A High-Affinity Fluorenone-Based β_2-Adrenergic Receptor Antagonist with a Photoactivatable Pharmacophore†

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ABSTRACT: To develop molecules capable of directly probing the catechol binding region of the β_2-adrenergic receptor (β_2AR), novel benzophenone- and fluorenone-based β_2AR antagonists were prepared as potential photoaffinity probes. While the benzophenone-containing ligands bound with relatively modest affinity, one of the fluorenone-based compounds, 4-(2-hydroxy-3-isopropylaminopropoxy)-7-amino-6-iodofluorenone (iodoaminoflisopolol, IAmF), showed very high affinity for the β_2AR, inhibiting [^{125}I]-ICYP binding with an apparent K_i of approximately 1×10^{-9} M. In comparison to the benzophenone ligands, the fluorenone ligands have one additional carbon−carbon bond that creates a planar unsaturated ring system and leads to a large increase in receptor binding affinity. Unlike previous β_2AR photoaffinity ligands, an attractive and unique feature of the fluorenone derivative IAmF is that the large planar unsaturated ring (believed to correspond to the catechol end of other β_2AR ligands) serves as both the binding pharmacophore and the photoreaction center for this molecule. With this potential for directly probing the catechol binding region of the β_2AR, we synthesized and tested IAmF in carrier-free radioiodinated form ([^{125}I]IAmF). When photoreduction was conducted at 350 nm for 20 min, [^{125}I]IAmF was able to produce cross-linked products in both triethylamine and methanol, with a reactivity pattern similar to that found in benzophenone photochemistry. As a final test of suitability as a photoaffinity label, specific labeling of the β_2AR in membranes (protectable by 10 μM alprenolol) was demonstrated. [^{125}I]IAmF represents a new class of β_2AR photoaffinity labels that can directly probe the catechol-analogous antagonist pharmacophore binding site in the β_2AR ligand binding pocket.

The β_2AR[1] is a member of the superfamily of the seven transmembrane G-protein coupled receptors. Activation of the β_2AR with agonists leads to G_s signaling, the stimulation of adenylyl cyclase, and the production of the second messenger cAMP, which regulates many important biological functions (*1*). Site-directed mutagenesis experiments (*2, 3*) of the hamster lung β_2AR have suggested that a number of amino acid residues are critical for receptor−ligand interactions. For instance, Asp-113 on transmembrane 3 (TM3) is required for high-affinity binding of both agonists and antagonists. It is believed that the carboxyl group of Asp-113 serves as a counterion for the amino group of bound ligands. Ser-204 and Ser-207 on TM5, on the other hand, are suggested to interact with the catechol portion of β_2AR ligands, again based on mutagenesis experiments. We sought to identify residues in the β_2AR ligand binding site using a direct biochemical approach. In this paper, we describe the development and application of a novel β_2AR antagonist photoaffinity label, [^{125}I]IAmF. This molecule represents a new class of photoaffinity label and has the unique feature that the large planar unsaturated ring, believed to correspond to the catechol end of β_2AR agonists, serves as both the binding pharmacophore and the photoreaction center for this molecule.

Because the concentration of receptors is generally very low, an effective photoaffinity ligand should have the features of high specific radioactivity and high-affinity binding in addition to having a photoreactive group that will covalently insert into the receptor binding site (*4*). A number of aryl azide-based β_2AR photoaffinity ligands have been previously reported (*5*), including iodoazidobenzylpindolol (IABP) (*6, 7*), azidoiodobenzylcarazolol (*8*), and azidobenzimidyliodocyanopindolol (*9*). Several additional non-azide-based photolabels have also been developed, including iodocyanopindolol diazirine (*10*). The β_2AR photoprobes developed thus far have their photosensitive moiety on the amine end, away from the pharmacophore region of the ligand. As a result, extended or folded conformations of the portions of these molecules that contain the photoreactive moiety are possible. Consequently, the amino acid residue or residues that the

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[1] Abbreviations: BPAP, 1-(benzophenol-3-yloxy)-3-(isopropylamino)-2-propanol; BPIAP, 1-(benzophenol-3-yloxy)-3-[1-(3-iodo-4-aminophenyl)-2-methyl-2-propylamino]-2-propanol; 3-BPISOP, 1-(benzophenol-3-yloxy)-3-isopropylamino)-2-propanol; IAmF, 4-(2-hydroxyl-3-isopropylaminopropoxy)-7-amino-6-iodofluorenone; IABP, iodoazidopindolol; ICYP, iodocyanopindolol; β_2AR, β_2-adrenergic receptor; TM, transmembrane; TEA, triethylamine; PMSF, phenylmethanesulfonyl fluoride.

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photolabel derivatizes are often outside the catechol binding region of the β_2AR ligand binding pocket, adding complexity to the understanding of the interactions of the "business end" of the molecule in the binding site. One such photoprobe, iodoazidobenzylpindolol ([^{125}I] IABP) (*8*), which has a photoactivatable azide group on a benzyl ring linked to the pindolol amino end, derivatized three sites (*11*) on the receptor: one on TM6−7, one on TM5, and the third in the region containing TM1−2. A likely explanation for this result is that [^{125}I]IABP exists in two conformation states: when it is extended, the azide derivatizes TM1−2, and when it is folded, the azide inserts into TM6−7 and TM5. In certain instances, there are valid reasons for placing the photoreactive moiety on the end of a long "tether". A salmeterol-based β_2AR agonist photoaffinity label recently developed in our laboratory is one such example. This photolabel, [^{125}I]-iodoazidosalmeterol, was used to investigate where the long aralkyl chain end of salmeterol (which confers the long-acting properties of this asthma drug) binds in the receptor (*12*).

Most azide-based ligands suffer from low photoinsertion yields during the photolysis (usually around 1%). When the photolabel is of very high specific radioactivity, this modest level of photoinsertion is sufficient for detection of radioactive photolabeled peptides. However, a potential advantage of benzophenone-based photoaffinity labels is that they have been reported to have photoinsertion yields as high as 80% (*13*−*15*). Another advantage of benzophenone photolabels is that the photoactivatable moiety can serve a dual role as an adrenergic pharmacophore. We hypothesized that a benzophenone-based photolabel could be developed that would allow direct probing of the business end of the β_2AR ligand binding site and not suffer from the disadvantage of earlier photolabels in which the photoreactive group was located at the extreme other end of the molecule. We prepared several benzophenone-based ligands but found that in general these had only modest affinity for the β_2AR. This result led us to further hypothesize that a coplanarity of the unsaturated rings may be important for high-affinity binding to the β_2AR and furthermore that perhaps a planar ring system would retain similar photochemical properties to that of benzophenone and be useful as a photolabel. Therefore, fluorenone-based ligands were subsequently prepared and tested, and one in particular was found to have very high-affinity binding. Fluorenone and benzophenone possess similar structural features, differing only by a single carbon−carbon bond that imposes planarity on the fluorenone ring system, but the affinities of our benzophenone- and fluorenone-based ligands for the β_2AR are dramatically different, reflecting the fact that the β_2AR ligand binding site has strict structural requirements for high-affinity binding. This observation suggests that, within the catechol region of binding site, planar unsaturated ring systems are favored, perhaps due to interactions with specific aromatic side chains in TM5 or TM6 (e.g., Phe-290 in TM6), as has been suggested from modeling (*16*, *17*).

