Synthesis of 13-Acetoxy-13-desmethylretinal, Its Pigment Formation with Bacterioopsin, and Apparent Dark-Inactivating Effect on the Pigment

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The title compound was designed to test for the involvement of nucleophilic catalysis, presumably by aspartate-212, in the dark cis–trans isomerizations of retinal in bacteriorhodopsin. β-Ionylidenacetaldehyde was condensed with acetylacetaldehyde dimethyl acetal in the presence of NaH in THF forming 1,1-dimethoxy-7-methyl-9-(2′,6′,6′-trimethyl-1-cyclohexen-1-yl)-4*E*,6*E*,8*E*-nonatrien-3-one (**4**) in good yield. **4** treated with LDA in THF followed by Ac_2O/DMAP yields 13-acetoxy-13-desmethylretinal dimethyl acetal (**5**). Careful, controlled hydrolysis of **5** in acetone, catalyzed by Bio-Rad AG 50W-X1, leads to 13-*cis*- and *all-trans*-13-acetoxy-13-desmethylretinal (**1**), and *cis*-1-acetoxy-7-methyl-9-(2′,6′,6′-trimethyl-1-cyclohexen-1-yl)-1,4*E*,6*E*,8*E*-nonatetraen-3-one (**6**). Both 13-*cis*-**1** and *all-trans*-**1** are highly unstable in the presence of weakly acidic material such as silica gel. Both are converted to **6** in the presence of acid. AM1 calculations indicate that **6** is 4.8 kcal/mol more stable than *all-trans*-**1**. 13-*cis*-**1** can be purified by HPLC on a cyano column and shown to form a pigment with bacterioopsin. The bacteriorhodopsin analogue, kept in the dark, slowly loses its absorption at 573 nm and in place develops absorption at 406 nm, signaling an alteration in the chromophore's binding to the protein as a protonated Schiff base (PSB). After a substantial drop in the 573 nm absorption, the retinal analogue can not be removed by the standard ethanol-delipidation method which would otherwise remove retinal, bound as a PSB, from native purple membrane or retinal oxime from photobleached purple membrane.

Introduction

Halobacteria halobium, in an environment of insufficient oxygen, generates a purple membrane which acts as a light-driven proton pump.[1] Protons are pumped out of the cell and the proton gradient that develops is used to drive the synthesis of ATP thereby replacing oxidative metabolism as an energy source. Thus light energy is converted to chemical energy. The purple membrane is a lipid bilayer which contains bacteriorhodopsin (bR), a protein of 248 amino acids. bR, a single polypeptide chain of high α-helical character, is woven into the membrane so as to form seven connected columns, approximately perpendicular to the membrane plane.[2,3] One equivalent of retinal is bound, as a protonated Schiff base (PSB), to lysine-216 and resides in a hydrophobic pocket of the protein. Light-adapted bR (bR^{LA}) exhibits a photocycle where the first step is the photoisomerization of the *all-trans*-retinal to its 13-*cis*-isomer. All subsequent steps of the photocycle do not require light. In succeeding nonphotolytic steps of the cycle, the isomerized PSB is sequentially deprotonated, reprotonated from the other side, and is thermally reisomerized to the *all-trans*-form to reset the system for a subsequent turnover. The initial photoisomerization is obligatory for proton pumping.[4] Consequently the dark reisomerization is necessary for repetitive pumping. This paper reports the synthesis and utilization of a retinal analogue designed to test the mechanism of dark cis–trans isomerization of retinal in bacteriorhodopsin.[5]

Thermal cis–trans isomerization also occurs upon dark-adaptation[6] and is dynamic in the dark-adapted state.[7] Contrasted with the thermal isomerization occurring in the photocycle, the chromophore, during dark-adaptation and while in the dark-adapted state, undergoes a one-step double cis–trans isomerization. bR^{LA} which has essentially all of its PSB in the *all-trans*,15-*anti* form, thermally isomerizes to a mixture of *all-trans*,-15-*anti* and 13-*cis*,15-*syn* in the absence of light.[8] The two isomers interconvert thermally in the dark by a reaction which appears to proceed through a bicycle-pedal mechanism.[9] Parallel studies in our laboratory with an enzyme/coenzyme system catalyzing cis–trans isomerizations have also uncovered one-step double cis–trans isomerizations.[10] Maleylacetoacetate cis–trans isomerase together with coenzyme glutathione catalyzes double isomerizations through a bicycle-pedal mechanism driven by nucleophilic addition. We have proposed a similar catalytic mechanism for the thermal cis–trans isomerizations in the bR photocycle and dark-adaptation reac-

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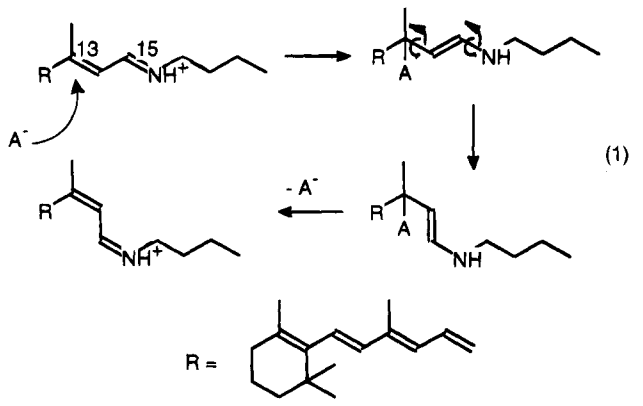
tions (see e.g. eq 1) and have suggested that aspartate-

