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Bacteriorhodopsin. The influence of the cyclohexene-ring methyls

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Abstract. Four ring-demethylated retinals, *viz.* 1,1′,5-tridemethylretinal,1,1′-didemethylretinal, 1,5--didemethylretinal and 1-didemethylretinal, have been synthesized via new and simple schemes. The properties of these modified retinals, their protonated *Schiff* bases and the corresponding bacteriorhodopsins have been studied and compared with the native system. These bacteriorhodopsin analogues have also been tested for their proton-pump efficiencies. A large decrease in proton-pump activity was found for the analogues lacking the 5-methyl group. On the whole, the opsin shifts of the modified bacteriorhodopsins were much lower than those of the native system. UV-Vis and ^1H NMR data support a planar 6-*s*-*trans* conformation for the demethylated retinals in solution rather than a twisted 6-*s*-*cis* conformation (torsion angle 40–60°) as found in retinal. This explains the lower opsin shift and the better fit of these demethylated retinals in bacteriorhodopsin's binding site, which, in its native form, contains a 6-*s*-*trans* chromophore.

1 Introduction

Chromo-proteins with a retinylidene chromophore such as bacteriorhodopsin, as well as visual pigments and retinochrome, are important in photobiology[1]. Bacteriorhodopsin (hereafter referred to as bR), the light-energy-converting protein of the purple membrane of the halophilic micro-organism *Halobacterium halobium*,occurs in a light--adapted (bR^{LA}_{568}; λ_{max} 568 nm) and in a dark-adapted form (bR^{DA}_{558}; λ_{max} 558 nm). The chromophore of the light--adapted form is an all-*trans* retinylidene moiety, bound as a protonated *Schiff* base to Lys 216[2,3]. In the dark-adapted form (bR^{DA}), a 3/2 equilibrium exists between 13-*cis* (bR_{548}) and all-*trans* (bR_{568}) isomers[2,3].

Model protonated *Schiff* bases of all-*trans* retinal (**1**) and *n*-butylamine in methanol absorb around 440 nm. It is clear that the interaction of the chromophore with the peptide chain in bR_{568} and bR_{548} causes the shift from 440 nm to 568 nm and 548 nm, resp. This difference in λ_{max} value of the protein, compared with the free chromophore, is called opsin shift (expressed in cm^{-1})[4].

^{13}C NMR experiments[5] have shown that in bR_{548} and bR_{568} the chromophores occur in planar C6–C7 *s*-*trans* conformation and that the positive charge on C5 is higher than in model protonated *Schiff* bases, due to a negative charge in

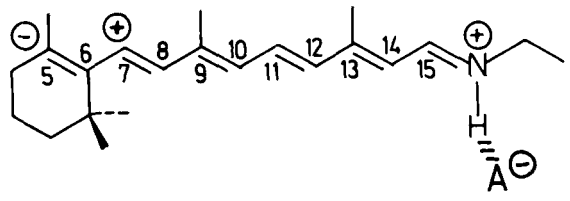


Fig. 1. The structure of the chromophore in bR_{568}.

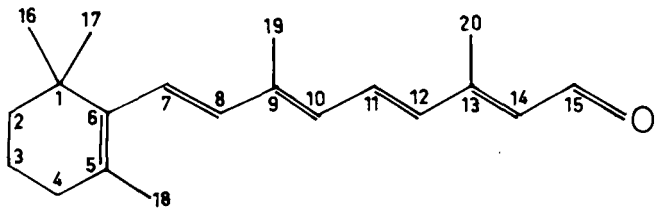


*Fig. 2. Structure of all-*trans *retinal* **1** *in solution having the prevalent twisted 6–7* s-cis *conformation; torsion angle* ca. *50°* [11].

the protein part close to C5 of the chromophore (see Fig. 1). Retinal **1** (in Fig. 2), its *Schiff* base and protonated *Schiff* base (SBH^+) occur mainly in a twisted (torsion angle 40°–60°) 6-*s-cis* conformation[6-8]. It is expected that the 1-, 1'- and 5-methyl groups have a steric influence on the conformation around the C6–C7 bond. Further, the 5-methyl group, being a weak electron-donating group, will have a pronounced influence on the charge density at C5.

In this paper we describe the various properties of the demethylated bacteriorhodopsins 1,1',5-didemethyl-bR ($= bR_a$), 1,1'-didemethyl-bR ($= bR_b$), 1,5-didemethyl-bR ($= bR_c$) and 1-demethyl-bR ($= bR_d$). The properties of 5-demethyl-bR ($= bR_e$) have been previously reported by ourselves and others[9,10].

The aim of the present study is to provide information about the possible influence of the 1-, 1'- and 5-methyl groups in bacteriorhodopsin on the bR formation, opsin shift, light–dark adaptation and the proton-pump action. For this reason, the properties of the bR analogues, having the four possible ring-demethylated chromophores, have been studied.

For these studies, we initially required the appropriate all-*trans* retinals **1a**, **1b**, **1c** and **1d**.

In this paper we describe a new efficient method of preparing retinal (**1**) starting from β-cyclocitral. This scheme is also used for the C_{10} extension of demethylcyclocitrals to **1a-d**. Efficient schemes have been designed for the preparation of the required demethylated cyclocitral synthons starting from cyclohexanone and simple derivatives.

The UV-Vis spectra of retinals **1a-d**, together with those of the corresponding *n*-butylamine-protonated *Schiff* bases (SBH^+), have been studied and this information used to evaluate the influence of the 1-, 1'- and 5-methyl groups on the opsin shift in bR.

