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SYNTHESIS AND CHARACTERIZATION OF 7-NITROBENZO-2-OXA-1,3-DIAZOLE (NBD)-LABELED FLUORESCENT OPIOIDS

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Abstract—Alkylation of sarcosine with 4-chloro-nitrobenzo-2-oxa-1,3-diazole (NBD-chloride) furnished a fluorescent tag that was coupled with a tetrahydrothebaine derivative and β-naltrexamine, respectively, to yield the fluorescent opioids 7α-(1*R*)-1-hydroxy-1-methyl-3-(4-hydroxyphenyl)-propyl]-6,14-endo-ethenotetrahydrothebaine NBD-sarcosinate (ASM-5-10) and *N*-cyclopropylmethyl-3-hydroxy-14β-hydroxy-6β-(NBD sarcosinyl)-amino-epoxymorphinan (ASM-5-67). The fluorescence intensity of the novel opioids allowed their detection at subnanomolar concentrations, and was dependent on the polarity of the solvent. Maximum quantum yield was obtained in ethyl acetate and ethanol, and minimal fluorescence in heptane and water. Compounds ASM-5-10 and ASM-5-67 displaced the opioid receptor binding of [^{3}H]Tyr-D-Ala-Gly-(Me)Phe-Gly-ol in monkey brain membranes with IC_{50} values of 8.4 and 1.5 nM, respectively. Whereas ASM-5-67 bound to μ, δ, and κ receptors with comparable affinities, ASM-5-10 was μ-selective, with selectivity indices (ratio of respective IC_{50} values) of 0.04 for both μ/δ and μ/κ. The sodium response ratio in binding revealed a pronounced agonist property of ASM-5-10. Both opioids were lipophilic, with octanol–water partition coefficients (log P_{app}) of 2.8 (ASM-5-10) and 1.0 (ASM-5-67). ASM-5-10 exhibited particularly strong membrane retention that was not reversible by four washes. Their favorable characteristics in fluorescence, receptor binding, and membrane interaction make these newly developed ligands useful molecular probes to study opioid receptor mechanisms.

The usefulness of fluorescent ligands to probe receptor mechanisms is well documented. The applications include the histochemical localization of receptors, their visualization on cell surfaces, quantitation of receptor mobility by the technique of fluorescence recovery after photobleaching, and fluorescent energy transfer experiments to characterize the receptor environment, e.g. the lipid boundary layer in membranes, or the topography of binding sites on isolated receptor molecules. Opioids containing different fluorophores, including dansyl [1], rhodamine [2], fluorescein [3], and pyrene [4], have been synthesized previously. The utility of dansylated compounds is limited due to intense autofluorescence of brain tissue [5] and the inhibition of opioid receptors at wavelengths required for the excitation of this fluorescent tag [6]. Rhodamine and fluorescein are bulky molecules likely to impair the lateral diffusion of the ligand-occupied receptor in the membrane, and the attenuation of pyrene fluorescence in solvents of intermediate polarity [7] may limit the contribution of signal derived from the occupation of membrane receptors by such ligand in proportion to that of free ligand.

The fluorescence characteristics and molecular properties of 7-nitrobenzo-2-oxa-1,3-diazole (NBD‖), when coupled to an amine, suggested that it may be a useful tag to study opioid receptor mechanisms. NBD fluorescence is complementary to rhodamine for fluorescent energy transfer, e.g. between receptor and GTP binding protein [8], and its dependence on the polarity of the respective environment favors the membrane receptor-bound ligand [9, 10]. NBD-labeled compounds have been used to visualize the γ-aminobutyric acid (GABA)-benzodiazepine receptor on rat spinal cord cells: fluorescence of bound ligand was enhanced 35-fold over background, allowing the measurement of receptor mobility by fluorescence recovery after photobleaching [11]. The NBD tag has also been attached to β-adrenergic antagonists [9, 12], and estrogen and progestin derivatives [10]. We have in this study synthesized two opioids containing the NBD residue, and have studied their characteristics as ligands of opioid receptors. The obtained results show that these novel fluorescent opioids represent valuable probes to study opioid receptor mechanisms.

