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(1*R*)- And (1*S*)-5-demethyl-8,16-methanobacteriorhodopsin and its properties. The synthesis and spectroscopy of 5-demethyl-8,16-methanoretinal in optically active and isotopic forms

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Abstract. (All-*E*)-5-demethyl-8,16-methanoretinal was prepared in both optical antipodes (1*R* and 1*S*), as well as in their 9-*Z* and 13-*Z* forms. The interaction of the 1*R* and 1*S* forms with bacterioopsin shows that there is a clear chiral recognition on the formation of the bacteriorhodopsin analogues, reflected in the rate of binding and ε_{max} values. The two bacteriorhodopsins with optically active chromophores have an identical λ_{max} value and show the same efficiency of the light-driven proton pump. The absence of the 5-methyl group leads to a 50% lower proton-pump efficiency than the native system. In addition, for the racemic 5-demethyl-8,16-methanoretinal, the 5-^{2}H, 7-^{2}H, 5,7-$^2H_2$ and 5,7,16a,16a-$^2H_4$ isotopomers in the various isomeric forms all-*E*, 9-*Z* and 13-*Z* were also prepared.

Introduction

The membrane protein bacteriorhodopsin (bR) functions as a light-driven proton pump in the purple membrane of *Halobacterium halobium*[1,2]. In the bacterium, light energy is converted into a proton gradient over the membrane, the energy of which is utilized by the bacterium to form ATP and to power its life processes[2].

The chromophore is all-*trans*-retinal bound to the ε-amino group of lysine 216 via a protonated Schiff base (PSB) linkage (Figure 1P)[3]. The λ_{max} value of light-adapted bR (568 nm) is much larger than that of the model PSB compound (440 nm in methanol) consisting of (all-*E*)-retinal and *n*-butylamine. The red shift in the absorption maximum of bR relative to its model PSB is due to interactions of the chromophore with the protein chain. This difference in wave numbers (5100 cm^{-1}) has been called the "opsin shift"[4]. One of the factors that contribute to the opsin shift is the conformation of the chromophore around the C6–C7 single bond[5]. According to solid-state ^{13}C NMR spectroscopy of ^{13}C5-bR, this conformation is planar *s-trans*[6,7], whereas it is reported to be predominantly twisted 6-*s-cis* for free retinal and derivatives in solution[8].

In a well established approach in bio-organic studies, bR (analogues) are regenerated by reaction of retinal (and its analogues) with the free protein bacterioopsin (bO), obtained by illumination of bR in the presence of hydroxylamine[9,10,11]. Our study of 8,16-methanobacteriorhodopsin (in which the chromophore is locked in an almost planar 6–7-*s-trans* conformation) (Figure 1Q) has given a wealth of new information about the contribution of the 6–7-*s-trans* conformation to the various properties of bR[11]. Analogous work with conformationally mobile ring demethylbacteriorhodopsins has shown that the 5-CH_3 group has a strong influence on the efficiency of the photochemical proton pump[12]. 5-Demethyl-8,16-methanobacteriorhodopsin (Figure 1T) is the system of choice in which the influence of the 5-CH_3 group can be studied in a system with well defined 6–7-*s-trans* conformation. The introduction of an 8,16-methano group introduces chirality into the system. In the previous study, only racemic retinals were obtained. For the synthesis of 5-demethyl-8,16-methanoretinal, 4,4a,5,6,7,8-hexahydro-4a-methyl-2(3*H*)-naphthalenone is available in its pure enantiomeric forms.

In this paper, we describe the synthesis of racemic 5-demethyl-8,16-methanoretinal and its pure 1*R* and 1*S* enantiomers (Figure 2: **1**, **1a**, **1b**), being the first retinoids that have been prepared in both pure enantiomeric forms. The access to two pure enantiomeric retinals has allowed us to investigate how chirality in the chromophore affects the

* IUPAC name: (all-*E*)-7-(3,4,4a,5,6,7-hexahydro-4a-methyl-2-naphthalenyl)-3-methyl-2,4,6-octatrienal.

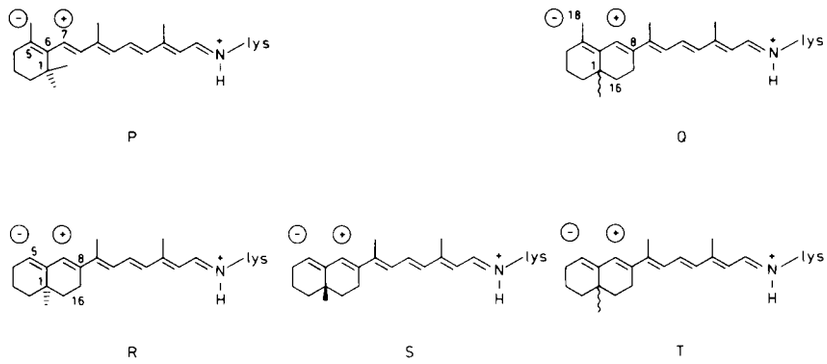


*Figure 1. Structures of chromophoric groups of P: bacteriorhodopsin (bR); Q: 8,16-methano-bR; R: (R)-5-demethyl-8,16-methano-bR [bR(**1a**)]; S: (S)-5-demethyl-8,16-methano-bR [bR(**1b**)]; T: (±)-5-demethyl-8,16-methano-bR [bR(**1**)]. The protein has electric charges near 5-C and 7-C (− and +, resp.).*

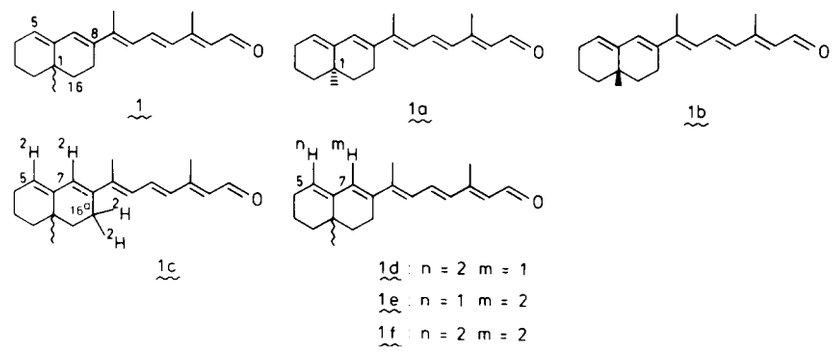
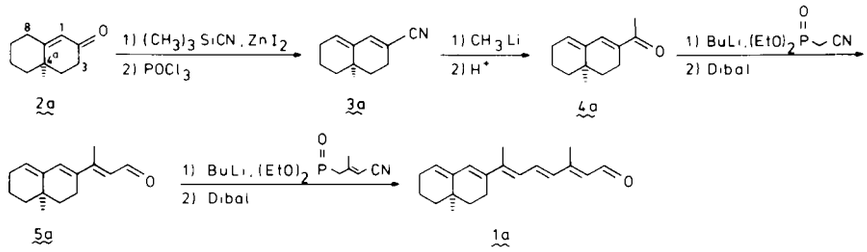


Figure 2. Structure and numbering of 5-demethyl-8,16-methanoretinal **1** *and its* **1a**: *(R)-*, **1b**: *(S)-*, **1c**: *(5,7,16a,16a-$^2H_4$)*, **1d**: *(5-^2H)*, **1e**: *(7-^2H)*, **1f**: *(5,7-$^2H_2$) isomer.*

various properties of the retinal–bO system, such as the binding and fitting in the active site, the λ_{max} and ε_{max} values and proton-pump activity. We also describe the synthesis of racemic (5,7,16a,16a-$^2H_4$)-, (5-^2H)-, (7-^2H)- and (5,7-$^2H_2$)5-demethyl-8,16-methanoretinal (Figure 2: **1c**, **1d**, **1e**, **1f**). These isotopically labeled systems will allow a detailed analysis of the vibrational characteristics of the 6–7 *s-trans* bond, the results of which will be described in a future paper.

