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6-*N*,*N*-Dimethylamino-2,3-naphthalimide: A New Environment-Sensitive Fluorescent Probe in δ- and μ-Selective Opioid Peptides

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A new environment-sensitive fluorophore, 6-*N*,*N*-(dimethylamino)-2,3-naphthalimide (6DMN) was introduced in the δ-selective opioid peptide agonist H-Dmt-Tic-Glu-NH_2 and in the μ-selective opioid peptide agonist endomorphin-2 (H-Tyr-Pro-Phe-Phe-NH_2). Environment-sensitive fluorophores are a special class of chromophores that generally exhibit a low quantum yield in aqueous solution but become highly fluorescent in nonpolar solvents or when bound to hydrophobic sites in proteins or membranes. New fluorescent δ-selective irreversible antagonists (H-Dmt-Tic-Glu-NH-$(CH_2)_5$-CO-Dap(6DMN)-NH_2 (**1**) and H-Dmt-Tic-Glu-Dap(6DMN)-NH_2 (**2**)) were identified as potential fluorescent probes showing good properties for use in studies of distribution and internalization of δ receptors by confocal laser scanning microscopy.

Introduction

Fluorescence spectroscopy has become one of the most valuable tools for the development of new probes for biochemical research,[1,2] being extensively used for monitoring ions,[3] small molecules,[4] and biological processes,[5] such as protein folding,[6] protein−protein interactions,[7] and phosphorylation events.[8] Whereas many fluorescence applications rely on the use of intrinsic fluorophores, the development of new extrinsic fluorophores remains an essential element for the design of new fluorescent probes. Environment-sensitive fluorophores are a special class of chromophores whose spectroscopic behavior depends on the physicochemical properties of its immediate environment.[9] These molecules generally exhibit a low quantum yield in aqueous solution but become highly fluorescent in nonpolar solvents or when bound to hydrophobic sites in proteins or membranes. Particularly useful are the solvatochromic fluorophores that display sensitivity to the polarity of the local environment, such as the 2-propionyl-6-dimethylaminonaphthalene (PRODAN),[10] 4-dimethylamino phthalimide (4-DMAP),[11] and 4-amino-1,8-naphthalimide derivatives.[12] Prodan and derivatives (Aladan, an alanine derivative of 6-(dimethylamino)-2-acyl-naphthalene)[13,14] still constitute the most widely used environment-sensitive fluorophores, regardless of certain limitations mainly resulting from the relatively intense fluorescence even in aqueous environments.

Peptide ligands for opioid receptors were previously labeled with fluorescent functionalities, such as rhodamine,[15] pyrene,[16] dansyl,[17,18] and fluorescein.[19,20] 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 receptor binding.[21] Other studies show that a nonpeptidic 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.[20] However, with the exception of the arylacetamide-derived fluorescent ligands,[22] 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) studied.

Schiller et al. reported the synthesis of the μ-opioid peptide [Dmt^1]DALDA (H-Dmt-D-Arg-Phe-Lys-NH_2) containing dansyl or anthranoyl fluorophores,[17,18] and subsequently, this group published the synthesis of a fluorescent δ-opioid peptide containing the environment-sensitive amino acid Aladan (H-Tyr-Tic-Aladan-Phe-OH) (K_i (δ) = 2.56 nM, selectivity μ/δ = 7930, Φ = 0.150 in Tris-HCl buffer at pH 6.6).[14] Furthermore, our group reported the synthesis of a δ-selective tripeptide containing fluorescein at the *C*-terminal (H-Dmt-Tic-Glu-NH-$(CH_2)_5$-NH-C(=S)-NH-fluorescein (K_i (δ) = 0.035 nM, selectivity μ/δ = 4370, Φ = 0.227 in Tris-HCl buffer at pH 6.6). [23]

Recently, Imperiali et al. published the synthesis of the new environment-sensitive fluorophore 6-*N*,*N*-(dimethylamino)-2,3-naphthalimide (6DMN) and the corresponding N-protected amino acid Fmoc-α-amino-β-(6-*N*,*N*-dimethylamino naphthalimide) propanoic acid (Fmoc-Dap(6DMN)-OH).[24] They demonstrated that the corresponding model compound methyl 2-(6-(dimethylamino)-1,3-dioxo-1*H*-benzo[*f*]isoindol-2(3*H*)-yl)-acetate (6DMN-Gly-OMe) had a red-shifted fluorescence in polar protic environments, with a maximum emission intensity that shifted from 491 nm in toluene to 592 nm in water, and the fluorescence quantum yield (Φ) decreased more than 100-fold from chloroform (0.225) to water (0.002). The 6DMN

* To whom correspondence should be addressed. Tel: +39-532-291-275. Fax: +39-532-291-296. E-mail: gbalboni@unica.it; bbg@unife.it.
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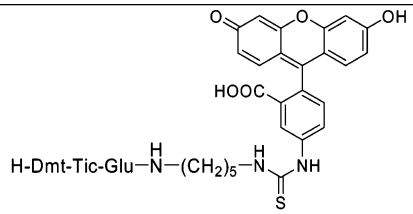
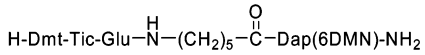
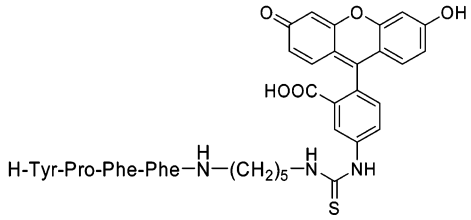
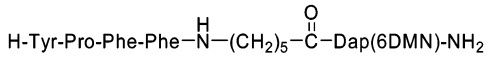
Table 1. Receptor Binding and Functional Bioactivity

