Recl. Trav. Chim. Pays-Bas 112, 303–308 (1993) 0165–0513/93/05303–06$06.00

The influence of the 5-methyl group in bacteriorhodopsin

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(Received December 21, 1992)

Abstract. All-*E*-5-bromo-5-demethylretinal was prepared in 6% yield starting from 4,4-dimethyl-2-cyclohexenone via 2-bromo-6,6-dimethyl-1-cyclohexenecarboxaldehyde. The crucial step in the preparation of the intermediate is a 2,3 Wittig rearrangement. 5-Bromo-5-demethylbacteriorhodopsin is easily formed from all-*E*-5-bromo-5-demethylretinal and bO. Due to the fact that the bromo substituent has about the same steric requirements as the 5-methyl group, the influence of electronic factors on the properties of bacteriorhodopsin could be studied. We conclude that the contribution of the 5-methyl group in bR to the proton-pump action and opsin shift is due to its electron-donating properties.

Introduction

The membrane protein bacteriorhodopsin (bR) belongs to the important class of retinal proteins. It is present in the purple membrane of *Halobacterium halobium*[1,2]. bR is folded into seven transmembrane helices; it functions as a light-driven proton pump that converts the energy of light into that of a proton gradient over the bacterial membrane. The bacterium uses this energy to generate ATP to drive its life processes. The chromophore is all-*E*-retinal, bound to the 6-amino group of lysine-216 via a protonated Schiff base (PSB) linkage[3], see Figure 1A.

The λ_{max} value of light-adapted bR (568 nm) is much larger than that of the model PSB compound from all-*E*-retinal and butylamine (440 nm in methanol). The red shift in absorption maximum of bR, relative to its model PSB, is due to interaction of the chromophore with the protein chain. The difference in wave numbers (5100 cm^{-1}) has been called the opsin shift[4]. Several factors contribute to the opsin shift, amongst them delocalization of the positive charge and interaction with the counter-ion[5].

In a well established, bioorganic approach, bR analogues are regenerated by reaction of retinal analogues with the free protein bacterioopsin (bO), obtained by bleaching of bR in the presence of hydroxylamine[6]. We have used this approach to regenerate bR analogues with a fixed 6–7-*s* conformation[7]. This study showed that 1200 cm^{-1} of the opsin shift is due to a change in conformation around the 6–7 single bond when retinal binds to opsin. We also found[8,9] that replacing the 5-methyl group by hydrogen reduces the proton-pump efficiency by 40% and reduces the opsin shift by 650 cm^{-1}.

We have now prepared the novel all-*E*-5-bromo-5-demethylretinal (**1**; see Figure 1B). **1** is sterically similar to retinal, the radius of the bromine atom being almost equal to the radius of the methyl group. Electronically, **1** differs from retinal, as the electron-donating methyl group has been replaced by the electron-withdrawing bromine. In bR, the isomorphous all-*E*-5-bromo-5-demethylretinal is expected to adopt the same conformation as native retinal. Study of 5-bromo-5-demethylbR will provide more information on the steric and electronic influence of the 5-methyl group on the proton-pump action and the opsin shift.

A molecular model of bR has only recently become available with the aid of high-resolution cryo-electron microscopy[10]. The resolution of this model is limited and the chromophore cannot be located with this technique. Bacteriorhodopsins with specifically deuterated chromophores have been used to obtain information on the location of the chromophore in bR by means of neutron-diffraction analysis[11]. 5-Bromo-5-demethyl-bR, having a heavy atom in a well-defined position, is a good model for studying the location of the chromophore in bR by means of neutron-diffraction analysis.

In this paper, we describe the synthesis and spectroscopy of all-*E*-5-bromo-5-demethylretinal and 5-bromo-5-demethylbacteriorhodopsin, and the effect of the 5-methyl group on the opsin shift and proton-pump activity.

Synthesis

For the preparation of all-*E*-5-bromo-5-demethylretinal (**1**), the novel 2-bromo-6,6-dimethyl-1-cyclohexenecarboxaldehyde (**9**) is the key intermediate, containing the 5-bromo group, that can be extended to **1** via a well documented four-step chain-elongation sequence that has been used in the synthesis of retinal and many modified retinals[12,13].

For the preparation of **9** we used commercially available 4,4-dimethyl-2-cyclohexenone (**2**) as starting material. Treatment of **2** with bromine and subsequent dehydrobromination by 1,5-diazabicyclo[4.3.0]non-5-ene (DBN) gave 2-bromo-4,4-dimethyl-2-cyclohexenone (**3**). The ketone function was reduced by diisobutylaluminum hydride (dibal) to the corresponding alcohol **4**, without affecting the vinylic bromine.

