Photoaffinity Labeling Studies of Bacteriorhodopsin with [15-^{3}H]-3-Diazo-4-keto-*all-trans*-retinal†

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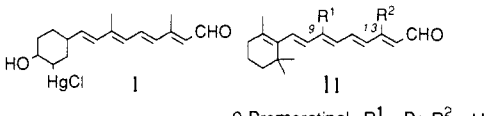
Abstract: Photoaffinity mapping can be used to help clarify the tertiary structures of the retinal proteins bacteriorhodopsin (bR) and rhodopsin (Rh). An efficient photoaffinity labeled retinal analogue, [15-^{3}H]-*all-trans*-3-diazo-4-ketoretinal (specific activity >4 Ci/mmol), has been developed as a probe for clarifying the binding site structure around the chromophoric ionone ring in bR. This analogue is functional (20% of the natural retinal) in bR and can be readily photolyzed, covalently binding the chromophore to amino acids in the receptor site. Enzymatic and chemical cleavages of labeled bR followed by HPLC purification and Edman degradation of the radiolabeled peptide fragments identified radiolabeled amino acids Ala126/Leu127 and Trp137/Trp138. The pattern of the labeled amino acid positions permits predictions of the structure of the retinal binding region in bR.

Bacteriorhodopsin (bR),[1-4] a membrane-bound protein consisting of 248 amino acids,[5,6] is the sole protein found in the purple membrane of *Halobacterium halobium*. It contains *all-trans*-retinal as the prosthetic group through a protonated Schiff base linkage[7,8] to Lys216.[9-11] Irradiation of bR initiates a photocycle leading to proton translocation across the membrane, which provides the energy for ATP synthesis. Tertiary structural studies of this membrane protein have progressed since the seminal electron diffraction measurements by Henderson and Unwin[12] disclosed that bR consisted of a cluster of seven transmembrane α-helices.[6] Additional tertiary structure studies have been performed by computations,[13] photoaffinity labeling,[14] neutron diffraction measurements of bR containing deuterated retinals,[15-17] site-specific mutations,[18] and X-ray measurements of bR containing Hg analogue I and Br analogues II.[19] In the following



Hg-Retinal

9-Bromoretinal R^1 = Br, R^2 = H

13-Bromoretinal R^1 = H, R^2 = Br

we report the synthesis of [15-^{3}H]-3-diazo-4-keto-*all-trans*-retinal (**1**) and results of photoaffinity studies with this chromatophore; the diazo ketone function promises to be a versatile photoaffinity label for retinal protein studies.

Photoaffinity labeling,[20] is a potentially useful tool for tertiary structural studies of proteins, especially membrane proteins such as retinal proteins, which are difficult to crystallize. The technique has been applied to bR previously. Khorana and co-workers incorporated [15-^{3}H]diazirinophenylretinal into bacterioopsin to give a bR analogue (nonfunctional), the enzymatic and chemical degradations of which showed the phenyl ring to be cross-linked to Ser193 and Glu194 in helix F.[14] We have also been exploring the use of 3-(diazoacetoxy)retinals as the photoaffinity label in both bR[21a,b] and rhodopsin.[21c,d] Recent incorporation and cleavage/sequencing results with the functional bR analogue derived from optically active 3(*S*)-([1-^{14}C]diazoacetoxy)-*trans*-retinal showed that the radioactive carbons of the 3-diazoacetoxy moiety, $O^{14}CO^{14}CHN_2$, had inserted into Thr121 and Gly122 in helix D,[22] presumably as a CH_2COOH group. Although the improved method[21b] has facilitated preparation of radioactive retinal diazoacetate, the ^{14}C label suffers from two serious drawbacks: (i) difficulty in manipulation when dealing with sensitive molecules such as retinal, particularly in the micromole range where the diazoacetoxylation yield is at best 20%;[21b] (ii) the [^{14}C]diazoacetoxy group is prepared from [$^{14}C_2$]oxalic acid, the specific activity of which is at the 10 mCi/mmol level. In most cases this activity level is insufficient to perform successive

