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SYNTHESIS OF A MULTIFUNCTIONAL RADIOIODINATABLE PHOTOAFFINITY PROBE

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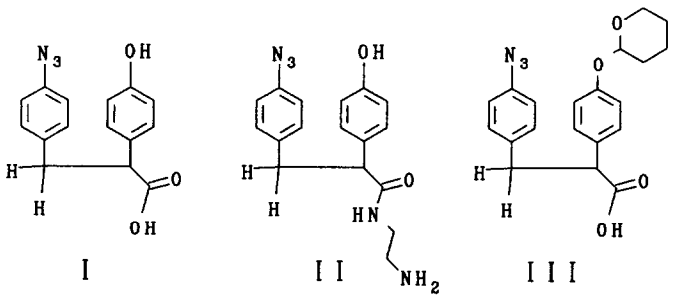
Abstract: Synthesis is described which results in an iodinatable photoaffinity probe and derivatives which are useful in the covalent coupling of the probe to amine, carboxylic acid, and alcoholic hydroxy function on specific ligands of biological importance. Specific examples of natural product derivatization from terrestrial and marine sources are given.

Photoaffinity labeling of natural products is important for investigations of specific binding sites in biological tissues, especially if the binding site is to be isolated and purified. We wish to report synthesis of a UV-activatable azido-photoaffinity probe which has broad utility in the covalent modification of ligands of biochemical importance. The core compound (I), 2-*p*-hydroxyphenyl-3-*p*-azidophenyl propionic acid, is useful for direct coupling with amines. By suitable modification of the core compound to either the ethylene diamine monoamide (II) or the *o*-tetrahydropyranyl derivative (III), carboxylic acids or alcoholic hydroxy functions may be coupled [1].



Synthesis of I: [*p*-acetoxyphenylacetic acid]. *p*-Hydroxyphenyl acetic acid (100 g) was dissolved in 72 mL acetic anhydride. The reaction was initiated by the addition of 2-3 drops concentrated sulfuric acid and was stirred. Upon cooling, the acetate separated. The acetate precipitate was redissolved in ether, washed 4 times with water, and was dried over Na_2SO_4. The solution was filtered and flash-evaporated, and the residue dried over phosphorus pentoxide *in vacuo* (yield 105 g, 82.5%).

[2-*p*-acetoxyphenyl *p*-nitro cinnamic acid] [2]. To 100 g *p*-acetoxyphenylacetic acid was added 100 g *p*-nitrobenzaldehyde, 182 mL acetic anhydride, and 91 mL triethylamine and the solution was refluxed for 2.5 hr. The resulting dark liquid was poured with stirring onto an ice-water mixture acidified with sulfuric acid. An oil separated, and solidified in 0.5 hr at 4°C. The solid was filtered by suction, washed thoroughly with ice water, and was recrystallized from 500 mL boiling glacial acetic acid containing 10 mL acetic anhydride. By precipitation with water, and recrystallization from 100 mL glacial acetic acid, a second yield of crystalline product was obtained (yield 145.5 g of yellow crystals, 67.8% mp 146°C).

[3-*p*-aminophenyl 2-*p*-hydroxyphenyl propionic acid hydrochloride]. Fifty grams of the preceding intermediate was hydrogenated in tetrahydrofuran solution in a Parr hydrogenator with 10% Palladium-charcoal, gently heating to prevent clogging of the gas inlet tube. After the theoretical amount of hydrogen was consumed, the warm solution was filtered and the filtrate was flash-evaporated. The residue was refluxed for 1.25 hr in 700 mL 1:1 aqueous HCl solution. After partial cooling, the solution was filtered on fluted filter paper and the filtrate was allowed to crystallize at 4°C. The product was filtered on a glass membrane filter and dried *in vacuo* over KOH (yield 36 g, 81.1%, mp 215°C).

