Highly Selective Fluorescent Analogue of the Potent δ-Opioid Receptor Antagonist Dmt-Tic

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A fluorescent tripeptide probe derived by coupling fluorescein to H-Dmt-Tic-Glu-NH_2 was developed to interact with δ-opioid receptors with high affinity (K_i = 0.035 nM) and selectivity (K_i^{μ}/K_i^{δ} = 4371). It acts as an irreversible δ-opioid receptor antagonist, and binding to NG108-15 cells is blocked by the standard nonpeptidic δ-opioid receptor antagonist naltrindole. This probe should prove useful in the study of the distribution of δ-opioid receptors in tissues and the internalization of opioid peptides during signal transduction.

Introduction

The availability of highly selective ligands for individual receptor types aids in the development of potential therapeutic agents. Such ligands, acting as agonists or antagonists, are valuable pharmacological tools for understanding the pharmacophoric requirements for binding and the various biological effects produced by individual receptor interactions.[2] Fluorescent ligands can be used to label receptors in cell culture or tissue preparations and studied by fluorescence microscopy, confocal laser microscopy, or flow cytometry. Strategically labeled ligands (e.g., with a fluorescent label) have been used as pharmacological tools to study receptor function and to help identify individual receptor types. They were utilized to assess the kinetics of receptor−ligand association and dissociation rates[3] and the interactions between ligands, receptors, and G proteins.[4,5] Other receptor properties, such as the localization of the receptor-binding domain,[6] have also been examined using fluorescently labeled ligands. Peptide ligands for opioid receptors were previously labeled with fluorescent functionalities, such as rhodamine,[7] pyrene,[8] dansyl,[9,10] and fluorescein.[11,12] These groups can be readily attached to a free carboxylic acid or an amino group on the peptides in one of two ways: (i) to a side chain functional group of a noncritical residue; or (ii) by extending the peptide backbone in a manner that has minimal influence on binding at the ligand-binding domain.[13]

A non-peptide fluorescent probe, derived from the naltrindole template for the δ-opioid receptor, is a potent δ-opioid receptor antagonist in the mouse vas deferens (MVD) (smooth muscle) assay and binds to the δ-opioid receptor with relatively high affinity (K_i = 1 nM) and selectivity.[12] However, with the exception of the arylacetamide-derived fluorescent ligands,[14] none of these compounds have been reported as molecular probes nor was their selectivity for any of the major opioid receptor types (δ, μ, κ) demonstrated. Recently, Schiller et al. reported highly potent fluorescent analogues of the μ-opioid receptor peptide [Dmt[1]]DALDA containing dansyl or anthranoyl fluorophores.[9,10]

Rationale

The goal was to develop potent and selective labeled opioid peptides as a pharmacological tool to study δ-opioid receptor structure and function. Among the diverse body of opioid ligands, the prototypic dipeptide Dmt-Tic,[15] which evolved from the weakly active Tyr-Tic[16] as a simplification of the TIP(P) class of compounds,[17] represents the minimal peptide sequence that selectively interacts with δ-opioid receptors with potent antagonist activity (K_i^{μ}/K_i^{δ} = 150 780; pA_2 = 8.2).[15] The high δ-opioid receptor affinity (K_i^{δ} = 0.022 nM) and selectivity exhibited by H-Dmt-Tic-OH made it an ideal candidate for fluorescein labeling. To label this dipeptide without reducing its δ-opioid receptor affinity and selectivity, we initially prepared a series of standard dipeptides extended at the C-terminus based on the general formula H-Dmt-Tic-L/D-Xaa. The third residue (Xaa) contained an acidic or an amide function at the C-terminus or in the side chain; a free carboxylic function is important to maintain high δ-opioid receptor selectivity,[15,18] and this amide tripeptide derivative could mimic the spatial positioning of the fluorescein binding site in the receptor.