MATERIALS AND METHODS

Materials

[^{3}H]Tyr - D - Ala - Gly - (Me) Phe - Gly - ol ([^{3}H]-

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‖ Abbreviations: NBD, 7-nitrobenzo-2-oxa-1,3-diazole; ASM-5-10, 7α-[(1*R*)-1-hydroxy-1-methyl-3-(4-hydroxyphenyl) - propyl] - 6,14 - endo - ethenotetrahydrothebaine NBD-sarcosinate; ASM-5-67, *N*-cyclopropylmethyl-3-hydroxy-14β-hydroxy-6β-(NBD sarcosinyl)-amino-epoxymorphinan; GABA, γ-aminobutyric acid; DAMGO, Tyr-D-Ala-Gly-(Me)Phe-Gly-ol; and DPDPE, [D-Pen^2,D-Pen^5]-enkephalin.

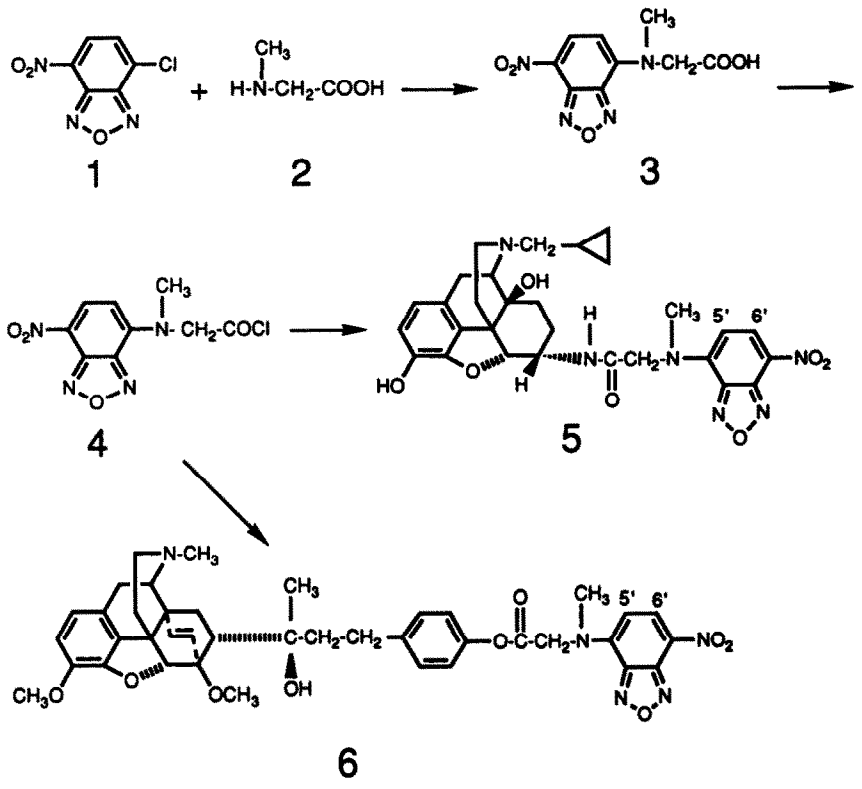

Fig. 1. Scheme of synthesis of fluorescent opioids. Details of the procedures are described under Materials and Methods. Compounds 5 and 6 represent the opioids ASM-5-67 and ASM-5-10 evaluated in this study.

(DAMGO) and [^{3}H][D-Pen^2,D-Pen^5]enkephalin ([^{3}H]DPDPE) were purchased from the Amersham Corp., and [^{3}H]U69,593 was obtained from the New England Nuclear Corp. The unlabeled opioids were obtained through the Narcotic Drug and Opiate Peptide Basic Research Center at the University of Michigan. The other biochemicals were from the Sigma Chemical Co.