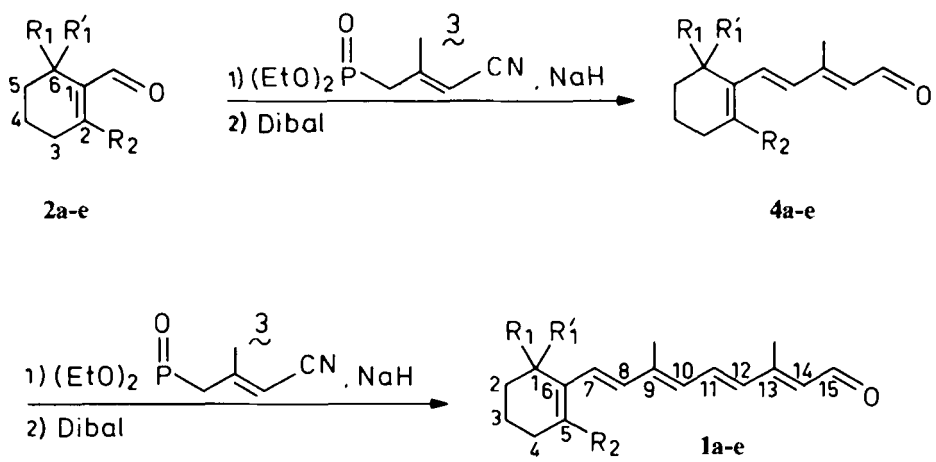
II Synthesis

For the preparation of the ring-demethylated retinals **1a-d** we required a simple procedure involving only a few steps. For the preparation of open-chain retinal analogues, we have found that repeated *Horner–Emmons* coupling with C_5-phosphonate **3**, and subsequent diisobutylaluminium hydride (Dibal) reduction, give a high yield and constitute a simple method for a ten-carbon extension to retinal analogues[12,13].

A similar method for the preparation of the ring-demethylated retinal is depicted in Scheme 1.

The cyclohexenecarbaldehydes **2a-e** are numbered according to the IUPAC nomenclature. For the retinals and intermediate C_{15} aldehydes **4a-e**, the IUPAC retinoid numbering is used[14].

It is known that *Horner–Emmons* couplings are sensitive to steric hinderance[15]. In order to test our method, the synthon with the greatest steric hindrance **2** (β-cyclocitral: the precursor for **1**) was submitted twice to the *Horner–Emmons* coupling with C_5-phosphonate **3** and Dibal reduction. Thus, **2** was found to be converted into retinal in 64% overall yield. Evidently, this novel method of preparing retinal does not suffer from possible steric hindrance, and we thus felt



Scheme 1. Synthesis of ring-demethylated retinals:

1 $R_1, R_1', R_2 = CH_3$; retinal
1a $R_1, R_1', R_2 = H$; 1,1',5-tridemethylretinal
1b $R_1, R_1' = H, R_2 = CH_3$; 1,1'-didemethylretinal
1c $R_1, R_2 = H, R_1' = CH_3$; 1,5-didemethylretinal
1d $R_1, R_2 = CH_3, R_1' = H$; 1-demethylretinal
1e $R_1, R_1' = CH_3, R_2 = H$; 5-demethylretinal

sure that the required demethylretinals could also be prepared via the same method using the appropriate cyclohexenecarboxaldehydes. The starting materials for 1,1',5-tri- (**1a**) and 1,1'-dimethylretinal (**1b**) are 1-cyclohexenecarboxaldehyde (**2a**) and 2-methyl-1-cyclohexenecarboxaldehyde (**2b**), resp. We prepared **2a** and **2b** via the novel and simple procedure starting from cyclohexanone (**5**) depicted in Scheme 2.

Cyclohexanone is converted into **7** in a one-pot, three-step reaction. The 2-formylation of cyclohexanone to the 2-formylate anion with base is a well known high-yield process[16]. The sodium salt **6**, treated subsequently with acetyl chloride and methanol, gives the dimethoxy acetal of 2-oxocyclohexanecarboxaldehyde (**7**) in 84% yield based on **5**. Reduction of the ketone function in **7** with lithium aluminium hydride ($LiAlH_4$) to give the secondary alcohol **8a**, followed by acid-catalysed dehydration and deprotection, lead to 1-cyclohexenecarboxaldehyde (**2a**). When the same procedure is used, but now with methyllithium (or methylmagnesium iodide), the required 2-methyl-1-cyclohexenecarboxaldehyde **2b** is obtained. In this way, both **2a** and **2b** are easily prepared from cyclohexanone in an overall yield of 57% and 45%, respectively. We realise that, according to this scheme, a wide range of 2-alkylated and 2-arylated 1-cyclohexenecarboxaldehydes, which are otherwise not easily accessible, can be made available.

The other necessary carboxaldehydes, namely 6-methyl-cyclohexenecarboxaldehyde **2c** and 2,6-dimethyl-1-cyclohexenecarboxaldehyde **2d**, cannot be obtained via Scheme 2. For the preparation of **2c** and **2d**, we therefore used the reaction sequence depicted in Scheme 3.

6-Methyl-1-cyclohexenecarbonitrile (**11c**) is obtained via a one-pot, two-step procedure[17]. The reaction of (commercial) trimethylsilyl cyanide [$(CH_3)_3SiCN$; **10**] and 2-methylcyclohexanone (**9**) gives an intermediate trimethylsilyloxy nitrile. This nitrile is directly treated with phosphorylchloride and pyridine to achieve elimination of the trimethylsiloxy group and production of the nitriles **11b** and **11c** in a 1/3 ratio. The nitriles were converted by Dibal reduction into the required 1-cyclohexenecarboxaldehydes **2b** and **2c**. The yield of the aldehydes **2b** and **2c** (**2b**/**2c** = 1/3) is 51% starting from **9**. Via the four-step sequence depicted in Scheme 1, aldehydes **2b** and **2c** are converted into 1,1'-didemethylretinal (**1b**) and 1,5-didemethylretinal (**1c**), respectively, in 64% yield. The overall yield of **1b** and **1c** (**1b**/**1c** = 1/3), based on the starting compound **9** is about 33%. **1c** is separated from **1b** by high-performance liquid chromatography (HPLC). The overall yield of pure **1c** is about 22% after 6 steps.

