Synthesis of a Photoaffinity-Labeled (11*Z*)-Retinal: Identification of Retinal/Rhodopsin Cross-Linked Sites along the Visual-Transduction Path

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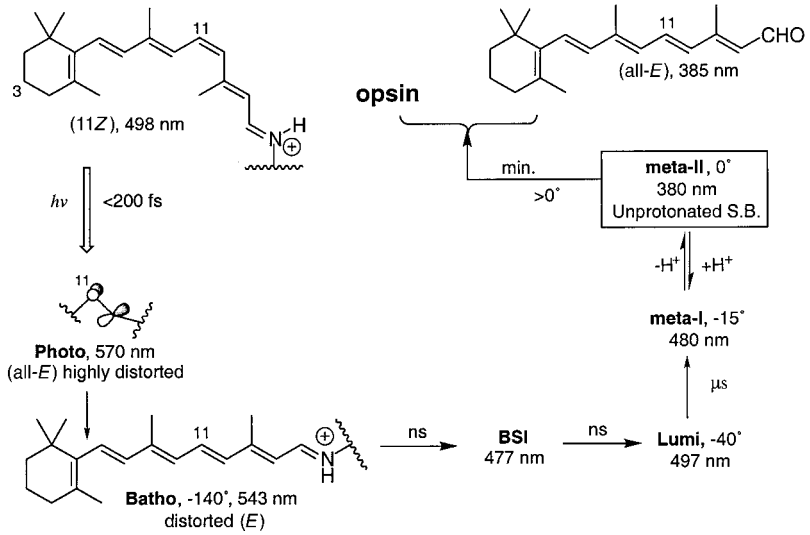
Dedicated to Professor *Albert Eschenmoser* on the occasion of his 75th birthday

The retinal chromophore (11*Z*)-3-diazo-4-oxoretinal (**1**) with two photo-labile moieties has been synthesized by semi-hydrogenation of an 11-yne precursor with activated Zn in aqueous media. Incorporation of **1** into opsin yielded diazoketo rhodopsin (DK-Rh), which, upon bleaching, gave rise to intermediates batho-Rh, lumi-Rh, meta-Rh, and meta-II-Rh corresponding to those of native Rh but at lower temperatures. Photoaffinity labeling of DK-Rh and these bleaching intermediates showed that the ionone ring cross-linked to Trp265 of helix F in DK-Rh and batho intermediate, and to Ala169 of helix D in lumi, meta-I, and meta-II intermediates. These results demonstrate the occurrence of large conformational changes along the visual transduction path, which, in turn, is responsible for activation of the G-protein.

Introduction. – Rhodopsin (Rh) is a seven-transmembrane α-helical G-Protein-Coupled Receptor (GPCR) composed of 348 amino acids and the chromophore, (11*Z*)-retinal, which is bound to Lys296 as a protonated *Schiff* base [1][2]. It is found in the outer segment of vertebrate rods, and is the photoreceptor that mediates dim vision. Visual transduction is triggered by photons which isomerize the (11*Z*)-chromophore to (all-*E*); this isomerization triggers a chain of conformational changes in the opsin, which induces an enzymatic cascade leading to vision [2–4]. *Scheme 1* depicts the intermediates present in the visual transduction process of Rh, identified by flash photolysis and various low-temperature spectroscopic measurements [4][5]. Light irradiation of Rh results in (11*Z*) → (11*E*) isomerization of the chromophore. This yields photo-Rh (femtosecond process), which is converted into batho-Rh, a primary product that can be sequestered at $-140°$; in contrast to Rh, λ_{max} 500 nm, batho-Rh absorbs at 543 nm and is considered to adopt a highly-strained (11*E*)-double bond [1][3]. As shown in *Scheme 1*, batho-Rh relaxes thermally to further photo intermediates, *i.e.*, lumi-Rh, that is in equilibrium with the blue-shifted intermediate, and then to meta-I-Rh and meta-II-Rh.

We have been interested in tracing the path of photo-isomerization and to identify the relative position of the chromophore with respect to Rh at each photo-intermediate. Results to date have shown that the C(3) of the ionone ring is in close contact with helix F of Rh in the dark prior to photo isomerization [4][5]. However, the contacts made by the chromophore after light activation, which subsequently lead to visual transduction, remain to be clarified. Recent site-directed spin labeling of rhodopsin mutants (and disulfide cross-linking) [6] have demonstrated that movements

Scheme 1. *The Photo-Isomerization of Rh*. Initially, the (11*Z*)-double bond is isomerized to a highly distorted (*E*)-intermediate (photo-Rh), which cannot be isolated. The batho-Rh, assumed to be a distorted (11*E*)-conformer, can be trapped at $-140°$ and, upon warming to $-40°$, is converted to lumi-Rh. A blue-shifted intermediate is in thermal equilibrium with the lumi-Rh intermediate but is not observed under cryogenic conditions; however, it can be observed with flash photolysis at ambient temperatures [25]. Meta-I-Rh is sequestered at $-15°$, which is in equilibrium with meta-II-Rh at 0°. Therefore, it is possible to trap each intermediate at specific temperatures and study the orientation and movement of the chromophore with respect to the protein. Photoaffinity label **1** (*cf. Scheme 3*) was synthesized to track the path of the β-ionone ring along the visual transduction path.



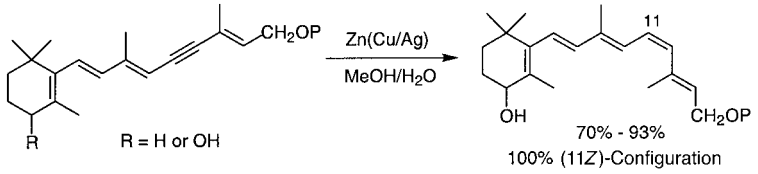
of helix F and C are involved in the light-activation process. This scheme has been further corroborated by *Sakmar* and co-workers, who have demonstrated that the relative movements of helices C and F is required for activation of the GPCR [7–9].

