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Dansylated analogues of the opioid peptide [Dmt^1]DALDA: *in vitro* activity profiles and fluorescence parameters★✪

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Dansylated analogues of the potent and selective μ opioid peptide agonist [Dmt^1]DALDA (H-Dmt-D-Arg-Phe-Lys-NH_2; Dmt = 2′,6′-dimethyltyrosine) were prepared either by substitution of N^{β}-dansyl-α,β-diaminopropionic acid or N^{ε}-dansyllysine for Lys^4, or by attachment of a dansyl group to the C-terminal carboxamide function *via* a linker. All three analogues displayed high μ agonist potency *in vitro* and the C-terminally dansylated one retained significant μ receptor selectivity. The three analogues showed interesting differences in their fluorescence emission max-

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Abbreviations: Boc, *tert*-butyloxycarbonyl; DALDA, H-Tyr-D-Arg-Phe-Lys-NH_2; DAMGO, H-Tyr-D-Ala-Gly-N$^{\alpha}$MePhe-Gly-ol; Dap, α,β-diaminopropionic acid; Dic, 1,3-diisopropylcarbodiimide; Dmt, 2′,6′-dimethyltyrosine; dns, dansyl (5-dimethylaminonaphthalene-1-sulfonyl); DSLET, H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH; EDT, 1,2-ethanedithiol; Fmoc, 9-fluorenylmethoxycarbonyl; GPI, guinea pig ileum, HOBt, 1-hydroxybenzotriazole; IBCF, isobutylchloroformate; Lys(dns), N^{ε}-dansyllysine; MVD, mouse vas deferens; NATA, *N*-acetyltryptophanamide; NMM, *N*-methylmorpholine, PhSMe, methylphenylsulfide; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; TFA, trifluoroacetic acid; TMSBr, trimethylsilyl bromide; U69,593, (5α,7α,8β-(–)-*N*-methyl-*N*[7-(1-pyorrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide.

ima and quantum yields, indicating that the dansyl group in two of them was engaged in intramolecular hydrophobic interactions. These dansylated [Dmt^1]DALDA analogues represent valuable tools for binding studies, cellular uptake and intracellular distribution studies, and tissue distribution studies.

The dermorphin tetrapeptide analogue [Dmt^1]DALDA (H-Dmt-D-Arg-Phe-Lys-NH_2; Dmt = 2′,6′-dimethyltyrosine) is a highly potent and selective μ opioid peptide agonist capable of producing a long-lasting antinociceptive effect (Schiller *et al.*, 2000; Zhao *et al.*, 2002). Furthermore, this peptide has been shown to be internalized into CACO-2 cells by a mechanism which does not involve receptor-mediated endocytosis (Zhao *et al.*, 2003). This internalization was visualized in a confocal laser fluorescence microscopy study using the fluorescent analogue [Dmt^1,Dap(dns)^4]DALDA [Dap(dns) = N^{β}-dansyl-L-α,β-diaminopropionic acid]. This dansylated peptide analogue retained subnanomolar μ opioid receptor binding affinity, very high μ opioid agonist activity in the guinea pig ileum (GPI) assay and extraordinarily high antinociceptive activity in the mouse tail-flick test (intrathecal administration) (Berezowska *et al.*, 2003). However, in comparison with the [Dmt^1]DALDA parent peptide, the dansylated analogue displayed much reduced μ opioid receptor binding selectivity (Table 1). A fluorescent [Dmt^1]DALDA analogue containing N^{β}-anthraniloyl-L-α,β-diaminopropionic acid in place of Lys^4 showed similarly low preference for μ over δ and κ opioid receptors (Berezowska *et al.*, 2003).

The steady-state fluorescence parameters of [Dmt^1,Dap(dns)^4]DALDA were compared with those of the *N*-acetylated and carboxamidated reference amino-acid N-Ac-Dap(dns)-NH_2, in which the fluorophore is completely exposed to the water. The fluorescent peptide showed the same fluorescence emission maximum (λ^{em}_{max} = 578 nm) as *N*-Ac-Dap(dns)-NH_2 and a similar fluorescence quantum yield (Table 3), indicating that its dansyl group is completely exposed to the aqueous environment and is not involved in any intramolecular interactions with peptide moieties that would affect the quantum yield and the location of the fluorescence emission maximum.