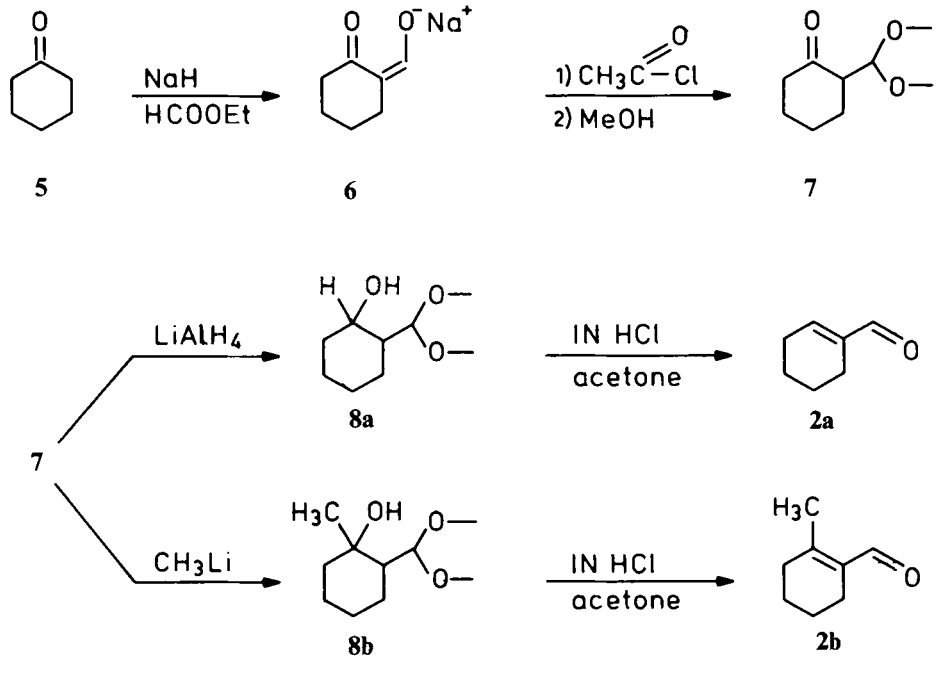
The preparation of 2,6-dimethyl-1-cyclohexenecarboxaldehyde (**2d**) proceeds analogously to the synthesis of **2c** (Scheme 3). 2,6-Dimethylcyclohexanone **12** is treated with **10** in the presence of potassium cyanide/18-crown-6 complex[18] as basic catalyst. In this case, the intermediate silyl ether **13** is isolated in 90% yield. Subsequently, **13** is treated with $POCl_3$ and pyridine to give nitrile **11d**, which affords carboxaldehyde **2d** after Dibal reduction. The yield of **2d** is 36% based on **12**. According to Scheme 1, **2d** is converted into 1-demethylretinal **1d** in 64% so that **1d** is prepared in 23% overall yield based on **12**.

The all-*trans* isomers of the different retinal analogues **1a-e**, needed for the preparation of the appropriate bR analogues, can easily be obtained by using HPLC. Their chemical purity and structure (configuration and conformation) have been checked by ^1H 300 HMz NMR, UV-Vis and single-focus mass spectroscopy. In this way, the all-*trans* isomers of **1a-e** have been prepared with >98% isomeric purity. The ^1H NMR and mass spectra are in agreement with the proposed chemical structure.

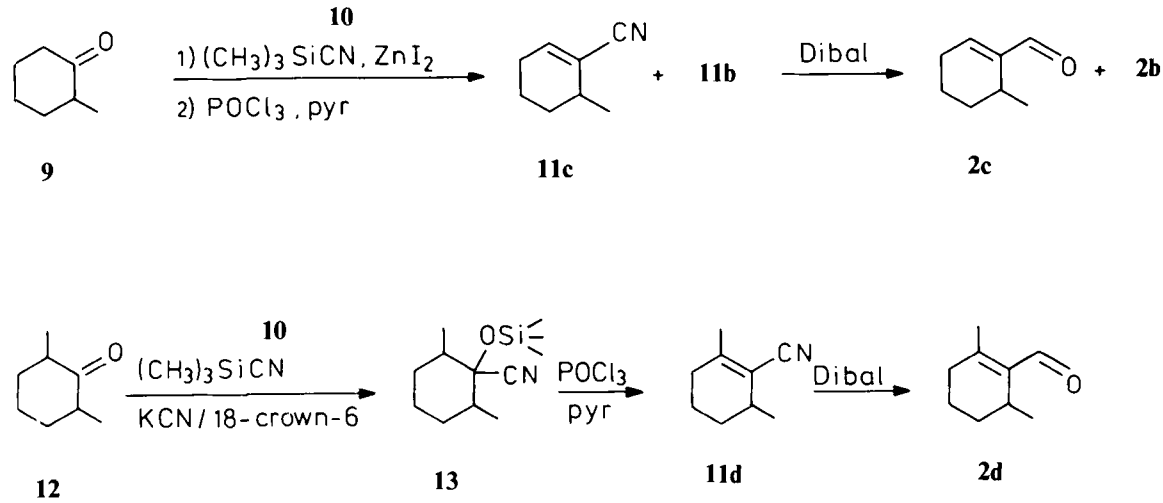
Looking at the ^1H NMRdata of the retinals **1a-e**, one can see that the δ values and coupling constants for the tail end (from C9 to C15) of the analogues are virtually identical with those of retinal **1**. In contrast, the ^1H NMR data for the ring part of the retinal analogues show considerable differences, as is to be expected on the basis of the different ring-substitution pattern and altered C6–C7 conformation.

Protonated Schiff *bases*

The chromophore of bR occurs as a protonated *Schiff* base in the protein. The so-called "opsin shift"[4], a measure for the interaction of the chromophore with the protein, is defined as the difference in wavenumbers (cm^{-1}) between the $(\lambda_{max})^{-1}$ values of the *n*-butylamine-protonated *Schiff* base (SBH^+) of the retinal (analogues) and the $(\lambda_{max})^{-1}$ values of the corresponding bR (analogues). In order to



Scheme 2. Synthesis of 1-cyclohexenecarboxaldehyde (**2a**) *and 2-methyl-1-cyclohexenecarboxaldehyde* (**2b**).



Scheme 3. Synthesis of 6-methyl-1-cyclohexenecarboxaldehyde (**2c**) *and 2,6-dimethyl-1-cyclohexenecarboxaldehyde* (**2d**).

determine the opsin shifts for the different bR analogues, the λ_{max} values of the corresponding BH^+'s have first to be determined. On treatment of the retinals **1a-e** with a small excess of *n*-butylamine and conc. HCl in methanol, the SBH^+'s form almost instantaneously. The λ_{max} values for the SBH^+'s in methanol are given in Table I (3rd column).

