A New Photo-Cross-Linking Reagent for the Study of Protein-Protein Interactions

John F. Resek, Santanu Bhattacharya,† and H. Gobind Khorana*

Departments of Biology and Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139

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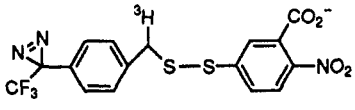
Rhodopsin is the membrane-bound photoreceptor of the vertebrate retinae.[1] Current structural models suggest that rhodopsin contains seven membrane-embedded α helices (Figure 1). The chromophore, 11-*cis*-retinal, located in the membrane, forms a Schiff base with Lys 296.[2] The absorption of a photon results in the isomerization of retinal to the *all-trans* conformation and this triggers the formation of a cascade of photointermediates, one of which, Meta II, interacts with and activates the retinal GTP binding protein transducin.[3] Previously, it has been shown that transducin binds to the cytoplasmic surface of Meta II rhodopsin.[4,5] To determine the nature of this chemical contact, we have sought to cross-link the two proteins and have synthesized a new cross-linking reagent 3-(4-(((4-nitro-3-carboxyphenyl)dithio)methyl-*t*)-phenyl)-3-(trifluoromethyl)-3*H*-diazirine, DTDA (1).



[^{3}H]-DTDA (1)

While a large number of photoactivatable reagents have been described in the literature,[6-8] the above carbene-generating reagent was designed with several specific features in view for determining the points of rhodopsin-transducin interaction. DTDA is able to form a disulfide bond with accessible cysteines. In combination with site-specific rhodopsin mutants it can be targeted to unique positions in the protein. In addition, following formation of the carbene, the radioactive label can be transferred to the site of insertion by cleaving the disulfide bond with a reducing agent. Activating DTDA with thionitrobenzoic acid (TNB) also gives the molecule a charge for selectively derivatizing residues located in the cytoplasmic domain. Finally, the small distance between the dithio linkage and the carbene (about 8 Å) allows residues in the immediate vicinity of the contact site to be cross-linked. In addition to determining the contact points between rhodopsin and transducin, DTDA will be generally useful for studying protein-protein interaction.

Synthesis of DTDA. The steps in the synthesis of DTDA (1) are shown in Figure 2. The bromobenzyl diazirine 2 was synthesized as previously described.[9-10] The introduction of a thiol group into the benzyl bromide was performed by reaction with potassium thioacetate. The acetyl thiobenzyl diazirine 3 was obtained from 2 in 90% yield. Incubation of 3 with 0.2 N NaOMe at room temperature for 5 min yielded the free thiol 4. The addition of dithionitrobenzoic acid, DTNB, yielded the final product, DTDA (1).

Synthesis of the [^{3}H]-DTDA started with the benzaldehyde 5, which was obtained from 2 by Kornblum oxidation.[11] The aldehyde 5 was reduced with a 1 molar equiv of NaB^3H_4 to generate the alcohol 6. This was activated with 2-fluoro-1-methylpyridinium tosylate (FMP)[12] and the intermediate reacted with potassium thioacetate to generate the tritiated thioacetate adduct 7. FMP was used to activate the alcohol, rather than tosyl chloride, because benzyl tosylates are highly unstable compounds which react rapidly to give undesirable side products.[13] Cleavage of the acetate with base followed by the addition of DTNB yielded the tritiated [^{3}H]-DTDA (1).

Derivatization of Rhodopsin. Bovine rhodopsin contains 10 cysteine residues (Figure 1). Of these only two, Cys 140 and 316 in the cytoplasmic domain, are derivatized with sulfhydryl reagents such as iodoacetic acid,[14] dithiopyridine,[15] and Ellman's reagent.[16] [^{3}H]-DTDA (1) reacts with these residues to form a disulfide bond and release 1 equiv of TNB. Figure 3A shows an SDS-PAGE fluorogram of rhodopsin cysteines titrated with [^{3}H]-DTDA. The extent of derivatization over time is shown in Figure 3B. The data indicate that at saturation rhodopsin had been derivatized with 1.73 ± 0.08 (n = 4) mol of label per mole of protein, consistent with derivatization of the two reactive rhodopsin cysteines. The biphasic titration curve observed with [^{3}H]-DTDA has been seen previously with other reagents and indicates that the two cysteines differ in their reactivity.[16]

To prepare rhodopsin for photo-cross-linking experiments, it was necessary to purify the native protein from excess [^{3}H]-DTDA after derivatization. This was done by derivatizing lauryl maltoside (LM) solubilized rhodopsin while it was bound to an antibody column.[17] The free label in the supernatant was removed by repeatedly washing the protein antibody complex. Following elution of bovine rhodopsin from the column, the protein concentration was determined from the UV-vis absorbance of the chromophore at 500 nm (ϵ_{500} = 40800) and the extent of labeling was measured by determining the tritium content of an aliquot of the solution. These data indicate

† Present Address: Department of Organic Chemistry, Indian Institute of Science, Bangalore, 560 012 India.

