

13-Acetoxy-13-desmethylretinal: Synthesis, Incorporation into Bacteriorhodopsin, and Its Apparent Inactivating Effect

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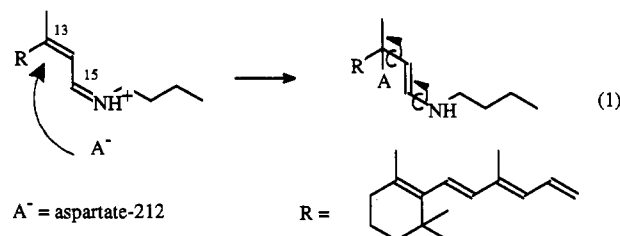
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Bacteriorhodopsin (bR), the protein pigment of the purple membrane (PM) light-driven proton pump, is a single polypeptide chain of 248 amino acids.¹ It traverses the membrane to form seven rods² of high α -helical character.³ PM's color results from the presence of an equivalent of retinal, bound as a protonated Schiff base (PRSB) at lysine 216, and its interaction with the protein. Light initiates a photocycle where the first step is a photoisomerization of *all-trans*-retinal to the 13-*cis* isomer. All subsequent steps in the cycle are thermal dark reactions. The *all-trans* \rightarrow 13-*cis* photoisomerization has been shown to be obligatory for proton pumping,⁴ and consequently, the thermal reisomerization of 13-*cis* \rightarrow *all-trans* in the latter part of the cycle is required for continual turnover. We report herein the synthesis and incorporation into bacteriorhodopsin of a novel analogue, 13-acetoxy-13-desmethylretinal, **1**, designed to probe the mechanism of dark *cis-trans* isomerization.

Thermal *cis-trans* isomerization also occurs upon dark adaptation⁵ (*all-trans*,15-*anti* \rightarrow 13-*cis*,15-*syn*) and is dynamic (13-*cis*,15-*syn* \rightleftharpoons *all-trans*,15-*anti*) while in the dark-adapted state (bR^{DA}).⁶ These double *cis-trans* isomerization reactions apparently proceed by a concerted one-step bicycle-pedal mechanism.⁷

The chromophore, except for the Schiff base proton, appears to be well shielded from solvent by the protein and lipid bilayer and suggests that the protein itself catalyzes dark *cis-trans* isomerization. We have noted previously that catalysis could be achieved by two mechanisms: (1) removal of the counteranion (aspartate 212) from the vicinity of the protonated Schiff base nitrogen and (2) the introduction of a negative charge or the addition of a nucleophile at C13 of the PRSB.⁸ Similar schemes with variation have been advanced by others.⁹ The catalytic effect of removing the counteranion has been demonstrated in a model system¹⁰ and more recently in a bR mutant where the counterion could be partially neutralized.¹¹ We have suggested,^{6,8} however, that the two types of catalytic enhancements could be ac-

complished in one act by the addition of aspartate 212¹² to the PRSB at C13 to provide an intermediate which could undergo internal rotation about the C15-N and/or the C13-C14 bonds (eq 1). Asp-212, shown by FTIR studies to be deprotonated,¹³



and Lys-216 are one above the other on the same side of the helix in close proximity. Recent structural data show Asp-212 as part of a complex counteranion of the positively charged Schiff base nitrogen where its nearest oxygen is 3.6 Å from nitrogen.¹⁴ That same oxygen is also within 4 Å of retinal's C13 and could, by a microconformational change, move closer to add to C13. Such a mechanism involving reversible addition of a nucleophile to C13 provides a rationale for the regiospecificity of isomerization (only 13-*cis* and *all-trans* are observed in this system) and is similar to the mechanism encountered in enzyme-catalyzed *cis-trans* isomerization where bicycle-pedal double isomerization has also been observed.¹⁵ Previous studies reported from this laboratory support a nucleophilic mechanism for thermal PRSB *cis-trans* isomerization.^{8,10,16}

We reasoned that if Asp-212 participated as proposed, a retinal analogue, **1**, possessing a good leaving group at C13, might trap the nucleophile and lead to a cross-linked chromophore to render the membrane inactive (Scheme 1). Nucleophilic addition of Asp-212's carboxyl to **1** at C13 would provide an almost symmetrical intermediate **2** where loss of acetate could compete with loss of aspartate. 13-Acetoxy-13-desmethylretinal (**1**) was synthesized from β -ionone according to Scheme 2.¹⁷ The C15 aldehyde (**3**), synthesized by methods reported in the literature,¹⁸ was treated with acetylacetaldehyde dimethyl acetal in THF and 2 equiv of NaH to obtain the 13-keto 15-dimethyl acetal (**4**). Treatment of **4** with lithium diisopropylamide generated its enolate, which was then acetylated with Ac₂O/DMAP to furnish 13-acetoxy-13-desmethylretinal 15,15-dimethyl acetal (**5**). Gentle hydrolysis of **5** in acetone, catalyzed by Bio-Rad AG 50W-X1 (H⁺ form) and monitored by HPLC, furnished a mixture of several products where two aldehyde components (δ 10.16, d, and 9.77, d) and the 13-keto 15-enol acetate (**6**, δ 8.22, d) were detected by NMR (acetone-*d*₆). Acids and acidic media, e.g., silica gel, readily catalyze the conversion of **1** to **6**. 13-*cis*-**1**, however, was purified by HPLC on a cyano column (Et₂O/hexane) end-capped with TMS groups.¹⁹ Hydrolysis at the keto dimethyl acetal (**4**)