III Modified bacteriorhodopsins

Formation and kinetics

When the retinal analogues **1a-e** are incubated at room temperature with bacterioopsin (bO), they all form the corresponding bR analogues very rapidly. In order to follow the incubation of bO with the retinals in more detail, the binding was carried out at lower temperature (1.0°C). Under these conditions, all the retinal analogues first form a complex[19]. (The complexes of the analogues have a λ_{max} at about 480 nm, while for retinal, this λ_{max} value of the complex lies at 430 nm[19,20].) These complexes are then converted in a slower step into the bR analogues. These phenomena are all very similar to those observed for the binding of retinal to bO[20]. The rate of formation of the bR analogues can be determined by measuring the absorption increase at their λ_{max} values against time. It is found that some of the analogues bind even faster to bO than does retinal itself (Table I); bR_a and bR_d formed about 1.5 times faster. In the case of bR_e, the formation is some 3 times more rapid[9,10].

When the different bR analogues are treated with excess of retinal **1**, no absorption increase at 568 nm (λ_{max} bR^{LA}) is detected. This indicates that each of the chromophore analogues occupies the same binding site as does retinal in the natural bR and further that the *Schiff*-base binding is stable towards displacement by retinal.

Light–dark adaptation

When the light-adapted (LA) form of the bR analogues is kept in the dark for a few hours at room temperature, the dark-adapted pigment is obtained. Illumination of this DA form with visible light results in the reformation of the LA form. As can be seen from Table I, the λ_{max} values of the LA form are always higher than that of the DA form. These effects correspond with those found for native bR. It is to be expected that, just as in bR^{DA}, the DA form of the bR analogues consists of a mixture of all-*trans* and 13,15-di-*cis* chromophores, respectively[21].

Table I Results of bR formation and proton-pump experiments[a].

All-*trans* retinal analogues **1**	λ_{max} (nm)				Opsin shift (cm^{-1})		Proton-pump activity [nmol H^+ (mg bR)$^{-1}$]	Relative efficiency[b] (in %)
	Aldehyde	SBH^+	bR					
			DA	LA	DA	LA		
1	380	440	558	568	4800	5100	186	100
a	387	458	539	544	3300	3500	106	57
b	401	474	553	564	3000	3400	179	96
c	392	465	537	549	3100	3300	74	40
d	396	470	558	566	3350	3600	184	99
e	388	453	540	548	3600	3800	130	70

[a] λ_{max} values of the retinals **1a-e** and their corresponding protonated *n*-butyl *Schiff* bases (SBH^+) were measured in MeOH. The λ_{max} values of the bR analogues [the light-adapted form (LA) as well as the dark-adapted form (DA)] were measured in H_2O. The opsin shift[4] is defined as:

$(\lambda_{max}SBH^+)^{-1} - (\lambda_{max}bR)^{-1}$ (in cm^{-1})

e.g.: $(440 \cdot 10^{-7})^{-1} - (568 \cdot 10^{-7})^{-1} = 5100\ cm^{-1}$.

[b] bR efficiency 100%, non-incorporated bO efficiency 0%.

Determination of the opsin shift

Now that the λ_{max} values of the SBH$^+$'s and corresponding bR analogues have been determined, the opsin shifts of the bR analogues can be calculated. The values of the opsin shift (of the DA and LA form) are presented in the fifth column of Table I, together with that of native bR. The opsin shifts of the bR^{LA} analogues are all considerably lower (3800 – 3300 cm^{-1}) than the opsin shift of native bR^{LA} (5100 cm^{-1}).

Proton-pump action

When illuminated with visible light, bR, in the cell membrane of Halobacterium halobium, pumps protons from the cytoplasmic side to the exterior of the cell. The trans--membrane proton gradient thus created is used by the enzym ATP-ase to make ATP from ADP and inorganic phosphate. To test their proton-pump efficiency, the bR analogues were incorporated into soyabean phospholipid vesicles and irradiated with blue-filtered light from a tungsten lamp. The proton efflux was measured using a sensitive pH electrode[22].

The relative proton-pump efficiencies of the bR analogues, compared with that of native bR_a, are given in the last column of Table I. It is clear that the presence of the 5-methyl group in bR, bR_b and bR_d is required for high proton-pump action (95–100%).

bR analogues lacking the 5-methyl group, as in bR_a, bR_c and bR_e, have proton-pump efficiencies of 70% or even less.

Discussion

The modified retinals **1a-e** can be easily prepared in high yield via the reactions given in Schemes 1, 2 and 3. The reactions in Scheme 2 represent an efficient method of preparing the otherwise poorly accessible 1-cyclohexenecarboxaldehyde **2a** and its 2-methyl derivative **2b** in two steps starting from cyclohexanone. We expect that the scope of this method for preparing a host of 2-alkyl- and 2-aryl--1-cyclohexenecarboxaldehydes is very wide, on account of the ready availability of the various alkyl- and arylmetal reagents (*e.g.* MeLi).

The preparation of **7** starting from cyclohexanone is analogous to the conversion of β-ionone into the dimethyl acetal of 3-oxo-5-(2,6,6-trimethyl-1-cyclohexenyl)-4-pentenal with sodium[23]. The use of NaH as base results in a more rapid reaction and easier work-up than does the reaction using sodium metal.

The preparation of **2a** with 26% overall yield via a difficult procedure starting from the commercially unavailable 1-chloro-2-cyclohexene has been described in the literature[24]. Due to its versatility and higher yield, our procedure is the method of choice for the preparation of compound **2a** and its analogues. Only one alternative (four-step) synthesis of **2d** has been previously published[25], but with little experimental detail. Our method gives **2d** in 3 steps and in good yield.

Via the novel sequence shown in Scheme 1, the 1-cyclohexenecarboxaldehydes were converted into the required retinal analogues in a 64% overall yield via a four-step procedure using only one C_5 synthon (**3**) as carbon source. The λ_{max} values of the retinal analogues **1a-e** and their protonated *Schiff* bases are all higher than those of the parent retinal (**1**) (see Table I). Electronically, the absence of methyl groups should lead to lower λ_{max} values[26], however, at the same time, it allows conformations in which the dihedral angle around the C6–C7 single bond is much more planar, thus leading to a better conjugation between the C5–C6 double bond and the rest of the conjugated chain[27].