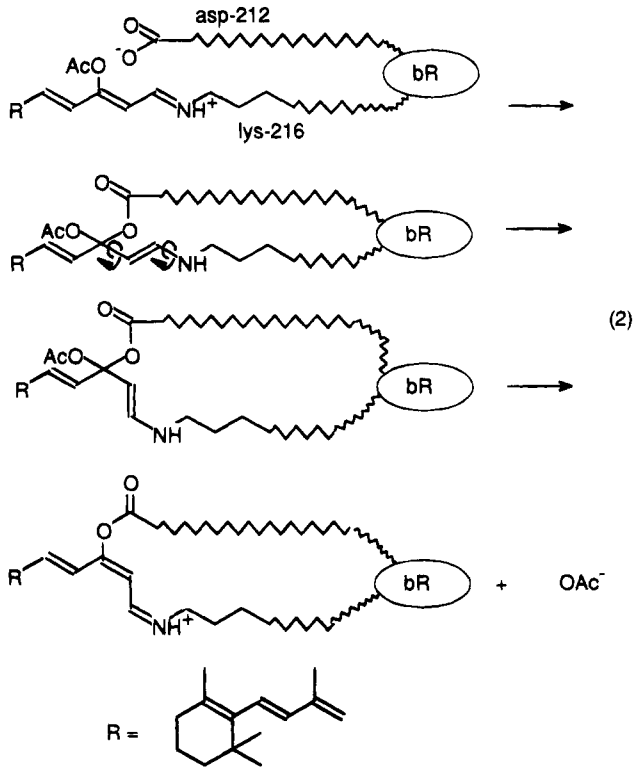


(1)

212 serves as the nucleophile adding to retinal's C13.[7] Previous studies in this laboratory involving secondary deuterium isotope effects,[11] model systems,[12] solvent isotope effects,[13] and quantum mechanical calculations[14] support a cis–trans isomerization of bR catalyzed by nucleophilic addition. Moreover, aspartate has been shown to participate as a nucleophile in other bioorganic reactions.[15]

Asp-212, located on the same side of the α-helix as lysine-216 and known to be deprotonated,[16] is believed to be part of a complex counterion to the positively charged Schiff base nitrogen. Its nearest oxygen is 3.6 Å from the nitrogen and within 4 Å of C13.[17] Previous quantum mechanical calculations show that the activation barrier for cis–trans isomerization is lowered when the counteranion is removed. This is due directly to the increased charge delocalization that develops in the remaining positively charged Schiff base.[14] The barrier is lowered further if a negatively charged nucleophile adds to C13 (eq 1). Both effects could be realized simultaneously, however, if asp-212 were to add to C13. The catalytic effect of removing the counteranion has already been demonstrated in a model system[12] and in a site-specific mutated bR.[18] Catalyzed cis–trans isomerization of a positively charged retinal Schiff base by a nearby carboxylate anion has also been reported.[12] The current study was initiated to test the hypothesis that nucleophilic addition of aspartate to retinal's C13 occurs during thermal cis–trans isomerization in bR.

A retinal analogue, 13-acetoxy-13-desmethylretinal (**1**) was synthesized and incorporated into bR in place of retinal. If aspartate-212 were to add to its C13, as suggested for the native system, an almost symmetrical intermediate would form (eq 2) where loss of acetate would be competitive with loss of aspartate. Loss of acetate would result in a cross-linked chromophore with an expected loss of the bR-analogue's activity.



(2)

Experimental Section

General. NMR, UV-vis, mass spectra, and high resolution mass spectra were obtained by methods described previously.[19] In this study UV-vis spectra were recorded with the aid of an integrating sphere. ^{1}H NMR chemical shifts are reported in ppm and coupling constants (J) in hertz, and peak assignments are given in the supplementary material.

2-Methyl-5-(2′,6′,6′-trimethyl-1-cyclohexen-1-yl)-2*E*,4*E*-pentadienecarbonitrile (2) was synthesized from β-ionone and cyanoacetic acid according to the methods of Young et al.[20a] and Huisman et al.[20b] The nitrile was reduced to β-ionylidenacetaldehyde (**3**) with DIBAL-H (84% yield) according to the method of Dugger and Heathcock.[20c]

The following synthetic procedures were performed under dim red light.

1,1-Dimethoxy-7-methyl-9-(2′,6′,6′-trimethyl-1-cyclohexen-1-yl)-4*E*,6*E*,8*E*-nonatrien-3-one (4). In a typical run 1.1g (23.1 mmol) of NaH (50% oil dispersion) was added to a dry flask with a magnetic stir bar, septum, and argon inlet. All subsequent additions were by syringe under an argon atmosphere. Four mL of dry THF was added, the mixture was stirred, and the solvent was removed. Then 8 mL of fresh THF was added and the mixture cooled in dry ice–acetone. Acetylacetaldehyde dimethyl acetal (1.2 mL, 9.0 mmol, Aldrich) was then added dropwise. After about 20 min the neat C15 aldehyde (2 mL, ~9.2 mmol) was added dropwise and the mixture was stirred and allowed to warm to about −2 to 0 °C when it was seen that the NaH was quickly consumed at this temperature and the reaction mixture darkened considerably. It was allowed to react at −2 to 0 °C for 10 min after which the reaction mixture was cooled to −25 °C, the septum was removed, 50 mL of petroleum ether (bp 30–60 °C) was added, and the mixture was transferred to a separatory funnel where 30 mL of 1 N HCl was added. The mixture was cautiously shaken, the layers were separated, and the organic layer was washed with saturated $NaHCO_3$ until the wash was neutral. The organic layer was dried over $MgSO_4$. The organic layer