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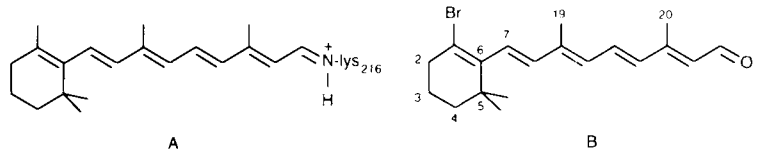


Fig. 1. Structure of chromophore in bR (A) and structure and numbering of **1** *(B).*

2-Bromo-4,4-dimethyl-2-cyclohexenol (**4**) was converted into alcohol **8** in a three-step one-pot sequence. The alcohol was deprotonated by potassium hydride and the anion was reacted with tributyl(iodomethyl)tin[14] (**5**) in an S_N2 reaction to give tin compound **6**. Treatment of **6** with butyllithium gives carbanion **7** and tetrabutyltin. Carbanion **7** undergoes 2,3 sigmatropic rearrangement to give 2-bromo-6,6-dimethyl-2-cyclohexenemethanol **8**. In this way, **8** was obtained from **4** in 45% yield at 72% conversion.

Oxidation of the alcohol function and shift of the double bond give the crucial 2-bromo-6,6-dimethyl-1-cyclohexenecarboxaldehyde (**9**). Oxidation was effected by a mild method that converts unconjugated primary alcohols into the corresponding aldehydes[15]. Alcohol **8** was treated with a complex of *N*-chlorosuccinimide and dimethyl sulfide in toluene, followed by treatment with triethylamine to give **9** in 84% yield based on **8**. The double bond was moved into conjugation by treatment of the unconjugated aldehyde with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). In this way, **9** was obtained in 23% yield based on **2** as starting material.

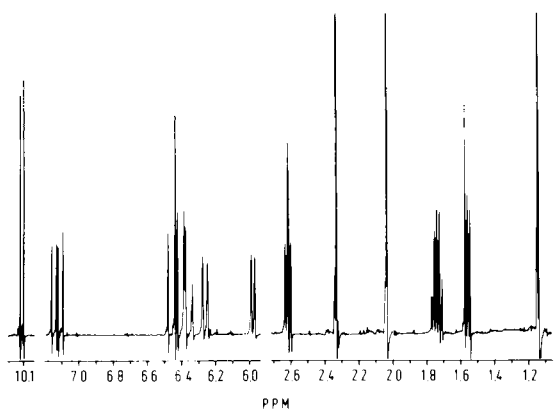


*Fig. 2. 400-MHz ^{1}H-NMR spectrum of all-*E*-5-bromo-5-demethylretinal (***1***) in $CDCl_3$.*

Aldehyde **9** was coupled in a Horner–Emmons reaction to 4-(diethylphosphono)-3-methyl-2-butenenitrile to give the β-ionylideneacetonitrile analogue. The all-*E* nitrile was separated and submitted to reduction by dibal. The resulting aldehyde **10** was reacted with 4-(diethylphosphono)-3-methyl-2-butenenitrile in a second Horner–Emmons reaction to produce the retinonitrile. Reaction of the retinonitrile with dibal gave an isomeric mixture with **1** as the main constituent. Isomerically pure all-*E*-5-bromo-5-demethylretinal (**1**) was obtained after SiO_2-column chromatography.

Spectroscopy

Mass spectrometry

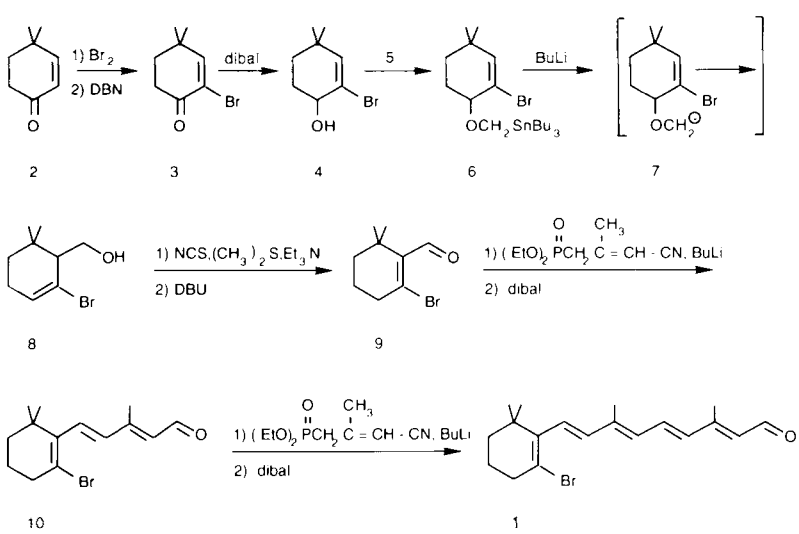
The high-resolution electron impact mass spectrum of all-*E*-5-bromo-5-demethylretinal (**1**) shows the molecular ion as parent peak at *m/z* 348.1094 (calculated for $C_{19}H_{25}OBr$: 348.1089).

The single-focus electron-impact spectrum (at 70 eV) shows the molecular ion at *m/z* 348 and 350 (with almost equal intensity), a clear indication of the presence of one bromine atom with its natural abundance of 50.7% ^{79}Br and 49.3% ^{81}Br. Other strong peaks appear at *m/z* 319 and 321 ($M^{\cdot+}$ – 29), 269, 251, 202, 177, 149, 106, 91, 77.

