Photoactivatable Opiate Derivatives as Irreversible Probes of the μ-Opioid Receptor

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The synthesis of aryldiazonium and arylazido derivatives of carfentanil, etonitazene, and naltrexone and of a triazaspirodecane derivative is described. The chemical stability and the spectral characteristics of these compounds were verified, and their binding affinity constants for the different opioid receptor classes were determined, in the absence of light, from competition experiments. With the exception of the naltrexyl derivatives, which remained nonselective, all compounds tested displayed a pronounced μ-binding selectivity with μ/δ and μ/κ ratios ranging from 12 to 1000. After irradiation, only the arylazido probes led to an irreversible μ-binding-site inactivation. This inactivation fulfilled the criteria for photoaffinity labeling such as protection against inactivation by other opiate ligands and absence of an effect of scavengers on the extent of the inactivation. Most of the photoactivatable probes formed long-lasting reversible complexes with the opioid binding sites: an efficient dissociation procedure was thus required to discriminate between pseudoirreversible and covalent complexes. The marked differences in labeling efficacy between aryldiazonium salts and their corresponding arylazido derivatives are discussed.

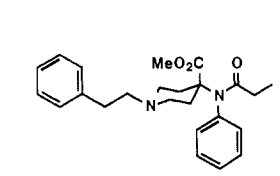
Affinity and photoaffinity labeling techniques consist of selective covalent-bond formation between a chemically reactive ligand analogue and the active site of a functional protein (enzyme or receptor). They have been widely used in the molecular identification of biological receptors for hormones and neurotransmitters.[1] Such analytical methods allow, when a radioactive probe is used, the detection of the target protein during its purification. Two major consequences arise: firstly, purification and microsequencing of a labeled peptide belonging to a protein of unknown sequence can help in conceiving molecular probes (antibodies and oligonucleotides) for gene cloning and sequencing;[2] secondly, positioning all the modified residues along a protein amino acid sequence can lead to the localization, and/or to a topographical analysis, of the ligand binding site.[3-5] In the latter case, however, irreversible labeling should be distributed among numerous amino acids of the active site regardless of their chemical reactivity: short-lived photogenerated species are therefore required.

Water-soluble, positively charged diazonium salts generate, upon irradiation, arylcations[6] which are highly reactive, as assessed by their reaction with carbon–carbon and carbon–hydrogen bonds,[7] as well as with molecular nitrogen.[8] They have been described, for instance, as suitable photoaffinity probes for the topographical analysis of the nicotinic acetylcholine receptor binding site.[4,5]

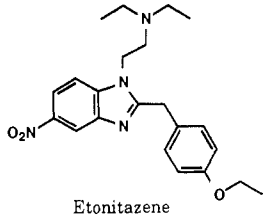
On the other hand, arylazido compounds, widely used as photolabels, are neutral, very stable in the dark, and much more hydrophobic than diazonium salts. The reactions of their photolysis products (arylnitrenes) have been extensively studied, and it appears that the nature of the substituent on the aromatic ring controls possible rearrangement reactions to less reactive species.[9]

Our goal was to design new photoaffinity labels of the μ-opioid receptor. Irreversible labeling techniques[10,11] were previously used to characterize the different types of the opioid receptor and to establish some possible structural differences among them. The most successful work in this field concerns the molecular characterization of the affinity[12] and photoaffinity[13] labeled δ-opioid receptor. However, depending on the probe and/or the source of receptor used (brain homogenates or NG 108 × 15 cultured cells), the molecular weight evaluation of the specifically

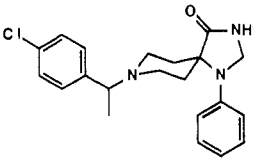
Chart I



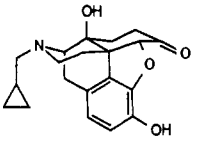
Carfentanil



Etonitazene



Triazaspirodecane derivative



Naltrexone

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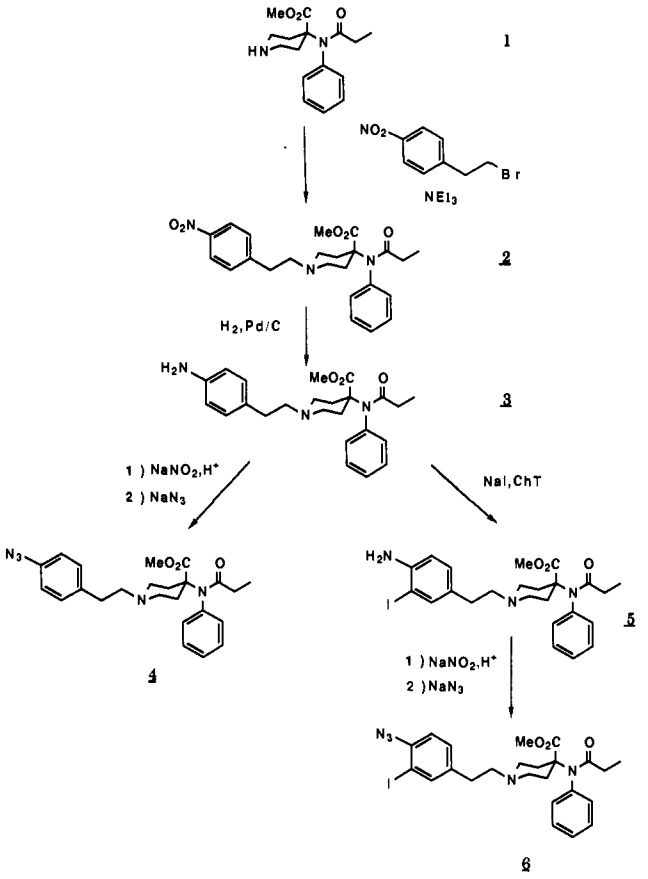
Scheme I



labeled polypeptides ranged from 33 to 71 kD. In one case, a polypeptide (58 kD) labeled by fentanyl isothiocyanate could be purified to apparent homogeneity.[14] Similarly, affinity labeling of the μ-type with agonist[15] or antagonist[16] derivatives, as well as crosslinkers,[11] led to the identification of an alkylated protein whose molecular weight was in the 38–58 kD range. Discrepancies between these results prevent the unambiguous characterization of the proteins associated with the μ- or δ-receptors and the establishment of whether they are different or not.