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provided 3.2 g of crude product which appeared to contain about 80% of **4** as determined by ^{1}H NMR. The product was generally used in the next step without further purification. A sample purified by flash chromatography (30% ether/hexane), however, exhibited the following ^{1}H NMR ($CDCl_3$): δ 1.03 (s), 1.72 (s), 2.06 (s), 2.90 (d, J = 5.5), 3.39 (s), 4.86 (t, J = 5.6), 6.17 (d, J = 11.7), 6.18 (d, J = 16.3), 6.20 (d, J = 15.0), 6.43 (d, J = 16.3), 7.64 (dd, J = 11.7, 15.0). MS: 332.2 (M^+).

13-Acetoxy-13-desmethylretinal Dimethyl Acetal (5). To 8 mL of dry THF in a flask fitted with magnetic stir bar, septum, and argon inlet was added 1.75 mL (12.5 mmol) of diisopropylamine, and the solution was cooled in ice. All subsequent additions were made by syringe. Five milliliters (12.5 mmol) of 2.5 M BuLi in hexane (Aldrich) was added dropwise. The mixture was cooled in dry ice–acetone, and the total acetal crude product from the previous step, diluted with 8 mL of dry THF, was added dropwise. The syringe was washed twice with 4 mL portions of THF, and the washings were added to the reaction mixture. The mixture was allowed to stir for 1 h after which 4 mL of Ac_2O containing 230 mg of DMAP was added dropwise. The mixture was allowed to stir while warming to 0 °C and then stirred for an additional hour at 0 °C. Then 100 mL of petroleum ether together with 15 mL of 2 N HCl were added while transferring the contents of the reaction mixture to a separatory funnel. The mixture was shaken and verified that the aqueous layer was acidic. The aqueous layer was discarded, and the organic layer was washed with saturated $NaHCO_3$ until the aqueous layer was near neutrality. The organic layer was dried over $MgSO_4$. Evaporation of the solvent yielded 3.4 g of crude product estimated by HPLC to contain about 35% of the target compound. The crude 13-acetoxy-13-desmethylretinal dimethyl acetal from a similar preparation, starting with 17.4 mmol of 1,1-dimethoxy-7-methyl-9-(2′,6′,6′-trimethyl-1-cyclohexen-1-yl)-nonatrien-3-one, was subjected to flash chromatography (SiO_2, 15% ether/hexane). Fractions were assayed by HPLC (SiO_2, 25 × 0.46 cm, 10% EtOAc/hexane, 2 mL/min, 313 nm detection). The fractions containing a substantial amount of the 7.4 min peak were pooled. Yield: 1.35 g of incompletely pure material. Rechromatography provided pure material. HRMS: theory ($^{12}C_{23}{}^1H_{34}{}^{16}O_4$), 374.24569; found, 374.24571. ^{1}H NMR (acetone-d_6): δ 1.01 (s), 1.69 (s), 1.94 (s), 2.25 (s), 3.22 (s), 4.99 (d, J = 6.0), 5.41 (d, J = 6.0), 6.14 (d, J = 17.4), 6.16 (d, J = 12.3), 6.21 (d, J = 16.6), 6.26 (d, J = 17.4), 6.70 (dd, J = 12.3, 16.6).

Acid-Catalyzed Hydrolysis of 13-Acetoxy-13-desmethylretinal Dimethyl Acetal (5). To 625 mg of purified 13-acetoxy-13-desmethylretinal dimethyl acetal in 20 mL of acetone was added 117 mg of Bio-Rad AG 50W-X1 and the mixture stirred magnetically at ambient temperature. Aliquots were examined periodically by HPLC, using the same conditions as above. There was a gradual decline in the reactant (7.4 min elution) and a rise of a peak at 8.4 min. At 85 min reaction time, when the area of the 8.4 min peak was about 70% of the total, the reaction was stopped by filtering the mixture through paper into 250 mL of petroleum ether. The petroleum ether was quickly washed with 2 × 25 mL of saturated $NaHCO_3$ and dried over Na_2SO_4 overnight. Three major products were present: *all-trans*- (*all-trans*-**1**) and 13-*cis*-13-acetoxy-13-desmethylretinal (13-*cis*-**1**), and *cis*-1-acetoxy-7-methyl-9-(2′,6′,6′-trimethyl-1-cyclohexen-1-yl)-1,4*E*,6*E*,8*E*-nonatetraen-3-one (**6**). Attempts to separate and purify the 13-acetoxy-13-desmethylretinal isomers on silica gel were unsuccessful because of their instability in the presence of acidic media. Consequently, the mixture was purified by HPLC on a cyano column (Machery-Nagle, Polygosil-CN, 18% ether/hexane) which had been end-capped with TMS-groups by several injections of a TMS-Cl/ether solution. The order of elution was **6**, *all-trans*-**1**, 13-*cis*-**1**. While 13-*cis*-**1** and **6** could be obtained in pure form by this method, *all-trans*-**1**, however, (δ 9.77, doublet) could not be separated from a coeluting contaminant. 13-*cis*-**1**: ^{1}H NMR (acetone-d_6): δ 1.03 (s), 1.71 (s), 2.05 (s), 2.33 (s), 5.72 (d, J = 7.3), 6.23 (s, J = 15.7), 6.35 (d, J = 10.8), 6.44 (d, J = 15.7), 7.22 (d, J = 14.9), 7.24 (dd, J = 10.8, 14.9), 10.16 (d, J = 7.3). HRMS: theory for $^{12}C_{21}{}^1H_{28}{}^{16}O_3$, 328.2043; found, 328.2038. UV (EtOH) 383 nm (log ϵ 4.61), 274 nm (log ϵ 4.86). ***cis*-1-Acetoxy-7-methyl-9-(2′,6′,6′-trimethyl-1-cyclohexen-1-yl)-1,4*E*,6*E*,8*E*-nonatetraen-3-one (6):** ^{1}H NMR (acetone-d_6): δ 1.02 (s), 1.71 (br s), 2.07 (br s), 2.22 (s), 6.24 (d, J = 15.2), 6.33 (d, J = 11.3), 6.44 (d, J = 12.5), 6.45 (d, J = 14.7), 6.50 (d, J = 15.2), 7.74 (dd, J = 11.3, 14.7), 8.22 (d, J = 12.5). MS: 328.3 (M^+). UV (EtOH) 375 nm (log ϵ 4.60), 274 nm (log ϵ 4.96).

