6.51 (s, 2H).

[Des-NH$_2$-α-Me-Dmt]-Tic-O*t*Bu. To a solution of des-NH$_2$-α-Me-Dmt-OH (0.1 g, 0.5 mmol) and H-Tic-O*t*Bu (0.14 g, 0.6 mmol) in DMF (10 mL) at 0 °C, were added HOBt (0.09 g, 0.6 mmol), WSC (0.11 g, 0.6 mmol) and TEA (0.08 mL, 0.6 mmol). The reaction mixture was stirred for 3 h at 0 °C and 24 h at room temperature. After evaporation of DMF, the residue was solubilized in ethyl acetate and washed with citric acid (10%), $NaHCO_3$ (5%) and saturated water. The organic phase was dried and evaporated to dryness. The residue was crystallized from petroleum ether: yield 0.14 g (73%); R_f (B) 0.83; HPLC $K' = 9.87$ and 9.96; mp 98−100 °C; $[\alpha]^{20}_D$ +5.3; MH^+ 424.

[Des-NH$_2$-α-Me-Dmt]-Tic-OH (3). [Des-NH$_2$-α-Me-Dmt]-Tic-O*t*Bu (0.1 g, 0.23 mmol) was treated with TFA (2 mL) for 0.5 h at room temperature. Diethyl ether was added to the solution until precipitation of the product occurred: yield 0.07 g (82%); R_f (A) 0.83; HPLC $K' = 3.78$; mp 127−129 °C; $[\alpha]^{20}_D$ +7.2; MH^+ 368.

TFA·H-*N*-Me-Tyr-Tic-OH (4). Title compound was obtained by condensation of Boc-*N*-Me-Tyr-OH with H-Tic-O*t*Bu as in the synthesis of **1**: yield 0.185 g (87%); R_f (A) 0.54; HPLC $K' = 3.78$; mp 144−146 °C; $[\alpha]^{20}_D$ +13.8; MH^+ 355.

Boc-*N*-Me-Dmt(Bzl)-Tic-O*t*Bu was prepared by condensation of Boc-*N*-Me-L-Dmt(Bzl)-OH, obtained in the same manner reported[14] using L-Dmt and H-Tic-O*t*Bu via WSC/HOBt: yield 0.14 g (78%); R_f (B) 0.93; HPLC $K' = 4.08$; oil; [αl^{20}D +11.2; MH^+ 629.

TFA·H-*N*-Me-Dmt-Tic-OH (5). Boc-*N*-Me-Dmt(Bzl)-Tic-O*t*Bu was deprotected at the benzyl group of Dmt by hydrogenation in presence of C/Pd (10%) and the product obtained was treated with TFA: yield 0.06 g (78%); R_f (A) 0.65; HPLC $K' = 4.08$; mp 157−159 °C; $[\alpha]^{20}_D$ +18.1; MH^+ 383.

TFA·H-*N*-Me-D-Dmt-Tic-OH (6) was prepared as **5** was using a racemic mixture of Boc-*N*-Me-Dmt(Bzl)-OH instead of the single enantiomer: yield 0.063 g (78%); R_f (A) 0.63; HPLC $K' = 4.83$; mp 160−162 °C; $[\alpha]^{20}_D$ +3.8; MH^+ 383.

TFA·H-*N*-Me-Dmt-Tic-NH$_2$ (7) was obtained as **5** was using H-Tic-NH$_2$ instead of H-Tic-O*t*Bu: yield 0.103 g (85%); R_f (A) 0.73; HPLC $K' = 4.68$; mp 145−147 °C; $[\alpha]^{20}_D$ +23.4; MH^+ 382.

TFA·H-*N*-Me-D-Dmt-Tic-NH$_2$ (8) was obtained by separation of the diastereomeric mixture derived by the condensation of Boc-*N*-Me-L-/D-Dmt(Bzl)-OH with H-Tic-NH$_2$ as reported for **6**: yield 0.091 g (83%); R_f (A) 0.71; HPLC $K' = 5.07$; mp 148−150 °C; $[\alpha]^{20}_D$ +19.2; MH^+ 382.

Boc-*N*-Me-Dmt(Bzl)-Tic-Ala-O*t*Bu was obtained as Boc-*N*-Me-Dmt(Bzl)-Tic-O*t*Bu starting from Boc-*N*-Me-Dmt(Bzl)-OH and H-Tic-Ala-O*t*Bu (obtained from Z-Tic-OH and H-Ala-O*t*Bu): yield 0.12 g (81%); R_f (B) 0.83; HPLC $K' = 11.93$; mp 91−93 °C; $[\alpha]^{20}_D$ +9.1; MH^+ 700.

TFA·H-*N*-Me-Dmt-Tic-Ala-OH (9) was obtained as **5** from deprotection of the preceding compound: yield 0.06 g (78%); R_f (A) 0.69; HPLC $K' = 4.15$; mp 147−149 °C; $[\alpha]^{20}_D$ +20.3; MH^+ 454.

TFA·H-*N*-Me-D-Dmt-Tic-Ala-OH (10) was obtained as **6**: yield 0.05 g (75%); R_f (A) 0.67; HPLC $K' = 4.31$; mp 153−155 °C; $[\alpha]^{20}_D$ +28.7; MH^+ 454.

TFA·*N*,*N*-(Me)$_2$-Dmt-Tic-OH (12). To a stirred solution of TFA·H-Dmt-Tic-OH (0.05 g, 0.11 mmol) in 37% aqueous formaldehyde (0.07 mL, 0.83 mmol) in acetonitrile (10 mL) was added sodium cyanoborohydride (0.016 g, 0.25 mmol). Glacial acetic acid (0.02 mL) was added over 10 min and the reaction was stirred at room temperature for 2 h. The reaction mixture was poured into 100 mL of ethyl acetate and then washed with brine. The organic solution was washed with three 20 mL portions of 1 N KOH and one 20 mL portion of brine. The ether solution was dried (Na_2SO_4) and evaporated *in vacuo* to give the crude product that was purified by preparative HPLC: yield 0.09 g (90%); R_f (A) 0.71; HPLC $K' = 4.72$; mp 145−147 °C; $[\alpha]^{20}_D$ +31.7; MH^+ 397.

