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Synthesis of nitroxide containing polyenes: two chemically modified retinals and their interaction with bacterioopsin

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Abstract. The synthesis and spectroscopic characterization of two retinal analogues is described, the paramagnetic 3-pyrrolin-1-yloxy analogue **1** and its diamagnetic equivalent, the 3-pyrroline analogue **2**. Various aspects of the synthesis of the aminoxy group containing polyenes are discussed. Upon interaction with bacterioopsin, both **1** and **2** are incorporated in the protein and form a system with λ_{max} 459 nm. Neither of the two bacteriorhodopsin analogues is photoactive. ESR spectroscopy data of the system containing **1** show that the ring part of the chromophore in the protein is rigidly fixed in orientation.

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]. bR is folded into seven transmembrane helices and functions as a light-driven proton pump that converts the energy of light into that of a proton gradient over the bacterial membrane[2]. The bacterium uses this energy to generate ATP to drive its life processes. The chromophore is all-*trans* retinal (**3**) which is bound to the 6-amino group of lysine-216 via a protonated Schiff base (PSB) linkage[3], see Figure 1. The λ_{max} value of light-adapted bR (568 nm) is much larger than that of the model PSB compound from all-*trans* 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 bound chromophore with the protein chain. The difference in wave numbers (5100 cm^{-1}) has been called opsin shift[4].
Recently, electron-cryomicroscopy data on bR have become available which has allowed the construction of a model of bR with a resolution of 2.8 Å in the membrane plane and approximately 6 Å perpendicular to this plane[5]. The chromophore cannot be detected with this technique. Few amino acids that interact with the chromophore have been identified[6], and there is no conclusive evidence on which amino acids form the binding pocket. Identification of those amino acids will provide a better understanding of the working principles of bR. ENDOR (electron nuclear double resonance) is a potent technique for extracting this information, but requires the presence of an electron spin and native bacteriorhodopsin does not contain a paramagnetic group or ion.
To enable ENDOR studies of bacteriorhodopsin we set out to prepare an electron-spin-labeled bacteriorhodopsin. In a bio-organic approach, bR analogues can be regenerated by reaction of retinal analogue with the free protein bacterioopsin (bO) obtained by bleaching of bR in the presence of hydroxylamine[7]. Replacement of the native chromophore with the 3-pyrrolin-1-yloxy analogue of all-*E* retinal (**1**; see Figure 2) provides a spin labeled bR analogue in which the location of the paramagnetic group is known with respect to the chromophore.
In this paper we describe the synthesis of the paramagnetic all-*E*-retinal analogue **1** and its novel diamagnetic analogue **2**, all-*E*-2-aza-4,4-dimethyl-3,18-dinorretinal (see Figure 2). **1** contains the paramagnetic aminoxy group fused into the ring part of the retinal skeleton. The diamagnetic retinal analogue **2** differs from **1** in the presence of a hydrogen atom on the nitrogen instead of the paramagnetic oxyl group. We also describe the interaction of **1** and **2** with bacterioopsin. A comparison of the characteristics and behavior of **1** and **2** enables us to differentiate between the effects of the presence of the electron spin and the steric changes of the altered carbon skeleton in binding studies.

Synthesis

We anticipated that the 3-pyrrolin-1-yloxy analogue **1** of retinal could be prepared by polyene chain elongation[8] from 3-formyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy (**4**), the nitroxide containing β-cyclocitral analogue, see Scheme 1. The synthesis of aminoxy aldehyde **4** has been reported in the literature[9], but these methods are not satisfactory in terms of yield and reproducibility on a one gram scale. We developed a high-yield method to prepare **4**, starting from 3-carboxy-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy (**5**), which is commercially available, or can be prepared as described by *Rozantsev*[10].
We converted aminoxy acid **5** into aldehyde **4** via the corresponding *N*-methoxy-*N*-methylamide intermediate. Treatment of **5** with thionyl chloride in the presence of

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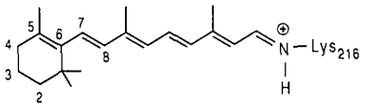
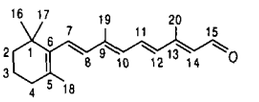


Figure 1. Structure of the chromophore in bacteriorhodopsin.

pyridine converts the acid function into the acid chloride, which was not isolated but treated *in situ* with *N*-methoxy-*N*-methylamine to give *N*-methoxy-*N*-methylcarboxamide **6**. Subsequent treatment of amide **6** with diisobutylaluminum hydride (Dibal) affords aldehyde **4** in 66% yield based on **5**. *N*-Methoxy-*N*-methylamides are known precursors of aldehydes and ketones, and we have previously reported on the advantage of their use as precursors of methyl ketones in the synthesis of isotopically labeled retinals[11].

For chain elongation of β-citral analogue **4** to retinal **1** we reacted **4** with the anion of ethyl 4-diethoxyphosphinyl-3-methyl-2-butenoate. Subsequent treatment of the resulting ester with lithium *N*-methoxy-*N*-methylamide gives a *E*/*Z* mixture of the corresponding *N*-methoxy-*N*-methylamide. The isomerically pure all-*E*-*N*-methoxy-*N*-methylamide **7** is obtained after SiO_2 column chromatography. Dibal converts the *N*-methoxy-*N*-methylamide **7** into isomerically pure all-*E* aldehyde **8** in 85% yield. Coupling the anion of ethyl 4-diethoxy-phosphinyl-3-methyl-2-butenoate with aldehyde **8** affords the required aminoxy containing carbon backbone of retinal **1**. Subsequent conversion of the ester group to the corresponding *N*-methoxy-*N*-methylamide is achieved by treatment of the ester with lithium *N*-methoxy-*N*-methylamide. Column chromatography affords the isomerically pure all-*E*-amide **9**. Dibal reduction gives the isomerically pure **1** in 16% overall yield from **5**. **1** was purified by recrystallization from tetrahydrofuran (THF). No stereochemical isomers were detected on analytical TLC.