In this paper, we describe the synthesis and characterization of several benzophenone- and fluorenone-based photoprobes for the β_2AR and demonstrate by alprenolol protectable photoaffinity labeling of the β_2AR in membranes that at least one of these labels, [^{125}I]IAmF, can be used in future studies to identify the precise amino acid(s) in the β_2AR that interact with antagonist pharmacophores.

MATERIALS AND METHODS

Chemicals. Carrier-free [^{125}I]NaI was purchased from DuPont-New England Nuclear. Phenanthraquinone, epichlorohydrin, isopropylamine, palladium, chloramine T, and organic solvents were obtained from Aldrich (Milwaukee, WI). Sulfuric acid, sodium nitrite, sodium iodide, and glass fiber filters (GF/A) were from Fisher Scientific (Chicago, IL). Precoated silica gel thin-layer chromatography plates (GF-254, type 60) were obtained from EM Sciences. Ex-Cell 401-defined medium was purchased from JRH Scientific (Woodland, CA). Antibiotics used in cell culture were from GIBCO BRL (Gaithersburg, MD). Sf9 cells were obtained from American Type Culture Collection (Rockville, MD). Cyanopindolol, S49 wild-type, and cyc$^-$ lymphocyte membranes were kind gifts from Dr. Richard Clark (University of Texas Health Science Center, Houston, TX).

Synthesis of Benzophenone-Based Photoprobes (Figure 1)

3-(2,3-Epoxypropoxy)benzophenone (**2**). The starting material 3-hydroxyl benzophenone **1** (1.0 g, 5 mmol) was dissolved in 80 mL of 0.2 N NaOH (16 mmol) at room temperature in a round-bottom flask equipped with a fast stirring magnetic bar. Epichlorohydrin (1.3 g, 15 mmol in 3 mL of methanol) was added dropwise. The reaction was allowed to proceed for 20 h at 4 °C. The product **2** precipitated and was collected by filtration of the reaction mixture and washed twice with 0.1 N NaOH. This epoxide compound was dissolved in ether, and impurities were removed with aqueous 0.1 N NaOH. The ether was removed by rotary evaporation to generate 1.0 mL of light yellow oil. The epoxide product **2** was crystallized from methanol at −20 °C and used without further characterization. The final yield was 820 mg (3.3 mmol, 65%). R_f 0.6 on a silica gel TLC plate developed in acetonitrile:toluene (3:7).

1-(Benzophenol-3-yloxy)-3-(N-isopropylamino)-2-propanol (**3**) (*3-BPISOP*). The epoxide **2** (40 mg, 0.16 mmol) was mixed with 3 mL of 20% (v/v) isopropylamine (400 mg, 6.7 mmol) in methanol. The reaction mixture was stirred at room temperature overnight. Isopropylamine and methanol were removed by rotary evaporation, and the product **3** was purified using a TLC silica plate to obtain 37 mg (0.12 mmol, 75% yield) colorless oil. R_f 0.65 on a silica gel TLC plate developed in methyl acetate:2-propanol:NH_4OH (1:1:0.1). ^{1}H NMR ($CDCl_3$) δ 7.8−7.2 (m, 9H), 4.0−4.2 (m, 3H), 2.7−3.05 (m, 3H), 2.01 (s, 1H), 1.3 (d, 6H).

1-(p-Aminophenyl)-2-methyl-2-propylamine (**6**). The diamine **6** was prepared following a method by Hass et al. (*18*) and Rashidbaigi and Ruoho (*7*). The ^{1}H NMR spectrum was consistent with a previous report (*7*).

1-(Benzophenol-3-yloxy)-3-[1-p-(aminophenyl)-2-methyl-2-propylamine]-2-propanol (**7**) (*BPAP*). The epoxide **2** (142 mg, 0.6 mmol) was dissolved in 5 mL of ethanol and then reacted with the diamine **6** (360 mg, 2.4 mmol) under argon in a sealed vial for 2 days at room temperature with rapid stirring. The product was purified using TLC silica gel plates to give **7** (25 mg, 10%). R_f 0.5 on a silica gel TLC plate developed in ethyl acetate:2-propanol:NH_4OH (1:4:0.1). ^{1}H NMR ($CDCl_3$), δ 7.2−7.8 (m, 9H), 7.0 (d, 2H), 6.6 (d, 2H), 4.1 (m, 1H), 3.3 (m, 2H), 3.0−2.8 (m, 4H), 1.1 (s, 6H).

Radiosynthesis of [^{125}*I*]*BPIAP*. Radioiodination of BPAP was performed according to the method of Rashidbaigi (*7*) to give product **8**.

Synthesis of Fluorenone-Based Photoprobes

2-Nitrophenanthraquinone (**10**). Nitration of phenanthraquinone **9** to obtain **10** has been described by Schmidt and Spoun (*19*). Briefly, 15 g of starting material **9** (72 mmol) was boiled in 450 mL (7 mol) of concentrated nitric acid (70%) on an oil bath for 20 min. The reaction mixture was cooled to room temperature, followed by addition of 1000 mL of water to generate a large amount of the yellow precipitate, which was filtered and washed with 500 mL of water. The yellow solid was then resuspended in 700 mL of ethanol and boiled for 5 min, followed by immediate filtration and wash with 200 mL of hot ethanol to give 7.5 g (29 mmol, 40% yield) of pure product **10**. (The filtrate, containing 4-nitrophenanthraquinone, was discarded.) R_f 0.07 on a silica gel TLC plate developed in a solvent system of hexane:ethyl acetate (4:1). This material was used in the next step without further characterization.

7-Nitrofluorenone-4-carboxylic Acid (**12**). Following the procedure of Moore and Huntress (*20*), **10** was oxidized to 4-nitrodiphenic acid **11** and then dehydrated to generate **12**. The yields for these reactions were over 87% and 93%, respectively. R_f 0.28 on a silica gel TLC plate developed in chloroform:methanol (9:1), mp 256−258C. The ^{1}H NMR spectrum was consistent with the report from Aldrich Chemical Co.

7-Nitro-4-hydroxyfluorenone (**13**). Synthesis of **13** was based on a general protocol described by Horner and Baston (*21*). Briefly, a mixture of 3 g (10 mmol) of **12** and 80 mL of thionyl chloride was refluxed for 2.5 h and treated with N_3/H_2O to generate an amine product. The amino group of fluorenone was converted to a hydroxyl in a Curtius rearrangement reaction to give **13** with approximately 60% yield. R_f 0.76 on a silica gel TLC plate developed in toluene:acetonitrile (1:1), mp 198−200 °C. MS *m*/*z* 242 (MH^+). ^{1}H NMR (CD_3COCD_3) δ 8.5 (dd, 1H), 8.3 (d, 1H), 8.2 (d, 1H), 7.35−7.2 (m, 3H).