Very recently, we reported the first experimental results that define the positioning and movement of the chromophore relative to the opsin along the transduction pathway [10]. This is critical for understanding Rh conformational changes triggered by the photo-isomerization, since it is these changes that eventually lead to the cascade of enzymatic reactions at the meta-II-Rh stage. By performing photoaffinity labeling with a photo-activatable Rh analog and identifying the cross-linked amino acids at respective intermediates, we have been able to trace changes in retinal/opsin interactions that accompany visual transduction. We have used DK-Rh (diazoketo-Rh) generated with (11*Z*)-3-diazo-4-oxoretinal (**1**; *cf. Scheme 3*), a chromophore with two photoactive moieties, the diazo-keto group activated by 254 nm light and the 11-ene moiety activated by 480 nm light. Cross-linking was first performed with restive Rh at $-196°$. Each transduction intermediates were then selectively populated and trapped by raising the temperature from the DK-batho-Rh stage, *i.e.*, batho-Rh (at $-196°$), lumi-Rh ($-80°$), meta-I-Rh ($-40°$), and meta-II-Rh (0°). The temperature at which each photo intermediate is populated was determined previously for DK-Rh [10]. Photo-cross-linking, proteolytic digestion, and sequencing of cross-linked

peptides performed at each intermediate determined the location of the chromophore β-ionone ring within the protein binding site. Herein, we report the synthesis of (11*Z*)-3-diazo-4-oxo-retinal (**1**), the biochemical protocols for photoaffinity labeling of rhodopsin, and isolation of photo-cross-linked peptide fragments.

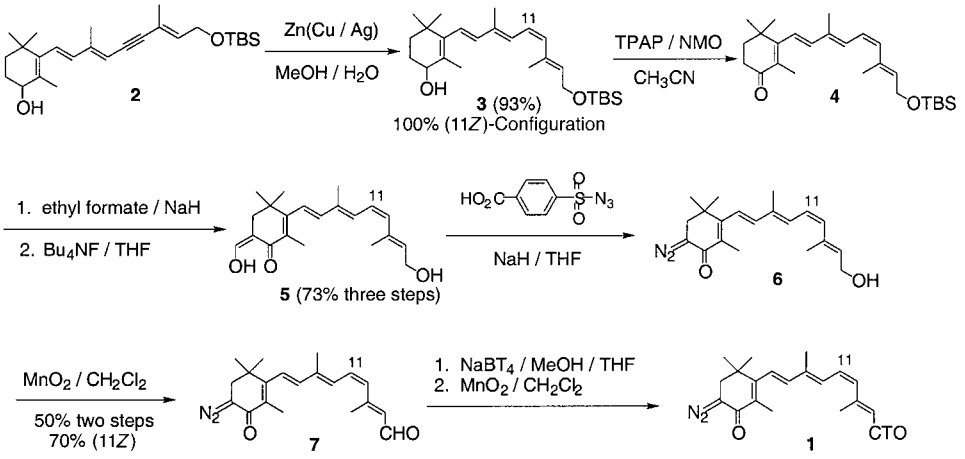
Results and Discussion. – The syntheses of (11*Z*)-retinal analogs are complicated by their unusual instability to light and temperature, and facile isomerization under conditions which most (*Z*)-double bonds survive; this is particularly the case with **1**, since it contains the additional photolabile diazo-keto moiety. The preparation of the labile **1** was first achieved by isomerization of the (all-*E*)-3-diazo-4-oxoretinal analog with retinochrome, the isomerase present in cephalopod visual cells [11][12]. Although the isomerization proceeded efficiently, and retinochrome could be recycled, this chemoenzymatic preparation was not suited for securing the quantity needed for the planned photo-cross-linking studies. However, we recently managed to devise a general synthetic method for preparation of various (11*Z*)-retinal analogs *via* semi-hydrogenation of 11-yne precursors with activated Zn (*Scheme 2*) [13]. Application of this protocol in the present studies was critical in achieving the synthesis of photoaffinity analog **1**.

Scheme 2. *Semi-Hydrogenation of 11-Yne Precursors of (11Z)-Retinal Analogs.* Most other semi-hydrogenation methods fail to deliver the desired compound, usually because of over-hydrogenation of tetrasubstituted C=C bonds [13].



Synthesis of the 11-yne precursor **2** has been demonstrated previously [13]. The crucial semi-hydrogenation of the acetylene is mediated by activated Zn powder in aqueous MeOH to secure the protected (11*Z*)-retinol analog **3** with high stereoselectivity (*Scheme 3*). Oxidation of **3** to yield the (11*Z*)-4-oxo-retinol analog **4** was attempted with activated MnO_2, CrO_3/hexamethylphosphoric triamide (HMPA), *Swern*, *Dess-Martin*, and DMSO/SO_3/pyridine. However, tetrapropylammonium perruthenate (TPAP)/*N*-methylmorpholine *N*-oxide (NMO) oxidation of **3** was superior in maintaining the (11*Z*)-configuration and delivering **4** in good yield. Formylation of the resulting ketone **4** with HCOOEt, and deprotection of the (*tert*-butyl)dimethylsilyl (TBS) ether yielded **5** in good overall yield. Diazotization of the formylated β-ionone ring with (4-carboxybenzene)sulfonazide afforded the diazoketone-containing compound **6**. Due to the instability of **6**, it was oxidized with MnO_2 immediately to secure product **7**. The tritium ([$^3H_1$]) label was introduced by reduction of aldehyde **7** with $NaBT_4$ (222.3 mCi/mmol), which was immediately reoxidized with MnO_2. The final compound **1** was purified on normal-phase HPLC (AcOEt/hexane 1:3). Starting with 100% (11*Z*)-isomer after semi-hydrogenation of **2**, we were able to obtain the final product with 70% (11*Z*)-configuration. It is desirable to undertake the semi-hydrogenation of the 11-yne bond as late as possible within the synthetic scheme

Scheme 3



in order to reduce the possibility of isomerization. In this case, if the reduction was performed at any of the later synthetic stages, the yields were either low, or desired products were not obtained.

