Direct Influence of C-Terminally Substituted Amino Acids in the Dmt−Tic Pharmacophore on δ-Opioid Receptor Selectivity and Antagonism

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A series of 17 analogues were developed on the basis of the general formula H-Dmt-Tic-NH-*CH(R)-R′ (* denotes chirality; R = charged, neutral, or aromatic functional group; R′ = −OH or $-NH_2$). These compounds were designed to test the following hypothesis: the physicochemical properties of third-residue substitutions C-terminal to Tic in the Dmt-Tic pharmacophore modify δ-opioid receptor selectivity and δ-opioid receptor antagonism through enhanced interactions with the μ-opioid receptor. The data substantiate the following conclusions: (i) all compounds had high receptor affinity [$K_i(\delta) = 0.034-1.1$ nM], while that for the μ-opioid receptor fluctuated by orders of magnitude [$K_i(\mu) = 15.1-3966$ nM]; (ii) δ-opioid receptor selectivity [$K_i(\mu)/K_i(\delta)$] declined 1000-fold from 22 600 to 21; (iii) a C-terminal carboxyl group enhanced selectivity but only as a consequence of the specific residue; (iv) amidated, positive charged residues [Lys-NH_2 (**6**), Arg-NH_2 (**7**)], and a negatively charged aromatic residue [Trp-OH (**11**)] enhanced μ-opioid affinity [$K_i(\mu) = 17.0$, 15.1, and 15.7 nM, respectively], while Gly-NH_2 (**8**), Ser-NH_2 (**10**), and His-OH (**12**) were nearly one-tenth as active; and (v) D-isomers exhibited mixed effects on μ-opioid receptor affinity (**2′** ≪ **3′** < **4′** < **1′** < **5′**) and decreased δ-selectivity in D-Asp-NH_2 (**1′**) and D-Lys(Ac)-OH (**5′**). The analogues exhibited δ-opioid receptor antagonism ($pA_2 = 6.9-10.07$) and weak μ-opioid receptor agonism ($IC_{50} > 1$ μM) except H-Dmt-Tic-Glu-NH_2 (**3**), which was a partial δ-opioid receptor agonist ($IC_{50} = 2.5$ nM). Thus, these C-terminally extended analogues indicated that an amino acid residue containing a single charge, amino or guanidino functionality, or aromatic group substantially altered the δ-opioid receptor activity profile (selectivity and antagonism) of the Dmt-Tic pharmacophore, which suggests that the C-terminal constituent plays a major role in determining opioid receptor activity as an "address domain".

Introduction[1]

It is well recognized that a major rationale in the design of ligands for the three major opioid receptor types (μ, δ, and κ) traditionally focused on the abatement of pain through the use of specific agonists. However, the elimination of addictive behaviors could be accomplished by the application of selective δ- or μ-opioid antagonists. For example, since the μ-opioid receptor system appears to be responsible for addiction and δ-opioid receptors apparently fail to elicit a dependency profile, selective δ-opioid antagonists were applied to alleviate some of these matters,[2] as well as to the treatment of cocaine addiction[3] and alcoholism.[4,5] Numerous peptide opioids[6] and non-peptide opiates[7−10] interact with the δ-opioid receptor with high affinities and exhibit either agonist or antagonist bioactivities. Among the diverse body of opioid ligands, the prototypic dipeptide Dmt-Tic,[11] which evolved from the weakly active Tyr-Tic[12] as a simplification of the TIP(P) class of compounds,[13] represented the minimal peptide sequence that selectively interacted with δ-opioid receptors with potent δ-opioid receptor antagonist activity.

To obtain chemically stable[14,15] opioid molecules with a higher bioactivity profile, the Dmt-Tic pharmacophore underwent extensive modifications that altered its properties, e.g., alkylation of the N-terminal amino function,[16] replacement of Tic with other constrained aromatic residues,[17] or substitutions on the aromatic ring of Tic,[18] in addition to transforming the C-terminal carboxylic function of Dmt-Tic into alcoholic, amide, and ether functions.[19,20] A carboxylic group on δ-opioid receptor ligands is required for selectivity because a negative charge nullifies the interaction with μ-opioid receptors.[11,21] Whereas additions to the pharmacophore at the C-terminal with L- or D-Ala did not substantially alter δ-opioid receptor affinity,[11] the presence of bulky uncharged hydrophobic groups greatly enhanced μ-opioid receptor affinity and drastically reduced δ-opioid selectivity.[22] In fact, recent data demonstrated the conversion of the Dmt-Tic pharmacophore from a δ-opioid receptor antagonist into potent δ-opioid agonist by transformation of the carboxyl function of H-Dmt-Tic-Gly-OH or H-Dmt-Tic-L/D-Ala-OH into a new template with the general formula H-Dmt-Tic-NH-*CH(R)-R′ [* denotes chirality; R = −H or $-CH_3$; R′ = 1*H*-benzimidazol-2-yl (Bid)].[19,20] On the other hand, C-

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Table 1. Receptor Binding and Functional Bioactivity of H-Dmt-Tic-Xaa Peptides

