Further Studies on the Dmt-Tic Pharmacophore: Hydrophobic Substituents at the C-Terminus Endow δ Antagonists To Manifest μ Agonism or μ Antagonism

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Twenty N- and/or C-modified Dmt-Tic analogues yielded similar K_i values with either [^{3}H]-DPDPE (δ_1 agonist) or [^{3}H]*N*,*N*(Me)$_2$-Dmt-Tic-OH (δ antagonist). N-Methylation enhanced δ antagonism while *N*-piperidine-1-yl, *N*-pyrrolidine-1-yl, and *N*-pyrrole-1-yl were detrimental. Dmt-Tic-X (X = −NHNH$_2$, −NHCH$_3$, −NH-1-adamantyl, −NH-tBu, −NH-5-tetrazolyl) had high δ affinities (K_i = 0.16 to 1 nM) with variable μ affinities to yield nonselective or weakly μ-selective analogues. *N*,*N*-(Me)$_2$Dmt-Tic-NH-1-adamantane exhibited dual δ and μ receptor affinities ($K_i\delta$ = 0.16 nM and $K_i\mu$ = 1.12 nM) and potent δ antagonism (pA$_2$ = 9.06) with μ agonism (IC$_{50}$ = 16 nM). H-Dmt-βHTic-OH (methylene bridge between C$_\alpha$ of Tic and carboxylate function) yielded a biostable peptide with high δ affinity (K_i = 0.85 nM) and δ antagonism (pA$_2$ = 8.85) without μ bioactivity. Dmt-Tic-Ala-X (X = −NHCH$_3$, −OCH$_3$, −NH-1-adamantyl, −NHtBu) exhibited high δ affinities (K_i = 0.06 to 0.2 nM) and elevated μ affinities (K_i = 2.5 to 11 nM), but only H-Dmt-Tic-Ala-NH-1-adamantane and H-Dmt-Tic-Ala-NHtBu yielded δ receptor antagonism (pA$_2$ = 9.29 and 9.16, respectively). Thus, Dmt-Tic with hydrophobic C-terminal substituents enhanced μ affinity to provide δ antagonists with dual receptor affinities and bifunctional activity.

Introduction[1]

The development of δ-opioid antagonists were an outgrowth of studies on truncated opioid peptides with enhanced hydrophobic properties: H-Tyr-Tic-Phe (Phe) [TIP(P)] and its reduced bond analogues, TIP(P)[ψ], exhibited greater biological potency than non-peptide opiate antagonists.[2] Removal of the Phe residues in those peptides engendered H-Tyr-Tic-OH and H-Tyr-Tic-Ala-OH, which were the first opioid peptides without Phe that had (albeit weak) δ-opioid selectivity and antagonist bioactivity.[3] In addition to the aromatic side chain at the third position in Tyr-Tic-Phe peptides,[2] compounds containing a residue with an aliphatic side chain in Tyr-Tic-Xaa analogues also augmented δ receptor binding.[3] Studies with 2′,6′-dimethyl-L-tyrosyl-*N*-(3-phenylpropyl)-D-alanine amide[4] demonstrated the feasibility of increasing the hydrophobicity by methylation of the phenolic side chain of Tyr while maintaining biological activity of the peptide. In fact, substitution of Tyr in H-Tyr-Tic-OH by Dmt created the dipeptide antagonist H-Dmt-Tic-OH which had exceptionally high δ affinity ($K_i\delta$ = 0.02 nM), ultraselectivity ($K_i\mu/K_i\delta$ =150 000), in vitro δ antagonist activity (K_e = 5.7 nM),[5] and acted systemically in vivo to reverse antinociception by a δ agonist.[6] Further enhancement of the hydrophobic properties of H-Dmt-Tic-OH through N-alkylation by methyl groups not only retained high δ affinity and δ receptor selectivity but also substantially enhanced bioactivity (K_e = 0.12 nM);[7,8] other N-alkylating agents affected $K_i\delta$ and decreased δ selectivity.[9] In toto, those observations demonstrated that the hydrophobicity imparted by 2′,6′-dimethylation of Tyr and N-alkylation was a substantial 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),[2] H-Tyr-Tic-OH,[3] and H-Dmt-Tic-OH[5,7] explored 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 (δ-opioid agonists).[10,11] Moreover, C-terminal amidation with a change of configuration to D-Tic produced weakly μ-selective compounds,[2,5] suggesting that discriminative modifications at the C-terminus might result in opioids with new properties.

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.[12] However, it should be noted that δ receptor agonists act as nonaddicting analgesic drugs,[13] which produce an antinociceptive response without the appearance of cross-tolerance to μ- or δ-opioid receptor agonists[14] and can lead to antinociception in the absence of the μ receptor.[15] The unique property of the δ receptor permitted the application of moderately δ-selective

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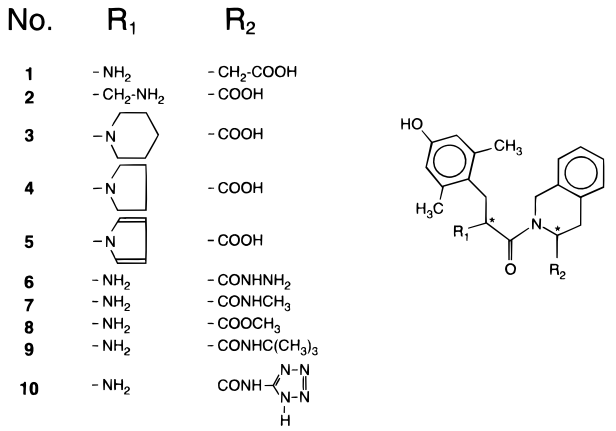

Figure 1. Dmt-Tic pharmacophore and the R_1 and R_2 substituents. The bold numbers refer to the analogues in Table 1. Asterisks denote chiral centers.

alkaloid antagonists in clinical trials; for example, the amelioration of the effects of alcoholism,[16] autism,[17] and Tourette's syndrome.[18] Using animal models, the δ-opiate antagonist naltrindole[19] inhibited the reinforcing properties of cocaine,[20] moderated the behavioral effects of amphetamines,[21] and brought about immunosuppression[22] suitable for organ transplantation.[23] These transplantation effects were also seen using another nonpeptide compound, 7-(benzylspiroindanyl)naltrexone.[24]

Rationale

The intractable membrane barriers, such as the blood-brain barrier, must be circumvented in order for peptide antagonists to express activity in vivo.[25] The requisite physicochemical properties of compounds capable of passing through this barrier include low molecular weight (<800 Da) and high octanol−water coefficient characteristics. Although systemically injected H-Dmt-Tic-OH was able to antagonize the analgesia of an icv administered δ_2 agonist (deltorphin B),[6] the augmentation of the hydrophobic properties of [D-Ala^2,Leu^5]-enkephalin with 1-adamantyl amide also permitted passage through the blood-brain barrier.[26] Thus, we sought to enhance the hydrophobic properties of H-Dmt-Tic-OH by altering the C-terminal function either with or without substitution at the N-terminus (Figure 1) in order to produce more potent δ-opioid antagonists.[27]

Results

Receptor Binding. Properties of N- and C-Terminally Modified Analogues Using a δ_1 Receptor Agonist. Elimination of the carboxylate function of H-Dmt-Tic-OH substantially increased the μ affinity of di- and tripeptide derivatives (**6**−**20**) (Figure 1 and Table 1). The net effect was the loss of δ-opioid selectivity and the appearance of compounds that were either essentially nonselective (**6**, **9**, **10**, and **15**), weakly μ-selective (**8**, **11**, and **16**), or moderately μ-selective (**12**). The δ affinity generally remained high for most C-terminally modified analogues, with K_i values ranging from 0.07 to 1 nM (Table 1). Nonetheless, the binding data revealed that several analogues lost high δ affinity, in particular the methyl ester derivative (**8**) and those containing the D-Tic enantiomer (**12** and **16**) as seen previously with amidated analogues of TIP(P)[2] or Dmt-Tic.[5,7]

Interposing a methylene spacer (**1**) between the C_α of the Tic residue and the carboxylate function (Figure 1) to prevent diketopiperazine formation had minimal effect on δ affinity, but it enhanced μ affinity (Table 1). The same chemical approach employing a methylene group between the amino group and C_α of Dmt (**2**) was more detrimental [compared to H-α(*R*,*S*)HDmt-Tic-OH]. The dipeptide analogues containing hydrazide (**6**), methyl amide (**7**), and tetrazole-5-yl (**10**) exhibited high δ affinities, but each with a marked gain in μ affinity, which was also observed with the Ala-containing tripeptide methyl ester (**18**) relative to its title compound (H-Dmt-Tic-Ala-NH_2).