Synthesis

The reaction sequence shown in Scheme 1, along which the enantiomers of 5-demethyl-8,16-methanoretinal are synthesized, was optimalized with racemic material. The starting compound was 4,4a,5,6,7,8-hexahydro-4a-methyl-2(3*H*)-naphthalenone (**2**), which was prepared via known methods[13]. Via a one-pot, two-step procedure[14], **2** was (in 76% yield) converted into the bicyclic conjugated nitrile **3** by reaction with trimethylsilyl cyanide[15] and a catalytic amount of ZnI_2, followed by treatment with $POCl_3$ in pyridine. The nitrile function in **3** was converted into a methylketone function by treating **3** with methyllithium to give, after acidic hydrolysis, the β-ionone derivative **4** in 55% yield. β-Ionone and modified β-ionones can be efficiently converted into retinal and retinal derivatives in high yield via a four-step procedure, as described in many of our previous publications[11,16-18]. Similarly, chain extension to **5** was performed by a Horner–Emmons coupling of **4** with diethyl (cyanomethyl) phosphonate[19] followed by diisobutylaluminum hydride (Dibal) reduction. Repeating this sequence using diethyl (3-cyano-2-methyl-2-propenyl)-phosphonate[20] gave 5-demethyl-8,16-methanoretinal (**1**, Figure 2). It was obtained in 60% yield based on **4** and consists mainly of (all-*E*)-**1**, (13-*Z*)-**1**, (9-*Z*)-**1** and a small amount of (9-*Z*,13-*Z*)-**1** (Figure 3). Preparative HPLC gave the all-*E*, 9-*Z*, 13-*Z* and 9-*Z*,13-*Z* isomers in a pure state. We then submitted (*R*)- and (*S*)-4,4a,5,6,7,8-hexahydro-4a-methyl-2(3*H*)-naphthalenone (**2a** and **2b**, respectively),



Scheme 1. Synthesis of (R)-5-demethyl-8,16-methanoretinal (**1a**).

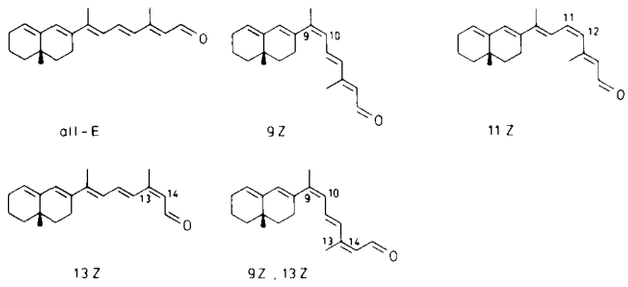
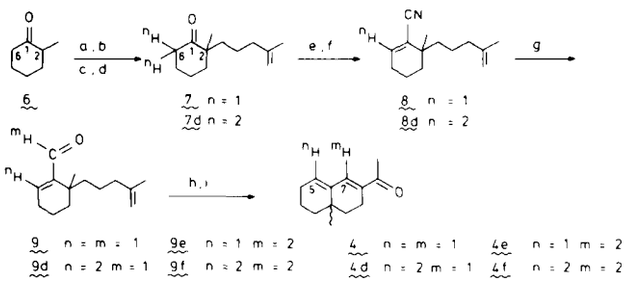


Figure 3. Structures of geometric isomers of (S)-5-demethyl-8,16-methanoretinal (**1b**).

which are commercially available in optically pure form, to the reactions of Scheme 1. The all-*E*, 9-*Z*, 13-*Z* and 9-*Z*,13-*Z* isomers of **1a** and **1b** were obtained (Figures 2 and 3).

For the preparation of the 5- and/or 7-deuterated forms of **1**, we first tried **2** as a suitable starting compound. The protons on carbons 1, 3 and 8 are, in principle, exchangeable with CH_3OD in the presence of methoxide. Treating **2** three times with deuterated methanol gave (1,3,3,8,8-$^2H_5$)-4,4a,5,6,7,8-hexahydro-4a-methyl-2(3*H*)-naphthalenone (**2c**) with high level of pentadeuterium incorporation. Using this compound as starting material for the reactions in Scheme 1 led to (5,7,16a,16a-$^2H_4$)5-demethyl-8,16-methanoretinal **1c** (Figure 2).

No conditions were found for selective introduction of deuterium into **2**. We, therefore, had to develop a new reaction scheme for the preparation of (5-^{2}H)-, (7-^{2}H)- and (5,7-$^2H_2$)5-demethyl-8,16-methanoretinal (**1d**, **1e**, **1f**, respectively, Figure 2): Scheme 2.



Scheme 2. Synthesis of synthons **4**, **4d**, **4e** *and* **4f**. *(a) NaH, EtOCOH; (b) LDA, $ICH_2CH_2CH_2C(=CH_2)CH_3$; (c) NaOH; (d) only for* **7d**: *NaH, CH_3O^2H; (e) $(CH_3)_3SiCN$, ZnI_2; (f) $POCl_3$; (g) Dibal-^{m}H* (m = 1, 2); *(h) OsO_4, $NaIO_4$; (i) KOH.*

The reactions in Scheme 2 start with commercially available 2-methylcyclohexanone (**6**), which was first converted into sodio-6-formyl-2-methylcyclohexanone[21]. The dianion of 6-formyl-2-methylcyclohexanone was then generated using lithium diisopropylamide. Treatment of the dianion with 2-methyl-5-iodo-1-pentene (which can be easily prepared, see experimental section) gave selective alkylation on position 2. After removal of the 6-formyl group, 2-methyl-2-(4-methyl-4-pentenyl)cyclohexanone (**7**) is obtained. Treatment of ketone **7** with $(CH_3)_3SiCN$ gave the trimethylsilated cyanohydrin, which on reaction with $POCl_3$ in pyridine gave the conjugated nitrile **8** in 63% yield. Dibal reduction converted **8** into the conjugated aldehyde **9** in 88% yield. The carbon–carbon double bond of the side chain must now be changed into a ketone function. This was effected by treating **9** with OsO_4 and $NaIO_4$ without apparent attack on the double bond of the enal system[22]. Subsequent base-catalyzed intramolecular aldol condensation of the enal and methyl ketone gave 5-demethyl-8,16-methano-β-ionone **4** in 44% yield.