In an effort to develop dansylated [Dmt^1]DALDA analogues with improved μ receptor selectivity, we prepared two peptides with the fluorophore in different locations of the molecule. One of them contains the dansyl group at the ε-amino group of the Lys^4 residue (H-Dmt-D-Arg-Phe-Lys(dns)-NH_2; Lys(dns) = N^{ε}-dansyllysine), whereas in the other one it is attached to the C-terminal carboxamide group *via* a linker (H-Dmt-D-Arg-Phe-Lys-NH-$(CH_2)_2$-NH-dns). These analogues will also permit an interesting comparison of their fluorescence parameters with those of the Dap(dns)^4-analogue and of the reference amino acid Ac-Dap(dns)-NH_2.

MATERIALS AND METHODS

Analytical methods. Precoated plates (silica gel 60 F_{254}, 250 μm; Merck, Darmstadt, Germany) were used for ascending TLC in the following solvent systems (all v/v): (I) *n*-BuOH/AcOH/H_2O (4:1:1); (II) *n*-BuOH/pyridine/AcOH/H_2O (15:10:3:12). Preparative reversed-phase HPLC was performed on a Vydac 218-TP1022 column (22 × 250 mm) with a linear gradient of 5–22% (first 20 min) and 22–30% (next 20 min) acetonitrile in 0.1% TFA at a flow rate of 13 mL/min. Analytical reversed-phase HPLC was performed on a Vydac 218TP54 column (5 × 250 mm) with a linear gradient of 5–30% acetonitrile in 0.1% TFA over 30 min at a flow rate of 1.5 mL/min. The same column was also used for the determination of the capacity factors K' under isocratic conditions (20% acetonitrile in 0.1% TFA). Molecular masses of compounds were determined by FAB mass spectrometry on an MS-50 HMTCTA mass spectrometer interfaced to a DS-90 data system

(Dr. M. Evans, Department of Chemistry, University of Montreal).

***Peptide synthesis*: *H-Dmt-D-Arg-Phe-Lys(dns)-NH$_2$*.** All reactions were carried out in the dark to prevent photodegradation of the dansyl group. The peptide was synthesized by the manual solid-phase technique using Boc-protection for the α-amino group, except for Dmt, which was Fmoc-protected. Side chain protection was as follows: tosyl (Arg), dansyl (Lys). Boc-Lys(dns)-OH was synthesized in a manner analogous to that reported for the preparation of Z-Lys(dns)-OH (Schwyzer *et al.*, 1971). 1,3-Diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole (HOBt) were used as coupling agents. The peptide was assembled on a *p*-methylbenzhydrylamine resin according to a published protocol (Schiller *et al.*, 2000). After peptide assembly was complete, Fmoc protection was removed with 30% (v/v) piperidine in dimethyl formamide (DMF) and the peptide was cleaved from the resin by HF/anisole treatment in the usual manner. The crude peptide was purified by preparative reversed-phase HPLC and was found to be at least 98% pure, as assessed by TLC and HPLC. TLC R_f 0.20 (I), 0.78 (II); K' 1.28; FAB-MS *m/e* 873.