† Dedicated to the 75th birthday of Professor G. B. Marini-Bettôlo.
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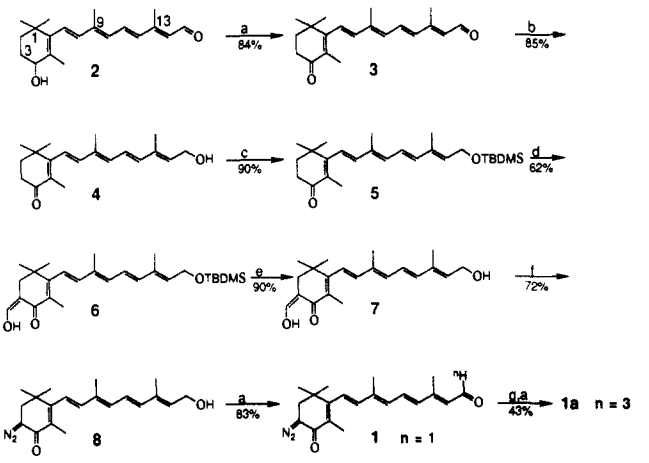

Figure 1. Reagents and conditions: (a) MnO_2, CH_2Cl_2, 0 °C; (b) $NaBH_4$, 1:1 MeOH–THF; (c) (TBDMS)Cl, DMAP, CH_2Cl_2; (d) NaH, EtOH, ethyl formate, THF 0 °C; (e) n-$Bu_4N^+F^-$, THF; (f) MsN_3, NaH, EtOH, THF; (g) NaB^3H_4, 1:1 MeOH–THF, 0 °C, room temperature.

cleavage reactions, e.g., Edman degradation, which are necessary to identify the labeled amino acid(s).[23]

We report the synthesis of [^{3}H]retinal analogue **1**, which yields a bR pigment, activity of which is 20% that of native bR (see below). Besides the >5000-fold increased specific activity of the ^{3}H over the ^{14}C label, which allows observation of the label after multiple Edman degradations, the diazo keto photoaffinity group offers a major advantage in retinal protein studies. Namely, the ease of its synthesis enables the preparation of retinal chromophores that carry this moiety at a variety of sites; this includes the important 11-cis analogues required for studies of the visual pigment rhodopsin.[24]

Chromophore Synthesis. Oxidation of 4-hydroxyretinal (**2**)[25,26] to 4-ketoretinal (**3**) with MnO_2 followed by reduction of the aldehyde in **3** (Figure 1) with $NaBH_4$ gave 4-ketoretinol (**4**). The protected retinyl TBDMS ether **5** with HCOOEt in THF was treated with NaH–EtOH (one drop) in THF to give 3-formyl ketone **6**, deprotection of which afforded formyl alcohol **7**. Treatment of a THF solution of **7** and NaH–EtOH (one drop) with mesyl azide[27,28] yielded crude diazo ketone **8**, MnO_2 oxidation of which gave the diazo ketone **1**, UV (MeOH) λ_{max} 385 nm (ϵ 50 500). [15-^{3}H]-3-Diazo-4-keto-retinal (**1a**) was synthesized by reducing aldehyde **1** in 1:1 MeOH–THF at 0 °C with NaB^3H_4 and subsequent MnO_2 treatment followed by HPLC purification.

Photolysis and Cleavage of Pigment Analogue. Diazo keto retinal **1a** was incorporated into bleached purple membrane[29] to yield the bR analogue, λ_{max} 497 nm. In order to avoid random cross-linking, 1 equiv of protein for 0.7 equiv of retinal was used. The proton pumping of bR derived from diazoretinal **1**, as measured by the pH change induced upon incorporation into the retinal-deficient white mutant and irradiation,[30] was $^1/_5$ that of natural retinal; however, the $^1/_5$ value is only approximate because of the possible occurrence of cross-linking during these measurements; i.e., a 1-min irradiation with a 475-nm cutoff filter led to a reduction in the 497-nm maximum and a rapid decrease in proton-pumping ability. Photolysis at 254 nm of bR analogue reconstituted with diazo ketone **1a** and cleavage with chymotrypsin gave two fragments, C1 and C2,[31] which were readily separable by HPLC.[32] Radioactivity counting of the radioactive fragments indicated 90–95% of the label to be in C1 and a 15% cross-linking. C1 was further cleaved at Met with CNBr to give fragments CNBr-6–CNBr-10 (Figure 2), which were partially separable by ODS HPLC;[31] radioactive counting indicated 65% of the radioactivity to be in CNBr-7 (amino acids 119–144, helices D and E, Figures 3 and 4), while 35% was in uncleaved CNBr-7/CNBr-8