This scheme to prepare the β-ionone derivative **4** is suited for the specific introduction of ^{2}H at either or both positions 5 and 7 in **4**: the two hydrogens on carbon 6 in the cyclohexanone **7** can be exchanged for ^{2}H by base-catalysed treatment with CH_3OD. Starting from (6-$^2H_2$)2-methyl-2-(4-methyl-4-pentenyl)cyclohexanone **7d**, this scheme leads to (5-^{2}H)5-demethyl-8,16-methano-β-ionone (**4d**). Treatment of the conjugated nitrile **8** with Dibal-D (prepared from diisobutylaluminumchloride and LiD[23] gave the deuterated conjugated aldehyde **9e**, which was next converted into (7-^{2}H)5-demethyl-8,16-methano-β-ionone (**4e**). Dibal-D reduction of **8d** gives the dideuterated conjugated aldehyde **9f**, which finally leads to (5,7-$^2H_2$)5-demethyl-8,16-methano-β-ionone (**4f**). Similarly to **4a** in Scheme 1, **4d-f** are elongated to the retinal stage to give (5-^{2}H)-, (7-^{2}H)- and (5,7-$^2H_2$)5-demethyl-8,16-methanoretinal, **1d**, **1e** and **1f**, respectively.

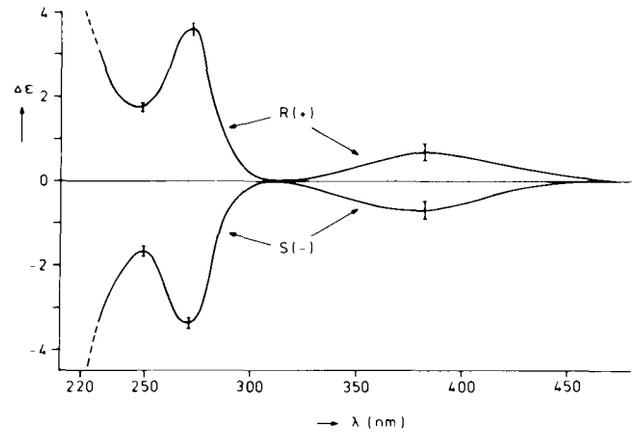


*Figure 4. CD spectra of all-*E *enantiomers of* **1a** *and* **1b** *(Δε in $l \cdot mol^{-1} \cdot cm^{-1}$, λ 220–330 nm: ε $7.3 \cdot 10^3$, concn. $6 \cdot 10^{-5}$ M; λ 330–550 nm: ε $47 \cdot 10^3$, concn. $2 \cdot 10^{-5}$ M).*

Photochemistry

Photochemical isomerization of retinals in polar solvents and subsequent HPLC isolation is a most effective way of preparing the *cis* isomers, including the (for visual studies important) 11-*cis* isomer, which is not or only present in small amounts in synthetic isomer mixtures[24,25]. Using a 100-W tungsten lamp, a dilute solution of pure (all-*E*)-5-demethyl-8,16-methanoretinal (**1**) in acetonitrile was irradiated in an argon atmosphere for 90 minutes. In the HPLC trace of the photostationary mixture, three peaks are present. Two of them are due to (all-*E*)-**1**, (13-*Z*)-**1** and the third peak consists of a 1:1 mixture of (9-*Z*)-**1** and (11-*Z*)-**1**. Using analytical HPLC, this latter peak could be resolved into two separate peaks. No observable amounts of (9-*Z*,13-*Z*)-**1** were present in the photostationary state. It is interesting that, in the photochemistry of **1**, the 11-*cis* form is found, whereas in other locked retinals, such as 8,16- and 8,18-methanoretinal, they are not formed[11,26,27].

Spectroscopic characterization

Mass spectrometry

The double-focus mass spectra (e.i. 70 eV) of the all-*E* isomers of **1**, **1a**, **1b**, **1c**, **1d**, **1e** and **1f** were measured. They all

*Table I Experimental and calculated mass values for all-*E *isomers of retinals* **1**, **1a**, **1b**, **1c**, **1d**, **1e** *and* **1f**.

	All-*E*						
	1	**1a**	**1b**	**1c**	**1d**	**1e**	**1f**
molecular formula	$C_{20}H_{26}O$	$C_{20}H_{26}O$	$C_{20}H_{26}O$	$C_{20}H_{22}D_4O$	$C_{20}H_{25}DO$	$C_{20}H_{25}DO$	$C_{20}H_{24}D_2O$
calculated mass	282.1983	282.1983	282.1983	286.2234	283.2046	283.2046	284.2109
experimental value	282.1984	282.1990	282.1992	286.2232	283.2043	283.2040	284.2104

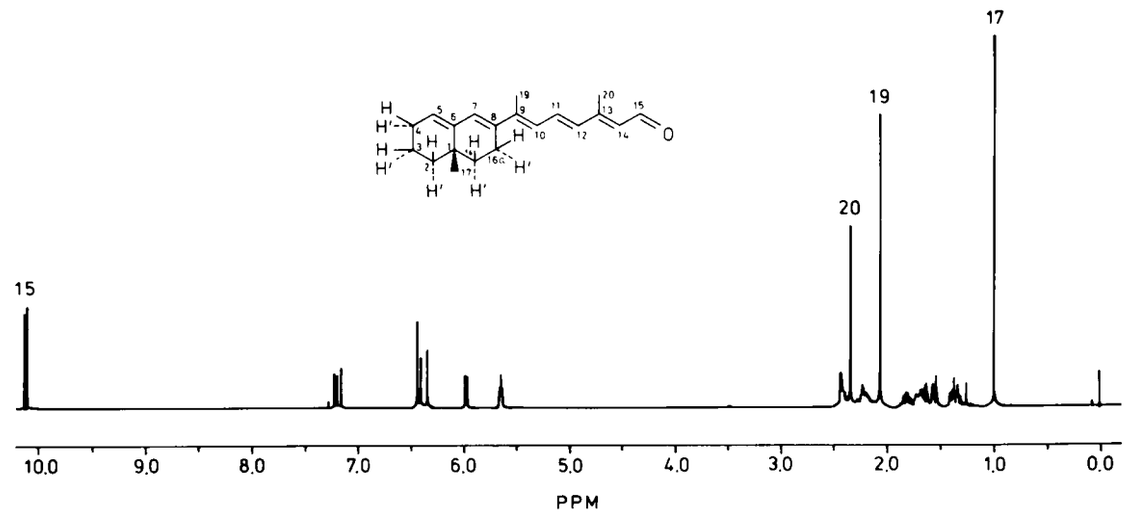


Figure 5. 400-MHz ^1H *NMR spectrum of (all-*E*)-5-demethyl-8,16-methanoretinal* (**1**) *in* $CDCl_3$ *with TMS as reference. Insert: numbering of all-*E-**1**; *the protons* trans *to 1-*CH_3 *are marked with an accent.*

show a strong molecular ion peak. The double-focus mass values are listed in Table I; within experimental error, they are fully in agreement with the calculated values of the corresponding molecular formulas. All the spectra show characteristic peaks at *m/z* $M^{+}*-15$, $M^{+}*-29$ and $M^{+}*-43$, due to the loss of CH_3, CHO and $CO+CH_3$ fragments, respectively. A similar fragmentation pattern is found in retinal and retinal analogues[28,29,11].