Photoconversion of *all-trans*-13-acetoxy-13-desmethylretinal to Its 13-*cis*-isomer. After removal of 13-*cis*-13-acetoxy-13-desmethylretinal (13-*cis*-**1**) and *cis*-1-acetoxy-7-methyl-9-(2′,6′,6′-trimethyl-1-cyclohexen-1-yl)-1,4*E*,6*E*,8*E*-nonatetraen-3-one (**6**) from the hydrolysate, the remaining *all-trans*-**1**, with its contaminant, was photolyzed at 18 °C in a 20% ether/hexane solution with light from a 500 W quartz halogen projection lamp filtered through Corning 3-72 glass. A photostationary state of the three compounds was generally established in about 1 h after which the mixture was resubjected to HPLC separation.

***cis*-1-Hydroxy-7-methyl-9-(2′,6′,6′-trimethyl-1-cyclohexen-1-yl)-1,4*E*,6*E*,8*E*-nonatetraen-3-one (7).** **4** (465 mg) in 3 mL of acetone containing 30 μL of water was mixed with 490 mg of Bio-Rad AG 50W-X1 resin. The mixture was agitated at 35 °C for about 24 h. The solvent was evaporated and 3 mL of fresh acetone and 30 μL of water were added and the process repeated. After two additional solvent evaporations and replacements, the mixture was filtered and the resin washed several times with acetone, and the washings and filtrate were combined. After evaporation of the solvent 358 mg of **7** was obtained which by ^{1}H NMR analyzed for 92% hydrolyzed product. Yield (corrected): 94%. ^{1}H NMR ($CDCl_3$): 1.072 (s), 1.730 (br s), 2.076 (br s), 5.590 (d, J = 3.2), 5.976 (d, J = 14.9), 6.187 (d, J = 16.2), 6.191 (d, J = 12.1), 6.434 (d, J = 16.2), 7.708 (dd, J = 12.1, 14.9), 8.642 (d, J = 3.2). MS: 286 (M^+).

Acetylation of 7. Crude **7** (122 mg) was diluted with 3 mL of acetic anhydride. Reaction was rapid at ambient temperature as monitored by HPLC. After 3 h the solvent was removed by rotoevaporation and the residue was subjected to flash chromatography (SiO_2, 20% ether/hexane). ^{1}H NMR ($CDCl_3$): 1.04 (s), 1.73 (br s), 2.08 (br s), 2.24 (s), 6.17 (d, J = 12), 6.19 (d, J = 16.0), 6.25 (d, J = 12.5), 6.38 (d, J = 14.9), 6.45 (br d, J = 16.3), 7.74 (dd, J = 12.0, 14.8), 8.33 (d, J = 12.5).

Purple Membrane. *Halobacteria halobium*, strain R1, were grown and purple membrane isolated according to the method of Oesterhelt and Stoeckenius.[21] The membranes were purified by the method of Becher and Cassim.[22] Purple membranes (~5 mg) were bleached in the presence of 0.4 M hydroxylamine in 0.03 M HEPES buffer, pH 7.3, with light from a 500 W quartz halogen projector lamp filtered through Corning 3-69 glass (>500 nm) at 18.5 °C which generally required 20 h irradiation.[23] The photolysate was centrifuged at 31 000*g* for 20 min. The pellet was washed twice with distilled water, suspended in 1 mL of distilled water, and stored in the dark at 4 °C.

Reconstitution was generally accomplished by adding 10–15 μL of a 95% ethanol solution of the retinal analogue to 1 mL of a 0.01 M HEPES solution containing 0.1 mL of bleached membrane stock solution.