NMR spectroscopy

The 400-MHz ^{1}H-NMR spectrum of all-*E*-5-bromo-5-demethylretinal (**1**) [a] (in $CDCl_3$) is reproduced in Figure 2. In the low-field part of the spectrum, the signals of the conjugated chain are easily recognized and in complete agreement with the all-*E* structure. 15-H and 14-H form an AX pattern (δ_{15H} 10.11 ppm, δ_{14H} 5.97 ppm, *J* 8.1 Hz),

[a] For signal designations in **1**, the IUPAC retinoid numbering [23] is used.



*Scheme 1. Synthesis of all-*E*-5-bromo-5-demethylretinal (***1***).*

Table I ^1H and ^{13}C chemical shifts of **1** *and difference of chemical shifts of* **1** *with that of all-E-retinal ($\Delta\{^1H\}$ and $\Delta\{^{13}C\}$)*

H	^1H	$\Delta\{^1H\}$	^{13}C	$\Delta\{^{13}C\}$
1	–		37.5	+3.0
2	1.56	+0.10	39.2	−0.4
3	1.74	+0.12	20.7	+1.5
4	2.61	+0.58	38.1	+4.9
5	–	–	123.7	−6.8
6	–	–	141.3	+3.7
7	6.35	+0.01	129.2	−0.5
8	6.46	+0.30	137.8	+0.7
9	–	–	140.5	−0.8
10	6.26	+0.07	130.8	+1.4
11	7.13	−0.01	132.1	−0.4
12	6.40	+0.03	135.2	+0.7
13	–	–	154.4	−0.4
14	5.97	0.00	129.2	+0.2
15	10.11	0.00	190.9	−0.2
16, 17	1.14	+0.10	28.5	−0.5
19	2.03	0.00	12.8	−0.2
20	2.33	0.00	13.0	−0.1

10-H, 11-H and 12-H form an ABC pattern (δ_{10H} 6.26 ppm, δ_{11H} 7.13 ppm, δ_{12H} 6.40 ppm, $J_{10H\text{-}11H}$ 11.5 Hz, $J_{11H\text{-}12H}$ 15.1 Hz). 7-H and 8-H form the pattern of a *trans*-ethene fragment (δ_{7H} 6.35 ppm, δ_{8H} 6.46 ppm, J 16.3 Hz) in which 7-H appears as a double multiplet, resulting from additional coupling to 4-H (J 1.7 Hz).

In the low–high-field part of the spectrum, the aliphatic signals arise. The signals of the 9-CH_3 and 13-CH_3 groups appear both as a doublet (δ_{19H} 2.03 ppm, $J_{19H\text{-}10H}$ 1.2 Hz; δ_{20H} 2.33 ppm, $J_{20H\text{-}14H}$ 1.2 Hz) and the two 1-CH_3 groups appear as one sharp singlet (δ 1.14 ppm). The remaining signals are the multiplets from the cyclohexene ring, 2-CH_2 (δ 1.56 ppm), 3-CH_2 (δ 1.74 ppm), 4-CH_2 (δ 2.61 ppm). The chemical shifts are collated in Table I. From comparing the spectra of **1** and all-*E*-retinal[16], it is clear that the introduction of bromine induces downfield shifts of the ring protons: 16,17-H (0.10 ppm), 2-H (0.10 ppm), 3-H (0.12 ppm), 4-H (0.58 ppm). The field effect of bromine shifts 8-H downfield by 0.30 ppm. The other signals are not affected by the introduction of bromine (see Table I).

^{13}C-NMR spectroscopy gives information about the backbone of the molecule. In the ^1H-noise-decoupled 75-MHz ^{13}C-NMR spectrum of **1** (in $CDCl_3$), 18 signals are present, in accordance with the 18 different carbon atoms. In the sp^2 region 11 signals are present. The signals of the proton-bearing carbons were assigned from the ^1H–^{13}C-COSY spectrum: 129.2 ppm (7-C), 137.8 (8-C), 130.8 (10-C), 132.1 (11-C), 135.2 (12-C), 129.2 (14-C) and 190.9 ppm (15-C). To assign the quaternary signals, the long-range ^1H–^{13}C-COSY spectrum ws recorded: δ 123.7 ppm (5-C), 141.3 (6-C), 140.5 (9-C) and 154.4 ppm (13-C).

In the sp^3 region, 7 signals are present. The signals from the 1-, 9- and 13-methyl groups are at δ 28.5, 12.8 and 13.0 ppm, respectively. These signals gave a negative peak in the APT spectrum. The four remaining signals are at δ 37.5 ppm (1-C), 39.2 (2-C), 20.7 (3-C) and 38.1 ppm (4-C), The chemical shifts of **1** were compared with those of all-*E*-retinal (see Table I). The largest shift is the α-effect on C-5 (6.8 ppm upfield shift). At C-4 and C-6 a β-effect of 4.9 and 3.7 ppm downfield shift, respectively, is observed and C-1 is shifted 3.0 ppm downfield. These shifts are in good agreement with those found for other alkenyl bromides[17].