The ^2H enrichment in **1c**, **1d**, **1e** and **1f** were determined from the single-focus mass spectra. These are for **1c** 78.6% $^2H_4$, 17.9% $^2H_3$, 1.9% $^2H_2$ and 1.6% ^2H; for **1d**: 93.9% ^2H; for **1e**: 97.0% ^2H and for **1f**: 92.0% $^2H_2$ and 7.1% ^2H. The mass values are, in each case, in agreement with the molecular composition within experimental error.

^1H *NMR spectroscopy*

The isomers with the lowest R_f values, which are present in larger amounts, have the all-*E* structure. In Figure 5, the 400-MHz ^1H NMR spectrum of racemic (all-*E*)-5-demethyl-8,16-methanoretinal (**1**) in $CDCl_3$ is reproduced. As expected, pure all-*E*-1*R* (**1a**) and all-*E*-1*S* (**1b**) enantiomers have identical ^1H NMR spectra. The ^1H NMR parameters of (all-*E*)-**1** and their assignments are given in Tables II and III.

The high-field region is in agreement with a structure containing one chiral carbon atom and it is very similar to that of racemic (all-*E*)-8,16-methanoretinal reported by us previously[11]. In this region, three methyl singlets are present, which are assigned to 1-CH_3 (δ 0.99 ppm), 9-CH_3 (δ 2.06 ppm) and 13-CH_3 (δ 2.34 ppm). In the vinyl region, the expected signals of six protons are present. At δ 5.63, the broad triplet of 5-H (*J* 4 Hz) is found, the doublet of 14-H [*J*(14-H–15-H) 8 Hz] at 5.97 ppm and the 7-H singlet at 6.33 ppm. The rest of the vinyl signals consist of a AMX spectrum of 10-H, 11-H and 12-H [δ 10-H 6.41, δ 12-H 6.40, δ 11-H 7.18; *J*(10-H–11-H) 11 Hz, *J*(11-H–12-H) 15.0 Hz]. At 10.11 ppm, the doublet of 15-H [*J*14-H–15-H) 8 Hz] is present. The chemical shift and coupling constant values are in complete agreement with an all-*E* structure for **1**[30,31]. Except for δ 7-H, all the chemical-shift values are, within 0.01 ppm, identical to those of (all-*E*)-8,16-methanoretinal[11]. The absence of a 5-CH_3 group in (all-*E*)-**1** releases the γ effect in 7-H, leading to a 0.44-ppm decrease in chemical shift compared with δ 7-H in (all-*E*)-8,16-methanoretinal.

Table II 300-MHz ^1H *NMR data (*$CDCl_3$, *TMS as reference) of geometric isomers of 5-demethyl-8,16-methanoretinal* (**1**).

Hydrogen atoms	Chemical shift, δ ppm				
	All-*E*	13-*Z*	11-*Z*	9-*Z*	9-*Z*,13-*Z*
5	5.63	5.64	5.63	5.50	5.50
7	6.33	6.34	6.31	5.88	5.88
10	6.41	6.44	6.78	6.05	6.08
11	7.18	7.08	6.73	7.03	6.93
12	6.40	7.32	5.94	6.27	7.18
14	5.97	5.89	6.09	5.94	5.80
15	10.11	10.21	10.10	10.08	10.19
17	1.00	1.00	0.99	1.06	1.06
19	2.06	2.06	2.02	1.96	1.97
20	2.33	2.15	2.39	2.23	2.04
^3J *coupling constants*					
4–5	4.1	4.1	3.8	3.9	3.9
10–11	11.3	11.3	12.4	11.1	10.9
11–12	15.0	14.8	11.8	15.4	15.3
14–15	8.2	7.8	7.6	8.2	8.1

*Table III Chemical-shift and coupling-constant (Hz) values of bicyclic part of all-*E-**1**.

H	δ (ppm)	$^2J_{HH}$		$^3J_{HH}$		$^4J_{HH}$		$^5J_{HH}$	
2	1.55	2–2′	– 12.9	2–3	3.3	2–4	– 0.8	16′–17	– 0.6
2′	1.33	3–3′	– 13.8	2–3′	3.4	3–5	– 0.2	16a–19	– 1.5
3	1.82	4–4′	– 19.6	2′–3	13.9	3′–5	– 1.0		
3′	1.71	16–16′	– 13.0	2′–3′	3.4	16a–7	– 1.2		
4	2.23	16a–16a′	– 17.3	3–4	6.3				
4′	2.16			3–4′	11.1				
16	1.63			3′–4	1.1				
16′	1.38			3′–4′	7.1				
16a	2.40			4–5	5.6				
16a′	2.43			4′–5	2.6				
				16–16a	5.5				
				16–16a′	1.7				
				16′–16a	12.4				
				16′–16a′	5.6				

The signals of the protons on 2-C, 3-C, 4-C, 16-C and 16a-C form a complex pattern. From the spectrum of (all-*E*)--tetradeuteroretinal (**1c**), the signals of the 16a-C protons are absent due to deuteration, those on 16-C form a simple AB pattern [δ 16-H 1.62, δ 16-H′ 1.36, *J*(16-H–16-H′ 13 Hz]. Starting from these values and a 600-MHz ^{1}H NMR spectrum of (all-*E*)-**1** the spectrum of the bicyclic part could be completely simulated using the Bruker PANIC program (see Figure 6). The chemical-shift and coupling-constant values are given in Table III.

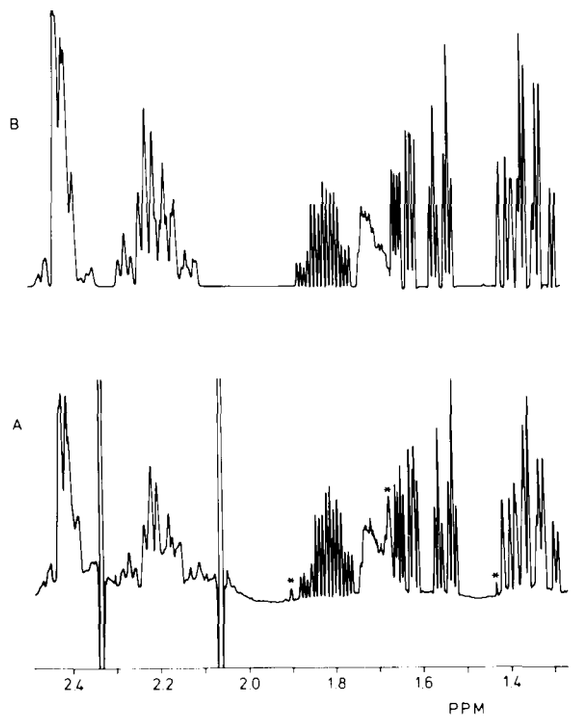


*Figure 6. A: high-field region of 400-MHz ^{1}H NMR spectrum of (all-*E*)-***1***.*
*B: simulation. * = impurity.*

In Figure 7, the vinyl parts of the 300-MHz ^{1}H NMR spectra of the all-*E* isomers of **1** (A) and its deuterated forms **1c** (B), **1d** (C), **1e** (D) and **1f** (E) are depicted. Trace B shows that, in (all-*E*)-**1c**, the signals of 5-H (5.63 ppm) and 7-H (6.33 ppm) have been reduced to less than 10%. For (all-*E*)-**1d**, the trace C shows that the signal of 5-H is almost reduced to zero. For (all-*E*)-**1e** (trace D), there is no observable signal from 7-H. Similarly, in trace E, no signals of 5-H and 7-H are present for (all-*E*)-**1f**. These spectra show the expected location of ^{2}H incorporation. The amount of ^{2}H incorporation agrees very well with the mass spectrometry data.