Ethanol Washes. An adaptation of the method of Orlando et al. was used.[24] The particular membrane, generally about 5 mg, was centrifuged and the pellet separated from its supernatant. The pellet was suspended in 2 mL of water and sonicated for 5 min at 4 °C in the dark. Ethanol (10.7 mL of 95%) was added to achieve a concentration of 80% (v/v) and the mixture vortexed and then sonicated for 5 min. It was allowed to stand in the dark at 4 °C overnight and then centrifuged at 31 000g for 1 h. The supernatant was removed

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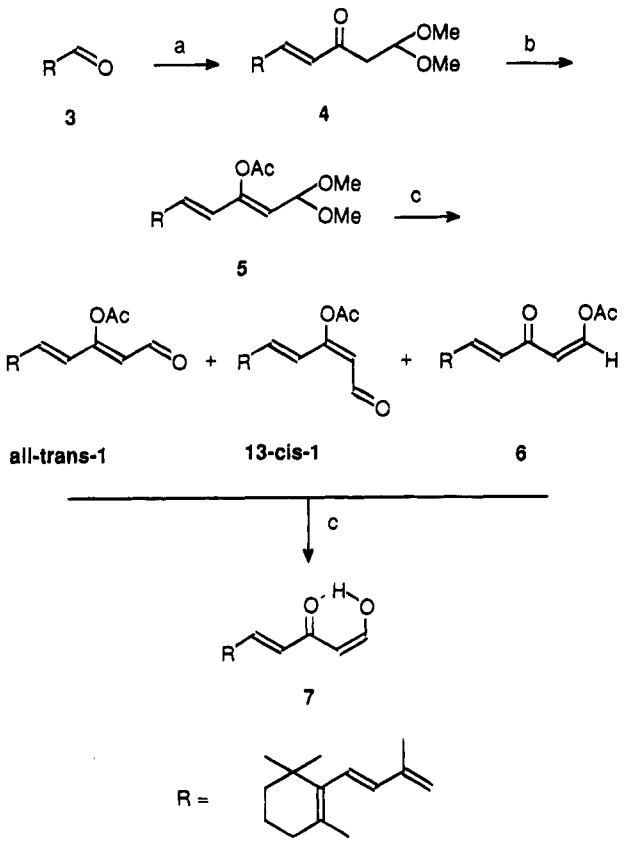
Scheme 1



[a] (a) $CH_3COCH_2CH(OCH_3)_2$/NaH/THF, (b) (1) LDA/THF, (2) Ac_2O/DMAP, (c) Bio-Rad 50W-X1/acetone.

and the procedure repeated. After the second centrifugation the pellet was placed in a vacuum desiccator and pumped on with house vacuum for 1 h. The protein was taken up in 1.5 mL of 88% formic acid/95% ethanol (3:7)[25] and its spectrum recorded vs a blank of the same solvent.

Results and Discussion

13-Acetoxy-13-desmethylretinal (**1**) was synthesized according to the sequence shown in Scheme 1. β-Ionylidenacetaldehyde (**3**) was prepared by reported methods.[20] Condensation of the C15 aldehyde with the anion of acetylacetaldehyde dimethyl acetal in THF provided 1,1-dimethoxy-7-methyl-9-(2′,6′,6′-trimethyl-1-cyclohexen-1-yl)-4*E*,6*E*,8*E*-nonatrien-3-one (**4**) in high yield. Acetylacetaldehyde dimethyl acetal is known to undergo base-catalyzed proton exchange, alkoxy exchange, and alcohol elimination, all presumably through the formation of the monoanion, $[CH_3COCHCH(OMe)_2]^-$.[26] If this is indeed the major reacting monoanion formed with NaH in THF, then its condensation with the C15 aldehyde would lead predominantly to an unwanted isomer of the oxocarbon skeleton necessary to form **1**. Two equivalents of NaH were used in an attempt to form an acetylacetaldehyde dimethyl acetal intermediate with *terminal* anionic character. Whether a dianion is formed under these condition is not known; however, these conditions provide the correct skeleton for retinal analogue formation.

4 was treated with 1 equiv of LDA in THF to form its enolate which, when treated with an excess of acetic anhydride and a catalytic amount of DMAP, was converted to the enol acetate dimethyl acetal **5**. **5** was purified by flash chromatography on silica gel with substantial loss due to its acid lability. Gentle acid-catalyzed hydrolysis of **5** to **1** was achieved through use of a small quantity of Bio-Rad AG 50W-X1 in acetone. Following Coppola,[27] Amberlite IR-120 was used and found to be suitable for deacetalization of acetylacetaldehyde dimethyl acetal but relatively inactive for deacetalization of **5**. Bio-Rad AG 50W-X1, however, was found to be quite efficient. Both resins are substituted phenylsulfonic acids; however, the reason for the lower activity of the Amberlite resin may be due to its higher degree of cross-linking preventing the longer retinal analogue molecule from reaching the catalytic sites in the interior of the bead.