In retinal itself, the conformation is twisted 6-*s-cis* (torsion angle about 60°)[6-8,11] diminishing the conjugation of the C5–C6 bond considerably. It has been found, using solid--state NMR[5], that the conformation of the chromophore in bR around the C6–C7 bond is planar *s-trans*. We have also studied 8,16-methano-retinal[28], which is locked in the planar 6 *s-trans* conformation. The opsin shift for the LA-form of its corresponding bR analogue amounts to 3800 cm^{-1}, a value much lower than for native bR (5100 cm^{-1}). Due to its constrained structure the chromophore now must remain planar upon binding to bO instead of going from twisted 6-*s-cis* to planar 6-*s-trans* as in bR. The opsin shifts of the bR analogues bR_{a-e} are also 3800 cm^{-1} or somewhat lower. This means that in all these cases binding does not invoke change in conformation upon binding.

Because the absence of methyl groups in the retinal analogues **1a-e** effects conformational changes around the C6–C7 bond, the ring-part in the retinal analogues has a significantly different ^1H NMR spectrum than in retinal. For example, the δ value for H7 varies between 6.29 ppm (in **1c**) and 6.92 ppm (in **1b**), while it is 6.34 ppm in retinal. This demonstrates that H7 is very sensitive to conformational changes around the C6–C7 bond. For **1b**, the high δ value for H7 (6.92 ppm), combined with its very high λ_{max} value (401 nm, see Table I), clearly points to a purely planar 6-*s-trans* conformation. The other retinal analogues also have higher λ_{max} values (387 nm to 396 nm) than does retinal (λ_{max} is 380 nm), indicating a large contribution of the planar 6-*s-trans* conformation. For **1a** (lacking all ring--methyl groups), this has been checked by NOE studies. Irradiation of proton H5 leads to a significant NOE on H7. In the case of 1-demethylretinal **1d**, NOE experiments likewise support the 6-*s-trans* conformation. The 5-demethyl analogue **1e** has also been examined for NOE's. Irradiation of H5 gives an enhanced intensity of both the H7 and the H8 signal. This indicates that, in solution at least, a considerable number of the molecules exist in the 6-*s-trans* conformation. Looking at the λ_{max} values of the bR analogues, it is clear that, as long as a 5-CH_3 group is present, the λ_{max} value is almost identical to that of bR. For bR_b and bR_d, λ_{max} is 564 nm and 567 nm, resp. The lack of the 5-methyl group gives λ_{max} values which are about 20 nm lower (as for bR_a, bR_c and bR_e). A similar correlation between the 5-CH_3 group and the proton pump action is very apparent, since bR_b and bR_d have a proton pump activity of effectively 96% and 99%, resp., which is close to that of bR. The lack of the 5-CH_3 group leads to a drastically lower proton-pump action, being at most 70% for bR_e or lower (bR_a and bR_c). This is also reflected in a substantially lower ATP-ase activity when ATP-ase is reconstituted into phospholipid vesicles with each of the bR analogues lacking the 5-CH_3 group[29]. This can be understood in terms of a lower *trans*-membrane proton gradient, which is needed for the functioning of ATP-ase. Upon binding to bO, the chromophore has to adopt a planar 6-*s-trans* conformation. The retinals studied can attain a planar conformation much more readily than retinal, which seems to be related to the more rapid binding of the retinal analogues (*cf.* refs. 19, 30).

In conclusion, this study shows that the 5-CH_3 group contributes significantly to the λ_{max} value of bR and that it has a very important role to play in the photochemical proton--pump action*.

* Recently, a study on 5-CF_3 retinal has been published[31]. After incubation with bO, this analogueformed a pigment with an unusually low λ_{max} value of 465 nm which showed no proton--pump action. In our opinion, this "pigment" is a random *Schiff* base and not a conventional bR analogue (the authors also suggest that this retinal analogue presumably occupies another binding site). Nevertheless, these findings support our interpretation that the substituent on C5 is very important for the efficient functioning of bacteriorhodopsin.

Experimental

Retinal synthesis

All experiments were carried out in a nitrogen atmosphere and those with polyenes under dim red light. Distilled dry solvents were used. Pet. ether refers to low-boiling petroleum ether 40°–60°C. Unless otherwise stated, purification was performed by flash chromatography[32] (Merck silica gel 60, 230–400 Mesh) using ether/pet. ether mixtures. TLC analysis were performed on Schleicher and Schuell F1500/LS254 silica-gel plates using ether/pet. ether mixtures. Evaporation of the solids was carried out *in vacuo* (10 Torr). The ^{1}H NMR spectra were recorded on a Bruker WM-300 (for the retinals) or a Jeol PS-100 spectrometer using tetramethylsilane (TMS: 0 ppm) as internal standard. The mass spectra were recorded using an AEI MS 902. IR spectra were obtained a Pye-Unicam SP 3–200 and the UV-Vis spectra using a Cary 219 spectrophotometer. HPLC separations were performed using a Dupont 830 equipped with a Dupont spectrophotometer (360 nm) and a 25 cm × 22.5 cm Zorbax Sil column. Elution was effected using 10% ether in pentane at a flow rate of 20 ml/min. ^{1}H NMR signals were assigned by comparison with those reported for other retinoids. Spectral designations for the cyclohexenecarboxaldehydes **2a-e** were based on the IUPAC Nomenclature, and for the C_{15} aldehydes **4a-e** and the retinals **1a-e** on the IUPAC retinoid numbering system[14].

2-(Dimethoxymethyl)cyclohexanone (**7**)

2.27 g of NaH (55% dispersion in oil, 52 mmol) were washed three times with pet. ether and suspended in 25 ml of dry ether. After this suspension had been cooled to 0°C, 5 g (51 mmol) of cyclohexanone (**5**) and 7.56 g (102 mmol) ethyl formate, dissolved in 25 ml ether, were added over a period of 10 min. Upon warming to room temperature, hydrogen evolution started and a yellow-orange precipitate (**6**) formed. After H_2 liberation had ceased, the mixture was refluxed for 25 minutes. After cooling to 0°C, 2.3 ml (39 mmol) of ethanol, 8 g (102 mmol) of freshly distilled acetyl chloride in 20 ml ether and 47 ml (1.17 mmol) of methanol were added. The mixture was stirred for 2 h at room temperature. Work-up was accomplished by pouring the mixture into 200 ml of 5% $NaHCO_3$ solution. After extraction with ether, the ether layers were washed with brine, dried over Na_2CO_3 and evaporated to give 7.4 g (84%) of **7** as a brown oil. ^{1}H NMR (100 MHz, $CDCl_3$): δ 1.4–2.0 (m, 6H, 3-CH_2, 4-CH_2 and 5-CH_2), 2.4 (m, 2H, 6-CH_2), 2.64 (m, 1H, 2-H), 3.44 (s, 6H, 2 × OCH_3), 4.72 [d, J_{HH} 6 Hz, 1H, $-CH(O-)_2$]. IR (KBr): 1710 cm^{-1} (C=O stretch), 1050–1100 cm^{-1} (C–O stretch).