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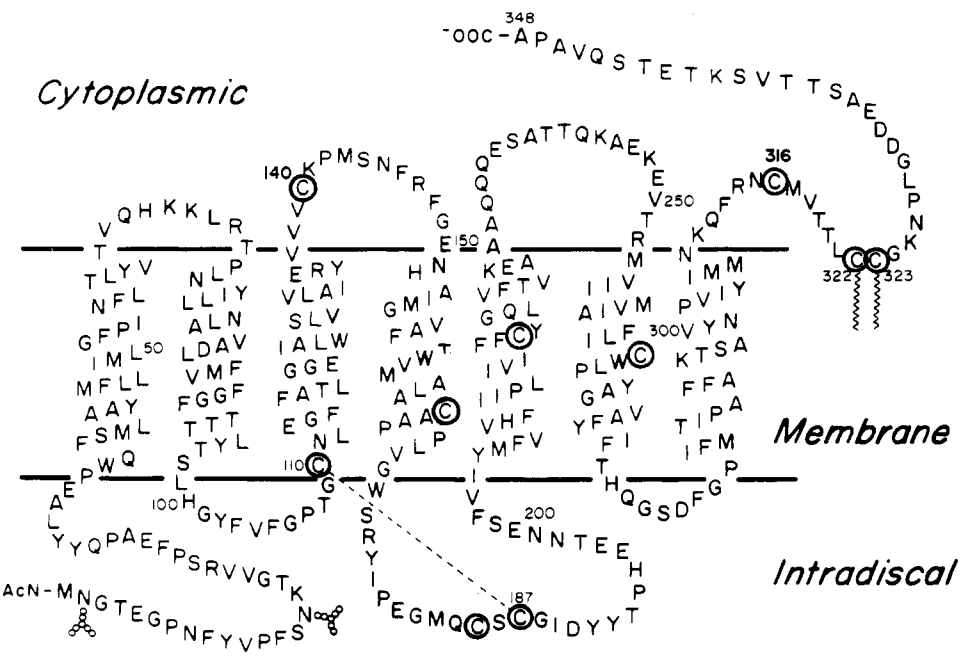

Figure 1. Secondary structure model of bovine rhodopsin. Single letter abbreviations are used for amino acids. The circled amino acids indicate the position of the cysteine residues. The dashed line between Cys-110 and Cys-187 on the intradiscal side shows a disulfide bond. Cys-322 and Cys-323 on the cytoplasmic face are palmitoylated. Residues 140 and 316 have been shown to react with sulfhydryl reagents.

that 1.88 ± 0.17 (n = 3) mol of tritiated diazirine was coupled to each mole of rhodopsin via a disulfide bond.

Rhodopsin derivatized with the photoaffinity label was incubated in the presence or absence of UV light and applied to a reducing SDS–PAGE. Fluorography indicated that in the presence of reducing agent the covalent association of ^{3}H was dependent upon photolysis (Figure 3C). These data demonstrate that the diazarine photoproduct had inserted into the protein via an intramolecular reaction.

Concluding Remarks. In this report we have presented the synthesis of a new sulfhydryl-directed photocross-linking reagent. The reagent has several features which make it generally useful for studying protein/protein interaction. These include (1) the ability to form a cleavable disulfide bond with accessible cysteines and (2) a minimal distance between the diazirine and the disulfide for determining the site of insertion near the disulfide. In addition, the reagent generates the carbene on photolysis and has been synthesized with tritium at a specific activity of 3 Ci/mmol. Finally, DTDA (1) was shown to derivatize the accessible cysteines of rhodopsin and insert into the protein following photolysis.