The largest increase in μ affinity occurred in the Dmt-Tic analogues C-terminally substituted with either *tert*-butyl amide (**9** and **14**) or 1-adamantyl amide (**11**, **12**, **15**, **16**, and **20**). In comparison to H-Dmt-Tic-OH, the μ affinities of compounds **11** and **12** increased 4350- and 12 600-fold, respectively, while the μ affinity of peptide **15** rose nearly 2200-fold relative to *N*,*N*(Me)$_2$-Dmt-Tic-OH. Comparisons between the amidated parental peptides to their C-terminal derivatives, however, indicated smaller changes in μ affinities (Table 1).

N-Alkylation of Dmt-Tic with piperidine-1-yl (**3**), pyrrolidine-1-yl (**4**), or pyrrole-1-yl groups (**5**) (Figure 1) decreased δ affinity, particularly the latter compound whose receptor binding was comparable to H-αDmt-Tic-OH (**2**), H-Dmt-Tic-OMe (**8**), and H-Dmt-D-Tic-NH-1-adamantane (**12**). Despite the bulky N-terminal substituents, the δ selectivities of **3** and **4** were analogous to other modified peptides (Table 1).

Receptor Binding. Competition against a Highly Selective δ-Opioid Antagonist. The receptor binding using the δ antagonist [^{3}H]*N*,*N*-(Me)$_2$-Dmt-Tic-OH yielded similar K_i values to those obtained with the agonist [^{3}H]DPDPE in over 80% of the peptides listed in Table 1. Exceptions (peptides whose K_i values differed by at least an order of magnitude) were the title peptide H-Dmt-Tic-OH, which is relatively unstable and forms a diketopiperazine,[29,30] and analogues **2**, **6**, **8**, and **10**.

Functional Bioactivity. Dmt-Tic analogues **1**, **9**, **13**, **15**, **19**, and **20** demonstrated the highest δ antagonist functional bioactivities (Table 2) as well as some of the highest δ receptor affinities (Table 1). Of greater interest, however, was the observation that several analogues acquired unusual bioactivity profiles. For example, both compounds **13** and **15** elicited excellent δ antagonism, yet the former was a weak μ antagonist and the latter clearly displayed μ agonism (Table 2). Inclusion of the D-Tic enantiomer (**12** and **16**) greatly reduced bioactivity on MVD with micromolar activity on GPI. Replacement of the N-terminal amine through alkylation by piperidine-1-yl (**3**), pyrrolidine-1-yl (**4**), or pyrrole-1-yl (**5**) was detrimental for all bioactivity measurements on MVD (Table 2). N-Alkylation by methyl groups to form secondary or tertiary amines was the only N-terminal substitution tolerated[9] (Table 2). Interestingly, the nonalkylated, C-terminally modified dipeptides **11** and **12** lacked δ antagonism; however, **11** surprisingly manifested a weak δ agonism and moderate μ agonism despite its high δ affinity (Table 1) while

Table 1. Membrane Receptor Binding of the Modified Dmt-Tic Pharmacophore

no.	peptide	$K_i\delta$ (nM) [^{3}H]DPDPE[a]	$K_i\delta$ (nM) [^{3}H]*N*,*N*(CH$_3$)$_2$-Dmt-Tic-OH[a]	[^{3}H]DAGO $K_i\mu$ (nM)[a]	agonist $K_i\mu/K_i\delta$	antagonist $K_i\mu/K_i\delta$
		Parental Compounds				
	H-Dmt-Tic-OH	0.022 ± 0.002 (6)	0.34 ± 0.05 (4)	3320 ± 440 (7)	151 000[b]	9 850
	H-Dmt-Tic-NH$_2$	1.22 ± 0.09 (6)	2.13 ± 0.12 (3)	277 ± 26 (3)	227[b]	130
	H-Dmt-Tic-Ala-OH	0.29 ± 0.03 (6)	0.29 ± 0.05 (3)	813 ± (4)	2 800[b]	2 800
	H-Dmt-Tic-Ala-NH$_2$	0.24 ± 0.02 (5)	0.69 ± 0.13 (3)	47 ± 3.4 (4)	195[b]	68
	N,*N*(Me)$_2$-Dmt-Tic-OH	0.12 ± 0.02 (3)	0.07 ± 0.01 (4)	2440 ± 460 (3)	20 300[c]	34 800
	H-(*R*,*S*)Dmt-Tic-OH	0.46 ± 0.001 (3)	0.80 ± 0.19 (4)	1160 ± 330 (3)	2 520	1 450
		Dipeptide Derivatives				
1	H-Dmt-βHTic-OH	0.85 ± 0.20 (5)	0.71 ± 0.04 (3)	418 ± 86 (3)	498	590
2	H-α(*R*,*S*)HDmt-Tic-OH	11.2 ± 3.5 (3)	475 ± 37 (4)	1740 ± 16 (3)	155	4
3	[des-NH$_2$-α-piperidine-1-yl]-Dmt-Tic-OH	1.18 ± 0.10 (3)	0.74 ± 0.30 (3)	2040 ± 260 (3)	1 730	2 760
4	[des-NH$_2$-α-pyrrolidine-1-yl]-Dmt-Tic-OH	1.62 ± 0.19 (3)	1.42 ± 0.14 (3)	814 ± 65 (3)	502	573
5	[des-NH$_2$-α-pyrrole-1-yl]- Dmt-Tic-OH	16.6 ± 2.5 (5)	9.94 ± 2.5 (3)	5590 ± 410 (3)	338	562
6	H-Dmt-Tic-NHNH$_2$	0.99 ± 0.04 (3)	42.0 ± 7.9 (5)	85.1 ± 7.3 (3)	86	2
7	H-Dmt-Tic-NHMe	0.47 ± 0.09 (3)	1.24 ± 0.15 (4)	85.5 ± 7.7 (3)	182	69
8	H-Dmt-Tic-OMe	9.64 ± 2.2 (3)	500 ± 90 (5)	423 ± 25 (3)	44	0.8
9	H-Dmt-Tic-NH-tBu	0.43 ± 0.07 (5)	0.93 ± 0.15 (4)	5.96 ± 0.82 (4)	14	6
10	H-Dmt-Tic-NH-tetrazole-5-yl	0.70 ± 0.03 (3)	9.75 ± 1.73 (5)	37.0 ± 4.5 (3)	53	4
11	H-Dmt-Tic-NH-1-adamantane	0.26 ± 0.05 (4)	1.01 ± 0.26 (3)	0.76 ± 0.05 (4)	3	0.8
12	H-Dmt-D-Tic-NH-1-adamantane	24.5 ± 4.9 (6)	70.3 ± 7.25 (3)	0.26 ± 0.08 (4)	0.01	0.004
13	*N*,*N*(Me)$_2$-Dmt-Tic-NHMe	0.54 ± 0.07 (3)	0.28 ± 0.02 (3)	359 ± 62 (3)	669	1270
14	*N*,*N*(Me)$_2$-Dmt-Tic-NH-tBu	0.61 ± 0.02 (3)	0.11 ± 0.04 (3)	226 ± 21 (3)	369	2130
15	*N*,*N*(Me)$_2$-Dmt-Tic-NH-1-adamantane	0.16 ± 0.02 (3)	0.12 ± 0.02 (3)	1.12 ± 0.10 (3)	7	9
16	*N*,*N*(Me)$_2$-Dmt-D-Tic-NH-1-adamantane	140 ± 30 (6)	120 ± 26 (3)	50.5 ± 2.6 (3)	0.36	0.42
		Tripeptide Derivatives				
17	H-Dmt-Tic-Ala-NHMe	0.058 ± 0.01 (3)	0.12 ± 0.04 (3)	5.75 ± 0.72 (3)	100	47
18	H-Dmt-Tic-Ala-OMe	0.23 ± 0.09 (3)	0.14 ± 0.04 (3)	11.3 ± 1.87 (3)	48	83
19	H-Dmt-Tic-Ala-NH-tBu	0.066 ± 0.01 (3)	0.08 ± 0.02 (3)	4.03 ± 0.21 (3)	61	48
20	H-Dmt-Tic-Ala-NH-1-adamantane	0.073 ± 0.02 (3)	0.04 ± 0.01 (3)	2.52 ± 0.56 (4)	35	72

[a] The numeric value in the parentheses indicates the number (*n*) of repetitions of independent binding assays using different synaptosomal preparations. [b] Salvadori et al. (1995) (ref 5). [c] Salvadori et al. (1997) (ref 7).