Incubation of HPLC-purified retinal analog **1** with bleached rod outer segment (ROS) membranes regenerated diazoketone-rhodopsin (DK-Rh). As depicted in *Fig. 1*, it gave a Rh analog that absorbed maximally at 467 nm, in contrast to the native chromophore, λ_{max} 500 nm. The UV indicated that full regeneration could be attained after 10 min, and that the chromophore is not displaced by addition of (11*Z*)-retinal. The CD spectrum of the regenerated pigment exhibited two positive peaks at 306 and 462 nm, closely matching the β and α peaks at 337 and 480 nm, respectively, of native Rh [14]. The rapid regeneration and spectroscopic data indicate that the overall conformation of DK-Rh closely matches that of the native pigment.

The latter DK-Rh sample prepared in 67% glycerol was subjected to the selective photo-isomerization and photo-cross-linking conditions. The glycerol functions as antifreeze, and provides a translucent and clear film of the protein solution necessary for efficient light penetration through the sample. Protocols were necessary for the isolation of the cross-linked protein from the glycerol solution, cyanogen bromide (CNBr) cleavage of the protein, and isolation of the cross-linked peptides. Recovery of rhodopsin from 67% glycerol solution proved to be problematic. A variety of isolation techniques such as precipitation with trichloroacetic acid (TCA), acetone/EtOH, and $(NH_4)_2SO_4$, dialysis, filtration with molecular cutoff membranes (*amicon*), and gel-sieve chromatography were attempted. In most cases, we were unable to recover a sufficient amount of the protein. In cases such as TCA precipitation, in which the protein was isolated in good yields, the isolate was soluble only in trifluoroacetic acid (TFA), and thus could not be derivatized (capping of cysteines) in the appropriate solvents prior to CNBr cleavage. This is probably due to the fact that Rh is a highly hydrophobic membrane-bound protein. The latter isolations seem to strip away the membrane and leave the protein unsolubilized.

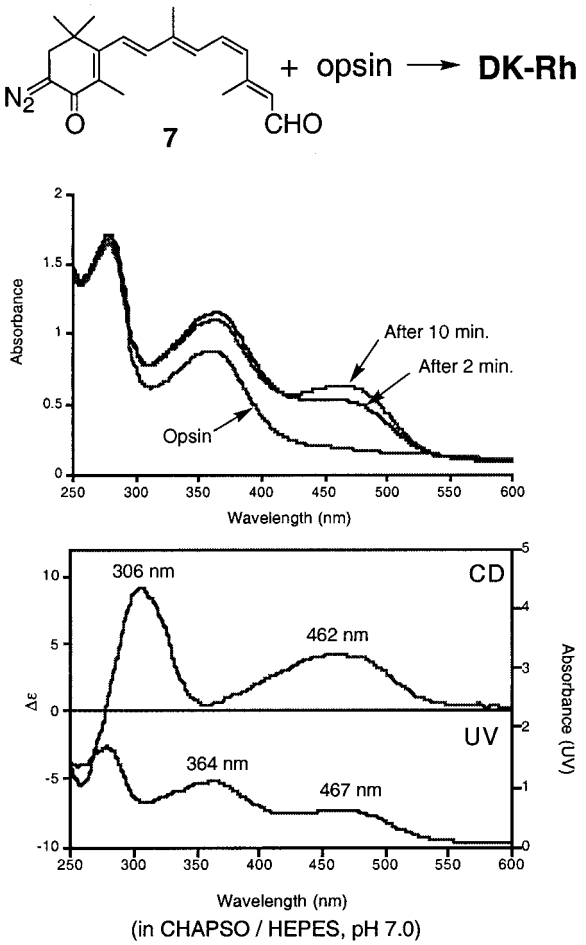


Fig. 1. *The incorporation of* **7** *into opsin to yield DK-Rh*. The newly bound pigment is not displaced by addition of (11*Z*)-retinal. UV/VIS Spectrum of DK-Rh exhibits λ_{max} at 467 nm. The CD with characteristic α- (462 nm) and β-bands (306 nm) is close to that of Rh (480 and 337 nm), indicating that binding of the photoaffinity retinal analog **7** is similar to that of native (11*Z*)-retinal.

To overcome the latter problem, it was necessary to perform all chemical manipulations prior to protein precipitation and digestion. *Scheme 4* depicts the sequence of steps used to isolate photo-cross-linked Rh dissolved in glycerol. The cysteines in Rh were capped by addition of Bu_3P and 4-vinylpyridine along with PrOH (used to better solubilize the reagents). Sonication of the latter solution for 1.5 h was sufficient, and the protein was precipitated with TCA, centrifuged, and washed with AcOEt and acetone. The protein pellet was then dissolved in 75% TFA. Addition of 40 equiv. of CNBr initiated the peptidic cleavage, and after 21 h the cleaved fragments were obtained by removing the TFA/CNBr solution under reduced pressure (*speed-vac*). The remaining residue was then dissolved in a mixture MeCN/TFA/H_2O 1:1:3.