The synthesis of the 3-pyrroline analogue **2** of retinal is based on chain elongation of the diamagnetic analogue of **4**: 2,2,5,5-tetramethyl-3-pyrroline-3-carboxaldehyde (**10**). To prepare aldehyde **10**, we started by treating 2,2,6,6-tetramethylpiperidin-4-one (**11**) with bromine in acetic acid to give 3,5-dibromo-2,2,6,6-tetramethylpiperidin-4-one[10,12] (**12**). This compound is known to undergo Favorskii rearrangement upon treatment with a sodium hydroxide solution and we anticipated that treatment with the *N*-methoxy-*N*-methylamide would give the corresponding



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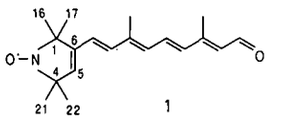

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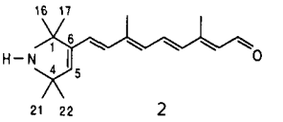
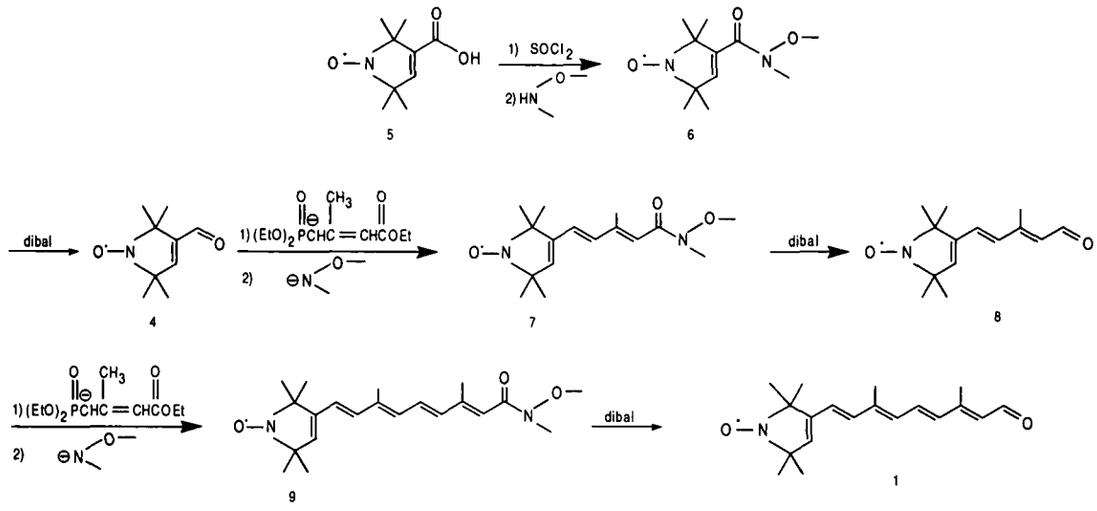


2

Figure 2. Structure and numbering of 1, 2 and 3.

N-methoxy-*N*-methylamide **13**. Treatment of **12** with one equivalent of *N*-methoxy-*N*-methylamine in the presence of triethylamine gives **13** in 90% yield. The use of triethylamine precludes the need for an excess of the expensive *N*-methoxy-*N*-methylamine by acting as a hydrogen bromide sink. Subsequently, **13** was reacted with dibal in THF to give the required **10**. Aldehyde **10** was converted into all-*E*-retinal **2** in the same way as **4** was converted into **1**. Thus, treatment of β-cyclocitral analogue **10** with the anion of ethyl 4-diethoxyphosphinyl-3-methyl-2-butenoate gives a *E*/*Z* mixture of the corresponding ester, which was treated with lithium *N*-methoxy-*N*-methylamide to afford the corresponding mixture of amides. It is important to separate the isomers in this stage and obtain the isomerically pure all-*E*-amide **14** by SiO_2 column chromatography. The separation of the isomeric mixture of the amides is easier than that of the preceeding mixture of esters. Separation of isomers cannot be carried out in the following stage, because the product is unstable. Dibal reduction of the all-*E*-*N*-methoxy-*N*-methyl amide **14** gives all-*E*-aldehyde **15**. Because aldehyde **15** was very unstable, care was taken to keep the compound in solution at or below room temperature. The reaction product was filtered, concentrated on a rotavap and immediately reacted in the next step with the anion of ethyl 4-diethoxyphosphinyl-3-methyl-2-butenoate to give an isomeric mixture of the corresponding ester. The ester function was converted into the *N*-methoxy-*N*-methylamide by treatment with litium *N*-methoxy-*N*-methylamide and the pure all-*E*-**16** was obtained after SiO_2 chromatography. Subsequent dibal reduction gives isomerically pure **2**. **2** is very unstable and care was taken to keep the compound below room temperature; it was stored in dilute solution under argon at −80°C.



Scheme 1. Synthesis of 1.

Spectroscopic characterization

Mass spectrometry

The high-resolution electron-impact mass spectra of the pyrrolinyloxy analogue **1** of retinal and the pyrroline analogue **2** show the parent peak at m/z 300.1964 (calculated for $C_{19}H_{26}NO_2$ 300.1964) and 285.2096 (calculated for $C_{19}H_{27}NO$ 285.2100), respectively. These values are within experimental error in agreement with the calculated values for the corresponding molecular formulae.

NMR spectroscopy

The 400 MHz ^{1}H-NMR spectrum of the pyrroline analogue **2** of retinal was recorded in CD_2Cl_2. In the low-field part of the spectrum the signals of the conjugated chain confirm the all-*E* structure, 15-H and 14-H form an AX pattern (δ_{15H} 10.06 ppm, δ_{14H} 5.91 ppm, J 8.1 Hz), 10-H, 11-H and 12-H form an AMX pattern (δ_{10H} 6.25 ppm, δ_{11H} 7.12 ppm, δ_{12H} 6.39 ppm, $J_{10H\text{-}11H}$ 12.3 Hz, $J_{11H\text{-}12H}$ 15.1 Hz) and 7-H and 8-H form the AB pattern of a *trans* ethene fragment (δ_{7H} 6.55 ppm, δ_{8H} 6.25 ppm, J 16.2 Hz). 5-H Appears as a broad singlet at 5.77 ppm.
In the high-field part of the spectrum the aliphatic signals confirm the presence of the pyrrolinyl ring. The signals of the two 1-CH_3 groups appear as a sharp singlet at δ 1.30 ppm, the two 4-CH_3 groups at δ 1.20 ppm. The assignment was confirmed by the NOE of 5-H on the 4,4-$(CH_3)_2$. The signals of the 9-CH_3 and 13-CH_3 groups appear at δ 1.98 ppm and δ 2.28 ppm, respectively, as sharp doublets. The 100-MHz ^{1}H-noise-decoupled ^{13}C-NMR spectrum of **2** (in CD_2Cl_2) shows 17 peaks, in accordance with the 17 chemically different carbon atoms. The eleven signals in the low-field region of the spectrum were assigned from the ^{1}H-^{13}C COSY spectrum and by comparison with the spectrum of all-*E*-retinal (**3**). In the high field region six signals appear. The signals of the methyl groups were assigned from the H-C COSY spectrum. The two remaining signals belong to the quaternary aliphatic carbons in the ring.
In order to obtain information on the structure of the paramagnetic **1**, we reduced **1** in the NMR tube to its corresponding diamagnetic hydroxylamine using phenylhydrazine according to the method of *Lee* and *Keana*[13]. The 200 MHz ^{1}H-NMR spectrum of the hydroxyl amine analogue of **1** was found to be very similar to the spectrum of **2**. Differences in chemical-shift values were found for 5-H (0.04 ppm upfield) and 4,4-$(CH_3)_2$ (0.01 ppm downfield).