		receptor binding[a]			MVD		GPI		
compd	Xaa-R′	$K_i(\delta)$ (nM)	$K_i(\mu)$ (nM)	μ/δ	pA_2[b] deltorphin	IC_{50}[c] (nM)	pA_2[b] dermorphin	IC_{50}[c] (μM)	ref
	Ala-OH	0.29 ± 0.03 (6)	5820 ± 675 (4)	20400	8.40			>10	11
	Ala-NH_2	0.24 ± 0.02 (5)	47.1 ± 3.4 (4)	195	8.00			4.74 ± 0.9	11
	D-Ala-NH_2	0.30 ± 0.02 (4)	47.5 ± 4.6 (3)	158	7.60			>10	20
1	Asp-NH_2	0.29 ± 0.02 (3)	2565 ± 290 (3)	8875	8.12			>10	20
1′	D-Asp-NH_2	0.44 ± 0.02 (3)	367 ± 59 (5)	834	8.14			>10	
2	Asn-OH	1.10 ± 0.26 (5)	80.3 ± 8.2 (4)	73	8.23			>10	20
2′	D-Asn-OH	0.74 ± 0.1 (4)	3996 ± 318 (4)	5400	8.23			>10	
3	Glu-NH_2	0.06 ± 0.008 (4)	1357 ± 268 (4)	22617		2.5 ± 0.8		>10	
3′	D-Glu-NH_2	0.053 ± 0.003 (3)	920 ± 159 (5)	17358	8.64		5.1		
4	Gln-OH	0.325 ± 0.052 (4)	465 ± 103 (5)	1431	8.98			>10	
4′	D-Gln-OH	0.45 ± 0.13 (5)	562 ± 80 (5)	1252	8.91			>10	
5	Lys(Ac)-OH	0.047 ± 0.013(4)	1051 ± 80 (5)	22361	10.07			>10	
5′	D-Lys(Ac)-OH	0.083 ± 0.01 (4)	86.8 ± 4 (3)	1046	9.29			1.5 ± 0.1	
6	Lys-NH_2	0.71 ± 0.13 (5)	17.0 ± 2.0 (4)	24	7.87			7.87 ± 0.6	
7	Arg-NH_2	0.73 ± 0.15 (4)	15.1 ± 2.3 (6)	21	6.90			6.6 ± 0.5	
8	Gly-NH_2	0.25 ± 0.05 (5)	140 ± 14 (5)	559	7.65			>10	
9	Ser-OH	0.27 ± 0.06 (4)	2830 ± 130 (3)	10481	8.88			>10	
10	Ser-NH_2	0.13 ± 0.02 (5)	140 ± 7 (3)	1120	8.07			>10	
11	Trp-OH	0.034 ± 0.002 (3)	15.7 ± 7.3 (5)	462	9.57			1.5	
12	His-OH	0.12 ± 0.04 (3)	174 ± 26 (3)	1403	8.72			>10	

[a] The K_i values (nM) were determined according to Chang and Prusoff[44] as detailed in the Experimental Section. The mean ± SE with *n* repetitions in parentheses is based on independent duplicate binding assays with five to eight peptide doses using different synaptosomal preparations. [b] pA_2 is the negative logarithm to base 10 of the molar concentration of an antagonist that makes it necessary to double the concentration of agonist needed to elicit the original submaximal response; the antagonistic properties of these compounds were tested using deltorphin C (δ agonist) or dermorphin (μ agonist) as agonists. [c] Agonist activity was expressed as IC_{50} obtained from dose−response curves. These values represent the mean ± SE of at least five fresh tissue samples. Deltorphin C and dermorphin were the internal standards for MVD (δ-opioid receptor bioactivity) and GPI (μ-opioid receptor bioactivity) tissue preparations, respectively.

terminal additions to the Tyr-Tic pharmacophore with one or two Phe residues yielded the δ-opioid selective antagonists H-Tyr-Tic-Phe-OH and H-Tyr-Tic-Phe-Phe-OH.[13]

Rationale

The aim of the current study was to test the hypothesis that a C-terminal moiety on the Dmt-Tic pharmacophore could influence δ-opioid receptor mediated activities. We asked if C-terminal substitutions and certain physicochemical characteristics of a third amino acid modify δ-opioid receptor selectivity through enhancement of the μ-opioid receptor affinity that could alter δ-opioid receptor antagonism. Thus, we prepared tripeptides of the general formula H-Dmt-Tic-Xaa, where Xaa is an amino acid with different chemical functions on the side chain containing a carboxylic or amide group at the C-terminus. In particular, the presence of an additional amino acid at the C-terminus containing either an acidic or a basic function would enable a detailed study on receptor−ligand binding, offering greater insight on receptor selectivity at membrane receptors and their interaction with opioid receptors embedded in functional biological tissues (i.e., mouse vas deferens and guinea pig ileum). Furthermore, the acidic or basic third residue could be used to attach chemical substituents (e.g., a fluorophore) in order to study receptor function and distribution. Earlier studies with deltorphin analogues revealed that while a negative charge was ineffective in altering δ-opioid affinity, it was critical in maintaining the high selectivity for this receptor; i.e., a negative charge repelled the δ-opioid ligand from interacting to any substantial degree with μ-opioid receptors.[21] These observations were also reported with a variety of analogues of the Dmt-Tic pharmacophore and in which elimination of a carboxyl group led to enhanced activity toward the μ-opioid receptor.[6,22] On the other hand, the presence of an additional negative charge in the sequence of a peptide ligand did not increase δ-receptor affinity and selectivity.[21]

Results and Discussion

Receptor Affinity Analysis. All of the tripeptides listed in Tables 1 and 2 exhibited high δ-opioid receptor affinity ($K_i \leq 1.1$ nM), and several analogues (**3**, **3′**, **5**, **5′**, **11**, **12**) demonstrated exceptional $K_i(\delta)$ values (≤0.12 nM) regardless of the composition of the C-terminal substituent. It appears that the Dmt-Tic pharmacophore remains the essential component of the peptide that preferentially interacts with the δ-opioid receptor[6,22,23] and that a C-terminal substituent can exert a subtle alteration in the overall receptor affinity of the opioid compound as well as its selectivity.