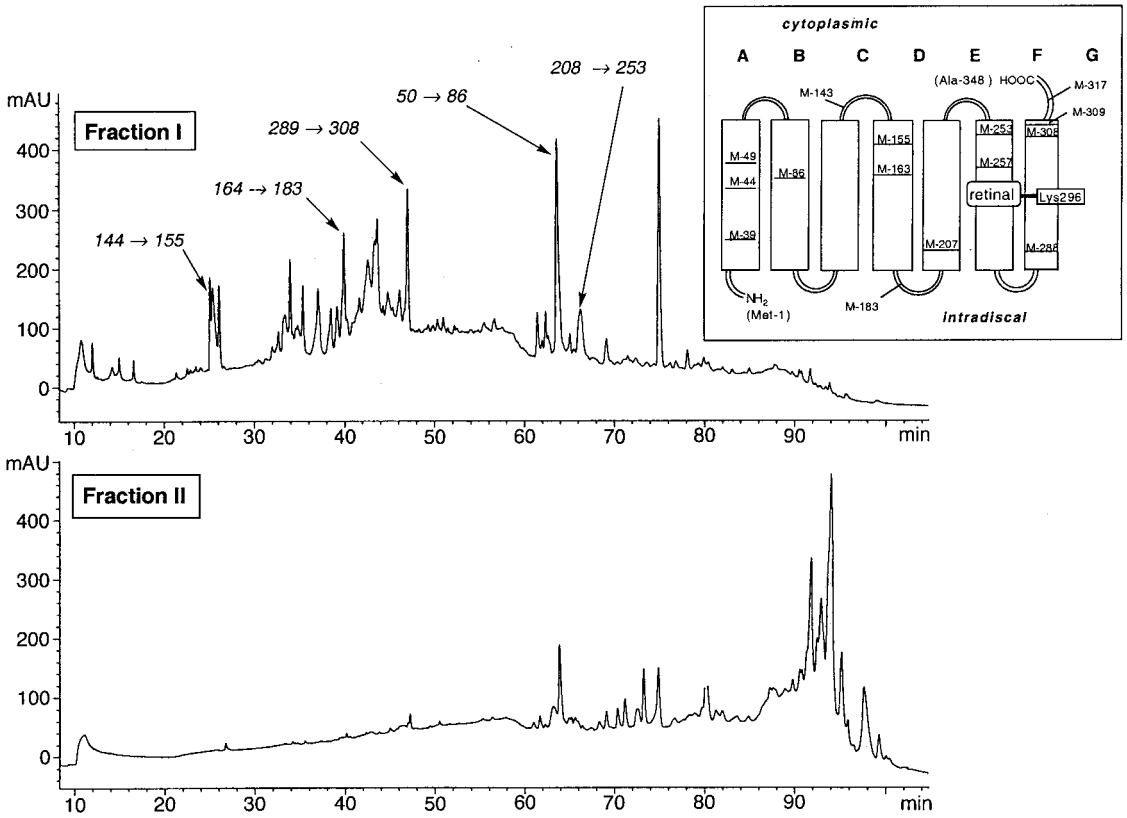


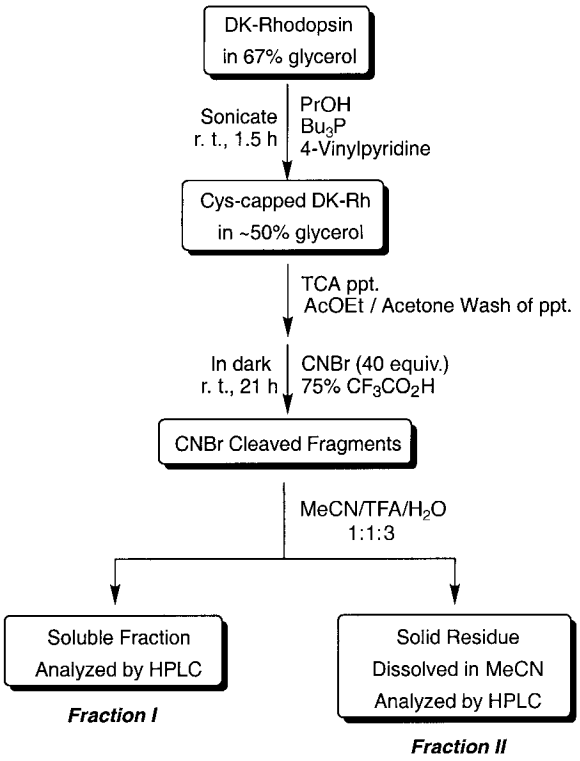
Fig. 2. *HPLC Traces for Fraction I and II*. A gradient system consisting of buffer *A* (0.06% CF_3COOH (TFA) in H_2O) and buffer *B* (80% MeCN, 0.052% TFA in H_2O) (0 → 2 min, 100% buffer *A*; 2 → 82 min, 100% buffer *A* → 30% buffer *A*; 82 → 92 min, 30% buffer *A* → 100% buffer *B*; 92 → 120 min, 100% buffer *B*; monitored at 215 nm) was used to separate the peptides. Five peaks were collected at random from *Fraction I*, and their masses were analyzed by MALDI-TOF. The observed masses matched the indicated peptide fragments expected for CNBr cleavage of Rh. *Insert:* The position of methionines in Rh are indicated, which correspond to the expected sites of CNBr cleavage.

The peptides soluble in this solvent system were separated from the insoluble fraction by centrifugation and analyzed directly by HPLC (labeled as *Fraction I*). The solid residue (*Fraction II*) was dissolved in MeCN and was also analyzed by HPLC.

As can be seen from the HPLC traces (*Fig. 2*), *Fraction I* contains early-eluting peaks as compared to *Fraction II*, which contains later-eluting peaks. This indicates a rough separation of peptidic fragments based on overall polarity into the two different fractions; *i.e.*, more hydrophilic fragments are found in *Fraction I*, whereas more hydrophobic fragments are found in *Fraction II*. This greatly simplified the protocols since, typically, the hydrophobic fragments can cause aggregation and reduce the resolution of the isolated peaks.

With a functional isolation and digestion protocol at hand, photoaffinity studies were undertaken. Initially, the resting state of the pigment was investigated by keeping

Scheme 4. *Chemical Derivatization of Rh*. The protein pellet was washed and subsequently dissolved in TFA and cleaved with CNBr. The cleaved peptidic fragments were then segregated based on their polarity by dissolving the more hydrophilic peptides in MeCN/TFA/H_2O 1:1:3 to yield *Fraction I*. The peptides insoluble in *Fraction I* were dissolved in MeCN (hydrophobic peptides).



the DK-Rh glycerol solution at $-196°$ in complete darkness and irradiating the sample with a 254-nm UV source. The cross-linked protein was pyridylethylated in the glycerol, precipitated with TFA, and cleaved with CNBr. The cleaved peptidic fragments were then fractionated into two as described above, and both fractions were analyzed by reversed-phase HPLC. Each peak isolated by HPLC was checked for radioactivity, and the radiolabeled peak(s) were sequenced by *Edman* degradation. The labeled amino acid was identified as Trp265 in fragment 13 resulting from CNBr cleavage, *i.e.*, CN-13 (Val258 to Phe287) of helix F (*Fig. 3*).