***Peptide synthesis: H-Dmt-D-Arg-Phe-Lys-NH-(CH$_2$)$_2$-NH-dns*.** For the preparation of TFA × H_2N-$(CH_2)_2$-NH-dns, $NaHCO_3$ (0.318 g, 3 mM) was added to a solution of *N*-Boc-ethylenediamine (0.256 mL, 1.62 nM) in water (5 mL), followed by addition of dansyl chloride (0.545 g, 2.02 mM) in acetone (10 mL) at room temp. After 2 h, the acetone was evaporated *in vacuo*, and ethyl acetate and aq. NaCl (sat.) were added. The ethyl acetate extract was dried over $MgSO_4$, filtered and evaporated to yield Boc-NH-$(CH_2)_2$-NH-dns as a yellow solid. N-Boc protection was removed by TFA treatment (30 min at 0°C) and after TFA evaporation, TFA × H_2N-$(CH_2)_2$-NH-dns was obtained in the form of yellow crystals. The peptide was assembled in solution using the symmetrical anhydride method and N^{α}-Fmoc protection. Side chain protection was as follows: Pmc (Arg), Boc (Lys). The coupling reagent was isobutylchloroformate (IBCF) in the presence of *N*-methylmorpholine (NMM). Coupling reactions were carried out in dry THF, initially at –20°C with subsequent gradual warming to room temp. over a period of 2 h. Fmoc protection was removed with 30% piperidine in DMF. After peptide assembly, Boc protection of Lys was removed with TFA (95% aq. TFA, 0°C, 1 h) and Pmc protection of Arg was removed by treatment with TMSBr/ PhSMe/EDT in TFA. The crude peptide was purified by preparative reversed-phase HPLC and was found to be at least 98% pure as assessed by TLC and HPLC. TLC R_f 0.08 (I), 0.73 (II); K' 1.57; FAB-MS *m/e* 916.

***Pharmacological testing in vitro*.** The guinea pig ileum (GPI) and mouse vas deferens (MVD) bioassays were carried out as reported in detail elsewhere (Schiller *et al.*, 1978; DiMaio *et al.*, 1982). A dose-response curve was determined with [Leu[5]]enkephalin as standard for each ileum and vas preparation, and IC_{50} values of the compounds being tested were normalized according to a published procedure (Waterfield *et al.*, 1979). Opioid receptor binding studies were performed as described in detail elsewhere (Schiller *et al.*, 1978). Binding affinities for μ and δ receptors were determined by displacing, respectively, [^{3}H]DAMGO (Multiple Peptide Systems, San Diego, CA, U.S.A.) and [^{3}H]DSLET (Multiple Peptide Systems) from rat brain membrane binding sites, and κ opioid receptor binding affinities were measured by displacement of [^{3}H]U69,593 (Amersham) from guinea pig brain membrane binding sites. Incubations were performed for 2 h at 0°C with [^{3}H]DAMGO, [^{3}H]DSLET and [^{3}H]U69,593 at respective concentrations of 0.72, 0.78 and 0.80 nM. IC_{50} values were determined from log dose-displacement curves, and K_i values were calculated from the obtained IC_{50} values by means of the equation of Cheng & Prusoff (1973), using values of 1.3, 2.6 and 2.9 nM for the dissociation constants

of [^{3}H]DAMGO, [^{3}H]DSLET and [^{3}H]U-69,593, respectively.

Fluorescence spectroscopy. Fluorescence emission spectra were recorded on a SLM-Aminco SPF-500C spectrofluorometer with 2 nm spectral resolution for excitation and emission. Solutions of peptides and reference amino acids in Tris/HCl buffer (pH 6.6) at a concentration of 2×10^{-5} M were used. The excitation wavelength was 350 nm. Fluorescence quantum yields (φ) were determined relative to *N*-acetyl-L-tryptophanamide (NATA) ($\varphi_{NATA} = 0.14$) as reference. The quantum yield was calculated based on the following equation:

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

where the subscripts S and R refer to the sample and reference compound (NATA), respectively. E is the integrated area under the corrected emission spectrum. A is the absorbance of the solution at the excitation wavelength ($A < 0.05$) and $(n_S/n_R)^2$ is the correction for the refractive index.