We are currently using [^{3}H]-DTDA in combination with site-directed cysteine mutants of rhodopsin to target the reagent to the cytoplasmic loops. This allows the diazirine to be linked to new and unique sites in the protein's cytoplasmic domain and has proven useful in identifying the contact points between rhodopsin and transducin.

Experimental Section

General Procedures. All solvents were freshly distilled or were HPLC grade. NMR data were obtained on a Brucker AC 250 or a Varian XL 300. Mass spectral analysis was performed at the Harvard Mass Spectral facility. [^{3}H]-NaB_3H_4 was purchased from NEN-Dupont at a specific activity of 12.6 Ci/mmol. 1D4-Sepharose was prepared as previously described.[18] Rhodopsin was purified from ROS membranes with ConA sepharose as described by Papermaster[19] and Litman.[20] Silica gel flash chromatography purifications were performed on silica gel (230–400 mesh) as described by Still.[21] Prior to purification, all solutions were dried with anhydrous $MgSO_4$ and concentrated by rotary evaporation. In the radiochemical synthesis, concentration of solvent was performed in a conical tube with a stream of argon gas. Photolysis was performed through a water-cooled quartz tube in a Raynoet RPR-100 photoreactor equipped with ten 3500-Å, 24-W lamps.

3-(4-((Acetylthio)methyl)phenyl)-3-(trifluoromethyl)-3*H*-diazirine (3). The diazirine benzyl bromide 2 (250 mg, 0.896 mmol) was dissolved in 10 mL of acetonitrile (CH_3CN) containing potassium thioacetate (173 mg, 1.52 mmol). After 60 min at room temperature with constant stirring, the solvent was removed *in vacuo* and the residue dissolved in 30 mL of ether (Et_2O). The Et_2O was then extracted 3 times with water. The organic layer was dried and concentrated. The product was purified by flash chromatography (hexane:$CHCl_3$ 50/50). TLC analysis of the oil in hexane/$CHCl_3$ (50:50) showed a single component (R_f = 0.8). The thioacetate 3 was obtained in 85% yield (0.762 mol). NMR and IR were performed on the crude material. ^{1}H NMR in $CDCl_3$: δ 2.3, 3H, s; 4.1, 2H, S; 7.1, 2H, d; J = 8.3 Hz, 7.3, 2H, d, J = 8.3 Hz. FT-IR: 1696 cm^{-1}, carbonyl band of the thioacetate.

3-(4-(((4-Nitro-3-carboxyphenyl)dithio)methyl)phenyl)-3-(trifluoromethyl)-3*H*-diazirine (DTDA, 1). The thioacetate diazarine 3 (60 mg, 0.218 mmol) was dissolved in 5 mL of MeOH under argon. Sodium methoxide (NaOMe) (0.4 M, 5 mL) was added to the solution and the reaction was allowed to incubate for 5 min at room temperature at which time 10 mL of 1 N HCl was added to the reaction. The aqueous solution was extracted 3 times with 10 mL of Et_2O and the organic layer was washed once with 10 mL of H_2O and dried. The Et_2O was concentrated and the residue dissolved in 5 mL of 200 mM sodium phosphate (NaPi) buffer (pH = 8.0) and incubated for 30 min with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (151 mg, 0.654 mmol).

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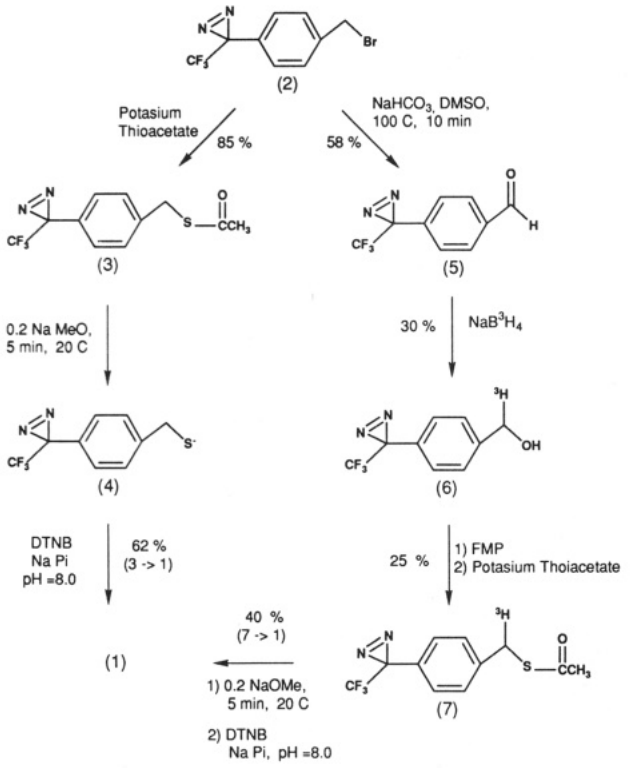