Table 2. Functional Bioactivity of the Modified Dmt-Tic Pharmacophore

no.	peptide	MVD pA$_2$ (range)[a]	MVD K_e (nM)	MVD ED$_{50}$ (μM)	GPI pA$_2$ (range)	GPI K_e (μM)	GPI ED$_{50}$ (μM)
	Dmt-Tic-OH	8.2	5.7	-	-	-	>10[b]
	Dmt-Tic-NH$_2$	7.2	42	-	-	-	>10[b]
	Dmt-Tic-Ala-OH	8.4	4.0	-	-	-	>10[b]
	Dmt-Tic-Ala-NH$_2$	8.0	9.0	-	-	-	4.74 ± 0.9
	N,*N*(Me)$_2$-Dmt-Tic-OH	9.4	0.28	-	-	-	>10[c]
	H-(*R*,*S*)Dmt-Tic-OH	8.17 (7.8−8.6)	6.76	-	-	-	>10
1	H-Dmt-βHTic-OH	8.8 (8.6−9.1)	1.41	-	-	-	>10
3	[des-NH$_2$-α-piperidine-1-yl]-Dmt-Tic-OH	7.31 (7.15−7.47)	49	-	-	-	>10
4	[des-NH$_2$-α-pyrrolidine-1-yl]-Dmt-Tic-OH	6.9 (6.9−7.1)	121	-	-	-	>10
5	[des-NH$_2$-α-pyrrole-1-yl]-Dmt-Tic-OH	6.39 (6.0−6.7)	408	-	-	-	>10
7	H-Dmt-Tic-NHMe	-[e]	>10 000	21.2 (9.0−45)	5.94 (4.2−7.6)	1.15	>10
9	H-Dmt-Tic-NH-tBu	8.24 (8.2−8.5)	1.74	-	-	-	1.04 (0.69−1.5)
10	H-Dmt-Tic-NH-tetrazole-5-yl	7.44 (7.1−7.7)	36.3	-	-	-	8.2 (2.7−24.9)
11	H-Dmt-Tic-NH-1-adamantane	-	>10 000	0.87 (0.8−1.2)	-	-	0.036 (0.019−0.068)
12	H-Dmt-D-Tic-NH-1-adamantane	-	>10 000	-	-	-	1.68 (1.23−2.3)
13	*N*,*N*(Me)$_2$-Dmt-Tic-NHMe	9.39 (8.8−9.9)	0.41	-	6.41 (6.3−6.5)	0.39	>10
14	*N*,*N*(Me)$_2$-Dmt-Tic-NH-tBu	7.85 (7.5−8.1)	14.1	-	6.52 (6.2−6.7)	0.30	>10
15	*N*,*N*(Me)$_2$-Dmt-Tic-NH-1-adamantane	9.06 (8.6−9.5)	0.87	-	-	-	0.016 (0.011−0.023)
16	*N*,*N*(Me)$_2$-Dmt-D-Tic-NH-1-adamantane	6.91 (6.8−7.0)	128	-	-	-	4.48 (3.17−6.32)
17	H-Dmt-Tic-Ala-NHMe	-	-	1.29 (1.18−1.42)	-	-	0.29 (0.18−0.45)
19	H-Dmt-Tic-Ala-NH-tBu	9.16 (8.7−9.5)	0.69	-	6.76 (6.6−6.9)	0.17	>10
20	H-Dmt-Tic-Ala-NH-1-adamantane	9.29 (9.0−9.5)	0.51	-	-	-	1.0 (0.7−3.0)

[a] pA$_2$ is the mean, and the range is in parentheses. [b] Salvadori et al. (1995) (ref 5). [c] Salvadori et al. (1997) (ref 7). [d] Temussi et al. (1994) (ref 3a). [e] A dash indicates the absence of pharmacological activity. Data were derived from at least four independent tissue samples and dose-response curves.

other analogues (**7**, **13**, **14**, and **17**) also produced anomalous biological activities on MVD and GPI (Table 2) relative to their receptor binding parameters (Table 1). H-Dmt-Tic-NHMe (**7**) had extraordinarily weak δ agonism with low μ antagonism, while its Ala-tripeptide derivative (**17**) gave very weak δ and μ agonism. Despite the high δ receptor affinity of compound **14**, the bioactivity data indicated only modest δ antagonism and weak μ antagonism. The tetrazole-5-yl-amide analogue (**10**) was a weak δ antagonist with very minimal activity on GPI (Table 2).

Discussion

Our results support and extend the observations that the Dmt-Tic pharmacophore represents a distinct class of δ-opioid antagonists.[5,7−9] The correlation between

competitive binding studies using a δ_1 agonist (DPDPE) and a δ antagonist [*N*,*N*(Me)$_2$-Dmt-Tic-OH] in 21 of 26 analogues appears to support the existence of a single binding site for agonists and antagonists in the ligand-binding domain of δ receptors. Further, the message domain of these peptides probably presents a similar conformation in order to fit the receptor cavity. [A series of nine other Dmt-containing di- and tripeptides with Tic or other heterocyclic residues at position 2 revealed no discrepancy in the binding between these two labeled ligands (Salvadori et al., unpublished data).] The minimum size of that message domain constitutes the dimensions of a dipeptide[3,7] which has a specific spatial geometry in solution[8,28] as seen in the crystallographic evidence for TIPP analogues[34] and *N*,*N*(Me)$_2$-Dmt-Tic-OH (Flippen-Anderson, J.; George, G., unpublished observations). Of course, until such time that a membrane receptor has been crystallized containing a bound agonist or antagonist, we are unable to absolutely verify the nature of the interaction between these two types of ligands within the δ receptor. However, the fact that the δ receptor embedded in a membrane vesicle can be equally labeled with either [^{3}H]DPDPE or [^{3}H]*N*,*N*(Me)2-Dmt-Tic-OH and whose binding can be reversed by graded dosages of unlabeled peptide at equilibrium conditions constitutes the basis of the radioreceptor assay. In contrast to ideal or hypothetical constructs, experimental data can often reveal discrepancies. Once such discrepancy was observed between the δ binding affinities of H-Dmt-Tic-OH and peptide **8** in competition against both labeled compounds that could be attributed to the well-documented rapid formation of a diketopiperazine.[29] Unfortunately, the assay of H-Dmt-Tic-OH immediately after solubilization demonstrated a rapid, time-dependent decline in δ binding, and even newly synthesized preparations revealed an instability that most likely led to the recorded incongruity between assays that were carried out weeks apart. The intrinsic δ binding capacity of *cyclo*(Dmt-Tic) (K_i ca. 10 nM) is considerably lower than the linear (noncyclic) peptides,[30] and increased levels of the diketopiperazine in the sample would inherently decrease the observed values for receptor affinity. The theoretical interpretation of this observation was in fact substantiated by the enhanced stability of peptide **1** (methylene bridge at C_α of Tic interposed before the carboxylate function; Figure 1) preventing the formation of the diketopiperazine to yield binding data equivalent to that of the parent peptide. An interesting observation relative to peptide **1** is the higher selectivity of H-Dmt-Tic-Ala-OH (parental peptide) that might indicate either a role for the aliphatic side chain of Ala in ligand-receptor binding mechanisms or in the modification of the solution conformation of the peptide.[31]

Since peptides **2**, **6**, and **10** have not been studied for either diketopiperazine formation or other possible intermediates, it would be inappropriate and premature for us to speculate on their observed discrepancy.

The differences observed between the δ-opioid receptor binding of Dmt-Tic peptides and their Tyr-Tic cognates[5,9,32] indicated that Dmt assumes a predominant role in the alignment or anchoring of the peptide within δ-, μ-, and κ-opioid receptor binding sites[8,28] or affecting solution conformation of the dipeptide antagonists.[28a,b] Furthermore, the spectrum of activity exhibited by the Tyr-Tic cognates of **8**, **17**, and **18** differed from the Dmt-Tic peptides.[9,32] For example, H-Tyr-Tic-Ala-OMe and H-Tyr-Tic-NH-1-adamantyl amide[9,32] greatly exceeded the δ-receptor binding properties of other Tyr-Tic di- and tripeptides[3,5,9,32] except for the TIP(P) series,[2] suggesting that the C-terminal address portion of the peptide can influence the message domain. In the peptides described herein, Dmt continued to enhance δ binding and maintain bioactivity in some compounds, even in the presence of the bulky C-terminal substituents. The loss of δ affinity and MVD bioactivity in **12** and **16** is comparable to the effect with other D-Tic-containing peptides,[2,5,7] implicating the importance of the spatial orientation of Tic on the interaction between aromatic rings in the peptide[8] and between peptide and receptor.