Among the C-terminal extended Dmt-Tic peptides examined, H-Dmt-Tic-Glu-NH_2 was the most selective ligand for the δ-opioid receptor (K_i^{δ} = 0.06 nM; K_i^{μ}/K_i^{δ} = 22 600).[19] To reduce the influence of fluorescein on potential interference with opioid receptor affinity,[9] H-Dmt-Tic-Glu and the fluorescein label were separated by an alkyl spacer.[11,14,12] Here, we describe

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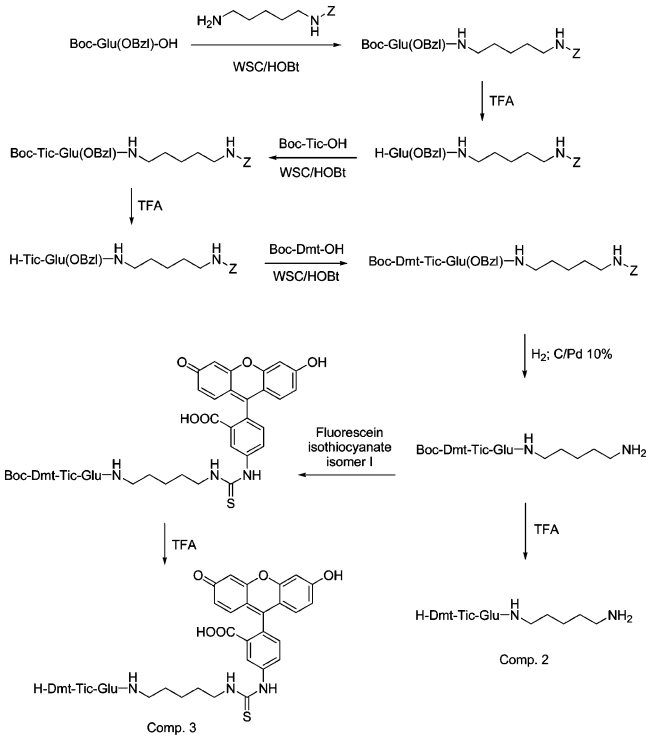
Scheme 1. Synthesis of Fluorescein-Bound Tripeptide Dmt-Tic-Glu



the synthesis, opioid activity profile, and fluorescence parameters of a H-Dmt-Tic-Glu-NH_2 analogue containing fluorescein linked at the C-terminus through a pentamethylene spacer.

Chemistry

The fluorescein-containing tripeptide was synthesized as seen in Scheme 1. Boc-Glu(OBzl)-OH was condensed with *N*-Z-1,5-pentanediamine via WSC/HOBt. After Boc deprotection with TFA, Boc-Tic-OH and Boc-Dmt-OH were added in succession. Catalytic hydrogenation (Pd/C, 10%) of Z and benzyl ester gave the free amino function suitable for the reaction with fluorescein isothiocyanate isomer I. Crude fluorescein-tripeptide was purified by preparative reversed-phase HPLC using a Waters Delta Prep 4000 system with Waters PrepLC 40 mm Assembly column C_{18} (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 using a Beckman System Gold and 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 a linear gradient from 0 to 50% B in 25 min. All analogues showed <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 systems: (A) 1-butanol/AcOH/H_2O (3:1:1, v/v/v); (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 cm × 70 cm, 0.7−1 g of material) was run on silica gel 60 (70−230 mesh, Merck) using the same eluent systems. Melting points were determined on a Kofler apparatus and are uncorrected. Optical rotations were determined at 10 mg/mL in 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 MS analyses 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 a α-cyano-4-hydroxycinnamic acid matrix.

Results

In Vitro Opioid Activity Profile. In the receptor binding assays, the fluorescent probe H-Dmt-Tic-Glu-NH-$(CH_2)_5$-NH-(C=S)-NH-fluorescein (**3**) displayed subnanomolar δ-opioid receptor binding affinity, which is the same order of magnitude as that of the reference compound H-Dmt-Tic-Glu-NH_2 (**1**), while the tripeptide (**2**), containing only the spacer at the C-terminus, exhibited only a 3.7-fold decrease in affinity for δ-opioid receptors. The μ-opioid receptor affinity increased 3.6- and ~9-fold for **2** and **3**, respectively, compared to the reference tripeptide (**1**). As a consequence, the δ-opioid receptor selectivity of fluorescent **3** fell 5-fold from 22 600 to 4370 and that of the tripeptide **2** decreased 13-fold compared to reference **1**.