We describe here the synthesis of four, structurally unrelated, opiate ligands derived from carfentanil, etoni-

Scheme II

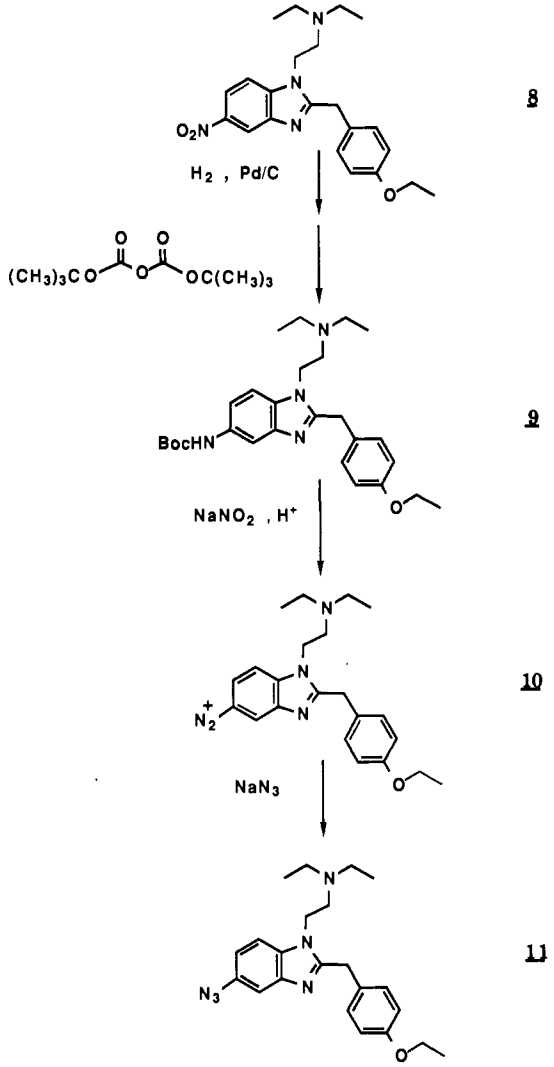


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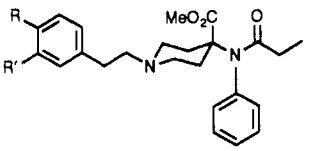
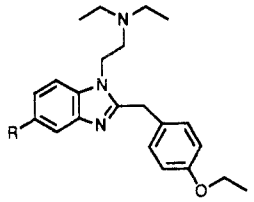
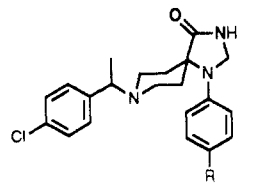
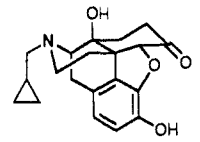
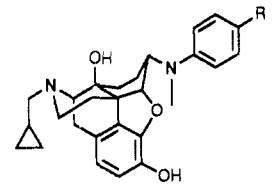
Table I. Structural Formula and Apparent Binding Affinity Constants at Opioid Receptors for Various Drugs

compound			drug affinity[a] (K_i, nM)			
			μ	δ	κ	T
	7	R = H, R′ = H	0.09[38]	9 ± 4	92 ± 30	0.25 ± 0.1
	4	R = N_3, R′ = H	8 ± 3	>1000	>1000	8 ± 5
	6	R = N_3, R′ = I	45 ± 15	>1000	>10000	27 ± 7
	8	R = NO_2	0.5[26]	>1000	ND	0.6 ± 0.2
	10	R = N_2^+	11 ± 5	>10000	ND	18 ± 8
	11	R = N_3	2 ± 2	400 ± 150	242 ± 87	4 ± 1
	13	R = H	4[38]	75 ± 20	ND	10 ± 4
	16	R = N_2^+	11 ± 5	>1000	ND	62 ± 30
	17	R = N_3	3 ± 1	100 ± 30	ND	5 ± 2
	18		1.8[39]	6.7[39]	8.7[39]	0.55[22]
	21	R = N_2^+	0.6 ± 0.2	0.6 ± 0.3	1 ± 0.5	1.7 ± 1
	22	R = N_3	0.6 ± 0.2	0.7 ± 0.2	0.7 ± 0.15	0.5 ± 0.1

[a] K_i values were determined from competition experiments performed against [^{3}H]DAGO (μ), [^{3}H]DADLE or [^{3}H]DTLET (δ), and [^{3}H]-diprenorphine (T on rat brain, κ on guinea pig cerebellum) as reported in the Experimental Section.

tazene, triazaspirodecane, and naltrexone (Chart I), bearing either aryldiazonium or arylazido groups, and their use as photoaffinity labels. Care was taken to introduce, when possible, aromatic rings with electron donating groups in the para position of the photoactivatable moiety. This conferred, in particular for the *p*-dialkylamino diazonium salts, an enhanced stability compatible with the photoaffinity labeling requirements.[17] In addition, photoactivation of such aminoaryl diazonium salts and azido derivative chromophores can be achieved at wavelengths longer than 300 nm, an experimental condition which strongly reduces the undesired light-promoted receptor inactivation.

The use of such opiate ligands modified at identical positions by two photoactivatable functions should lead to variations in their crosslinking reactions with the receptor protein. Moreover, the availability of structurally unrelated opiate photolabels should help in probing different regions of the opioid receptor binding site.

Results

Chemistry. The compounds which were synthesized and tested are shown in Table I. Synthesis of azidocarfentanil derivatives **4** and **6** is outlined in Scheme I. The common 4-anilido-4-carbomethoxypiperidine moiety **1** was obtained in five steps from 1-benzyl-4-piperidone as described.[18] Reaction of **1** with phenethyl bromide yielded carfentanil **7** (Table I). The nitro carfentanil derivative **2** was obtained by a similar reaction with 4-nitrophenethyl bromide in the presence of triethylamine. Catalytic hydrogenation of **2** gave amine **3** which was either directly converted to the azido **4** or iodinated with sodium iodide in the presence of chloramine T[19] (ChT) to give the amine precursor **5** of the azido derivative **6**.

Scheme II describes the synthesis of derivatives from etonitazene **8**. The nitro group was reduced by catalytic hydrogenation, and the resulting oxidation-sensitive amine was characterized (**9**) after its protection by a *tert*-butoxycarbonyl (Boc) group. Alternatively, **9** could be directly obtained in one step from etonitazene when the protecting reagent was added to the reaction mixture before reduction.

The protected amine **15** of triazaspirodecane derivative was obtained by following the synthetic route outlined in Scheme III. After reductive amination of the commercially available 1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one **12** with *p*-chloroacetophenone, the nitrosation reaction gave, beside the expected chromophoric *p*-nitroso derivative **14**, an equal amount of a byproduct, whose structure, according to mass spectrometry and ^{1}H NMR spectroscopy, corresponded to the 2,3-dehydro compound (results not shown). Compound **15** was obtained after a one-pot