2-(Dimethoxymethyl)cyclohexanol (**8a**)

4 g (23.3 mmol) of compound **7** in 50 ml ether were added slowly to a cooled (ice-bath) and stirred suspension of 0.9 g (22.5 mmol) $LiAlH_4$ in 10 ml ether. After removal of the ice-bath, the mixture was stirred for 30 min at room temperature. After completion, a little ice together with a saturated NH_4Cl solution (pH 4–5) were added slowly. This mixture was extracted with 50 ml ether (3 ×). The ether layers were washed with brine, dried over $NaCO_3$, filtered and evaporated to give 3.6 g (90%) of **8a**. ^{1}H NMR (100 MHz, $CDCl_3$): δ 1.0–2.0 (m, 9H, 2-H, 3-CH_2, 4-CH_2, 5-CH_2 and 6-CH_2), 3.46 and 3.37 (2 × s, 6H, 2 × OCH_3), 3.52 (m, 1H, 1-H), 4.1 (bs, 1H, OH), 4.3 [d, J_{HH} 6 Hz, 1H, $-CH(O-)_2$]. IR (KBr): 3450 cm^{-1} (OH stretch), 1100 cm^{-1} (C–O stretch).

2-(Dimethoxymethyl)-1-methylcyclohexanol (**8b**)

To a cooled (0°C) and stirred solution of 19 mmol CH_3Li (12 ml 1.55 M solution in ether; Aldrich), 2 g of compound **7**, dissolved in 25 ml ether, were added over a period of 20 min. After the addition, stirring was continued for 75 min at room temperature. The mixture was then cooled to 0°C and 200 ml of 0.1 N HCl were added slowly. After extraction with ether (3 ×), the ether layers were washed with water, dried over Na_2CO_3 and evaporated to yield 1.56 g (69%) of **8b**. According to NMR and IR data, in some instances a considerable amount of the 2-hydroxy-2-methylcyclohexanecarboxaldehyde had already formed. ^{1}H NMR (100 MHz, $CDCl_3$): δ 1.34 (s, 3H, 1-CH_3), 1.0–2.0 (m, 9H, 2-H, 3-CH_2, 4-CH_2, 5-CH_2 and 6-CH_2), 3.38 and 3.25 (2 × s, 6H, 2 × OCH_3). IR (KBr): 3450 cm^{-1} (OH stretch), 1050–1100 cm^{-1} (C–O stretch).

1-Cyclohexenecarboxaldehyde (**2a**)

3.5 g (20.1 mmol) of **8a** were dissolved in 35 ml of acetone and heated to reflux. 0.6 ml of 1 N HCl were then added via a syringe and the mixture was refluxed for about 2½ hours. After cooling, the mixture was extracted with ether. The ether layer was washed with brine (2 ×), dried over $MgSO_4$ and evaporated after filtration to give 2.5 g of crude aldehyde **2a** (> 100%). Purification by column chromatography (silica gel; pet. ether/ether = 85/15) yielded 1.1 g (50%) of **2a**. ^{1}H NMR (100 MHz, $CDCl_3$): δ 1.7 (m, 4H, 4-CH_2 and 5-CH_2), 2.3 (m, 4H, 3-CH_2 and 6-CH_2), 6.8 (m, 1H, 2-H), 9.5 (s, 1H, aldehyde-H).

2-Methyl-1-cyclohexenecarboxaldehyde (**2b**)

1.56 g of **8b** was dissolved in 25 ml of acetone and heated to reflux. To the refluxing mixture, 0.4 ml of 1 N HCl was added slowly via a syringe. The refluxing was continued for a further 2 h. After cooling, 50 ml of water were added. Extraction was performed with pet. ether (4 ×). The pet. ether layers were washed with water, dried over $MgSO_4$ and distilled *in vacuo*. The yield consisted of 843 mg (82%) of **2b** as a red-brown oil. ^{1}H NMR (100 MHz, $CDCl_3$): δ 1.6 (m, 4H, 4-CH_2 and 5-CH_2), 2.16 (s, 3H, 2-CH_3), 2.16 (m, 4H, 3-CH_2 and 6-CH_2), 10.06 (s, 1H, aldehyde-H).

The method of synthesis of the intermediates **4a,b**, including an *Emmons–Horner* coupling and Dibal reduction, has been described in earlier publications[12,13]. The spectroscopic assignments of these compounds, based on the IUPAC retinoid numbering system[14], are presented below.

(2E,4E)-5-(1-Cyclohexenyl)-3-methyl-2,4-pentadienal (**4a**). ^{1}H NMR (100 MHz, $CDCl_3$): δ 1.72 (m, 4H, 2-CH_2 and 3-CH_2), 2.24 (m, 4H, 1-CH_2 and 4-CH_2), 2.32 (s, 3H, 9-CH_3), 5.96 (d, J_{HH} 8 Hz, 1H, 10-H), 6.24 (d, J_{HH} 16 Hz, 1H, 8-H), 6.76 (d, J_{HH} 16 Hz, 1H, 7-H), 10.08 (d, J_{HH} 8 Hz, 1H, 11-H).

(2E,4E)-3-Methyl-5-(2-methyl-1-cyclohexenyl)-2,4-pentadienal (**4b**). ^{1}H NMR (100 MHz, $CDCl_3$): δ 1.65 (m, 4H, 2-CH_2 and 3-CH_2), 1.88 (s, 3H, 5-CH_3), 2.14 (m, 4H, 1-CH_2 and 4-CH_2), 2.30 (s, 3H, 9-CH_3), 5.94 (d, J_{HH} 8 Hz, 1H, 10-H), 6.20 (d, J_{HH} 16 Hz, 1H, 8-H), 7.20 (d, J_{HH} 16 Hz, 1H, 7-H), 10.04 (d, J_{HH} 8 Hz, 1H, 11-H).