Synthesis of fluorescent opioids

Melting points were taken on a laboratory Mel-Temp apparatus. The ^{1}H NMR spectra were run on a Varian XL200 spectrometer using $(CH_3)_4Si$ as the internal standard. IR spectra were run on a Perkin–Elmer model 298 infrared spectrophotometer, and microanalyses were performed by Atlantic Microlab, Inc., Norcross, GA.

Preparation of N-4-(7-*nitrobenzo*-2-*oxa*-1,3-*diazolyl*)-*sarcosine (Fig.* 1, *compound* 3). A solution of 500 mg (2.5 mmol) of 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-chloride, Fig. 1, compound 1) in 20 mL methanol was added to the stirred solution of 225 mg (2.5 mmol) sarcosine (Fig. 1, compound 2) and 210 mg (2.5 mmol) of sodium bicarbonate in 5 mL of water. The mixture was stirred at 55° for 3 hr, the methanol was evaporated *in vacuo*, and the residue was disolved in 20 mL of water containing 265 mg (2.5 mmol) sodium bicarbonate. The basic solution was then washed with 50 mL ethyl acetate, and the aqueous phase was filtered and neutralized with 7.5 mL (7.5 mmol) of 1 N HCl. After concentration of the neutral solution at room temperature, the desired NBD-sarcosine crystallized. The precipitate was filtered, washed with a little water and then with ether, and dried to give 300 mg of product with a melting point of 182–183°. Further concentration of the mother liquor gave a second crop of desired product. Total yield: 375 mg (60%), which melted at 185–187° after recrystallization from methanol. Calculated analysis (in percent) for $C_9H_8N_4O_5$: C, 42.86; H, 3.20; N, 22.22. Found: C, 42.96; H, 3.20; N, 22.16.

Preparation of NBD-sarcosine-acid chloride (Fig. 1, *compound* 4). NBD-sarcosine (34 mg; 0.135 mmol) was suspended in 5 mL of dry methylene chloride, and 0.5 g (4.0 mmol) of thionyl chloride was added. The mixture was heated under reflux for 20 min. The resulting clear solution was then distilled *in vacuo* until all excess of thionyl chloride was removed. The oily residue was dissolved in 10 mL of dry methylene chloride and again evaporated *in vacuo* to remove traces of thionyl chloride. The desired acid chloride was obtained as a dark red film and used without further purification.

Preparation of ASM-5-67, N-*cyclopropylmethyl*-3-*hydroxy*-14β-*hydroxy*-6β-*(NBD sarcosinyl)*-*amino-epoxymorphinan (Fig.* 1, *compound* 5). In a flask equipped with a magnetic stirrer and protected from moisture, 50 mg (0.12 mmol) of β-naltrexamine·HCl was suspended in 10 mL of dry

methylene chloride and then 40 mg (0.4 mmol) triethylamine was added. To the stirred mixture a solution of the above acid chloride (0.13 mmol) in 5 mL of dry methylene chloride was added dropwise during 5 min at room temperature, and stirring was continued for 24 hr. The reaction solution was diluted with methylene chloride, and washed with saturated sodium bicarbonate solution and with water. The methylene chloride phase was filtered and evaporated *in vacuo* to give 56 mg crude product that was chromatographed on preparative TLC plates (silica gel/ethyl acetate : methanol 9 : 1) to give 35 mg (51%) of TLC-pure desired amide. Melting point: 230–240° (decomposition). Calculated analysis (in percent) for $C_{29}H_{32}N_6O_7 \cdot H_2O$: C, 58.58; H, 5.76; N, 14.14. Found: C, 59.01; H, 5.74; N, 14.07.

