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Letters

Naphthalene Dicarboxaldehyde as an Electrophilic Fluorogenic Moiety for Affinity Labeling: Application to Opioid Receptor Affinity Labels with Greatly Improved Fluorogenic Properties

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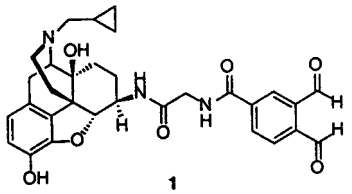
Abstract: To develop ligands with fluorogenic properties amenable for following the kinetics of cross-linking to receptors, a naphthalene dicarboxaldehyde moiety has been attached to an opiate pharmacophore **2** and evaluated in μ opioid receptors. The fluorescence of the benzo[*f*]isoindole formed upon cross-linking of μ opioid receptors by **2** permitted the time-course of covalent bonding to be followed. This demonstrated proof-of-concept suggests the usefulness of naphthalene dicarboxaldehyde-containing affinity labels as kinetic probes.

Affinity labels have been employed as tools to investigate the pharmacological and structural features of enzymes and receptors.[1] Most of these ligands contain an electrophilic moiety with covalent bonding potential. Recently, a new approach to affinity labeling was developed with the demonstration that opiate **1** is capable of specifically cross-linking opioid receptors with the concomitant formation of a fluorophore.[2] This type of ligand was named a "reporter" affinity label because its *o*-phthalaldehyde (OPA) moiety reports the cross-linking of neighboring lysine and cysteine residues through the generation of an isoindole fluorophore. This approach offers an advantage over conventional affinity labels because the fluorescence generated from the formation of a covalently bound receptor can be detected in cultured cells using flow cytometry.



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A problem associated with OPA-derived affinity labels is the laser-induced autofluorescence of cells that overlaps partially with the fluorescence of the isoindole fluorophore, thereby reducing the sensitivity of the method. This has limited the use of OPA-based affinity labels as fluorogenic probes because the kinetics of covalent bonding cannot be easily followed using standard flow cytometers. Here, we report on the utilization of the naphthalene dicarboxaldehyde (NDA) moiety as a fluorogenic component to circumvent this shortcoming and permit such ligands to be employed as tools in monitoring the kinetics of site-directed covalent cross-linking.

Given that NDA has reactivity similar to that of OPA (Figure 1) and since the benzo[*f*]isoindole fluorophore derived from NDA possesses an excitation maximum at 480 nm (rather than 325 nm for the corresponding OPA-derived isoindole), excitation and detection of the generated fluorophore were possible without interference from the autofluorescence using standard flow cytometers to follow the kinetics of cross-linking.[2−4] As another advantage, the fluorescence quantum yield of the NDA-derived fluorophore ($\phi_f = 0.58$) is improved over that of isoindole ($\phi_f = 0.42$).[3] For these reasons, NDA is considered superior to OPA as a fluorogenic reagent for the routine detection of amino acids and in labeling enzymes.[3,4]

To demonstrate proof of concept for this approach, we have synthesized affinity label **2** that contains the NDA moiety linked to an opioid antagonist pharmacophore. The synthesis of compound **2** (as its hydrated dihydroxybenzophthalan) required 6,7-diformyl-2-naphthoic acid **6** and β-naltrexamine[5] **7** as intermediates. Com-

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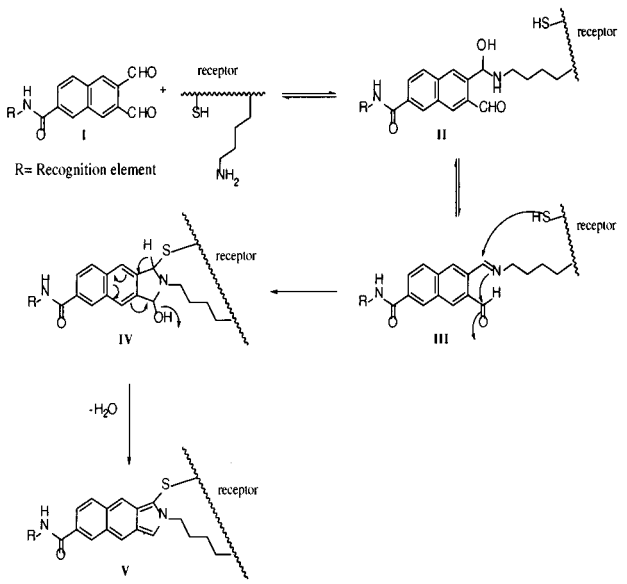


Figure 1. Mechanism for benzo[*f*]isoindole formation at a recognition site. The reaction of the NDA moiety (**I**) with the ϵ-amino group of lysine affords a carbinolamine (**II**), which then dehydrates to give the imine (**III**). This is attacked by a neighboring SH group of cysteine to form an intermediate (**IV**) that undergoes dehydration to yield the fluorescent benzo[*f*]-isoindole (**V**).