1,1',5-Tridemethylretinal (**1a**). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.68 (m, 4H, 2-CH_2 and 3-CH_2), 2.01 (s, 3H, 9-CH_3), 2.19 (m, 4H, 1-CH_2 and 4-CH_2), 2.32 (s, 3H, 13-CH_3), 5.90 (bt, J_{HH} 6.6 Hz, 1H, 5-H), 5.97 (d, J_{HH} 8.1 Hz, 1H, 14-H), 6.24 (d, J_{HH} 11.4 Hz, 1H, 10-H), 6.25 (d, J_{HH} 16.3 Hz, 1H, 8-H), 6.36 (d, J_{HH} 15.1 Hz, 1H, 12-H), 6.42 (d, J_{HH} 16.3 Hz, 1H, 7-H), 7.13 (dd, J_{HH} 15.1 Hz, J_{HH} 11.4 Hz, 1H, 11-H), 10.10 (d, J_{HH} 8.1 Hz, 1H, 15-H). Mass spectrum: *m/z* 242 (M^+).

1,1'-Didemethylretinal (**1b**)

^{1}H NMR (300 MHz, $CDCl_3$): δ 1.66 (m, 4H, 2-CH_3 and 3-CH_2),1.86 (s, 3H, 5-CH_3), 2.04 (s, 3H, 9-CH_3), 2.14 (m, 2H, 1-CH_2), 2.21 (m, 2H, 4-CH_2), 2.33 (s, 3H, 13-CH_3), 5.98 (d, J_{HH} 8.1 Hz, 14-H), 6.24 (d, J_{HH} 11.8 Hz, 1H, 10-H), 6.29 (d, J_{HH} 15.5 Hz, 1H, 8-H), 6.36 (d, J_{HH} 15.1 Hz, 1H, 12-H), 6.92 (d, J_{HH} 15.5 Hz, 1H, 7-H), 7.14 (dd, J_{HH} 15.1 Hz, J_{HH} 11.8 Hz, 1H, 11-H), 10.10 (d, J_{HH} 8.1 Hz, 1H, 15-H). Mass spectrum: *m/z* 256 (M^+).

6-Methyl-1-cyclohexenecarbonitrile (**11c**)

For this one-pot, two-step procedure care has to be taken that glassware, solvents and reagents are dried thoroughly. A mixture of 2-methylcyclohexanone (0.19 g; 1.7 mmol), commercial trimethylsilyl cyanide (**10**; 0.20 g; 2.0 mmol; Merck) and zinc iodide (*ca.* 13 mg) in benzene (1 ml) was stirred at room temperature until completion of the addition reaction (overnight; TLC analysis). Pyridine (2.7 ml) and freshly distilled $POCl_3$ (5 mmol) were added and the whole mixture was heated to reflux for about 10 h. The cooled dark solution was then poured into ice-cold hydrochloric acid. After extraction, the solvent was removed and the product was purified using silica-gel column chromatography. The yield of nitrile (**11b/11c** = 1/3) was 128 mg (60%). This mixture of **11b** and **11c** was not separated until the retinal stage, when the all-*trans*

isomers of the two compounds could easily be separated with HPLC. ^{1}H NMR (of **11c**, 100 MHz, $CDCl_3$): δ 1.19 (d, J_{HH} 6 Hz, 3H, 1-CH_3), 1.4–2.7 (m, 7H, 3-CH_2, 4-CH_2, 5-CH_2 and 6-H), 6.54 (m, 1H, 2-H).

6-Methyl-1-cyclohexenecarboxaldehyde (**2c**)

A stirred solution of 128 mg (1 mmol) of nitrile (**11b** + **11c**) in pet. ether was cooled to −60°C. 1.5 ml of a solution (1.5 mmol) of diisobutylaluminium hydride (Dibal) was then added dropwise. After completion (1–2 h at 0°C), 2 g of silica gel, moistened with 0.4 ml of water and slurried in ether/pet. ether (1/1), were added slowly and the mixture was stirred at 0°C for *ca.* 1 h. After drying ($MgSO_4$), the solids were filtered off and washed with ether. Evaporation of the solvents yielded 109 mg (85%) of **2c** and **2b** as a 3/1 mixture. ^{1}H NMR (of **2c**, 100 MHz, $CDCl_3$): δ 1.3 (d, J_{HH} 7.5 Hz, 3H, 6-CH_3), 6.59 (t, J_{HH} 4 Hz, 1H, 2-H), 9.9 (s, 1H, aldehyde-H). The aldehyde mixture (**2c**/**2b** = 3/1) was converted into the corresponding retinals **1c** and **1b** (3/1 mixture) by the same procedure as shown in Scheme 1. The relevant spectroscopic assignments are presented below.

(2E,4E)-3-Methyl-5-(6-methyl-1-cyclohexenyl)-2,4-pentadienal (**4c**). ^{1}H NMR (100 MHz, $CDCl_3$): δ 1.1 (d, J_{HH} 7 Hz, 1H, 1-CH_3), 2.3 (s, 3H, 9-CH_3), 5.92 (bs, 1H, 5-H), 5.96 (d, J_{HH} 8 Hz, 1H, 10-H), 6.26 (d, J_{HH} 16 Hz, 1H, 8-H), 6.58 (d, J_{HH} 16 Hz, 1H, 7-H), 10.10 (d, J_{HH} 8 Hz, 1H, aldehyde-H).

1,5-Didemethylretinal (**1c**)

^{1}H NMR (300 MHz, $CDCl_3$): δ 1.12 (d, J_{HH} 7 Hz, 1H, 1-CH_3), 1.5–1.8 (m, 5H, 1-H, 2-CH_2 and 3-CH_2), 2.01 (s, 3H, 9-CH_3), 2.18 (m, 2H, 4-CH_2), 2.33 (s, 3H, 13-CH_3). 5.83 (t, J_{HH} 4.2 Hz, 1H, 5-H), 5.97 (d, J_{HH} 8.1 Hz, 1H, 14-H), 6.24 (d, J_{HH} 11.6 Hz, 1H, 10-H), 6.28 (d, J_{HH} not determined, 1H, 8-H), 6.29 (d, J_{HH} not determined, 1H, 7-H), 6.37 (d, J_{HH} 15.0 Hz, 1H, 12-H), 7.14 (dd, J_{HH} 15.0 Hz, J_{HH} 11.6 Hz, 1H, 11-H), 10.11 (d, J_{HH} 8.1 Hz, 1H, 15-H). Mass spectrum: *m/z* 228 (M^+).