Figure 2. Synthesis of DTDA. The bromobenzyl trifluorodiazirine **2** was synthesized as previously described. The introduction of a thiol group was performed by alkylation with a potassium thioacetate to produce the thioacetate diazirine **3**. Incubation of **3** with 0.2 N NaOMe yielded the free thiol **4** which was converted to the final product DTDA (**1**) with the addition of dithionitrobenzoic acid. To synthesize [^{3}H]-DTDA the benzaldehyde **5** was obtained from **2** by Kornblum oxidation. This was reduced with 1 molar equiv of NaB^3H_4 to generate **6**. The alcohol **6** was activated with 2-fluoro-1-methylpyridinium tosylate (FMP) and reacted with potassium thioacetate to generate the tritiated thioacetate adduct **7**. Cleavage of the acetate with base followed by the addition of DTNB yielded the tritiated [^{3}H]-DTDA (**1**).

The solution was then acidified with 1 N HCl until pH < 1 and extracted into Et_2O. The organic layer was concentrated and purified by flash chromatography ($CHCl_3$/acetic acid (AcOH) 20:1). The purified material showed one UV (+) spot on TLC ($CHCl_3$/AcOH (20/1). The yield was 62% (50 mg, 0.135 mmol) from the thioacetate. ^{1}H NMR in $CDCl_3$: δ 4.0, 2H, s; δ 7.1, 1H, d; J = 10.3 Hz; 7.3, 1H, d, J = 10.3 Hz, 7.55, 1H, dd, J = 11.0, 2.5 Hz; 7.75, 1H, d, J = 2.5 Hz, 7.84, 1H, d, J = 11.0 Hz, 9.35, 1H, S. Mass spectrum: M^-, 428.0003 (calcd 427.9987).

3-(4-(Formylphenyl)-3-(trifluoromethyl)-3*H*-diazirine (5). The benzyl bromide diazirine **2** (331 mg, 1.18 mmol) was added to 10 mL of DMSO which was heated to 100 °C and in which $NaHCO_3$ (1.32 g, 15.7 mmol) had been dissolved. The reaction was incubated for 10 min and then allowed to cool to room temperature and added to 100 mL of ice water saturated with NaCl. The aqueous solution was extracted twice with 50 mL of Et_2O and the organic layer was washed once with 25 mL of $NaHCO_3$ saturated water and once with 25 mL of NaCl-saturated water. The Et_2O was concentrated and purified by flash chromatography ($CHCl_3$/hexane 50/50). The pure product was obtained in 58% yield (146 mg, 0.682 mmol). ^{1}H NMR in $CDCl_3$: δ 7.41, 2H, d; J = 11.0 Hz, 7.95, 2H, d, J = 11.0, 16.1, 1H, S. Mass spectrum: M^+, 215.0000 ($C_9H_6N_2OF_3$).

3-(4-(Hydroxymethyl-*t*)phenyl)-3-(trifluoromethyl)-3*H*-diazirine (6). Reduction of the aldehyde **5** to the alcohol **6** was performed in a conical tube with a screw cap. The [^{3}H]-NaB_3H_4 (500 mCi, 12.6 Ci/mmol, 3.96×10^{-2} mmol) was dissolved in 200 μL of (2-propanol/0.01 N NaOH 70/30) to which was added **5** (2.74 mg, 3.96×10^{-2} mmol) dissolved in 50 μL of (2-propanol/0.01 N NaOH 70/30). The reaction was incubated for 1 h at room temperature, at which time it was quenched with 2 mL of Et_2O and 2 mL of 0.1 N acetic acid. The aqueous layer was washed with Et_2O and the combined Et_2O fractions were washed with 5% $NaHCO_3$ and water. The organic phase was then dried with Na_2CO_4. TLC analysis (CH_2Cl_2/hexane 3/1) followed by fluorography showed one radioactive spot. The yield (30%, 42.4 mCi of tritium) was determined by counting aliquots of the Et_2O layer.