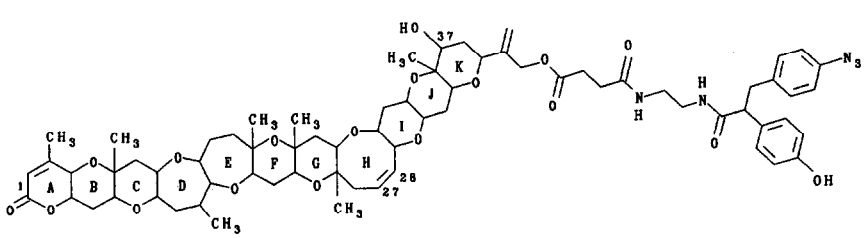
[3-*p*-azidophenyl 2-*p*-hydroxyphenyl propionic acid] [3]. Thirty-seven grams (0.125 mole) of amine hydrochloride

intermediate was dissolved in a mixture of 25.8 mL sulfuric acid and 143 mL water. On cooling, a suspension results. Aqueous sodium nitrite (11.92 g in 109 mL water) was added dropwise to the cold stirred suspension, which resulted in full solubilization. Excess nitrous acid was decomposed by the addition of solid urea. The solution was treated with Norite and was filtered by suction. Sodium azide (15.6 g in 80.3 mL water) was added dropwise to the cold stirred solution, and the azidophenyl compound precipitated. After 1 hr at 4°C on ice, the product was filtered by suction and was washed with cold water and dried *in vacuo* over KOH. The dried product (I) was recrystallized from 115 mL boiling glacial acetic acid, washed with cold glacial acetic acid, and was dried *in vacuo* over KOH (yield 27.8 g, 78.6%, mp 182°C).

Synthesis of II: [2-*p*-hydroxyphenyl 3-*p*-azidophenyl propionyl ethylene diamine monoamide]. 27.4 g of (I) and 22 g *p*-nitrophenol were dissolved in 40 mL dimethylformamide [4]. Dicyclohexyl carbodiimide (16.2 g in 10 mL dimethylformamide) was added to the stirred solution dropwise, and stirring was continued for 2 more hr on ice. With stirring, the solution was allowed to equilibrate to room temperature and after 1 hr, 10 mL glacial acetic acid was added and the suspension was stirred 15 min longer. The urea was removed by filtration, and was washed with 35 mL tetrohydrofuran. The combined filtrates were poured onto ice and immediately a total volume of 600 mL oil separated. The oil was decanted and extracted with benzene; the benzene layer was washed four times with 10% NaCl solution, and was dried over sodium sulfate. A solution composed of 20 mL ethylene diamine and 100 mL benzene was cooled on ice, and the dried oil extract was added dropwise under vigorous stirring over a period of two hr. A yellow precipitate was filtered by suction, the solid was suspended in a minimum volume of 10% v/v sulfuric acid, and was extracted repeatedly with ethyl acetate to remove *p*-nitrophenol from the yellow gummy layer. The dark aqueous layer formed crystals upon standing at 4°C. The product is the sulfate salt of (II) (yield based on (I) is 51.6%, mp 145°C). This salt is poorly soluble in water compared to the hydrochloride. Preparation of the free base can be accomplished by adding a saturated sodium carbonate solution to a stirred aqueous suspension of the sulfate salt until the solution becomes alkaline. The free base preciptated and after drying was recrystallized from hot ethanol (mp 196°C).

Synthesis of III: [2-*p*-tetrahydropyranoxy-phenyl, 3-*p*-azidophenyl propionic acid]. 3.4 g (I) was suspended in 10 mL dihydropyrane and stirred with a glass rod moistened with *p*-toluene sulfonic acid. Mixture produced an exothermic reaction, resulting in a single phase. One hr later, 10 mL of 2M alcoholic KOH was added and the solution was heated in a water bath at 60°C for two hr. The solution was flash-evaporated and 200 mL 5% potassium sulfate solution was added. The solution was mixed with 400 mL ethyl acetate and the mixture was cooled to 0°C. To this solution was added 10 mL cold concentrated sulfuric acid, and the mixture was shaken. The ethyl acetate layer was washed three times with ice-water to pH neutrality, and was dried by the addition of solid sodium sulfate. The dried solution was flash-evaporated and the residue was redissolved in 40 mL acetone, and applied to preparative silica gel thin-layer chromatography plates. Following chromatography using cyclohexane:ethyl acetate (75:25) as mobile phase, the product was identified (R_f = 0.1) and was eluted from the silica gel with acetone. Exposure of eluted compound (III) to 6M HCl in acetone for three hr at room temperature resulted in conversion to I, thus confirming identity of the derivative.