No.	Structure	Receptor affinity [a] (nM) K_i^δ	K_i^μ	μ/δ	δ/μ	Functional bioactivity (nM) MVD IC_{50} [b]	MVD K_e [c]	GPI IC_{50} [b]
	 *Reference Compound*	*0.035* [d]	*152* [d]	*4370* [d]			*Irreversible antagonist* [d]	*>1000* [d]
1		0.158±0.027 (3)	41.3±1.09 (3)	261			Irreversible antagonist > 0.3 nM	244±35
2	H-Dmt-Tic-Glu-Dap(6DMN)-NH_2	7.80±0.29 (4)	7880±1139 (4)	1010			Irreversible antagonist > 10 nM	Partial agonist (max 40%) IC_{50} = 100 nM
3		668.4 ±123 (6)	251.7±90 (6)		2.7	Partial agonist (max 20%) IC_{50} = 200 nM		120±8
4		5937±1396 (4)	244.5±14 (5)		24	Partial agonist (max 29%) IC_{50} = 300 nM		253±15
5	H-Tyr-Pro-Dap(6DMN)-Phe-NH_2	839±38 (3)	3420±817 (3)	4.1		Partial agonist (max 22%) IC_{50} = 500nM		389±12

[a] The K_i values (nM) were determined according to Chang and Prusoff[37] 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] Agonist activity was expressed as the IC_{50} values obtained from dose−response curves. These values represent the mean ± SE for at least five fresh tissue semples. Deltorphin C and dermorphin were the internal standards for MVD (δ-opioid receptor bioactivity) and GPI (μ-opioid receptor bioactivity) tissue preparation, respectively. [c] The K_e (equilibrium dissociation constant) values of opioid antagonists against the agonists (deltorphin C and dermorphin) were determined by the method of Kosterlitz and Watt.[40] [d] Data taken from Balboni, G. et al.[23]

fluorophore combines some of the advantageous fluorescence properties of PRODAN with the extreme sensitivity to the local polarity exhibited by the 4-aminophthalimide family of the environment-sensitive fluorophores.[11] Such environment-sensitive molecules could be useful in confocal microscopy studies, where flurescence will be higher for opioid−receptor interactions and lower for nonspecific binding.

In this article, we evaluate the opportunities offered by this new fluorescent amino acid, starting from the data we collected with our reference compound H-Dmt-Tic-Glu-NH-$(CH_2)_5$-NH-C(=S)-NH-fluorescein.[23] We report the synthesis, biological evaluation, and in situ visualization of δ-opioid receptors in mouse neuroblastoma cells of μ- and δ-selective opioid peptides derived from endomorphin-2[25] and Dmt-Tic pharmacophore, respectively (Table 1).[26]

Chemistry

The peptides, including the fluorescent-sensitive amino acid (**1**, **2**, **4**, and **5**), were synthesized using standard Fmoc solid-phase peptide synthesis (SPPS), cleaved/deprotected from the solid support, and purified by HPLC as detailed in the Experimental Section. Compound **3**, containing fluorescein, was prepared by standard solution peptide synthesis as reported in Scheme 1, Supporting Information. Boc-Tyr-Pro[27] was condensed with H-Phe-Phe-OBzl[28] via WSC/HOBt. After *C*-terminal benzyl ester deprotection by catalytic hydrogenation (Pd/C, 10%), *N-Z*-1,5-pentanediamine was condensed (WSC/HOBt). The *N-Z* deprotection by catalytic hydrogenation (Pd/C, 10%) gave the intermediate Boc-Tyr-Pro-Phe-Phe-NH-$(CH_2)_5$-NH_2 suitable for the reaction with fluorescein 5-isothiocyanate.[23] Final *N*-Boc-deprotection with TFA gave the final crude product (**3**) that was purified by preparative HPLC as detailed in the Supporting Information.

Results and Discussion

In Vitro Opioid Activity Profile. Receptor binding and functional bioactivity are reported in Table 1. In comparison to the reference δ-selective fluorescent compound derivative,[23] **1** is characterized by the substitutions of 1,5-pentanediamine, introduced to reduce the potential interference with the opioid receptors, with 6-aminohexanoic acid (the same number of methylene units) and fluorescein by the new fluorescent probe Dap(6DMN). These modifications are detrimental for the binding and selectivity of δ-opioid receptors. In fact, the affinity for δ-opioid receptors decreases 4.5-fold, whereas that for μ-opioid receptors increases 3.7-fold, yielding a loss of selectivity of about 17-fold. Unexpectedly, **2**, the same analogue without the pentamethylene spacer, decreased δ-affinity by 49-fold but increased selectivity by 3.9-fold (K_i^δ = 7.8 nM, selectivity = 1010) relative to that of **1**. Compounds **3**−**5** depict three attempts to synthesize fluorescent derivatives selective for μ-opioid receptors; only few examples are reported in the literature. In fact, the last active but not selective μ peptides synthesized were reported by Schiller et al.;[17,18] the fluorescent analogues of [Dmt[1]]DALDA were endowed with high μ affinity (K_i^μ ranging from 0.508 to 0.589 nM) but exhibited very low selectivities (K_i^δ/K_i^μ = 3.2−6.1). Compd **4** coincides with **1**, where the δ-selective tripeptide Dmt-Tic-Glu is substituted by the μ-selective tetrapeptide Tyr-Pro-Phe-Phe corresponding to the sequence of endomorphin-2. This new analogue (**4**), despite its low μ affinity (K_i^μ = 244.5 nM), is characterized by a selectivity (K_i^δ/K_i^μ = 24) better than that of other published μ-selective