Preparation of ASM-5-10,7α-[(1R)-1-hydroxy-1-methyl-3-(4-hydroxyphenyl)-propyl]-6,14-endo-ethenotetrahydrothebaine NBD-sarcosinate (Fig. 1, compound 6). To the stirred solution of 54 mg (0.198 mmol) NBD-sarcosine-acid chloride in 15 mL of dry methylene chloride, a solution of 90 mg (0.178 mmol) of 7α-[(1*R*-1-hydroxy-1-methyl-3-(4-hydroxyphenyl) - propyl] - 6,14 - endo - etheno-tetrahydrothebaine in 10 mL of dry methylene chloride was added at room temperature followed by the addition of 25 mg (0.2 mmol) of 4-dimethylaminopyridine. Stirring at room temperature with exclusion of moisture was continued overnight. The reaction mixture was then diluted with methylene chloride and washed with saturated sodium bicarbonate solution and with water. The organic layer was evaporated *in vacuo* and the residue was chromatographed on silica gel plates using ethyl acetate as the developing solvent to give 96 mg (73%) of TLC-pure phenolic ester. The latter was dissolved in ethyl acetate, filtered and triturated with ether to give an orange-colored solid that was filtered, washed with diethyl ether and dried. Melting point: 145–155° (decomposition). Calculated analysis (in percent) for $C_{40}H_{43}N_5O_9 \cdot 1/4\ H_2O$: C, 64.72; H, 5.90; N, 9.43. Found: C, 64.62; H, 5.92; N, 9.40.

Membrane isolation

Membranes from rhesus monkey cortex were prepared as previously described [13]. The protein concentration of the final membrane suspensions was approximately 0.6 mg/mL as determined by the method of Lowry *et al.* [14] using bovine serum albumin as standard.

Opioid receptor assay

The procedure that was followed was described previously in detail [13, 15]. The assay is based on the displacement of a receptor-selective radiolabeled opioid by the tested compounds. Specific binding of the radioligand was defined as the difference between binding in the absence and presence of an appropriate excess of the corresponding unlabeled opioid. After incubation at 25° to reach binding equilibrium, the samples were quickly filtered, washed, and subjected to liquid scintillation counting. The affinity of the investigated ligands in binding to opioid receptor was expressed as the concentration that inhibited 50% of maximal binding of a given radiolabeled ligand (IC_{50}). Binding selectivity was expressed as selectivity index, representing the ratio of IC_{50} values of a compound in inhibiting the specific binding of two selective radiolabeled ligands. The sodium response ratio for a given compound corresponded to the ratio of the respective IC_{50} values obtained in the presence and absence of 150 mM NaCl.

Membrane retention

Sixteen milliliters of a suspension of brain membranes in 50 mM Tris·HCl, pH 7.4, was incubated with a 100 nM concentration of the fluorescent opioids for 60 min at 25°. Aliquots were then removed for receptor binding and protein assay, and the residual suspension was diluted with 25 mL of ice-cold buffer and centrifuged at 20,000 *g* for 15 min. The resulting pellet was resuspended with buffer to the original protein concentration and an aliquot removed for the determination of bound ligand and protein concentration. The washes were repeated subsequently and samples removed for binding and protein assays after each wash. Receptor binding was carried out by the displacement of 0.5 nM [^{3}H]DAMGO as described above.

Lipid–water partition coefficients (log P_{app})

These parameters were determined by a recently developed micromethod requiring minute amounts of the opioids [16]. Briefly, following the partitioning of the opioid (low- or sub-micromolar solution) between *n*-octanol and Tris·HCl buffer, pH 7.4, at 25°, the concentration remaining in the aqueous phase was determined using an opioid receptor binding assay based on the displacement by the tested opioids of bound [^{3}H]DAMGO in brain membranes.

Fluorescence spectra

Fluorescence was measured on a PTI Alphascan fluorometer with excitation and emission slits at 5 nm. The fluorescent opioids were solubilized with either 50 mM Tris·HCl, pH 7.4, 100% ethanol, ethyl acetate, or heptane. As shown previously [9, 10], the excitation wavelengths were solvent specific: their values are listed in the legend to Fig. 2.