To achieve photoaffinity labeling at the batho-Rh stage, the glycerol DK-Rh solution was irradiated with 480 nm light at $-196°$ in order to populate the batho stage. The photo-isomerized sample was then subjected to photo-cross-linking conditions (254 nm, $-196°$), and the labeled amino acid was determined as described above. Cross-linking indeed occurred at $-196°$ and again yielded clear-cut results, namely, cross-link occurred solely at Trp265 (*Fig. 3*). This is the first conclusive experimental evidence showing that interaction between the β-ionone ring moiety and the opsin hardly changes at the batho stage [15], for which an (all-*E*) chromophoric geometry

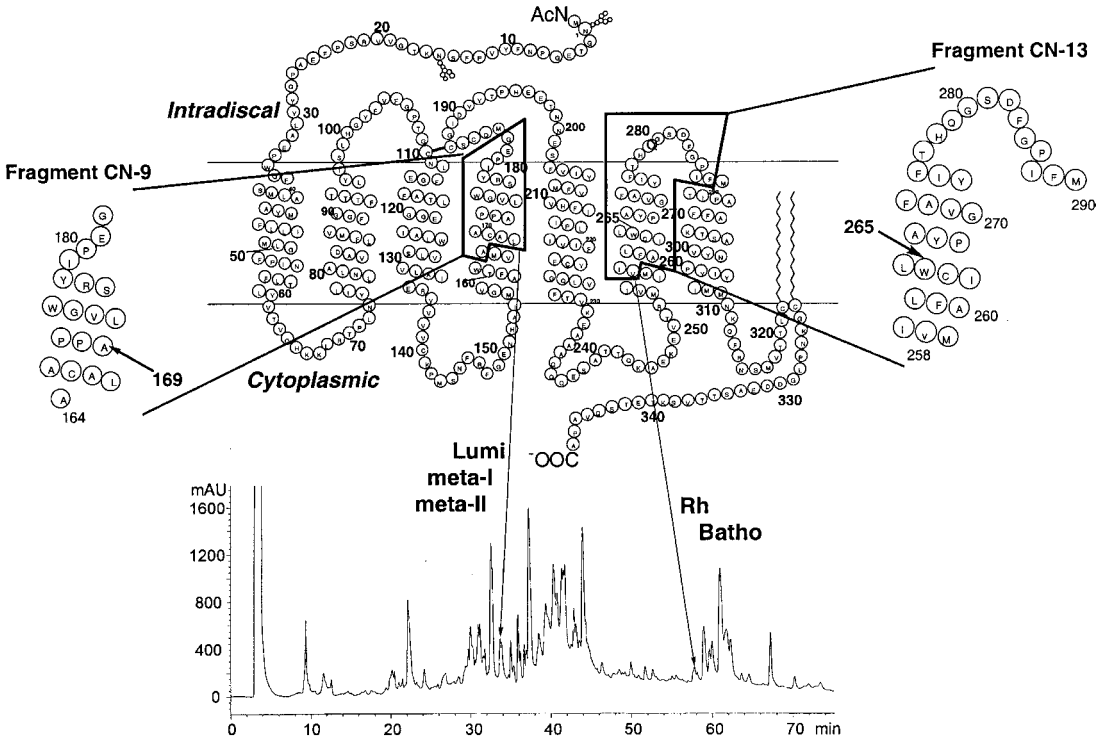


Fig. 3. *DK-Rh and other cross-linked photo-intermediates.* The cross-linked proteins were digested, fractionated, and analyzed by HPLC. One-minute aliquots were collected in silanized glass tubes. The radioactive fractions were identified by liquid-scintillation counting, and the peptide and cross-linked amino acids were identified by amino-acid sequencing. All of the radioactive peptidic fragments were found in *Fraction I*. DK-batho-Rh photo-cross-linked to Trp265 (helix F), the same amino acid which also photo-cross-linked in DK-Rh. DK-lumi-Rh, DK-meta I-Rh, and DK-meta II-Rh cross-linked to Ala169 (helix D). The arrows point to the peptides with the highest radioactivity obtained in each photoaffinity-labeling experiment. In most cases, these were the only 'hot' fragments. However, occasionally other peptidic fragments contained some radioactive counts. Upon analysis of these minor fragments, it was shown in all cases that they contain the same amino-acid sequences as the major 'hot' fragments, but are different as the result of either incomplete digestion or oxidation of some of the amino-acid side chains.

had been suggested by other spectroscopic means [16–21]. The present photo-cross-linking results together with the NMR and vibrational-spectroscopic data demonstrate that the $(11Z) \rightarrow (11E)$ isomerization occurs with minimal movement of the β-ionone ring or minimal changes in the ligand/receptor (opsin) interaction, thus leading to the very large strain. The conversion of Rh to batho-Rh gives rise to a large bathochromic shift, which is accompanied by inversion of the positive CD 480-nm α band in Rh to an intense negative CD band at 520 nm in batho-Rh [1][2]. These changes could possibly be accounted for by the high strain, especially in the '(E)'-11-ene of the chromophore/protein complex estimated to be 30–36 kcal/mol of the photon energy absorbed by Rh [22–24].

The protocols for photoaffinity labeling of further intermediates were identical except for the temperature at which each state was populated. After cooling the

glycerol DK-Rh solution to $-196°$ in complete darkness, it was photo-isomerized to batho-Rh by irradiation with 480-nm light. The protein solution was then warmed in complete darkness to the appropriate temperatures to populate the specific photo intermediates, *i.e.*, $-80°$ for lumi-Rh, $-40°$ for meta-I-Rh, and $0°$ for meta-II-Rh. The solutions were then cooled back to $-196°$ to freeze the protein conformation, and then irradiated with 254-nm light to perform the photo-cross-linking. Photolysis of native Rh at room temperature forms an intermediate BSI (blue-shifted intermediate) that is in equilibrium with batho-Rh before decaying to form lumi-Rh [25][26]. However, it was predicted that BSI would not be observable in Rh photolysis performed at temperatures below $-100°$ [25], and as this was the case in the present low-temperature experiments, BSI will not be discussed here.