4-(2,3-Epoxypropoxy)-7-nitrofluorenone (**14**). The hydroxyl starting material **13** (550 mg, 2.3 mmol) was dissolved in 60 mL of 0.4 N NaOH in a 250-mL round-bottom flask equipped with a stirring bar. The maroon-colored solution was cooled on an ice bath, and 3.7 g (40 mmol) of epichlorohydrin in 5 mL of methanol was slowly added. After a 4-h reaction on ice, an additional 3.7 g of epichlorohydrin in 5 mL of methanol was added, and the reaction was allowed to proceed for 2 days at 4 °C with constant stirring. The epoxide product was extracted three times with 50 mL of diethyl ether. The combined extract was washed with 0.2 N KOH and brine (40% NaCl) and dried with anhydrous magnesium sulfate. The solvent was removed with rotary evaporation, and a yellow solid **14** was obtained with a total yield of 180 mg (0.61 mmol, 27%). R_f 0.17 on a silica gel TLC plate developed in toluene:acetonitrile (9:1), mp 174−176 °C. MS *m*/*z* 298 (MH^+). ^{1}H NMR (CD_3COCD_3) δ 8.02−7.94 (d, 1H), 7.64−7.56 (m, 2H), 7.4−7.25 (m, 4H), 4.6 (dd, 1H), 4.1 (dd, 1H), 3.5 (m, 1H), 3.15 (m, 2H).

4-(2-Hydroxyl-3-isopropylaminopropoxy)-7-nitrofluorenone (*7-Nitroflisopolol*) (**15**). A mixture of 180 mg (0.61 mmol) of **14** and 1.4 g (24.4 mmol) of isopropylamine was refluxed for 4 h. Following removal of the unreacted isopropylamine, the residue was dissolved in 20 mL of warm 1.0 N HCl and washed with ether. The aqueous solution was neutralized with 2 N KOH and extracted three times with 20 mL of ethyl acetate. The combined extract was washed with brine and dried with anhydrous magnesium sulfate. The solvent was then removed by rotary evaporation to give 130 mg (0.37 mmol, 60% yield) of **15**. R_f 0.33 on a silica gel TLC plate developed in methyl acetate:2-propanol:NH_4OH (10:10:1), mp 162−164 °C. MS *m*/*z* 357 (MH^+). IR 1730 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 7.34−8.50 (m, aromatic), 4.01−4.3 (m, 3H), 2.70−2.80 (m, 3H), 1 (d, 6H).

4-(2-Hydroxyl-3-isopropylaminopropoxy)-7-aminofluorenone (*7-Aminoflisopolol*) (**16**). Conversion of nitroflisopolol to amino flisopolol without reduction of the ketone moiety was performed using a standard hydrogen reduction reaction. A mixture of 100 mg (0.3 mmol) of **15**, 10 mg of palladium on charcoal (10%), and 4 mL of methanol was hydrogenated at room temperature with a low flow of hydrogen at ambient pressure for 20 min. The mixture became dark colored. The charcoal particles were subsequently removed by filtration, and the filtrate was rotary evaporated to give a dark solid, which was dissolved with 3 mL of 1.0 N HCl and lyophilized to give 70 mg (0.18 mmol, 59% yield) of **16**. R_f 0.5 on a silica gel TLC plate developed in methanol:ethyl acetate:triethylamine (10:10:1). MS *m*/*z* 328 (MH^+). IR 1730 cm^{-1}. ^{1}H NMR (D_2O) δ 7.7 (d, 1H), 7.3 (d, 2H), 6.56−7.08 (m, 3H), 4.15 (m, 3H), 3.4 (dd, 2H), 3.1 (m, 1H), 1.2 (d, 6H).

4-(2-Hydroxyl-3-isopropylaminopropoxy)-7-amino-6-iodofluorenone (*6-Iodo-7-aminoflisopolol*) (**17**). Iodination of **16** was conducted by using thallium trichloride as described by Rashidbaigi and Ruoho (*6*, *7*). In a 100-mL round-bottom flask equipped with a magnetic stir bar, 50 mg (0.13 mmol) of **16** was dissolved in 10 mL of sodium acetate buffer (0.1 M, pH 4.0). To this solution was added 200 μL of 97.5 mg/mL NaI (0.13 mmol), followed by the addition of 1.0 mL of 40 mg/mL thallium trichloride. The flask was placed on a steam bath for 15 min, and the reaction was terminated by adding 1.0 mL of $Na_2S_2O_3$ (20 mg/mL in H_2O). The reaction mixture was made alkaline with 4 mL of 30% $NaHCO_3$ and extracted three times with 20 mL of ethyl acetate. The combined extract was washed with water and brine and dried with anhydrous magnesium sulfate, and the solvent was removed by rotary evaporation. The resulting residue was purified by a preparative silica gel TLC plate (20 × 20 cm; 500 mm) developed in butanol:water:glacial acetic acid (4:1:1) to give 7 mg (0.016 mmol, 12%) of **17** as a purple powder. R_f 0.70 on a silica gel TLC plate developed in butanol:water:glacial acetic acid (4:1:1). MS *m*/*z* 452 (MH^+). ^{1}H NMR (DMSO) δ 7.98 (s, 1H), 7.59 (s, 1H), 7.02−7.25 (m, 3H), 4.15 (m, 3H), 3.4 (dd, 2H), 3.1 (m, 1H), 1.2 (d, 6H).

4-(2-Hydroxyl-3-isopropylaminopropoxy)-6-iodofluorenone (*6-Iodoflisopolol*) (**18**). In a 50-mL round-bottom flask equipped with a magnetic stirring bar, 10 mg (0.026 mmol) of **16** was dissolved in 1.5 mL of 3% sulfuric acid and cooled in a 5 °C water bath. $NaNO_2$ (35 mg, 0.52 mmol in 50 μL of precooled water) was added. The reaction was allowed to proceed for 5 min, and then 86 mg (0.52 mmol) of KI in 50 μL of precooled water was added. After 60 min iodination with KI at 5 °C, the reaction was quenched with $Na_2S_2O_3$ (60 mg in 100 μL of water), followed by ethyl acetate extraction to give 4.5 mg of **18** (0.01 mmol, 40%). R_f 0.58 on a silica gel TLC plate developed in methanol:ethyl acetate:triethylamine (10:10:1). MS *m*/*z* 439 (MH^+).

Radiosynthesis of [*125I*]*IAmF*. In a glass test tube, the following were added sequentially: 10 μL of [^{125}I]NaI (2.5 mCi in 0.1 N NaOH, 1.125 nmol), 10 μL of 0.1 N HCl, 20 μL of sodium acetate buffer (pH 5.6, 0.5 M), and 10 μL of 7-aminoflisopolol hydrochloride **16** (4 μg, 10 nmol). Iodination was initiated by the addition of 10 μL of chloramine T (0.3 mg/mL in H_2O) at room temperature. The reaction was terminated after 1 min with 100 μL of $Na_2S_2O_3$ (40 μg/mL in H_2O). The radiolabeled compound was extracted with ethyl acetate and then purified on a silica gel TLC plate (10 $\times$ 20 cm, 25 mm) using a solvent system of methanol:ethyl acetate:triethylamine (1:1:0.1) to give 0.32 mCi (13% yield) of the radiolabeled product **17** in carrier-free form, which was stored in methanol at -20 °C in the dark. This material comigrated with authentic cold **17** (6-iodo-7-aminoflisopolol).