FT-IR spectroscopy

The FT-IR spectrum was recorded and a tentative assignment was made by comparison with the IR spectrum of all-*E*-retinal, which has been completely analysed[18]. At 731 cm^{-1}, the C–Br stretch vibration is present. All-*E*-retinal does not show absorptions in this region. The strong signal from the C=O stretch vibration arises at 1658 cm^{-1}. Strong C=C stretch vibration absorption is present at 1569 cm^{-1}. This frequency is shifted 7 cm^{-1} towards a lower wavenumber relative to its value in all-*E*-retinal. The single-bond-stretch vibrations give medium peaks in the 1202–1119 cm^{-1} region. At 976 and 961 cm^{-1}, the hydrogen-out-of-plane vibrations of the disubstituted *trans*-alkenes absorb weakly (966 cm^{-1} in all-*E*-retinal); at 871 cm^{-1} (876 and 887 cm^{-1} in all-*E*-retinal)[18] the hydrogen-out-of-plane vibrations of the trisubstituted alkenes can be found.

UV/Vis spectroscopy

The electronic absorption spectrum of all-*E*-5-bromo-5-demethylretinal (**1**) (in ethanol) shows a broad bell-shaped curve without vibrational fine structure, with λ_{max} at 374 nm. This is a 9-nm hypsochromic shift relative to all-*E*-retinal (383 nm in ethanol). This shift is probably entirely due to electronic changes, assuming that both **1** and all-*E*-retinal adopt the same conformation in solution.

Analogue pigment studies

Bacterioopsin reacts easily with all-*E*-5-bromo-5-demethylretinal (**1**) at room temperature to form, within seconds, the bR analogue bR(**1**) with a λ_{max} at 535 nm (ϵ_{max} $54 \cdot 10^3$). With only a slight excess of **1**, the conversion of bacterioopsin to bR(**1**) is complete after 20 minutes. If bR(**1**) is treated with excess of all-*E*-retinal, no increase in absorption at 568 nm is detected, indicating that regeneration is complete and stable towards displacement by retinal. This means that the retinal analogue occupies the binding pocket.

BR(**1**) does show light–dark adaptation. After leaving to stand in the dark for a few hours, the λ_{max} value has shifted to 529 nm (ϵ_{max} $60 \cdot 10^3$). It completely reverts to the light-adapted form (λ_{max} 535 nm, ϵ_{max} $54 \cdot 10^3$) upon exposure to visible light.

From the λ_{max} values of light-adapted bR(**1**) and the *n*-butylamine-protonated Schiff base of **1** (λ_{max} 430 nm), the opsin shift of bR(**1**) is calculated to amount to 4560 cm^{-1}, only 540 cm^{-1} less than that of the parent compound.

Proton-pump action

We reconstituted bacteriorhodopsin and bR(**1**) in soybean-phospholipid vesicles. Illumination of the vesicles with visible light rapidly increased the pH of the external medium. The resulting proton gradient decreases the velocity of proton uptake asymptotically until a steady state is reached, in which light-driven proton uptake equals the passive back-leakage. The extent of proton uptake is measured as the difference between the pH in the outside medium before illumination and the pH reached at the steady state, expressed as nmol H^+/mg bR. When the light is then turned off, the protons re-equilibrate until the proton gradient has disappeared.

bR(**1**) as well as native bR and bO were reconstituted in liposomes. For natural bR, a proton-pump activity of 211 nmol H^+/mg was recorded, with an initial velocity of 530 nmol $H^+/(mg \cdot min)^{-1}$. Under the same conditions, for bR(**1**) a value of 27 nmol H^+/mg was recorded, only 13% of the value recorded for natural bR. The initial velocity of proton uptake of bR(**1**) amounts to 60 nmol $H^+/(mg \cdot$

min)$^{-1}$, only 11% of the value recorded for natural bR. The liposomes containing bacterioopsin showed no proton-pump activity.

Discussion

We prepared all-*E*-5-bromo-5-demethylretinal (**1**) on a 100-mg scale in 6% overall yield based on 4,4-dimethyl-2-cyclohexenone (**2**). **2** was first converted into the corresponding 5-bromo derivative by a bromination and dehydrobromination sequence. During upscaling of this reaction to the 10-gram scale, a substantial amount of bromination of the sp^3 carbon atom α to the carbonyl group occurs. Treatment of **2** with phenylselenyl bromide in the presence of pyridine gives pure **3** on the 10-gram scale. We prepared the corresponding iodide via treatment of **2** with diphenyl diselenide and iodine. However, this compound could not be converted into the corresponding 2-iodo-6,6-dimethyl-1-cyclohexenecarboxaldehyde: the 2,3 Wittig rearrangement of the iodo analogue to the iodo analogue of **6** did not take place. One-pot conversion of **4** into **8**, including the 2,3 Wittig rearrangement, gave **8** in 36% after optimization. It is known that the 2,3 Wittig rearrangement does not proceed quantitatively for sterically hindered compounds[19].