UV / Vis spectroscopy

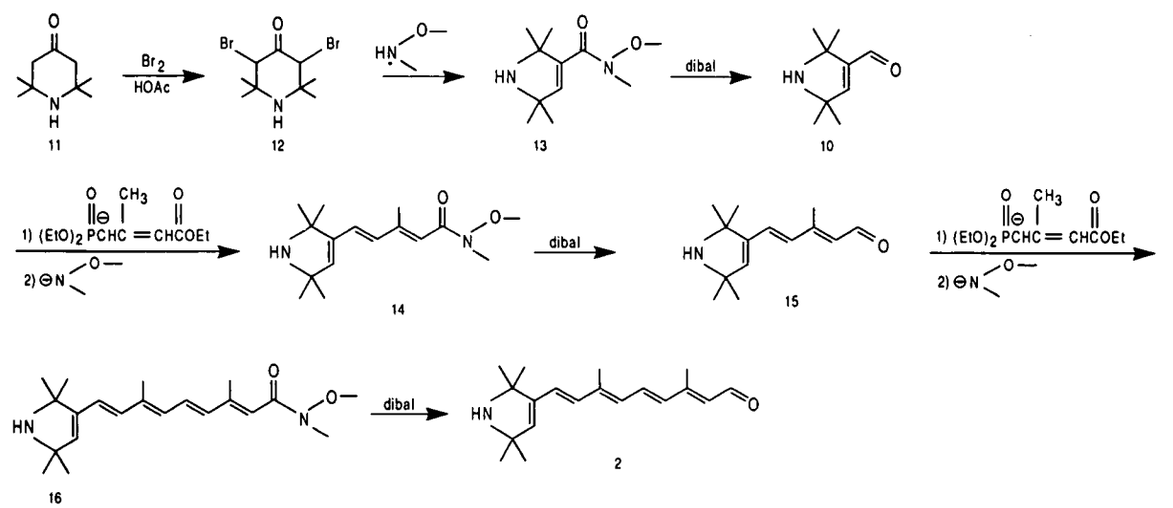
The electronic absorption spectrum of **1** shows a broad bell-shaped absorbance curve without vibrational fine structure. The λ_{max} in the absorbance is at 384 nm in ethanol, and 366 nm in n-hexane. Aminoxy groups are known to have a $n \rightarrow \pi^*$ transition absorption[14] with a λ_{max} around 420–460 nm and a small ϵ_{max} value (< 20). This absorption is not observed in the electronic absorption spectrum of **1** because of the much stronger $\pi \rightarrow \pi^*$ transition of the conjugated polyene[15]. The electronic absorption spectrum of **2** also shows a broad bell-shaped absorbance curve without vibrational fine structure and a λ_{max} in the absorbance at 384 nm in ethanol, and 366 nm in *n*-hexane. **1** and **2** are indistinguishable by UV/Vis spectroscopy.

ESR spectroscopy

The 9 GHz ESR spectrum of **1** in dilute benzene solution shows the characteric isotropic nitroxide pattern with a g value of 2.0055 and a hyperfine splitting (a_N) of 14.6 mT, confirming the presence of the free radical. The g and a_N values are typical for a dialkylnitroxide[16]. Calibration of the signal intensity against that of a known amount of DPPH [2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl] confirmed the presence of 1.0 electron spins per molecule.
The ESR spectrum of a single crystal of **1** displays a single absorption. The hyperfine interaction of the electron spins with the ^{14}N nucleus is averaged out by the electron spin-spin interaction. The spectrum is highly anisotropic, the line shape and the g value depend on the orientation of the crystal in the external magnetic field. The 9 GHz ESR spectrum of **1** in the crystalline state and in dilute benzene solution is reproduced in Figure 3.

Interaction with bacterioopsin

Bacterioopsin (bO) reacts with a slight excess of all-*E*-retinal (**3**) at room temperature to form, within seconds, bacteriorhodopsin (bR; λ_{max} 568 nm, ϵ_{max} 63.10^3). After 30 minutes, the conversion is complete. Upon standing in the dark for a couple of hours, dark-adapted bR is formed (λ_{max} 558 nm). It completely reverts to the light-adapted



Scheme 2. Synthesis of 2.

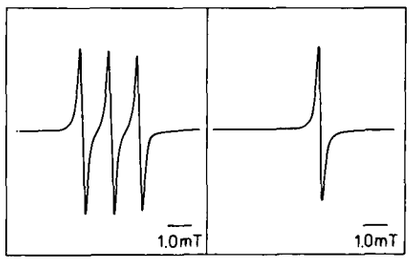


Figure 3. ESR spectrum of 1, a) in benzene solution, b) as a single crystal at an arbitrary orientation.

form upon exposure to visible light.

A slight excess of paramagnetic retinal analogue **1** reacts with bO to form, within minutes, a system with λ_{max} 459 nm, bR′(**1**). The opsin shift of this state amounts to 1000 cm^{-1}. This system does not show light-dark adaptation. Upon incubation of this bR′(**1**) with all-*E*-retinal (**3**), the analogue chromophore is replaced by the native chromophore. This process is complete in approximately 48 hours.