Similarly, the ^{1}H NMR spectra of the 9-*Z*, 11-*Z*, 13-*Z* and 9-*Z*,13-*Z* forms were recorded; the chemical-shift and coupling-constant values are listed in Table II. The signals of the bicyclic part in the *Z* isomers are similar to those in the all-*E* form; these were not analysed.

^{13}*C NMR spectroscopy*

^{13}C NMR spectroscopy is a powerful method for obtaining structural information. We recorded proton-noise-decoupled 75-MHz ^{13}C NMR spectra of the all-*E* isomers of **1**, **1c**, **1d**, **1e** and **1f**. The spectrum of (all-*E*)-**1** shows the expected

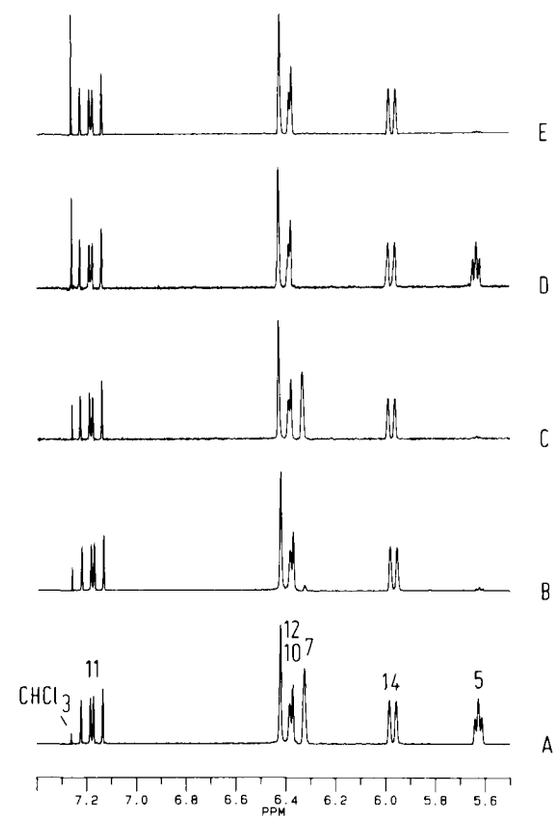


*Figure 7. Vinyl region of 300-MHz ^{1}H NMR spectrum of all-*E *isomers of* **1** *(A),* **1c** *(B),* **1d** *(C),* **1e** *(D),* **1f** *(E).*

twenty signals, nine of which are in the sp^3 region and eleven in the sp^2 region. The signals from the proton-bearing carbon atoms (except 10-C and 12-C) could be unambiguously assigned from the $^1H-^{13}C$-correlated 2D NMR spectrum. The quaternary carbon atoms and 10-C and 12-C are easily assigned by comparison with the data of (all-*E*)--retinal and (all-*E*)-8,16-methanoretinal[32,11].

Table IV 75-MHz ^{13}C NMR chemical-shift values in $CDCl_3$ of (all-E)-5-demethyl-8,16-methanoretinal (**1**).

C	δ (ppm)	C	δ (ppm)
1	31.7	11	133.1
2	37.0	12	134.4
3	18.2	13	154.8
4	26.1	14	128.7
5	127.3	15	190.9
6	141.7	16	36.8
7	128.6	16a	23.0
8	135.0	17	23.2
9	141.9	19	14.1
10	124.5	20	13.0

The spectrum of (all-*E*)-**1c** gives additional support to the assignment of 5-C, 7-C and 16a-C. A deuterated carbon signal has a low intensity, due to the absence of NOE and the fact that the signal is split up into a triplet and quintet for a mono- and dideuterated carbon, respectively[33].

In the spectrum of (all-*E*)-**1c**, two triplets from 5-C [126.9 ppm, $J(^{13}C-^2H)$ 23 Hz] and 7-C [128.3 ppm $J(^{13}C-^2H)$ 23 Hz] and a multiplet of very low intensity from 16a-C [22.7 ppm, $J(^{13}C-^2H)$ 18 Hz] can be seen. In addition to the triplet for $5\text{-}C-^2H$, a small singlet (127.3 ppm) is present; no singlet is observed at 128.6 ppm (7-C) nor at 23.0 ppm (16a-C). This shows that, within experimental error, deuteration is complete at 7-C and 16a-C and that a small amount of ^1H is still present at 5-C in **1c**.

The spectra of (all-*E*)-**1d**, (all-*E*)-**1e** and (all-*E*)-**1f** show the expected $^{13}C-^2H$ triplets at δ 127.0, δ 128.3 and δ 127.0 + 128.3 ppm, respectively. No signals for residual protonated carbons are observed at these positions. This reflects high ^2H incorporation and is in agreement, within experimental error, with the ^1H NMR and mass data.

UV-Vis and CD spectroscopy

The UV-Vis spectrum of (all-*E*)-**1** in ethanol consists of a broad peak without vibrational fine structure, with λ_{max} 397 nm ($\varepsilon\ 47 \cdot 10^3$) and a small shoulder at 281 nm ($\varepsilon\ 7 \cdot 10^3$). Almost the same λ_{max} value has been found for 8,16-methanoretinal (402 nm)[11] and a 14-nm lower value for retinal (383 nm)[25]. The longer wavelength is in agreement with the planar 6–7-*s-trans* conformation and better conjugation in (all-*E*)-**1** and (all-*E*)-8,16-methanoretinal, whereas retinal has a twisted 6–7-*s-cis* conformation leading to restricted conjugation of the 5–6 double bond. The 5-nm contribution of the 5-methyl group to the λ_{max} is about the value expected for a methyl group on a conjugated chain[34,35]. The λ_{max} value for (13-*Z*)-**1** (389 nm) is close to that of (all-*E*)-**1**. However, the λ_{max} for (9-*Z*)-**1** (360 nm) and (9-*Z*,13-*Z*)-**1** (352 nm) differ considerably from that of the all-*E* isomer. In both 9-*Z* isomers, there is steric interference between 11-H and the two 16a protons. This prevents the molecule from attaining a planar 8–9 conformation leading to decreased conjugation and a lower λ_{max} value. This has also been found in other retinal analogues in which steric hindrance occurs[26].

