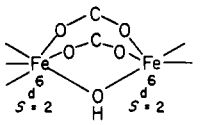
most reasonable choice is a hydroxo (or oxo) bridge, although a large protein rearrangement could lead to additional possibilities. No hydroxo or oxo bridged binuclear ferrous complexes are presently known, but it can be estimated that exchange in these systems should be somewhat larger than that of analogous Fe(III) dimers.[17] Values of $-J = 17$ and $\cong 100$ cm^{-1} have been reported for similar ferric model complexes[18] with hydroxide and oxo bridges, respectively. Thus while the actual magnitude of the exchange coupling is dependent on a number of factors, such as bridging angle, these values do indicate that a hydroxo or oxo bridging ligand is capable of mediating a $-J \geq 13$ cm^{-1} in a binuclear Fe(II) site. Thus the variable-temperature MCD is consistent with the presence of a hydroxo (or possibly oxo) bridge in deoxyhemerythrin. This, combined with an analysis of the near-IR–CD spectrum[8] which indicates one six- and one five-coordinate Fe(II), leads to a preliminary model for the deoxy active site.



Pertubations of the d–d bands in the CD spectrum (compare Figure 1, parts A amd B, zero field spectra) show that N_3^- and OCN^- ($K_B \cong 70$ M^{-1} at pH 7.7) and also F^- ($K_B \cong 7$ M^{-1}) bind to deoxy-Hr, making both irons six coordinate.[8] No other ligands (including Cl^-, Br^-, SCN^-, NCN^{2-}, or CN^-) bind with $K_B \geq 0.5$ M^{-1}. The MCD of these ligand-bound forms is dramatically different from that of deoxy-Hr, as shown for deoxy N_3^- in Figure 1b. The intensity now increases upon going to low temperatures requiring a paramagnetic ground state. In addition, the signal saturates very easily at low temperatures and high fields, generating a saturation–magnetization curve[19] consistent with $g_{eff} > 8$. This behavior indicates the ground state of N_3^-–deoxy-Hr is dominated by a large negative ZFS. Although some contribution from exchange coupling may also be present, its magnitude cannot be accurately determined from the present data. This ground state can give rise to EPR signals if limited rhombic splitting exists and relaxation is slow.[20] We do in fact observe an EPR signal (Figure 1C) that behaves as expected for an $M_S = \pm 2$ doublet, having $g_{eff} \cong 13$ and broadening rapidly with increasing temperature, finally disappearing above ~40 K.

It should be noted that these results provide a reasonable explanation for exchange of ^{18}O into the oxo bridge in the met derivatives, which is observed with only certain exogenous ligands present.[21] The ligands that lead to rapid ^{18}O exchange when the met species are formed (a deoxy intermediate has been implicated[22] in this process) are those that we find to bind strongly to deoxy-Hr and drastically alter the ground-state properties. This is consistent with exogenous ligand binding labilizing an endogenous hydroxo bridge.

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(17) This estimate results from the relation of the many-electron J to the one-electron J_{ij}, $J = (1/n^2) \sum_{ij} J_{ij}$, where i and j are the electrons on each iron center and n is the number of unpaired electrons on each Fe. The J for Fe(II) can be estimated to be on the order of 25/16 of J for Fe(III).

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Neither the Retinal Ring nor the Ring Double Bond Is Required for Proton Pumping in Bacteriorhodopsin: Acyclic Retinal Bacterioopsin Analogues

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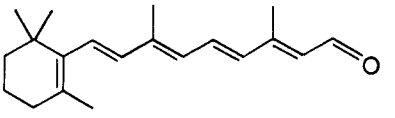
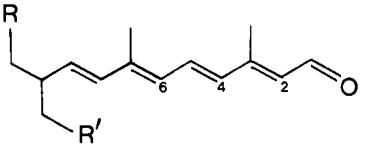
The light-transducing pigment bacteriorhodopsin is the protein in the purple membrane of the halophilic bacteria *Halobacterium halobium* responsible for proton pumping. Like the visual pigment rhodopsin, its chromophore, retinal **1**, binds with the apomembrane



(1)