The novel all-*E*-bromo-5-demethylretinal (**1**) reacts with bacterioopsin to form bR(**1**) as efficiently as normal bR, with an opsin shift of 4560 cm^{-1}, which is 540 cm^{-1} smaller than that of bR. The light-driven proton-pump activity of bR(**1**) is only 13% of native bR and the initial velocity of proton translocation was found to be 11% of the value recorded for native bR. Because the Van-der-Waals radius of the bromo substituent is similar to that of the methyl group, this decrease in opsin shift and proton-pump activity must be related to the electron-withdrawing effect of the bromine compared to the electron-donating effect of the 5-methyl group. This result makes it plausible that the contribution of the 5-methyl group to the proton-pump activity is related to the electron-donating effect of the 5-methyl group.

bR(**1**) forms two-dimensional crystals, just as native bR. An excellent neutron-diffraction pattern has been recorded from a sample of oriented crystals. The structural analysis of the diffraction pattern will be the subject of a future publication.

18,18,18-trifluoro-bR[20], having the sterically more demanding strongly electron-withdrawing CF_3 group, does not show light-induced proton-pump activity. Only one bR analogue has been prepared with an electron-donating substituent stronger than the 5-methyl group, namely 5-demethyl-5-methoxy-bR[21]. Its proton-pump activity amounts to 20% of that of the native system. In this case, the steric demands of the large methoxy group may compensate for electronic factors.

Experimental

General

Chemicals were purchased as reagent-grade from Janssen Chimica (Belgium) or Aldrich (MO, USA). The following solvents were distilled prior to use: tetrahydrofuran (THF; from $LiAlH_4$), diethyl ether (from P_2O_5), petroleum ether (b.p. 40–60°C; from P_2O_5), CH_2Cl_2 (from CaH_2).

Reactions were carried out in a nitrogen atmosphere. Diisobutylaluminum hydride (dibal) was used as an 1.0 M solution in hexanes, *n*-butyllithium (BuLi) as an 1.6M solution in hexanes. These solutions were introduced into the reaction mixture via a syringe.

NMR spectra were run in $CDCl_3$ (with tetramethylsilane as internal standard, δ 0) at a Jeol FX-200 or a Bruker MSL-400 (operating at 199.5 MHz and 400.1 MHz, respectively, for ^{1}H and 50.1 MHz and 100.6 MHz for ^{13}C). UV/Vis spectra were run on a Varian DMS-200, using ethanol or *n*-hexane (spectroscopic grade) as solvent. Infrared spectra were run on a Bruker IFS-113v, with samples in CsI. The regeneration experiments were followed spectroscopically on a Pharmacia NovaspecII, connected to a Macintosh PC. Bacterioopsin was prepared according to published procedures[22].

Electron-impact (EI) mass spectra were recorded at 70 eV and 15 eV on a V.G. Micromas ZAB-2HF mass spectrometer, an instrument with reverse geometry, fitted with a high-field magnet and coupled to a V.G. 11/250 data system. The samples were introduced via a direct insertion probe into the ion source. The ion-source temperature was generally 150°C. During the high-resolution EIMS measurements a resolving power of 20000 (10% valley definition) was used.

Evaporation of solvents was performed *in vacuo* (20 mmHg). Purification was performed by flash SiO_2-column chromatography, using ether/petroleum ether as eluent, unless stated otherwise.

2-Bromo-4,4-dimethyl-2-cyclohexenone (**2**)

4,4-Dimethyl-2-cyclohexenone (2.0 g, 16 mmol) was dissolved in 20 ml of $CHCl_3$ and cooled to 0°C. A solution of 2.6 g (16 mmol) in 10 ml of $CHCl_3$ was added dropwise. After stirring for $\frac{1}{2}$h at 0°C, 4.0 g (32 mmol) of 1,5-diazabicyclo[4.3.0]non-5-ene was added dropwise. The mixture was stirred for 3 h at room temperature and 60 ml of a 1M solution of HCl were then added. The organic layer was separated and the water layer was extracted thrice with ether. The combined organic layers were washed with brine and dried over $MgSO_4$; the solvents were evaporated. Yield 3.1 g (95%) of **2**. ^{1}H NMR (200 MHz), δ: 7.14 ppm, s, 3-CH; 2.66, t, J 6.8 Hz, 6-CH_2; 1.93, t, J 6.8 Hz, 5-CH_2; 1.23, s, 2 4-CH_3.

2-Bromo-4,4-dimethyl-2-cyclohexenol (**4**)

2-Bromo-4,4-dimethyl-2-cyclohexenone (3.1 g, 15 mmol) was dissolved in 15 ml of CH_2Cl_2 and 15 ml of petroleum ether and cooled to −80°C. Dibal (20 mmol) was added via a syringe and the mixture was allowed to warm to room temperature (1 h). A slurry of 15 g of SiO_2 and 10 ml of water was then added. The mixture was stirred for 1 h at room temperature. $MgSO_4$ was then added and the solids were filtered off. After evaporation of the solvents, 2.3 g (73%) of **4** was obtained. m.p. 49–50°C. ^{1}H NMR (200 MHz). δ: 5.94, s, 3-CH; 4.16, dt, J 5.1 Hz, J 4.1 Hz, 1-CH; 2.22, d, J 4.1 Hz, −OH; 2.11–1.80, m 6-CH_2; 1.69–1.39, m, 5-CH_2; 1.06, s, 1-CH_3; 1.01, s, 1-C'H_3. ^{13}C NMR (50 MHz), δ: 141.9, 3-C; 124.5, 2-C; 69.7, 1-C; 36.0, 4-C; 32.2; 29.0, 28.9, 2 1-CH_3; 27.9. IR: 3270, 3020, 1640, 1045, 880, 770 (=C–Br stretch).