The amino acid labeled in the lumi stage was identified in fragment CN-9 (Ala164-Gly182) of the D-helix as Ala169 (*Fig. 3*). There was no evidence for any labeling in fragment CN-13, which was labeled in DK-Rh and in DK-batho-Rh. Therefore, the β-ionone ring is stationary during the Rh to batho-Rh transition, but the batho to lumi-Rh transition is accompanied with a large β-ionone ring movement. This results in a substantial blue shift (543 nm $\rightarrow$ 497 nm), which can be rationalized by chromophoric movements as it relieves the torsional strain. Even more intriguing is the site of lumi cross-linking. According to the 3D models for restive Rh, helix D does not appear to be close to the position occupied by the isomerized retinal β-ionone ring [27–30]. The cross-linked amino acid in the two intermediates after lumi-Rh, *i.e.*, meta-I-Rh and meta-II-Rh, is again only Ala169. These results suggest that a large protein conformational change must occur in order to move helix D close enough to photo-cross-link with the chromophore.

In summary, we report a successful chemical synthesis of (11*Z*)-3-diazo-4-oxoretinal (**1**). This was accomplished through the semi-hydrogenation methodology developed for reduction of 11-yne precursors of (11*Z*)-retinal analogs. Binding of **1** with opsin proceeded smoothly; however, new protocols were necessary in order to re-isolate the protein from the glycerol solution. Utilizing a crude fractionation, we were able to separate the more hydrophobic fragments away prior to HPLC analysis. Radiolabeled peptides were isolated, and the cross-linked amino acid was determined by *Edman* degradation. Our data have demonstrated that the chromophore does not move with respect to Rh (other than (*Z*)/(*E*) isomerization of the 11-ene) at the batho stage. However, a major conformational change occurs at the batho-Rh to lumi-Rh stage, which induces subsequent conformational changes of the pigment, leading to visual transduction. To the best of our knowledge, this is the first tracing of GPCR substrate/helix movements along the signal-transduction path.

Experimental Part

General. All the commercially purchased chemicals were obtained from *Aldrich*. Radioactive $NaBT_4$ was purchased from *NEN Life Sciences*. The solvents used in the reactions were freshly distilled to dryness and used under an Ar atmosphere. The reactions of compounds with more than 3 conjugated C=C bonds were performed in the dark room with minimal red lighting (photographic safety lamps). (11*Z*)-Retinoids are unstable compounds and must be stored in absolute darkness. They can easily isomerize in the presence of trace acids. We have found that they store very well as frozen solutions of benzene at $-78°$. *Edman* degradation was performed at Columbia University, Medical Center. Flash column chromatography (FC): *ICN* silica gel (32–63 mesh). A *Hewlett Packard 1100* HPLC system equipped with column-temp. compartment and a diode-array detector was

used for separation of peptidic fragments. ^{1}H-NMR Spectra: *Bruker DMX 500* or *400* MHz instrument, the residual protic solvent ($CDCl_3$ or C_6D_6) as internal reference. ^{13}C-NMR Spectra: at 75 MHz on a *Bruker DMX 300* instrument. Low-resolution and high-resolution (HR) FAB-MS: *JEOL JMS-DX303 HF* mass spectrometer, with a glycerol matrix and Xe ionizing gas.

*3-[(1*E*,3*E*,5*Z*,7*E*)-9-[(*tert*-Butyl)dimethylsilyloxy]-3,7-dimethylnona-1,3,5,7-tetraenyl]-2,4,4-trimethylcyclohex-2-en-1-ol* (**3**). Activated Zn dust was prepared as described by *Boland et al.* [31]. Ar was bubbled through a suspension of Zn dust (10 g) in distilled H_2O (60 ml) for 15 min. $Cu(OAc)_2$ (1 g) was added, flask was immediately sealed, and the mixture was stirred vigorously for 15 min. $AgNO_3$ (1 g) was then added (exothermic), and the mixture was stirred for 30 min. The activated Zn was then filtered and was washed with H_2O, MeOH, acetone, and Et_2O, successively. The moist activated Zn was immediately transferred to the reaction flask containing solvents (H_2O (20 ml) and MeOH (20 ml)). *3-[(1*E*,3*E*,7*E*)-9-[(*tert*-Butyl)dimethylsilyloxy]-3,7-dimethylnona-1,3,7-trien-5-ynyl]-2,4,4-trimethylcyclohex-2-en-1-ol* (**2**; 170 mg, 0.41 mmol) was added to this mixture, and the mixture was stirred at r.t. in the dark for 21 h. The Zn dust was filtered through *Celite* with Et_2O and H_2O, and the org. material was washed with sat. NaCl soln. Product was then dried (Na_2SO_4) to give **3** (93%) with 100% (*Z*)-stereoselectivity. ^{1}H-NMR (500 MHz, $CDCl_3$): 0.08 (*s*, 6 H); 0.91 (*s*, 9 H); 1.01 (*s*, 3 H); 1.04 (*s*, 3 H); 1.43 (*m*, 1 H); 1.64 (*m*, 1 H); 1.70 (*m*, 1 H); 1.83 (*s*, 3 H); 1.84 (*s*, 3 H); 1.88 (*m*, 1 H); 1.92 (*s*, 3 H); 4.00 (*t*, $J = 4.6$, 1 H); 4.32 (*d*, $J = 6.3$, 2 H); 5.63 (*t*, $J = 6.2$, 1 H); 5.89 (*d*, $J = 11.7$, 1 H); 6.13 (*s*, 2 H); 6.32 (*t*, $J = 11.9$, 1 H); 6.57 (*d*, $J = 12.0$, 1 H). ^{13}C-NMR (75 MHz, $CDCl_3$): −5.1; 12.2; 17.2; 18.3; 18.6; 26.0; 27.5; 28.5; 29.1; 34.5; 34.8; 60.4; 70.2; 124.4; 125.7; 127.4; 129.5; 132.0; 133.5; 133.9; 136.3; 139.1; 142.0. HR-MS: 416.3121 ($C_{26}H_{44}O_2Si^+$; calc. 416.3111).