While a direct comparison between the δ-opioid receptor selectivity of our fluorescent probe and that of other fluorescent opioid molecules found in the literature may not be wholly compatible because of inherent differences in assay methods, it is instructive to compare them when inconsistencies exceed orders of magnitude; **3**, for example, was 115- and 857-fold more selective than fluorescent naltrindole derivatives.[12,28] Similarly, the labeling of the δ-opioid receptor agonist [D-Ala^2]-deltorphin I with Alexa 488 and BODIPY TR caused a precipitous loss of δ-selectivity from 9000 to >128 and 16, respectively. Moreover, TIPP, another δ-opioid selective antagonist labeled with Alexa 488, exhibited a marked change in selectivity from >20000 to 84.[29]

In the in vitro functional bioactivity profiles of **1**−**3**, there was negligible activity in the GPI preparations (IC_{50} > 1 μM). In the MVD assay, tripeptide **1** is a partial δ-opioid agonist,[19] and C-terminal amidation with a spacer, as demonstrated with **2**, transforms the intrinsic δ-opioid agonist activity into δ-opioid antagonist activity; it behaves as a competitive antagonist producing parallel displacing of [D-Ala^2]deltorphin I dose−response curves without alteration of the maximal response, from which equiactive dose ratios can be calculated and used in the Schild equation (pA_2 = 8.8). Interestingly, the fluorescent derivative **3** in the MVD had nonequilibrium antagonist activity (Figure 1). The log dose−response curves of [D-Ala^2]deltorphin I in the presence of increasing concentrations of **3** reflected a reduction in the apparent efficacy and Hill slope ([D-Ala^2]deltorphin I = 1.4; +1 nM **3** = 1.1; +5 nM **3** = 0.5). The compound bound tightly and dissociates very slowly from the tissue preparation; antagonism could not be reversed by washing the tissue with a drug-free

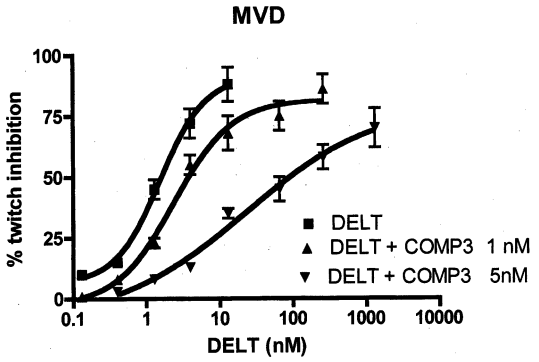


Figure 1. Functional bioactivity of **3** on MVD. Inhibition of the twitch by DELT using two concentrations of **3** were conducted as given in Experimental Section using MVD preparations.

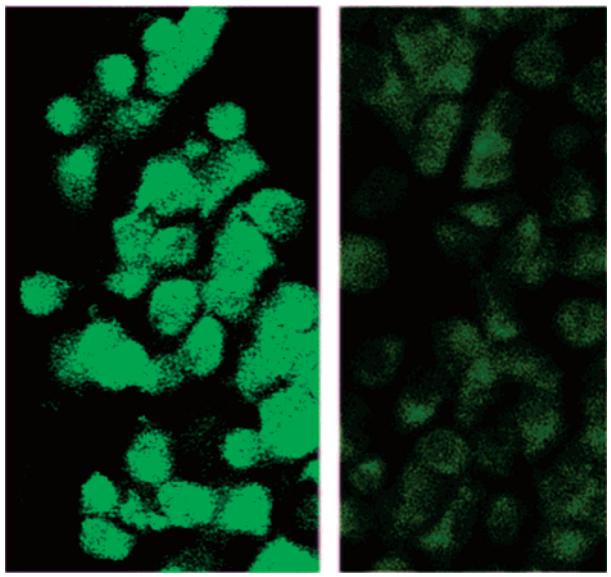


Figure 2. Confocal microscopic visualization of the fluorescence of **3** in NG108-15 cells: (left) NG108-15 cells with fluorescence compound **3**; (right) cells preincubated with the δ-opioid receptor antagonist naltrindole.

solution for over 3 h; moreover, the longer the compound is in contact with the tissue, the greater was the magnitude of the observed antagonism.