Enhancement of μ receptor binding occurred due to the hydrophobicity of the substituents at the C-terminus of the Dmt-Tic pharmacophore. Compounds containing C-terminal *tert*-butyl amide and 1-adamantyl amide were δ antagonists with high dual binding affinities and biological activity toward both δ and μ receptor types (Tables 1 and 2). 1-Adamantyl amide, with its high hydrophobic constant, high steric constant, and van der Waals volume,[33] affects peptide activity and as a consequence elevated μ affinity to yield nonselective analogues (Table 1). Similarly, the elimination of the negative charge in δ agonists[10,11] or δ antagonists either by amidation[2,5] or reduction of the acid to an alcohol[5] also augmented binding to μ receptors. Therefore, the heightened activity of these analogues toward μ receptors was not unexpected since the negatively charged carboxylate anion accounts for δ selectivity due to repulsion from the μ receptor.[5,10,11,38] However, the methyl amide moiety (**7** and **17**) appears to impart a negative effect and an anomalous bioactive behavior to the peptides. Thus, while high-affinity binding to μ receptors increased in peptides devoid of a carboxylate anion and which are physically larger than the Dmt-Tic dipeptide, the di- or tripeptide analogues containing either 1-adamantyl amide (**11**, **12**, **15**, and **20**) or *tert*-butyl amide (**9**, **14**, and **19**) also interacted effectively with the δ receptor. Considering that both *N*,*N*(Me)$_2$-Dmt-Tic-NH-1-adamantane (**15**) and H-Dmt-Tic-Ala-NH-1-adamantane (**20**) exhibited exceptional antagonist activity on MVD and only **15** displayed μ agonism demonstrated that different biological activities can occur in the same, small opioid peptide, somewhat analogous to that found with the non-peptide opiate naltrindole.[19] Furthermore, these two analogues disclose the subtle interplay between the N- and C-termini in the elicitation of bioactivty.

Earlier studies revealed that increased hydrophobicity by N-methylation generally enhanced the biological properties of the peptide analogue without significant change in receptor affinity.[7,9] For example, δ antagonism of *N*,*N*(Me)$_2$-Dmt-Tic-OH increased 20-fold (K_e = 0.28 nM) while retaining high δ-receptor affinity (K_i = 0.12 nM) and selectivity ($K_i\mu/K_i\delta$ = 20 600).[7] On the other hand, N-alkylation by piperidine-1-yl (**3**), pyrrolidine-1-yl (**4**), and especially pyrrole-1-yl (**5**) led to substantial losses in δ-receptor binding and bioactivity. Several possible explanations can be attributed to this effect,

such as the loss of the protonated nitrogen in compound **5** (comparable to that observed with the diketopiperazine derivative[30]), the derivatives exceeded the correct spatial volume in the receptor pocket, an inability to form nonionic bonds with side chains of residues in the receptor, or a misalignment of Dmt that prevented optimal orientation of the crucial hydroxyl group on the phenol side chain. Thus, in support of other data,[7,9] N-alkylation of Dmt-Tic was less favorably tolerated than the covalent addition of a third amino acid (Ala) or presence of bulky hydrophobic groups at the C-terminus. This observation fits with the common message domain discovered between deltorphins and dermorphins, opioid peptides with bioactivities for δ and μ receptors, respectively.[11,27]

Conclusions

Our data provide additional evidence that δ-opioid agonists and antagonists interact within the same ligand-binding domain in opioid receptors and that hydrophobic substituents at the C-terminus of the Dmt-Tic pharmacophore augment μ-opioid receptor affinity. The consequence of this alteration in some analogues resulted in the formation of nonselective or μ-selective analogues. Two analogues containing 1-adamantyl amide (**15** and **20**) exhibited very high δ receptor binding and potent δ antagonism with a MVD bioassay, and the former was a μ-opioid agonist as well. Thus, a single opioid peptide can possess antagonist and agonist activity toward different receptor types through the modification of the C-terminus; the dichotomy in the unusual activity of this chimeric opioid molecule must be related to the distinct physicochemical properties imparted by 1-adamantyl amide.[33] Considering the effectiveness of non-peptide opiates in transplantation studies,[23,24] these biologically stable, dual activity opioids should also be exploited for their potential pharmacological and clinical effectiveness.[9]

Experimental Section

Materials. H-Dmt-OH was synthesized as reported and compared to a sample generously supplied by Dygos et al.[37] Boc-Tic-OH was obtained from Bachem Feinchemikalien AG. [^{3}H]DPDPE (32.0 Ci/mol) 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.[38] Dipeptides were obtained by condensation of Boc-Dmt-OH with Tic derivatives (H-Tic-OtBu, H-Tic-NHMe, H-Tic-NH-1-adamantane, and H-βHTic-OMe) or by condensation of Boc-Dmt-Tic-OH with *tert*-butylamine or 5-aminotetrazolyl via DCC/HOBt. Tripeptides were obtained by condensation of Boc-L-Dmt-Tic-OH with Ala derivatives (H-Ala-NHMe, H-Ala-NH-1-adamantane, H-Ala-NH-tBu, and H-Ala-OMe) via DCC/HOBt. Final products were obtained, when necessary, by ester function hydrolysis with 1 N NaOH and removal of the Boc protecting group in TFA. N-Alkylated di- and tripeptide derivatives were obtained by reductive alkylation of the corresponding deprotected linear peptides with aldehydes (formaldehyde, glutaraldehyde, succinaldehyde) and $NaBH_3CN$ in acetonitrile.[39] HCl·Pt-βHTic-OMe was prepared by reduction of H-Tic-OMe with $LiAlH_4$ to the corresponding 3-hydroxymethyl-Tic that was transformed first in 3-bromomethyl-Tic and then in 3-cyano-Tic. Treatment of the cyano group with HCl-methanol gave the final product.[40] H-α(*R*,*S*)-HDmt-Tic-OH was obtained by condensation of (*R*,*S*)-2-cyano-3-(4-hydroxy-2,6-dimethylphenyl)-propanoic acid with H-Tic-OtBu via DCC/HOBt. In turn, (*R*,*S*)-2-cyano-3-(4-hydroxy-2,6-dimethylphenyl)-propanoic acid was prepared from ethyl cyanoacetate and *O*-carbethoxy-3,5-dimethyl-4-chloromethylphenol.[41]

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 50 mL/min using the following mobile phases: solvent A (10% acetonitrile in 0.1% TFA, v/v) and solvent B (60% acetonitrile in 0.1% TFA, v/v). 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 UV variable wavelength detector. Recording and quantification were accomplished with a chromatographic data processor coupled to an Epson computer system (QX-10). Analytical determinations were 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/AcOH/H_2O (3:1:1, v/v/v) and (B) CH_2Cl_2/toluene/methanol (17:1:2, v/v/v). Ninhydrin (1%, Merck), fluorescamine (Hoffman-La Roche) and chlorine reagents 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.

Melting points were determined on a Kofler apparatus and are uncorrected. Optical rotations were determined at 10 mg/mL in methanol with a Perkin-Elmer 241 polarimeter with a 10 cm water-jacketed cell. All ^{1}H 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.

Boc-Dmt-Tic-OMe. To a solution of Boc-Dmt-OH (0.4 g, 1.29 mmol) and H-Tic-OMe[42] (0.25 g, 1.29 mmol) in DMF (10 mL) at 0 °C were added HOBt (0.22 g, 1.42 mmol) and DCC (0.29 g, 1.42 mmol). The reaction 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/Pe (1:1, v/v): yield 0.52 g (83%); R_f (B) 0.81; HPLC $K' = 7.91$; mp 135−137 °C; $[\alpha]^{20}_D$ −13.2; MH^+ 483; ^{1}H NMR (DMSO) $\delta = 1.37-1.46$ (d, 9H), 2.16 (s, 6H), 2.96−3.01 (m, 2H), 3.08−3.13 (m, 2H), 3.46−3.56 (m, 1H), 3.72 (s, 3H), 4.34−4.88 (m, 3H), 6.46 (s, 2H), 7.18−7.21 (m, 4H), 8.32 (bs, 1H).

Boc-Dmt-Tic-NHNH$_2$. To a solution of Boc-Dmt-Tic-OMe (0.26 g, 0.54 mmol) in MeOH (10 mL) was added NH_2NH_2·H_2O (1 mL). The reaction mixture was stirred for 24 h at room temperature. After evaporation of the solvent, the residue was crystallized from Et_2O/Pe (1:1, v/v): yield 0.25 g (94%); R_f (B) 0.75; HPLC $K' = 6.83$; mp 154−156 °C; $[\alpha]^{20}_D$ −14.5; MH^+ 483; ^{1}H NMR (DMSO) $\delta = 1.35-1.44$ (d, 9H), 2.16 (s, 6H), 3.07−3.40 (s, 4H), 3.79 (m, 1H), 4.29−4.78 (m, 5H), 6.34 (m, 2H), 6.95 (bs, 1H), 7.14 (3, 4H), 8.22 (bs, 1H).

TFA·H-Dmt-Tic-NHNH$_2$ (6). Boc-Dmt-Tic-$NHNH_2$ (0.2 g, 0.41 mmol) was treated with TFA (1 mL) for 0.5 h at room temperature. Et_2O/Pe (1:1, v/v) were added to the solution until the product precipitated: yield 0.18 g (91%); R_f (A) 0.69; HPLC $K' = 2.52$; mp 143−145 °C; $[\alpha]^{20}_D$ +13.5; MH^+ 382. Anal. ($C_{21}H_{26}N_4O_3$·TFA) C, H, N.