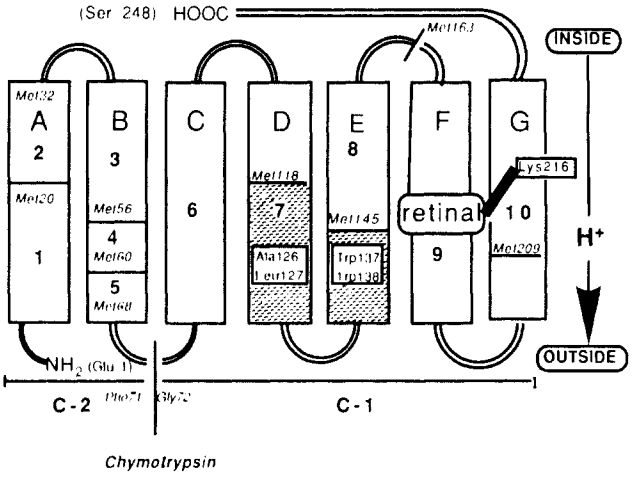

Figure 2. Schematic 2D drawing of the seven α-helices, A–G. The numbers 1–10 represent the CNBr fragments cleaved after each Met residue. The chymotrypsin cleavage site is also shown.

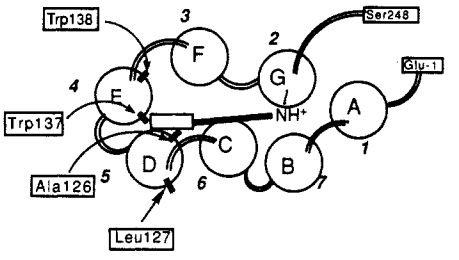

Figure 3. The seven density rods in the 2D electron density map, viewed from the inside of the membrane;[12] numbering of rods follows standard convention. Location of the chromophore is from neutron diffraction[15-17] and X-ray results.[19] Photoaffinity labeled sites are also indicated. The rectangle and bar within the helices denote the ring and polyene moieties, respectively, of the chromophore.

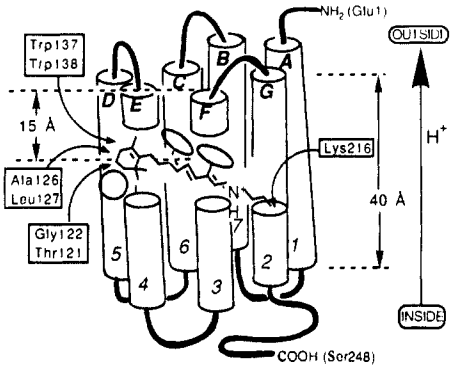

Figure 4. Schematic representation of bR. The 15-Å distance between C-9 and membrane surface is from ref 34. The Hg and Br atoms[19] are represented by hatched circle and ellipses, respectively.

(23) The current photoaffinity studies originally started as a collaboration with Professor G. Khorana's group several years ago. After painstaking preparation, the sample of bR incorporating *dl*-[^{14}C]-3-(diazoacetox)retinal was sent to MIT and submitted to sequencing studies. However, after a chymotrypsin digest and iodosobenzoic acid cleavage, the low radioactivity level of the peptide did not allow identification of the labeled amino acid.

(24) Several diazoketoretinal analogues, including those of 11-*cis*-retinal, have been or are being made for studies of tertiary structure and other purposes.

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(amino acids 119–163), which coeluted with CNBr-9 (helix F).[33] Edman degradation of CNBr-7 followed by radioactive counting of the collected fragments showed 57% and 43% of the radioactivity to be associated with Ala126/Leu127 and Trp137/Trp138, respectively, when adjusted for a ca. 90% repetitive yield for each Edman degradation step. Some or all of the radioactivity of Leu127 and Trp138, i.e., the second amino acids in each pair (Figure 3), could arise from incomplete cleavage/elution following each cycle, or all-trans → 13-cis photoisomerization of this biochemically active probe.

The presence of two insertion sites helices D and E on CNBr-7 agrees with other data[12-16] indicating the ionone ring to be located between these two helices, pointing toward the exterior of the membrane (Figures 3 and 4). The results also agree with the data from two other photoaffinity labeling studies with diazirinophenylretinal and diazoacetylretinal; the labeled sites are in close proximity, suggesting that chromophoric orientations in these analogues are not all that different.