Fluorescence Spectroscopic Parameters. The fluorescence emission spectra of H-Dmt-Tic-Glu-NH-$(CH_2)_5$-NH-(C=S)-NH-fluorescein and the reference amino acid derivative Ac-Glu-NH$(CH_2)_5$-NH-(C=S)-NH-fluorescein show a maximum at 515 nm in Tris-HCl buffer (pH 6.6). This indicated that the fluorescein label of the tripeptide was located in a completely aqueous environment and does not engage in any significant intramolecular interactions. This was also verified by the similar fluorescence quantum yields calculated for the fluorescein-tripeptide ($\varphi = 0.227$) and for the reference fluorescein-amino acid derivative Ac-Glu-NH$(CH_2)_5$-NH-(C=S)-NH-fluorescein.

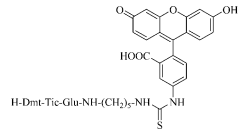
Fluorescence Detection. Visualization of δ-opioid receptor sites with our fluorescent probe was obtained by incubating (15 min at 35 °C) the fluorescent tripeptide **3** (0.2 nM) with the NG108-15 (mouse N18 neuroblastoma × rat C6 glioma) cell line, which expresses mouse δ-opioid receptors. The left panel of Figure 2 reveals the fluorescent photomicrograph obtained from the confocal scanning laser microscope, while in the right panel the fluorescent photomicrograph is that of cells preincubated with the δ-opioid receptor antagonist naltrindole (0.2 μM) before addition of the fluorescent probe (0.2 nM); naltrindole essentially eliminated the fluorescence bound to δ-opioid receptors.

Discussion

Fluorophores are stable molecules that generate UV−visible radiation only when excited by a primary source. An example is fluorescein, one of the most commonly used fluorescent labels in the biosciences. This molecule is optimally excited at 490 nm and emits at 520 nm. Peptides can be labeled with this reagent through an α-side chain amino group or, in some cases, by a spacer located at the C-terminal of the template, which provides the amino function to covalently bind fluorescein via an isothiocyanate moiety. Attachment of fluorescein, which is considered a relatively small fluorophore, to a ligand for a GPCR constitutes an increase in molecular weight and corresponding molecular volume of the ligand. Also, derivatization of the peptide ligand with fluorophores with high molar absorptivity and fluorescence quantum yields tends to introduce a significant amount of hydrophobicity into the ligand.

The δ-opioid receptor fluorescent derivative described herein retains a high δ-opioid receptor affinity and only a 5-fold decrease in δ-opioid receptor selectivity compared to the reference H-Dmt-Tic-Glu-NH_2. Although elongation at the C-terminus with the spacer and fluorescein increased μ-opioid receptor affinity 10-fold compared to the reference tripeptide and was similar to other C-terminal modified Dmt-Tic analogues,[18,19,21] the ratio of δ- versus μ-opioid receptor selectivity of the fluorescent derivative remained very high, more than 4000, exceeding the selectivity of other fluorescently labeled compounds.[12,28,29] In the MVD assay, the fluorescent compound bound δ-opioid receptors quite tightly and in a manner similar to an irreversible antagonist (Table 1). Thus, the spacer and fluorescein modify the partial δ-opioid agonist activity of H-Dmt-Tic-Glu-NH_2, respectively, into a potent δ-opioid receptor antagonist and an irreversible antagonist. The fluorescent parameters of **3** at pH 6.6 and 20 °C are $\lambda_{max} = 515$ nm with a high fluorescence quantum yield (φ) very similar to that of the reference Ac-Glu-NH−(C=S)-NH-fluorescein. An interpretation of this result is that the fluorophore is exposed to the aqueous environment and is not interacting with the chemical function of the peptide (e.g., binding mechanism). Localization and visualization of opioid receptor binding sites were obtained by incubating the fluorescent probe with the NG108-15 cells as shown in the fluorescent photomicrograph obtained from the confocal scanning laser microscope (Figure 2, left). Preincubation of the cells with δ-opioid receptor antagonist NTI for 5 min prior to adding the fluorescent probe **3** produced considerable blockage of the fluorescence (Figure 2, right). Similar results were obtained when the competition experiments were performed with other δ-opioid receptor antagonists, such as *N*,*N*′$(CH_3)_2$-Dmt-Tic-OH and TIPP (data not shown). However, the residual fluorescence (~10%) suggests a minor nonspecific binding of the probe to NG108-15 cell membranes or a residual staining of a fraction of