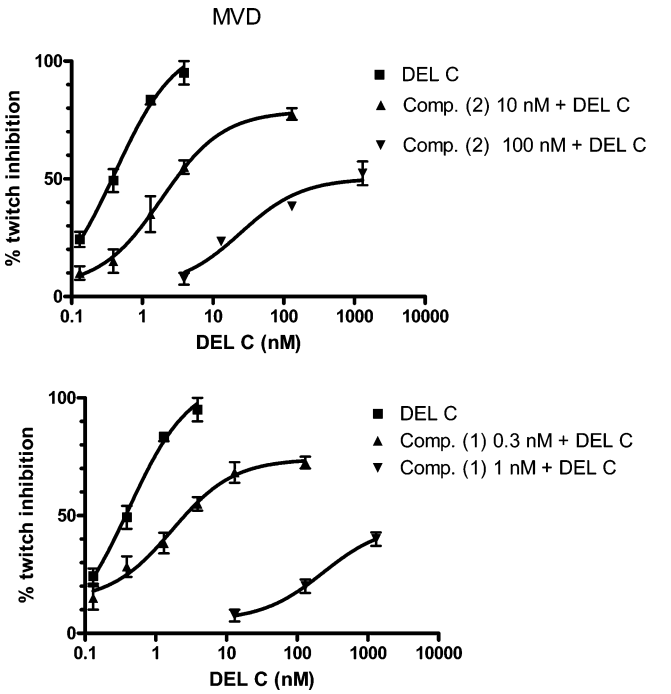


Figure 1. Functional bioactivity of compounds **1** and **2** on MVD. The inhibition of DEL C-induced twitch by (**2**) (10 and 100 nM) and (**1**) (0.3 and 1 nM) were conducted as given in the Experimental Section using MVD preparations.

compounds.[29] However, **3** is comparable to the reference compound, where the δ-selective tripeptide Dmt-Tic-Glu was substituted by the μ-selective tetrapeptide Tyr-Pro-Phe-Phe. The endomorphin-2 analogue (**5**) (H-Tyr-Pro-Dap(6DMN)-Phe-NH_2) is derived from a similar modification reported in the δ-selective tetrapeptide TIPP, where Phe^3 is substituted by the fluorescent amino acid Aladan to yield [$Aladan^3$]TIPP.[14] Considering this modification, we substituted Dap(6DMN) in the third position of the endomorphin-2 sequence; however, neither of these two compounds (**4** and **5**) had negligible affinity and selectivity for μ- and δ-opioid receptors.

Compounds **1**−**5** were tested for functional bioactivity in MVD and GPI preparations (Table 1). Interestingly, these fluorescent derivatives (**1** and **2**) and the reference compound in the MVD assay demonstrated nonequilibrium antagonist activity (Figures 1 and 2). The log dose−response curves of deltorphin C (δ agonist) in the presence of increasing concentrations of **1** or **2** reduced the apparent efficacy and Hill slope (deltorphin C = 1.1; +0.3 nM (**1**) = 0.7 and at +1 nM, (**1**) = 0.5; at +10 nM, (**2**) = 0.9 and at +100 nM, (**2**) = 0.6). Both compounds bind tightly and dissociate very slowly in the tissue preparation. The δ antagonism could not be reversed by washing the tissue with a drug-free solution for over a 3 h period of time. Moreover, the longer the compound remained in contact with the tissue, the greater was the magnitude of the observed δ antagonism. Compd **1**, containing the pentamethylene spacer between the opioid sequence and the fluorescent amino acid, demonstrated a δ-irreversible antagonism starting from a concentration of 0.3 nM, whereas the corresponding derivative, lacking the pentamethylene spacer (**2**), exhibited an irreversible δ-antagonist activity that began at a 30-fold higher concentration (10 nM), confirming the importance of the spacer between the opioid pharmacophore and the fluorophore.

In the GPI assay, **1** and **2** are endowed with a weak agonist or partial agonist activity, which is in agreement with their affinity data and selectivity. Compd **3**, projected as an analogue of the reference compound modified to have potential μ-opioid selectivity, and peptides **4** and **5** failed to elicit opioid activity, confirming the difficulty in obtaining biologically functional fluorescent μ-ligands based on an endomorphin scaffold.

Fluorescence Detection. The visualization of δ-opioid receptors in situ with our fluorescent probe was obtained by incubating (15 min at 35 °C) the fluorescent compound (**1**) (20 nM) with the NG108-15 (mouse N18 neuroblastoma × rat C6 glioma) cell line, which expresses mouse δ-opioid receptors. Figure 2 shows the fluorescent photomicrographs obtained using **1** in the absence (left panel) or in the presence (right panel) of the nonspecific opioid-receptor antagonist naloxone (20 μM); preincubation with naloxone (10 min) essentially abolished the fluorescence bound to δ-opioid receptors. This illustrates the advantage of using environment-sensitive fluorophores with low Φ in the unbound state. This results in a lower background signal from the free probe and, therefore, better sensitivity and spatial resolution.