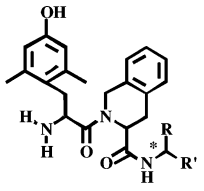
Structures of the compounds are noted in Figure 1, and opioid binding data are listed in Table 1. Data from compounds containing a negatively charged third residue in the side chain at the C-terminus, except compounds **6**−**8** and **10**, confirmed the importance of a negative charge in the H-Dmt-Tic-Xaa tripeptides for high δ-opioid receptor selectivity. Analogues **3** and **5** with μ/δ selectivity ratios of 22 617 and 22 361, respectively, are the best δ-opioid selective tripeptides in this study, although several other compounds exhibited high δ-opioid receptor selectivity (e.g., **3′**, **9**, **1**, and to a lesser extent **2′**); δ-opioid receptor affinity improved 5- to 6-fold compared to **1** and the reference compound (H-Dmt-Tic-Ala-OH) where the negative charge is at the C-terminus.[11]

While H-Dmt-Tic-Ala-NH_2 drastically improved μ-opioid receptor affinity by 123-fold compared to H-Dmt-Tic-Ala-OH, thereby reducing δ-opioid receptor selectivity by 100-fold,[11] analogues **3** and **3′** with a negative charge in the side chain and an amide function at the

Table 2. Analytical Data of H-Dmt-Tic-Xaa Analogues[a]

compd	Xaa-R′	mp (°C)	$[\alpha]^{20}_D$	R_f	K'	MH^+, *m/z*	analytical data
1′	D-Asp-NH_2	152−154	+26.5	0.72	7.45	483	($C_{25}H_{30}N_4O_6$·TFA) C, H, N
2′	D-Asn-OH	153−155	+28.7	0.73	7.66	483	($C_{25}H_{30}N_4O_6$·TFA) C, H, N
3	Glu-NH_2	148−150	+28.2	0.78	8.72	497	($C_{26}H_{32}N_4O_6$·TFA) C, H, N
3′	D-Glu-NH_2	149−151	+26.9	0.80	8.84	497	($C_{26}H_{32}N_4O_6$·TFA) C, H, N
4	Gln-OH	152−154	+25.9	0.78	9.27	497	($C_{26}H_{32}N_4O_6$·TFA) C, H, N
4′	D-Gln-OH	153−155	+24.2	0.80	9.35	497	($C_{26}H_{32}N_4O_6$·TFA) C, H, N
5	Lys(Ac)-OH	155−157	+24.8	0.74	8.93	539	($C_{29}H_{38}N_4O_6$·TFA) C, H, N
5′	D-Lys(Ac)-OH	156−158	+18.6	0.75	8.99	539	($C_{29}H_{38}N_4O_6$·TFA) C, H, N
6	Lys-NH_2	150−152	+27.5	0.71	8.82	496	($C_{27}H_{37}N_5O_4$·2TFA) C, H, N
7	Arg-NH_2	164−166	+21.8	0.68	8.41	524	($C_{27}H_{37}N_7O_4$·2TFA) C, H, N
8	Gly-NH_2	142−144	+36.8	0.62	8.01	425	($C_{23}H_{28}N_4O_4$·TFA) C, H, N
9	Ser-OH	158−160	+34.8	0.58	8.15	456	($C_{24}H_{29}N_3O_6$·TFA) C, H, N
10	Ser-NH_2	145−147	+35.2	0.63	8.09	455	($C_{24}H_{30}N_4O_5$·TFA) C, H, N
11	Trp-OH	158−160	+40.5	0.77	9.41	555	($C_{32}H_{34}N_4O_5$·TFA) C, H, N
12	His-OH	165−167	+31.7	0.67	7.31	506	($C_{27}H_{31}N_5O_5$·2TFA) C, H, N

[a] The details can be found in the Experimental Section.



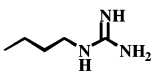
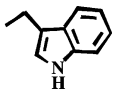
no.	R	*	R'
1	CH_2-COOH	S	$CONH_2$
1'	CH_2-COOH	R	$CONH_2$
2	CH_2-$CONH_2$	S	COOH
2'	CH_2-$CONH_2$	R	COOH
3	$(CH_2)_2$-COOH	S	$CONH_2$
3'	$(CH_2)_2$-COOH	R	$CONH_2$
4	$(CH_2)_2$-$CONH_2$	S	COOH
4'	$(CH_2)_2$-$CONH_2$	R	COOH
5	$(CH_2)_4$-NH-Ac	S	COOH
5'	$(CH_2)_4$-NH-Ac	R	COOH
6	$(CH_2)_4$-NH_2	S	$CONH_2$
7		S	$CONH_2$
8	H		$CONH_2$
9	CH_2-OH	S	COOH
10	CH_2-OH	S	$CONH_2$
11		S	COOH
12		S	COOH

Figure 1. General structure of H-Dmt-Tic-Xaa peptides defined in the side chain (R) and in the C-terminal (R′) of the Xaa residue. The asterisk denotes chirality.

C-terminus represented ideal candidate peptides as design templates for δ-opioid receptor selective compounds.[20] A change in the chirality of the analogues containing Asp (**1**, **1′**) and Lys(Ac)-OH (**5**, **5′**) exhibited a decreased δ-opioid receptor selectivity of approximately 10- and 20-fold, respectively, because of improvement of μ-opioid receptor affinities. The opposite occurred with Asn (**2**, **2′**), in which δ-opioid receptor selectivity rose 74-fold, and only small changes were observed with Glu (**3**, **3′**) and Gln (**4**, **4′**) (Table 1). It is well-known that the chirality of Tic plays a dominant role in the activity of these compounds[11,12] as well as in the TIP(P) peptides.[13,24,25]

Changing the position of the negative charge and amide function from the side chain to the C-terminus in analogues **2** and **4** decreased δ-opioid receptor selectivity 120-fold for the former and 16-fold for the latter compared to **1** and **3** because of an improvement of μ-opioid receptor affinity. The binding profiles of the corresponding Asn and Gln distereoisomers (**2′** and **4′**) revealed that δ-opioid receptor selectivity is partially dependent on the chirality of the residue; i.e., H-Dmt-Tic-D-Asn-OH was 74-fold more selective than its L-Asn analogue (**2**), while D-Gln had a δ-opioid selectivity comparable to its distereoisomer (**4**). While most selective analogues in the C-terminal extended TIP(P) series containing D-Gln-OH,[25] the C-terminal position of this residue would spatially differ from **2′** and **4′**, presumably by interacting with different receptor pocket side chains; i.e., our compounds being tripeptides in comparison to the TIP(P) tetra- or pentapeptides are substantially smaller. However, the difference in activity could also be attributable to the presence of Dmt in lieu of Tyr at the N-terminus, which is known to cause distinct shifts in the spectrum of peptide activity.[6,26−29]

The presence of a positively charged residue, Arg (**7**) or Lys (**6**), and the acetylation of the amino function of the side chain of Lys (**5**, **5′**) were investigated for their effects on μ-opioid activity, since a positive charge has been shown to play a pivotal role in some dermorphin analogues to increase μ-opioid receptor selectivity.[30] Arg and Lys also serve as potential residues for attachment of other functional groups (infra vide). Dmt-Tic-Lys(Ac)-OH (**5**) had high δ-opioid receptor selectivity comparable to **3**, while that for the corresponding D-Lys diastereoisomer (**5′**) was reduced about 20-fold mainly because of a large increase in μ-opioid receptor interaction. As anticipated, the nonacetylated, C-terminally amidated Lys analogue (**6**) and H-Dmt-Tic-Arg-NH_2 (**7**) exhibited considerably higher affinity for μ-opioid receptors and thus were only marginally δ-opioid receptor selective (Table 1).