Z-Tic-NHMe. To a solution of Z-Tic-OH[41] (1 g, 3.21 mmol) and HCl·H_2NMe (0.22 g, 3.21 mmol) in DMF (10 mL) at 0 °C were added NMM (0.35 mL, 3.21 mmol), HOBt (0.54 g, 3.53 mmol), and DCC (0.73 g, 3.53 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/Pe (1:1, v/v): yield 0.90 g (87%); R_f (B) 0.97; HPLC K' = 8.56; mp 137−139 °C; $[\alpha]^{20}_D$ +13.5; MH^+ 325; ^1H NMR (DMSO) δ = 2.44−2.46 (d, 3H), 3.12−3.24 (m, 2H), 4.36−4.46 (m, 3H), 5.17 (s, 2H), 7.18−7.21 (m, 5H), 7.36−7.39 (m, 4H), 7.74−7.77 (m, 1H).

H-Tic-NHMe. To a solution of Z-Tic-NHMe (0.90 g, 2.78 mmol) in MeOH (30 mL) was added C/Pd (5%, 0.05 g), and H_2 was bubbled for 1 h at room temperature. After filtration, the solution was evaporated to dryness. The residue was crystallized form Et_2O/Pe (1:1, v/v): yield 0.49 g (92%); R_f (B) 0.38; HPLC K' = 5.03; mp 123−125 °C; $[\alpha]^{20}_D$ +18.7; MH^+ 191.

Boc-Dmt-Tic-NHMe. This product was obtained by condensation of Boc-Dmt-OH with H-Tic-NHMe via DCC/HOBt as reported for Boc-Dmt-Tic-OMe: yield 0.13 g (85%); R_f (B) 0.73; HPLC K' = 6.34; mp 142−146 °C; $[\alpha]^{20}_D$ −15.3; MH^+ 482; ^1H NMR (DMSO) δ = 1.35−1.44 (d, 9H), 2.16 (s, 6H), 2.44−2.46 (d, 3H), 3.05−3.41 (m, 4H), 3.79 (m, 1H), 4.29−4.78 (m, 3H), 6.34 (s, 2H), 6.95 (bs, 1H), 7.14 (s, 4H), 8.22 (bs, 1H).

TFA·H-Dmt-Tic-NHMe (7). Boc-Dmt-Tic-NHMe was treated with TFA as reported for TFA·H-Dmt-Tic-$NHNH_2$ (**6**): yield 0.12 g (93%); R_f (A) 0.69; HPLC K' = 3.10; mp 152−154 °C; $[\alpha]^{20}_D$ −22.0; MH^+ 382. Anal. ($C_{22}H_{27}N_3O_3$·TFA) C, H, N.

TFA·*N,N*-(Me)$_2$-Dmt-Tic-NHMe (13). This compound was obtained by exhaustive methylation of TFA·H-Dmt-Tic-NHMe as reported for TFA·*N,N*-(Me)$_2$-Dmt-Tic-NH-1-adamantane (**15**): yield 0.12 g (96%); R_f (A) 0.71; HPLC K' = 3.19; mp 156−158 °C; $[\alpha]^{20}_D$ −19.3; MH^+ 410. Anal. ($C_{24}H_{31}N_3O_3$·TFA) C, H, N.

TFA·H-Dmt-Tic-OMe (8). Boc-Dmt-Tic-OMe was treated with TFA as reported for TFA·H-Dmt-Tic-$NHNH_2$ (**6**): yield 0.25 g (92%); R_f (A) 0.81; HPLC K' = 3.83; mp 118−120 °C; $[\alpha]^{20}_D$ −24.0; MH^+ 382. Anal. ($C_{22}H_{26}N_2O_4$·TFA) C, H, N.

Boc-Dmt-*β*HTic-OMe. This substance was obtained by condensation of Boc-Dmt-OH with HCl·*β*HTic-OMe[40] [R_f (B) 0.38, HPLC K' = 2.31, mp 153−155 °C, $[\alpha]^{20}_D$ −37.5; MH^+ 206] via DCC/HOBt as reported for Boc-Dmt-Tic-OMe: yield 0.42 g (97%); R_f (B) 0.93; HPLC K' = 9.27; mp 94−96 °C; $[\alpha]^{20}_D$ +33.9; MH^+ 497; ^1H NMR (DMSO) δ = 1.82 (s, 9H), 2.35 (s, 6H), 2.80−3.50 (m, 6H), 3.70 (s, 3H), 3.90 (m, 1H), 4.30 (m, 2H), 4.40 (dd, 1H), 6.43 (s, 2H), 7.20 (m, 6H), 9.50 (bs, 1H).

Boc-Dmt-*β*HTic-OH. To a solution of Boc-Dmt-*β*HTic-OMe (0.42 g, 0.90 mmol) in MeOH (10 mL) was added 1 N NaOH (1.34 mL). The reaction mixture was stirred for 24 h at room temperature. After evaporation of the solvent, the residue was solubilized in EtOAc and washed with citric acid (10%) and brine. The organic phase was dried and evaporated to dryness. The residue was crystallized from Et_2O: yield 0.42 g (98%); R_f (B) 0.35; HPLC K' = 7.54; mp 117−119 °C; $[\alpha]^{20}_D$ +36.2; MH^+ 483.

TFA·H-Dmt-*β*HTic-OH (1). Boc-Dmt-*β*HTic-OH was treated with TFA as reported for TFA·H-Dmt-Tic-$NHNH_2$ (**6**): yield 0.26 g (68%); R_f (A) 0.82; HPLC K' = 4.9; mp 118−120 °C; $[\alpha]^{20}_D$ +95.0; MH^+ 383; ^1H NMR (DMSO) δ = 2.2 (s, 6H), 2.80−3.0 (m, 4H), 3.4 (s, 2H), 4.1 (m, 1H), 4.3 (m, 2H), 4.5 (m, 1H), 6.43 (s, 2H), 7.20 (m, 4H), 9.2 (bs, 3H). Anal. ($C_{22}H_{26}N_2O_4$·TFA) C, H, N.

Boc-Dmt-Tic-OH. To a solution of Boc-Dmt-Tic-OMe (0.52 g, 1.08 mmol) in MeOH (10 mL) was added 1 N NaOH (1.3 mL). The reaction mixture was stirred for 24 h at room temperature and treated according to the methods reported for Boc-Dmt-*β*HTic-OH: yield 0.45 g (89%); R_f (B) 0.36; HPLC K' = 6.71; mp 147−149 °C; $[\alpha]^{20}_D$ −15.6; MH^+ 469.

Boc-Dmt-Tic-NH-tetrazole-5-yl. To a solution of Boc-Dmt-Tic-OH (0.4 g, 0.85 mmol) and 5-aminotetrazole monohydrate (0.1 g, 0.85 mmol) in DMF (10 mL) at 0 °C were added HOBt (0.14 g, 0.94 mmol) and DCC (0.19 g, 0.94 mmol). The reaction mixture was stirred for 3 h at 0 °C and 24 h at room temperature. After evaporation of the DMF, it was treated according to the procedure reported for Boc-Dmt-*β*HTic-OH; however, the residue was crystallized from Et_2O/Pe (1:1, v/v): yield 0.19 g (42%); R_f (B) 0.75; HPLC K' = 7.18; mp 146−148 °C; $[\alpha]^{20}_D$ −23.1; MH^+ 537; ^1H NMR (DMSO) δ = 1.36−1.45 (d, 9H), 2.16 (s, 6H), 2.96−3.0 (m, 2H), 3.08−3.13 (m, 2H), 3.46−3.56 (m, 1H), 4.34−4.88 (m, 3H), 6.46 (s, 2H), 7.18−7.21 (m, 4H), 8.32 (bs, 1H), 8.45 (bs, 1H), 14.39 (bs, 1H).

TFA·H-Dmt-Tic-NH-tetrazole-5-yl (10). Boc-Dmt-Tic-NH-tetrazole-5-yl was treated with TFA as reported for TFA·H-Dmt-Tic-$NHNH_2$ (**6**): yield 0.17 g (87%); R_f (A) 0.79; HPLC K' = 3.75; mp 131−133 °C; $[\alpha]^{20}_D$ −18.3; MH^+ 436; ^1H NMR (DMSO) δ = 2.18 (s, 6H), 2.81−3.03 (m, 2H), 3.12−3.18 (m, 2H), 3.66−3.76 (m, 1H), 4.38−4.52 (m, 3H), 6.45 (s, 2H), 7.21−7.25 (m, 4H), 8.27 (bs, 3H), 8.9 (bs, 3H), 14.39 (bs, 1H). Anal. ($C_{22}H_{25}N_7O_3$·TFA) C, H, N.