RESULTS

Synthesis and fluorescent properties

The fluorescent opioids were prepared as described under Materials and Methods and depicted in Fig. 1. The spectral properties of fluorescent opioids were determined in solvents of various polarities (Fig. 2). Compared to solutions of intermediate polarity, the excitation and emission maxima of the compounds were shifted to longer wavelengths in polar and apolar solvents. The intensity of NBD fluorescence was minimal in Tris·HCl buffer, pH 7.4, and heptane, but was greatly increased in ethanol and ethyl acetate. Compared to Tris buffer, the enhancement factor was 12- and 23-fold and 10- and 7-fold for ASM-5-10 and ASM-5-67 in ethanol and ethyl acetate, respectively. Relative to heptane, the quantum yield of fluorescence in ethanol and ethyl acetate was amplified 60- and 108-fold for ASM-5-10 and 24- and 16-fold for ASM-5-67. In

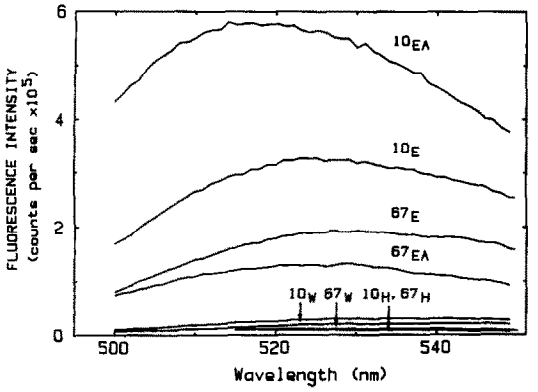

Fig. 2. Fluorescence spectra of novel opioids. Shown are the fluorescence intensities of compounds ASM-5-10 (10) and ASM-5-67 (67) dissolved at a 100 nM concentration in ethyl acetate (EA), ethanol (E), heptane (H) and aqueous buffer (Tris) medium (W). The corresponding wavelengths for the excitation and emission maxima were (nm): 486, 526 (E); 496, 536 (H); 480, 533 (W). In EA the two wavelengths were 456 and 517 for ASM-5-10 and 456 and 526 for ASM-5-67.

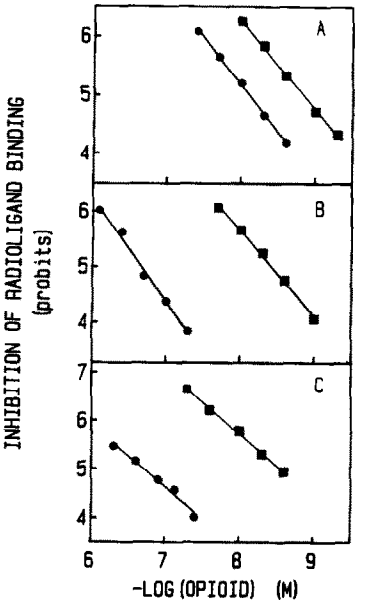

Fig. 3. Displacement of receptor-selective opioids by the fluorescent compounds. Shown is the inhibition by ASM-5-10 (●) and ASM-5-67 (■) of the specific binding of 0.5 nM [^{3}H]DAMGO (A), 1.5 nM [^{3}H]DPDPE (B), and 1.5 nM [^{3}H]U69,593 (C) in monkey brain membranes. Plotted is extent inhibition at different concentrations of the fluorescent opioids. The corresponding IC_{50} values are listed in Table 1. Shown are mean values of three experiments, each carried out in duplicate.

ethanol, the limit of detection of the fluorescent signal was less than 1 nM for both ligands.

Receptor binding

While ASM-5-67 bound with high affinity to μ, δ, and κ opioid receptors, ASM-5-10 exhibited considerable selectivity for the μ receptor (Fig. 3 and Table 1). The slightly higher slope factors of ASM-5-10 are likely to reflect the strong lipophilicity of this compound, as discussed later. The corresponding selectivity indices showed that ASM-5-10 preferred μ sites over those of δ or κ specificity to a similar extent. The results of ligand binding are expressed as IC_{50} in order to allow direct comparison with binding affinities and receptor selectivities of various opioids determined in our laboratory [13]. The concentration of radioligand in the binding assay was selected to be low relative to its K_D in order to minimize the divergence between the obtained IC_{50} and true K_i. Indeed, the binding selectivities of fluorescent opioids calculated on the basis of their K_i, rather than IC_{50}, values were virtually identical to the results listed in Table 1: μ/δ and μ/κ indices for ASM-5-10 were 0.036 and 0.043, and for ASM-5-67 they were 0.396 and 0.525, respectively. Sodium attenuated the binding affinity of ASM-5-10 at the μ receptor (Table 1) to an extent similar to that obtained in the binding of the opioid agonists DAMGO or sufentanil: these compounds had sodium ratios of 1.36 and 1.38, respectively, in contrast to naloxone whose ratio was 0.49 [13].