Neutral amino acids at the C-terminus of the Dmt-Tic pharmacophore included Gly (**8**) or Ser (**9**, **10**); analogues containing Ala are the reference peptides in Table 1. Although all three analogues (**8**−**10**) exhibited high δ-opioid receptor affinities, the amidated compounds (**8** and **10**) had increased μ-opioid affinity that resulted in weaker δ-opioid receptor selective ligands. That H-Dmt-Tic-Ser-OH (**9**) was 10-fold more δ-opioid receptor selective than the corresponding amide further

emphasizes the role of a carboxylate anion in repelling the ligand from interaction with the μ-opioid receptor.[11,21,31] Furthermore, the fact that **9** exhibited half the δ-opioid receptor selectivity of the reference compound (H-Dmt-Tic-Ala-OH) suggests that the hydroxyl function in the side chain of Ser increased μ-opioid receptor affinity.

Last, maintaining the required aromaticity in residues C-terminal to Tic in the Dmt-Tic pharmacophore,[17−20] Trp (**11**) and His (**12**) were then investigated as residues that are aromatic yet whose the properties differ from those of Phe.[13,32−34] Although His was noted to be important for the high activity of deltorphin A (a δ-opioid receptor heptapeptide agonist), the highly flexible nature of this peptide[35] might permit an approximate alignment of the His residues in the Dmt-Tic tripeptide analogue that could interact within a similar ligand-binding domain within the receptor. Although both analogues (**11** and **12**) showed high δ-opioid receptor affinity as noted in other Dmt-Tic analogues containing C-terminal aromatic or hydrophobic substituents[22] in comparison to the reference compound H-Dmt-Tic-Ala-OH, they displayed an increased affinity to the μ-opioid receptor by 370-fold and 33-fold, respectively. This observation further substantiates the hypothesis that aromatic or hydrophobic groups[22] serve as requirements for ligands that preferentially interact within a receptor site or region within the pocket that better fits the contours of the μ-opioid receptor.[36]

Functional Bioactivity Analysis of H-Dmt-Tic-Xaa Analogues. The compounds were tested in the electrically stimulated MVD and GPI assays for intrinsic activity. We and other investigators have previously discussed the discrepancy of the correlation between receptor binding affinities and functional bioactivity. Unfortunately, we have neither a definitive nor comprehensive answer for this observation.[21b]

Our data reveal that all the analogues were inactive as antagonists in the GPI assay except for **3′**. Furthermore, they exhibited antagonism against the δ-opioid receptor reference agonist deltorphin C (ref 1) in the MVD assay except compound **3**, which exhibited δ-opioid agonism. The results of these biological assays are presented in Table 1.

As expected, most of the compounds exhibited fairly potent δ-opioid receptor antagonism, except analogue **3**, which was a partial δ-opioid receptor agonist, and analogues **6**−**8**, which had modest δ-opioid receptor antagonist activity comparable to that of the reference compound (H-Dmt-Tic-D-Ala-NH_2). The most potent analogue was H-Dmt-Tic-Lys(Ac)-OH (**5**), which displaced the deltorphin C dose−response curve to the right ($pA_2 = 10.07$ or $K_e = 0.085$ nM), while the GPI remained unresponsive at 10 μM. Furthermore, being 50-fold more potent than H-Dmt-Tic-Ala-OH, this compound could be an ideal candidate to further develop modified analogues coupled to the ϵ-amino function of the C-terminal lysine in the formation of fluorescent molecules.[37]

On the other hand, H-Dmt-Tic-Glu-NH_2 (**3**) inhibited the electrically evoked twitch in a concentration-dependent manner from 2 to 160 nM (Figure 2, top). It behaved as a partial δ-receptor agonist, however, with a maximum effect of only 60% inhibition. Although

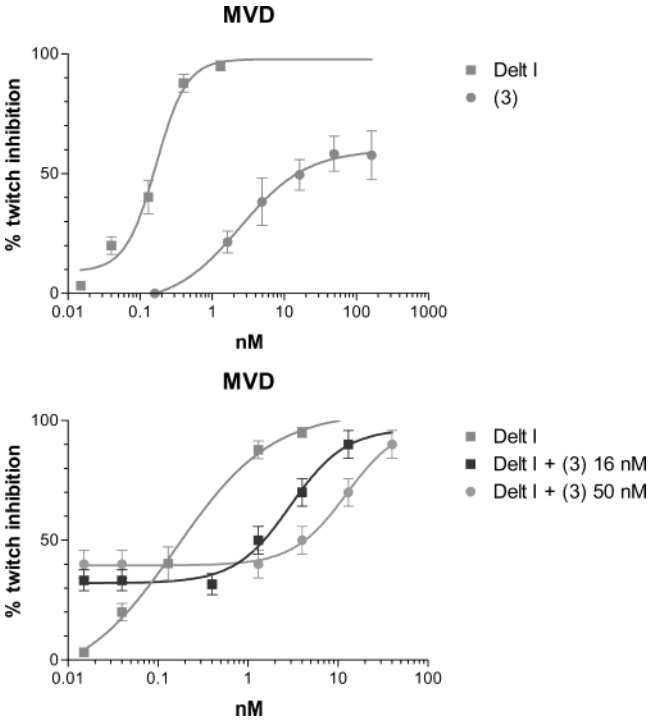


Figure 2. Action of [D-Ala^2]deltorphin I (deltorphin C) and **3** as δ-opioid receptor agonists in the electrically stimulated MVD (top) and the inhibition (antagonism) by **3** on [D-Ala^2]-deltorphin I relaxation of electrically stimulated MVD (bottom).