Tributyl(iodomethyl)tin (**5**)

Copper(II) acetate (0.37 g, mmol) was warmed up in 40 ml of glacial acetic acid to 100°C. Zinc dust (24 g, 0.37 mol) was then added. The mixture was stirred for 2 min; the acetic acid was then decanted; finally 40 ml of fresh acetic acid was added. The mixture was stirred again and decanted again. The zinc–copper couple was washed thrice with ether and, subsequently, the vessel containing the couple was flame-dried under flushing with N_2. THF (200 ml) was added. A solution of 100 g (0.37 mol) of diiodomethane in 60 ml of THF was added over 11 h. The mixture was then left to stirr at room temperature for 12 h. Tributyltin chloride (60 g, 0.19 mol) was added and the mixture was stirred for 24 h at room temperature. Petroleum ether (0.5 l) was then added and the mixture was washed thrice in brine, dried over $MgSO_4$ and filtered over celite. The solvents were evaporated. The residue was distilled to give 65 g of product; b.p. 125°C at 0.4 mmHg. ^{1}H NMR (200 MHz), δ: 1.93 ppm, s, 2H, −CH_2I; 1.57–1.49, m, 6H; 1.38–1.30, m, 6H; 1.02–0.87, m, 15H.

2-Bromo-6,6-dimethyl-2-cyclohexene methanol (**8**)

KH (1.3 g, 11 mmol) was added to 2.0 g (9.8 mmol) of **4** in 25 ml of THF and the mixture was stirred for 30 min. 18-Crown-6 (0.5 g, mmol) and 5.7 g (13 mmol) of organotin compound **5** were added and the mixture was stirred for 4 h at reflux to form **6**. The mixture was then cooled to −90°C and 16 ml of BuLi was added and the mixture was stirred for a further 1 h at −90°C. Satd. NH_4Cl solution was added and the water layer was extracted thrice with ether. The combined ethereal layers were washed with brine and dried over $MgSO_4$. After evaporation of the solvents, the product was purified, yielding 0.71 g (33%) of **8** and 0.55 g (28%) of **4**. ^{1}H NMR (200 MHz), δ: 6.22, t, J 3.7 Hz 3-H; 3.94–3.83, m, α-H; 2.14–2.03, m, 1-H + 4-H; 1.78–1.63, m, 5-H; 1.07, s, CH_3; 1.00, s, CH_3. ^{13}C NMR

(50 MHz), δ: 131.0, 3-C; 123.2, 2-C; 61.2, 1-C; 55.7, α-C; 34.1, 6-C; 31.6; 27.5, CH_3, 27.2, CH_3; 25.0.

2-Bromo-6,6-dimethyl-1-cyclohexene (**9**)

N-Chlorosuccinimide (0.6 g, 4.5 mmol) was dissolved in 25 ml of toluene (by heating to 60°C) and at −10°C, 0.47 g (7.5 mmol) of dimethylsulfide was added. A white precipitate formed immediately. After stirring for 30 min, the mixture was cooled to −25°C and 0.35 g (1.6 mmol) of **8** in 5 ml of toluene was added dropwise. The mixture was stirred for 4 h at −40 to −25°C. Triethylamine (0.45 g) was added and the reaction mixture was warmed to room temperature. After 30 min, 1M HCl solution was added and the water layer was extracted thrice with diethyl ether. The combined ethereal layers were washed with water (twice) and brine and dried over $MgSO_4$. The solvents were evaporated and the product was purified, yielding 0.29 g (84%). ^{1}H NMR (50 MHz), δ: 9.56, d, *J* 4.4 Hz, CHO; 6.39, dt, *J* 4.0 Hz, 1.0 Hz, 3-H; 2.92, dd, *J* 4.4 Hz, 4.0 Hz, 5-H; 2.27–2.16, m, 3-H; 1.64–1.37, m, 1-H; 1.04, s, CH_3; 1.03, s, CH_3. ^{13}C NMR (50 MHz), δ: 200.7; 132.2; 116.2; 65.9; 35.4; 32.4; 27.7; 25.6; 25.1.
Aldehyde was added to this (0.28 g, 1.3 mmol) in 25 ml diethyl ether. After stirring for 1 h, 40 ml of 1M HCl solution was added. The aqueous layer was extracted thrice with ether and the combined organic layers were washed with brine and dried over $MgSO_4$. The solvents were evaporated and the product was purified, yielding 0.24 g of **9** (86%). ^{1}H NMR (200 MHz), δ: 10.05, s, CHO; 2.75, t, *J* 6.3 Hz, 3-H; 1.75, m, 2-H; 1.53, m, 1-H; 1.22, s, CH_3. ^{13}C NMR (50 MHz), δ: 195.0; 145.5; 141.5; 40.2; 39.8; 36.7; 27.2; 20.5. UV/Vis, λ_{max} (ethanol): 258 nm.