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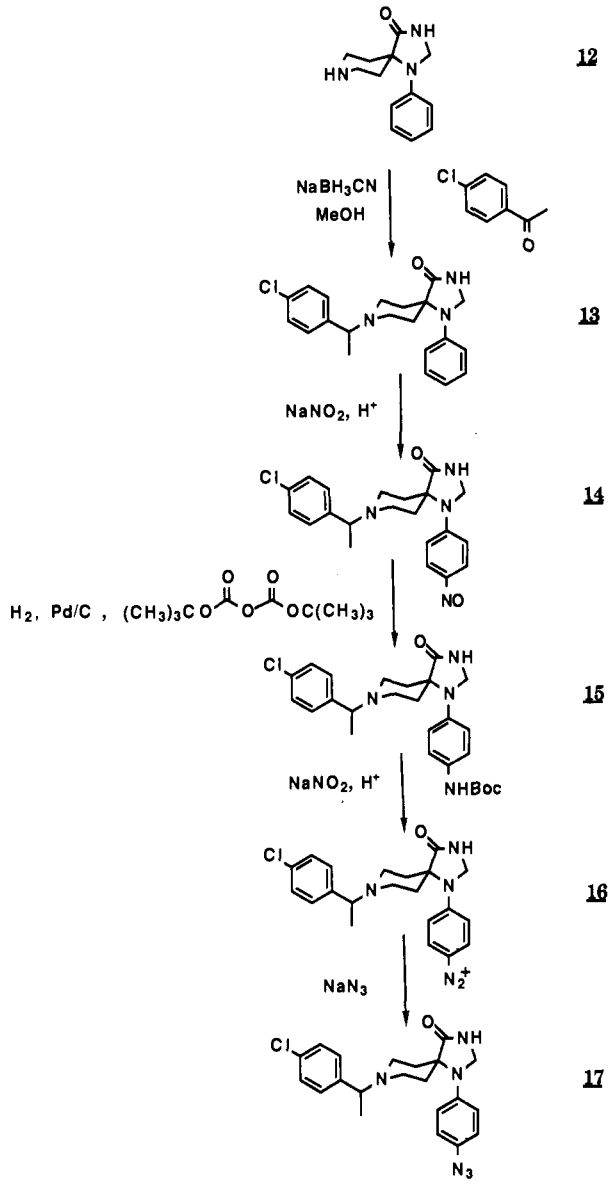
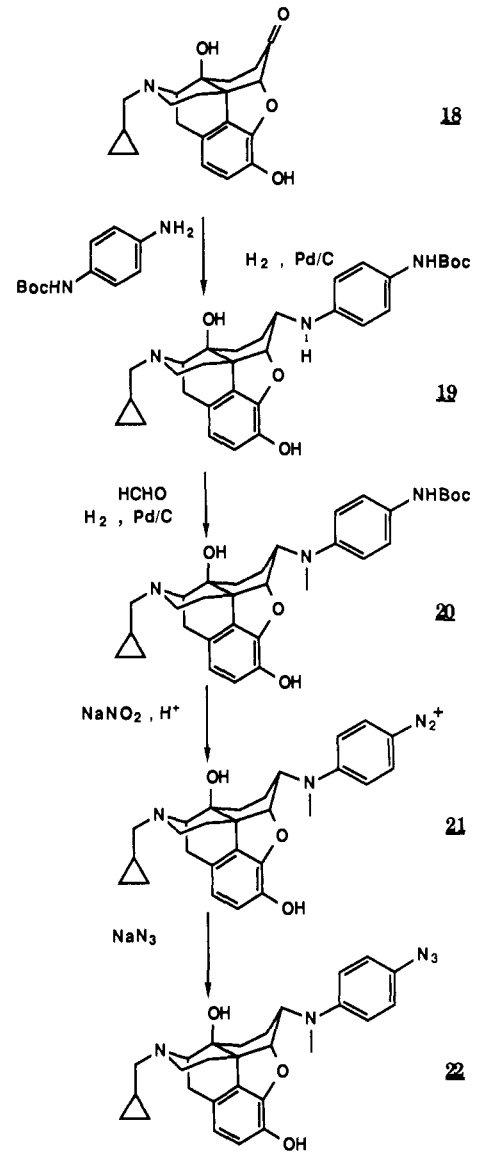
Scheme III



Scheme IV



reduction and protection of the intermediate amine. The synthesis of diazonium salt **16** has been also achieved in one step from aniline **13** by reaction with a large excess of sodium nitrite according to Tedder[20] with an overall yield of 25%. The azido derivative **17** was obtained from **16** in good yields by sodium azide treatment.

Finally, the synthesis of the derivatives in the naltrexone series is outlined in Scheme IV. The modification at position 6 of naltrexone was selected since it did not result in a significant loss of affinity.[21] The substituted aromatic moiety was introduced by reductive amination of the 6-keto moiety of naltrexone **18** with use of a mono protected *p*-phenylenediamine derivative. The resulting secondary amine **19** was methylated with formaldehyde under catalytic hydrogenation conditions leading to compound **20**. It should be possible to take advantage of this methylation reaction to incorporate tritium in this molecule. The proposed α-configuration at the 6-position of **19** was assigned by ^{1}H NMR spectroscopy by comparison with the known 6α- and 6β-naltrexamine.[22] In particular, the observed coupling constant between H_5 and H_6 is identical with the one described for α-naltrexamine; $J_{H_5-H_6}$ = 4 Hz versus 7.4 Hz for the β-epimer. The photoactivatable derivative **21** was obtained by using a general diazotization procedure, adapted to small-scale reactions (1–2 μmol), which generally started from the Boc derivatives. The reaction was performed in strongly acidic medium (1:1 mixture of trifluoroacetic acid (TFA)/concentrated HCl) at low temperature (−25 °C) in order to avoid nitrosation of amide bonds[23] or nitrosative cleavage of tertiary amines.[24] The homologous azido derivative **22** was obtained from the isolated and purified parent diazonium salt through classical sodium azide treatment.

All these derivatives were isolated and purified by HPLC and their physicochemical properties were studied (Table II). With the exception of *p*-alkyl substituted derivatives, azido compounds absorbed light with a maximum in the 270–300 nm region and their molecular extinction coefficients ϵ ranged from 7000 to 19 000 M^{-1} cm^{-1}. Except for compounds **4** and **6**, which were irradiated at 254 nm, such properties allowed irradiation at 315 nm. The spectral

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Table II. Chemical Stability and Spectral Properties of Diazonium and Azido Probes

compound	maximal absorption wavelength, λ_{max}, nm	molar extinction coefficient, ϵ, M^{-1} cm^{-1}	half-life,[a] $T_{1/2}$, h
azidocarfentanil (4)	252	9600[c]	>24
iodoazidocarfentanil (6)	259	7800[c]	>24
etonitazenediazonium (10)	312	11750[b]	0.8
azidoetonitazene (11)	300	7000[c]	>24
spirodecanonediazonium (16)	369	37500[b]	5
azidospirodecanone (17)	273	19000[c]	>24
naltrexonediazonium (21)	382	38000[b]	5
azidonaltrexone (22)	285	19000[c]	>24

[a] The chemical stability of these various derivatives was estimated in the absence of light, in 50 mM Tris-HCl–1 mM EDTA (pH 7.4) buffer. [b] Molar extinction coefficients, ϵ, were calculated by extrapolation with related model aromatic diazonium salts, obtained in a pure crystalline form. [c] Molar extinction coefficients, ϵ, were measured after HPLC purification.

characteristics of aryldiazonium salts (λ_{max} = 310–380 nm; ϵ = 11000–38000 M^{-1} cm^{-1}) allowed irradiation at their maximal absorption wavelength.

Chemical stability (in absence of light, in Tris-EDTA buffer pH 7.4) of the diazonium salts was measured (Table II). The half-life of the derivatives used in photoaffinity labeling experiments was more than 45 min; the diazonium precursors of 4 and 6, which had half-lives shorter than 30 min, could not be studied. We checked that, as expected, the azido derivatives remained unchanged in the same incubation conditions (not shown).

Biochemistry. Reversible Binding at Opioid Receptors. Binding affinity constants of our derivatives were determined from equilibrium competition experiments, in the absence of light, either on rat brain homogenates against μ-selective ([^{3}H]DAGO), δ-selective ([^{3}H]DTLET and [^{3}H]DADLE), or nonselective ([^{3}H]diprenorphine) radioligands or on guinea pig cerebellum membranes against [^{3}H]diprenorphine (κ-sites).