Fluorescence Quantum Yield Measurements. To probe the environment affecting the sensitivity of the fluorescent peptides, the emission spectra and the fluorescent quantum yields of **1** were recorded in solvents of different polarity: water (Tris-HCl buffer at pH = 6.6), acetonitrile, and dichloromethane. Fluorescence quantum yields were calculated with respect to quinine sulfate in 0.5 M H_2SO_4 as a standard (Φ = 0.546).[30] Steady-state fluorescence parameters of **1** and **2** are reported in Table 2. Solutions of both the samples and the reference compound were prepared by dilution in the appropriate solvent from the primary solutions whose absorbance was below 0.2 at the same excitation wavelength (350 nm). Fluorescent measurements were taken for each solution with the same instrument parameters, and the fluorescence spectra were corrected for instrumental response before integration. The integrated corrected emission spectra values were plotted against the absorbance data, and least-squares fit was used to fit the data to a straight line. The slope of the best-fit line was assumed to be proportional to the emission quantum yield. The yield for each sample was calculated as detailed in the Experimental Section. For samples in aqueous solution, the correction for refractive index was assumed to be insignificant. The spectroscopic behavior of **1** resembled that of the model compound 6DMN-GlyOMe reported by Imperiali et al.;[24] the lower energy absorption maxima, around 380 nm, showed only a small dependence on solvent polarity, whereas emission maxima followed the trend observed for 6DMN-GlyOMe in CH_3CN and CH_2Cl_2. The emission maxima found in water at pH 6.6 for **1** and **2** (λ_{em} = 467 and 464 nm, respectively) are significantly blue shifted with respect to those expected on the basis of the previously reported behavior of the fluorophore in protic solvents and water at neutral pH. This could be attributed to the role of protonation inside the compound, which might have a strong influence on the electronic structure of the excited state.

Conclusion

The incorporation of the amino acid H-Dap(6DMN)-OH containing the environment-sensitive fluorophore 6-*N*,*N*-(dimethylamino)-2.3-naphthalimide (6DMN) into compounds containing the δ-opioid antagonist pharmacophore Dmt-Tic yielded selective probes (K_i^{δ}/K_i^{μ} = 260 and 1000 relative to that of the μ-opioid receptor), which bound as apparent irreversible antagonists, to δ-opioid receptors in the membranes of NG108-15 cells. The maintenance of the opioid binding properties and fluorescence parameters indicated that the fluorophore had a weak effect on δ-receptor interaction. Our fluorescent conjugates **1** and **2** could be applied as specific probes to study the in vitro

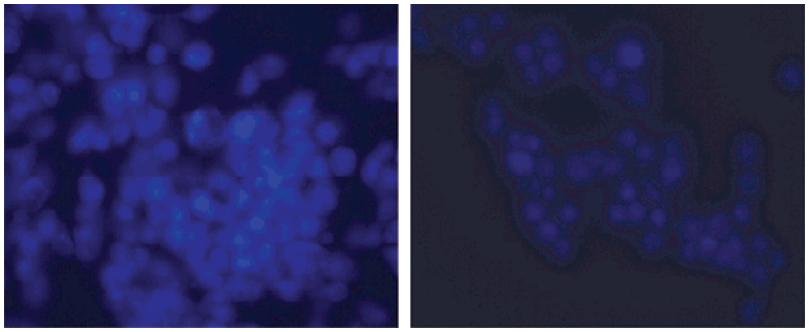


Figure 2. Microscopic visualization of compound **1** in NG108-15 cells. Left panel: NG108-15 cells with fluorescence compound. Right panel: nonspecific binding obtained by preincubation with the opioid receptor antagonist naloxone.

Table 2. Steady State Fluorescence Parameters of Compounds **1** and **2**[a]

no.	λ_{em} (nm)	$\lambda_{em,corr}$ (nm)	Φ
reference	520[a]	522[a]	0.24 ± 0.01[a]
	464[a]	464[a]	0.012 ± 0.001[a]
1	557[b]	571[b]	0.194 ± 0.01[b]
	534[c]	541[c]	0.396 ± 0.01[c]
	467[a]	467[a]	0.022 ± 0.001[a]
2	554[b]	567[b]	0.192 ± 0.01[b]
	531[c]	538[c]	0.394 ± 0.01[c]

[a] Recorded in 50 mM Tris-HCl buffer (pH 6.6, 20 °C); λ = 350 nm. [b] Recorded in CH_3CN. [c] Recorded in CH_2Cl_2.

localization of δ-opioid receptors in tissues, receptor internalization, and the trafficking in live cells in real time by using confocal microscopy.[29] It is important to emphasize that environment-sensitive fluorophores become highly fluorescent in either nonpolar solvents or when bound to hydrophobic sites in proteins or membranes. Therefore, there will be some background signal resulting from the nonspecific binding of the probes to lipid membranes that will have to be taken into account. Such environment-sensitive molecules could be useful in confocal microscopy studies, where flurescence will be higher for interactions between the opioid ligand and its receptor and lower for nonspecific binding.

The comparison between the environment-nonsensitive fluorophore (reference compound) and the confocal microscopic images obtained after treatment with the environment-sensitive fluorophore **1** could be useful for various applications, including studies on membrane interactions, binding to receptors, cellular uptake and intracellular distribution, and tissue distribution.