Synthesis of 4-(*2-Hydroxy-3-isopropylaminopropoxy*)*fluorenone* (*Flisopolol*). Flisopolol (structure shown in Figure 4B) was synthesized from 4-hydroxyl-fluorenone (Aldrich) by coupling with epichlorohydrin to obtain the epoxide, followed by reaction with isopropylamine to generate flisopolol. The synthetic strategies were similar to the related protocols as described above. ^{1}H NMR (CD_3COCD_3) δ 8.0 (s, 3H), 7.6$-$7.25 (m, 4H), 4.4$-$4.1 (m, 3H), 3.0 (m, 3H), 1.2$-$1.3 (d, 6H).

Photolysis of Radioactive Iodoaminoflisopolol ([*125I*]*IAmF*). [^{125}I]IAmF (approximately 460 000 cpm) was combined with 50 μL of either methanol or triethylamine (TEA) in sealed polypropylene vials. Photoreduction was performed by irradiation with 350 nm light for 15 min at 4 °C using a Rayonet biochemical photoreactor (model RPR-100, The Southern New England Ultraviolet Co., Branford, CT; approximate output 400 W). The distance between the lamps and the sample tubes was approximately 2 cm. After photolysis, the photoproducts were developed on a TLC silica gel plate with methanol:ethyl acetate:triethylamine (1:1:0.1). The results were analyzed by autoradiography.

β_2AR Ligand Binding Assays, Adenylyl Cyclase Assays, and Photolabeling

Membrane Preparation. Sf9 cells were cultured in serum-free Ex-Cell 401 medium plus 2.5 μg/mL gentamicin, 2.5 μg/mL streptomycin, and 2.5 units/mL penicillin G. Cells were maintained in suspension in Erlenmeyer flasks at 27 °C on a rotating platform at 110 rpm. Cells were grown to a density of $4 \times 10^6$$-$$5 \times 10^6$ cells/mL and infected with a recombinant baculovirus containing the hamster β_2AR cDNA at an MOI of approximately 1.0. After a 1-h incubation with the virus at room temperature, the cells were diluted with fresh medium to a density of approximately 1×10^6 cells/mL and grown for 2 days. The infected cells (about 15 million) were collected by centrifugation (1000*g* for 10 min) and resuspended in 1.5 mL of I_3 buffer (10 mM Tris-HCl, pH 7.4, 5 mM EGTA, 100 μM benzamidine, 100 μM PMSF, 5 μg/mL soybean trypsin inhibitor, and 20 μg/mL leupeptin). The cells were sheared by passage three times through a 27 gauge needle, and the suspension was centrifuged at 15000*g* for 30 min. The membrane-containing pellet was resuspended in 1.5 mL of I_3 buffer, aliquoted, and stored at -80 °C. The protein concentration of the membranes was 2.0 mg/mL, as measured by the Bradford method (*22*) using bovine serum albumin as a standard.

Preparation of [*125I*]*ICYP*. In a glass test tube, 10 μL of [^{125}I]NaI in 0.1 N NaOH was neutralized with 10 μL of 0.1 N HCl. To this solution, 20 μL of 1.0 M potassium phosphate buffer (pH 7.6) was added, followed by the addition of 10 μL of cyanopindolol hydrochloride (2 mg/mL in H_2O). Iodination was initiated by adding 20 μL of chloramine T (0.34 mg/mL in H_2O). After 4 min at room temperature, the reaction was quenched by addition of 300 μL of $Na_2S_2O_3$ (1 mg/mL in H_2O). The radioiodinated product, [^{125}I]ICYP, was then extracted with ethyl acetate and purified by paper chromatography developed in 0.1 M ammonium formate and 0.01% phenol.

Preparation of [*125I*]*IABP*. Preparation of [^{125}I]IABP and [^{125}I]IABP photolabeling of the β_2AR in membranes was performed according to the method of Rashidbaigi and Ruoho (*7*).

[*125I*]*ICYP Displacement Binding Assay*. Sf9 membranes were diluted in 200 μL of B1 buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 2 mM EDTA) to a final protein concentration of 10 μg/mL. The binding assay was initiated by adding [^{125}I]ICYP (final concentration 100 pM) to the incubation mixture in the presence of various concentrations of the ligands to be tested. The incubation was carried out at 30 °C for 30 min. The unbound [^{125}I]ICYP was separated from the bound [^{125}I]ICYP/receptor complex by filtration through Whatman GF/A glass microfiber filters and washed twice with 10 mL of the ice cold buffer containing 10 mM Tris-HCl, pH 7.4, and 4 mM $MgCl_2$. The amount of [^{125}I]-ICYP bound to each filter was determined using a Packard 800C gamma counter. The K_i for each ligand was calculated from the Cheng$-$Prusoff equation [$K_i = IC_{50}/(1 + L/K_D)$], where IC_{50} is the concentration of nonradioactive ligand that half-maximally inhibits [^{125}I]ICYP binding, L is the concentration of [^{125}I]ICYP used in the assay, and K_D is the equilibrium dissociation constant of [^{125}I]ICYP determined from Scatchard analysis, which was 50 pM based on our experimental data.

Photolabeling the Sf9 Membrane-Bound β_2AR. Photolabeling of the β_2AR expressed in Sf9 cell membranes was performed by incubating the membranes at 30 °C for 30 min with the radioligand [^{125}I]IAmF, in the absence or presence of 10 μM ($-$)-alprenolol to assess nonspecific labeling. The membranes (approximately 20 μg of protein) were suspended in a polypropylene test tube with 100 μL of the B1 buffer and approximately 1 nM [^{125}I]IAmF. Photoreduction of the radioactive ligand at the β_2AR binding site was performed by irradiation with 350 nm light for 15 min at 4 °C using a Rayonet biochemical photoreactor (model RPR-100, The Southern New England Ultraviolet Co., Branford, CT; approximate output 400 W). The distance between the lamps and the sample tubes was approximately 2 cm. The photolyzed samples were then combined with 30 μL of SDS sample buffer (60 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 15 mM 2-mercaptoethanol, and 0.1% bromophenol blue) and analyzed by SDS$-$PAGE on a 12% Laemmli gel (*23*).