2 behaves very similarly to **1** in its interaction with bO, generating a system with a λ_{max} of 459 nm bR′(**2**) and which does not show light-dark adaptation. Also bR′(**2**) is unstable in the presence of **3**. These results suggest that the presence of the paramagnetic aminoxy group in **1** has no influence on the electronic absorption and binding characteristics of bR′(**1**) when compared to the diamagnetic amine in bR′(**2**).

We reconstituted native bR, as well as bR′(**1**) and bR′(**2**) in soybean phospholipid vesicles. Illumination of the vesicles containing bR with visible light rapidly increases the pH of the external medium. The resulting proton gradient then decreases the velocity of proton uptake asymptotically until a steady state is reached in which the light-driven proton uptake equals the passive back-leakage. The extent of proton uptake can be measured as the difference between the pH before illumination and the pH reached at the steady state. When the light is turned off, the protons reequilibrate until the proton gradient has disappeared.

For the vesicles containing bR′(**1**) or bR′(**2**), no change in pH of the external medium could be detected, showing that neither bR′(**1**) nor bR′(**2**) is active as a proton pump.

Native bacteriorhodopsin can be converted into a highly oriented, hydrated thin film by evaporation of a concentrated membrane suspension of bR on a glass plate[17]. We have prepared such a film containing bR′(**1**). The 9-GHz ESR spectrum of the bR′(**1**) film is highly anisotropic, indicative of a high degree of orientation of the bR molecules in the film. The spectrum has two well-separated (6.7 mT) outer hyperfine extrema, see Figure 4.

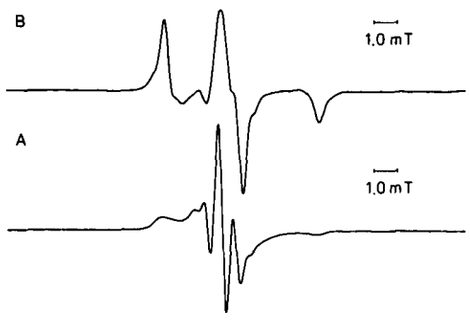


Figure 4. ESR spectrum of bR′(1), with the plane of the membrane oriented a) along the magnetic field and b) perpendicular to the magnetic field.

This is typical for a slow-motion nitroxide spectrum near the rigid limit. It proves that the head-end group is rigidly fixed in the binding site.

Discussion

We have prepared the pyrrolinyloxy analogue **1** of retinal in 16% based on **5** and the pyrroline analogue **2** in 14% based on **11**. Initially we envisioned introducing the paramagnetic group in the retinal analogue in one of the last steps by mild oxidation of the amine. Clearly, retinal **2** is not a suitable precursor for **1**, as oxidation inevitably involves the aldehyde group. We tried to oxidize the amine in *N*-methoxy-*N*-methylamide **16** selectively, but methods using 3-chloroperoxybenzoic acid, monoperoxyphthalic acid, or based on a two-phase system (the classical reagent sodium wolframate, *eg.*, is only soluble in water, whereas **16** only dissolves in organic solvents) were found to be ineffective or to oxidize double bonds before an aminoxy group could be detected.

Chain elongation reactions in polyene synthesis are often carried out by coupling of a phosphonate to an aldehyde. In our group, one of the reasons we use (diethoxyphosphinyl)acetonitrile and 4-diethoxyphosphinyl-3-methyl-2-butenitrile as a first choice of chain-elongation synthons is that the resulting nitrile can be easily converted into the corresponding aldehyde in a high-yield two-step one-pot reaction *viz* reduction with diisobutylaluminum hydride (Dibal). Addition of dibal to a petroleum ether or dichloromethane solution of the unsaturated nitrile at −60°C proceeds to give an aluminum complex within 15 minutes, which can be hydrolysed under very mild conditions without isomerization of double bonds. (In THF, the reduction of nitriles using Dibal fails, because the intermediate does not break down to the aldehyde under the hydrolysis conditions that we use.) This simple method failed for the nitriles containing the aminoxy group: a small excess of dibal left the starting material unchanged and addition of more Dibal and elevation of the reaction temperature eventually led to the destruction of the starting material without detection of the expected aldehyde. The reduction of the esters corresponding to the nitriles and subsequent MnO_2 oxidation of the resulting unsaturated alcohols is another procedure often used to prepare the aldehydes. In the case of ester intermediates corresponding to **6**, **7** and **9**, containing the aminoxy group, the reduction of the ester group with Dibal or lithium aluminum hydride ($LiAlH_4$; in THF or diethyl ether) was found to be capricious and gave low and irreproducible yields. Reduction of the *N*-methoxy-*N*-methyl amides in petroleum ether was also unsuccesful, but in THF the reduction to the corresponding aluminum intermediate and mildly acidic hydrolysis to the aldehyde went to completion rapidly and in high yield without isomerization of the double bonds. It is as yet unclear how the presence of the aminoxy group influences the otherwise problemless reductions so drastically.

In the corresponding intermediates in the synthesis of **2** the reductions posed little problem. In this case, we found that the *N*-methoxy-*N*-methyl intermediates were much easier to separate into the isomerically pure compounds than were their ester and nitrile analogues. As already pointed out, separation is crucial in the step before the aldehyde, because the aldehydes were very unstable, and little or no yield was obtained after attempted silica gel chromatography. No silicagel was used in the hydrolysis of the aluminum complex. Instead, the hydrolysis was carried out with water and ammonium chloride, and salts were filtered off using celite. The instability of the amine aldehydes **15** and **2** is likely to be due to base catalysed

condensation by the amine in the pyrroline ring. Amine aldehyde **10** was found to be considerably more stable than **15** or **2** and no special precautions were necessary to prevent undesirable reactions.

A mixture of **1** and its isomers could not be separated by normal phase isocratic HPLC because the resolution was too low; attempts using reverse phase HPLC were also unpromising. The pure **1** crystallizes easily from THF (+4°C) and single crystals can be obtained.

A different synthesis of **1** was first reported by *R.K. Crouch* et al.[9c] in 1981 on a milligram scale; this synthesis was found to be unsuitable for large scale purposes. We have now developed a high-yield chain elongation method for aminoxy retinal **1** and its novel amine equivalent, and it is clear that a host of aminoxy-labeled polyenes has become routinely accessible on a 100 mg scale. The yields are sufficiently high to allow incorporation of ^{13}C labels in these molecules, thereby enabling ENDOR studies.