Table 1. Receptor Binding and Functional Bioactivity[a]

		receptor affinity[a] (nM)			functional bioactivity		
no.	compd	K_i^δ	K_i^μ	μ/δ	MVD IC_{50}[c] (nM)	MVD pA_2[b]	GPI IC_{50}[c] (μM)
1	H-Dmt-Tic-Glu-NH_2	0.06 ± 0.008 (4)[d]	1360 ± 268 (4)[d]	22600[d]	2.58 ± 0.8[d]		>1
2	H-Dmt-Tic-Glu-NH-$(CH_2)_5$-NH_2	0.22 ± 0.04 (4)	380 ± 65 (4)	1700		8.8	>1
3		0.035 ± 0.01 (4)	152 ± 44 (4)	4370		irreversible antagonist	>1

[a] The K_i values (nM) were determined according to Chang and Prusoff[24] 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 several different synaptosomal preparations. [b] pA_2 is the negative logarithm$_{10}$ of the molar concentration of an antagonist that is necessary to double the concentration of agonist needed to elicit the original submaximal response; the antagonist properties of these compounds were tested using deltorphin C (δ-opioid receptor agonist) or dermorphin (μ-opioid receptor agonist). [c] Agonist activity was expressed as IC_{50} obtained from dose−response curves. These values represent the mean ± SE for 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. [d] Data taken from Balboni et al.[19]

δ-opioid receptors due to the essentially irreversible kinetics of the fluorescent probe. The high lipophilicity of fluoroscein may contribute to this nonspecific binding to NG108-15 lipid membranes and to the distinct pharmacological behavior of the fluorescent probe in the MVD assay.

Conclusion

The incorporation of the fluorescein molecule into a compound containing the potent δ-opioid antagonst pharmacophore Dmt-Tic yielded a highly selective probe (>4000 relative to the μ-opioid receptor) that bound as an apparent irreversible antagonist to δ-opioid receptors in NG108-15 cells. The retention of the opioid binding properties by **3**, and its unique fluorescence parameters and high quantum yield, clearly indicated that the fluorescein moiety had minimal effect on receptor interaction. Therefore, our fluoroscent conjugate **3** could be applied as a specific probe to study the in vitro localization of δ-opioid receptors in tissues, receptor internalization, and trafficking in live cells in real time by use of confocal microscopy[29] or in the formulation of a nonradiolabeled competitive binding assay.

Experimental Section

Peptide Synthesis. Boc-Glu(OBzl)-NH$(CH_2)_5$-NH-Z. To a solution of Boc-Glu(OBzl)-OH (0.30 g, 0.90 mmol) and *N*-Z-1,5-pentanediamine hydrochloride (0.24 g, 0.90 mmol) in DMF (10 mL) at 0 °C were added NMM (0.10 mL, 0.90 mmol), HOBt (0.15 g, 0.99 mmol), and WSC (0.19 g, 0.99 mmol). The mixture was stirred for 3 h at 0 °C and for 24 h at room temperature. After DMF was evaporated, the residue was solubilized in EtOAc and washed with 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 (1:9, v/v): yield 0.47 g (94%); R_f(B) 0.94; HPLC K' = 9.15; mp 141−143 °C; $[\alpha]^{20}_D$ +20.4; MH^+ 556; ^1H NMR (DMSO) δ 1.29−1.55 (m, 15 H), 2.18−2.25 (m, 4H), 2.96−3.20 (m, 4H), 4.53−5.34 (m, 5H), 7.11−7.29 (m, 10H).