Experimental Section

All peptide synthesis reagents and amino acid derivatives were purchased from GL Biochem (Shanghai) and Novabiochem. *C*-terminal amide peptides were synthesized on Fmoc-PAL-PEG-PS resin from Applied Biosystems, and all other chemicals were purchased from Aldrich or Fluka. High-performance liquid chromatography was performed using an Agilent 1100 series Liquid Chromatograph Mass Spectrometer system. Analytical HPLC was run using LiChrospher RP-18 (5 μm) 4.6 × 150 mm analytical column from Merck. The purification of the peptides was performed on a ZORBAX ODS C_{18}, 94 × 250 mm reverse-phase column. The standard gradient used for analytical and preparative HPLC was 90:10 to 20:80 over 30 min (water/acetonitrile, 0.1% TFA).

Electrospray Ionization Mass Spectrometry (ESI/MS) was performed with an Agilent 1100 Series LC/MSD model in positive scan mode using direct injection of the purified peptide solution into the MS. ^{1}H NMR (δ) spectra were measured, when not specified, in DMSO-d_6 (Bruker AC-200 spectrometer), and the peaks are parts per million downfield from tetramethylsilane (internal standard). 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/methanol/toluene (17:1:2), and ninhydrin (1%, Merck), fluorescamine (Hoffman-La Roche), and chlorine reagents were used as sprays. The 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.

Peptide synthesis used standard SPPS protocols on a 0.02 to 0.05 mmol scale using a 0.21 mmol/g loading PAL-PEG-PS solid support. Amino acids were purchased as protected Fmoc amino acids with the standard side chain protecting scheme: Fmoc-6-aminohexanoic acid, Fmoc-Glu(OtBu)-OH, Fmoc-Tic-OH, Fmoc-Phe-OH, Fmoc-Pro-OH. Boc-Tyr(tBu)-OH and Boc-Dmt-OH[31] were used as *N*-terminal amino acids. The synthesis of Fmoc-Dap-(6DMN)-OH fluorescent building block was made using previously reported procedures.[24]

Amino acids were manually coupled in 4-fold excess using a mixture of *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium (HBTU) and 1-hidroxybenzotriazole (HOBt) and *N*,*N*-diisopropylethylamine (DIEA) in DMF as activating agents. The coupling of Fmoc-Dap(6DMN)-OH was performed using 2-(1*H*-7-aza-benzotriazol-1-*yl*)-1,1,3,3-tetramethyluronium hexafluorophosphate methanaminium (HATU) but otherwise using the same protocol as the other amino acids. Each amino acid was activated for two minutes in DMF before being added onto the resin. Peptide-bond-forming couplings were conducted for 1 h and monitored using the TNBS test.[32] The deprotection of the temporal Fmoc protecting group was performed by treating the resin with 20% piperidine in DMF solution for 15 min.

The peptides were cleaved from the resin, and side-chain protecting groups were simultaneously removed by treatment with the following cleavage mixture: 50 μL of dichloromethane, 25 μL of triisopropyl silane, 25 μL of water, and 950 μL of TFA (1 mL of mixture/50 mg of resin) for 1.5−2 h at room temperature. All peptides were precipitated with diethyl ether (4 °C) and further purified by reverse-phase HPLC.

Deprotection. To 0.05 mmol of Fmoc-HN-$(aa)_n$ in solid support, piperidine (3 mL, 20% in DMF) was added, and nitrogen was passed through the mixture for 15 min. The resin was then filtered and washed with DMF (3 × 3 mL × 3 min), and the TNBS test was run with a small resin sample to confirm that deprotection was successful.

Coupling. Fmoc-aa-OH (0.2 mmol) was dissolved in an HOBt/HBTU solution (1 mL of 0.2 M HBTU, 0.2M HOBt in DMF), and DIEA (1.5 mL of 0.195 M solution in DMF) was added. The resulting mixture was activated for 2 min and then added over the previously deprotected resin. Nitrogen was passed through the resin suspension for 1 h, when the TNBS test of a small resin sample was negative. The resin was then washed with DMF (3 × 3 mL × 3 min) and subjected to the following deprotection/coupling cycles in a similar way.

Coupling of Fmoc-Dap(6DMN)-OH. Fmoc-Dap(6DMN)-OH (55 mg, 0.1 mmol) and HATU (38 mg, 0.1 mmol) were dissolved in DMF (0.5 mL), and DIEA (0.75 mL of 0.195 M solution in DMF) was added, the resulting mixture was activated for 2 min,

and then added over the resin. Nitrogen was passed through the resin suspension for 1 h, when the TNBS test of a small resin sample was negative. The resin was then washed with DMF (3 × 3 mL × 3 min) and recoupled with Fmoc-Dap(6DMN)-OH following the same protocol described above. The resin was then subjected to the following deprotection/coupling cycles using the HOBt/HBTU coupling scheme.

Cleavage. The resin-bound peptide dried overnight (0.05 mmol) was placed in a 50 mL flask, to which was added 5 mL of the cleavage cocktail (250 μL of CH_2Cl_2, 125 μL of water, 125 μL of TIS, and TFA to 5 mL), and the resulting mixture was shaken for 2 h, the resin was filtered, and the TFA filtrate was concentrated under an argon current until a volume of 2 mL. Then it was added to ice-cold ethyl ether (40 mL). After 5 min, the peptide was centrifuged and washed again with 20 mL of ice-cold ether. The solid residue was dried under Ar and redissolved in acetonitrile/water 1:1 (2 mL), and purified by preparative reverse-phase HPLC. The collected fractions were lyophilized and stored at −20 °C.