Table I presents affinity constants of the photoactivable analogues in the four series along with those of the unmodified compounds. K_i values against [^{3}H]diprenorphine (T), which are generally considered as good estimates of the affinity for the total opioid receptor population,[25] ranged from 0.2 to 90 nM. Comparison of the K_i values deduced from [^{3}H]DAGO and [^{3}H]diprenorphine competition experiments indicates that the more the derivative is selective for the μ-binding sites, the more its K_i value against [^{3}H]-diprenorphine is close to that obtained against [^{3}H]DAGO (compounds 4–17). On the other hand, when the probe is nonselective toward the different opioid classes, its K_i value against [^{3}H]diprenorphine reflects a mean affinity value for the whole opioid binding sites (compounds 21 and 22). All compounds displaced radioligands bound to the μ-sites at nanomolar concentrations (0.2–40 nM) and K_i values for the μ-sites were not affected by charge or hydrophobicity differences between diazonium and azido groups. Selectivity of agonist derivatives for the μ- over the δ-sites ranged from 12- to 1000-fold (6 and 10, respectively) while the μ/κ affinity constant ratio was always greater than 100. Derivatives of the nonselective antagonist naltrexone remained unselective.

Reversibility of the photosensitive drug binding from μ-sites was quantitatively assayed by a filter dissociation technique[26] (Table III). All photoactivatable derivatives were fully reversible in the absence of light, a property allowing their use in further photolabeling experiments. An exception was compound 17 which could not be dissociated at all, most probably because of its very poor solubility in water. Note that when the washing buffer was deprived of sodium chloride, the efficiency of the filter procedure was diminished (compound 21).

Table III. Irreversible Occupancy of μ-Opioid Receptor Sites by Various Photoactivatable Derivatives[a]

compd	concn, M	specific [^{3}H]sufentanil binding (% of control)		
		$-h\nu$	$+h\nu$	$+h\nu$ + naloxone 10^{-6} M
naloxone	10^{-6}	111	105	–
4	3×10^{-7}	96	108	115
6	4×10^{-7}	100	75	94
10	10^{-7}	105	109	105
11	3×10^{-7}	95	19	69
16	10^{-7}	88	93	105
17	3×10^{-7}	0	–	–
21	3×10^{-7}	55[b]	96[b]	–
22	3×10^{-8}	96	58	76

[a] Rat brain membranes were incubated for 20 min at 37 °C, in the dark, with the indicated concentration of ligand (column 1) and then submitted (columns 3 and 4) or not (column 2) to irradiation (20 min at 5 °C) as reported under the Experimental Section. Irradiation steps were carried out in the absence (column 3) or the presence of 10^{-6} M naloxone (column 4). Unbound ligand was removed by using the filter dissociation technique[26] and the recovered μ-binding sites were measured by specific [^{3}H]sufentanil binding as described earlier. Reported values are referred to similarily treated samples, deprived of photosensitive probe (controls). Each experiment was performed at least in duplicate. [b] Drug dissociation was undertaken using Tris–EDTA buffer deprived of 1 M NaCl.

Irreversible Binding at Opioid Receptors. Ligands were preincubated in the dark with rat brain membranes and irradiated at appropriate wavelengths in a quartz cell. Treated samples were then adsorbed onto filters and submitted to the dissociation procedure at high ionic strength. Recovered μ-opioid binding sites were estimated by specific binding of [^{3}H]sufentanil (0.5 nM). This ligand showed, respectively, about 100- and 1000-fold greater binding affinity for the μ-opioid receptor (K_d = 0.25 nM)[26] than for δ-[27] and κ-binding sites.[28] Thus under our experimental conditions, only the μ-opioid binding sites were quantified. Whatever the photolabeling conditions, naloxone alone never led to μ-receptor inactivation and was therefore used to protect the opioid binding sites against inactivation by the photoactivatable drugs.

Results of a typical photoaffinity labeling experiment are shown in Figure 1. The reversibility, in the dark, of the binding of 11 was verified (A compared to B) after a 40-min dissociation period on filters. In the irradiated control samples (membranes alone (C) or with 10^{-6} M naloxone (D)), less than 10% of the ^{3}H-sufentanil binding sites were lost. 5-Azidoetonitazene (11) produced a concentration-dependent (not shown) μ-receptor inactivation reaching 82% at 3.10^{-7} M (E) which was not modified by performing a prolonged washing step on filters. Previous addition of an excess of naloxone (10^{-6} M) to the incubation medium allowed substantial protection of the receptor sites against inactivation (F). The nitrene scavenger *p*-aminobenzoic acid (PAB) (up to 10^{-3} M) did not inhibit labeling by 11, suggesting that its photoreactive species was not in contact with the aqueous medium.

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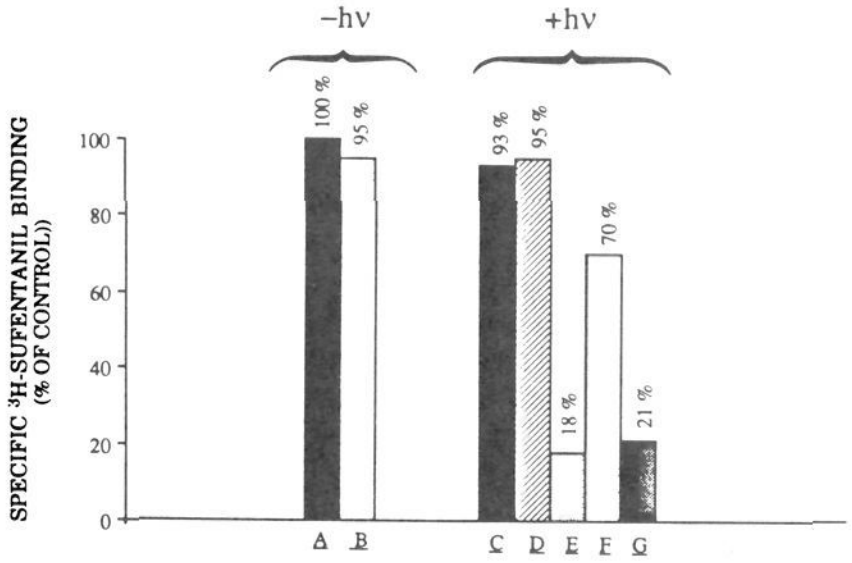

A : Membranes
B : Membranes + **11** (3.10^{-7} M)
C : Membranes
D : Membranes + Naloxone (10^{-6} M)
E : Membranes + **11** (3.10^{-7} M)
F : Membranes + **11** (3.10^{-7} M) + Naloxone (10^{-6} M)
G : Membranes + **11** (3.10^{-7} M) + PAB (10^{-3} M)

Figure 1. Photoinactivation of μ-opioid receptors by the azido derivative **11**. Membranes and drugs, at the indicated concentrations, were incubated for 20 min at 37 °C and subsequently irradiated (+$h\nu$) or not (−$h\nu$) for 20 min at 315 nm. Thereafter, the filter dissociation procedure and the [^{3}H] sufentanil binding assay proceeded as described. Controls (A) are from unirradiated samples, deprived of probe **11**.

Results of similar experiments using the other photoactivatable opiates are presented in Table III. We first checked that the binding of all probes (at the indicated concentration) was fully reversible in the dark. Interestingly, the complex formed by the interaction of **21** with the μ-opioid receptor was stable in the dark, while after irradiation its dissociation rate increased markedly. A reasonable explanation for this phenomenon could be the transformation of the bound derivative into the corresponding phenol which dissociated more rapidly (result not shown). Aryldiazonium salts **10**, **16**, and **21**, irradiated at their maximum absorption wavelength, did not lead to any detectable receptor inactivation (less than 5%). On the other hand, some of the azido compounds irreversibly occupied μ-binding sites in a naloxone-sensitive manner. Compounds **6**, **11**, and **22** inactivated specific [^{3}H]sufentanil by 25%, 80%, and 38%, respectively. The extent of these inactivations was not modified by addition of PAB to the incubation medium, indicating that no pseudoaffinity labeling occurred.[29] It should be noted that, unlike its iodinated counterpart **6**, the carfentanil derivative **4** did not alkylate the binding sites. Finally, compound **22** due to its absence of selectivity could possibly inactivate δ- and κ-receptors; this point was not explored further.