TFA·H-Glu(OBzl)-NH$(CH_2)_5$-NH-Z. Boc-Glu(OBzl)-NH-$(CH_2)_5$-NH-Z (0.47 g, 0.85 mmol) was treated with TFA (2 mL) for 30 min at room temperature. Et_2O/Pe (1:5, v/v) was added to the solution until the product precipitated: yield 0.46 g (94%); R_f(A) 0.77; HPLC K' = 6.89; mp 153−155 °C; $[\alpha]^{20}_D$ +23.9; MH^+ 456.

Boc-Tic-Glu(OBzl)-NH$(CH_2)_5$-NH-Z. To a solution of Boc-Tic-OH (0.22 g, 0.80 mmol) and TFA·H-Glu(OBzl)-NH$(CH_2)_5$-NH-Z (0.46 g, 0.80 mmol) in DMF (10 mL) at 0 °C were added NMM (0.09 mL, 0.80 mmol), HOBt (0.13 g, 0.88 mmol), and WSC (0.17 g, 0.88 mmol). The mixture was stirred for 3 h at 0 °C and for 24 h at room temperature. After DMF was evaporated, the residue was treated as reported above for Boc-Glu(OBzl)-NH$(CH_2)_5$-NH-Z: yield 0.51 g (89%); R_f(B) 0.95; HPLC K' = 9.26; mp 143−145 °C; $[\alpha]^{20}_D$ +16.7; MH^+ 615; ^1H NMR (DMSO) δ 1.29−1.55 (m, 15 H), 2.18−2.25 (m, 4H), 2.96−3.20 (m, 6H), 4.22−5.34 (m, 8H), 6.96−7.19 (m, 14H).

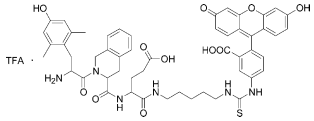
TFA·H-Tic-Glu(OBzl)-NH$(CH_2)_5$-NH-Z. Boc-Tic-Glu(OBzl)-NH$(CH_2)_5$-NH-Z (0.51 g, 0.71 mmol) was treated with TFA (2 mL) for 30 min at room temperature. Et_2O/Pe (1:5, v/v) was added to the solution until the product precipitated: yield 0.49 g (94%); R_f(A) 0.79; HPLC K' = 6.85; mp 156−158 °C; $[\alpha]^{20}_D$ +18.1; MH^+ 615.

Boc-Dmt-Tic-Glu(OBzl)-NH$(CH_2)_5$-NH-Z. To a solution of Boc-Dmt-OH (0.21 g, 0.67 mmol) and TFA·H-Tic-Glu(OBzl)-NH$(CH_2)_5$-NH-Z (0.49 g, 0.67 mmol) in DMF (10 mL) at 0 °C were added NMM (0.07 mL, 0.67 mmol), HOBt (0.11 g, 0.74 mmol), and WSC (0.14 g, 0.74 mmol). The mixture was stirred for 3 h at 0 °C and for 24 h at room temperature. After DMF was evaporated, the residue was treated as reported above for Boc-Glu(OBzl)-NH$(CH_2)_5$-NH-Z: yield 0.40 g (88%); R_f(B) 0.87; HPLC K' = 8.94; mp 140−142 °C; $[\alpha]^{20}_D$ +17.1; MH^+ 905; ^1H NMR (DMSO) δ 1.29−1.55 (m, 15 H), 2.06−2.35 (m, 10H), 2.96−3.20 (m, 8H), 4.40−5.34 (m, 9H), 6.29 (s, 2H), 6.96−7.19 (m, 14H).