Proton-pumping studies with retinal analogues having C-9 side chains of various lengths containing ω-sulfate groups (which cannot submerge into the helices)[34] showed that *a distance of ca. 15 Å from the membrane surface to C-9 is necessary for the 15-CHO to reach Lys216*; this corresponds to an angle of 20° with the membrane surface. These results are in accord with linear dichroic measurements,[35] which show the polyene to be inclined by 25° with respect to the membrane surface, as well as with an energy-transfer experiment[36] and a second harmonic generation experiment,[37] which suggest that the chromophore is tilted toward the exterior. Figure 4 incorporates results from the present studies and our previous results from photoaffinity labeling[22] and from Hg- and Br-containing retinals,[19] as well as the vast evidence from other laboratories.[6-18,34-37] The 5-, 9-, and 13-methyl groups are depicted as pointing "out" on the basis of our earlier chemical observations;[34] however, this is only tentative because spectroscopic measurements, i.e., linear dichroism[38] and neutron diffraction,[39] lead to the opposite direction.

The diazo keto group offers several advantages as a photoprobe in retinal protein studies. (i) The synthesis of several hundred millicuries is relatively simple. (ii) Efficient incorporation of the radiolabel in the second to the last step permits the specific activity to be maximized, an important aspect for the Edman degradation since it allows one to perform multiple cleavages; e.g., Trp138 is obtained after the 20th cleavage. (iii) The simple diazo ketone functionality can be inserted into a variety of other sites, including a long aliphatic chain attached to the retinal. That the present labeled analogue is functional, although at only 15–20% efficiency, shows the conformational similarity of the diazo keto analogue to native bR; however, the affinity probes necessarily introduce additional steric and electronic effects, an inherent aspect of photoaffinity studies. Usage of analouges of this probe for tertiary structural and other studies of bR and rhodopsin by photoaffinity labelling is ongoing.

Experimental Section

General Procedures. Solvents employed were reagent grade. Anhydrous solvents were dried by distillation from appropriate drying agents (THF, Na/benzophenone; CH_2Cl_2, CaH_2). Chromatography solvents were HPLC grade and degassed under vacuum and sonication. All retinals were synthesized under dim red light. Reactions were followed by thin-layer chromatography on glass plates with UV fluorescent indicator (Analtech Silica Gel GHLF, 250 μm). Flash column chromatography employed 32–63 mesh silica gel from ICN. HPLC was performed on a JA1 liquid chromatograph system equipped with a JA1 UV detector operated at 254 nm, and a YMC-Pack S-5 silica, 25 × 250 mm preparative column. Radioactive retinals were separated on a Beckman 331 HPLC system using a semipreparative Lichrosorb silica 5 μm 10 × 250 mm column, as were peptide separations, using a preparative size-exclusion TSK 3000 column and an analytical ODS Vydac C_{18} 10-μm column with the detector operated at 280 nm. NMR spectroscopy was performed on a Bruker WM-250 spectrometer operating at 250.13 MHz for observation of hydrogen. Only the chemical shifts for the all-trans isomers are reported. FTIR spectra were obtained on a Perkin-Elmer 1600 series spectrometer and UV spectra were obtained on a Perkin-Elmer 320 spectrophotometer. Low-resolution and high-resolution FAB mass spectra were measured on a JOEL JMJ-DX303 HF mass spectrometer using Xe for bombardment gas and glycerol matrix. Scintillation counting was performed on a Beckman LS 3801 scintillation counter using ScintiVerse II as the scintillation cocktail. Edman degradation was carried out on an Applied Biosystem 470A gas-phase sequencer equipped with a Model A PTH analyzer. Protein (7–11 nmol, 2 × 10^6 cpm) was applied to the sequencer, 40% of which was analyzed by the analyzer and the rest of which was collected for radioactive counting. The repetitive yield for each step was 89%. Glassware used for anhydrous conditions was flame-dried and cooled under vacuum immediately prior to use. Reagents were obtained from Aldrich Chemical and were reagent grade.