Lipophilicity

On the basis of their *n*-octanol/water partitions, the fluorescent opioids were found to be strongly lipophilic with log *P* values of 2.8 and 1.0 for compounds ASM-5-10 and ASM-5-67, respectively. The corresponding values for etorphine and morphine, determined by the identical method, were 2.79 and −0.21 [16].

Considering the strongly hydrophobic nature of the fluorescent opioids, their retention in the membrane was assessed by the recovery of [^{3}H]-DAMGO binding after repeated washing of membranes incubated with receptor-saturating concentrations of fluorescent ligand. While ASM-5-67 was readily washed out from membranes, opioid receptor binding in membranes incubated with ASM-5-10 was still inhibited by 75% after four washes (Fig. 4).

DISCUSSION

Fluorescent ligands of various receptor systems, including the α- and β-adrenergic, adenosine, glucagon, steroid, dopamine, and benzodiazepine have been used successfully to study receptor function [e.g. Ref. 17]. Opioids tagged with different fluorophores were also synthesized and their properties evaluated. While providing interesting new insights, e.g. the clustering of opioid receptors in neuroblastoma cells using a rhodamine-tagged enkephalin derivative [2], the major drawback of most of these compounds was their impaired affinity and opioid character in binding to opioid receptors. The attachment of fluorescein to the C-6 position of oxymorphone, naltrexone, or naloxone resulted in a 16- to 20-fold attenuation of receptor binding affinity [3]. In addition, the attachment of fluorescein

Table 1. Receptor binding of fluorescent opioids

Compound	IC_{50} (nM)			Selectivity index		Sodium ratio
	μ	δ	κ	μ/δ	μ/κ	
ASM-5-10	8.40 ± 0.71 (1.10)	216 ± 2.31 (1.28)	193 ± 4.05 (1.12)	0.04	0.04	1.55
ASM-5-67	1.53 ± 0.10 (1.02)	3.81 ± 0.12 (1.09)	2.83 ± 0.09 (0.98)	0.40	0.54	1.00

The effects of compounds ASM-5-10 and ASM-5-67 on the opioid receptor binding of [^{3}H]-DAMGO (μ-selective), [^{3}H]DPDPE (δ-selective)aand [^{3}H]U69,593 (κ-selective) in monkey brain membranes were determined, in the absence of NaCl, as described under Materials and Methods and in the legend to Fig. 3. The IC_{50} values and slope factors were determined by linear regression analysis from corresponding Hill plots, relating percent inhibition of specific radioligand binding to five concentrations of the competing opioid. All the regression coefficients (r^2) were higher than 0.98. Shown are the mean ± SEM values for IC_{50} and corresponding slope factors (in parentheses) obtained in three experiments, each carried out in duplicate. The selectivity indices represent the ratio of respective IC_{50} values of a compound in inhibiting the binding of radiolabeled selective opioids, e.g. [^{3}H]DAMGO/[^{3}H]DPDPE for the μ/δ index. Also listed is the sodium ratio, calculated as the ratio of IC_{50} obtained in displacing 0.5 nM [^{3}H]DAMGO in the presence and absence of 150 mM NaCl.