Discussion

The existence of different opioid receptor types is well documented even though their structural identities as well as their biological functions continue to be areas of active research.[30] During the last decade, the search for new ligands, as selective as possible for each receptor class, became very intense and led to extensive structure–activity studies. This facilitated the design of (photo)affinity probes as useful tools to study, at a molecular level, the polymorphism of the opioid receptors.

We took advantage of the existence of several classes of opiate compounds, structurally unrelated, to design and synthesize the probes. Four series of chemicals were selected according to (i) their good binding affinities for the opioid receptor sites, (ii) their pharmacological properties which should enable receptor polymorphism studies, and (iii) the possibility of modifying an existing aromatic group by introducing the diazonium or the corresponding azide functions without altering the general ligand structure, shape and pharmacological characteristics. From these considerations, we selected three μ-selective agonists, namely carfentanil (**7**), etonitazene (**8**), and a triazaspirodecane derivative (**13**), each displaying a pronounced μ-binding selectivity, as well as a high affinity, nonselective antagonist naltrexone (**18**).

In the carfentanil series, the synthesis of the diazonium salt and consequently of its azido derivative could not be achieved from the corresponding amine on the propioanilido aromatic ring. We therefore designed two *p*-alkyl arylazido carfentanil derivatives, with (**6**) or without (**4**) an iodine atom on the phenethyl side chain in the position ortho to the photosensitive function. Both compounds retained a fairly good affinity and selectivity for the μ-opioid type (Table I). When irradiated in the presence of rat brain membranes, only compound **6** was able to partially inactivate the μ-binding site. This fact might be explained either by a difference of chemical reactivity between the two corresponding nitrenes or by different positioning of the ligand in the binding site. *p*-Alkyl nitrenes, such as those obtained after irradiation of compound **4** almost quantitatively rearrange to electrophilic azacycloheptatetraenes which react with amines and to a lesser extent with sulfhydryl or hydroxyl groups.[31] Conversely, para-iodinated arylnitrenes intercross from the singlet to the triplet state and react as diradicals with the surrounding species.[9] No such data, however, are available for the *o*-iodo isomers.

Another possible explanation would be that the presence of a bulky iodine atom on the aromatic ring induces a different ligand positioning in the binding site. This can be correlated to the larger decrease in binding affinity of compound **6**, as compared to **4**, for the μ-receptor type. Furthermore, the absence of irreversible inactivation of the μ-class by compound **4** can be linked to the labeling characteristics of fentanil isothiocyanate (FIT):[12b] both molecules carry at the same position of the phenethyl aromatic ring a substituent of comparable size and shape and are unable to covalently label μ-opioid receptor sites. This suggests the absence of nucleophilic residues in the probed area.

The etonitazene azido derivative **11** displayed a pronounced μ-binding selectivity with μ/δ and μ/κ binding affinity ratios of greater than 2 orders of magnitude. In the absence of light, **11** was much more stable in Tris–EDTA buffer ($t_{1/2}$ > several days at pH 7.4, Table II) than its parent diazonium **10** and completely dissociated from the receptor site when the filter technique was used. Its irradiation with membranes led to an efficient μ-receptor inactivation. This labeling was sensitive to the presence of an opiate competing ligand (naloxone) and was not modified by a large excess of PAB. It may thus be considered as a good candidate for further labeling studies.

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The triazaspirodecane derivatives **16** and **17** were also good ligands for the μ-receptor, but they showed a lesser μ/δ selectivity as compared to the etonitazene series. The diazonium derivative **16** displayed the typical spectral properties of *p*-(*N*,*N*-dialkylamino) aryldiazonium derivatives and was chemically stable in the dark. Interestingly, its aqueous photogenerated phenol derivative lost the high affinity binding property for the μ-receptor sites (not shown), a fact which might help in preventing a sterile occupancy of the binding sites during the photolabeling reaction. Unfortunately, a poor inactivation of the μ-opioid receptor was observed when this diazonium derivative **16** was irradiated in the presence of membranes. On the other hand, the pseudoirreversible character of the corresponding azido derivative **17** in the dark, precluded any safe photolabeling experiments to be performed. Discrimination between an affinity labeling and a very slow dissociating process, possibly due to an enhanced hydrophobic character, would require the use of a radioactive probe and a sodium dodecyl sulfate polyacrylamide gel analysis of the alkylation pattern.

Finally the naltrexyl derivatives **21** and **22** displayed, as expected, a high affinity and no μ-, δ-, or κ-selectivity. The partial reversibility of the binding of **21** might be explained by the mild dissociation conditions that were used (no added salt in the washing buffer). The occurrence of an affinity labeling of the receptor is also unlikely since the ligand was completely removed after irradiation of the drug–receptor complex at a wavelength appropriate to diazonium photodecomposition (382 nm). On the contrary, the homologous azido derivative **22** produced, upon irradiation, a significant receptor blockade, sensitive to naloxone, but not to the nitrene scavenger PAB. In this case again, it is interesting to note that irradiation of *p*-(*N*,*N*-dialkylamino)aryl azides leads to nitrenes which do not rearrange to azacycloheptatetraenes but quantitatively intercross to the triplet state.[9b] Thus, the reactive species of the naltrexone derivative **22** is most probably the triplet state of the arylnitrene which could cross-link with proteic residues as recently suggested.[32] Finally, this compound **22** binds equally well in the presence or absence of sodium chloride to its binding sites (not shown) and might therefore help in characterizing purified receptors.

The use of four structurally unrelated photoactivatable opiates led to the conclusion that alkylation of μ-receptors was observed exclusively with azido probes, but never significantly with aryldiazonium salts. This result is totally unexpected according to the relative reactivity of the photogenerated species and raises several questions. On one hand, we are dealing with hyperreactive aryl cations for which an efficient coupling to the receptor molecule is expected, assuming that the photosensitive probe and the protein are in close proximity in the reversible complex. On the other hand, alkylation with the less reactive nitrenes, even though in their triplet state, should be much more dependent on the chemical environment.

A chemical instability of the diazonium salts, in the presence of membranes, seems unlikely since no inactivation was observed even at high concentrations of diazonium ligands. It seems more reasonable to assume that, according to their structure and/or their respective physicochemical properties, the probes are differently oriented in the binding site. The more liposoluble azido probes could effectively be partially buried in a hydrophobic environment (apolar amino acid residues and/or lipids). Such a positioning would explain the lack of quenching observed in the presence of scavenger. On the other hand, the charged diazonium salts are more likely to be oriented toward a nonproteic polar surrounding (membrane surface, outer medium) and therefore unable to react with the receptor at least at the binding site.

Recent works emphasized the crucial role of a lipid environment for the binding to opioid receptors and in particular correlated the binding to μ-sites to the presence of negatively charged phospholipids.[33] A strong ionic interaction could therefore be expected between the diazonium moiety and these polar heads. A description of this binding site, partially buried in a lipidic environment, where the probes could adopt a flexible positioning, remains the most satisfying explanation of our irreversible labeling results.