*3-[(1*E*,3*E*,5*Z*,7*E*)-9-[(*tert*-Butyl)dimethylsilyloxy]-3,7-dimethylnona-1,3,5,7-tetraenyl]-2,4,4-trimethylcyclohex-2-en-1-ol* (**4**). Solid tetrapropylammonium perruthenate (TPAP; 0.04 equiv.) was added in one portion to a stirred mixture of **3** (142.0 mg, 0.34 mmol), 4-methylmorpholine *N*-oxide (NMO; 60.9 mg, 0.52 mmol), and activated 4-Å molecular sieves (500 mg/mmol) in MeCN (4 ml). The mixture was stirred at r.t. under Ar in the dark for 5 h. The reaction was then quenched by removal of the solvent under reduced pressure, the residue was dissolved in Et_2O, and the insoluble metal complexes were filtered through *Celite*. The crude product was chromatographed (silica gel; hexanes/AcOEt 9 : 1) to yield **4** (124.0 mg, 88%). (11*Z*)/(11E) 6.2 : 1. ^{1}H-NMR (400 MHz, $CDCl_3$): (11*Z*)-isomer: 0.08 (*s*, 6 H); 0.90 (*s*, 9 H); 1.18 (*s*, 6 H); 1.43 (*m*, 1 H); 1.63 (*m*, 1 H); 1.85 (*s*, 3 H); 1.86 (*s*, 3 H); 1.95 (*s*, 3 H); 2.50 (*t*, $J = 6.9$, 2 H); 4.31 (*d*, $J = 6.3$, 2 H); 5.63 (*t*, $J = 6.1$, 1 H); 5.95 (*d*, $J =$ 11.7, 1 H); 6.23 (*d*, $J = 7.3$, 1 H); 6.30 (*d*, $J = 6.1$, 1 H); 6.35 (*t*, $J = 11.7$, 1 H); 6.66 (*d*, $J = 12.0$, 1 H). HR-MS: 414.2950 ($C_{26}H_{44}O_2Si^+$; calc. 414.2954).

*3-[(1*E*,3*E*,5*Z*,7*E*)-9-Hydroxy-3,7-dimethylnona-1,3,5,7-tetraenyl]-6-(hydroxymethylidene)-2,4,4-trimethylcyclohex-2-en-1-one* (**5**). To a suspension of NaH (60% in mineral oil; 62.4 mg, 2.60 mmol) in dry THF (3 ml) was added HCOOEt (96.2 mg, 1.30 mmol) at 0° under Ar. Ketone **4** (107.4 mg, 0.26 mmol) in THF (3 ml) and abs. EtOH (200 proof, 100 µl) were subsequently added, and the mixture was stirred for 45 min at r.t. Reaction was then quenched with aq. NH_4Cl and extracted with Et_2O (2 ×). The org. material was washed with sat. NaCl soln., dried (Na_2SO_4), and the solvent was removed under reduced pressure. The crude formylated derivative was dissolved in THF (4 ml) and treated with excess of Bu_4NF (1.04 ml, 1M in THF, 1.04 mmol). The mixture was stirred at r.t. for 2 h, then it was extracted with Et_2O, washed with H_2O (2 ×) and saturated NaCl soln., dried (Na_2SO_4), filtered, concentrated, and chromatographed (SiO_2; hexanes/AcOEt 3 : 2) to yield **5** (72.5 mg, 85%). (11*Z*)/(11*E*) 3 : 1. ^{1}H-NMR (400 MHz, C_6D_6): (11*Z*)-isomer: 0.91 (*s*, 6 H); 1.63 (*s*, 3 H); 1.74 (*s*, 3 H); 1.90 (*s*, 3 H); 2.02 (*s*, 2 H); 3.95 (*d*, $J = 6.6$, 2 H); 5.70 (*t*, $J = 6.6$, 1 H); 5.95 (*d*, $J = 11.7$, 1 H); 6.28 (*t*, $J = 11.0$, 1 H); 6.29 (*d*, $J = 5.7$, 1 H); 6.33 (*d*, $J = 5.7$, 1 H); 6.80 (*d*, $J = 12.0$, 1 H); 7.26 (*s*, 1 H). HR-MS: 328.2044 ($C_{21}H_{28}O_3^+$; calc. 328.2038).

*6-Diazo-3-[(1*E*,3*E*,5*Z*,7*E*)-8-Formyl-3,7-dimethylocta-1,3,5,7-tetraenyl]-2,4,4-trimethylcyclohex-2-en-1-one* (**7**). To a suspension of NaH (60% in mineral oil; 20.6 mg, 0.86 mmol) in dry THF (2 ml) was added (4-carboxybenzene)sulfonazide (48.8 mg, 0.21 mmol), and the mixture was stirred for 5 min at r.t. Compound **5** (28.2 mg, 0.086 mmol) in THF (2 ml) and abs. EtOH (200 proof, 75 µl) were then added, and the mixture was stirred at r.t. for 50 min. Reaction was quenched with 1N NaOH and extracted with Et_2O (2 ×). The org. material was washed with H_2O, sat. NaCl soln., and dried (Na_2SO_4). After removal of the solvent under reduced pressure, the crude product **6** (14 mg, 0.043 mmol) was dissolved in anh. CH_2Cl_2 (2 ml), and MnO_2 (93.5 mg, 1.07 mmol) was added at 0°. After 1 h, the mixture was charged with additional MnO_2 (93.5 mg, 1.07 mmol). The reaction was complete in 3.5 h and quenched by filtration through *Celite* with Et_2O to give crude **7**. After FC (SiO_2; hexanes/AcOEt 3 : 2), the isomeric mixture was further separated by normal-phase HPLC (*Cosmosil Si-60*; 4.6 × 250 mm; hexane/AcOEt 4 : 1, 3 ml/min, detection at 370 nm) to give **7** (14 mg, 50% for two steps). All spectroscopic data were in agreement with previously reported data for **7** [12]. ^{1}H-NMR (500 MHz, C_6D_6): 0.81

(*s*, 6 H); 1.62 (*s*, 3 H); 1.72 (*s*, 3 H); 1.94 (*s*, 2 H); 2.05 (*s*, 3 H); 5.60 (*d*, $J = 11.8$, 1 H); 5.97 (*d*, $J = 16.2$, 1 H); 6.05 (*d*, $J = 7.6$, 1 H); 6.10 (*d*, $J = 16.2$, 1 H); 6.29 (*t*, $J = 12.0$, 1 H); 6.52 (*d*, $J = 12.0$, 1 H); 9.88 (*d*, $J = 7.6$, 1 H). HR-MS: 325.1918 ($C_{20}H_{25}O_2N_2^+$; calc. 325.1916).