Receptor-Stimulated Adenylyl Cyclase Activity Assays. Receptor-stimulated adenylyl cyclase activity assays were performed according to procedures described by Salomon et al. (*24*). Typically, an assay mixture of 100 μL contained 50 mM Tris-HCl, pH 7.4, 1.0 mM $MgCl_2$, 0.1 mM ATP, 50 μM GTP, 2.0 mM creatine phosphate, 16 units creatine

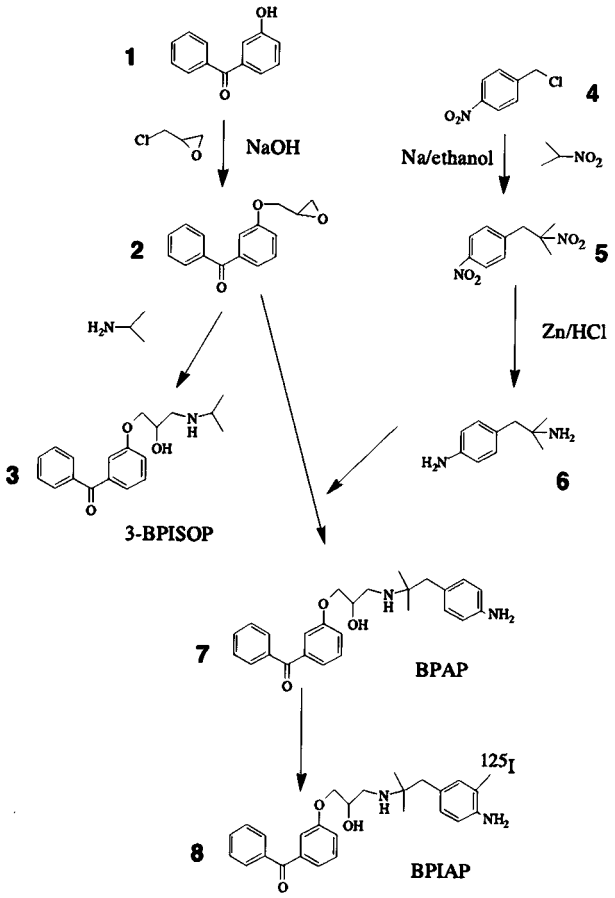


FIGURE 1: Synthetic scheme for benzophenone-based derivatives.

kinase, 1 mM IBMX (3-isobutyl-1-methylxanthine), 0.1 mM cAMP, 5×10^6 cpm [α-^{32}P]ATP, 100 μM isoproterenol, and 50 μg of S49 wild-type lymphoma membrane proteins. The membranes were added to initiate the assay. Incubation proceeded at 30 °C for 30 min, at which point cAMP production was terminated by adding 1.0 mL of stop solution (2% SDS, 40 mM ATP, and 1.4 mM cAMP at pH 7.5 and approximately 20 000 cpm [^{3}H]cAMP). In the experiments to test antagonist activities, antagonists at various concentrations were incubated in the assay mix first for 10 min at 30 °C, followed by the addition of 100 μM isoproterenol and incubation for an additional 20 min. After the stop solution was added, the tubes were vortexed gently and decanted into columns (4×0.7 cm) containing 1.0 mL of Dowex resin (AGW50 × 4, BioRad), which had been equilibrated with water. The column was washed with 2 mL of water, and the cAMP was then eluted with 3 mL of water collected directly onto a second series of columns (4×0.7 cm) containing 1 g of aluminum oxide. The alumina columns were washed with 5 mL of 0.1 N imidazole. cAMP was eluted with 2 mL of 0.1 N imidazole collected directly into vials and mixed with cocktail for liquid scintillation counting.

RESULTS

Synthesis of 3-BPISOP, BPAP, and [^{125}I]BPIAP (*Figure 1*). Two general steps were involved in the preparation of the benzophenone derivatives as adrenergic ligands: (a) coupling of epichlorohydrin to the hydroxyl group of the benzophenone under alkaline conditions and (b) condensation of an amine to the epoxide to give the final compounds.

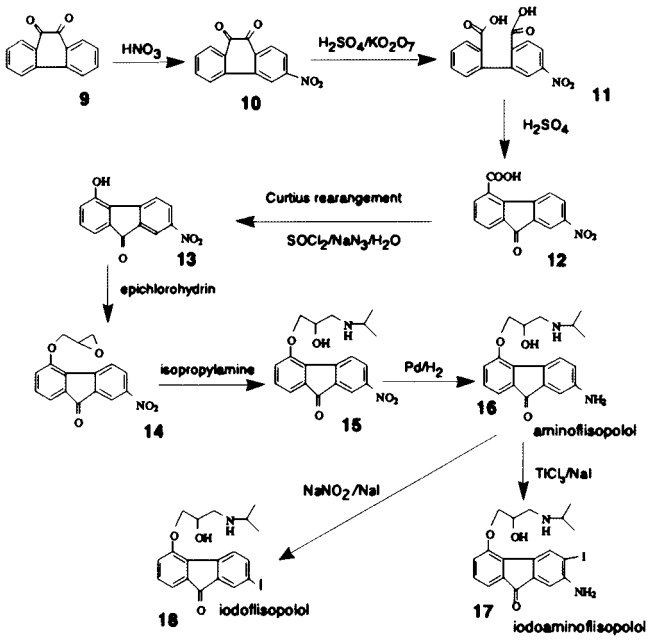


FIGURE 2: Synthetic scheme for fluorenone-based derivatives.

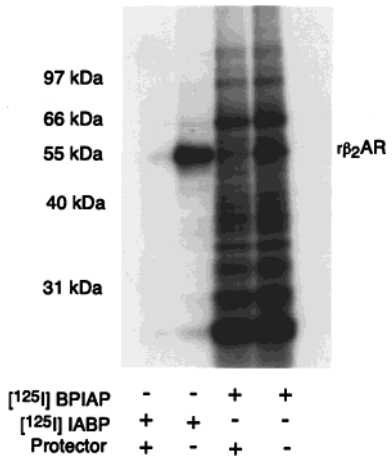


FIGURE 3: Photolabeling of the β_2AR with [^{125}I]IABP and [^{125}I]-BPIAP. Recombinant baculovirus-infected Sf9 cell membranes (20 μg of protein/lane) were incubated with 1 nM [^{125}I]BPIAP and photolyzed (two right lanes). The results were analyzed by SDS−PAGE and autoradiography. As a comparison, [^{125}I]IABP, a well-characterized photoprobe, was used to label the same membrane preparation (two left lanes). The concentration of [^{125}I]IABP was 1 nM. Specificity of labeling was assessed by adding 10 μM (−)-alprenolol for protection of the binding site. The β_2AR is the protectably labeled 55-kDa polypeptide.

BPAP was prepared by coupling the diamine compound **6** to the benzophenone epoxide **2** (Figure 1). This was a slow, low-yield reaction, most likely due to steric hindrance of the amine in the coupling reaction. Radioiodination of BPAP with [^{125}I]NaI in the presence of chloramine T generated [^{125}I]-BPIAP, which was purified in carrier-free form (specific activity 2200 Ci/mmol).