naloxone (3 μM) inhibited the agonist activity of **3** (data not shown), this analogue managed to shift the response curve of deltorphin C to the right (Figure 2, bottom). Interestingly, the binding assays revealed that **3** had the highest δ-opioid receptor selectivity ($\mu/\delta = 22\,617$). From the GPI activity profiles of these analogues (Table 1), they were either inactive at 10 μM or very weak μ-opioid receptor agonists (**5′**, **6**, **7**, **11**), and only **3′** elicited marginal μ-opioid antagonist activity against dermorphin with a K_e of about 10 μM ($pA_2 = 5.1$).

Conclusions

Our data demonstrated that additions at the C-terminus of the Dmt-Tic pharmacophore with different amino acids gave analogues with a δ-opioid receptor affinity comparable to or higher than the reference tripeptide H-Dmt-Tic-Ala-OH. The carboxylic function in the side chain or at the C-terminal of H-Dmt-Tic-Xaa was important for δ-opioid receptor selectivity; the D-stereochemistry of the third residue was ineffective for **4′** and **3′** but effective for three analogues (**1′**, **2′**, **5′**). H-Dmt-Tic-Glu-NH_2 (**3**) and H-Dmt-Tic-Lys(Ac)-OH (**5**) exhibited the highest δ-opioid receptor selectivities, however, with partial δ-opioid agonist and potent δ-opioid antagonist bioactivity, respectively. On the basis of these data, these two peptides (**3** and **5**) emerged as templates for potential derivatization and the synthesis of new opioid ligands with various new functional groups that might serve to further differentiate between agonist and antagonist activity and between δ- and μ-opioid receptors.

Experimental Section

Chemistry. All tripeptides were synthesized according to published methods using standard solid-phase synthesis techniques[38] with a Milligen 9050 synthesizer and Fmoc/tBu

strategy. Protected amino acids and chemicals were purchased from Bachem, Novabiochem, and Fluka (Switzerland), while Dmt was prepared as reported[39] and used in the solid-phase synthesis without protection of the side chain but protected at the amino function as a *tert*-butyloxycarbonyl derivative (Boc).[16]

For the synthesis of the C-terminal amide peptides, the resin [5-(4′-Fmoc-aminomethyl-3′,5′-dimethoxyphenoxy)valeric acid] on a poly(ethylene glycol)/polystyrene support (Fmoc-PAL-PEG-PS; Millipore Waltham, MA) was employed. Other peptides were obtained with the resin preloaded with the C-terminal amino acid on the poly(ethylene glycol)/polystyrene support [Fmoc-Xaa-PEG-PS; Xaa = L- or D-Asn(Trt), L- or d-Gln(Trt), Trp(Boc), His(Trt), Ser(tBu); Millipore Waltham, MA]. For the synthesis of the peptides containing the L- or D-Lys(Ac), the preloaded resin was obtained by condensing Fmoc-L- or Fmoc-D-Lys(Ac)-OH residue on Wang resin according to published methods.[40] The Fmoc-PAL-PEG-PS-resin, Fmoc-Xaa-PEG-PS-resin, and Fmoc-L- or D-Lys(Ac)-Wang-resin (0.1 mequiv in all syntheses) were treated with piperidine (20%) in DMF and linked with the first C-terminal amino acids: Fmoc-L-/D-Asp(OtBu)-OH, Fmoc-L- or D-Glu(O*t*Bu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Gly-OH, and Fmoc-Ser(tBu)-OH, in the case of the C-terminal amide peptides, followed by Fmoc-Tic-OH and Boc-Dmt-OH (4-fold excess) by using *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU)[41] (4-fold excess) in DMF as coupling reagent with a reaction time of 1 h.

The peptide resin was washed with methanol and dried in vacuo to yield the protected peptide-PEG-PS-resin. All the protected peptides were cleaved from the resin by treatment with 10 mL of TFA/H_2O/Et_3SiH (88:5:7, v/v/v) per 0.1 mequiv of resin at room temperature for 1 h. After filtration of the exhausted resin, the solvent was concentrated in vacuo and the residue was triturated with ethyl ether. The resulting solid was collected by centrifugation and purified by preparative HPLC.

Crude peptides were purified by preparative reversed-phase high-performance liquid chromatography (HPLC) using a Waters Delta Prep 4000 system with Waters Prep LC 40 mm Assembly column C18 (30 cm × 4 cm, 15 μm particle size column). The column was perfused at a flow rate of 40 mL/min with mobile phase solvent A (10% acetonitrile in 0.1% TFA, v/v), and a linear gradient from 0% to 50% of solvent B (60% acetonitrile in 0.1% TFA, v/v) in 25 min was adopted for the elution of the products. Analytical HPLC analyses were performed with a Beckman System Gold with a Beckman ultrasphere ODS column (250 mm × 4.6 mm, 5 μm particle size). Analytical determinations and capacity factor (k') of the products were determined using HPLC conditions in the above solvent systems (solvents A and B) programmed at a flow rate of 1 mL/min using the linear gradient from 0% to 50% B in 25 min. All analogues showed less than 1% impurities when monitored at 220 and 254 nm.

TLC was performed on precoated plates of silica gel F254 (Merck, Darmstadt, Germany) using the following solvent system: 1-butanol/AcOH/H_2O (3:1:1, v/v/v), ninhydrin (1%, Merck), fluorescamine (Hoffman-La Roche), and chlorine reagents used as sprays.

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. Molecular weights of the compounds were determined by a MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight) analysis using a Hewlett-Packard G2025A LD-TOF system mass spectrometer and α-cyano-4-hydroxycinnamic acid as a matrix.