TFA·*N*,*N*-(Me)$_2$-Tyr-Tic-OH (11) was prepared as **12** by exhaustive methylation of TFA·H-Tyr-Tic-OH (obtained from the condensation of Boc-Tyr-OH and H-Tic-O*t*Bu via WSC/HOBt): yield 0.09 g (90%); R_f (A) 0.63; HPLC $K' = 3.47$; mp 138−140 °C; [α]^{20}D +26.8; MH^+ 369.

TFA·*N*,*N*-(Me)$_2$-D-Dmt-Tic-OH (13) was obtained as **12** after the separation of the diastereoisomeric mixture: yield 0.05 g (88%); R_f (A) 0.73; HPLC $K' = 4.56$; mp 151−153 °C; $[\alpha]^{20}_D$ +8.3; MH^+ 397.

TFA·*N*,*N*-(Me)$_2$-Dmt-Tic-NH$_2$ (14) was prepared as **12** by methylation of TFA·H-Dmt-Tic-NH$_2$ (obtained from Boc-Dmt-OH and H-Tic-NH$_2$): yield 0.07 g (82%); R_f (A) 0.78; HPLC $K' = 4.83$; mp 138−140 °C; $[\alpha]^{20}_D$ +28.4; MH^+ 396.

TFA·*N*,*N*-(Me)$_2$-Dmt-Tic-Ala-OH (15) was obtained by treating TFA·H-Dmt-Tic-Ala-OH (derived from the condensation of Boc-Dmt-OH with H-Tic-Ala-O*t*Bu) under the same conditions as **12**: yield (87%); R_f (A) 0.74; HPLC $K' = 4.91$; mp 153−155 °C; $[\alpha]^{20}_D$ +17.8; MH^+ 468.

TFA·*N*,*N*-(Me)$_2$-D-Dmt-Tic-Ala-OH (16) was prepared by the separation of the corresponding diastereoisomeric mixture as reported for **13**: yield (83%); R_f (A) 0.72; HPLC $K' = 5.23$; mp 147−149 °C; $[\alpha]^{20}_D$ +28.2; MH^+ 468.

Radioligand Binding. Synaptosomal membranes were prepared from whole brains (minus cerebellum) taken from Sprague−Dawley rats and preincubated to remove endogenous opioids.[4] The radioligand displacement for δ and μ receptors used [^{3}H]DPDPE and [^{3}H]DAGO, respectively, under equilibrium conditions and conducted in duplicate with three or more different membrane preparations using 2 μM unlabeled ligand to suppress nonspecific binding as described previously.[4,7,9,37] Peptides varied over 3−4 orders of magnitude in concentration. The data are presented as the mean ± SEM and the number of repetitions of independent experiments with different synaptosomal preparations (n values) are listed in parentheses in Table 1. Affinity constants (K_i) were determined according to Cheng and Prusoff.[58]

Functional Pharmacological Bioassays. Electrically induced smooth muscle contraction *in vitro* used a single MVD and the GPI longitudinal muscle-enteric plexus suspended in Krebs' solution according to the method of Kosterlitz and Watt.[59] The μ agonist activity was compared to dermorphin [$IC_{50} = 1.82$ nM (range 1.43−2.31 nM)]. The μ antagonism was derived by the inhibition of dermorphin action and δ antagonist activity determined through the inhibition of deltorphin C [δ_1 receptor agonist, $IC_{50} = 0.54$ nM (range 0.43−0.68 nM)] and compared to the nonpeptide opiate antagonist naltrindole.[7] All values for MVD and GPI were obtained from at least four independent tissue samples based on dose−response curves. In each tissue (MVD and GPI) concentration−response curves of the agonist were performed in the absence (control) and in the presence of increasing concentrations of the antagonist, and the pA_2 values were calculated according to the method of Arunlakshana and Schild[60] (Figure 2). Data were statistically analyzed using a software package.[61] The antagonists examined responded to the following criteria: (i) In presence of the antagonist, the log agonist concentration−effect curve shifted to the right in a parallel fashion. (ii) The relationship

between the extent of the shift and the concentration of the antagonist followed the Schild equation,[61] and the subsequent plots were linear with a slope not significantly different from 1.0.

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References

(1) Symbols and abbreviations used in this paper follows the IUPAC−IUB Commission on Biochemical Nomenclature (*J. Biol. Chem*. **1985**, *260*, 1442). Boc, *tert*-butyloxycarbonyl; DAGO, [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]enkephalin; deltorphin C, [D-Ala^2]-deltorphin I (H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2); DMSO, dimethyl sulfoxide; Dmt, 2′,6′-dimethyl-L-tyrosine; DPDPE, [D-Pen^{2,5}]enkephalin; Et_2O, diethyl ether; EtOAc, ethyl acetate; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; K_e, the antilog of pA_2 in molar concentration; Me, methyl group; MeOH, methanol; MVD, mouse vas deferens; pA_2, the negative log of the molar concentration necessary to double the concentration of agonist needed to elicit the original response; *t*Bu, *tert*-butyl; TEA, triethylamine; TFA, trifluoroacetic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TLC, thin-layer chromatography; WSC, 1-ethyl-3-(3′-(dimethyl)aminopropyl)carbodiimide hydrochloride; Z, benzyloxycarbonyl.

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