Boc-Dmt-Tic-Ala-OMe. To a solution of Boc-Dmt-Tic-OH (0.2 g, 0.42 mmol) and HCl·H-Ala-OMe (0.06 g, 0.42 mmol) in DMF (10 mL) at 0 °C were added NMM (0.05 mL, 0.42 mmol), HOBt (0.07 g, 0.46 mmol), and DCC (0.09 g, 0.46 mmol). The reaction was stirred for 3 h at 0 °C and 24 h at room temperature. After evaporation of DMF, the residue was treated according to Boc-Dmt-Tic-NH-tetrazole-5-yl: yield 0.2 g (85%); R_f (B) 0.77; HPLC K' = 7.68; mp 124−126 °C; $[\alpha]^{20}_D$ +40.1; MH^+ 555; ^1H NMR (DMSO) δ = 1.37−1.46 (m, 12H), 2.16 (s, 6H), 2.96−3.01 (m, 2H), 3.08−3.13 (m, 2H), 3.46−3.56 (m, 1H), 3.73 (s, 3H), 4.03−4.07 (q, 1H), 4.34−4.88 (m, 3H), 6.46 (s, 2H), 7.18−7.21 (m, 4H), 8.32 (bs, 1H), 8.51 (bs, 1H).

TFA·H-Dmt-Tic-Ala-OMe (18). Boc-Dmt-Tic-Ala-OMe was treated with TFA as reported for TFA·H-Dmt-Tic-$NHNH_2$ (**6**): yield 0.19 g (92%); R_f (A) 0.64; HPLC K' = 3.72; mp 130−132 °C; $[\alpha]^{20}_D$ +90.1; MH^+ 454. Anal. ($C_{25}H_{31}N_3O_3$·TFA) C, H, N.

Boc-Tic-NH-1-adamantane. To a solution of Boc-Tic-OH (0.5 g, 1.8 mmol) and 1-amino adamantane hydrochloride (0.34 g, 1.8 mmol) in DMF (10 mL) at 0 °C were added NMM (0.20 mL, 1.8 mmol), HOBt (0.30 g, 1.98 mmol), and DCC (0.41 g, 1.98 mmol). The reaction was stirred for 3 h at 0 °C and 24 h at room temperature. After evaporation of DMF, the residue was treated as reported for Boc-Dmt-Tic-NH-tetrazole-5-yl: yield 0.6 g (81%); R_f (B) 0.89; HPLC K' = 9.68; mp 127−129 °C; $[\alpha]^{20}_D$ +15.3; MH^+ 412; ^1H NMR (DMSO) δ = 1.39−1.46 (d, 9H), 1.65 (s, 6H), 1.93−2.07 (m, 9H), 3.08−3.13 (m, 2H), 4.34−4.88 (m, 3H), 7.17−7.20 (m, 4H), 8.08 (bs, 1H).

TFA·H-Tic-NH-1-adamantane. Boc-Tic-NH-1-adamantane (0.6 g; 1.46 mmol) was treated with TFA (2 mL) for 0.5 h at room temperature. The mixture Et_2O/Pe (1:1, v/v) was added to the solution until the product precipitated: yield 0.57 g (92%); R_f (A) 0.73; HPLC K' = 5.80; mp 157−159 °C; $[\alpha]^{20}_D$ +18.4; MH^+ 311.

Boc-Dmt-Tic-NH-1-adamantane. This peptide was obtained by condensation of Boc-Dmt-OH with TFA·H-Tic-NH-1-adamantane via DCC/HOBt as reported for Boc-Dmt-Tic-OMe: yield 0.16 g (85%); R_f (B) 0.93; HPLC K' = 9.28; mp 142−144 °C; $[\alpha]^{20}_D$ +28.1; MH^+ 603; ^1H NMR (DMSO) δ = 1.38−1.45 (d, 9H), 1.64 (s, 6H), 1.93−2.08 (m, 9H), 2.17 (s, 6H), 2.96−3.01 (m, 2H), 3.08−3.13 (m, 2H), 3.47−3.54 (m, 1H), 4.34−4.88 (m, 3H), 6.46 (s, 2H), 7.17−7.20 (m, 4H), 8.27 (bs, 1H), 8.47 (bs, 1H).

TFA·H-Dmt-Tic-NH-1-adamantane (11). Boc-Dmt-Tic-NH-1-adamantane was treated with TFA as reported for TFA·H-Dmt-Tic-$NHNH_2$ (**6**): yield 0.15 g (94%); R_f (A) 0.64; HPLC K' = 6.98; mp 180−182 °C; $[\alpha]^{20}_D$ −2.7; MH^+ 502. Anal. ($C_{31}H_{39}N_3O_3$·TFA) C, H, N.

Boc-D-Tic-NH-1-adamantane. This compound was obtained by condensation of Boc-D-Tic-OH with 1-amino adamantane hydrochloride as reported for Boc-Tic-NH-1-adamantane: yield 0.6 g (81%); R_f (B) 0.89; HPLC K' = 9.68; mp 127−129 °C; $[\alpha]^{20}_D$ −15.3; MH^+ 412; ^1H NMR (DMSO) δ = 1.39−1.46 (d, 9H), 1.65 (s, 6H), 1.93−2.07 (m, 9H), 3.08−3.13 (m, 2H), 4.34−4.88 (m, 3H), 7.17−7.20 (m, 4H), 8.08 (bs, 1H).

TFA·H-D-Tic-NH-1-adamantane. Boc-D-Tic-NH-1-adamantane was treated with TFA as reported for TFA·H-Tic-NH-1-adamantane: yield 0.57 g (92%); R_f (A) 0.73; HPLC K' = 5.80; mp 157−159 °C; $[\alpha]^{20}_D$ −18.4; MH^+ 311.

Boc-Dmt-D-Tic-NH-1-adamantane. This substance was obtained by condensation of Boc-Dmt-OH with TFA·H-D-Tic-NH-1-adamantane via DCC/HOBt as reported for Boc-Dmt-Tic-OMe: yield 0.16 g (85%); R_f (B) 0.87; HPLC K' = 9.54; mp 135−137 °C; $[\alpha]^{20}_D$ +14.2; MH^+ 603; ^1H NMR (DMSO) δ =

1.39−1.46 (d, 9H), 1.63 (s, 6H), 1.94−2.09 (m, 9H), 2.16 (s, 6H), 2.97−3.03 (m, 2H), 3.05−3.14 (m, 2H), 3.49−3.56 (m, 1H), 4.41−4.91 (m, 3H), 6.41 (s, 2H), 7.18−7.20 (m, 4H), 8.31 (bs, 1H), 8.43 (bs, 1H).

TFA·H-Dmt-D-Tic-NH-1-adamantane (12). Boc-Dmt-D-Tic-NH-1-adamantane was treated with TFA as reported for TFA·H-Dmt-Tic-$NHNH_2$ (**6**): yield 0.15 g (94%); R_f (A) 0.58; HPLC $K' = 7.24$; mp 164−166 °C; $[\alpha]^{20}_D$ +27.5; MH^+ 502. Anal. ($C_{31}H_{39}N_3O_3$·TFA) C, H, N.

Boc-Ala-NH-1-adamantane. To a solution of Boc-Ala-OH (0.34 g, 1.8 mmol) and 1-amino adamantane hydrochloride (0.34 g, 1.8 mmol) in DMF (10 mL) at 0 °C were added NMM (0.20 mL, 1.8 mmol), HOBt (0.30 g, 1.98 mmol), and DCC (0.41 g, 1.98 mmol). The reaction was stirred for 3 h at 0 °C and for 24 h at room temperature. After evaporation of DMF, the residue was treated according to Boc-Dmt-Tic-NH-tetrazole-5-yl: yield 0.52 g (89%); R_f (B) 0.85; HPLC $K' = 7.73$; mp 107−109 °C; $[\alpha]^{20}_D$ +5.9; MH^+ 324; ^{1}H NMR (DMSO) δ = 1.38−1.45 (m, 12H), 1.65 (s, 6H), 1.93−2.07 (m, 9H), 4.04−4.07 (q, 1H), 8.08 (bs, 1H), 8.42 (bs, 1H).

TFA·H-Ala-NH-1-adamantane. Boc-Ala-NH-1-adamantane (0.52 g, 1.6 mmol) was treated with TFA (2 mL) for 0.5 h at room temperature. The mixture Et_2O/Pe(1:1, v/v) was added to the solution until the product precipitated: yield 0.5 g (92%); R_f (A) 0.82; HPLC $K' = 4.95$; mp 131−133 °C; $[\alpha]^{20}_D$ +6.8; MH^+ 224.

Boc-Dmt-Tic-Ala-NH-1-adamantane. This compound was obtained by condensation of Boc-Dmt-Tic-OH with TFA·H-Ala-NH-1-adamantane via DCC/HOBt as reported for Boc-Dmt-Tic-Ala-OMe: yield 0.12 g (87%); R_f (B) 0.91; HPLC $K' = 9.32$; mp 137−139 °C; $[\alpha]^{20}_D$ +15.5; MH^+ 674; ^{1}H NMR (DMSO) δ = 1.38−1.45 (d, 12H), 1.64 (s, 6H), 1.93−2.08 (m, 9H), 2.17 (s, 6H), 2.96−3.01 (m, 2H), 3.08−3.13 (m, 2H), 3.47−3.54 (m, 1H), 4.03−4.07 (m, 1H), 4.34−4.88 (m, 3H), 6.46 (s, 2H), 7.17−7.20 (m, 4H), 8.27 (bs, 1H), 8.47 (bs, 1H), 8.53 (bs, 1H).