RESULTS AND DISCUSSION

As was reported previously (Berezowska *et al.*, 2003), the Dap(dns)^4-analogue retained high μ receptor binding affinity, being about four times less potent than the [Dmt^1]-DALDA parent peptide (Table 1). However, this compound displayed only slight preference for μ receptors over δ receptors. Extension of the 4-position side chain in the latter peptide, as achieved by substitution of N^ϵ-dansyllysine, resulted in a compound, H-Dmt-D-Arg-Phe-Lys(dns)-NH_2, which showed about the same high μ receptor binding affinity (K_i^μ = 0.506 nM) but somewhat improved μ *vs.* δ receptor selectivity (K_i^δ/K_i^μ = 8) and still considerable μ *vs.* κ selectivity (K_i^κ/K_i^μ = 24) (Table 1). In comparison with the [Dmt^1]DALDA parent, the Lys(dns)^4-analogue displayed about the same high agonist potency in the GPI assay and about 3-fold higher potency in the MVD assay. Attachment of the dansyl group to the C-terminal carboxamide function of [Dmt^1]DALDA produced a compound, H-Dmt-D-Arg-Phe-Lys-NH-$(CH_2)_2$-NH-dns, which retained high μ receptor binding affinity (K_i^μ = 0.657 nM), but showed markedly decreased δ receptor affinity (K_i^δ = 47.8 nM) and, therefore, improved μ *vs.* δ receptor selectivity (K_i^δ/K_i^μ = 73) (Table 1). Its μ *vs.* κ selectivity (K_i^κ/K_i^μ = 26) is similar to that of the Lys(dns)^4-analogue. In the GPI assay, this compound displayed similarly high agonist potency as the [Dmt^1]DALDA parent and the other two dansylated analogues.

The [Dmt^1]DALDA parent peptide carries a net positive charge of 3+, whereas the positive charge of the Dap(dns)^4- and Lys(dns^4)-analogues is only 2+ at physiological pH. As observed in an earlier study, gradual augmentation of the positive charge from 1+ to 3+ in a series of dermorphin-(1–4) tetrapeptide analogues produced a progressive increase in μ receptor selectivity (Schiller *et al.*, 1989). The decrease in μ *vs.* δ receptor selectivity observed with the Dap(dns)^4- and Lys(dns)^4-analogues as compared to the parent peptide is in agreement with the results of the latter study. Like the [Dmt^1]DALDA parent, the C-terminally dansylated analogue H-Dmt-D-Arg-Phe-Lys-NH-$(CH_2)_2$-NH-dns carries a net charge of 3+. This may explain its relatively higher μ *vs.* δ selectivity as compared to the Dap(dns)^4- and Lys(dns)^4-analogues.

The fluorescence emission maximum of the dansyl group is known to be strongly dependent on the polarity of its environment. While the emission maximum of N^ϵ-dansyllysine and of N^β-dansyl-L-α,β-diaminopropionic acid or its N-acetylated and carboxamidated derivative (Ac-Dap(dns)-NH_2) is at 578 nm (Schiller *et al.*, 1972; Berezowska *et al.*, 2003), a hypsochromic shift of up to 80 nm is observed with the dansyl fluorophore in lipophilic sur-

Table 1. Opioid receptor binding affinities of dansylated [Dmt[1]]DALDA analogues

Compound	K_i^{μ} [nM]	K_i^{δ} [nM]	K_i^{κ} [nM]	K_i ratio μ/δ/κ
H-Dmt-D-Arg-Phe-Dap(dns)-NH_2	0.589 ± 0.023	1.88 ± 0.19	35.0 ± 6.5	1/3/59
H-Dmt-D-Arg-Phe-Lys(dns)-NH_2	0.506 ± 0.057	3.97 ± 0.85	12.1 ± 0.3	1/8/24
H-Dmt-D-Arg-Phe-Lys-NH-$(CH_2)_2$-NH-dns	0.657 ± 0.063	47.8 ± 5.2	16.8 ± 0.4	1/73/26
H-Dmt-D-Arg-Phe-Lys-NH_2	0.143 ± 0.015	2100 ± 310	22.3 ± 4.3	1/14700/156
Morphine	1.00 ± 0.04	32.6 ± 3.7	217 ± 49	1/33/217

Data represent the mean ±S.E.M. from 3–6 independent experiments.