Because bR′(**1**) and bR′(**2**) are photospectrometrically identical and neither constitute an active proton pump, it can be assumed that **1** and **2** occupy the same position in the binding site and that the introduction of the paramagnetic group itself has no influence on the properties of the resulting pigment. bR′(**2**) can be regarded as a true diamagnetic analogue of bR′(**1**).

We have shown earlier that retinal analogues with a locked 6-7-s-*cis* conformation react with bO to give the corresponding bR analogues, which rapidly convert to systems with a smaller opsin shift (~ 600 cm^{-1}) and which do not pump protons[8b,18]. It has been suggested that in the 6-7-s-*cis* conformation, the dimethyl group interferes with residues in helix D and F[6]. Although the five-membered pyrrolidinyl ring in **1** and **2** is somewhat smaller than the six-membered ring in the native **3** and lacks a 5-methyl group, the presence of two bulky dimethyl groups was expected to provoke a similar steric interaction. Indeed, a very small opsin shift (1000 cm^{-1}) was observed for the system formed by reaction of **1** or **2** with bacterioopsin. (We did not observe the initial formation of a system with a larger opsin shift.) Since both **1** and **2** are replaced upon incubation with the native chromophore, it is clear that the analogues **1** and **2** bind only loosely to the protein. The ESR spectrum, however, shows that retinal analogue **1** has a well defined orientation in the protein and that the resulting bR′(**1**) is not a random pigment. Anticipated ENDOR experiments, with the electron spin on the retinal and ^{13}C nuclear spin labels in the peptide chain, are expected to shed light on the nature of the binding of these chromophores and the interaction with specific amino acid residues.

Experimental

General

Chemicals were purchased from Janssen Chimica (Belgium) or Aldrich (MA, USA). Tetrahydrofuran (THF) was distilled prior to use from lithium aluminum hydride ($LiAlH_4$), diethyl ether from P_2O_5, petroleum ether (bp. 40–60) from P_2O_5, *n*-hexane from P_2O_5, and ethyl acetate from P_2O_5. Other chemicals (reagent grade) were used without purification. *N*-methoxy-*N*-methylhydroxylamine hydrochloride was prepared according to *Goel* and *Kroll*[19].

Reactions were generally carried out in a nitrogen atmosphere. Glassware of appropriate size was flame-dried prior to use. A magnetic stirring bar was used to stir the solutions. The course of reaction was followed by analytical TLC.

Diisobutyl aluminum hydride (dibal) was used as a 1.0M solution in hexanes, *n*-butyllithium (BuLi) as a 1.6M solution in hexanes. These solutions were introduced into the reaction mixture dropwise via a syringe.

After completion of the reaction, the products were isolated as follows, unless stated otherwise: water was added to the reaction mixture. The layers were separated and the aqueous phase was extracted three times with diethyl ether. The combined organic layers were washed with brine and dried with sufficient $MgSO_4$. The solids were filtered off over a glass fritted funnel. The residue was rinsed with diethyl ether. The solvents in the filtrate were evaporated.

Evaporation of solvents was performed *in vacuo* (20 mmHg). Unless stated otherwise, purification was performed by flash column SiO_2 chromatography, using petroleum ether/ethyl acetate/triethylamine (5%) mixtures as eluent.

NMR spectra were run in $CDCl_3$ or CD_2Cl_2 (for aldehydes) with tetramethylsilane (δ 0 ppm) as internal standard on a Jeol FX-200, a Bruker WM-300 or a Bruker MSL-400 (operating at 199.5 MHz, 300.1 MHz and 400.1 MHz, respectively, for proton NMR and 50.1 MHz, 75.4 MHz and 100.4 MHz, respectively, for carbon-13). Spectral signal designations are based on the retinoid numbering system[20].

NMR spectra of paramagnetic compounds were recorded using the method described by *Lee* and *Keana*[13]. In short, a known amount of the paramagnetic compound was dissolved in a suitably deuterated solvent and transferred to the NMR tube. Then, a solution of a known concentration of phenylhydrazine was added in equimolar amount. Under ideal conditions, the paramagnetic aminoxy is reduced to its hydroxylamine and phenylhydrazine is converted into benzene and nitrogen. Frequently, however, the formation of side products prevented the identification of resonances in the ^{13}C-NMR spectra; in the ^{1}H-NMR spectra, the peaks arising from side products could usually be distinguished from the peaks arising from the molecule under investigation.

UV/Vis spectra were run on a Varian DMS-200, using methanol or *n*-hexane as solvents. The EI mass spectra were recorded on a Finingan MAT 900 (ion source temperature 150°C). The ESR spectra were recorded on a Jeol JES-RE2, operating at 9.15 GHz. *g*-Values were calibrated against 2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl.

N-*methoxy*-N-*methylamine*

N,O-Dimethylhydroxylamine hydrochloride (40.0 g, 0.41 mol) was dissolved in 80 ml (0.6 mol) of *sym*-collidine. The mixture was heated, first at an oil bath temperature of 115°C, then increasing to 178°C. The free *N,O*-dimethylhydroxylamine was distilled and collected in an ice-cooled receiver flask. Bp. 44°C; density at 23°C: 0.832 g/ml. Yield 22.7 g (91%). ^{1}H NMR (200 MHz): δ 6.50 ppm, br. s. (N-H); 3.51, s (-O-CH_3); 2.70, s (-N-CH_3).

3-Carboxy-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy (**5**)

12 88 g (0.19 mol) was added to a mixture of 200 ml of 35% aqueous ammonia. The mixture was stirred for 2 h. The mixture was then saturated with KOH and the product was filtered off. Yield 29.7 g (80%). 29.7 g (0.18 mol) of 2,2,5,5-tetramethyl-3-pyrroline-3-carboxamide, 300 ml of water, 1.5 g of titriplex-II and 1.5 g of $Na_2WO_4 \cdot 2H_2O$ were mixed and 30 ml of 30% H_2O_2 were added. The mixture was left to stand in the dark at room temperature and was occasionally swirled. After 10 days, the bright yellow crystals were filtered off and used without further purification. Yield 26.3 g (81%) of 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy. Mass *m/z* 183 (M+). A mixture of 5 g (27 mmol) of this compound and 60 ml of N NaOH was refluxed until the evolution of ammonia had ceased. After cooling, the mixture was acidified with N HCl and 100 ml of dichloromethane was added. The pale yellow product, **5**, was isolated. Yield 4.2 g (85%). Mass *m/z* 184 (M+).