Experimental Section

Melting points were obtained on a Mettler FP1 apparatus and are given as uncorrected values. All ^{1}H NMR experiments were performed with $CDCl_3$ as solvent except where indicated otherwise. They were recorded on either a Bruker Model WP60 (60 MHz) or Bruker Model WP SY200 (200 MHz) spectrometers with $SiMe_4$ as an internal reference. Chemical shifts are reported in δ (ppm). Mass spectra were recorded either on a LKB Model 9000S instrument with an electron impact (EI) source (70 eV) or a Thomson Model THW 208 instrument with a chemical ionization (CI) source (NH_3, CH_4). UV spectra were recorded on a Kontron UVIKON 860 spectrophotometer. HPLC was conducted on a WATERS model Automated Gradient Controlled instrument with μBondapak reverse-phase columns. The compounds were analyzed for C, H, and N by the Service de Microanalyse du CNRS de l'Université Louis Pasteur (Strasbourg), and the results were within 0.4% of the theoretical value.

The sources of tritiated ligands were as follows: [^{3}H]diprenorphine (38.9 Ci/mmol) and [^{3}H]DADLE ([^{3}H-D-Ala^2-D-Leu^5]enkephalin, 58 Ci/mmol), Amersham; [^{3}H]DAGO ([^{3}H-D-Ala^2-MePhe^4-Gly-ol^5]enkephalin derivative, 43 Ci/mmol), and [^{3}H]DTLET([tyrosyl-(3,5-^{3}H)-D-Thr-Gly-Phe-Leu-Thr]enkephalin derivative, 61 Ci/mmol), C.E.A. (Saclay, France). [^{3}H]Sufentanil (12 Ci/mmol) was from Janssen Pharmaceutica (Beerse, Belgium).

Morphiceptine was purchased from Bachem, naloxone and naltrexone from du Pont de Nemours (Glenolden). Etonitazene was a gift of Ciba-Geigy (Basel, Switzerland), U 50 488 of Upjohn Company (Kalamazoo, MI) and diprenorphine of Reckitt and Colman (Kingston, England).

Carfentanil (7), mp 188–189 °C (lit. mp 189.5 °C), was synthesized according to a previously described synthetic route[18,34] with an overall yield of 11%.

Methyl 4-[*N*-(1-Oxopropyl)-*N*-phenylamino]-1-[2-(4-nitrophenyl)ethyl]-4-piperidinecarboxylate (2). An acetonitrile solution (20 mL) containing 5.38 mmol (1.56 g) of methyl 4-[*N*-(1-oxopropyl)-*N*-phenylamino]-4-piperidinecarboxylate (1),[1] 8 mmol (820 mg) of triethylamine, and 16 mmol (3.71 g) of 4-nitrophenyl bromide was stirred at 70 °C for 24 h. The solvent was evaporated, and the solid residue was dissolved in ethyl acetate, washed twice with water, and dried over sodium sulfate. After concentration in vacuo, the precipitate was recrystallized from ether to give 1.13 g (48%) of **2**: mp 146–147 °C; ^{1}H NMR (200 MHz) δ 8.1 (d, 2 H), 7.44–7.27 (m, 7 H), 3.8 (s, 3 H), 2.86–2.17 (m, 10 H), 1.85 (q, 2 H), 1.63 (m, 2 H), 0.96 (t, 3 H). Anal. $C_{24}H_{29}N_3O_5$: C, H, N.

Methyl 4-[*N*-(1-Oxopropyl)-*N*-phenylamino]-1-[2-(4-aminophenyl)ethyl]-4-piperidinecarboxylate (3). A solution of 0.45 mmol (200 mg) of **2** in 5 mL of methanol containing 20 mg of 10% Pd/C was submitted to catalytic hydrogenation (4 atm) for 3 h. After removal of the catalyst by filtration and evaporation of the solvent, the mixture was partitioned between water and ethyl acetate. The extract was dried and concentrated

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under vacuum. The oily residue was dissolved in ether and converted to the oxalate salt to yield after recrystallization, 224 mg (99%) of **3**: mp 167–168 °C; ^{1}H NMR (200 MHz) δ 7.48–7.35 (m, 5 H), 6.9 (d, 2 H), 6.6 (d, 2 H), 3.8 (s, 3 H), 3.6 (s, 2 H), 2.86–2.17 (m, 10 H), 1.85 (q, 2 H), 1.63 (m, 2 H), 0.96 (t, 3 H). Anal. $C_{26}H_{33}N_3O_7$ (oxalate): C, H, N.

Methyl 4-[*N*-(1-Oxopropyl)-*N*-phenylamino]-1-[2-(3-iodo-4-aminophenyl)ethyl]-4-piperidinecarboxylate (5). A solution of the amine **3** (0.24 mmol, 100 mg) in methanol (16 mL) was added to a 0.5 N solution (160 mL) of sodium acetate buffer (pH 5.6). The mixture was treated in rapid succession with 0.36 mmol (54 mg) of NaI in 136 mL of 0.1 N NaOH followed by 0.36 mmol (101 mg) of chloramine T.[18] After 5 min the reaction was stopped by the addition of 0.72 mmol (137 mg) of $Na_2S_2O_5$. The aqueous mixture was extracted with dichloromethane. The organic layer was subjected to chromatography on silica gel and eluted with ethyl acetate. Conversion to the oxalate salt and crystallization from ether yielded 65 mg (51%) of the monoiodo compound **5**: mp 158–159 °C; ^{1}H NMR (200 MHz) δ 7.47–7.27 (m, 6 H), 6.91 (d, 1 H), 6.64 (dd, 1 H), 3.8 (s, 3 H), 3.6 (s, 2 H), 2.86–2.17 (m, 10 H), 1.85 (q, 2 H), 1.63 (m, 2 H), 0.96 (t, 3 H). Anal. $C_{26}H_{32}N_3O_7I$ (oxalate): C, H, N.

1-[2-(*N*,*N*-Diethylamino)ethyl]-2-(*p*-ethoxybenzyl)-5-[*N*-(*tert*-butoxycarbonyl)amino]benzimidazole (9). A solution of 0.5 mmol (200 mg) of etonitazene hydrochloride (**8**) in 5 mL of methanol containing 20 mg of 10% Pd/C was submitted to catalytic hydrogenation (2.5 atm) for 3 h. After removal of the catalyst by filtration and evaporation of the solvent, the mixture was partitioned between saturated aqueous sodium carbonate solution and ethyl acetate. The extract was dried and concentrated under vacuum, and the oily residue was dissolved in tetrahydrofuran. After addition of 0.75 mmol (165 mg) of di-*tert*-butyl dicarbonate, the mixture was stirred at 50 °C for 3 h. The solvent was evaporated, and the protected amine was chromatographed on silica gel (10 g). Elution with ethyl acetate gave 160 mg (70%) of the compound **9** which was recrystallized in ethyl acetate/hexane: mp 147–148 °C; ^{1}H NMR (60 MHz) δ 7.50 (s, 1 H), 7.35 (d, 1 H), 7.21 (m, 1 H), 7.12 (d, 2 H), 6.76 (d, 2 H), 6.55 (s, 1 H), 4.25 (s, 2 H), 4.00 (q + dd, 4 H), 2.43 (q, 6 H), 1.52 (s, 9 H), 1.37 (t, 3 H), 0.90 (t, 6 H). Anal. $C_{27}H_{38}N_4O_3$: C, H, N.