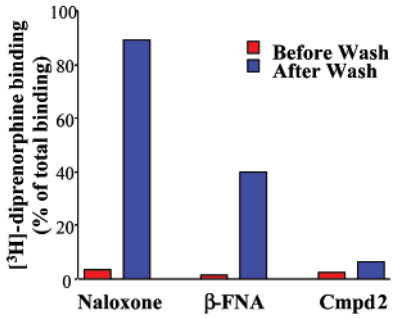
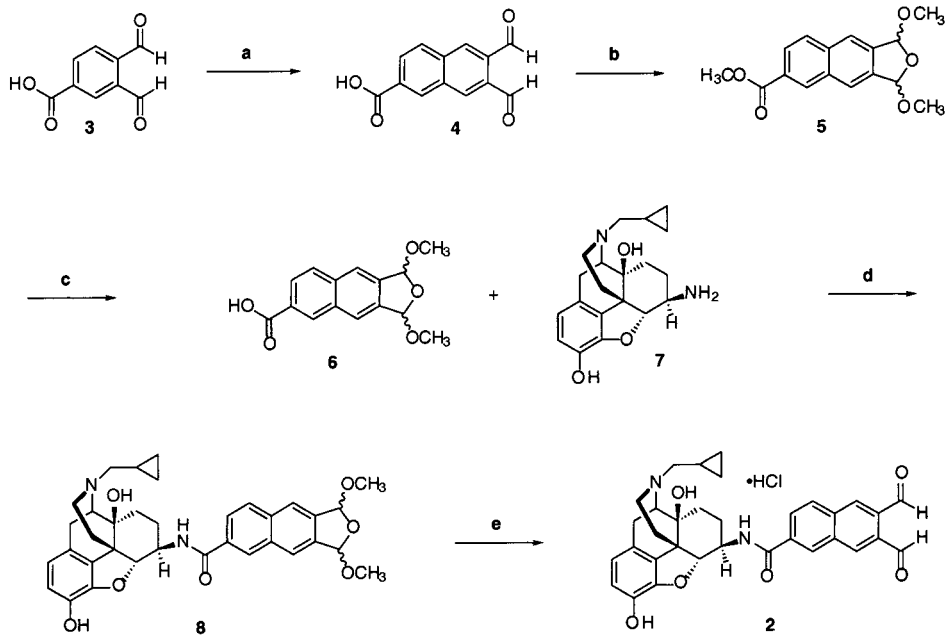


Figure 2. Irreversible binding of **2** and β-FNA to μ opioid receptors. Membranes of HEK cells stably transfected with μ opioid receptors were pretreated with different ligands (1 μM) at 37 °C for 15 min. Free receptor sites were determined in the presence of [^{3}H]diprenorphine (1 nM) before and after washing. The values represent the means of one experiment performed in duplicate.

pound **6** was prepared from the previously reported 3,4-diformylbenzoic acid **3**[2] as outlined in Scheme 1. A double aldol condensation of **3** with 3,5-dimethoxytetrahydrofuran in the presence of a catalytic amount of piperidine in a mixture of glacial acetic acid and water (2:1) afforded the 6,7-diformyl-2-naphthoic acid **4**.[6] Treatment of **4** in refluxing absolute methanol under Dean−Stark conditions produced the dimethoxybenzophthalan methyl ester **5**, which was hydrolyzed with lithium hydroxide to provide the free acid **6** as the key intermediate. Coupling of **6** to β-naltrexamine **7** with EDCI/HOBT afforded the corresponding amide **8**.[7] Hydrolysis of **8** under acidic conditions gave the hydrochloride salt of **2** as the hydrated dihydroxybenzophthalan.

Receptor binding of **2** was conducted on μ opioid receptors stably expressed in human embryonic kidney (HEK) cells. Compound **2** inhibited [^{3}H]diprenorphine receptor binding with an apparent K_i[8] value of 0.744 ± 0.308 nM. Experiments to determine irreversible binding of **2** were carried out on membranes from HEK cells containing stably expressed μ opioid receptors (Figure 2). Incubation of **2** or the standard μ opioid receptor affinity label, β-funaltrexamine,[9] at a concentration of 1 μM with receptor membranes for 15 min (37 °C) followed by extensive washing, reduced the binding of [^{3}H]diprenorphine relative to that of the control. These results suggested that compound **2** bound irreversibly

Scheme 1[a]



[a] Reagents and conditions: (a) 3,5-dimethoxytetrahydrofuran, HOAc, H_2O, piperidine, reflux, 18 h, 65%; (b) MeOH, *p*-TsOH, Dean−Stark, 12 h, 30%; (c) LiOH, THF, H_2O, 1 h, 99%. (d) (1) EDCI, HOBt, DMF, N_2, room temp, 18 h, (2) MeOH, K_2CO_3, 63%; (e) HCl (1 N), N_2, acetone, room temp, 5 days, 55%.