*3-(*N-*Methoxy*-N-*methylcarbamoyl)-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy* (**6**)

5 (3.0 g, 16 mmol) and 2.6 g (32 mmol) of pyridine were dissolved in CH_2Cl_2. $SOCl_2$ (2.2 g in CH_2Cl_2) was added dropwise at 0°C. The mixture was stirred for 1 h at room temperature, then 1.2 g (20 mmol) of *N*-methoxy-*N*-methylamine in CH_2Cl_2 were added dropwise. The mixture was stirred for 1 h, then the solids were filtered off. Excess pyridine was removed by washing with N HCl. The product was isolated and purified. Yield 2.8 g (75%). ^{1}H NMR (Ph-NH-NH_2, $CDCl_3$, 200 MHz): δ 6.10 ppm, s (5-H); 3.63, s (O-CH_3); 3.24, s (N-CH_3); 1.42, s (2 CH_3); 1.30, s (2 CH_3). Mass *m/z* 227 (M+).

3-Formyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy (**4**)

6 (2.0 g, 8.8 mmol) was dissolved in THF and reduced with 20 mmol of Dibal at −60°C. After stirring for 1 h, 10 ml of a saturated ammonium chloride solution were added at 0°C. When the mixture

warms up to room temperature, hydrolysis takes place and much heat is generated. Care should be taken to avoid boiling of the solvent. After 2 h, $MgSO_4$ was added and the solids were filtered off over a layer of celite on a glass fritted funnel. The solvents were evaporated and the product was purified, yielding 1.3 g (88%) of **4**. ^{1}H NMR (Ph-NH-NH_2, $CDCl_3$, 200 MHz): δ 9.68 ppm, s (7-H); 6.59, s (5-H); 1.38, s (2 CH_3); 1.30, s (2 CH_3). Mass *m/z* 168 (M+).

*3-[(1E,3E)-5-(*N*-Methoxy-*N*,-methylamino)-3-methyl-5-oxopenta-1,3-dienyl]-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy* (**7**)

Methyl 5-diethoxyphosphinyl-3-methyl-2-pentenoate (7.0 mmol) was dissolved in THF at −60°C and 6.6 mmol of BuLi was added dropwise via a syringe. After stirring for 30 min at −60°C, 1.0 g (6.0 mmol) of **4** was added and the mixture was stirred overnight. Then the product was isolated to yield 1.4 g (83%). *N*-methoxy-*N*-methylamine hydrochloride (0.98 g, 10 mmol) was dissolved in THF and BuLi (20 mmol) was added at −20°C. After stirring for 15 min, a solution of 1.4 g (5.0 mmol) of the ester in THF was added dropwise. The mixture was stirred at room temperature for 3 h. Then the product was isolated and purified, yielding 1.1 g of **7** (75%). Mass *m/z* 293 (M+). ^{1}H NMR (Ph-NH-NH_2, $CDCl_3$, 200 MHz): δ 6.55 ppm, d, *J* 16.2 Hz (7-CH); 6.40 d, *J* 16.2 Hz (8-CH); 6.27, s (10-CH); 5.81, s (5-CH); 3.71, s (N-CH_3); 3.23, s (O-CH_3); 2.29, s (9-CH_3); 1.35, s (2 CH_3); 1.24, s (2 CH_3).

3-[(1E,3E)-3-methyl-5-oxopenta-1,3-dienyl]-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy (**8**)

7 (0.6 g, 2.0 mmol) was reduced with 4 mmol of Dibal at −60°C in THF. After stirring for 1 h, 3 ml of a saturated ammonium chloride solution were added at 0°C. When the mixture warms up to room temperature, hydrolysis takes place and much heat is generated. Care should be taken to avoid boiling. After 3 h, $MgSO_4$ was added and the solids were filtered off over a layer of celite on a glass fritted funnel. The solvents were evaporated and the product was purified, yielding 0.41 g (85%). Mass *m/z* 234 (M+). ^{1}H NMR (Ph-NH-NH_2, $CDCl_3$, 200 MHz): δ 10.13 ppm, d *J* 8.2 Hz, (11-CH); 6.7.1, d, *J* 16.4 Hz (7-CH); 6.61, d *J* 16.4 Hz, (8-CH); 6.04, d, *J* 8.2 Hz (10-CH); 5.96, s (5-CH); 2.31, s (19-CH_3); 1.37, s (2 CH_3); 1.27, s (2 CH_3).

*2E,4E,6E,8E-*N*-(2,2,5,5-tetramethyl-3-pyrrolin-1-ox-2-yl)-2,4,6,8-nonatetraenamide* (**9**)

Methyl 5-diethoxyphosphinyl-3-methyl-2-pentenoate (2.0 mmol) was dissolved in THF at −60°C and 1.9 mmol of BuLi was added dropwise via a syringe. After stirring for 30 min at −60°C, 0.40 g (1.7 mmol) of **8** was added and the mixture was stirred overnight. Then the product was isolated, yielding 0.41 g (68%). Mass *m/z* 344 (M+). *N*-methoxy-*N*-methylamine hydrochloride (0.49 g, 5 mmol) was dissolved in THF and BuLi (10 mmol) was added at −20°C. After stirring for 15 min, a solution of 0.41 g (1.2 mmol) of the ester in THF was added dropwise. The mixture was stirred at room temperature for 3 h. Then the product was isolated and purified, yielding 0.38 g of **9** (89%). Mass m/z 359 (M+). ^{1}H NMR (Ph-NH-NH_2, $CDCl_3$, 200 MHz): δ 7.73 ppm, dd, *J* 12.3 Hz, *J* 15.1 Hz (11-CH); 6.60, d, *J* 15.4 Hz (7-CH); 6.38, d, *J* 15.0 Hz (12-CH); 6.28, d, *J* 13.4 Hz (10-CH); 6.28, d, *J* 16.4 Hz (8-CH); 6.08, s (14-CH); 5.80, br.s (5-CH); 3.71, s (O-CH_3); 3.27, s (N-CH_3); 2.34, s (13-CH_3); 1.98, s (9-CH_3); 1.33, s (2 CH_3); 1.23, s (2 CH_3).