3-(4-((Acetylthio)methyl-*t*)phenyl)-3-(trifluoromethyl)-3*H*-diazirine (7). The organic solvent Et_2O, containing the alcohol **6** (42.2 mCi), was concentrated and the residue dissolved in 100 μL of CH_3CN containing diisopropylethylamine (2.5 mg, 1.92×10^{-2} mmol) and 2-fluoro-1-methylpyridinium *p*-toluenesulfonate (FMP) (5.4 mg, 1.92×10^{-2} mmol). The reaction was incubated for 30 min at which time 100 μL of CH_3CN containing potassium thioacetate as a suspension (2.5 mg, 2.2×10^{-2} mmol) was added. The reaction was incubated for 1 h and then 1 mL of 1 N HCl was added to the solution and the aqueous layer extracted with CH_2Cl_2. The CH_2Cl_2 was dried with Na_2SO_4 and the solvent removed with a stream of argon gas. The residue was

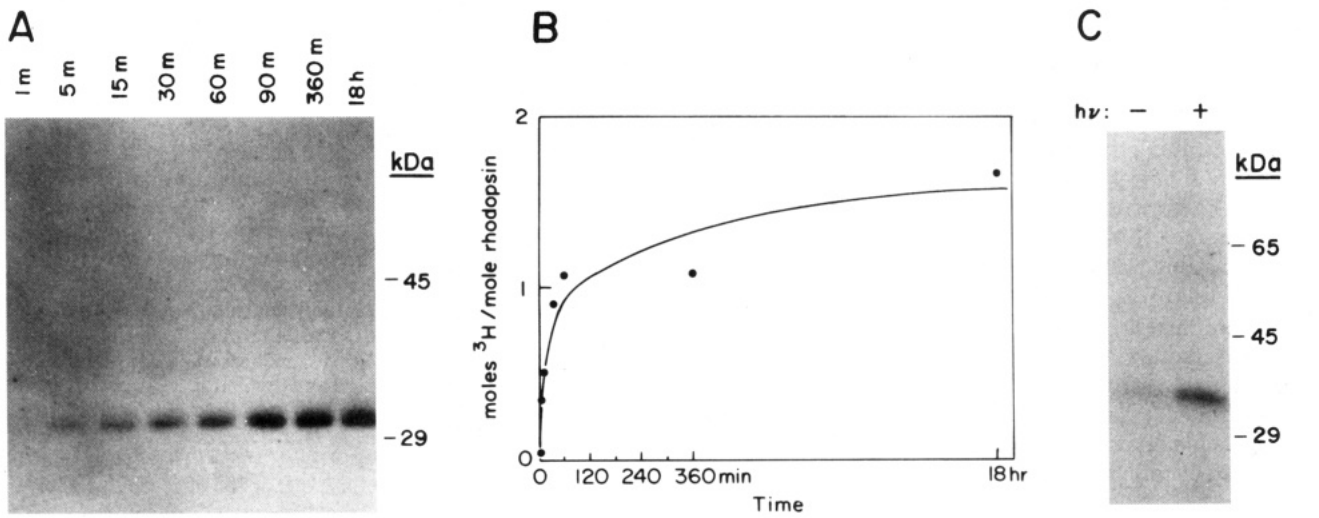

Figure 3. Reactivity of [^{3}H]-DTDA with rhodopsin. The reagent DTDA (**1**) reacts with cysteine residues in proteins to form a disulfide and release 1 equiv of TNB. (A) Fluorogram of rhodopsin cysteines titrated with [^{3}H]-DTDA. The gel was run under nonreducing conditions. (B) Radioactive content of each protein band determined by counting the tritium content of the gel slice. From four separate experiments it was determined that 1.73 ± 0.08 mol of cysteines per mole of rhodopsin had been labeled with [^{3}H]-DTDA. (C) Native rhodopsin purified by 1D4-sepharose following derivatization and incubated in the presence (+) or absence (−) of UV light (λ = 350 nm) prior to reducing SDS-PAGE. Fluorography indicated that photolysis resulted in the label being covalently associated with the protein via the diazirine photoproduct.

spotted on a TLC plate (hexane/$CHCl_3$ 50/50). Overnight autoradiography indicated one major radioactive band. The silica gel corresponding to the tritiated material was removed and the product extracted with CH_2Cl_2. The radioactive product 7 was shown by TLC to comigrate with the nonradioactive thioacetate analog 3 and to separate from the starting benzyl alcohol. Yield of the tritium was determined to be 25.7% (10.4 mCi).