(2) R=R′=CH_3

(3) R=H; R′=CH_3

(4) R=R′=H

by a protonated Schiff base linkage with the ϵ-amino group of a lysine residue. However, the mechanism of the recombination and interaction of retinal with the respective apoproteins, bacterioopsin and opsin, may be quite different. In the case of the rhodopsin, a specific binding site for one or more of the ring methyl groups has been suggested based on the data that β-ionone[1] and methylated cyclohexanes[2] inhibit the rate of rhodopsin regeneration and that the acyclic retinals lacking "ring methyl groups" do not form pigments with opsin.[3] However, β-ionone and cyclocitral have no inhibitory effect on the regeneration rate of bacteriorhodopsin.[4] Moreover, several retinal analogues such as the spin-labeled retinal[5] and phenyl retinal,[6] which have drastic modifications of the ring, do form pigments with bacteriorhodopsin although the λ_{max} are significantly blue shifted. These shifts may be due to a disruption of the secondary interaction of the chromophore with the charged group in the ring region, which was proposed to account for the red shift of bacteriorhodopsin.[7] All

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Table I. Absorption and Stability of Bacteriorhodopsin and Acyclic-Bacterioopsin Pigments[a]

retinal		pigment				
		λ_{max}, nm				
compd	λ_{max} in ethanol, nm	da	la	M	NH_2OH degradation of da, h	*trans*-retinal displacement of da, h
all-trans-**1**	381	560	570	412		
13-*cis*-**1**	377	560	570			
all-trans-**2**	373	487	487	372	5	72
2-*cis*-**2**	368	476	476		5	72
all-trans-**3**	364	487	487	363	5	72
2-*cis*-**3**	361	477	477		5	72
all-trans-**4**	364	487	487	365	5	24
2-*cis*-**4**	358	477	477		5	24

[a] λ_{max} ±3 nm. da, dark adapted pigment; la, light adapted pigment; M, intermediate formed on flash of actinic light >450 nm.

of these observations indicate that there might not be a specific chromophore ring binding site in bacterioopsin. We present here further evidence showing that there is no cyclohexyl ring binding site in bacterioopsin, on the basis of the formation of pigments with three acyclic retinal analogues, and we further describe experiments suggesting that the cyclohexyl ring and the ring double bond are not essential factors for photochemical and light-induced pH changes in the pigment.

The acyclic retinals **2–4** were synthesized and purified as previously reported.[3] The 2-cis, 6-cis, 2,6-dicis, and all-trans isomers of each analogue (corresponding respectively to the 13-cis, 9-cis, 9,13-dicis, and all-trans isomers of retinal) were isolated by high-pressure liquid chromatography (HPLC) and identified by nuclear magnetic resonance as previously described.[3] Pigments were regenerated from bleached purple membrane[8] and the acyclic retinals by reported procedures.[9] When the all-trans or 2-cis isomers of **2–4** are combined with bacterioopsin in the dark, an increase in absorption with a λ_{max} of 487 nm is observed indicating pigment formation. The rate of pigment formation (as measured by $t_{1/2}$ under pseudo-first-order conditions with respect to the retinal) is approximately the same as for regenerated bacteriorhodopsin for *all-trans*-**2** and -**3** ($t_{1/2}$ = 160 ± 20 s) but significantly slower for *all-trans*-**4** ($t_{1/2}$ = 285 ± 25 s). Extractions of the pigment chromophore with methylene chloride followed by HPLC analysis for identification of the attached chromophore show the dark-adapted pigments formed with *all-trans*-**2**, -**3**, or -**4** to contain the all-trans isomer of the respective chromophore. The dark-adapted 2-cis pigments likewise contain the 2-cis isomer with small quantities of the corresponding all-trans isomers.

The reaction of these acyclic pigments with the native chromophore, *all-trans*-retinal, and with hydroxylamine was investigated as a measure of pigment stability (Table I). 2-cis-**2** and -**3** or *all-trans*-**2** and -**3** pigments are reasonably stable to an excess of *all-trans*-retinal, showing a slow decrease in absorption at 480 nm and increase at 560 nm over 72 h. The *all-trans*- and 2-*cis*-**4** pigments are less stable, the acyclic chromophore being completely displaced after 24 h. All the acyclic pigments show some sensitivity to hydroxylamine (10 mM), decomposing over 5 h as determined by a decrease in absorption at 487 nm.