Pharmacology. Competitive Binding Assays. Receptor binding assays were conducted as described in considerable detail elsewhere using rat brain synaptosomes (P_2 fraction).[20−22,42] This membrane preparation was preincubated to eliminate endogenous opioid peptides and stored at −80 °C in buffered 20% glycerol.[21,43] Each analogue was analyzed in duplicate assays using five to eight dosages and independent repetitions with different synaptosomal preparations (*n* values are listed in Table 1 in parentheses and results are the mean ± SE). Unlabeled peptide (2 μM) was used to determine nonspecific binding in the presence of 5.53 nM [^{3}H]DPDPE (34.0 Ci/mmol, PerkinElmer, Boston, MA; K_D = 4.5 nM) for δ-opioid receptors and 3.5 nM [^{3}H]DAMGO (50.0 Ci/mmol, Amersham Biosciences, Buckinghamshire, U.K.; K_D = 1.5 nM) for μ-opioid receptors. Glass fiber filters (Whatman GFC) were soaked in 0.1% polyethylenimine in order to enhance the signal-to-noise ratio of the bound radiolabeled-synaptosome complex, and the filters were washed thrice in ice-cold buffered BSA.[21] The affinity constants (K_i) were calculated according to Cheng and Prusoff.[44]

Activity in Isolated Organ Preparations. Preparations of myenteric plexus longitudinal muscle obtained from male guinea pig ileum (GPI, rich in μ-opioid receptors) and preparations of mouse vas deferens (MVD, rich in δ-opioid receptors) were used for field stimulation with bipolar rectangular pulses of supramaximal voltage.[45] Agonists were evaluated for their ability to inhibit the electrically evoked twitch. The biological potency of the compounds was compared with that of the μ-opioid receptor agonist dermorphin in GPI and with that of the δ-opioid receptor agonist deltorphin C in MVD. The results are expressed as the IC_{50} values obtained from concentration−response curves (Prism, GraphPad). The IC_{50} values (nM) represent the mean of not less than six tissue samples ± SE. Competitive antagonist activities were evaluated for their ability to shift the deltorphin C (MVD) and dermorphin (GPI) log(concentration)−response curve to the right; pA_2 values were determined using the Schild Plot.[46]

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References

(1) Abbreviations. In addition to the IUPAC-IUB Commission on Biochemical Nomenclature (*J. Biol. Chem*. **1985**, *260*, 14−42), this paper uses the following symbols and abbreviations: Ac, acetyl; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]enkephalin; Bid, 1*H*-benzimidazol-2-yl; Boc, *tert*-butyloxycarbonyl; deltorphin C, [D-Ala^2]deltorphin I (Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2); Dmt, 2′,6′-dimethyl-L-tyrosine; DPDPE, *cyclic*[D-Pen^{2,5}]enkephalin; Fmoc-PAL-PEG-PS, 5-(4′-Fmoc-aminomethyl-3′,5′-dimethoxyphenoxy)valeric acid bound on a poly(ethylene glycol)/polystyrene support; GPI, guinea pig ileum; HATU, *O*-(17-azabenzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MVD, mouse vas deferens; O*t*Bu, *tert*-butyl ester; Ph, phenyl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; pA_2, negative log of the molar concentration required to double the agonist concentration to achieve the original response; *t*Bu, *tert*-butyl; TFA, trifluoroacetic acid; THA, tetrahydofurane; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TIP(P), H-Tyr-Tic-Phe-(Phe)-OH; TLC, thin-layer chromatography; Trt, trityl; WSC, 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide·HCl.

(2) Fundytus, M.; Schiller, P.; Shapiro, M.; Weltrowska, G.; Coderre, T. Attenuation of morphine tolerance and dependence with the highly selective δ-opioid receptor antagonist TIPP[ψ]. *Eur. J. Pharmacol.* **1995**, *286*, 105−108.

(3) Menkens, K.; Bilsky, E.; Wild, K.; Portoghese, P. S.; Reid, L.; Porreca, F. Cocaine place preference is blocked by the δ-opioid receptor antagonist, naltrindole. *Eur. J. Pharmacol.* **1992**, *219*, 345−346.

(4) Hill, K. G.; Kiefer, S. W. Naltrexone treatment increases the aversiveness of alcohol for outbred rats. *Alcohol.: Clin. Exp. Res.* **1997**, *21*, 637−641.

(5) Stomberg, M. F.; Casale, L.; Volpicelli, L.; Volpicelli, J. R.; O'Brien, C. P. A comparison of the effects of the opioid antagonists naltrexone, naltrindole, and β-funaltrexamine on ethanol consumption in the rat. *Alcohol* **1998**, *15*, 281−289.

(6) Bryant, S. D.; Jinsmaa, Y.; Salvadori, S.; Okada, Y.; Lazarus, L. H. Dmt and opioid peptides: a potent alliance. *Biopolymers* **2003**, *71*, 86−102.

(7) Portoghese, P. The design of δ-selective opioid receptor antagonists. *Farmaco* **1993**, *48*, 243−251.

(8) Farouz-Grant, F.; Portoghese, P. S. Pyrrolomorphinans as δ opioid receptor antagonists. The role of steric hindrance in conferring selectivity. *J. Med. Chem*. **1997**, *40*, 1977−1981.

(9) Nagase, H.; Kawai, K.; Hayakawa, J.; Wakita, H.; Mizusuna, A.; Matsuura, H.; Tajima, C.; Takezawa, Y.; Endoh, T. Rational drug design and synthesis of a highly selective nonpeptide δ-opioid antagonist, (4aS*,12aR*)-4a-(3-hydroxyphenyl)-2-methyl-1,2,3,4,4a,5,12a-ocatahydropyridol[3,4-*b*]acridine (TAN-67). *Chem. Pharm. Bull.* **1998**, *46*, 1695−1702.

(10) Calderon, S. N.; Rothman, R. B.; Porreca, F.; Flippen-Anderson, J. L.; McNutt, R. W.; Xu, H.; Smith, L. E.; Bilsky, E. J.; Davis, P.; Rice, K. C. Probes for narcotic receptor mediated phenomena. 19. Synthesis of (+)-4-[(α*R*)-α-((2*S*,5*R*)-4allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide (SNC 80): a highly selective, nonpeptide δ opioid receptor agonist. *J. Med. Chem.* **1994**, *37*, 2125−2128.