Table 2. Guinea pig ileum (GPI) and mouse vas deferens (MVD) assay of dansylated [Dmt[1]]DALDA analogues

Compound	GPI IC_{50} [nM]	MVD IC_{50} [nM]	MVD/GPI IC_{50} ratio
H-Dmt-D-Arg-Phe-Dap(dns)-NH_2	2.52 ± 0.41	3.02 ± 0.49	1.20
H-Dmt-D-Arg-Phe-Lys(dns)-NH_2	1.23 ± 0.31	6.99 ± 1.97	5.68
H-Dmt-D-Arg-Phe-Lys-NH-$(CH_2)_2$-NH-dns	2.93 ± 0.34	3.98 ± 0.65	1.35
H-Dmt-D-Arg-Phe-Lys-NH_2	1.41 ± 0.29	23.1 ± 2.0	16.4
Morphine	29.3 ± 2.2	155 ± 31	5.29

Data represent the mean ±S.E.M. from 3–5 independent experiments.

Table 3. Steady-state fluorescence parameters of dansylated [Dmt[1]]DALDA analogues

Compound	λ_{max}^{em} [nm]	φ
H-Dmt-D-Arg-Phe-Dap(dns)-NH_2	578	0.025
H-Dmt-D-Arg-Phe-Lys(dns)-NH_2	570	0.045
H-Dmt-D-Arg-Phe-Lys-NH-$(CH_2)_2$-NH-dns	572	0.036
Ac-Dap(dns)-NH_2	578	0.030

roundings (Chen, 1967; Parker *et al.*, 1967). Like the reference amino-acids H-Lys(dns)-OH and Ac-Dap(dns)-NH_2, the fluorescence emission spectrum of [Dmt[1],Dap(dns)[4]]-DALDA shows a maximum at 578 nm (Table 3) in Tris/HCl buffer (pH 6.6). This result indicates that the dansyl group of this peptide is completely exposed to the aqueous environment and does not engage in any intramolecular interactions with peptide moieties that would affect the location of the fluorescence emission maximum. Interestingly, the analogue H-Dmt-D-Arg-Phe-Lys(dns)-NH_2 shows a hypsochromic shift of 8 nm of its dansyl fluorescence emission maximum (λ_{max}^{em} = 570 nm) (Table 3), indicating the existence of pep-

tide conformers in which the fluorophore is located in a somewhat hydrophobic environment. This is also indicated by the 50% increase of the dansyl fluorescence quantum yield seen with this peptide (φ = 0.45) as compared to the reference amino-acid Ac-Dap-(dns)-NH_2 (φ = 0.30). Obviously, the long, flexible side chain of the dansylated lysine residue permits interactions with one or several lipophilic moieties in the peptide. In contrast, the shortness of the dansylated side chain of the N^{β}-dansyl-α,β-diaminopropionic acid residue in H-Dmt-D-Arg-Phe- Dap(dns)-NH_2 does not permit such interactions. With the C-terminally dansylated analogue H-Dmt-D-Arg-Phe-Lys-NH-$(CH_2)_2$-NH- dns a slightly smaller hypsochromic shift (6 nm) in the dansyl fluorescence emission maximum and a smaller (20%) increase in the fluorescence quantum yield (φ = 0.036) are observed (Table 3). These fluorescence parameters indicate that the dansyl group in this peptide is also involved in intramolecular hydrophobic interactions with lipophilic moieties, but to a lesser degree than it is the case with the dansyl group in H-Dmt-D-Arg-Phe- Lys(dns)-NH_2.

In conclusion, we prepared three highly potent, dansylated analogues of the μ opioid peptide agonist [Dmt^1]DALDA. One of these analogues, H-Dmt-D-Arg-Phe-Lys-NH-$(CH_2)_2$-NH-dns displayed improved μ *vs.* δ receptor selectivity as compared to the other two which were relatively non-selective. Two of the analogues showed blue-shifts of the dansyl fluorescence emission maximum and increases in the fluorescence quantum yield. These findings represent the first evidence to indicate that fluorophores contained even in small peptides may engage in significant intramolecular interactions with lipophilic moieties. These dansylated [Dmt^1]DALDA analogues are expected to be useful as pharmacological tools for various applications, including binding studies with receptors and other biopolymers, cellular uptake and intracellular distribution studies by confocal laser scanning microscopy and tissue distribution studies.

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