1-Phenyl-8-[1-(*p*-chlorophenyl)ethyl]-1,3,8-triazaspiro[4.5]decan-4-one (13). A mixture of 10 mmol (2.31 g) of 1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one (**12**) and 60 mmol (9.28 g) of *p*-chloroacetophenone in 25 mL of methanol (acidified with 4% acetic acid) was treated with 7 mmol (0.44 g) of sodium cyanoborohydride at room temperature.[35] The resulting solution was stirred for 72 h. After evaporation of the solvent, the solid residue was dissolved in ethyl acetate and washed with water. The organic phase was dried over sodium sulfate and chromatographed on silica gel (230 g). Elution with ethyl acetate gave 1 g (27%) of pure **13** recrystallized in ether: mp 207 °C (lit.[36] mp 206.5 °C); ^{1}H NMR (200 MHz, acetone-d_6) δ 7.60–7.30 (m, 6 H), 7.1 (d, 2 H), 6.93 (t, 1 H), 4.84 (s, 2 H), 3.67 (q, 1 H), 2.80–2.70 (m, 6 H), 1.80–1.60 (m, 2 H), 1.47 (d, 3 H); MS *m/e* 369 (M^+).

1-(*p*-Nitrosophenyl)-8-[1-(4-chlorophenyl)ethyl]-1,3,8-triazaspiro[4.5]decan-4-one (14). Solid sodium nitrite (2.6 mmol, 0.179 g) was added, over a period of 45 min, to a cooled (0 °C) solution of 2.5 mmol (0.922 g) of **13** in 10 mL of concentrated HCl. An orange color appeared almost immediately. After 60 min of additional stirring at room temperature, the reaction was stopped by neutralization with aqueous sodium carbonate. The green solution was extracted with ethyl acetate. After evaporation of the solvent, the crude product was recrystallized from ethyl acetate and methanol to give 410 mg (40% yield) of green crystals (**14**): mp 201 °C; ^{1}H NMR (200 MHz, CD_3OD) δ 7.85 (d, 2 H), 7.40–7.30 (m, 4 H), 7.15 (d, 2 H), 4.80 (s, 2 H), 3.84 (q, 1 H), 3.00–2.80 (m, 6 H), 1.90–1.80 (m, 2 H), 1.48 (d, 3 H); MS *m/e* 398 (M^+). Anal. $C_{23}H_{26}N_4O_4Cl$ (acetate): C, H, N.

1-[4-[*N*'-(*tert*-Butoxycarbonyl)amino]phenyl]-8-[1-(4-chlorophenyl)ethyl]-1,3,8-triazaspiro[4.5]decan-4-one (15). A solution of 1 mmol (398 mg) of **14** in 20 mL of methanol containing 40 mg of 10% Pd/C was submitted to catalytic hydrogenation (3 atm) for 2 h. Then 1.1 mmol (240 mg) of di-*tert*-butyl dicarbonate was added to the reaction medium, and the mixture was stirred overnight. After filtration of the catalyst and evaporation of the solvent, the crude residue was chromatographed on a dry silica gel column eluted with 1-butanol/water/acetic acid (25/10/4) to give 210 mg (72%) of white crystals (**15**): mp 146 °C; ^{1}H NMR (200 MHz, acetone-d_6) δ 7.50–7.30 (m, 6 H), 7.0 (d, 2 H), 4.65 (s, 2 H), 3.5 (q, 1 H), 2.80–2.70 (m, 6 H), 1.80–1.65 (m, 2 H), 1.48 (s, 9 H), 1.30 (d, 3 H); MS *m/e* 484 (M^+). Exact mass: 484.2248 (theor 484.2241). Anal. $C_{26}H_{33}N_4O_3Cl$ (oxalate): C.H.

***N*-(6α-Naltrexyl)-*N*'-(*tert*-butoxycarbonyl)-*p*-phenylenediamine (19).** A mixture containing 0.66 mmol (250 mg) of naltrexone **18** and 1.37 mmol (300 mg) of *N*-(*tert*-butoxycarbonyl)-*p*-phenylenediamine in 5 mL of methanol was hydrogenated in the presence of 50 mg of 10% Pd/C at atmospheric pressure for 24 h. After removal of the catalyst by filtration and evaporation of the solvent, the mixture was partitioned between aqueous sodium carbonate solution and ethyl acetate. The organic phase was washed with water and dried over Na_2SO_4. The crude mixture was adsorbed on silica gel (2 g) and chromatographed on a dry silica column (150 g) by elution with a mixture of 1-butanol/acetic acid/water (25/4/10). The fractions containing the compound **19** were collected and concentrated under vacuum. The solid residue was resuspended in water and filtered successively through paper and C_{18} Sep-pack cartridges (Waters Associates). Lyophilization gave 133 mg (23% yield) of crystalline **19**: mp 118–119 °C; ^{1}H NMR (200 MHz) δ 7.14 (d, 2 H), 6.72 (d, 1 H), 6.65 (d, 2 H), 6.54 (d, 1 H), 6.30 (s, 1 H), 4.75 (d, 1 H), 4.1–4.0 (m, 1 H), 1.50 (s, 9 H); MS *m/e* 548 ($M + NH_4^+$). Anal. $C_{33}H_{43}N_3O_7$ (acetate): C, H. N.

***N*-Methyl-*N*-(6α-naltrexyl)-*N*'-(*tert*-butoxycarbonyl)-*p*-phenylenediamine (20).** A solution of methanol (1 mL) containing a mixture of 0.018 mmol (10 mg) of **19** (as the acetate) and 0.5 mL of a 30% aqueous solution of formaldehyde was hydrogenated in the presence of 20 mg of Pd/C for 4 h. After removal of the catalyst by filtration and evaporation of the solvent, the mixture was dissolved in ethyl acetate and washed with aqueous sodium carbonate solution and dried over Na_2SO_4. After concentration under reduced pressure, the product was chromatographed by TLC eluted with 1-butanol/water/acetic acid (25/10/4). The spot of R_f = 0.3 was eluted from silica with ethyl acetate. The organic phase was concentrated, and the residue resuspended in acetonitrile and filtered over paper and C_{18} Sep-pack cartridges leading to 8 mg (76%) of **20**: mp 110 °C; ^{1}H NMR (200 MHz) δ 7.22 (d, 2 H), 6.85 (d, 2 H), 6.74 (d, 1 H), 6.54 (d, 1 H), 6.32 (s, 1 H), 4.81 (d, 1 H), 4.3–4.2 (m, 1 H), 2.88 (s, 3 H), 1.51 (s, 9 H); MS *m/e* 548 (M^+). Exact mass: 547.3030 (theor 547.3046). Anal. $C_{34}H_{45}N_3O_7$ (acetate): C, H.

Deprotection, Diazotization, and Azidation of Aromatic Amines. All the following experiments were carried out in the absence of any prejudiciable light source.

Diazonium Salts. In a typical experiment, 1.9 μmol (1 mg) of the protected amine **20** was dissolved in 150 μL of a 1/1 mixture of TFA and concentrated HCl. After 10 min of stirring at room temperature, the solution was cooled to −20 °C, and 210 μL of a 10^{-2} M aqueous sodium nitrite solution was added over a period of 30 min. After 30 min of additional stirring, the acids were evaporated under vacuum at room temperature and the residue solubilized in water. The diazonium salt **21** was purified on a Waters μBondapak (3.9 × 300 mm) column and eluted at a flow rate of 2 mL/min under gradient conditions (100% H_2O, 0.05% TFA to 100% CH_3CN in 40 min). Elution of the products was monitored by absorbance at 229 nm. All the other aryldiazonium salts were obtained similarly with over 95% estimated yield.