4-Ketoretinal (3), [9-(2,6,6-Trimethyl-3-keto-1-cyclohex-1-yl)-3,7-dimethyl-2,4,6,8-nonatetraen-1-al]. To *all-trans*-4-hydroxyretinal (**2**)[25,26] (10 g, 33.3 mmol) in CH_2Cl_2 (250 mL) at 0 °C was added MnO_2 (50 g, 575 mmol) and the suspension was stirred for 3 h. After filtration through Florisil, and concentration of the filtrate, the residue was chromatographed (SiO_2, 10–20% EtOAc–hexanes) and crystallized from 10:1 hexane–EtOAc to give 95% *all-trans*- and 5% 13-*cis*-4-ketoretinal **3**: 8.3 g, 27.9 mmol, 84% yield; TLC 1:5 EtOAc–hexanes, R_f = 0.19; FAB-MS (M + 1) 299; UV (MeOH) 373 nm; IR (neat) 1661 (CHO), 1658 (C=O); ^{1}H - NMR ($CDCl_3$) δ 1.18 (s, 1-$(CH_3)_2$), 1.82 (s, 5-CH_3), 1.83 (t, J = 7 Hz, 2-CH_2), 2.04 (s, 9-CH_3), 2.32 (s, 13-CH_3), 2.51 (t, J = 7 Hz, 3-CH_2), 5.99 (d, J = 8 Hz, 14-CH), 6.28 (d, J = 11 Hz, 10-CH), 6.35 (dd, J = 3 Hz, 7,8-CH), 6.44 (d, J = 11 Hz, 12-CH), 7.11 (dd, J = 15, 11 Hz, 11-CH), 10.11 (d, J = 8 Hz, 15-CH).

4-Ketoretinol (4), [9-(2,6,6-Trimethyl-3-keto-1-cyclohexen-1-yl)-3,7-dimethyl-2,4,6,8-nonatetraen-1-ol]. To 4-ketoretinal (**3**; 2 g, 6.71 mmol of 95% all-trans, 5% 13-cis) in 1:1 THF–MeOH (50 mL) at 0 °C was slowly added $NaBH_4$ (70 mg, 1.84 mmol) in 0.1 N aqueous NaOH solution (2 mL). The reaction was warmed to room temperature with stirring for 1 h, diluted with 200 mL of ether and 100 mL of brine, and extracted three times with ether. The organics were dried over anhydrous $MgSO_4$, filtered, concentrated, and purified by chromatography (SiO_2, 20% EtOAc–hexanes) to give keto alcohol **4** (1.82 g, 6.07 mmol, 90% yield) as a mixture of 95% all-trans and 5% 13-cis, which was used in the next step without further purification: TLC 1:1 EtOAc–hexanes, R_f = 0.47; FAB-MS (M + 1) 301; UV (MeOH) 348 nm; FTIR (neat) 3408 (OH), 1649 (C=O); ^{1}H NMR ($CDCl_3$) δ 1.16 (s, 1-$(CH_3)_2$), 1.84 (s, 5-CH_3), 1.84 (t, J = 7 Hz, 2-CH_2), 1.85 (s, 9-CH_3), 1.96 (s, 13-CH_3), 2.48 (t, J = 7 Hz, 3-CH_2), 4.30 (d, J = 7 Hz, 15-CH_2), 5.72 (t, J = 7 Hz, 14-CH), 6.20 (d, J = 16 Hz, 8-CH), 6.21 (d, J = 11 Hz, 10-CH), 6.31 (d, J = 16 Hz, 7-CH), 6.34 (d, J = 15 Hz, 12-CH), 6.58 (dd, J = 15, 11 Hz, 11-CH).

4-Ketoretinyl *tert*-Butyldimethylsilyl Ether (5), [9-(2,6,6-Trimethyl-3-keto-1-cyclohexen-1-yl)-3,7-dimethyl-1-[(*tert*-butyldimethylsilyl)oxy]-2,4,6,8-nonatetraene]. To 4-ketoretinol (**4**; 1.82 g, 6.07 mmol) in CH_2Cl_2 (10 mL) was added (TBDMS)Cl (1.37 g, 9.11 mmol) and DMAP (1.11 g, 9.11 mmol). The reaction was stirred at room temperature overnight and concentrated and the crude retinyl TBDMS ether purified (SiO_2, 5% EtOAc–hexanes) to give retinyl TBDMS ether **5** (2.44 g, 5.89 mmol, 97% yield) as an 11:1 mixture consisting of all-trans and 13-cis isomers: TLC 1:5 EtOAc–hexanes, R_f = 0.49; FAB-MS (M) 414; UV (MeOH) 325 nm; IR (neat) 1660 cm^{-1} (C=O); ^{1}H NMR δ ($CDCl_3$) 0.06 (s, $(CH_3)_2$Si), 0.89 (s, $(CH_3)_3$CSi), 1.16 (s, 1-$(CH_3)_2$), 1.80 (s, 5-CH_3), 1.84 (t, J = 7 Hz, 2-CH_2), 1.85 (s, 9-CH_3) 1.95 (s, 13-CH_3), 2.48 (t, J = 7 Hz, 3-CH_2), 4.33 (d, J = 6 Hz, 15-CH_2), 5.16 (t, J = 6 Hz, 14-C*H*), 6.19 (d, J = 10 Hz, 10-C*H*), 6.26 (d, J = 16 Hz, 8-C*H*), 6.33 (d, J = 16 Hz, 7-C*H*), 6.35 (d, J = 10 Hz, 12-C*H*), 6.55 (dd, J = 10, 11 Hz, 11-C*H*).