3-[(1E,3E,5E,7E)-3,7-Dimethyl-9-oxonona-1,3,5,7-tetraenyl]-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy (**1**)

9 (0.30 g, 0.84 mmol) was dissolved in THF and 2.0 mmol of Dibal were added at −60°C. The mixture was stirred for 30 min, then 2 ml of a saturated ammonium chloride solution was added at 0°C. When the mixture warms up to room temperature, hydrolysis takes place and much heat is generated. Care should be taken to avoid boiling. After 2 h, $MgSO_4$ was added and the solids were filtered off over a layer of celite on a glass fritted funnel. The solvents were evaporated and the product was purified, yielding 0.19 g (76%) of **1**. The product was redissolved in THF and crystallized at +4°C in large yellow crystals. The spectroscopic characterization is described in the text. In the single focus mass spectrum (EI) major peaks appear at *m/z* 300(M+.), 286, 270, 241, 227, 161, 159, 145, 133, 128. The peak at *m/z* 270 probably represents loss of an N-O fragment.

3,5-Dibromo-2,2,6,6-tetramethylpiperidin-4-one hydrobromide (**12**)

To 100 g (0.52 mol) of 2,2,6,6-tetramethylpiperidin-4-one (**11**) in 400 ml of acetic acid, 160 g (1.0 mol) of bromine were added dropwise. The mixture was stirred and left to stand overnight. Then the product was collected on a glass fritted funnel and washed successively with 100 ml of acetic acid, 100 ml of water and 100 ml of diethyl ether. Yield 192 g (93%) of **12**, which was used without further purification.

N-*Methoxy-*N*,2,2,5,5-pentamethyl-3-pyrroline-3-carboxamide* (**13**)

12 (88 g, 0.19 mol) was added to a mixture of 200 ml of water, 200 ml of triethylamine and 21.6 g (0.22 mol) of *N,O*-dimethylhydroxylamine hydrochloride. The mixture was stirred for 2 h at 50°C. Then the mixture was allowed to cool down and the product was isolated, yielding 34 g (86%) of **13**. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 6.14 ppm, br. s. (5-H); 3.65, s. (-N-CH_3); 3.26, s (O-CH_3); 1.41, s (2 CH_3); 1.31, s (2 CH_3).

2,2,5,5-Tetramethyl-3-pyrroline-3-carboxaldehyde (**10**)

13 (5.0 g, 24 mmol) was dissolved in THF and reduced with 50 mmol of Dibal at −60°C. After stirring for 1 h, 10 ml of a saturated ammonium chloride solution were added at 0°C. When the mixture warms up to room temperature, hydrolysis takes place and much heat is generated. Care should be taken to avoid boiling. After 2 h, $MgSO_4$ was added and the solids were filtered off over a layer of celite on a glass fritted funnel. The solvents were evaporated and the product was purified, yielding 3.1 g (87%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.80 ppm, s (7-H); 6.67, s (5-H); 2.01, br.s (N-H); 1.40, s (2 CH_3); 1.35, s (2 CH_3).

*(2E,4E)-*N*-Methoxy-*N*,3-methyl-5-(2,2,5,5-tetramethyl-3-pyrrolin-3-yl)-2,4-pentadienamide* (**14**)

Methyl 5-diethoxyphosphinyl-3-methyl-2-pentenoate (23.0 mmol) was dissolved in THF at −60°C and 22 mmol of BuLi was added dropwise via a syringe. After stirring for 30 min at −60°C, 3.1 g (20 mmol) of **10** was added and the mixture was stirred overnight. Then the product was isolated, yielding 4.68 g (87%). *N*-Methoxy-*N*-methylamine hydrochloride (2.94 g, 30 mmol) was dissolved in THF and BuLi (60 mmol) was added at −20°C. After stirring for 15 min, a solution of 4.6 g (17.0 mmol) of the ester in THF was added dropwise. The mixture was stirred at room temperature for 3 h. Then the product was isolated and purified, yielding 3.4 g of **14** (69%). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 6.55 ppm, d, *J* 16.4 Hz (7-CH); 6.41, d, *J* 16.4 Hz (8-CH); 6.27, s (10-CH); 5.85, s (5-CH); 3.71, s (N-CH_3); 3.23, s (O-CH_3); 2.29, s (9-CH_3); 1.37, s (2 N-C-CH_3); 1.27, s (2 N-C-CH_3).

(2E,4E)-3-Methyl-5-(2,2,5,5-tetramethyl-3-pyrrolin-3-yl)-2,4-pentadienal (**15**)

14 (3.4 g, 12 mmol) was dissolved in THF and reduced with 20 mmol of Dibal at −60°C. After stirring for 1 h, 10 ml of a saturated ammonium chloride solution were added at 0°C. When the mixture warms up to room temperature, hydrolysis takes place and much heat is generated. Care should be taken to avoid boiling. After 2 h, $MgSO_4$ was added and the solids were filtered off over a layer of celite on a glass fritted funnel. Yield 2.2 g (82%). The pure **15** is unstable and tends to polymerize rapidly. It should not be exposed to temperatures higher than 25°C, and should immediately be reacted in the next step. We found that **15** deteriorates even upon storage under argon atmosphere in dilute petroleum ether solution at −80°C. ^{1}H NMR (CD_2Cl_2, 200 MHz): δ 10.13 ppm, d *J* 8.2 Hz, (11-CH); 6.64, d, *J* 16.4 Hz (7-CH); 6.54, d *J* 16.4 Hz (8-CH); 5.99, d, *J* 8.2 Hz (10-CH); 5.96, s (5-CH); 2.30, s (19-CH_3); 1.38, s (2× CH_3); 1.28, s (2× CH_3). ^{13}C-NMR (50 MHz): δ 190.9 ppm (C=O); 154.0 (C-9), 144.4 (C-6), 139.8, 132.5, 129.5, 128.7, 65.7 (N-C), 63.2 (N-C), 30.3 (2 CH_3), 29.9 (2 CH_3), 12.6 (19-CH_3).