H-Dmt-Tic-Glu-NH-$(CH_2)_5$-(C=O)-Dap(6DMN)-NH_2 (1). *Rf*(A) 0.72; HPLC *K′* 7.91; mp 161−163 °C; $[\alpha]^{20}_D$ −7.8; MH^+ 920; ^{1}H NMR (DMSO-d_6) δ 1.29−1.57 (m, 6H), 2.06−3.20 (m, 24H), 3.95−5.16 (m, 8H), 6.29−8.42 (m, 11H).

H-Dmt-Tic-Glu-Dap(6DMN)-NH_2 (2). *Rf*(A) 0.65; HPLC *K′* 7.38; mp 157−159 °C; $[\alpha]^{20}_D$ −9.2; MH^+ 807; ^{1}H NMR (DMSO-d_6) δ 2.06−3.17 (m, 20H), 3.95−5.16 (m, 8H), 6.29−8.42 (m, 11H).

TFA·H-Tyr-Pro-Phe-Phe-NH-$(CH_2)_5$-NH-(C=S)-NH-fluorescein (3). *Rf*(A) 0.66; HPLC *K′* 7.26; mp 153−155 °C; $[\alpha]^{20}_D$ −15.1; MH^+ 1047; ^{1}H NMR (DMSO-d_6) δ 1.29−1.55 (m, 6H), 1.92−2.34 (m, 4H), 2.92−3.29 (m, 8H), 3.41−3.51 (m, 4H), 3.95−4.92 (m, 4H), 6.11−7.28 (m, 23H).

H-Tyr-Pro-Phe-Phe-NH-$(CH_2)_5$-(C=O)-Dap(6DMN)-NH_2 (4). *Rf*(A) 0.74; HPLC *K′* 7.96; mp 165−167 °C; $[\alpha]^{20}_D$ −13.8; MH^+ 995; ^{1}H NMR (DMSO-d_6) δ 1.29−1.57 (m, 6H), 1.92−2.34 (m, 6H), 2.85−3.20 (m, 14H), 3.41−3.51 (m, 2H), 3.95−5.16 (m, 7H), 6.68−8.37 (m, 19H).

H-Tyr-Pro-Dap(6DMN)-Phe-NH_2 (5). *Rf*(A) 0.69; HPLC *K′* 7.48; mp 160−162 °C; $[\alpha]^{20}_D$ −14.3; MH^+ 735; ^{1}H NMR (DMSO-d_6) δ 1.92−2.34 (m, 4H), 2.92−3.17 (m, 10H), 3.41−3.51 (m, 2H), 3.95−5.16 (m, 6H), 6.68−8.42 (m, 14H).

Elemental analysis of compounds **1**-**5** is reported in Supporting Information, Table 3.

Competitive Receptor Binding Assays. These assays were conducted as described in detail elsewhere using rat brain synaptosomes (P2 fraction).[31,33−35] Membrane preparations were preincubated to eliminate endogenous opioid peptides and stored at −80 °C in buffered 20% glycerol.[34,36] 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). An unlabeled peptide (2 μM) was used to determine nonspecific binding in the presence of 1.9 nM [^{3}H]deltorphin C (45.0 Ci/mmol, Perkin-Elmer, Boston, MA; K_D = 1.4 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 were washed thrice in ice-cold buffered BSA.[34] The affinity constants (K_i) were calculated according to Cheng and Prusoff.[37]

Functional Bioactivity in Isolated Organ Preparations. The preparations of myenteric plexus-longitudinal muscle obtained from the male guinea pig ileum (GPI, enriched in μ-opioid receptors) and the preparations of MVD (containing δ-opioid receptors) were used for field stimulation with bipolar rectangular pulses of supramaximal voltage.[38] Agonists were evaluated for their ability to inhibit the electrically evoked twitch. The biological potency of the compounds was compared against the activity of the μ-opioid receptor agonist dermorphin in GPI and with MVD for the δ-opioid receptor measured agonist deltorphin C. The results are expressed as IC_{50} values obtained from dose−response curves (Prism, GraphPad). To evaluate antagonism, the compounds were added to the bath and allowed to interact with tissue receptor sites for 5 min before adding the standard peptide. 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.[39] IC_{50} (nM, mean ± SE) as well as the pA_2 values were obtained from at least six experiments conducted with fresh tissues.

Fluorescence Spectroscopy. Fluorescence quantum yields were calculated with respect to quinine sulfate (Fluka) in 0.5 M H_2SO_4 as a standard ($\phi = 0.546$).[30] Solutions of both the sample and the reference were prepared by dilution with the appropriate solvent of mother solutions whose absorbance was below 0.2 at the same excitation wavelength (350 nm). Fluorescence measurements were taken for each solution with the same instrument parameters, and the fluorescence spectra were corrected for instrumental response before integration. The integrated corrected emission spectra values were plotted against the absorbance, and data were least-squares fitted to a straight line. The slope of the best-fit line was assumed to be proportional to the emission quantum yield. The yield for each sample was calculated according to the formula

$$\phi_S = \phi_R \frac{m_S}{m_R}\left(\frac{\eta_S}{\eta_R}\right)^2$$

where ϕ is the emission quantum yield, m is the slope of the best-fit line, and η is the refractive index of the solvent for the sample (S) and the reference (R), respectively. Absorption spectra were recorded with a Jasco V-570 UV−Vis-NIR spectrophotometer, fluorescence spectra were obtained with a Jobin-Yvon FluoroMax-2 spectrofluorometer. To detect the fluorescent signal, a Nikon Optiphot microscope (Hoechest filter EX 350 nm, EM 467 nm) was used with a 40× magnification.

