A Bifunctional Photoaffinity Probe for Ligand/Receptor Interaction Studies

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Photoaffinity labeling has become a powerful tool for investigating ligand/receptor interactions since its introduction by Westheimer.[1,2] However, despite the progress in the design of photolabile groups,[3] and methods for separating cross-linked peptide fragments and identifying cross-linking sites,[4,5] the cases in which cross-linked amino acids have been identified are relatively limited.[4,6] This is particularly true with membrane-bound proteins, including the philanthotoxin/nicotinic acetylcholine (nACh) receptor complex.[7] With retinal/rhodopsin and retinal/bacteriorhodopsin, it was possible to characterize cross-linked amino acids, but with considerable difficulty.[8−14]

A bifunctional photoaffinity probe (BPP) with a photoaffinity label (site **A**) *and* a photocleavable moiety (site **B**) has been prepared to streamline the tedious photoaffinity labeling process. The concept is shown in Figure 1: (i) The ligand−BPP molecule is bound to the receptor and photolyzed, and the receptor is cleaved enzymatically or chemically; (ii) the cross-linked and non-cross-linked peptide fragments are separated (a biotin tag linked to the ligand may facilitate separation);[15] (iii) the ligand (with the biotin tag) is detached from the cross-linked peptides by site **B** photocleavage; (iv) the mixture of peptides with the nitrophenolic marker is sequenced by tandem MS.

Tandem MS can directly sequence the respective peptide fragments, including those from membrane-bound proteins,[16] at the femtomole level,[17] without separation of the mixture; moreover, no radioisotope is required.[18] However, it is desirable that the bulk of the ligand moiety (including biotin tag) be removed from the peptides before MS sequencing to avoid complications in MS analysis (Figure 1). This led to the design of the probe shown in Figure 1, which carries photolabeling (site **A**) *and* photocleavable sites (site **B**), and which can derivatize various ligands.

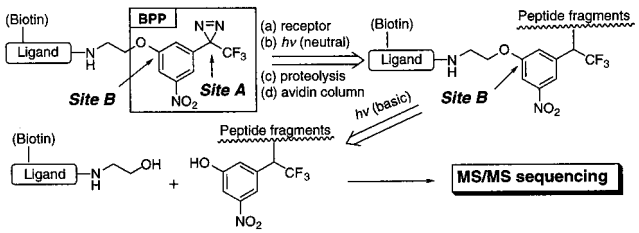

Figure 1. Conceptual outline of BPP.

For site **A**, the 3-(trifluoromethyl)-3-phenyldiazirine moiety[19] was chosen as the photolabel because of its outstanding photo-cross-linking results[13,20] and because irradiation at its 350 nm absorption band would not destroy the protein, λ_{max} 280 nm; it is also stable to mild reductive, acidic, and basic conditions.[3,4] With respect to site **B**, the linkage should withstand the manipulations associated with photoaffinity labeling experiments, but should also be readily cleavable for subsequent MS measurements. A comparison of various linkages and cleavage conditions in model systems[21] led to the "aromatic photosubstitution" system studied by Havinga.[22] Namely, as shown in Scheme 1 (I), irradiation in mild base displaces the OCH_3 group of *m*-nitroanisole **1** by $^{18}OH^-$ to yield **2**; however, in neutral media no photochemistry occurs.[23] Thus, irradiation of 3-nitroanisole **1** and 3-(4-methylphenyl)-3-(trifluoromethyl)-1,2-diazirine **3** in CD_3OD at 365 nm for 180 min gave adduct **4**, characterized by ^{1}H NMR, ^{19}F NMR, and GC−MS (Scheme 1, II). Whereas diazirine **3** reacted completely, anisole **1** remained intact. It is therefore possible to selectively excite the photolabeling site **A** in the presence of the phenoxyl ether site **B**.