In Figure 4, the circular dichroism spectra of (all-*E*)-**1a** and (all-*E*)-**1b**, determined in ethanol, are displayed. Within experimental error, the curves are mirror images, demonstrating that (all-*E*)-**1a** and (all-*E*)-**1b** are enantiomers with the same optical purity. The CD of the *R* enantiomer shows the presence of a small but distinct positive. Cotton effect in the long-wavelength region (382 nm, Δε 0.7) as well as a more intense band near 272 nm (Δε 3.5) of the same sign. One can distinguish two contributions to the CD: that due to a possible twist in the polyenal (inherently dissymmetrical chromophore) and that due to the chiral aliphatic carbon skeleton (induced CD)[36]. Long-wavelength Cotton effects of similar low intensity have been reported for chiral polyenals and derivatives with inherently symmetrical chromophores[37,38]. If we assume that the induced CD in the

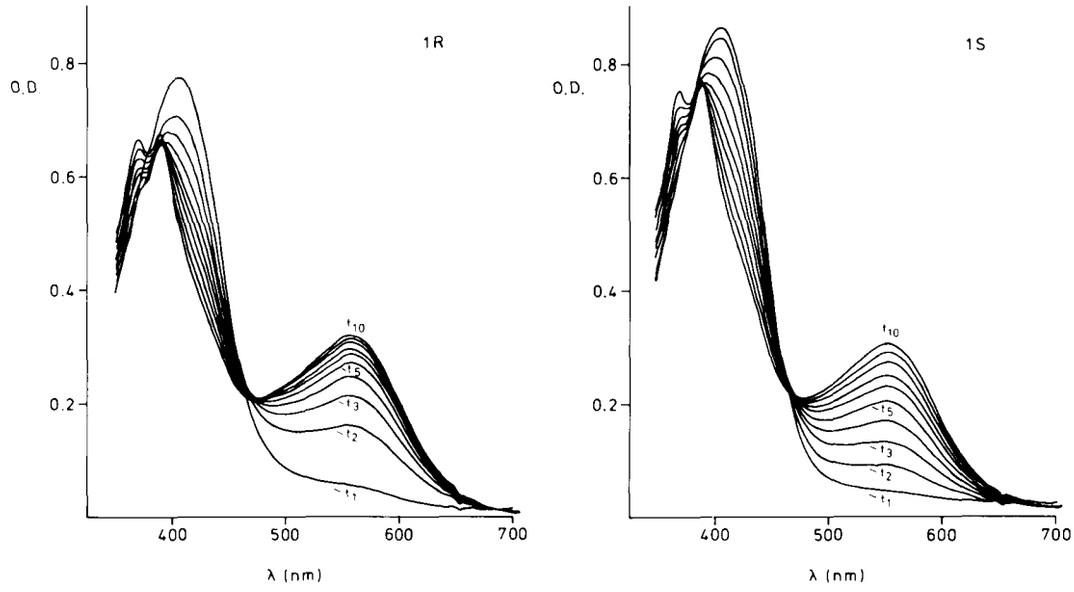


Figure 8. Time-dependent absorption spectra of reaction of excess of (all-E)-(R)- and (all-E)-(S)-5-demethyl-8,16-methanoretinal with bO (concn. $7.5 \cdot 10^{-6}$ M). $\lambda_{max}[bR(\mathbf{1a})] = \lambda_{max}[bR(\mathbf{1b})] = 552$ nm. $t_1 = 11''$, $t_2 = 1'.24''$, $t_3 = 2'.37''$, $t_5 = 6'.11''$, $t_{10} = 19'.11''$.

long-wavelength band of those compounds is of similar magnitude as in our compound, the contribution of inherent twist to the long-wavelength CD of (all-*E*)-**1a** is, at most, of the order $\Delta\varepsilon \approx -1$. This points to a nearly planar π system in the molecule, including the bicyclic moiety.

Binding properties

bO reacts with a slight excess of racemic (all-*E*)-**1** at room temperature to form, within a few seconds, the bacteriorhodopsin analogue bR(**1**) (λ_{max} 552 nm, $\varepsilon\ 57 \cdot 10^3$) (Figure 1, T). After 20 minutes, the conversion is complete. Similar incubation with the optically pure (all-*trans*)-(1-*R*)-retinal (**1a**) gives the bacteriorhodopsin analogue bR(**1a**) (λ_{max} 552 nm, $\varepsilon\ 56 \cdot 10^3$); with (1-*S*)-(all-*E*)-**1b**, bacteriorhodopsin bR(**1b**) (λ_{max} 552 nm, $\varepsilon\ 62 \cdot 10^3$) is formed (Figure 1, *R* and *S*, respectively). Interestingly, the three bacteriorhodopsins have, within experimental error, the same λ_{max} values; however, the ε_{max} values of bR(**1a**) and bR(**1b**) differ significantly. The ε_{max} of the racemic form is very close to that of bR(**1a**).

In order to study the binding kinetics, the reaction was carried out at a lower temperature (4°C). Upon addition of the retinal to bO, complexes of bO with (all-*E*)-**1**, -**1a**, and -**1b** (λ_{max} 438, 441 and 436 nm, respectively) are formed within a few seconds, without a resolved vibrational fine structure. These complexes are converted into the corresponding bR analogues with first-order kinetics. BR(**1a**) is initially formed 2.1 times more rapidly than bR(**1b**). Figure 8 nicely illustrates this difference in regeneration kinetics. The initial rate of formation of bR(**1**) is somewhat slower (0.8 ×) than that of bR(**1a**). All three bR analogues bR(**1**), bR(**1a**) and bR(**1b**) are formed more rapidly than bacteriorhodopsin is formed from (all-*E*)-retinal: bR(**1**) 2 times faster, bR(**1a**) 2.6 times faster and bR(**1b**) 1.3 times faster.

The bR analogues bR(**1**), bR(**1a**) and bR(**1b**) show dark adaptation. The λ_{max} values of the dark-adapted forms are at 547 nm. They completely revert to light-adapted forms (λ_{max} 552 nm) upon exposure to light.

From the λ_{max} values of light-adapted bR(**1**) (552 nm) and the *n*-butylamine-protonated Schiff base of (all-*E*)-**1** (467 nm), the opsin shift of bR(**1**) can be calculated at 3300 cm^{-1}, *i.e.*, 1800 cm^{-1} less than for natural bR (5100 cm^{-1})[4].

Proton-pump action

Illumination of bR(**1**) incorporated into soybean phospholipid vesicles results in a rapid increase of the pH of the external medium. The resulting proton gradient ($\Delta\tilde{\mu}(H^+)$) 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 before ilumination and the pH reached at the steady state, expressed as nmol H^+/mg bR(**1**). When the light is then turned off, the protons re-equilibrate until $\Delta\tilde{\mu}(H^+)$ has disappeared. In the presence of valinomycin [2 μg/mg bR(**1**)], a K^+ ionophore, the ΔpH can reach higher values because the membrane potential ($\Delta\psi$) is absent; initial velocity and re-equilibration are faster and extent is larger. If valinomycin as well as nigericin (K^+/H^+ exchange) are present [both 2 μg/mg bR(**1**)], the back leakage of protons almost equals the amount of light-driven transport, so that there is almost no net proton uptake.

Each of the three bR analogues bR(**1**), bR(**1a**), bR(**1b**) as well as unmodified bR and bO, were reconstituted in liposomes 2 or 3 times and, for each reconstitution, 3 photocycles were measured. In Table V, the results of the experiments are listed. The proton-pump activity of the liposomes with the three different bR analogues are equal within experimental error, at approximately 50% of the value recorded for unmodified bR liposomes (270 nmole H^+/mg bR).

Table V Extent of net proton uptake and initial proton uptake upon illumination of bR and (analogues)–liposomes; n = number of reconstitutions. All values are represented as mean ± S.D. (standard deviation).

	Extent [nmole H^+/(mg bR)]	*n*	Initial velocity [(nmole H^+) · min^{-1} · (mg bR) $^{-1}$]
bR[a]	270 ± 25	3	690 ± 180
bR(**1**)	140 ± 23	2	310 ± 60
bR(**1a**)	130 ± 29	3	360 ± 140
bR(**1b**)	120 ± 18	2	320 ± 80
bO	0	2	0

[a] Prepared from bacteriorhodopsin and (all-*E*)-retinal.