TFA·H-Dmt-Tic-Ala-NH-1-adamantane (20). Boc-Dmt-Tic-Ala-NH-1-adamantane was treated with TFA as reported for TFA·H-Dmt-Tic-$NHNH_2$ (**6**): yield 0.11 g (92%); R_f (A) 0.65; HPLC $K' = 6.91$; mp 163−165 °C; $[\alpha]^{20}_D$ +20.1; MH^+ 574. Anal. ($C_{34}H_{44}N_4O_4$·TFA) C, H, N: C, 71.3; H, 7.74; N, 9.78. Anal. ($C_{34}H_{44}N_4O_4$·TFA) C, H, N.

TFA·*N,N*-(Me)$_2$-Dmt-Tic-NH-1-adamantane (15). To a stirred solution of TFA·H-Dmt-Tic-NH-1-adamantane (0.7 g, 0.11 mmol) in acetonitrile (10 mL) were added NMM (0.01 mL, 0.11 mmol), 37% aqueous formaldehyde (0.07 mL, 0.83 mmol), and 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 evaporated in vacuo to give a crude product that was purified by preparative HPLC: yield 0.06 g (90%); R_f (A) 0.64; HPLC $K' = 7.44$; mp 118−120 °C; $[\alpha]^{20}_D$ −58.01; MH^+ 530. Anal. ($C_{33}H_{43}N_3O_3$·TFA) C, H, N.

TFA·*N,N*-(Me)$_2$-Dmt-D-Tic-NH-1-adamantane (16). The peptide was obtained by exhaustive methylation of TFA·H-Dmt-D-Tic-NH-1-adamantane (**12**) as reported for TFA·*N,N*-(Me)$_2$-Dmt-Tic-NH-1-adamantane (**15**): yield 0.06 g (90%); R_f (A) 0.61; HPLC $K' = 7.53$; mp 134−136 °C; $[\alpha]^{20}_D$ +8.2; MH^+ 530. Anal. ($C_{33}H_{43}N_3O_3$·TFA) C, H, N.

Boc-Dmt-Tic-Ala-NHMe. This compound was obtained by condensation of Boc-Dmt-Tic-OH with HCl·H-Ala-NHMe via DCC/HOBt as reported for Boc-Dmt-Tic-Ala-OMe: yield 0.25 g (84%); R_f (B) 0.78; HPLC $K' = 8.90$; mp 131−133 °C; $[\alpha]^{20}_D$ +42.7; MH^+ 553; ^{1}H NMR (DMSO) δ = 1.37−1.46 (m, 12H), 2.16 (s, 6H), 2.44−2.46 (d, 3H), 2.96−3.01 (m, 2H), 3.08−3.13 (m, 2H), 3.46−3.56 (m, 1H), 4.03−4.07 (q, 1H), 4.34−4.88 (m, 3H), 6.46 (s, 2H), 7.18−7.35 (m, 4H), 8.32 (bs, 1H), 8.43 (bs, 1H), 8.51 (bs, 1H).

TFA·Dmt-Tic-Ala-NHMe (17). Boc-Dmt-Tic-Ala-NHMe was treated with TFA as reported for TFA·H-Dmt-Tic-$NHNH_2$ (**6**): yield 0.24 g (91%); R_f (A) 0.58; HPLC $K' = 4.29$; mp 142−144 °C; $[\alpha]^{20}_D$ +28.5; MH^+ 453. Anal. ($C_{25}H_{32}N_4O_4$·TFA) C, H, N.

TFA·[des-NH_2-α-piperidine-1-yl]-Dmt-Tic-OH (3). To a stirred solution of TFA·H-Dmt-Tic-OH[12] (0.15 g, 0.31 mmol) in acetonitrile (10 mL) were added NMM (0.07 mL, 0.62 mmol), 50% aqueous glutaraldehyde (0.38 mL, 2.36 mmol), and sodium cyanoborohydride (0.45 g, 0.73 mmol). Glacial acetic acid (0.06 mL) was added over 10 min, and the reaction was stirred at room temperature for 2 h. The reaction mixture was evaporated in vacuo to give a crude product that was purified by preparative HPLC: yield 0.16 g (90%); R_f (A) 0.74; HPLC $K' = 3.66$; mp 205−207 °C; $[\alpha]^{20}_D$ −14.2; MH^+ 437. Anal. ($C_{22}H_{32}N_2O_4$·TFA) C, H, N.

TFA·[des-NH_2-α-pyrrolidine-1-yl]-Dmt-Tic-OH (4) and TFA·[des-NH_2-α-pyrrole-1-yl]-Dmt-Tic-OH (5). These two compounds were obtained by reductive alkylation of TFA·H-Dmt-Tic-OH with succinaldehyde as reported for TFA·[des-NH_2-α-piperidine-1-yl]-Dmt-Tic-OH. Analytical data for TFA·[des-NH_2-α-pyrrolidine-1-yl]-Dmt-Tic-OH (**4**): yield 0.016 g (20%); R_f (A) 0.81; HPLC $K' = 2.68$; mp 168−170 °C; $[\alpha]^{20}_D$ +45.2; MH^+ 423. Anal. ($C_{25}H_{30}N_2O_4$·TFA) C, H, N.

Analytical data for TFA·[des-NH_2-α-pyrrole-1-yl]-Dmt-Tic-OH (**5**): yield 0.1 g (80%); R_f (A) 0.67; HPLC $K' = 5.85$; mp 185−187 °C; $[\alpha]^{20}_D$ −56.4; MH^+ 421. Anal. ($C_{25}H_{26}N_2O_4$·TFA) C, H, N.

Boc-Dmt-Tic-NHtBu. This intermediate was obtained by condensation of Boc-Dmt-Tic-OH with *tert*-butylamine via DCC/NMM as reported for Boc-Dmt-Tic-NH-tetrazole-5-yl: yield 0.26 g (87%); R_f (B) 0.88; HPLC $K' = 7.02$; mp 153−155 °C; $[\alpha]^{20}_D$ −8.2; MH^+ 524; ^{1}H NMR (DMSO) δ = 1.23−1.44 (m, 18H), 2.16 (s, 6H), 3.05−3.41 (m, 4H), 3.79 (m, 1H), 4.29−4.78 (m, 3H), 6.34 (s, 2H), 6.95 (bs, 1H), 7.14 (s, 4H), 8.22 (bs, 1H).

TFA·H-Dmt-Tic-NHtBu (9). Boc-Dmt-Tic-NHtBu was treated with TFA as reported for TFA·H-Dmt-Tic-$NHNH_2$ (**6**): yield 0.12 g (93%); R_f (A) 0.74; HPLC $K' = 5.35$; mp 150−152 °C; $[\alpha]^{20}_D$ −15.6; MH^+ 424. Anal. ($C_{25}H_{33}N_3O_3$·TFA) C, H, N.

TFA·*N,N*-(Me)$_2$-Dmt-Tic-NHtBu (14). This N,N-alkylated peptide was obtained by exhaustive methylation of TFA·H-Dmt-Tic-NHtBu (**9**) as reported for TFA·H-Dmt-Tic-NH-1-adamantane (**11**): yield 0.12 g (88%); R_f (A) 0.78; HPLC $K' = 6.01$; mp 157−159 °C; $[\alpha]^{20}_D$ −18.7; MH^+ 452. Anal. ($C_{27}H_{37}N_3O_3$·TFA) C, H, N.

Boc-Dmt-Tic-Ala-NHtBu. This compound was obtained by condensation of Boc-Dmt-Tic-OH with HCl·H-Ala-NHtBu[43] via DCC/HOBt as reported for Boc-Dmt-Tic-Ala-OMe: yield 0.2 g (87%); R_f (B) 0.85; HPLC $K' = 8.09$; mp 150−152 °C; $[\alpha]^{20}_D$ +28.2; MH^+ 595; ^{1}H NMR (DMSO) δ = 1.23−1.48 (m, 21H), 2.16 (s, 6H), 3.05−3.41 (m, 4H), 3.79 (m, 1H), 4.29−4.78 (m, 4H), 6.34 (s, 2H), 6.95 (bs, 1H), 7.14 (s, 4H), 8.22 (bs, 1H), 8.36 (bs, 1H).

TFA·Dmt-Tic-Ala-NHtBu (19). Boc-Dmt-Tic-Ala-NH-tBu was treated with TFA as reported for TFA·H-Dmt-Tic-$NHNH_2$ (**6**): yield 0.18 g (91%); R_f (A) 0.64; HPLC $K' = 5.35$; mp 150−152 °C; $[\alpha]^{20}_D$ +25.7; MH^+ 495. Anal. ($C_{28}H_{38}N_4O_4$·TFA) C, H, N.