(11Z)-3-Diazo-4-oxo[15-$^3H_1$]retinol (**1**). $NaBT_4$ (5 mCi, 222.3 mCi/mmol) was suspended in THF/MeOH 1:1 (1.0 ml) at 0°. The unlabeled compound **7** (4.1 mg, 0.0126 mmol) dissolved in THF/MeOH 1:1 (1.0 ml) was added to the reaction vial and stirred at 0° for 5 min. The reaction was quenched by addition of aq. NH_4Cl and extracted with Et_2O (3×). The org. layer was then washed with sat. NaCl soln. and dried (Na_2SO_4). After filtering the salt and removing the solvent under reduced pressure, the residue was dissolved in CH_2Cl_2 (1.5 ml), and MnO_2 (30 equiv., 0.38 mmol, 33 mg) was added at 0°. The mixture was stirred at 0° for 25 min, at which time the reaction was complete based on TLC. *Celite* filtration of the mixture afforded crude product **1**, sufficiently pure for HPLC purification. Normal-phase HPLC (*Cosmosil Si-60*; 4.6 × 250 mm; hexane/AcOEt 3:1, 3 ml/min, detection at 370 nm) of crude product yielded **1** (0.865 mCi, 205 mCi/mmol) in high purity. Less than 10% isomerization of the (11*Z*)-configuration was observed (HPLC), and the final product co-eluted on TLC and HPLC with authentic unlabeled *(11Z)-3-diazo-4-oxoretinal* (**7**).

Regeneration of DK-Rh with Opsin and **7**. Incubating **7** with bleached rod outer segment (ROS) membranes regenerated DK-Rh. Rod outer segments were isolated from bovine retinae according to a standard procedure [32] with minor modifications [33]. The bleaching was performed after resuspension in phosphate buffer (67 mM, pH 7.0) containing hydroxylamine (100 mM) at room-light and ice-bath temp. All the following manipulations were performed in a dark room with minimal red lighting. Reconstitution was effected by the addition of an ethanolic stock soln. of **7** (OD 4.0) to opsin (OD 4.0) in 10 mM CHAPSO/10 mM HEPES (1.5 ml, pH 7.0). The protein soln. was incubated at r.t., and UV and CD spectra were recorded at indicated time points (*Fig. 1*). The incorporation of **7** into opsin to yield DK-Rh proceeded rapidly and was complete within 10 min. The newly bound pigment is not displaced by addition of (11*Z*)-retinal, as evident by the lack of a peak present at 500 nm.

Photoaffinity Procedures and Isolation of Cross-Linked Peptides. Incubating **1** with bleached rod outer segment (ROS) membranes regenerated DK-Rh. Rod outer segments were isolated from bovine retinae according to a standard procedure [32] with minor modifications [33]. The bleaching was performed after resuspension in phosphate buffer (67 mM, pH 7.0) containing hydroxylamine (100 mM) at room-light and ice-bath temp. All the following manipulations were performed in a dark room with minimal red lighting. Reconstitution was effected by the addition of an ethanolic stock soln. of **1** (30 µl, 3.75 OD, 1.5 µCi) to opsin (OD 4.0) in 10 mM CHAPSO/10 mM HEPES (1.5 ml, pH 7.0). The protein soln. was incubated at r.t. for 10 min, and glycerol (2.75 ml) was added to serve as antifreeze during the cooling of the protein sample. The glycerol/DK-Rh soln. was then placed in a *Petri* dish (5-cm diameter) yielding a thin transparent layer, which was cooled to −196° with liquid N_2. The glass-like glycerol layer was then irradiated for 10 min with a 1000-W tungsten lamp fitted with a 480-nm band pass filter while keeping the sample cooled at −196°. The *Petri* dish containing the protein was then irradiated for 20 min with two low-pressure Hg lamps equipped with a 254-nm band pass filter to effect photo-activation of the diazo-ketone (−196°). The photo-cross-linked sample was then warmed to r.t. and transferred to a test tube. *Tris* buffer (0.5 ml, 500 mM, pH 8.5) and PrOH (2 ml) were added, and the cysteine residues were pyridylethylated under Ar atmosphere with 4-vinylpyridine (100 µl) and Bu_3P (100 µl) for 1.5 h in a sonicator. The protein was then precipitated in glass centrifuge tubes with addition of Cl_3CCOOH (90%, 15 ml) and H_2O (50 ml), and was isolated by centrifugation (45 min, 12,000 rpm). The unbound retinal was removed by successively washing the protein precipitate with AcOEt, acetone, and H_2O (2× each). The protein was then dissolved in 75% TFA soln. and digested with excess CNBr (40 equiv.) for 21 h. The peptidic fragments were purified by HPLC (buffer *A* 0.06% TFA in H_2O; buffer *B* 80% MeCN, 0.052% TFA in H_2O; 0 → 2 min, 100% buffer *A*; 2 → 82 min, 100% buffer *A* → 30% buffer *A*; 82 → 92 min, 30% buffer *A* → 100% buffer *B*; 92 → 120 min, 100% buffer *B*; monitored at 215 nm). The HPLC column was incubated at 60°, which resulted in much better peak resolution as compared to HPLC runs performed at ambient temp. One-min aliquots were collected in silanized glass tubes. The radioactive fractions were identified by liquid scintillation counting, and the peptide and cross-linked amino acids were identified by amino-acid sequencing. For analysis of DK-Rh (initial state of the (11*Z*)-retinal chromophore prior to isomerization), the glycerol/protein soln. was irradiated with low-pressure Hg lamps at −196° without initial photo-isomerization of the pigment with 480-nm light. For all other photo-intermediates, DK-lumi, DK-meta-I, and DK-meta-II, the glycerol/protein solution was warmed and incubated for 20 min at the appropriate temp. for populating each state after photo-isomerization with 480-nm light at −196°. The sample was then re-cooled to −196° and photo-cross-linked by irradiation with 254-nm UV light. On a separate occasion, DK-Rh was irradiated with only 480-nm light for 20 min, but this yielded no cross-linked peptides; this served as a crucial control demonstrating that 480-nm

irradiation does not activate the photolabel. The percent cross-linking calculated based on isolated radioactive peptides were between 0.65 – 1.50%.

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