2,6-Dimethyl-1-cyclohexenecarbonitrile (**11d**)

For this reaction, the same precautions have to be taken as for the preparation of **11c** to guarantee absolutely dry conditions. Commercial 2,6-dimethylcyclohexanone **12** (mixture of isomers; 0.5 g; 4 mmol) was dissolved in 2 ml of benzene. With the aid of a syringe, 0.52 g (5.2 mmol) trimethylsilylcyanide **10** was added, followed by a catalytic amount of potassium/18-crown-6 complex. This mixture was stirred over night at room temperature in a closed argon-filled round-bottom fask. After addition of 5 ml of pentane, washing the pentane layers with saturated sodium bisulfite solution and drying over sodium sulfate, 0.8 g (89%) of **13** was isolated on evaporation of the solvent.

This silyl ether **13** (0.8 g; 3.6 mmol) was dissolved in pyridine (5.6 ml) and refluxed for 7 h in the presence of freshly distilled $POCl_3$ (10.7 mmol). The same work-up procedure as for **11c** yielded 0.24 g (50%) of **11d**. ^{1}H NMR (100 MHz, $CDCl_3$): δ 1.19 (d, J_{HH} 7.0 Hz, 3H, 6-CH_3), 1.4–2.0 (m, 4H, 4-CH_2 and 5-CH_2), 2.02 (s, 3H, 2-CH_3), 1.9–2.4 (m, 3H, 1-H and 3-CH_2).

2,6-Dimethyl-1-cyclohexenecarboxaldehyde (**2d**)

The nitrile **11d** (0.24 g; 1.78 mmol) was dissolved in pet. ether and cooled to −60°C. Treatment with 2.6 ml of a Dibal solution in hexane (2.6 mmol) and hydrolysis after completion with 1.0 ml of water adsorbed on 4.2 g of silica gel as a suspension in ether/pet. ether (1/1), yielded 0.2 g (80%) of **2d**. ^{1}H NMR (100 MHz, $CDCl_3$): δ 1.26 (d, J_{HH} 7 Hz, 3H, 6-CH_3), 1.6 (m, 4H, 4-CH_2 and 5-CH_2), 2.0–2.3 (m, 3H, 1-H and 3-CH_2), 2.13 (s, 3H, 2-CH_3), 10.04 (s, 1H, aldehyde-H).

Compound **2d** was twice extended with the C_5 phosphonate **3** according to Scheme 1. The spectroscopic data of the intermediate C_{15} aldehyde **4d** and retinal analogue **1d** are given below.

(2E,4E)-5-(2,6-Dimethyl-1-cyclohexenyl)-3-methyl-2,4-pentadienal (**4d**). ^{1}H NMR (100 MHz, $CDCl_3$): δ 1.09 (d, J_{HH} 7 Hz, 3H, 1-CH_3), 1.4–1.9 (m, 5H, 1-H, 2-CH_2 and 3-CH_2), 1.88 (s, 3H, 5-CH_3), 2.15 (m, 2H, 4-CH_2), 2.36 (s, 3H, 9-CH_3), 5.92 (d, J_{HH} 8.0 Hz, 1H, 10-H), 6.24 (d, J_{HH} 16 Hz, 1H, 8-H), 7.07 (d, J_{HH} 16 Hz, 1H, 7-H), 10.04 (d, J_{HH} 8.0 Hz, 1H, aldehyde-H).

1-Demethylretinal (**1d**). ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.08 (d, J_{HH} 6.9 Hz, 3H, 1-CH_3), 1.4–1.9 (m, 5H, 1-H, 2-CH_2 and 3-CH_2), 1.83 (bs, 3H, 5-CH_3), 2.0–2.2 (m, 2H, 4-CH_2), 2.05 (s, 3H, 9-CH_3), 2.33 (s, 3H, 13-CH_3), 5.97 (d, J_{HH} 8.2 Hz, 1H, 14-H), 6.25 (d, J_{HH} 11.,3 Hz, 1H, 10-H), 6.31 (d, J_{HH} 16.1 Hz, 1H, 8-H), 6.37 (d, J_{HH} 15.0 Hz, 1H, 12-H), 6.79 (d, J_{HH} 16.1 Hz,1H, 7-H), 7.15 (dd, J_{HH} 15.0 Hz, J_{HH} 13.1 Hz, 1H, 11-H), 10.10 (d, J_{HH} 8.2 Hz, 1H, 15-H). Mass spectrum: *m/z* 270 (M^+).

Formation of Schiff *base (SB) and* SBH^+

The protonated *n*-butyl *Schiff* bases of retinal (analogues) were protonated on a cuvet scale by addition of excess (a few drops) *n*-butylamine, followed by one drop of conc. HCl, to a $10^{-4} - 10^{-5}$ M solution of the retinal (analogue) in 2 ml of methanol. The λ_{max} values were directly determined.

Preparation of bO

Halobacterium halobium (low carotenoid strain S9, R1 mutant) was grown and purple membrane was isolated as described in the literature[33]. bO was prepared following known procedures (refs. 34 and 35).

Binding experiments and opsin shift

Binding experiments were performed as described in the literature at room temperature and at 1.0°C (for kinetic experiments)[22,23]. The regeneration was followed in 1-cm-pathlength cuvettes using a Cary 219 spectrophotometer. To obtain an impression of the effect of the bacteriorhodopsin binding site on the absorption spectrum of the chromophore, the opsin shift (O.S.) was determined for all of the bR analogues. The opsin shift[4] is defined as the difference between the wavenumber of the SBH^+ of all-*trans* retinal and that of dark-adapted bR:

$$\text{O.S.} = (\lambda_{max}SBH^+)^{-1} - (\lambda_{max}bR)^{-1} \quad (\text{in cm}^{-1})$$

Light–dark adaptation and stability of bR analogues

Light–dark adaptation was performed according to the literature[22]. The stability of the bR analogues was tested by adding an excess of normal all-*trans* retinal **1** to the regenerated bR pigment. After two days at 5°C, the occurrence of possible chromophore exchange was checked by measuring the O.D. at 568 nm (λ_{max} of normal bR).

Incorporation of bR into phospholipid vesicles and proton pump

The incorporation of the bR analogues into the phospholipid vesicles was performed and the light-driven proton-pump action was determined as described in the literature[22].

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