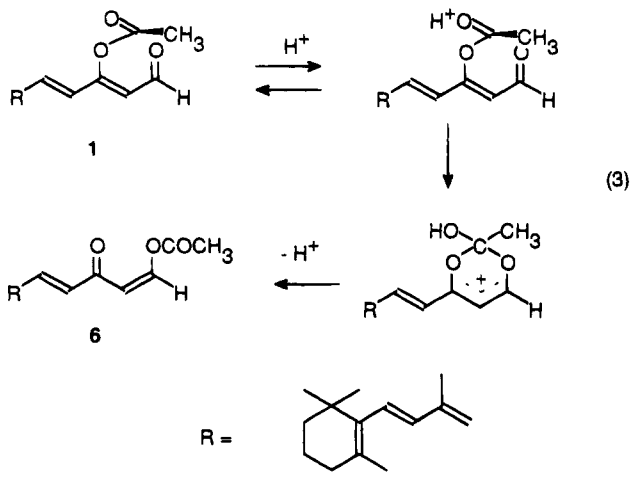
Doublets at δ 9.77 (J = 7.5) and 10.16 (J = 7.3), generally with areas in the ratio of 2:1, respectively, appear in the hydrolysate indicating that two aldehydes are formed in the hydrolysis of **5**. Both doublets decay when the hydrolysate comes in contact with weakly acidic media such as silica gel (*vide infra*). The sensitivity toward silica gel necessitated the use of a cyano adsorbent which had been endcapped with TMS groups in order to separate isomers with minimal loss. Preliminary purification through a 25 cm and then rechromatography through a 50 cm-length HPLC cyano column provides a pure sample of the δ 10.16 aldehyde but not of the δ 9.77 aldehyde. Up to now we have been unable to separate the latter aldehyde from a coeluting impurity.

In this study the vinyl regions of the ^{1}H NMR of new compounds were analyzed using PANIC, Bruker's iteration program. The resulting calculated chemical shifts and coupling constants are given in the Experimental Section and their observed and simulated spectra are presented in the supplementary material. The ^{1}H NMR indicates that the δ 10.16 aldehyde is 13-*cis*-**1**. The chemical shifts and coupling constants are in accord with this structure but most telling is its H-12 proton at δ 7.22 which compares quite well with H-12 chemical shifts for other 13-*cis*-retinals:[28] 13-*cis*, 7.28; 7,13-*dicis*, 7.22; 9,-13-*dicis*, 7.25; 7,9,13-*tricis*, 7.12; however, 9,11,13-*tricis*, 5.99. Trans geometry at C13 leads to chemical shifts for H-12 which are shifted upfield by about 1 ppm relative to their 13-*cis*-isomers. Although structural information on the δ 9.77 aldehyde is incomplete we tentatively assign the *all-trans*-**1** structure to that compound. That these two aldehydes are formed from the same precursor and that they elute very closely in HPLC suggests they are isomeric. In addition to characteristic resonances (in acetone-d_6), e.g. at δ 7.68 (dd, J = 11.9, 14.9, H-11), 6.68 (d, J = 14.9, H-7), 6.8–6.2 (m), 5.89 (d, J = 7.5, H-14), 1.71 (br s, 5-Me), and 1.02 (s, 1,1-Me_2), *all-trans*-**1** exhibits a singlet at δ 2.42 suggesting that the 13-acetoxy group is intact. Additional resonances, however, of an HPLC-purified sample at 7.53 (d, J = 7.25), 7.23 (dd, J = 11.5, 14.9), 5.69 (d, J = 7.5), 1.70 (s) and 1.03 (s) indicate an additional retinoid present as a contaminant. Moreover, the integrated area of the vinyl peaks in the δ 6.2–6.8 was always found to be in substantial excess of what would be expected based on the area of the aldehyde doublet at 9.77 were the compound to be pure.

In addition to the formation of *all-trans*-**1** and 13-*cis*-**1**, a rearranged product, *cis*-1-acetoxy-7-methyl-9-(2′,6′,6′-

(25) Gerber, G. E.; Khorana, H. G. *Methods Enzymol.* **1982**, *88 (Part I)*, 56–74.
(26) Antus, S.; Boross, F.; Nogradi, M. *J. Chem. Soc. Chem. Comm.* **1977**, 333–334. *Liebigs Ann. Chem.* **1978**, 107–117.
(27) Coppola, G. M. *Synthesis* **1984**, 1021–1023
(28) Liu, R. S. H.; Asato, A. E. *Methods Enzymol.* **1982**, *88 (Part I)*, 506–516.

trimethyl-1-cyclohexen-1-yl)-1,4*E*,6*E*,8*E*-nonatetraen-3-one (**6**) is formed during acid-catalyzed hydrolysis of **5**. The H-1,H-2 coupling constant of 12.5 Hz suggests a cis arrangement for these protons.[29] The formation of **6** is best rationalized as arising directly from an acid-catalyzed 1,5-sigmatropic shift of the acetyl group in a 14-*s*-*cis* conformation of *all-trans*-**1** (eq 3).[30] MM2 fol-



lowed by AM1 calculations, to optimize the structures of *all-trans*-**1** and **6**, indicate that **6** is 4.8 kcal/mol *more stable* than *all-trans*-**1**. Although greater stability for a linearly conjugated system such as in **1** is anticipated when compared to its cross-conjugated isomer **6**, the predominant effect in ordering the stabilities appears to be caused by the inherent greater stability of a ketone compared to an aldehyde. The large calculated difference in thermodynamic stability provides a rationalization for the high lability of **1** in the presence of very weak acids such as silica gel.

Continued hydrolysis with AG 50W-X1 leads to deacetylization of **1** and **6** yielding *cis*-1-hydroxy-7-methyl-9-(2′,6′,6′-trimethyl-1-cyclohexen-1-yl)-1,4*E*,6*E*,8*E*-nonatetraen-3-one (**7**). An intramolecular hydrogen bonded ketoenol structure is suggested by the H-1,H-2 coupling constant of 3.2 Hz. Acetylation of **7** with acetic anhydride, in the absence of base, leads to **6**.