Discussion

The all-*E* isomers of both (1*R*)- and (1*S*)-5-demethyl-8,16-methanoretinal (**1a** and **1b**, resp.) could be prepared in good overall yield (25%) via Scheme 1. The CD spectra of (all-*E*)-**1a** and (all-*E*)-**1b** show opposite Cotton effects, just showing that they are enantiomers with, within experimental error, the same optical purity. The Cotton effect of the long-wavelength absorption (397 nm) is small. This is in agreement with an almost planar structure for the polyene chromophore. The 13-*Z*, 11-*Z*, 9-*Z* and 9-*Z*,13-*Z* forms of **1a** and **1b** were also obtained. In addition, the racemic form of (all-*E*)-5-demethyl-8,16-methanoretinal (**1**) and its various *Z* isomers have been prepared. Access to the first pair of enantiomeric retinals and its racemic form has allowed us to probe the chiral recognition upon bacteriorhodopsin formation with bacterioopsin. Both (1*R*)-(all-*E*)-**1a** and (1*S*)-(all-*E*)-**1b** (and the racemic form) combine efficiently with bacterioopsin to form bR analogues: bR(**1a**), bR(**1b**) and bR(**1**). All three have, within experimental error, the same λ_{max} value: 552 nm, which is 18 nm shorter than that of 8,16-methano-bR[5]. Comparison with the much smaller λ_{max} difference between the corresponding free retinals (5 nm) shows that the methano bridge has a negligible influence on the λ_{max} value. The opsin shift of bR(**1**) [and bR(**1a**) and bR(**1b**)] amounts to 3300 cm^{-1}, which is 650 cm^{-1} less than the opsin shift of 8,16-methano-bR[5]. Both bR analogues have a rigid 6–7-*s-trans* conformation, so that there is a pronounced influence of the 5-CH_3 group. The 552-nm λ_{max} value of bR(**1**) [bR(**1a**), bR(**1b**)] is very close to that of 5-demethyl-bR (548 nm)[12]. Similarly, the λ_{max} value of 8,16-methanoretinal (570 nm) is very close to that of bR (568 nm). In bR(**1**) and 8,16-methano-bR, both chromophores occur in a rigid 6–7-*s-trans* conformation. The absence of a 5-methyl group in bR(**1**) leads to an 18-nm lower λ_{max} value compared to 8,16-methano-bR. This is considerably larger than in the corresponding free retinals (5 nm) or the free protonated Schiff bases (2 nm). Presumably, this is related to the fact that, in the chromophore in bR (and analogues), more positive charge is present on C5 than in corresponding model systems. The electron-donating ability of the 5-CH_3 stabilises the charge in 8,16-methano-bR much more than in bR(**1**).

However, bR(**1a**), bR(**1b**) and bR(**1**) differ significantly in rate of formation and in ε_{max} values. The initial rate of

reaction of bO with the racemic form is twice as rapid as that with (all-*trans*)-retinal. Racemic 8,16-methanoretinal binds 1.5 times slower than retinal, whereas racemic 5-demethylretinal binds three times as rapidly[11,12]. This shows that the absence of a 5-methyl group and the presence of the 8,16-methylene group leads to opposing influences in the binding of a retinal to bO. The presence of an 8,16-methano bridge leads to a decrease in binding kinetics (by 1.5), the absence of a 5-methyl group leads to an increase by a factor of 3 in each case.

The 1-*R* form binds 2.6 times more rapidly than retinal (it binds more rapidly than the racemic form). The 1-*S* form binds 1.3 more rapidly than retinal. This is a factor of two slower than the 1-*R* form. This factor of two is a clear indication of the chiral recognition by the chiral active site of bO. As expected, the rate of binding of racemic **1** is in between that of **1a** and **1b**, somewhat closer to that of **1a**. The ε_{max} value of bR(**1a**) is $56 \cdot 10^3$, distinctly different from the ε_{max} of bR(**1b**) ($62 \cdot 10^3$). The ε_{max} value of the racemic form is in between that of bR(**1a**) and bR(**1b**), closer to that of bR(**1a**) ($57 \cdot 10^3$).

It is clear that only due to access to the pure enantiomer, these subtle differences in chiral recognition could be observed. The activity of the light-driven proton pump of both bR(**1a**) and bR(**1b**) was studied. Both have, within experimental error, the same proton pump action, namely about 50% of that of bacteriorhodopsin itself. It is clear that the chirality of the chromophore has no effect on the proton-pump action. The proton-pump action of 5-demethyl-bR is about 60% of that of bR[12], whereas the proton-pump action of 8,16-methano-bR (90%) is only somewhat lower than that of bR[11]. This clearly shows that the 5-methyl group has an important contribution (50%) to the proton-pump action. It is, at this moment, not clear why the 5-methyl group in retinal has such a pronounced effect on the proton-pump activity. One factor may be that the 5-methyl group gives better stability to the positive charge on C5. (From solid-state-NMR spectroscopy, it could be established that in the chromophore in bacteriorhodopsin C5 bears more positive charge than free, protonated Schiff base due to the presence of a negative charge in the protein near C5.)

In this paper, we also describe the synthesis of the (5-^{2}H), (7-^{2}H) and (5,7-$^2H_2$) isotopomers of 5-demethyl-8,16-methanoretinal in the various isomeric forms.

Experimental

All experiments were carried our under a nitrogen atmosphere and the purified polyenes were handled in 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[39] (Merck silica gel 60, 230–400 mesh) using ether/pet.-ether mixtures. TLC analyses were performed on Schleicher and Schuell F 1500/LS 254 silica gel plates using ether/pet.-ether mixtures. Evaporation of the solvents was carried out *in vacuo* ($1.4 \cdot 10^3$ Pa). The ^{1}H NMR spectra were recorded on a Jeol FX-200, a Bruker WM-300, MSL-400 and AM-600 NMR spectrometer using tetramethylsilane (TMS; 0 ppm) as internal standard. The ^{13}C NMR spectra were recorded on a Jeol NM FX-200 at 50.1 MHz or on a Bruker WM-300 spectrometer at 75.5 MHz.

Exact mass and label determinations were carried out using a Kratos MS 9/50 mass spectrometer (source conditions: electron energy 70 eV, T 425 K) and GC/MS spectra were measured on a ITD 700 Finnigan MAT apparatus. The IR spectra were obtained using a Pye-Unicam SP 3-200 spectrophotometer and UV-Vis spectra were recorded at 4°C on a Cary 219, at room temperature on a DMS-200 spectrophotometer. The CD spectra were recorded with a Jobin Yvon M III dichrograph.

Preparative HPLC separations were performed using a Dupont 830, equipped with a Dupont UV spectrophotometer (detection a 370 nm), and a 250 × 22.5 mm Zorbax Sil column. Elution was effected using 15% ether in pentane at a flow rate of 20 ml/min. Analytical HPLC separations were performed using a LKB 2150 instrument equipped with a LKB 2141 UV spectrophotometer (detection at 370 nm) and a 250 × 4.6 mm Lichrosorb Si-60-5 column (10% ether in pentane, 1 ml/min).