Photolabeling the β_2AR with [^{125}I]BPIAP. [^{125}I]BPIAP was incubated with β_2AR containing Sf9 membranes, photolyzed, and electrophoresed on an SDS−PAGE gel, followed by autoradiography. As shown in lanes 3 and 4 of Figure 3, [^{125}I]BPIAP covalently derivatized a polypeptide with an apparent molecular mass of 55 kDa. Photolabeling was blocked by the β_2AR antagonist (−)-alprenolol. The polypeptide labeled by [^{125}I]BPIAP migrates at the same position as

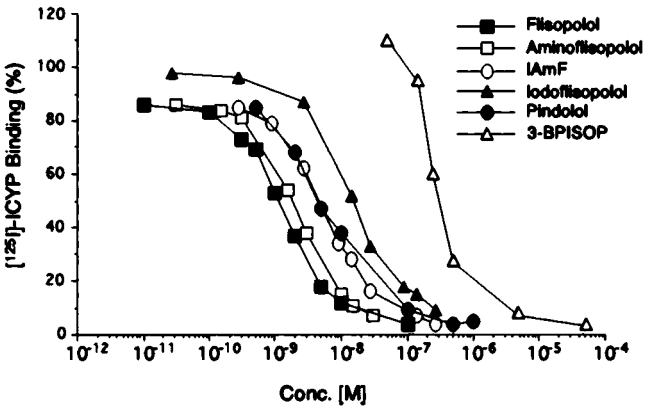
that identified by [^{125}I]IABP photolabeling, as shown in lanes 1 and 2 of Figure 3. However, [^{125}I]BPIAP showed only modest selectivity for the β_2AR, as evidenced by nonspecific labeling of nonreceptor peptides.

Synthesis of Iodoaminoflisopolol (**17**, *see Figure 2*). For ease of discussion, the acronym flisopolol is used in this paper for this novel ligand because it contains a fluorenone moiety and an isopropylamine moiety and (as shown below) is a potent β-adrenergic antagonist. The synthetic scheme for fluorenone-based β_2AR ligands is summarized in Figure 2. 2-Nitrophenanthraquinone was prepared via a nitration reaction from the starting material phenanthraquinone. The nitro compound was oxidized and dehydrated to generate 4-carboxylic 7-nitrofluorenone with high yield. Through a Curtius rearrangement reaction, the carboxylic group was converted to a hydroxyl, which provided a link to form the fluorenone epoxide. The fluorenone epoxide was synthesized from 7-nitro-4-hydroxylfluorenone. The epoxide was reacted with isopropylamine to generate 4-(2-hydroxy-3-isopropylaminopropoxy)-7-nitrofluorenone or nitroflisopolol. Nitroflisopolol was reduced to aminoflisopolol by hydrogen reduction catalyzed with palladium. IR analysis showed a sharp peak at 1730 cm^{-1}, indicating that the ketone group remained unreduced. Mass spectroscopy demonstrated a molecular weight consistent with the fluorenone structure. The amino group on flisopolol activated the ring system for iodination.

Characterization of Flisopolol and Its Derivatives. The displacement curves in Figure 4A show the ability of flisopolol and its derivatives to compete for receptor binding in S49 cyc$^-$ membranes with the radioligand [^{125}I]ICYP. Flisopolol has a higher affinity for the β_2AR than pindolol, iodoaminoflisopolol, or aminoflisopolol, all the latter compounds showing similar affinity (see Figure 4B). The K_i value of iodoflisopolol for the displacement of [^{125}I]ICYP binding was 20-fold higher than flisopolol. These results indicate that flisopolol and its derivatives have K_i values in the nanomolar range and further suggest that the position of the iodine may play an important role in determining affinity (see Figure 4B). The amino group on the fluorenone moiety may also provide additional ionic interactions or hydrogen bonds within the binding pocket. The benzophenone derivative 3-BPISOP showed the lowest affinity for the β_2AR, with a K_i of approximately 100 nM.

Inhibition of β_2AR-Stimulated Adenylyl Cyclase Activity by Aminoflisopolol and Its Analogues. The fluorenone-based ligands were found to be functional antagonists in an adenylyl cyclase assay using wild-type S49 lymphocyte membranes. As shown in Figure 5, isoproterenol (100 μM) stimulated cyclase activity was inhibited by aminoflisopolol in a concentration-dependent manner with an IC_{50} of approximately 3 nM. Other compounds, including flisopolol and pindolol, were also tested, and all showed inhibition of isoproterenol-stimulated cyclase activity under the same assay conditions (data not shown). Flisopolol showed the highest affinity for the β_2AR and also showed the most potent inhibition of receptor-stimulated cAMP production. As seen in Figure 5, increasing concentrations of aminoflisopolol completely eliminated receptor-stimulated adenylyl cyclase activity. Furthermore, flisopolol alone did not activate adenylyl cyclase (data not shown), indicating that flisopolol and its derivatives are antagonists and not partial agonists.

A.



B.

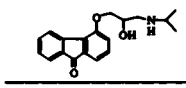
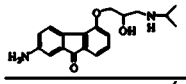
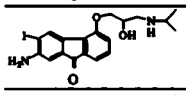
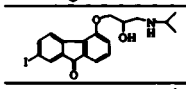
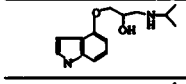
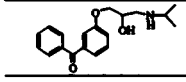
Structure	Name	K_i
	Flisopolol	1.0 x 10^{-9} M
	Aminoflisopolol	2.0 x 10^{-9} M
	Iodoaminoflisopolol (IAmF)	4.0 x 10^{-9} M
	Iodoflisopolol	2.0 x 10^{-8} M
	Pindolol	4.0 x 10^{-9} M
	3-BPISOP	1.34 x 10^{-7} M

FIGURE 4: Competitive displacement of [^{125}I]ICYP binding with fluorenone-based and related β_2AR ligands. (A) Competitive displacement curves. S49 cyc$^-$ membranes (approximately 80 μg of protein/mL) were incubated with 100 pM [^{125}I]ICYP in the presence of ligands at various concentrations as indicated. (B) Summary of structures and K_i values for β_2AR antagonists tested. K_i for each antagonist was calculated from the Cheng−Prusoff equation, as described in Materials and Methods.

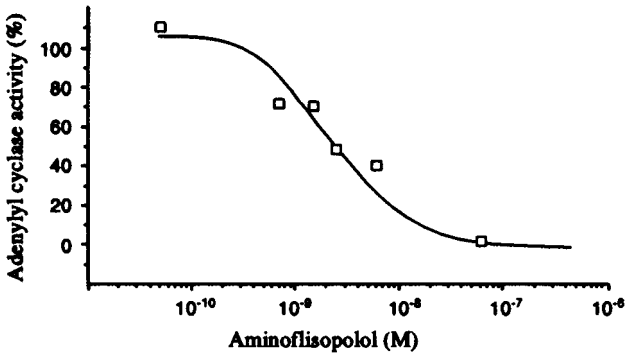


FIGURE 5: Inhibition of β_2AR-stimulated adenylyl cyclase activity in S49 wild-type lymphocyte membranes by aminoflisopolol. The inhibition of cyclase activity was performed as described in Materials and Methods by adding aminoflisopolol at various concentrations to the assay mixture prior to addition of 100 μM isoproterenol.