Coupling to Sodium Channel Activator Brevetoxin: Brevetoxins, biosynthesized by the Florida red tide dinoflagellate *Ptychodiscus brevis* (=*Gymnodinium breve*), bind to Site 5 associated with the α-subunit of the voltage-sensitive sodium channel in excitable membranes, inducing an inward ion current at normal resting membrane potential [5].



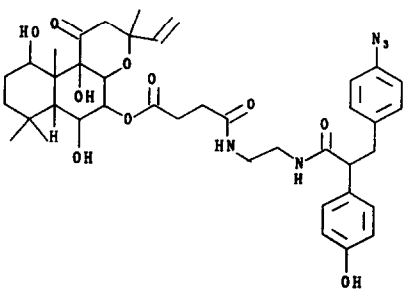
IV

CAUTION: Brevetoxins are potent neurotoxins and should be handled only by persons familiar with proper precautions for handling and treatment [6]. Studies carried out using brevetoxin PbTx-2 (=brevetoxin B) photoaffinity probes indicate a 1:1 stoichiometry with α-subunit, a competitive ability to displace radioactive brevetoxin from its specific binding site, and a concentration-dependent increase in specific binding. Further preliminary studies employing equations of Cheng and Prusoff [7] indicated that brevetoxin photoaffinity probe displacement was truly competitive in nature, with a derived inhibition constant (K_i) of 10.3 nM, which compares favorably with a 2.4 nM inhibition constant determined for PbTx-3 against itself. A complete description of ^{125}I-labeled brevetoxin photoaffinity binding to the α-subunit of the voltage-sensitive sodium channel shall be described elsewhere.

Dry PbTx-2 (5.0 mg) and 0.147 mL of a 0.4M cerium chloride solution in methanol were mixed together under constant stirring [6]. An equal volume of methanol was added, followed by slow addition of 17.1 μL of 0.01 M $NaBH_4$ solution in dimethyl formamide, repeating addition of reductant every 20 minutes until the PbTx-2 was completely reduced to PbTx-3, as adjudged by thin-layer chromatography, and complete PbTx-2 solubilization. The product volume was diluted with water to twice volume, products were extracted with diethyl ether, dried and subjected to reverse phase hplc (C-18 column, 85% isocratic methanol, 215 nm ultraviolet detection), resulting in crystalline PbTx-3 (4.294 mg, 87.4% yield). Proton nmr spectra were obtained on a Varian VXR-300 spectrometer, using $CDCl_3$ (99.96% enriched) and using TMS as internal standard. Reduction of PbTx-2 resulted in the loss of an aldehyde signal at 9.55 ppm and the shift of the α-methylene signals from 6.09 ppm (trans) and 6.36 ppm (cis) to 4.94 and 5.11 ppm, respectively. Purified PbTx-3 was dissolved in 0.4 mL redistilled pyridine, and a ten-fold molar excess of succinic anhydride in pyridine was added with stirring. The reaction vial was sealed, and the solution was heated to 85°C for two hr with stirring. Solvent was removed under a stream of nitrogen and products were redissolved in methanol and chromatographed on silica gel tlc plates using ethyl acetate/light petroleum (70/30) as mobile phase. Previous experiments using tritiated brevetoxin PbTx-3 indicated that the desired conjugate migrated with an R_f of 0.25, and tested positive with bromcresol green indicator spray reagent.