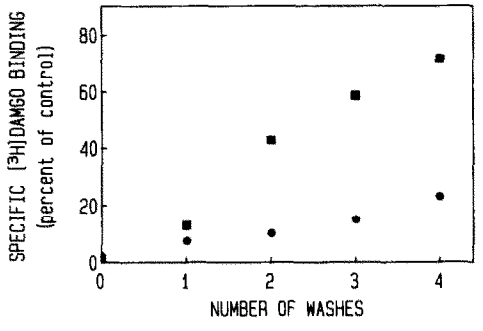

Fig. 4. Membrane retention of fluorescent opioids. A suspension of brain membranes in Tris·HCl buffer, pH 7.4, was incubated with a 100 nM concentration of ASM-5-10 (●) or ASM-5-67 (■). After incubation for 60 min at 25°, the suspension was centrifuged, the pellet was resuspended with 50 mM Tris·HCl, pH 7.4, and aliquots were included in the opioid receptor assay using [^{3}H]-DAMGO. The residual membrane suspension was subjected to repeated sequences of the washout procedure and opioid receptor assay, as described under Materials and Methods. In the initial control membranes, the specific binding of [^{3}H]DAMGO was 73 fmol/mg protein. Shown are the averages of results obtained in two experiments, each carried out in duplicate.

to the opioid antagonists naloxone and naltrexone attenuated their antagonist property [18]. The incorporation of the fluorescent amino acid L-1-pyrenylalanine into leucine-enkephalin at positions 4 or 5 preserved recognition at μ or δ opioid receptors although, again, with diminished affinity: in displacing [^{3}H]DAMGO, the 4- and 5-substituted enkephalins had IC_{50} values of 60 and 113 nM, respectively [4]. Interestingly, the opioid nature of the enkephalin molecule was also preserved following the substitution of its tyrosyl residue by pyrenylalanine [7]. L-Tyrosine in position 1 was considered essential for recognition of enkephalins at the opioid receptor.

In the work described here, we have synthesized two fluorescent opioid alkaloids containing the NBD residue. The favorable fluorescent characteristics and molecular properties of this fluorophore have previously led to its successful use in probes of the adrenergic [9] and GABA-benzodiazepine receptors [11]. As shown in the latter studies, as well as in our work (Table 1), a common feature of the NBD-labeled fluorescent ligands appears to be the preservation of high affinity in binding to the respective receptors. The molecular feature likely to contribute to this favorable characteristic of the NBD-labeled compounds is the small size of the NBD fluorophore, particularly when compared to fluorescein or rhodamine. The limited bulk of the NBD residue should also offer considerable advantage in the use of such ligands to study receptor dynamics in the plasma membrane. As observed in this study, the NBD fluorescence has a strong dependence on solvent polarity, a property that aids in discriminating between the fluorescence of receptor-bound, membrane-solubilized, and free ligand in the assay medium. In addition, the fluorescence intensity of the two synthesized opioid alkaloids enables their detection at subnanomolar concentrations.

The two NBD-labeled opioids exhibited high binding affinity and, in part, strong selectivity towards the μ opioid receptor. The respective properties of ASM-5-10 compared favorably with those of morphine [13], a finding not unexpected since this fluorescent opioid is an *N*-methyl compound and should, like morphine, be μ-selective. On the basis of the sodium response ratio in binding, ASM-5-67 displayed mixed agonist–antagonist properties, but ASM-5-10 showed agonist character. While both compounds were hydrophobic, the octanol–water partition coefficient of ASM-5-10 was particularly high. As shown with buprenorphine, opioids of such

pronounced lipophilicity exhibit unusual receptor kinetics and time-course of action [19]. To what extent the washout pattern of ASM-5-10 reflects slow dissociation from receptor or retention in hydrophobic membrane compartments remains unresolved at this time. It is conceivable to expect that high concentrations of a ligand in membrane regions around the receptor influence the respective IC_{50} value. On the other hand, a comprehensive study of morphinomimetic opioids belonging to various chemical classes revealed no correlation between binding affinity in rat brain membranes and lipophilicity of the compounds [20]. Indeed, the characteristics of ASM-5-10 identify it as a useful probe to study membrane modulation of opioid receptor mechanisms [21].

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