Photoreactivity of [^{125}I]IAmF. To our knowledge, only one other fluorenone-based photoaffinity label has been reported

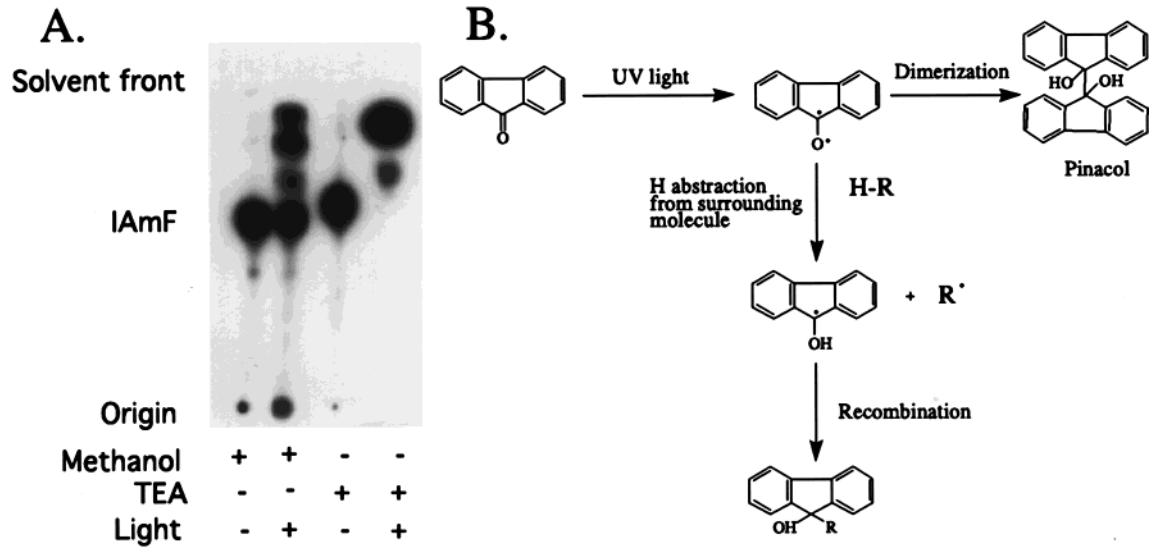


FIGURE 6: Photoreduction of [^{125}I]IAmF. (A) Photolysis of [^{125}I]IAmF in methanol and triethylamine. Shown is an autoradiogram of a silica gel TLC analysis of light-induced photoreaction products of [^{125}I]IAmF. Radioactive [^{125}I]IAmF in carrier-free form was photolyzed for 20 min at 350 nm in either methanol or triethylamine and chromatographed on a silica gel TLC plate developed in methanol:ethyl acetate:triethylamine (1:1:0.1). The photoproducts are shown migrating above the starting material [^{125}I]IAmF. Without light treatment, no reaction occurrs and [^{125}I]IAmF remains intact. (B) Mechanism of fluorenone photoreduction. Photoreduction occurs when UV light is applied to fluorenone molecules, resulting in disproportionation of the electrons in the carbonyl group, as illustrated. The electron on the oxygen abstracts a proton from a surrounding molecule (e.g., an amino acid side chain), and the resulting carbon free radical combines to form a cross-linked product. R, in the case of photolysis of [^{125}I]IAmF in solvent, is the carbon side chain of triethylamine, probably the carbon α to the amino group.

for a biological target (*25*), although the benzophenone moiety has been intensively studied and utilized in the identification of receptors, enzymes, and polypeptides. To test the photoreactive properties of a fluorenone-based photolabel, [^{125}I]IAmF was dissolved in either methanol or triethylamine and photolyzed for 20 min at 4 °C in a Rayonet photochemical reactor. The results were analyzed using silica gel thin-layer chromatography developed with a solvent system of methanol:ethyl acetate:triethylamine (10:10:1) and visualized by autoradiography (Figure 6A). The photoproducts migrated with a higher R_f than the starting material [^{125}I]-IAmF, and no reaction took place in the absence of light. In a separate experiment, fluorenone, aminoflisopolol, and nitroflisopolol were irradiated with UV light, and these compounds also all formed new photoreaction products, as analyzed by TLC (data not shown). It is apparent that triethylamine is a better reactant than methanol for the generation of [^{125}I]IAmF photoproducts, as has also been reported for benzophenone photochemistry (*26*). The photoproducts were not chemically characterized but are likely to be the result of hydrogen abstraction and covalent bond formation between the carbonyl carbon of the fluorenone moiety and the ethyl side chain carbon of triethylamine (Figure 6B).

Time Course of [^{125}I]IAmF Photolysis. Photoinsertion of [^{125}I]IAmF into the β_2AR occurred after a 30-s photolysis and reached a plateau at approximately 10 min, as shown in Figure 7A. Figure 7B shows the Coomassie-stained SDS−PAGE gel from which the autoradiogram in panel A was obtained. Clearly, the β_2AR expressing Sf9 membranes contain numerous proteins, but only two polypeptides (of 55 and 58 kDa) were photolabeled by [^{125}I]IAmF. The lower band of 55 kDa is the β_2AR, and labeling of this band was protected by addition of the antagonist (−)-alprenolol. Labeling of the upper band (58 kDa) was not protectable by excess β_2AR ligand (alprenolol), and we conclude that this band is not likely to be the β_2AR. The position of this nonprotectable labeled band corresponds to a prominent Coomassie-stained protein band from Sf9 membranes, which we conclude is labeled nonspecifically.

Photoaffinity Labeling of the β_2AR in S49 Cyc$^-$ Lymphocyte Membranes. Historically, S49 lymphoma cells have been used extensively in the investigation of the β_2AR/G protein/adenylyl cyclase signaling system. In this study, ligand binding inhibition curves and receptor-stimulated adenylyl cyclase assays were performed using S49 cell membranes. Therefore, an important extension of these experiments was to also demonstrate [^{125}I]IAmF photolabeling of the β_2AR in S49 cell membranes. Membranes prepared from S49 cyc$^-$ lymphoma cells were incubated with [^{125}I]IAmF (in the same manner as described for Sf9 membranes) and photolyzed. The pattern of photolabeled bands appeared as a doublet, as described in a previous report (*27*), of 55 and 60−65 kDa (Figure 7C). The reason for the doublet in the S49 cell membranes is very likely due to differential glycosylation. Photolabeling of both of the S49 cyc$^-$ β_2AR bands was protected when 10 μM (−)-alprenolol was present during the incubation, demonstrating the specificity of photolabeling.