The bifunctional photoaffinity probe should be compact and carry a *m*-nitrophenoxyl ether function and a trifluoromethyl diazirine side chain, and both positions ortho to the diazirine side chain should be unsubstituted to avoid intramolecular cross-linking. This led to BPP **11**, prepared as in Scheme 2. Aldehyde **5**[24] was converted into trifluoroacetyl ketone **6** by trifluoromethylation with Ruppert's reagent[25] and oxidation of the resulting alcohol with Dess−Martin periodinane.[26] Heating of **6** with $NH_2OH\cdot HCl$ in pyridine at 60 °C gave a mixture of *syn*- and *anti*-oximes **7**. Saponification of methyl ester **7** with aqueous base followed by Curtius rearrangement with DPPA in *t*-BuOH[27]

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(24) Aldehyde **5** was readily prepared from dimethyl 5-hydroxyisophthalate in four steps with 55% overall yield: (i) allyl bromide, K_2CO_3, acetone, reflux; (ii) NaOH, H_2O/MeOH; (iii) isopropyl chloroformate; $NaBH_4$, THF/H_2O; (iv) Dess−Martin periodinane, CH_2Cl_2. For details, see Supporting Information.
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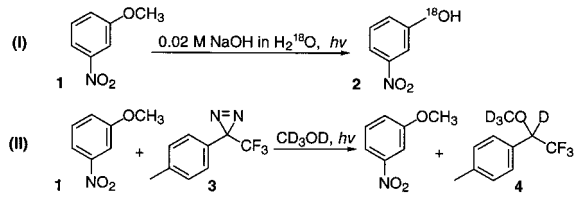
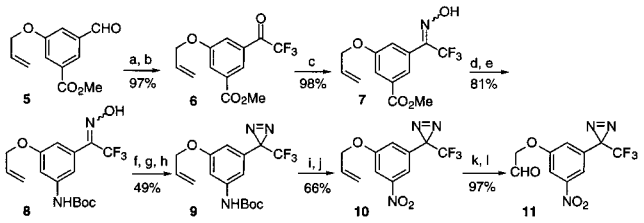
Scheme 1

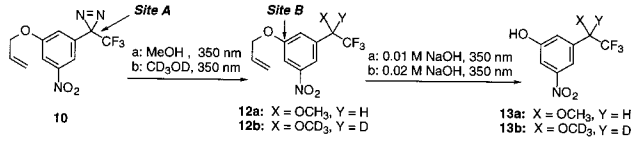


Scheme 2[a]



[a] Conditions: (a) $TMSCF_3$, catalytic TBAF, THF; then aqueous HCl. (b) Dess−Martin periodinane, TFA, CH_2Cl_2. (c) $NH_2OH{\cdot}HCl$, pyridine, 60 °C. (d) NaOH, MeOH/H_2O. (e) DPPA, Et_3N, *t*-BuOH, reflux. (f) TsCl, pyridine, reflux. (g) NH_3(liq), ether, sealed tube. (h) I_2, Et_3N, CH_2Cl_2. (i) TMSOTf, CH_2Cl_2. (j) 2,2-dimethyldioxirane. (k) Catalytic OsO_4, NMO, acetone/H_2O. (l) $NaIO_4$, silica gel, H_2O/CH_2Cl_2.

Scheme 3



provided *N*-Boc aniline derivative **8**. Standard manipulations[28,29] furnished diazirine **9** in three steps, overall yield 49%. Successive TMS triflate deprotection of Boc[30] and dioxirane oxidation[31,32] yielded nitro compound **10** (HRMS calcd for $C_{11}H_8F_3N_3O_3$ 287.0518, found 287.0528). Further oxidation with OsO_4/NMO and then with $NaIO_4$/wet silica gel in CH_2Cl_2[33] gave BPP **11**[34] in 97% yield.

Due to the tendency of the aldehyde group in BPP **11** to undergo hydration, the photochemistry was checked with allylic ether **10** (Scheme 3, Figure 2). Irradiation of **10** (6.2 mg, 21 mmol) in deoxygenated MeOH (20 mL) in Pyrex culture tubes in a Rayonet photoreactor[35] at 350 nm for 150 min gave **12a** (2.5 mg, 42%, HRMS calcd for $C_{12}H_{12}NO_4F_3$ 291.0718, found 291.0711), while further irradiation of **12a** (1.6 mg, 5.5 mmol) at 350 nm in 0.01 M NaOH (14 mL) for 120 min gave **13a** (HRMS calcd for $C_9H_8NO_4F_3$ 251.0405, found 251.0401) in quantitative yield. These results demonstrate that sites **A** and **B** can be selectively excited as expected. Similarly irradiation of **10** in CD_3OD in an NMR tube yielded **12b**, and then **13b**.