(11) Salvadori, S.; Attila, M.; Balboni, G.; Bianchi, C.; Bryant, S. D.; Crescenzi, O.; Guerrini, R.; Picone, D.; Tancredi, T.; Temussi, P. A.; Lazarus, L. H. δ Opioidmimetic antagonists: prototypes for designing a new generation of ultraselective opioid peptides. *Mol. Med.* **1995**, *1*, 678−689.

(12) Temussi, P. A.; Salvadori, S.; Amodeo, P.; Bianchi, C.; Guerrini, R.; Tomatis, R.; Lazarus, L. H.; Tancredi, T. Selective opioid dipeptides. *Biochem. Biophys. Res. Commun.* **1994**, *198*, 933−939.

(13) Schiller, P. W.; Nguyen, T. M.-D.; Weltrowska, G.; Wilkes, B. C.; Marsden, J.; Lemieux, C.; Chung, N. N. Differential stereochemical requirements of μ vs δ opioid receptors for ligand binding and signal transduction: development of a class of potent and highly δ-selective peptide antagonists. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 11871−11875.

(14) Carpenter, K. A.; Weltrowska, C.; Wilkes, B. C.; Schmidt, R.; Schiller, P. W. Spontaneous diketopiperazine formation via end-to-end cyclization of a nonactivated linear tripeptide: an unusual chemical reaction. *J. Am. Chem. Soc.* **1994**, *116*, 8450−8458.

(15) Capasso, S.; Sica, F.; Mazzarella, L.; Balboni, G.; Guerrini, R.; Salvadori, S. Acid catalysis in the formation of dioxopiperazines from peptides containing tetrahydroisoquinoline-3-carboxylic acid at position 2. *Int. J. Pept. Protein Res.* **1995**, *45*, 567−573.

(16) Salvadori, S.; Balboni, G.; Guerrini, R.; Tomatis, R.; Bianchi, C.; Bryant, S. D.; Cooper, P. S.; Lazarus, L. H. Evolution of the Dmt-Tic pharmacophore: N-terminal methylated derivatives with extraordinary δ opioid antagonist activity. *J. Med. Chem.* **1997**, *40*, 3100−3108.

(17) Balboni, G.; Salvadori, S.; Guerrini, R.; Bianchi, C.; Santagada, V.; Calliendo, G.; Bryant, S. D.; Lazarus, L. H. Opioid pseudopeptides containing heteroaromatic or heteroaliphatic nuclei. *Peptides* **2000**, *21*, 1663−1671.

(18) Santagada, V.; Balboni, G.; Caliendo, G.; Guerrini, R.; Salvadori, S.; Bianchi, C.; Bryant, S. D.; Lazarus, L. H. Assessment of substitution in the second pharmacophore of Dmt-Tic analogues. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2745−2748.

(19) Balboni, G.; Guerrini, R.; Salvadori, S.; Bianchi, C.; Rizzi, D.; Bryant, S. D.; Lazarus, L. H. Evaluation of the Dmt-Tic pharmacophore: conversion of a potent δ-opioid receptor antagonist into a potent δ-agonist and ligands with mixed properties. *J. Med. Chem.* **2002**, *45*, 713−720.

(20) Balboni, G.; Salvadori, S.; Guerrini, R.; Negri, L.; Giannini, E.; Yunden, J.; Bryant, S. D.; Lazarus, L. H. New potent δ-opioid agonists based on the Dmt-Tic pharmacophore. *J. Med. Chem.* **2002**, *45*, 5556−5563.

(21) (a) Lazarus, L. H.; Salvadori, S.; Santagada, V.; Tomatis, R.; Wilson, W. E. Function of negative charge in the "address domain" of deltorphins. *J. Med. Chem.* **1991**, *34*, 1350−1359. (b) Salvadori, S.; Bianchi, C.; Lazarus, L. H.; Scaranari, V.; Attila, M.; Tomatis, R. Para-substituted Phe[3] deltorphin analogues: enhanced selectivity of halogenated derivatives for δ opioid receptor sites. *J. Med. Chem.* **1992**, *35*, 4651−4657.

(22) Salvadori, S.; Guerrini, R.; Balboni, G.; Bianchi, C.; Bryant, S. D.; Cooper, P. S.; Lazarus, L. H. Further studies on the Dmt-Tic pharmacophore: Hydrophobic substituents at the C-terminus endows δ antagonists to manifest μ agonism or μ antagonism. *J. Med. Chem.* **1999**, *42*, 5010−5019.

(23) Bryant, S. D.; George, C.; Flippen-Anderson, J.; Salvadori, S.; Balboni, G.; Guerrini, R.; Lazarus, L. H. Solid-state structure of analogues containing the Dmt-Tic pharmacophore. *J. Med. Chem.* **2002**, *45*, 5506−5513.

(24) Schiller, P. W.; Nguyen, T. M.-D.; Weltrowska, G.; Wilkes, B. C.; Marsden, B. J.; Schmidt, R.; Lemieux, C.; Chung, N. N. TIPP opioid peptides: development of extraordinarily potent and selective δ antagonists and observation of astonishing structure−intrinsic activity relationships. *Peptides: Chemistry and Biology*; ESCOM: Leiden, The Netherlands, 1994; pp 483−486.

(25) Kumar, V.; Murray, T. F.; Aldrich, J. V. Extended TIP(P) analogues as precursors for labelled δ-opioid receptor ligands. *J. Med. Chem.* **2000**, *43*, 5050−5054.

(26) Guerrini, R.; Capasso, A.; Sorrentino, L.; Anacardio, R.; Bryant, S. D.; Lazarus, L. H.; Attila, M.; Salvadori, S. Opioid receptor selectivity alteration by single residue replacement: synthesis and activity profile of [Dmt[1]]deltorphin B. *Eur. J. Pharmacol.* **1996**, *302*, 37−42.