Although *all-trans*-**1** could not be separated from a coeluting impurity it could be photolyzed with >440 nm light to form a photostationary state which contained *all-trans*-**1**, **6**, and 13-*cis*-**1** in the ratio of 1.8:1.3:1.0 as determined by NMR. 13-*cis*-**1** could be cleanly isolated by HPLC from this mixture. The remaining isomers were rephotolyzed and in this way 13-*cis*-**1** was continually stripped from each photolysate. Since 13-*cis*-**1** could be obtained in pure form and *all-trans*-**1** could not, an attempt was made to photolyze pure 13-*cis*-**1** in the hope

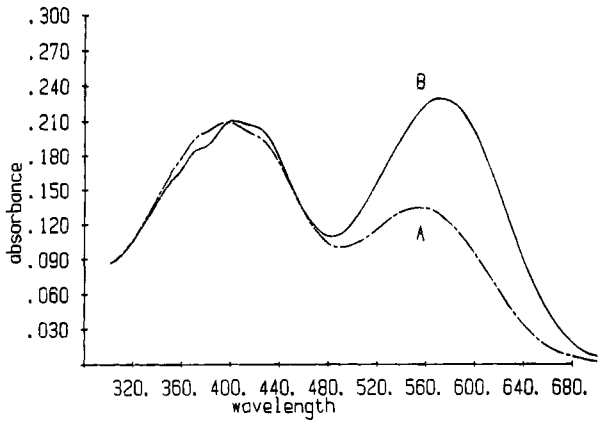


Figure 1. Purple membrane, initially 4.6 mg/mL, was photobleached and washed (see Experimental Section). One-tenth of the resulting preparation was added to 1 mL of 0.01 M HEPES, pH 7.3, and its UV-vis spectrum measured with the aid of an integrating sphere. A 95% EtOH solution (10 μL) of 13-*cis*-**1** was added to the apomembrane in the dark and its spectrum recorded immediately at 100 nm/min. Curve A is the difference spectrum between the pigment forming solution and that of the apomembrane solution. Curve B is the same as A taken 45 min later while kept in the dark.

that the pure *all-trans*-isomer might be obtained. This method was unsuccessful because the coeluting impurity appears also to be formed during photolysis.

The formation of **4** from ethyl formate and 6-methyl-8-(2′,6′,6′-trimethyl-1-cyclohexen-1-yl)-3*E*,5*E*,7*E*-octatrien-2-one, followed by acetal formation with H_2SO_4/MeOH, has been reported previously.[31] The acetal, to our knowledge, however, was not characterized. The condensation of the C18 ketone with ethyl formate reportedly leads to the 13-keto-15-enol **7**. Our ^{1}H NMR of **7**, prepared by a different route (*vide supra*), suggests that this is an intramolecular hydrogen bonded ketoenol. All of our attempts to acetylate the ketoenol in acidic media have led to the 13-keto-15-enol acetate. It is somewhat surprising, therefore, that the reaction with methanol is reported to lead to the acetal rather than the 13-keto-15-enol methyl ether.

Attempted Pigment Formation with *all-trans*-1. Addition of *all-trans*-**1** to the bleached membrane resulted in the slow formation of a very weakly absorbing pigment at 574 nm indicating a very low degree of reconstitution (data not shown). It may be that the impurity present blocks pigment formation.

Pigment Formation with 13-*cis*-1. A new pigment (**13-Ac-bR**) quickly forms upon addition of 13-*cis*-**1** to the bleached membrane. Initially the absorption maximum is at 559 nm (Figure 1, curve A) but within 1 h in the dark the maximum moves to 573 nm (Figure 1, curve B). The shift to longer wavelength is believed to be due to dark-adaptation (i.e. dark cis–trans isomerization). Normally dark-adaptation of bR^{LA} results in a shift to shorter wavelength. However, if the native membrane is reconstituted with 13-*cis*-retinal, dark-adaptation to the same 13-*cis*/*all-trans*-retinal mixture as in bR^{DA} results in a shift to longer wavelength because the absorption maximum for *all-trans*-retinal-bR is at longer wavelength than that for its 13-*cis*-isomer.

(29) A referee has pointed out that $J_{1,2}$ = 12.5 Hz for **6** may also be consistent with a trans H-1,H-2 arrangement. Two other observations (see below in text), however, support a cis-1,2 configuration for **6**: (a) The reasonable suggestion of a 1,5-sigmatropic shift of the acetyl group for the conversion of *all-trans*-**1** to **6** predicts the initially formed product to have a cis-1,2 arrangement; (b) **7** undoubtedly has a cis-1,2 geometry ($J_{1,2}$ = 3.2 Hz). Acetylation of **7** yields **6**. If **6** were to have a trans-1,2 geometry the acetylation would have had to proceed with a cis-trans isomerization which is less likely.