3-Formyl-4-ketoretinyl *tert*-Butyldimethylsilyl Ether (6), [9-(2,6,6-Trimethyl-3-keto-4-formyl-1-cyclohexen-1-yl)-3,7-dimethyl-1-[(*tert*-butyldimethylsilyl)oxy]-2,4,6,8-nonatetraene]. To an anhydrous THF solution (5 mL) containing sodium hydride (174 mg, 7.25 mmol) and ethanol (one drop) at 0 °C was added retinyl TBDMS ether (**5**; 1 g, 2.4 mmol) together with ethyl formate (585 μL, 7.25 mmol) in anhydrous THF (3 mL). The reaction was stirred at 0 °C for 1 h and then warmed to room temperature for 2 h followed by addition of water (1 mL) and dilution with ether (100 mL). Water was added, and the organics were

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extracted three times with ether, dried over anhydrous $MgSO_4$, concentrated, and chromatographed (SiO_2, 10% EtOAc–hexanes) to give formyl derivative **6** (660 mg, 1.49 mmol, 62% yield) as a 4:1 mixture of all-trans and 13-cis: TLC 1:5 EtOAc–hexanes, R_f = 0.50; FAB-MS (M) 442; UV (MeOH) 368 nm; IR (neat) 1636 (C=O); ^{1}H NMR δ 0.06 (s, $(CH_3)_2Si$), 0.89 (s, $(CH_3)_3CSi$), 1.10 (s, 1-$(CH_3)_2$), 1.80 (s, 5-CH_3), 1.91 (s, 9-CH_3), 1.96 (s, 13-CH_3), 2.26 (s, 2-CH_2), 4.34 (d, J = 6 Hz, 15-C*H*), 5.65 (t, J = 6 Hz, 14-C*H*), 6.15 (d, J = 16 Hz, 8-CH), 6.21 (d, J = 13 Hz, 10-CH), 6.34 (d, J = 16 Hz, 7-CH), 6.35 (d, J = 14 Hz, 7-CH), 6.59 (dd, J = 11, 15 Hz, 11-C*H*), 7.47 (d, J = 8 Hz, 3-CC*H*OH).

3-Formyl-4-ketoretinol (7), [9-(2,6,6-Trimethyl-3-keto-4-formyl-1-cyclohexen-1-yl)-3,7-dimethyl-2,4,6,8-nonatetraen-1-ol]. To a solution of formyl derivative **6** (660 mg, 1.49 mmol) in anhydrous THF (5 mL) at 0 °C was added 1 M n-$Bu_4N^+F^-$ solution in THF (3 mL, 3 mmol) and the reaction warmed to room temperature for 2 h. Addition of water (3 mL), extraction with ether, drying over anhydrous $MgSO_4$, concentration, and chromatography (SiO_2, 20–50% EtOAc-hexanes) gave retinol derivative **7** (448 mg, 1.37 mmol, 92% yield) as a 4:1 mixture of all-trans and 13-cis: TLC 1:1 EtOAc–hexanes, R_f = 0.23; FAB-MS (M + 1) 329; UV (MeOH) 335 nm; IR (neat) 3394 (OH), 1629 (C=O); ^{1}H NMR ($CDCl_3$) δ 1.09 (s, 1-$(CH_3)_2$), 1.86 (s, 5-CH_3), 1.92 (s, 9-CH_3), 1.97 (s, 13-CH_3), 2.26 (s, 2-CH_2), 4.31 (d, J = 7 Hz, 15-CH_2), 5.73 (t, J = 7 Hz, 14-C*H*), 6.17 (d, J = 16 Hz, 8-C*H*), 6.22 (d, J = 11 Hz, 10-C*H*), 6.35 (d, J = 16 Hz, 7-C*H*), 6.36 (d, J = 15 Hz, 12-C*H*), 6.58 (dd, J = 15, 11 Hz, 11-C*H*), 7.47 (br s, 3-CC*H*OH).