(27) Guerrini, R.; Capasso, A.; Marastoni, A.; Bryant, S. D.; Cooper, P. S.; Lazarus, L. H.; Temussi, P. A.; Salvadori, S. Rational design of dynorphin A analogues with δ-receptor selectivity and antagonism for δ- and κ-receptors. *Bioorg. Med. Chem.* **1998**, *6*, 57−62.

(28) Sasaki, Y.; Suto, T.; Ambo, A.; Ouchi, H.; Yamamoto, Y. Biological properties of opioid peptides replacing Tyr at position 1 by 2,6-dimethyl-Tyr. *Chem. Pharm. Bull.* **1999**, *47*, 1506−1507.

(29) Schiller, P. W.; Nguyen, T. M.-D.; Berezowska, I.; Dupuis, S.; Weltrowska, G.; Chung, N. N.; Lemieux, C. Synthesis and in vitro opioid activity profiles of DALDA analogues. *Eur. J. Med. Chem.* **2000**, *35*, 895−901.

(30) Schiller, P.; Nguyen, T.; Chung, N.; Lemieux, C. Dermorphin analogues carrying an increased positive net charge in their "message" domain display extremely high μ opioid receptor selectivity. *J. Med. Chem.* **1989**, *32*, 698−703.

(31) Lazarus, L. H.; Salvadori, S.; Attila, M.; Grieco, P.; Bundy, D. M.; Wilson, W. E.; Tomatis, R. Interaction of deltorphin with opioid receptors: molecular determinants for affinity and selectivity. *Peptides* **1993**, *14*, 21−28.

(32) Schiller, P. W.; Weltrowska, C.; Nguyen, T. M.-D.; Lemieux, C.; Chung, N. N.; Marsden, B. J.; Wilkes, B. C. Conformational restriction of the phenylalanine residue in a cyclic peptide analogue: effects of receptor selectivity and stereospecificity. *J. Med. Chem.* **1991**, *34*, 3125−3132.

(33) Salvadori, S.; Bryant, S. D.; Bianchi, C.; Balboni, G.; Scaranari, V.; Attila, M.; Lazarus, L. H. Phe[3]-Substituted analogues of deltorphin C. Spatial conformation and topography of the aromatic ring in peptide recognition by δ opioid receptors. *J. Med. Chem.* **1993**, *36*, 3748−3756.

(34) Capasso, A.; Amodeo, P.; Balboni, G.; Guerrini, R.; Lazarus, L. H.; Temussi, P. A.; Salvadori, S. Design of μ selective opioid dipeptide antagonists. *FEBS Lett.* **1997**, *417*, 141−144.

(35) (a) Bryant, S. D.; Salvadori, S.; Attila, M.; Lazarus, L. H. Topographical conformations of the deltorphins predicate δ opioid receptor affinity. *J. Am. Chem. Soc.* **1993**, *115*, 8503−8504. (b) Salvadori, S.; Guerrini, R.; Forlani, V.; Bryant, S. D.; Attila, M.; Lazarus, L. H. Prerequisite for His[4] in deltorphin A for high δ opioid receptor selectivity. *Amino Acids* **1994**, *7*, 291−304.

(36) Okada, Y.; Tsuda, Y.; Fujita, Y.; Yokoi, T.; Sasaki, Y.; Ambo, A.; Konishi, R.; Nagata, M.; Salvadori, S.; Yunden, J.; Bryant, S. D.; Lazarus, L. H. Unique synthetic μ-opioid receptor agonists with central- and systemic-mediated analgesia. *J. Med. Chem.* **2003**, *46*, 3201−3209.

(37) Berezowska, I.; Chung, N. N.; Lemieux, C.; Zelent, B.; Szeto, H. H.; Schiller, P. W. Highly potent fluorescent analogues of the opioid peptide Dmt(1) DALDA. *Peptides* **2003**, *24*, 1195−1200.

(38) Atherton, E.; Sheppard, R. C. *Solid Phase Peptide Synthesis: A Practical Approach*; IRL Press: Oxford, U.K., 1989.

(39) Dygos, J. H.; Yonan, E. E.; Scaros, M. G.; Goodmonson, O. J.; Getman, D. P.; Periana, R. A.; Beck, G. R. A convenient asymmetric synthesis of the unnatural amino acid 2,6-dimethyl-L-tyrosine. *Synthesis* **1992**, *8*, 741−743.

(40) Sieber, P. An improved method for anchoring of 9-fluorenylmethoxycarbonyl-amino acids to 4-alkoxybenzyl alcohol resins. *Tetrahedron Lett.* **1987**, *28*, 6147−6150.

(41) Carpino, L. A. 1-Hydroxy-7-azabenzotriazole. An efficient peptide coupling additive. *J. Am. Chem. Soc.* **1993**, *115*, 4397−4398.

(42) Balboni, G.; Salvadori, S.; Guerrini, R.; Negri, L.; Giannini, E.; Bryant, S. D.; Jinsmaa, Y.; Lazarus, L. H. Synthesis and opioid activity of *N*,*N*-dimethyl-Dmt-Tic analogues: acquisition of potent δ antagonism. *Bioorg. Med. Chem.* **2003**, *11*, 5435−5441.

(43) Lazarus, L. H.; Wilson, W. E.; de Castiglione, R.; Guglietta, A. Dermorphin gene sequence peptide with high affinity and selectivity for δ-opioid receptors. *J. Biol. Chem.* **1989**, *264*, 3047−3050.

(44) Cheng, Y.-C.; Prusoff, W. H. Relationships between the inhibition constant (K_i) and the concentration of inhibition which cause 50% inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099−3108.

(45) Melchiorri, P.; Negri, L.; Flaconieri-Erspamer, G.; Severini, C.; Corsi, R.; Soaje, M.; Erspamer, V.; Barra, D. Structure−activity relationships of the δ-opioid-selective agonists, deltorphins. *Eur. J. Pharmacol.* **1991**, *195*, 201−207.

(46) Tallarida, R. J.; Murray, R. B. *Manual of Pharmacological Calculation*, 2nd ed.; Springer-Verlag: New York, 1986.

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