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Supporting Information Available: Experimental details for the synthesis of compound **3**and references cited herein; elemental analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Abbreviations: in addition to the IUPAC-IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* **1985**, *260*, 14−42), this article uses the following additional symbols and abbreviations: Ac, acetyl; Bid, 1*H*-benzimidazol-2-yl; Boc, *tert*-butyloxycarbonyl; DAMGO, [D-Ala^2,*N*-Me-Phe^4, Gly-ol^5] enkephalin; DEL C, deltorphin C, (H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2); DER, dermorphin (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH2); DMF, *N*,*N*-dimethylformamide; DMSO-d_6, hexadeuteriodimethyl sulfoxide; Dmt, 2′,6′-dimethyl-L-tyrosine; 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; NMM, 4-methylmorpholine; 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; TIPP, H.-Tyr-Tic-Phe-Phe-OH.; TIS, triisopropylsilane; TLC, thin-layer chromatography; TNBS, 2,4,6-trinitrobenzene sulfonic acid; WSC = EDC, 1-ethyl-3-[3′-dimethyl)aminopropyl]-carbodiimide hydrochloride; Z, benzyloxycarbonyl.

(2) Zhang, J.; Campbell, R. E.; Ting, A. Y.; Tsien, R. Y. Creating new fluorescent probes for cell biology. *Nat. Rev. Mol. Cell. Biol.* **2002**, *3*, 906−918.
(3) Jiang, P.; Guo, Z. Fluorescent detection of zinc in biological systems: recent development on the design of chemosensors and biosensors. *Coord. Chem. Rev.* **2004**, *248*, 205−229.
(4) Chan, P.-H.; Liu, H.-B.; Chen, Y. W.; Chan, K.-C.; Tsang, C.-W.; Tsang, C.-W.; Leung, Y.-C.; Wong, K. Y. Rational design of a novel fluorescent biosensor for β-lactam antibiotics from a class A β-lactamase. *J. Am. Chem. Soc.* **2004**, *126*, 4074−4075.
(5) Lippincott-Schwartz, J.; Patterson, G. H. Development and use of fluorescent protein markers in living cells. *Science* **2003**, *300*, 87−91.
(6) Heyduk, T. Measuring protein conformational changes by FRET/LRET. *Curr. Opin. Biotechnol.* **2002**, *13*, 292−296.
(7) Jameson, D. M.; Croney, J. C.; Moens, P. D. J. [1] Fluorescence: basic concepts, practical aspects, and some anecdotes. *Methods Enzymol.* **2003**, *360*, 1−43.
(8) Chen, C.-A.; Yeh, R.-H.; Yan, X.; Lawrence, D. S. Biosensors of protein kinase action: from in vitro assays to living cells. *Biochim. Biophys. Acta* **2004**, *1697*, 39−51.
(9) Valeur, B. *Molecular Fluorescence: Principles and Applications*; Wiley-VCH: Weinheim, Germany, 2002.
(10) Weber, G.; Farris, F. J. Synthesis and spectral properties of a hydrophobic fluorescent probe 6-propionyl-2-(dimethylamino)naphthalene. *Biochemistry* **1979**, *18*, 3075−3078.
(11) (a) Saroja, G.; Soujanya, T.; Ramachandran, B.; Samanta, A. 4-Aminophthalimide derivatives as environment-sensitive probes *J. Fluoresc.* **1998**, *8*, 405−410. (b) Vázquez, M. E.; Rothman, D. M.; Imperiali, B. A new environment-sensitive fluorescent amino acid for Fmoc-based solid-phase peptide synthesis. *Org. Biomol. Chem.* **2004**, *2*, 1965−1966.
(12) Grabchev, I.; Chovelon, J.-M.; Qian, X. A copolymer of 4-*N*,*N*-dimethylaminoethylene-*N*-allyl-1,8-naphthalimide with methylmethacrylate as a selective fluorescent chemosensor in homogeneous systems for metal cations. *J. Photochem. Photobiol., A* **2003**, *158*, 37−43.
(13) (a) Cohen, B. E.; McAnaney, T. B.; Park, E. S.; Jan, Y. N.; Boxer, S. G.; Jan, L. Y. Probing protein electrostatics with a synthetic fluorescent amino acid. *Science* **2002**, *296*, 1700−1703. (b) Vázquez, M. E.; Nitz, M.; Stehn, J.; Yaffe, M. B.; Imperiali, B. Fluorescent caged phosphoserine peptides as probes to investigate phosphorylation-dependent protein associations. *J. Am. Chem. Soc.* **2003**, *125*, 10150−10151.
(14) Chen, H.; Chung, N. N.; Lemieux, C.; Zelent, B.; Vanderkooi, J. M.; Gryczynski, I.; Wilkes, B. C.; Schiller, P. W. [Aladan^3]TIPP: a fluorescent δ-opioid antagonist with high δ-receptor binding affinity and δ selectivity *Biopolymers* **2005**, *80*, 325−331.
(15) Hazzum, E.; Chang, K. J.; Shecter, Y.; Wilkinson, S.; Cautrecasas, P. Fluorescent and photoaffinity enkephalin derivatives: preparation and interaction with opiate receptors. *Biochem. Biophys. Res. Commun.* **1979**, *88*, 841−846.
(16) Mihara, H.; Lee, S.; Shimohigashi, Y.; Aoyagi, H.; Kato, T.; Izumiya, N.; Costa, T. μ and δ Opioid receptor probes: fluorescent enkephalins with high receptor affinity and specificity. *FEBS Lett.* **1985**, *193*, 35−38.