At room temperature there is an apparent lack of light/dark adaptation of these pigments. However, at 4 °C, a red shift in the absorption is observed. The light-adapted acyclic pigments return to the dark-adapted form rapidly after warming, indicating that the rate of light–dark adaption is too rapid to be measured at room temperature. On a short flash of light,[10] the all-trans pigments show an increase in absorbance within 0.2 ms at wavelengths shorter than the λ_{max} of the pigment (Table I). This

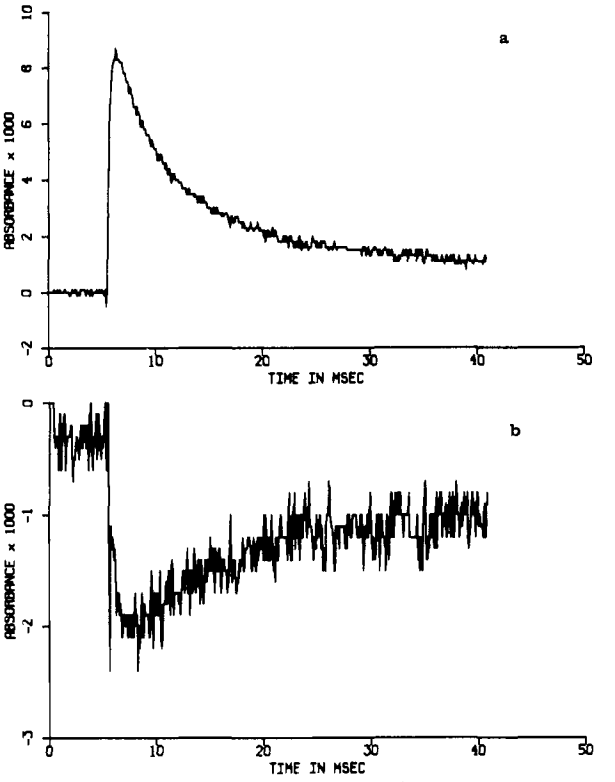

Figure 1. Flash-induced absorbance changes of (a) the M intermediate and (b) *p*-nitrophenol for *all-trans*-**2** pigment sheets suspended in 0.5 M KCl. Each trace is an average of six flashes: actinic > 450 nm (Xe flash plus Corning CS 3-72 filter); measuring at 380 nm; pH 6.68 ± 0.50; pigment $OD_{490} \simeq 0.25$; *p*-nitrophenol 38 μM; temperature 20 °C.

new short-wavelength species decays with a half-life of ~5 ms, which is similar to the decay of the short-wavelength M intermediate of bacteriorhodopsin (e.g., Figure 1a) for all-trans pigment. These M intermediates are somewhat blue shifted from the M intermediate of native bacteriorhodopsin, which is reasonable as these analogues contain one less double bond. For determination of the number of moles of M intermediate, an extinction coefficient of 28 000 was used for all three trans pigments.

Flash-induced proton release and uptake were determined as described earlier[10] by measuring the flash-induced absorbance change at 380 nm in the absence and presence of a pH-sensitive dye, *p*-nitrophenol. Absorption change (ΔA dye) of the dye was obtained by subtracting ΔA with dye from ΔA without dye (e.g., Figure 1b) for *all-trans*-**2** pigment. The number of moles of protons released was determined by calibration of ΔA dye with HCl. The ratio of released protons to M is calculated to be approximately 2.2–2.9 for all three pigments, indicating fully active pigments.

The resonance Raman spectrum of the *all-trans*-**2** pigment shows a band at 1652 cm^{-1}, corresponding to the C–N stretching vibration which shifts 15-cm^{-1} downward upon suspension in D_2O. This result indicates that the Schiff base C=N covalent linkage between the retinal analogue and the apomembrane is a protonated Schiff base as in bacteriorhodopsin. Moreover, the magnitude of the deuteration shift is the same as for bacteriorhodopsin and other bacteriorhodopsin analogues. We have previously argued that the size of the isotope effect on the Schiff base frequency is indicative of its local environment.[11] Thus the present result suggests that the environment of this acyclic **2** pigment near the Schiff base is like that of bacteriorhodopsin. In photostationary measurements of the *all-trans*-**2** pigment at 20 °C, two major ethylenic C–C stretch bands were observed, at 1558 and 1573 cm^{-1}. The 1573-cm^{-1} band disappeared for measurements on

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samples at 80 K. The C–C band at 1558 cm^{-1} represents the initial all-trans pigment while that at 1575 cm^{-1} almost certainly arises from the short-wavelength M-type intermediate absorbing at ca. 380 nm. The results agree with previously determined correlation between λ_{max} and C=C stretching frequency and expected temperature dependence of M formation.[12]