3-Diazo-4-ketoretinol (8), [9-(2,6,6-Trimethyl-3-keto-4-diazo-1-cyclohexen-1-yl)-3,7-dimethyl-2,4,6,8-nonatetraen-1-ol]. To a solution of NaH (88 mg, 3.67 mmol) and EtOH (one drop) in anhydrous THF (6 mL) was added retinol derivative **7** (400 mg, 1.22 mmol) followed by mesyl azide[27,28] (444 mg, 3.67 mmol). After 3 h at room temperature, water was added, the organics were extracted with ether, dried over anhydrous $MgSO_4$, filtered concentrated, and the residue was chromatographed (SiO_2, 50% EtOAc–hexanes) to give the 3-diazo-4-ketoretinol **8** (265 mg, 0.81 mmol, 66% yield) as a 4:1 mixture of all-trans and 13-cis: TLC 1:1 EtOAc–hexanes, R_f = 0.35; FAB-MS (M + 1) 327; UV (MeOH) 362 nm; IR (neat) 3400 (OH), 2077 (diazo), 1655 (C=O); ^{1}H NMR ($CDCl_3$) δ 1.21 (s, 1-$(CH_3)_2$), 1.86 (s, 5-CH_3), 1.92 (s, 9-CH_3), 1.98 (s, 13-CH_3), 2.65 (s, 2-CH_2), 4.30 (d, J = 7 Hz, 15-CH_2), 5.75 (t, J = 7 Hz, 14-C*H*), 6.13 (d, J = 11 Hz, 10-C*H*), 6.15 (d, J = 5 Hz, 8-C*H*), 6.23 (d, J = 5 Hz, 7-C*H*), 6.35 (d, J = 16 Hz, 12-C*H*), 6.69 (dd, J = 16, 11 Hz, 11-C*H*).

***all-trans*-3-Diazo-4-ketoretinal (1). [9-(2,6,6-Trimethyl-3-keto-4-diazo-1-cyclohexen-1-yl)-3,7-dimethyl-2,4,6,8-nonatetraen-1-al].** To 3-diazo-4-ketoretinol (**8**; 265 mg, 0.81 mmol) in CH_2Cl_2 (5 mL) at 0 °C was added MnO_2 (1.33 g, 15.3 mmol). The suspension was stirred at 0 °C for 1 h, filtered through a pad of Florisil, concentrated and chromatographed (SiO_2, 30–50% EtOAc–hexanes), resulting in a 4:1 mixture of all-trans and 13-cis retinals, which were separated by preparative HPLC (SiO_2, 25 × 250 mm, 30% EtOAc–hexanes) to give *all-trans*-diazoketoretinal **1** (218 mg, 0.67 mmol, 87% yield), as orange-brown crystals: TLC 1:1 EtOAc–hexanes, R_f = 0.33; upon heating with vanillin stain a characteristic purple-red color appears; HRFAB-MS 325.1920 (M + 1), calcd 325.1916; UV (MeOH) 385 nm (ϵ 50 500); FTIR (neat) 2071 (diazo), 1656 cm^{-1} (CHO); ^{1}H NMR ($CDCl_3$) δ 1.21 (s, 1-$(CH_3)_2$), 1.93 (s, 5-CH_3), 2.04 (d, J = 1 Hz, 9-CH_3), 2.33 (d, J = 1 Hz, 13-CH_3), 2.67 (s, 2-CH_2), 5.99 (d, J = 8 Hz, 14-C*H*), 6.29 (dd, J = 3 Hz, 7,8-C*H*), 6.31 (d, J = 11 Hz, 10-C*H*), 6.44 (d, J = 15 Hz, 12-C*H*), 7.11 (dd, J = 15, 11 Hz, 11-C*H*), 10.11 (d, J = 8 Hz, 15-C*H*).