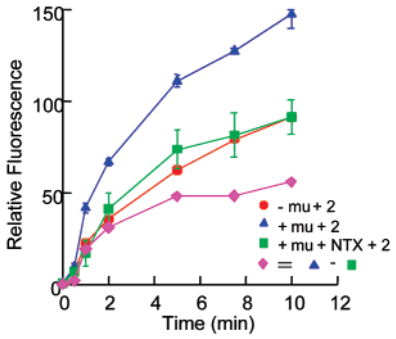


Figure 3. Time-course of relative fluorescence intensity of μ-opioid-receptor-transfected HEK cells exposed to **2** (100 nM). The kinetics were followed in nontransfected HEK cells (red ●, $-\mu + 2$), HEK cells transfected with μ opioid receptors (blue ▲, $+\mu + 2$), and naltrexone-pretreated HEK cells transfected with μ opioid receptors (green ■, $+\mu$ + NTX + **2**). Specific fluorescence (red ◆) was determined by subtracting the relative fluorescence values of tranfected cells from those of NTX-pretreated transfected cells (blue ▲ − green ■) at the same time points.

to μ opioid receptors and demonstrated a reactivity greater than that of β-FNA.

Experiments were carried out to evaluate the fluorescence that is generated upon interaction of **2** with μ opioid receptors. This was accomplished with a Becton−Dickinson FACS Vantage equipped with an argon laser for excitation at 488 nm using a band-pass filter of 530 ± 15 nm for detection.[10] A baseline autofluorescence was measured for HEK cells containing stably expressed μ opioid receptors suspended in HEPES buffer (pH = 7.5). Upon addition of **2** (100 nM)[11] to the suspension and monitoring over 10 min, the fluorescence intensity rapidly increased (<30 s) and plateaued at ∼7.5 min (Figure 3).[12] To determine if the fluorescence was due to covalent association in the vicinity of the receptor binding pocket (specific fluorescence), protection studies were carried out by pretreating HEK cells containing stably expressed μ opioid receptors with the opioid antagonist naltrexone (1 μM). The finding that the fluorescence of the naltrexone-protected, receptor-transfected HEK cells was essentially equivalent to that of the nontransfected cells suggests that **2** formed covalent bonds with lysine and cysteine residues in the region of the opioid recognition site (Figure 1). The specific fluorescence was then determined by subtracting the relative fluorescence values of the receptor-transfected HEK cells from those of the NTX-protected receptor-transfected HEK cells at the same time points. The kinetics of covalent association was determined from the resultant curve (Figure 3).

The truly distinct advantage of the NDA-derived reporter affinity label **2** over OPA and conventional affinity labels is the ability to follow the kinetics of covalent association in cells using one-color flow cytometry. Assuming that the reversible binding step is very rapid at the concentration of **2** employed, the rate-limiting step should be covalent cross-linking of the neighboring lysine and cysteine residues at the recognition site, resulting in the formation of the benzo[*f*]-isoindole. Therefore, pseudo-first-order kinetics of covalent bonding can be determined from the time-dependent, specific fluorescence intensity increase.

In conclusion, as proof of concept, an NDA-based affinity label **2** has been synthesized and determined to have superior fluorogenic properties that enable it to be employed to follow the kinetics of μ opioid receptor cross-linking. The improved fluorogenic properties of the NDA moiety make this a viable approach for the development of fluorogenic affinity labels in general and opioid receptor probes in particular. In this regard, reporter affinity label **2** is a potentially useful probe for exploring the ligand recognition sites of the opioid receptor family. Mutagenesis studies to determine the specific lysine and cysteine residues that are cross-linked to form the benzo[*f*]isoindole moiety are presently underway.

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Supporting Information Available: Experimental details for the synthesis of compounds **2, 4**−**6**, and **8** and their spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

Note Added after ASAP Posting. This manuscript was released ASAP on 6/12/2002 with errors in the artwork for **1**. The correct version was posted on 6/27/2002.

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(7) A minor byproduct of the coupling reaction produced the desired **8** as well as the N- and O-acylated byproducts. These side reaction products were hydrolyzed to **8** by treating the crude reaction with a methanolic solution of potassium carbonate during workup.

(8) The apparent K_I reflects both the reversible and irreversible binding components.
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(10) Preliminary studies indicated that **2** is nonfluorescent at the wavelengths used for detection of the resultant benzo[*f*]isoindole. Furthermore, the optimal emission and absorption wavelength values used in the flow cytometric experiments are in the same range as those reported for the benzo[*f*]isoindole fluorophore.[3,4]
(11) On the basis of the development of FACS assay conditions, the 100 nM concentration of **2** worked best to demonstrate a kinetic distinction between specific and nonspecific binding. Lower concentrations also demonstrated an increase in median fluorescence, but the viability of the cells did not permit study of the time frames necessary to complete the reaction. Higher concentrations saturated the system to the point of immediate reaction completion demonstrating the endpoint of a kinetic analysis.
(12) The stability of the whole cells in this assay did not permit time points to be acquired past 10 min. In theory, the total fluorescence curve and nonspecific fluorescence curve would eventually converge to the same endpoint.

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