2E,4E-5-(2-Bromo-6,6-dimethyl-1-cyclohexenyl)-3-methyl-2,4-pentadienal (**10**)

BuLi (1.6 mmol) was added to 0.43 g (2.0 mmol) 4-(diethylphosphono)-3-methyl-2-butenenitrile dissolved in 15 ml of THF at 0°C. After 15 min stirring, 0.23 g (1.0 mmol) of **9** in 3 ml of THF was added. The mixture was stirred for 19 h at room temperature. Satd. NH_4Cl was then added and the aqueous layer was extracted thrice with ether. The combined organic layers were washed with brine and dried over $MgSO_4$. The solvents were evaporated and the nitrile product was purified, yielding 0.20 g of a mixture of nitriles (67%). ^{1}H NMR (200 MHz); δ: (ring positions primed): 6.57, dd, *J* 16.3, 1.7 Hz, 5-H; 6.39, d, *J* 16.3 Hz, 4-H; 5.25, s, 2-H; 2.61, dt, *J* 6.3, 1.7 Hz, 2-H); 2.21, d, *J* 0.8 Hz, 3′-CH_3; 1.80–1.69, m, 4-H); 1.60–1.53, m, 5′-H); 1.12, s, 2 6′-CH_3. The product (0.20 g, 0.71 mmol) was dissolved in 10 ml of petroleum ether and at −70°C 1.4 mmol of dibal was added. The mixture was warmed to −20°C and a slurry of 2 g of SiO_2 and 0.5 ml of water was added. After stirring for 1 h, $MgSO_4$ was added and the solids were filtered off. The solvents were evaporated and the residue was purified to yield 0.11 g of isomerically pure **10** (54%). ^{1}H NMR (200 MHz); δ: 10.14 ppm, d, *J* 7.9 Hz, CHO; 6.74, d, *J* 16.5 Hz, 5-H; 6.46, d, *J* 16.5 Hz, 4-H; 5.99, d, *J* 7.9 Hz, 2-H; 2.62, dt, *J* 6.2, 1.4 Hz, 3′-H; 2.32, d, *J* 1.3 Hz, 3-CH_3; 1.81–1.68, m, 4′-H; 1.60–1.54, m, 5-H; 1.14, s, 2 6′-CH_3.

All-E-5-bromo-5-demethylretinal (**1**)

BuLi (0.8 mmol) was added to 0.22 g (1.0 mmol) 4-(diethylphosphono)-3-methyl-2-butenenitrile dissolved in 10 ml of THF at 0°C. After 15 min stirring, 0.11 g (1.0 mmol) of **10** in 3 ml of THF was added. The mixture was stirred for 4 h at room temperature. Satd. NH_4Cl was then added and the aqueous layer was extracted with ether thrice. The combined organic layers were washed with brine and dried over $MgSO_4$. The solvents were evaporated and the product was purified, yielding 0.12 g of a mixture of nitriles (89%). ^{1}H NMR (200 MHz), δ (retinoid numbering[23]): 6.94, dd, *J* 11.5, 15.0 Hz, 11-H; 6.45, d, *J* 16.4 Hz, 8-H; 6.33, br. d, 7-H + 12-H; 6.19, d, *J* 11.5 Hz, 10-H; 5.20, s, 14-H; 2.61, t, *J* 6.3 Hz, 4-H; 2.21, d, *J* 0.8 Hz, 13-CH_3; 2.01, s, 9-CH_3; 1.77–1.69, m, 3-H; 1.58–1.52, m, 2-H; 1.13, s, 1-CH_3. These nitriles (0.12 g (0.35 mmol) were dissolved in 10 ml of petroleum ether and at −70°C 0.7 mmol of dibal was added. The mixture was warmed to −20°C and a slurry of 1 g of SiO_2 and 0.2 ml of water was added. After stirring for 1 h, $MgSO_4$ was added and the solids were filtered off. The solvents were evaporated and the residue was purified to yield 0.10 g (84%) of a 13*Z*/13*E* mixture of 5-bromo-5-demethylretinal. Isomerically pure **1** was obtained after a second SiO_2-column chromatographic purification; yield 84 mg.

Schiff base and protonated Schiff base

The Schiff base was prepared in a cuvette in the UV/Vis photospectrometer by addition of excess of *n*-butylamine to a dilute solution of **1** in methanol. Addition of a drop of concentrated hydrochloric acid to this methanol solution completely converted the Schiff base into the protonated form.

Binding experiments

Binding experiments were performed as described earlier at room temperature. Regeneration was followed in 2-mm path-length cuvettes. Light–dark adaptation was performed as described earlier[9].