The different chemicals were identified by UV spectroscopy (Table II), after purification by HPLC. The following retention times, in the above mentioned conditions, were observed: **10**, 8 min; **16**, 19 min; and **21**, 9 min. These chemicals have not been otherwise characterized.

Azido Derivatives. A 10^{-2} M aqueous solution of the diazonium salt **21** was adjusted to pH 7.5 with Na_2CO_3. The mixture was treated with 10 equiv of solid NaN_3 at room temperature. After 30 min stirring, the floculate was dissolved in ethyl acetate.

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The organic phase was washed with water, dried on sodium sulfate, and concentrated under vacuum. The crude product **22**, resuspended in 1 mL acetonitrile, was purified by HPLC on a Waters μBondapak (3.9 × 300 mm) column eluted at a flow rate of 2 mL/min (100 % H_2O, 0.05% TFA to 50% CH_3CN in 40 min).

Each azido derivative was verified by UV spectroscopy (Table II) and by HPLC retention time in elution conditions described above: **4**, 34 min; **6**, 37 min; **11**, 18 min; **17**, 35 min; **22**, 14 min. The presence of azido group was confirmed by an IR absorption band at 2100 cm^{-1} and by ^1H NMR (200 MHz): (**4**) δ 7.45 (m, 3 H), 7.24 (m, 2 H), 7.13 (d, 2 H), 6.95 (d, 2 H); (**6**) δ 7.58 (s, 1 H), 7.45 (m, 3 H), 7.40 (m, 2 H), 7.19 (dd, 1 H), 7.02 (d, 1 H); (**11**) δ 7.42 (s, 1 H), 7.25 (d, 1 H), 7.14 (d, 2 H), 6.92 (dd, 1 H), 6.82 (d, 2 H). The molecular weight of each isolated product was determined by using fast-atom bombardment ionization technique on a Varian mass spectrometer. Exact mass **4**: 436.2412 (theor 436.2348); **6**: 562.1371 (theor 562.1317); **11**: 393.2432 (theor 393.2403).

Opioid Receptor Binding. Membrane Preparation. (i) **Rat Brain Membranes.** A crude membrane fraction from rat brain (minus cerebellum) was prepared as follows. Brain tissue samples were homogenized in 0.25 M ice-cold sucrose by using a Duall homogenizer. After centrifugation at 1100*g* for 10 min, the supernatant was diluted to 45 volumes (mL/g wet weight of tissue) in 50 mM Tris-HCl–1 mM EDTA buffer (pH 7.4) and centrifuged at 35000*g* for 30 min at 4 °C. The pellet was resuspended in 5 volumes 0.32 M ice-cold sucrose and stored at –70 °C until needed.

(ii) **Guinea Pig Cerebellum Membranes.** Cerebellum tissue samples were homogenized in 50 mM Tris-HCl buffer (pH 7.4) by using a Potter-Elvehjem homogenizer. After two successive centrifugations at 100000*g* for 35 min, the pellet was suspended in the same buffer to a final concentration of 5.5 mg of protein/mL. Membrane preparations were used directly in binding assays.

Binding Assays. (i) **Rat Brain Membranes.** Prior to equilibrium binding experiments, membrane preparations were thawed and diluted in Tris–EDTA buffer to obtain a final protein concentration of 0.15 mg/mL ([^3H]diprenorphine binding assay) or 0.27 mg/mL (all other binding assays).

To assess μ- and δ-site binding, the drug binding affinity constants were determined by competition experiments, performed in triplicate, in the absence of light, with [^3H]DAGO (1 nM), [^3H]DTLET (1 nM), and [^3H]DADLE (1 nM). The μ-opioid binding component of [^3H]DADLE was blocked by 500 nM morphiceptine. [^3H]Diprenorphine (0.5 nM), possessing almost equal binding affinity constants at μ-, δ-, or κ-sites, was used to assess the overall opioid binding affinities of the drugs. Nonspecific binding levels of the tritiated ligands were determined in the presence of 10^{-5} M ([^3H]DADLE binding assay) or of 10^{-6} M naloxone (all other binding assays).

(ii) **Guinea Pig Cerebellum Membranes.** The membranes suspension was diluted in Tris–EDTA buffer to a final concentration of 0.29 mg of protein/mL. The κ-site affinity constants of the different ligands were determined by competition experiments, performed in triplicate, against [^3H]diprenorphine (0.3 nM). Nonspecific binding was determined in the presence of 10^{-5} M diprenorphine. To assess κ-sites binding, competition experiments by U-50488 were performed and indicated that about 85% of the binding sites were of κ-type.

Samples (1 mL final volume) were incubated at 25 °C for 90 min with the appropriate ^3H-labeled ligand and various concentrations of the assayed cold drugs. Incubation was stopped by filtering the 1 mL of assay mixture over GF/B (Whatman) filters and rapidly rinsing twice with 3 mL of ice-cold buffer and counted for radioactivity in Biofluor (New England Nuclear) scintillation cocktail.

Apparent binding affinity constants (K_i values) of drugs were calculated from IC_{50} values, determined in competition experiments, according to the Cheng and Prusoff[37] relation: $K_i = IC_{50}/(1 + L/K_d)$ where L and K_d are respectively, the radioligand concentration and its equilibrium dissociation constant which was determined experimentally ([^3H]diprenorphine, 0.1 nM (rat brain) or 0.3 nM (guinea pig cerebellum); [^3H]DAGO, 1.3 nM; [^3H]-DADLE, 1 nM; [^3H]DTLET, 1.1 nM).

The filter dissociation procedure combined to a [^3H]sufentanil binding assay was described previously.[26]

Photolabeling Experiments. Monochromatic light was obtained from a 1000-W xenon–mercury lamp (Hanovia) connected to a grating monochromator (Jobin-Yvon). The light intensity was measured (in volts) with a thermopile (Kipp and Zohnen) and adjusted through an iris diaphragm at desired intensity. The light beam was focused through a quartz lens on the refrigerated assay cell to form a 10-mm high and 2-mm-wide spot. Aliquots of membrane preparation (0.8 mg/mL) in 50 mM Tris-HCl–1 mM EDTA buffer (pH 7.4) were incubated for 20 min at 37 °C with the indicated concentrations of photosensitive substances with or without previous addition of naloxone. Thereafter, 3-mL aliquots of incubation mixture, were irradiated at 5 °C, with gentle stirring for 20 min. Diazonium salts were irradiated at their maximal absorption wavelengths (**10**, 312 nm; **16**, 369 nm; **21**, 382 nm) and azido derivatives at 315 nm except for **4** and **6** which were irradiated at 250 nm. Irradiated membrane suspensions (3 mL) were diluted 4-fold with Tris–EDTA buffer (pH 7.4) and incubated for 10 min at 37 °C. Samples (2 mL) were then adsorbed under partial vacuum on GF/B (Whatman) glass-fiber filters and submitted to repeated washings to remove the unbound ligands. The dissociation procedure was followed by the [^3H]-sufentanil filter binding assay, performed according to ref 26.

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