[15-^{3}H]-*all-trans*-3-Diazo-4-ketoretinal (1a), (9-(2,6,6-Trimethyl-3-keto-4-diazo-1-cyclohexen-1-yl)-3,7-dimethyl-2,4,6,8-nonatetraen-1-[^{3}H]al). To diazoketoretinal **1** (32 mg, 0.099 mmol) in 1:1 MeOH–THF (5 mL) at 0 °C was slowly added NaB^3H_4 (500 mCi, 22 Ci/mmol, 0.023 mmol) in 0.1 N aqueous NaOH (100 μL) and the resultant mixture was stirred for 1 h at 0 °C and warmed to room temperature. The reaction was diluted with ether (10 mL) and washed with brine and the organics were extracted three times with ether. The ethereal solution was dried over anhydrous $MgSO_4$ and concentrated in vacuo to give 400 mCi of tritiated 3-diazo-4-ketoretinol, which was used directly in the next step. Treatment of tritiated 3-diazo-4-ketoretinol in CH_2Cl_2 (20 mL) at 0 °C with MnO_2 (200 mg, 2.30 mmol), 2 h stirring, filtration through Florisil and HPLC (SiO_2, 10 × 250 mm, 30% EtOAc–hexanes), gave [^{3}H]-*all-trans*-diazoketone **1a** (12 mg, 0.04 mmol, 164 mCi, 43% chemical yield, 33% radiochemical yield, specific activity 4.8 Ci/mmol) as an orange-brown solid, which coeluted with authentic cold diazoketoretinal **1**.

Photolysis and Cleavage of Pigment Analogue. To bleached purple membrane[29] (100 OD, ca. 1500 nmol) in water (100 mL) was added diazoketoretinal **1a** (1000 nmol) in EtOH (100 uL). UV measurements indicated that approximately 70% of bR analogue (λ_{max} 497 nm) was reconstituted within 1 h in the dark and no further increase in absorbance was observed, suggesting that all the retinal **1a** was incorporated. Irradiation of labeled bR (70 OD) at 254 nm, 2 min, 4 °C, led to photolysis of the diazo ketone group (weakening of the 497-nm band). Labeled bR was concentrated by centrifugation (35 000 rpm, 30 min), bleached, and cleaved with chymotrypsin to give two fragments, C1 and C2.[31] After concentration the protein was bleached again, followed by washing and centrifugation three times with water. The protein was resuspended in aqueous 0.1 M NH_2OH (15 mL) and 10% SDS (1 mL) was added. The mixture was stirred for 2 h and the protein was precipitated by addition of MeOH (20 mL) and TFA (5 mg). Centrifugation (15 000 rpm, 10 min) followed by resuspension and centrifugation of the protein with 2:1 MeOH–water to remove free radiolabeled retinals gave a pale yellow protein, which was further purified by size-exclusive HPLC. Fragments C1 and C2 were separated by HPLC (4 × 5 mg dissolved in 250 μL of 7:3 HCOOH–H_2O were injected on a TSK 3000 column eluted with MeOH–$CHCl_3$–H_2O–TFA 5/2/2/0.1; 4 mL/min, 280 nm).[32] Radioactivity counting indicated 90–95% of the label to be in C1 and a 15% cross-linking.

C1 was further cleaved at Met with CNBr (1000-fold excess, 7:3 HCOOH–H_2O, 24 h, dark) to give fragments CNBr-6–CNBr-10 (Figure 2). Separation by ODS HPLC[31] (3 × 2 mg of crude protein in 100 μL of 7:3 HCOOH–H_2O were injected on a Vydac C_{18} column and eluted with 5% HCOOH–H_2O–5% HCOOH–EtOH by a linear gradient from 30% to 100% in 1 h, 2 mL/min) indicated 65% of the radioactivity to be in CNBr-7 (amino acids 119–114, helices D and E, Figure 3); 35% was with CNBr-9 (helix F) and uncleaved CNBr-7/CNBr-8 (amino acids 119–163), which all coeluted.[33]

The proton pumping of bR derived from diazoretinal **1** was measured by following the method of Racker et al.,[30] using a retinal-deficient white mutant and irradiating with a 475-nm cutoff filter. The pH change was $^1/_5$ that of natural retinal; however, the ability to pump protons decreased rapidly after 1 min of irradiation due to photolysis of the diazo ketone functionality.

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