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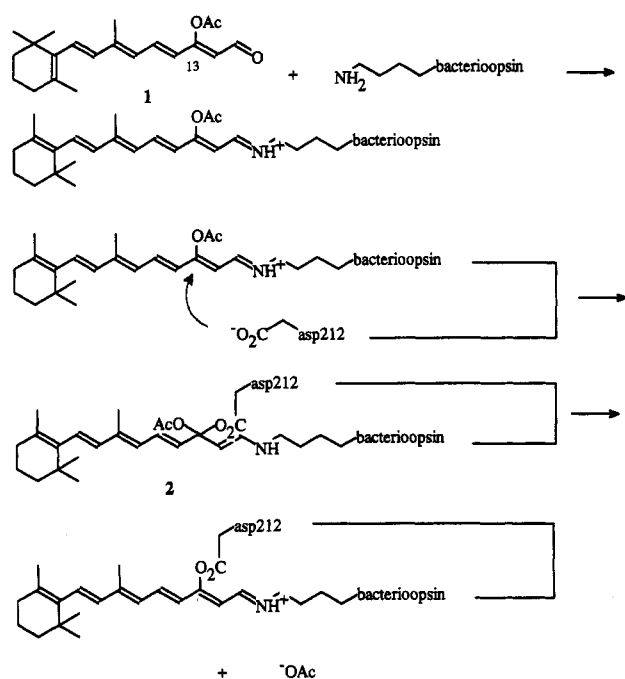
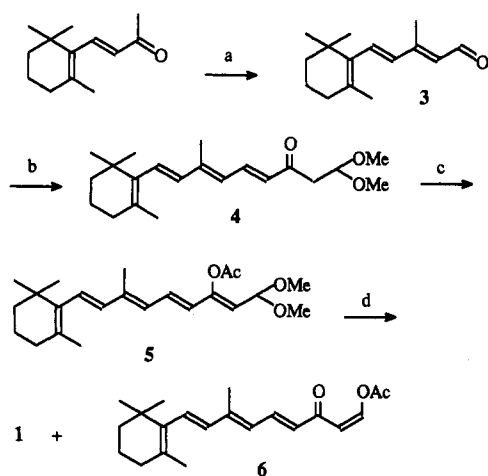
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Scheme 1

Scheme 2^a

^a (a) Reference 17; (b) $\text{CH}_3\text{COCH}_2\text{CH}(\text{OMe})_2/\text{NaH}$ (2 equiv)/THF, -78°C , 10 min at 0°C ; (c) (1) LDA/THF, -78°C , (2) $\text{Ac}_2\text{O}/\text{DMAP}$; (d) Bio-Rad AG 50W-X1/acetone.

stage and attempted acetylation of the anticipated 13-keto,15-enol/15-aldo,13-enol mixture under varied conditions, however, resulted instead in only the formation of the 13-keto 15-enol acetate (6). MM2 molecular mechanics coupled with AM1 semiempirical quantum calculations indicate that 6 is 4.8 kcal/mol more stable than 1, providing a rationale for the ready conversion of 1 to 6.

13-*cis*-1 forms a pigment (13-Ac-bR) with bacterioopsin in the dark which initially absorbs at 559 nm, but within 1 h in the

(19) The elution order is 6, *all-trans*-1, 13-*cis*-1. H-12 for 13-*cis*-1 is at $\delta 7.22$, indicative of a 13-*cis* geometry.²⁰ *all-trans*-1 has not yet been successfully separated from a coeluting impurity.

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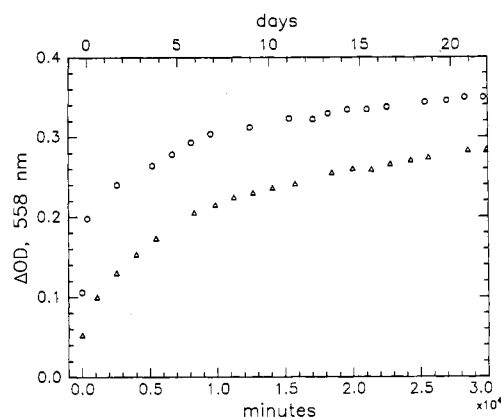


Figure 1. The course of replacement of 1 in 13-Ac-bR by *all-trans*-retinal. All operations are carried out in the dark under dim red light. 13-Ac-bR is fully formed, and the sample is divided exactly in half. To one (circles) is added excess *all-trans*-retinal immediately. The other (triangles) is allowed to sit for a week before the same excess amount of *all-trans*-retinal is added.

dark its maximum moves to 573 nm, 15 nm red-shifted with respect to that for native bR^{DA}. The λ_{max} (EtOH) of 13-*cis*-1 is 383 nm. The substantial red shift indicates protonated Schiff base formation between 13-*cis*-1 and the protein and that the chromophore fits well into the retinal binding pocket.²¹ The 559 \rightarrow 573 shift is reminiscent of the shift observed upon dark adaptation of native bR initially formed from 13-*cis*-retinal. Synthetic bR chromophores often undergo replacement by *all-trans*-retinal. Addition of excess *all-trans*-retinal to 13-Ac-bR results in the slow replacement of 1 by the native chromophore as evidenced by a shift of the maximum to 558 nm and an increase in absorbance. The non-pseudo first order kinetics exhibit a half-time of about 12 h at ambient temperature.

Upon long standing in the dark the 573 nm absorption of 13-Ac-bR slowly decreases with a concomitant increase in absorption at 406 nm indicative of a transformation. At ambient temperature about half of the 573 nm absorption is lost in 8 days. Addition of equal amounts of excess *all-trans*-retinal to equal aliquots of (a) freshly formed 13-Ac-bR and (b) an identical sample after 1 week in the dark indicates that the 13-Ac-bR aliquot suffering a substantial loss of its 573 nm absorption also loses a substantial amount of its ability to bind *all-trans*-retinal (Figure 1) by forming an intermediate which is not readily hydrolyzed.²² The nature of this inactivation reaction is under investigation and will be reported in the future.

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