Boc-Dmt-Tic-Glu-NH$(CH_2)_5$-NH_2. To a solution of Boc-Dmt-Tic-Glu(OBzl)-NH$(CH_2)_5$-NH-Z (0.4 g, 0.44 mmol) in methanol (30 mL) was added C/Pd (10%, 0.07 g), and H_2 was bubbled for 1 h at room temperature. After filtration, the solution was evaporated to dryness. The residue was crystallized from Et_2O/Pe (1:9, v/v): yield 0.27 g (90%); R_f(A) 0.58; HPLC K' = 3.87; mp 161−163 °C; $[\alpha]^{20}_D$ +19.4; MH^+ 682.

2TFA·H-Dmt-Tic-Glu-NH$(CH_2)_5$-NH_2 (2, Table 2). Boc-Dmt-Tic-Glu-NH$(CH_2)_5$-NH_2 (0.05 g, 0.07 mmol) was treated with TFA (1 mL) for 30 min at room temperature. Et_2O/Pe (1:5, v/v) was added to the solution until the product precipitated: yield 0.06 g (95%); R_f(A) 0.59; HPLC K' = 4.21; mp 163−165 °C; $[\alpha]^{20}_D$ +19.4; MH^+ 582; ^1H NMR (DMSO) δ 1.29−1.55 (m, 6 H), 2.05−3.20 (m, 18H), 3.95−4.92 (m, 5H), 6.29 (s, 2H), 6.96−7.02 (m, 4H).

Table 2. Physicochemical Properties and Elemental Analysis of **2** and **3**[a]

compd	MH^+, *m/z*	elemental analysis
2TFA·H-Dmt-Tic-Glu-NH-$(CH_2)_5$-NH_2	582	$(C_{31}H_{43}N_5O_6\cdot 2TFA)$ C, H, N
	971	$(C_{52}H_{54}N_6O_{11}S\cdot TFA)$ C, H, N, S

[a] Only the analysis of the new compounds, detailed in the Experimental Section, are included.

5-(3-{5-[2-({2-[2-*tert*-Butoxycarbonylamino-3-(4-hydroxy-2,6-dimethylphenyl)propionyl]-1,2,3,4-tetrahydroisoquinoline-3-carbonyl}amino)-4-carboxybutyrylamino]pentyl}thioureido)-2-(6-hydroxy-3-oxo-3*H*-xanten-9-yl)benzoic Acid [Boc-Dmt-Tic-Glu-NH(CH_2)$_5$-NH-(C=S)-NH-fluorescein]. With stirring at 25 °C under argon, fluorescein isothiocyanate isomer I (0.06 g, 0.15 mmol) was added to a mixture of Boc-Dmt-Tic-Glu-NH(CH_2)$_5$-NH_2 (0.1 g, 0.15 mmol) and triethylamine (2.5 mL) in freshly distilled THF (10 mL) and absolute ethanol (15 mL). The mixture was stirred in the dark, at room temperature for 24 h. After solvent evaporation, the residue was purified by preparative HPLC: yield 0.07 g (49%); R_f(B) 0.74; HPLC K' = 8.03; mp 157−159 °C; $[\alpha]^{20}_D$ +8.2; MH^+ 1070; ^{1}H NMR (DMSO) δ 1.29−1.55 (m, 15 H), 2.06−2.35 (m, 10H), 3.05−3.45 (m, 8H), 4.40−4.92 (m, 5H), 6.11−7.26 (m, 15H).[14]

5-(3-{5-[2-({2-[2-Amino-3-(4-hydroxy-2,6-dimethylphenyl)propionyl]-1,2,3,4-tetrahydroisoquinoline-3-carbonyl}-amino)-4-carboxybutyrylamino]pentyl}thioureido)-2-(6-hydroxy-3-oxo-3*H*-xanten-9-yl)benzoic Acid. [TFA·H-Dmt-Tic-Glu-NH(CH_2)$_5$-NH−(C=S)-NH-fluorescein] (3). Boc-Dmt-Tic-Glu-NH(CH_2)$_5$-NHCSNH-fluorescein (0.07 g, 0.07 mmol) was treated with 66% TFA (1 mL) for 30 min at room temperature. Et_2O/Pe (1:5, v/v) was added to the solution until the product precipitated: yield 0.067 g (94%); R_f(A) 0.71; HPLC K' = 5.47; mp 169−171 °C; $[\alpha]^{20}_D$ +9.7; MH^+ 971; ^{1}H NMR (DMSO) δ 1.29−1.55 (m, 6H), 2.05−2.35 (m, 10H), 3.05−3.95 (m, 9H), 4.46−4.92 (m, 4H), 6.11−7.28 (m, 15H).[11]