DISCUSSION

An effective photoaffinity label for the study of a receptor binding site should have the properties of high affinity for the binding site and a suitable combination of reasonable photoinsertion yield and/or high specific radioactivity. [^{125}I]-IAmF satisfies these criteria and in addition has the unique property that derivatization of the ligand binding site occurs precisely at the site where the planar aromatic pharmacophore is bound in the receptor and which, by analogy to other β_2-

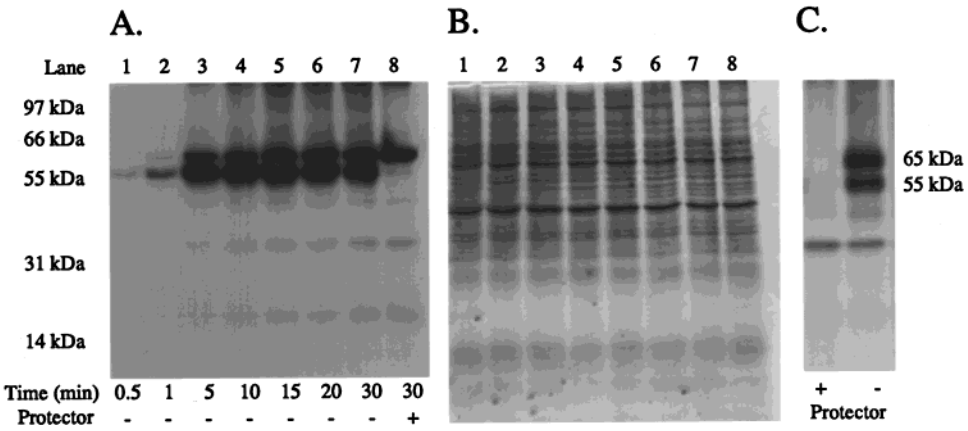


FIGURE 7: [^{125}I]IAmF photoaffinity labeling of the β_2AR in Sf9 and S49 cell membranes. (A) An autoradiogram of an SDS−PAGE gel showing a time course of photolabeling of the β_2AR expressed in Sf9 cell membranes with [^{125}I]IAmF. The β_2AR containing Sf9 membranes were photolyzed for the times indicated. (−)-Alprenolol (10 μM) was added to the reaction in lane 8 to assess nonspecific photolabeling. The β_2AR was identified as the 55-kDa protectable polypetide (the lower of the two labeled bands). The upper labeled band at approximately 58 kDa is a nonspecifically labled protein (not the β_2AR), as demonstrated by failure of 10 μM (−)-alprenolol to protect the photolabeling of the upper band (lane 8). (B) The Coomasie Blue stained SDS−PAGE gel from which the autoradiogram in panel A was obtained. (C) An autoradiogram of an SDS−PAGE gel showing [^{125}I]IAmF photoaffinity labeling of the β_2AR in S49 lymphoma cyc$^-$ cell membranes. (−)-Alprenolol (10 μM) was used as a protector. Unlike the β_2AR from Sf9 membranes (panel A) but consistent with a previous report (*27*), the β_2AR in S49 membranes is manifest as two photolabeled polypeptides of molecular masses 55 and 60−65 kDa.

AR ligands, is believed to be the catechol binding region of the β_2AR. The fluorenone derivative flisopolol and the benzophenone derivative 3-BPISOP have almost identical structures (shown in Figure 4B), but their affinities for the β_2AR are dramatically different, reflecting the fact that the β_2AR ligand binding site has strict structural requirements for high-affinity binding. In fluorenone, the ortho carbons on each phenyl ring relative to the carbonyl carbon are directly linked, resulting in a rigid planar fused unsaturated ring system, while in benzophenone, the two phenyl rings are only connected through the carbonyl carbon and cannot assume coplanarity. A reasonable hypothesis based on reports from molecular modeling and site-directed mutagenesis (*2*, *16*) is that a rigid planar three-ring system, such as in flisopolol, intercalates by ring stacking and $\pi-\pi$ electrostatic interactions between aromatic rings of side chains (perhaps Phe-290 and Try-286 on TM6). For a noncoplanar ring system, such as in 3-BPISOP, this type of intercalation may be less favorable. The benzophenone-based radioactive photolabel [^{125}I]BPIAP specifically derivatized the β_2AR, but it also nonspecifically labeled many other proteins (Figure 3). The dilemma in the practical application of the benzophenone-based ligands as photoprobes is their relatively poor affinity, which reduced any benefit gained from their high photoinsertion yields. We have not precisely measured the photoinsertion yield of [^{125}I]IAmF, but the photoinsertion yield appears to be intermediate between the yields of benzophenones and the yields of aryl azides (10−50%) and is certainly sufficient for easy detection of photolabeled peptides when using a high specific radioactivity photolabel.

Experiments were conducted to assess the photoreactivity of fluorenone and its derivatives. As shown in Figure 6A, photoreduction of [^{125}I]IAmF was light dependent and reactant dependent: upon photolysis of [^{125}I]IAmF in TEA, all the starting material disappeared, and photoproducts were generated. The photoproducts in methanol and in TEA showed differing patterns on a silica gel TLC plate. These solvent-dependent differences indicated that the light-induced photoproducts were not the result of random degradation or dimerizations between two photolabel molecules (i.e., pinacol formation; see Figure 6B) but are cross-linked products between [^{125}I]IAmF and solvent molecules. Fluorenone and benzophenone have been demonstrated to react via similar mechanisms of photoreduction, as shown in Figure 6. UV irradiation causes a disproportionation of electrons in the carbonyl group in a fashion similar to that of benzophenone (*26*, *28*). The electron on the oxygen abstracts a proton from surrounding molecules, such as solvent or an amino acid side chain, and the resulting carbon free radicals combined to form a new cross-linked product. When a high concentration (e.g., mM) of fluorenone is UV-irradiated, a fluorenone dimer (pinacol) can be generated in addition to cross-linked products. This is not likely to be the case in the use of fluorenone-based compounds as photoaffinity labels because the concentration of the photoprobe is very low (approximately 1 nM), and there is less of a chance for two fluorenone molecules to form a pinacol dimer.

Cohen and Gutternplan (*26*) studied the effects of the solvent on the photoreduction of several fluorenone derivatives. They found that fluorenone was not photoreduced in 2-propanol or cyclohexane but was readily photoreduced by 2-butylamine or triethylamine, resulting in cross-linked products. The data in Figure 6 demonstrate that methanol is also able to photoreact with [^{125}I]IAmF, although to a lesser degree as compared with triethylamine. Photoreduction of fluorenone is expected to follow the same mechanism as that of benzophenone. Viewed from the point of the lowest triplet state, fluorenone is of the $\pi-\pi^*$ configuration, whereas benzophenone is of the $n-\pi^*$ configuration (*26*). Fluorenone is thus somewhat less photoactive than benzophenone, but as we have demonstrated by direct [^{125}I]IAmF photolabeling of the β_2AR in cell membranes, the sensitivity provided by carrier-free radioiodine allows the use of fluorenone-based derivatives, such as [^{125}I]IAmF, as effective photoaffinity probes for the β_2AR ligand binding site. The feasibility of using fluorenone-based photolabels is further evidenced by the recent report of a fluorenone-containing parathyroid hormone photoaffinity label (*25*).

In conclusion, we have synthesized and tested a series of benzophenone- and fluorenone-based compounds for their suitability as β_2AR photoaffinity labels. One of these compounds, [^{125}I]iodoaminoflisopolol, had particularly favorable properties and was synthesized in carrier-free, radioiodinated form and found to specifically label the β_2AR in cell membranes in an (−)-alprenolol protectable manner. [^{125}I]IAmF is a unique photolabel for the β_2AR in which the photoreactive group and planar unsaturated pharmacophore are combined together in the same portion of the molecule and can be used to identify an amino acid or amino acids that contribute to the β_2AR antagonist binding site.

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