Incorporation of bR analogues in phospholipid vesicles and light-driven proton-pump action

bR (2 mg) was regenerated. bR, bR(**1**) and bO were then precipitated, the pellet was taken up in Millipore-filtered water and precipitated. This was repeated twice. The precipitate was then taken up in 3 ml of a solution of 0.15M KCl and 2mM EDTA (pH 7). The concentration was determined photospectroscopically. The bR solution (2 ml) was added to 50 mg of purified soybean phospholipids (asolectin)[24]. The suspension was then sonified using a MSE probe-type ultrasonifier (probe diameter 2 mm, freq. 21 kHz, ampl. 5 μm) for 15 s, followed by 45 s of cooling for 1 h[25]. The mixture was kept under nitrogen and cooled in ice during sonication. Using this procedure, liposomes containing bO, bR and bR(**1**) were prepared. Light-dependent pH changes were measured in a 2.5-ml temperature-controlled multi-purpose cuvette (25°C) equipped with a stirring device and containing 200 μl freshly prepared (modified) bR liposomes and 1.8 ml of a solution of 0.15M KCl, 0.2mM EDTA and 4 μg valinomycin at pH 7. The pH of the medium was measured continuously using an Ingold glass calomel electrode connected to an amplifier (Radiometer PHM 63) and recorded on a Pantos U-228 unicorder. The cuvette was illuminated with a cold light source. The pH changes upon illumination were calibrated by the addition of 50-nmol oxalic acid.

Acknowledgments

We would like to thank *R.H. Fokkens* (University of Amsterdam) for recording the mass spectra, *G.A. van Albeda* for recording the FT-IR spectra and Dr. *M.P. Heyn* (Freie Universität Berlin) for his interest in this work and for recording of the neutron-diffraction pattern.

References

1 *R.R. Birge*, Annu. Rev. Biophys. Bioenerg. **10**, 315 (1981).
2 *W. Stoeckenius* and *R.A. Bogomolni*, Annu. Rev. Biochem. **51**, 587 (1982).
3 *A. Lewis, J. Spoonhower, R.A. Bogomolni, R.H. Lozier* and *W. Stoeckenius*, Proc. Natl. Acad. Sci. USA **71**, 4462 (1974).
4 *K. Nakanishi, V. Balogh-Nair, M. Arnaboldi-Tanis, K Tsujimoto* and *B. Honig*, J. Am. Chem. Soc. **102**, 7947 (1980).
5 *H.J.M. de Groot, G.S. Harbison, J. Herzfeld* and *R.G. Griffin*, Biochemistry **28**, 3346 (1989).
6 *H.J.M. de Groot, S.O. Smith, J.L.M. Courtin, E.M.M. van den Berg, C. Winkel, J. Lugtenburg, R.G. Griffin* and *J. Herzfeld*, Biochemistry **29**, 6873 (1990).
7 *R. van der Steen, P.L. Biesheuvel, C. Erkelens, R.A. Mathies* and *J. Lugtenburg*, Recl. Trav. Chim. Pays-Bas **108**, 83 (1989).
8 *M.B. Spijker-Assink, G.W. Robijn, J.H. Ippel, J. Lugtenburg, B.H. Groen* and *K. van Dam*, Recl. Trav. Chim. Pays-Bas **111**, 29 (1992).
9 *M. Muradin-Sweykowska, L.J.P. van Amsterdam, L.J.M. Rodenburg, J. Lugtenburg, R.L. van der Bend* and *K. van Dam*, FEBS Lett. **154**, 180 (1983).
10 *R. Henderson, J.M. Baldwin, T.K. Ceska, F. Zemlin, E. Beckmann* and *K.H. Downing*, J. Mol. Biol. **213**, 899 (1990).
11 *T. Hauß, S. Grzesiek, H. Otto, J. Westerhausen* and *M.P. Heyn*, Biochemistry **29**, 4904 (1990).
12 *R.S.H. Liu* and *A.E. Asato*, Tetrahedron **40**, 1931 (1984).
13 *M. Groesbeek* and *J. Lugtenburg*, Photochem. Photobiol. **56**, 903 (1992).
14 *D. Seyferth* and *S.B. Andrews*, J. Organometallic Chem. **30**, 151 (1971).
15 *E.J. Corey, C.U. Kim* and *P.F. Misco*, Org. Synth. **58**, 122.
16 *D.J. Patel*, Nature **221**, 825 (1969).
17 *E. Breitmaier* and *W. Voelter*, Carbon-13 NMR spectroscopy, VCH Verlag, Weinheim, Germany 1987.

[18] *B. Curry, I. Palings, A.D. Broek, J.A. Pardoen, J. Lugtenburg* and *R.A. Mathies*, Adv. in infrared and Raman spectroscopy **12**, 115 (1985).
[19] *W.C. Still* and *A. Mitra*, J. Am. Chem. Soc. **100**, 1927 (1978).
[20] *V.J. Rao, F. Derguini, K. Nakanishi, T. Taguchi, A. Hosoda, Y. Hanzawa, Y. Kobayashi, C.M. Pande* and *R.H. Callender*, J. Am. Chem. Soc. **108**, 6077 (1986).
[21] *E. Kölling, D. Oesterhelt, H. Hopf* and *N. Krause*, Angew. Chem. Int. Ed. Engl. **26**, 580 (1987).
[22] *D. Oesterhelt* and *L. Schumann*, FEBS Lett. **44**, 262 (1974).
[23] IUPAC-IUB Joint commission on Biochemical Nomenclature, Eur. J. Biochem. **129**, 1 (1982).
[24] *Y. Kawaga* and *E. Racker*, J. Biol. Chem. **246**, 5477 (1971).
[25] *K.J. Hellingwerf*, Ph.D. Thesis, University of Amsterdam (1979), The Netherlands.