Competitive Receptor Binding Assays. These assays were conducted as described in detail elsewhere using rat brain synaptosomes (P_2 fraction).[18,20−22] Membrane preparations were preincubated to eliminate endogenous opioid peptides and stored at −80 °C in buffered 20% glycerol.[20,23] Each analogue was analyzed in duplicate using five to eight dosages of peptide and independent repetitions with different synaptosomal preparations (n values are listed in Table 1 in parentheses and the results are listed as 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, Perkin-Elmer, Boston, MA; K_D = 4.5 nM) for δ-opioid receptors, and for μ-opioid receptors, 3.5 nM [^{3}H]DAMGO (50.0 Ci/mmol, Amersham Biosciences, Buckinghamshire, U.K.; K_D = 1.5 nM) was used. Glass fiber filters (Whatman GFC) were soaked in 0.1% polyethylenimine to enhance the signal/noise ratio of the bound radiolabeled-synaptosome complex, and the filters washed thrice in ice-cold buffered BSA.[20] The affinity constants (K_i) were calculated according to Cheng and Prusoff.[24]

Functional Bioactivity in Isolated Organ Preparations. Preparations of myenteric plexus-longitudinal muscle obtained from male guinea pig ileum (GPI, enriched in μ-opioid receptors) and preparations of MVD (containing δ-opioid receptors) were used for field stimulation with bipolar rectangular pulses of supramaximal voltage.[25] 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} obtained from dose−response curves (Prism, GraphPad). To evaluate antagonistic properties, **2** and **3** were added to the bath and allowed to interact with tissue receptor sites 5 min before adding deltorphin C. The IC_{50} values (nM) represent the mean of not less than six fresh 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.[26] IC_{50} and pA_2 (nM) are the mean ± SE of at least six experiments conducted with fresh tissues.

Fluorescence Spectroscopy. Fluorescence emission spectra were recorded on a Jobin Yvon-Spex FluoroMax-2 spectrofluorometer with 1 nm spectral resolution for excitation and emission. A peptide solution at 2×10^{-5} M in Tris-HCl buffer, pH 6.6, was used. The excitation wavelength was 350 nm. Fluorescence quantum yield (φ) was determined relative to quinine sulfate (Fluka) in 1 N H_2SO_4 (φ = 0.546 [27]) as a reference. The quantum yield was calculated according to

$$\varphi_S = \varphi_R \frac{E_S A_R}{E_R A_S}\left(\frac{n_S}{n_R}\right)^2$$

where the subscripts S and R refer to the sample and reference compounds, respectively, E is the integrated area under the corrected emission spectrum, and A is the absorbance of the solution at the excitation wavelength. Absorbance values were kept below 0.02 to minimize inner filter and self-quenching effects. Since the sample and the reference were in aqueous solution, the correction for the refractive index $(n_S/n_R)^2$ was considered to be of no significant relevance.

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: DAMGO, [D-Ala^2,*N*-Me-Phe^4, Gly-ol^5]enkephalin; Bid, 1*H*-benzimidazol-2-yl; Boc, *tert*-butyloxycarbonyl; DELT or deltorphin C, [D-Ala^2]deltorphin I (Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2); 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; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MVD, mouse vas deferens; pA_2, negative log of the molar concentration required to double the agonist concentration to achieve the original response; TFA, trifluoroacetic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TIP(P), H-Tyr-Tic-Phe-(Phe)-OH; TLC, thin-layer chromatography; WSC, 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide·HCl.

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