3-(4-(((4-Nitro-3-carboxyphenyl)dithio)methyl-*t*)phenyl)-3-(trifluoromethyl)-3*H*-diazirine (1). The volume of CH_2Cl_2 extracts containing the [^{3}H]-thioacetate (2.8 mCi) was concentrated. NaOMe (50 μL, 0.6 N) was added and the reaction was incubated for 4 min at room temperature. The reaction was neutralized with 15 μL of 2 N HCl and added to 300 μL of methanol containing DTNB (10 mg, 2.52×10^{-4} mol). The solution was then added to 3.5 mL of 200 mM NaPi, 10 mM EDTA, pH 8.0, and incubated for 30 min under argon. Then 2 mL of 2 N HCl was added and the radioactivity extracted 3 times with 2 mL of Et_2O. The volume of Et_2O was decreased with an argon stream and spotted onto a TLC plate which was developed on $CHCl_3$/AcOH 20/1). Following fluorography, the radioactive band was extracted with MeOH. The yield was 40.4% from the thioacetate (1.13 mCi). TLC analysis showed a single radioactive spot which comigrated with the nonradioactive DTDA ($CHCl_3$/AcOH 20/1). The specific activity of [^{3}H]-DTDA was determined from the absorbance at λ = 412 nm following the release of this nitrobenzoic acid in the presence of cysteine. The absorbance was used to measure the number of moles of reagent in a given aliquot. A determination of the tritium in this aliquot was then used to calculate the specific activity (3.0 Ci/mmol).

Radioactive Assay for [^{3}H]-DTDA Titration of Rhodopsin Sulfhydryls. ConA-purified rhodopsin (25 μg, 6.57×10^{-10} mol) was incubated in 600 μL of buffer (0.02% LM, 20 mM NaPi, pH = 8.0, 1 mM EDTA) in the presence of [^{3}H]-DTDA (6.57×10^{-9} mol). Aliquots (2.5 μg, 6.57×10^{-11} mol) were recovered at 1, 5, 15, 30, 60, 90, 360, and 1100 min and added to 10 μL of buffer containing the nonradioactive DTDA (6.57×10^{-8} mol). The aliquots were then subjected to SDS-PAGE and stained with Commassie blue and fluorography was performed. The SDS-PAGE utilized dihydroxylethylenebis(acrylamide) as a cross-linker, allowing the gel to be dissolved with sodium periodate. The radioactive content of the Coomassie-stained bands was then determined by scintillation counting. The data indicated that a saturating 1.73 ± 0.08 (n = 4) mol of label were incorporated per mole of protein.

Derivitization of Rhodopsin on the 1D4 Antibody Column. ConA-purified rhodopsin (25 μg, 6.57×10^{-10} mol) was bound to 1D4-sepharose (350 μL, 10 μg/100 μL capacity) in 20 mM NaPi, pH = 8.0, 1 mM $MgCl_2$, 0.02% LM. After 3 h the 1D4-sepharose was centrifuged and a UV–vis spectrum taken on the supernatant. The spectrum confirmed that no rhodopsin was in the supernatant. The 1D4-sepharose was then incubated at room temperature in the dark overnight with 350 μL of buffer containing [^{3}H]-DTDA (6.57×10^{-9} mol). The free label was then removed by repeated washing of the antibody column and rhodopsin eluted two times with a peptide corresponding to the carboxyl terminal 18 amino acids of rhodopsin. The recovery of rhodopsin was 68 ± 8% (n = 3); 1.88 ± 0.17 (n = 3) mol of [^{3}H] were incorporated per mole of rhodopsin.

Photolysis of the [^{3}H]-DTDA-Labeled Rhodopsin. The labeled rhodopsin was incubated in a buffer (0.02% LM, 10 mM NaPi, pH 7.5, 5 mM $MgCl_2$) and cooled to 4 °C. Photolysis was performed for 2 min and the sample was then removed and subjected to SDS-PAGE in the presence of 5 mM βME. Overnight fluorography of the dried gel was then performed.

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Supplementary Material Available: ^{1}H NMR spectral data for compounds 1 and 5 as well as 2-D ^{1}H NMR for the aromatic region of compound 1 (3 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.