On combination of the 6-cis and 2,6-dicis isomers of **2–4** with bacterioopsin, a broad absorption is noted with a λ_{max} of 450–460 nm with a shoulder at 510 nm. Extraction of the pigment with methylene chloride yields the respective 6-cis or 2,6-dicis isomer. These pigments are rapidly (~5 min) destroyed by hydroxylamine or *all-trans*-retinal. On irradiation, the absorption shifts to 487 nm, increases in intensity, and is identical with that of the all-trans pigments. Only the respective all-trans isomer is obtained when these irradiated pigments are extracted with methylene chloride. These results indicate that an unstable associate of the 6-*cis*- and 2,6-*dicis*-**2–4** retinals and bacterioopsin is formed, possibly allowed by the lack of the cyclohexyl ring and thus the greater flexibility of the chromophore.

The above experiments demonstrate that these acyclic chromophores can form pigments with bacterioopsin which show light-induced absorption and pH changes. As these retinal derivatives lack both a cyclohexyl ring and the fifth C=C, neither of these structural elements are evidently essential for these functions of the pigment. A similar but more restrictive conclusion has been proposed for the 5,6-ethylene bond by our studies of the 5,6-dihydroretinal pigments.[13] These results are consistent with isomerization and charge separation being the primary event[14] and indicate that the proton pumping and photocycling are dependent on the polyene chain portion of the retinal chromophore.

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Synthetic Studies on the Avermectins: Substituent Effects on Intramolecular Diels–Alder Reactions of *N*-Furfurylacrylamides and Further Reactions of the Cycloadducts

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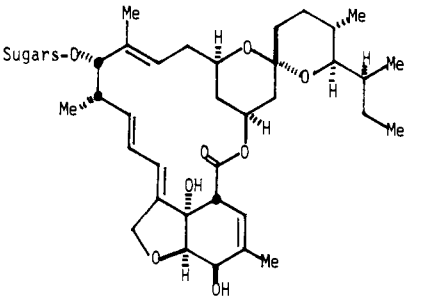
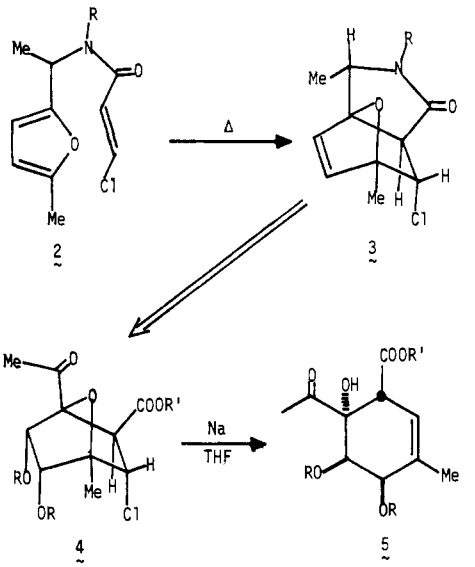
The avermectins[1] and milbemycins[2] are a recently discovered class of pentacyclic lactones having potent biological activity. Those compounds with a dihydroxyhexahydrobenzofuran unit have greater insecticidal activity than those with a tetrasubstituted-benzene unit,[3] with ivermectin (**1**) being the best broad-spectrum



1

antiparasitic agent. Although two excellent syntheses of milbemycin β_3 have been published[4] as well as routes to the top part of the molecule and attached sugars,[5] essentially no work has appeared on preparing properly substituted polyhydroxytetrahydrobenzoic acids for conversion into the bottom part of the avermectins. We now describe a process in which the intramolecular Diels–Alder adducts of *N*-furfuryl-β-chloroacrylamides can be reductively eliminated to give hydroxylated tetrahydrobenzoates as a model for the bottom part of the avermectins.

Our approach to the bottom half of **1** has two key constructive steps: an intramolecular cycloaddition of an *N*-furfuryl-β-chloroacrylamide **2** to give **3** and the reductive elimination of the chloro ether **4**, derived from **3**, to give **5**. The internal Diels–Alder



2 **3** **4** **5**

reactions of furans is a quite useful process, with tertiary *N*-furfurylacrylamides giving the adducts in high yield.[6–8] We have found that a methyl group on the connecting chain greatly accelerates the Diels–Alder reaction, affording one diastereomer in pure form.

The *N*-furfurylacrylamides **8a–h** were prepared from the corresponding 2-acetylfurans or furaldehydes **6a** by a simple

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