(30) For other examples of thermal 1,5-sigmatropic shifts of acyl, nitrile, ester, and nitro groups, see: (a) Jones, D.; Marmon, R. J. *J. Chem. Soc. Perkin Trans. 1* **1990**, 3271–3275. (b) Schiess, P.; Funfschilling, P. *Tetrahedron Lett.* **1972**, 5191–5194. (c) Franck-Neumann, M.; Buchecker, C. *Tetrahedron Lett.* **1972**, 937–940. (d) Bapat, G. S.; Fischer, A.; Henderson, G. N.; Raymahassay, S. *J. Chem. Soc. Chem. Commun.* **1983**, 119–120. (e) Habraken, C. L.; Cohen-Fernandes, P. *J. Chem. Soc. Chem. Commun.* **1972**, 37–38.

(31) (a) Shchablinskii, A. N.; Sirotkina, L. I.; Shemaeva, L. I.; Gazizullina, L. G. *Khim-Farm. Zh.* **1982**, *16*, 602–604. (b) See also: Danshina, S. V.; Drachev, A. L.; Drachev, L. A.; Eremin, S. V.; Kaulen, A. D.; Khitrina, L. V.; Mitsner, B. I. *Arch. Biochem. Biophys.* **1990**, *279*, 225–231.

On further long standing in the dark the pigment slowly loses its absorption at 573 nm and gains absorption at 406 nm in a non-first-order process indicating that a reaction takes place in the absence of light. About half of its absorption is lost in 12 h. We have not yet observed the long wavelength absorption of the pigment decay to zero; however, samples that have been kept in the dark and followed for a month still show some small absorption at >500 nm albeit as a shoulder on the 406 nm peak. This behavior is to be contrasted with native bR^{DA} which is very stable in the dark for long periods of time.

The loss of the 573 nm absorption with a concomitant increase of optical density at 406 nm signals an alteration in bonding and/or environment of the chromophore in **13-Ac-bR**. That the bonding of the chromophore to the protein has been affected has been shown by competitive binding experiments between the chromophore in **13-Ac-bR** and *all-trans*-retinal.[5] In the dark, added *all-trans*-retinal slowly replaces **1** from **13-Ac-bR** to form bR^{DA}. The extent of replacement is considerably diminished for **13-Ac-bR** which has undergone a substantial prior loss of its 573 nm absorption suggesting that the retinal binding site becomes blocked as a result of the aging reaction.[5]

Experiments to remove the chromophore from aged **13-Ac-bR** also point to a new covalent binding of the chromophore which is not readily hydrolyzable. Native purple membrane which is photobleached in the presence of hydroxylamine, contains retinal oxime in its membrane. Two washes with 80% ethanol efficiently removes the lipids and retinal oxime as shown by the protein's very low residual absorption at 360−400 nm (Figure 2, curve A). Similarly, unbleached purple membrane, containing retinal bound to the protein through a protonated Schiff base, when washed with 80% ethanol yields residual protein with only a very small absorption around 380 nm (Figure 2, curve B). A sample of **13-Ac-bR**, however, allowed to sit in the dark for 48 days during which time its 573 nm absorption decayed almost to zero, when washed twice with 80% ethanol in the same way, yields a protein with strong residual absorption at 396 nm (Figure 2, curve C) indicating that the chromophore analogue remains covalently bound inspite of the severe treatment. Whether the chromophore remains bound through an asp-212 covalent bond to C13 of the analogue (see eq 2) or in some other covalency, cannot be ascertained at this time. Studies are in progress to elucidate the site and mode of binding and will be reported in the future.

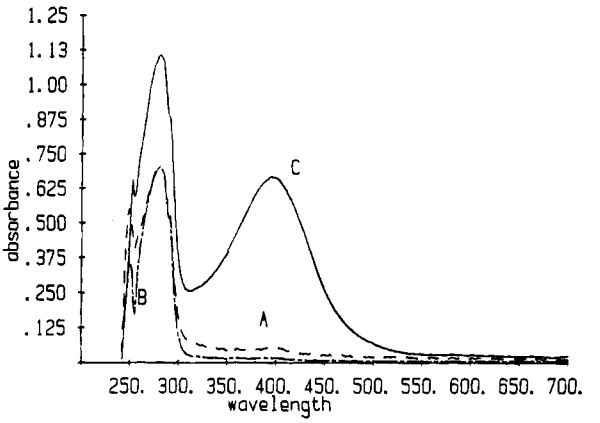


Figure 2. All operations were carried out under dim red light at 4 °C. (A) Purple membrane, initially 4.6 mg/mL, was photobleached, washed (see Experimental Section), and centrifuged. The pellet, in 2 mL of water, was sonicated for 5 min, and then 10.7 mL of 95% EtOH was added. It was mixed and sonicated for another 5 min and allowed to stand overnight. The mixture was centrifuged, and the water/EtOH treatment was repeated with the resulting pellet. The final pellet was dissolved in 1.5 mL of formic acid/EtOH (3:7) and its spectrum taken with the aid of an integrating sphere. (B) Similar treatment for half the quantity of native unbleached membrane in half the volume of EtOH-washing solutions but dissolved in 1.5 mL of formic acid/EtOH. Its absorbance has been multiplied by 2. (C) Similar treatment for **13-Ac-bR** formed from the same concentration of apomembrane as in A but allowed to sit in the dark for 48 days before EtOH extraction. A spectrum with formic acid/EtOH (3:7) in both sample and reference cuvettes has been subtracted from each.

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Supplementary Material Available: Observed and simulated ^1H NMR vinyl region-spectra for 13-*cis*-**1**, **4**, **5**, **6**, and **7** (5 pages). The 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.

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