(*R,S*)-2-Cyano-3-(4-hydroxy-2′,6′-dimethylphenyl)-propanoic Acid Ethyl Ester. To a solution of sodium ethoxide (Na, 0.17 g, 7.2 mmol; anhydrous EtOH, 12 mL) were added ethyl cyanoacetate (0.62 mL, 7.08 mmol) and, after 10 min, *O*-carbethoxy-3,5-dimethyl-4-chloromethylphenol (1.8 g, 7.42 mmol). The reaction mixture was refluxed for 2 h, cooled, and filtered. The solution was evaporated in vacuo. The residue was crystallized from H_2O/acetone (5:1, v/v). The product was purified by column chromatography [SiO_2; Et_2O/AcOEt (1:1, v/v)]: yield 1.05 g (60%); R_f (B) 0.74; HPLC $K' = 5.38$; mp 132−134 °C; MH^+ 248; ^{1}H NMR (DMSO) δ = 1.25−1.81 (t, 3H), 2.23 (s, 6H), 3.08−3.14 (m, 2H), 4.14−4.27 (m, 3H), 6.44 (s, 2H), 9.17 (s, 1H).

(*R,S*)-2-Cyano-3-(4-hydroxy-2′,6′-dimethylphenyl)-propanoic Acid. To a solution of (*R,S*)-2-cyano-3-(4-hydroxy-2′,6′-dimethylphenyl)-propanoic acid ethyl ester (1.05 g, 4.25 mmol) in EtOH (10 mL) was added 1 N NaOH (4.68 mL, 4.68 mmol). The reaction mixture was stirred for 24 h at room temperature. After evaporation of the solvent, the residue was dissolved in EtOAc and washed with citric acid (10%) and brine. The organic phase was dried and evaporated to dryness. The

residue was crystallized from Et_2O/PtEt (1:2, v/v): yield 0.79 g (85%); R_f (B) 0.31; HPLC $K' = 3.54$; mp 154−156 °C; MH^+ 220.

[Des-NH$_2$-α-cyano-(*R,S*)]-Dmt-Tic-OtBu. To a solution of (*R,S*)-2-cyano-3-(4-hydroxy-2′,6′-dimethylphenyl)-propanoic acid (0.12 g, 0.53 mmol) and H-Tic-OtBu (o.12 g, 0.53 mmol) in DMF (10 mL) at 0 °C were added HOBt (0.09 g, 0.58 mmol) and DCC (0.12 g, 0.58 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 treated as reported for Boc-Dmt-Tic-NH-tetrazole-5-yl: yield 0.18 g (80%); R_f (B) 0.82; HPLC $K' = 8.40$; mp 140−142 °C; $[\alpha]^{20}_D$ −13.85; MH^+ 435.

[Des-NH$_2$-α-cyano-(*R,S*)]-Dmt-Tic-OH. [Des-NH$_2$-α-cyano-(*R,S*)]-Dmt-Tic-OtBu (0.18, 0.42 mmol) was treated with TFA (1 mL) for 0.5 h at room temperature. Et_2O/Pe (1:1, v/v) was added until the product precipitated: yield 0.15 g (96%); R_f (A) 0.68; HPLC $K' = 5.55$; mp 142−144 °C; $[\alpha]^{20}_D$ −15.31; MH^+ 379; IR (KBr) 3420 (OH), 2360 (nitrile), 1636 (C═O, amide), 1734 (C═O, acid), 1142 (C−O, carboxylate anion) cm^{-1}.

H-α(*R,S*)HDmt-Tic-OH (2). To a solution of [des-NH$_2$-α-cyano-(*R,S*)]-Dmt-Tic-OH (0.15 g, 0.4 mmol) in EtOH (30 mL) were added 1 N HCl (4.5 mL) and PtO_2 (0.05 g), and H_2 was bubbled for 8 h at room temperature. After filtration, the solution was evaporated to dryness. The residue was crystallized from Et_2O/Pe (1:1, v/v): yield 0.15 g (98%); R_f (A) 0.39; HPLC $K' = 2.51$; mp 157−159 °C; $[\alpha]^{20}_D$ −18.7; MH^+ 383; IR (KBr) 3432 (NH_3^+), 1683 (C═O, amide), 1616 (C═O, carboxylate anion), 1203 (C−O, carboxylate anion) cm^{-1}. Anal. ($C_{22}H_{26}N_2O_4$·TFA) C, H, N.

Receptor Binding Assays. Peptides were assayed with a rat brain synaptosomal preparations (P_2) previously preincubated in 0.1 M NaCl, 0.4 mM GDP, 50 mM HEPES, pH 7.5, and 50 μg/mL soybean trypsin inhibitor for 60 min at room temperature to remove endogenous opioids.[44] After extensive washing in ice cold buffer containing protease inhibitor, the material was resuspended in buffered inhibitor containing 20% glycerol, aliquoted, and stored at −80 °C.[44] The assays were conducted as described in detail elsewhere.[5,7,45] Briefly, the agonists [^{3}H]DPDPE (30−60 Ci/mmol, NEN-DuPont) and [^{3}H]-DAGO (30−60 Ci/mmol, Amersham) were used to label δ and μ sites, respectively, under saturation binding conditions (2 h at 22 °C). Excess unlabeled peptide (2 μM) established nonspecific binding levels. The labeled membranes were rapidly filtered on Whatman GF/C glass fiber filters, thoroughly washed, and dried, and the radioactivity was determined using CytoScint (ICN). The δ antagonist [^{3}H]*N,N*-$(CH_3)_2$-Dmt-Tic-OH was catalytically dehalogenated from a diiodo intermediate to a specific activity of 59.88 Ci/mol and binds to δ-receptors with a $K_d = 0.39$ nM.[46] The receptor binding and biological properties of the unlabeled peptide were described previously.[7] All analogues were analyzed in duplicate using 5−9 dosages, and at least three independent repetitions using different synaptosomal preparations were conducted for each peptide (actual *n* values are listed in Table 1 in parentheses), with results given as mean ± SEM. The affinity constants (K_i) were calculated according to Cheng and Prusoff.[47]

Pharmacological Bioassays. The specifics of the standard functional bioassays using mouse vas deferens (MVD) and guinea pig ileum (GPI) for δ and μ activity, respectively, were published.[5,48] Briefly, a 2−3 cm segment of GPI was placed in 20 mL tissue bath containing Kreb's solution, 70 μM hexamethonium bromide, and 0.125 μM mepyramine and aerated with 95% O_2/5% at 36 °C. Transmural stimulation of GPI was by means of a square-wave electrical pulses of 0.5 ms duration at a frequency of 0.1 Hz. A single MVD was suspended in 4 mL modified Kreb's solution aerated with 95% O_2/5% at 33 °C. An isometric transducer recorded the twitch induced by field stimulation (0.1 Hz for 1 ms at 40 V). Dose-response curves were obtained[5] for both tissues. The μ agonist activity was compared against dermorphin ($IC_{50} = 1.82$ nM), and δ antagonism was determined through the inhibition of deltorphin C (δ_1 receptor agonist, $IC_{50} = 0.54$ nM) in comparison to the nonpeptide δ antagonist naltrindole. Data were derived from at least four independent tissue samples and dose-response curves from which the pA_2 values were determined[7] according to Arunlakshana and Schild.[49]

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References

(1) Abbreviations. In addition to the IUPAC-IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* **1985**, *260*, 1442), this paper uses the following symbols and abbreviations: Boc, *tert*-butyloxycarbonyl; DAGO, [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]enkephalin; Dmt, 2′,6′-dimethyl-L-tyrosine; DPDPE, *cyclo*[D-Pen^{2,5}]enkephalin; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; K_e, the antilog of pA_2 in molar concentration; MeOH, methanol; MVD, mouse vas deferens; pA_2, negative log of the molar concentration required to double the agonist concentration to achieve the original response; tBu, *tert*-butyl; TFA, trifluoroacetic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; H-β Tic, 1,2,3,4-tetrahydroisoquinoline-3-yl acetic acid; H-αDmt-OH, 2-methylamino-3-(2′,6′-dimethyl-4-hydroxyphenyl)-propionic acid; TIP(P), H-Tyr-Tic-Phe-(Phe)-OH; TLC, thin-layer chromatography; WSC, 1-ethyl-3-[3′-dimethyl)aminopropyl]carbodiimide hydrochloride; Z, benzyloxycarbonyl, TEA, triethylamine; NH-tBut, *tert*-butylamine; Me, methyl; OMe, methyl ester; DCC, *N,N*′-dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; DMF, *N,N*-dimethylformamide; $LiAlH_4$, lithium aluminum hydride; $NaBH_3CN$, sodium cyanoborohydride; DMSO, dimethyl sulfoxide; EtPt, petroleum ether; Et_2O, diethyl ether.

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