PbTx-3-succinate was dissolved in 1 mL pyridine, mixed with two-fold molar excess of dicyclohexyl-carbodiimide in 0.5 mL pyridine, and solid equimolar (II). The mixture was sealed in a screw-capped tube and was heated for 2 hr at 85°C in a Nujol bath. Following reaction, the bath was cooled, the pyridine evaporated under vacuum, redissolved in methanol and subjected to silica gel tlc using ethyl acetate/light petroleum (80/20) as mobile phase. The complete PbTx-3-linked 2-*p*-hydroxyphenyl 3-*p*-azidophenyl propionyl ethylene diamine monoamide (IV) was visualized on the plate with iodine vapor, was scraped and eluted with methanol. Probe was stable for 6 months at -20°C in methanol solution.

Coupling to Adenylate Cyclase Activator Forskolin: Adenylate cyclase (EC 4.6.1.1) is an important enzyme in regulatory biology as the enzyme responsible for cAMP synthesis which in turn regulates phosphorylation of a number of key proteins and enzymes *via* activation of cAMP-dependent protein kinases [8]. Forskolin, a bioactive diterpene with pronounced cardiovascular activity, is isolated from the terrestrial plant *Coleus forskohlii*.



V

The photoaffinity analog of forskolin possessed 8% of the adenylate cyclase activating activity of the original unaltered forskolin; a value determined to be due to activity of purified photoaffinity-forskolin and not an 8% contamination of photoprobe by forskolin. UV-activation of membrane-bound forskolin photoaffinity probe inhibited activation of the normal activity by forskolin, indicating a block of the activity site for unaltered forskolin. Iodinated forskolin-photoprobe specifically binds to the forskolin receptor site on the catalytic subunit of adenylate cyclase, as evidenced by autoradiography of sodium dodecyl sulfate polyacrylamide gels, and its specific displacement by unlabeled forskolin [8].

In a small screw-capped glass vessel, 160 μmoles (II) and 111 μmoles forskolin hemisuccinic acid were dissolved in 10 mL dioxane. To this solution was added 111 μmoles of dicyclohexyl carbodiimide. The reaction mixture was incubated for 2 hr, after which time, the solvent was evaporated using a stream of nitrogen. The product mixture was dissolved in minimal dioxane, and was purifed by silica gel thin-layer chromatography using dioxane/ethyl acetate/cyclohexane (66/17/17) as mobile phase. the complete forskolin photoaffinity probe (V) exhibited a UV-fluorescent band (254 nm), and a R_f of 0.60-0.65.

Photoaffinity probes provide for solubilization, electrophoresis, and chemical and enzymatic degradation to determine the primary character of the specific site, without the dissociation which would take place using radioactive ligand alone. Detailed quantitative structure-activity relationships (QSAR) of ligands and agonists have been described in the literature and with this literature background, it is possible to design appropriate derivatives using the three photoprobes described in this communication. Based on the size and character of the photoaffinity probes described herein, we expect that these derivatives will be most useful in identifying specific membrane-bound proteins which contain receptors for ligands. Aryl azides are particularly useful in labeling membrane-bound receptors [9], based on their selectivity in hydrophobic regions when reacting with CH bonds. The more pronounced distance between "native" ligand and the aryl azide function in our probes, when compared to other photoaffinity probes like simple azidobenzoic acid derivatives or derivatives without succinic acid bridges, may seem large. However, the interest in "nearest neighbor" labeling and the ability of these probes to "fold back" upon themselves may actually lead to increased specificity. Minor modifications in the length of the "bridge" between ligand and azide should provide for full description of nearest neighbor analysis, much like the earlier work with photoaffinity labeling of Na,K-ATPase by cardiac glycoside photoaffinity probes [10] and the specific labeling of α-thrombin sites in CHO cells [11]. The two natural product photoaffinity probes described represent the first active photoaffinity probes of brevetoxin and forskolin, and each successfully labels a specific polypeptide we believe is in close proximity to the specific binding site of each ligand.

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