(17) 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.
(18) Berezowska, I.; Lemieux, C.; Chung, N. N.; Zelent, B.; Schiller, P. W. Dansylated analogues of the opioid peptide [Dmt1]DALDA: in vitro activity profiles and fluorescence parameters. *Acta Biochim. Pol. (Engl. Transl.)* **2004**, *51*, 107−113.
(19) Goldstein, A.; Nestor, J. J.; Naidu, A.; Newman, S. R. "DAKLI": a multipurpose ligand with high affinity and selectivity for dynorphin (κ opioid) binding sites. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 7375−7379.
(20) Kshirsagar, T.; Nakano, A. H:, Law, P.-Y.; Elde, R.; Portoghese, P. S. NTI4F: a non-peptide fluorescent probe selective for functional delta opioid receptors. *Neurosci. Lett.* **1998**, *249*, 83−86.
(21) Kumar, V.; Murray, T. F.; Aldrich, J. V. Extended TIP(P) analogues as precursors for labeled δ-opioid receptor ligands. *J. Med. Chem.* **2000**, *43*, 5050−5054.
(22) Chang, A.-C.; Chao, C. C.; Takemori, A. E.; Gekker, G.; Hu, S.; Peterson, P. K.; Portoghese, P. S. Arylacetamide-derived fluorescent probes: synthesis, biological evaluation, and direct fluorescent labeling of δ opioid receptors in mouse microglial cells. *J. Med. Chem.* **1996**, *39*, 1729−1735.
(23) Balboni, G.; Salvadori, S.; Dal Piaz, A.; Bortolotti, F.; Argazzi, R.; Negri, L.; Lattanzi, R.; Bryant, S. D.; Jnsmaa, Y.; Lazarus, L. H. Highly selective fluorescent analogue of the potent δ-opioid receptor antagonist Dmt-Tic. *J. Med. Chem.* **2004**, *47*, 6541−6546.
(24) Vázquez, M. E.; Blanco, J. B.; Imperiali, B. Photophysics and biological applications of the environment-sensitive fluorophore 6-*N*,*N*-(dimethylamino)-2.3-naphthalimide. *J. Am. Chem. Soc.* **2005**, *127*, 1300−1306.
(25) Zadina, J. E.; Hackler, L.; Ge, L. J.; Kastin, A. J. A potent selective endogenous agonist for the mu-opiate receptor. *Nature* **1997**, *386*, 499−502.
(26) Balboni, G.; Salvadori, S.; Guerrini, R.; Negri, L.; Giannini, E.; Bryant, S. D.; Jinsmaa, Y.; Lazarus, L. H. Direct influence of *C*-terminally substituted amino acids in the Dmt-Tic pharmacophore on δ-opioid receptor selectivity and antagonism. *J. Med. Chem.* **2004**, *47*, 4066−4071.
(27) Salvadori, S.; Marastoni, M.; Balboni, G.; Borea, P.; Tomatis, R. Opioid peptides: synthesis and biological properties of dermorphin related hexapeptides. *Eur. J. Med. Chem.* **1990**, *25*, 171−177.
(28) Oya, M.; Takahashi, T. The steric hindrance of the stepwise reaction of N-carboxy alpha amino acid anhydride with the alpha amino acid ester *Bull. Chem. Soc. Jpn.* **1981**, *54*, 2705−2707.
(29) Arttamangkul, S.; Alvarez-Maubecin, V.; Thomas, G.; Williams, J. T.; Grandy, D. K. Binding and internalization of fluorescent opioid peptide conjugates in living cells. *Mol. Pharmacol.* **2000**, *58*, 1570−1580.
(30) Meech, S. R.; Phillips, D. Photophysics of some common fluorescence standards. *J. Photochem.* **1983**, *23*, 193−217.
(31) Salvadori, S.; Guerrini, R.; Balboni, G.; Bianchi, C.; Bryant, S. D.; Copper, P. S.; Lazarus, L. H. Further studies on the Dmt-Tic pharmacophore: Hydrophobic substituents at the *C*-terminus endow δ antagonists to manifest μ agonism or μ antagonism. *J. Med. Chem.* **1999**, *42*, 5010−5019.
(32) Hancock, W. S.; Battersby, J. E. A new micro-test for the detection of incomplete coupling reactions in solid-phase peptide synthesis using 2,4,6-trinitrobenzenesulphonic acid. *Anal. Biochem.* **1976**, *15*, 260−264.
(33) Balboni, G.; Salvadori, S.; Guerrini, R.; Negri, L.; Giannini, E.; Jinsmaa, Y.; Bryant, S. D.; Lazarus, L. H. Potent δ-opioid receptor agonists containing the Dmt-Tic pharmacophore. *J. Med. Chem.* **2002**, *45*, 5556−5563.
(34) 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.
(35) 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-NH-CH(R)-R′ analogues: acquisition of potent δ antagonism. *Bioorg. Med. Chem.* **2003**, *11*, 5435−5441.
(36) 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.
(37) 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.
(38) Melchiorri, P.; Negri, L.; Falconieri-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.
(39) Tallarida, R. J.; Murray, R. B. *Manual of Pharmacological Calculation*, 2nd ed.; Springer-Verlag: New York, 1986.
(40) Kosterlitz, H. W.; Watt, A. J. Kinetic parameters of narcotic agonists and antagonists, with particular reference to N-allylnoroxymorphone (naloxone). *Br. J. Pharmacol.* **1968**, *33*, 266−276.

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