*(2E,4E,6E,8E)-*N*-Methoxy-*N*,3,7-trimethyl-9-(2,2,5,5-tetramethyl-3-pyrrolin-3-yl)-2,4,6,8-nonatetraenamide* (**16**)

Methyl 5-diethoxyphosphinyl-3-methyl-2-pentenoate (11 mmol) was dissolved in THF and BuLi (10 mmol) was added dropwise at −60°C via a syringe. After stirring for 30 min at −60°C, 2.0 g (9.1 mmol) of freshly prepared **15** was added and the mixture was stirred overnight. Then the product was isolated, yielding 2.6 g (88%). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.02 ppm, dd (11-H); 6.60–6.10, vinylic signals; 5.80, s, (14-H); 5.78, s, (5-H); 4.12, q (O-CH_2); 2.32, s (20-CH_3); 1.96, s (19-CH_3); 1.32, s (2 CH_3); 1.28, t (CH_3); 1.22, s (2 CH_3). *N*-methoxy-*N*-methylamine hydrochloride (1.96 g, 20 mmol) was dissolved in THF and BuLi (40 mmol) was added at −20°C. After stirring for 15 min, a solution of 2.6 g (7.9 mmol) of the ester in THF

was added dropwise. The mixture was stirred at room temperature for 3 h. Then the product was isolated and purified, yielding 1.7 g of **16** (62%). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.77 ppm, dd, J 12.1 Hz, J 14.9 Hz (11-CH); 6.61, d, J 14.9 Hz (7-CH); 6.38, d, J 15.0 Hz (12-CH); 6.27, d, J 13.4 Hz (10-CH); 6.27, d, J 16.2 Hz (8-CH); 6.08, s (14-CH); 5.77, br.s (5-CH); 3.71, s (O-CH_3); 3.27, s (N-CH_3); 2.34, s (13-CH_3); 2.01, s (9-CH_3); 1.33, s (2 CH_3); 1.22, s (2 CH_3).

(2E,4E,6E,8E)-3,7-Dimethyl-9-(2,2,5,5-tetramethyl-3-pyrrolin-3-yl)-2,4,6,8-nonatetraenal (**2**)

16 (1.7 g, 4.9 mmol) was dissolved in THF and reduced with 10 mmol of Dibal at −60°C. After stirring for 1 h, 4 ml of a saturated ammonium chloride solution were added at 0°C. When the mixture warms up to room temperature, hydrolysis takes place and much heat is generated. Care should be taken to avoid boiling. After 2 h, $MgSO_4$ was added and the solids were filtered off over a layer of celite on a glass fritted funnel. The solvents were evaporated. Yield 1.1 g (78%). The pure **2** is unstable and tends to polymerize rapidly. It should not be exposed to temperatures higher than 25°C. It should be freshly prepared from **16** when needed. We found that **2** deteriorates even upon storage under argon atmosphere in dilute *n*-pentane solution at −80°C. The spectroscopic characterization of **2** is described in the text. In addition, in the single focus mass spectrum (EI), major peaks appear at m/z 285 (M+.), 270 (M+. −15), 240, 226, 190, 161, 133, 108 and 95.

Schiff bases and protonated Schiff bases

The Schiff bases were prepared in a cuvette in the UV/Vis photospectrometer by addition of an excess of *n*-butylamine to a dilute solution of **1** and **2** in methanol. The λ_{max} of the n-butyl imine of **1** is at 365 nm. The absorption spectrum of the *n*-butylimine of **2** was very similar to that of **1**, with the same λ_{max} value. Addition of a drop of concentrated hydrochloric acid to these methanolic solutions completely converted the Schiff bases into their protonated form.

Bacterioopsin

Bacteriorhodopsin (bR) was obtained from *Halobacterium halobium* cultures (strain R1S9) as published before[21]. Bacterioopsin was prepared from bR by bleaching at 37°C in the presence of hydroxylamine (per 100 mg of bR: 100 ml of water, 300 mg of N-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] and 3.5 g of hydroxylamine hydrochloride, adjusted with KOH to pH 8.0). The solution was centrifuged at 18.000 rpm for 10 min to remove the salts. The supernatant was decanted and the pellet was resuspended in a few ml of water and pelleted again. Then the supernatant was resuspended, washed three times with *n*-hexane and pelleted. Finally the pellet was resuspended in water at a concentration of 2 mg/ml and used for regeneration experiments.

*Preparation of hydrated bR'(**1**) film*

A solution of approximately 2 mg of bR'(**1**) patches was pelleted at 10.000 rmp (Dupont Sorvall RBC-5, SS-34 rotor). The pellet was washed 10 times with an aqueous bovine serum albumin (BSA, Fraction IV, Sigma Chemical Co, MO; 20 g/l) solution to remove any excess of retinals, then 10 times with millipore water to remove all BSA. After the last rinse, the supernatant was discarded completely and a small amount of water [10–30 μl/mg of bR'(**1**)] was added. The pellet was not stirred, but left to swell for two days. If necessary, a little more water was added to make the suspension fluid enough to take it up with a pipet without getting bubbles. Bubbles would interfere with spreading evenly on a glass plate. The suspension was transferred onto a 4 mm × 20 mm quartz plate. The suspension on the plate was left to dry for three days at room temperature in a closed argon atmosphere in the presence of a saturated KCl solution. Samples were stored in this environment.

Binding experiments

Binding experiments were performed at room temperature as described earlier[22]. Regeneration was followed in 2-mm pathlength cuvettes. Light-dark adaptation was performed as described earlier[22]. Both bR'(**1**) and bR'(**2**) did not show light-dark adaptation.

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

bR (10 mg) was bleached to bO. The solution was pelleted and taken up in 8 ml of millipore water. All-*E*-retinal (**3**), **1** and **2** (in 10 μl ethanol) were each added to 2 ml of the bO solution. After overnight incubation equal amounts of bR, bR'(**1**), bR'(**2**) and bO were obtained and subsequently precipitated. Each pellet was taken up in 3 ml of a solution of 0.15M KCl and 2mM EDTA (pH 7). Two ml of the bR solution were added to 50 mg of soybean phospholipids (from Sigma, MO, USA). The suspension was then sonified using a MSE probe type ultrasonifier (probe diameter 2 mm, freq. 21 kHz, ampl. 5 μm) for 15-s periods followed by 45 s of cooling for 1 h[23]. The mixture was kept under nitrogen and cooled in ice during sonication. Using this procedure, liposomes containing bO, bR, bR'(**1**) and bR'(**2**) were prepared.

The 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 bR liposomes and 1.8 ml of a solution of 0.15M KCl and 2mM EDTA 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 (20 V). The pH changes upon illumination were calibrated by the addition of 50 nmol oxalic acid.

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