BPP can be readily incorporated into ligands through a lysine side chain or other primary amino groups. For example, **11** was coupled to the peptidomimetic *N*α-Cbz-lysine isopropyl amide through reductive amination to give compound **14** (60%, HRMS calcd for $C_{27}H_{34}N_6O_6F_3$ $(MH)^+$ 595.2492, found 595.2487) (Scheme 4). Under similar conditions, **11** was coupled to philanthotoxin-433 (PhTX433), a noncompetitive antagonist of nACh and Glu receptors isolated from the venom of the digger wasp *Philanthus triangulum*,[36] to yield PhTX433-BPP **15** (66%, HRMS calcd for $C_{33}H_{47}N_8O_6F_3$ 709.3649 $(MH)^+$, found 709.3651). It is worth noting that in a [^{3}H]TCP (1-(1-(2-thienyl)cyclohexyl)-piperidine) competition assay, PhTX433-BPP **15** demonstrated >30-fold stronger binding activity toward nACh receptor as compared to PhTX343, the reference in analogue SAR studies.[37] Since diazirine is stable under reductive conditions, **11** can possibly be converted into a tosylate by reduction of the aldehyde followed by tosylation, and coupled to ligands via hydroxyl or phenoxyl groups.

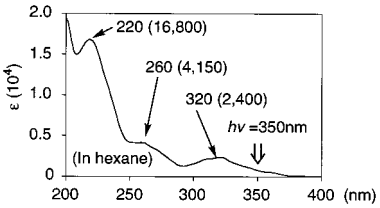
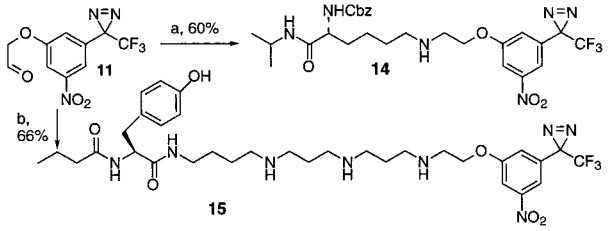


Figure 2. λ_{max} (ϵ) of BPP precursor **10**. Unfilled arrow denotes irradiation wavelength.

Scheme 4[a]



[a] Conditions: (a) *N*α-Cbz-lysine isopropyl amide, $NaCNBH_3$, MeOH. (b) PhTX433, $NaCNBH_3$, MeOH.

In summary, two photolabile functions which can be selectively activated are incorporated into the aromatic ring of the bifunctional photoaffinity probe **11**. The UV band above 300 nm can also serve as an HPLC marker for purification of the labeled proteins/peptides.[38] This bifunctional probe, in combination with tandem MS, is currently being employed to investigate ligand/receptor interactions;[39] it should find wide applications in other areas. The photo-cross-linking and sequencing results will be reported in due course.

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Supporting Information Available: Experimental procedures for model reactions, photochemistry, and synthesis of compounds and ^{1}H NMR, ^{19}F NMR, and HRMS data of compounds (10 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(34) ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.84 (1H, s), 7.72 (1H, dd, J = 2.2, 2.0 Hz), 7.69 (1H, s), 7.11 (1H, s), 4.73 (2H, s). ^{19}F NMR (282 MHz, $CDCl_3$, $CFCl_3$ = 0.00 ppm) δ −65.49. HRMS calcd for $C_{10}H_6F_3N_3O_4$ 289.0310, found 289.0305.
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(39) It has been reported that *p*-nitroanisole undergoes photochemical nucleophilic aromatic substitution with the ϵ-amino group of lysine at pH 8: Jelenc, P. C.; Canto, C. R.; Simon, S. R. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 3564−3568. It is unlikely that such a reaction would occur in the present case; however, if it does take place, its product will not contain the ligand and hence could be readily removed by the avidin treatment.