(*R*)(−)- and (*S*)(+)-4,4a,5,6,7,8-hexahydro-4a-methyl-2(3*H*)-naphthalenone (**2a**, **2b**, respectively) with 97% purity were purchased from Aldrich, Li^{2}H (>98% ^{2}H) and CH_3O^2H (>99.5% ^{2}H) from Janssen Chimica, and soybean phospholipid from Fluka. Spectral signal designations for the C_{15} aldehydes **5**, **5a-f** and the 5-demethyl-8,16-methanoretinals **1**, **1a-f** are based on the IUPAC retinoid numbering system[40]; those of the other compounds on IUPAC nomenclature. For the labeled compounds, only the spectral changes relative to the unlabeled compounds are given.

*4,4a,5,6,7,8-Hexahydro-4a-methyl-2(3*H*)-naphthalenone* (**2**)

This compound (**2**) was synthesized according to published procedures[13]. ^{1}H NMR (200 MHz): δ 1.24 (s, 3H, 4a-CH_3), 1.1–2.1 (m, 8H, 4-H_2, 5-H_2, 6-H_2 and 7-H_2), 2.1–2.8 (m, 4H, 3-H_2 and 8-H_2), 5.71 (s, 1H, 1-H). ^{13}C NMR (50 MHz): δ 21.5 (s, 4a-CH_3), 21.2, 26.4, 31.4, 32.8, 37.3, 41.1 (s, 3-C, 4-C, 5-C, 6-C, 7-C and 8-C), 35.3 (s, 4a-C), 123.5 (s, 1-C), 169.7 (s, 8a-C), 198.7 (s, 2-C).

*(1,3,3,8,8-$^2H_5$)***2** (**2c**). Sodium hydride (NaH, 46 mg, 1.1 mmol, 55% in mineral oil) was rinsed three times with dry pet. ether to remove the mineral oil and dried by means of an electric hairdryer. Using a syringe, CH_3OD was added slowly until no more gas evolution was seen. The remainder of 2 ml CH_3OD was then added, followed by **2** (397 mg, 2.4 mmol). The solution was stirred at 40°C for 7 h. After cooling, the solution was diluted with D_2O and the mixture extracted three times with ether. The organic layers were washed with brine, dried over $MgSO_4$, filtered over silica gel and concentrated. This procedure was repeated three times, yielding virtually pure **2c** (323 mg, 1.9 mmol, 79%). ^{1}H NMR (200 MHz): as for **2** except that the signal of 1-H (5.7 ppm) is reduced to less than 5% and the allylic signals (2.1–2.8 ppm) have been reduced to about 5%. ^{13}C NMR: as for **2**, except that three peaks have disappeared. A small triplet is present at 123.3 ppm [J(C−^{2}H) 24 Hz, 1-C] and two multiplets at 30.6–35.0 ppm (3-C and 8-C).

3,4,4a,5,6,7-Hexahydro-4a-methylnaphthalene-2-carbonitrile (**3**)

A mixture of **2** (864 mg, 5.3mmol), trimethylsilyl cyanide (678 mg, 6.9 mmol)[15] and zinc iodide (42 mg, 0.13 mmol) in benzene (2 ml) was stirred for 8 h at 35°C. After cooling, a supplement of TMSCN (180 mg, 1.8 mmol) and benzene (0.5 ml) was added, and the suspension was stirred overnight at room temperature. Pyridine (4 ml) and $POCl_3$ (1.6 ml, 17 mmol) were added and the mixture was heated to 95°C. After it had been allowed to react for 22 h at this temperature, the reaction mixture was cooled, poured into ice-cold 1.3N hydrochloric acid and extracted six times with ether. The combined organic layers were washed with saturated aqueous $NaHCO_3$ solution and brine, successively, dried over $MgSO_4$ and concentrated. The crude oil was purified by means of column chromatography (7% ether/pet.-ether) to yield **3** (697 mg, 4.0 mmol, 76%). ^{1}H NMR (200 MHz): δ 0.99 (s, 3H, 4a-CH_3), 1.1–2.0 (m, 6H, 4-H_2, 5-H_2 and 6-H_2), 2.0–2.6 (m, 4H, 3-H_2 and 7-H_2), 5.79 (t, J_{HH} 4.0 Hz, 1H, 8-H), 6.66 (d, J_{HH} 1.7 Hz, 1H, 1-H). GC/MS: *m/z* 173 (calcd. for $C_{12}H_{15}N$ 173). IR: 2220 cm^{-1} (C≡N stretch).

(R)-**3** (**3a**). Starting with 300 mg **2a** and following the same procedure as for **3**, yielded **3a** (226 mg, 71%). The ^{1}H NMR spectrum of **3a** is identical to that of **3**.

(S)-**3** (**3b**). Starting with 300 mg **2b** and following the same procedure as for **3**, yielded **3b** (238 mg, 75%). The ^{1}H NMR spectrum of **3b** is identical to that of **3**.

*(1,3,3,8-$^2H_4$)***3** (**3c**). Starting with 323 mg **2c** and following the same procedure as for **3**, yielded **3c** (244 mg, 72%). ^{1}H NMR (100 MHz): as for **3**, except for the signals at δ 6.66, 5.79 and 2.0–2.6 ppm which are 95–99% reduced in intensity.

1-(3,4,4a,5,6,7-Hexahydro-4a-methyl-2-naphthalenyl)ethanone (**4**)

CH_3Li (1.2 ml 1.6M solution in ether, 1.9 mmol) was added slowly at −60 °C to a stirred solution of **3** (302 mg, 1.7 mmol) in dry THF (15 ml), using a syringe. The temperature was raised to −20 °C over 40 min, after which 8 ml of cooled 1.3N HCl solution was added slowly. The reaction mixture was stirred for another 40 min at 0 °C. Solid $NaHCO_3$ was then added cautiously and the mixture was extracted six times with ether. The combined organic layers were washed with brine, dried over $MgSO_4$ and evaporated. To complete mild hydrolysis a suspension of 1/5 water/silica-gel in 50% ether/pet.-ether was added to the crude oil and the mixture was stirred for 1 h at 0 °C. After drying over $MgSO_4$, the solids were filtered off and washed with ether. After removal of solvents and purification of the product by means of column chromatography (20% e/p), the yield of **4** was 182 mg (0.97 mmol, 55%). ^{1}H NMR (200 MHz): δ 0.96 (s, 3H, 4a-CH_3), 1.1–2.0 (m, 6H, 4-H_2, 5-H_2 and 6-H_2), 2.0–2.7 (m, 4H, 3-H_2 and 7-CH_2), 2.32 (s, 3H, $COCH_3$), 5.88 (t, J_{HH} 4 Hz, 1H, 8-H), 6.92 (s, 1H, 1-H).

(R)-**4** (**4a**). As above, **4a** (129 mg, 52%) was prepared from 226 mg **3a**. The ^{1}H NMR spectrum of **4a** is identical to that of **4**.

(S)-**4** (**4b**). As above, **4b** (143 mg, 55%) was prepared from 238 mg **3b**. The ^{1}H NMR spectrum of **4b** is identical to that of **4**.

*(1,3,3,8-$^2H_4$)***4** (**4c**). As above, **4c** (147 mg, 55%) was prepared from 244 mg **3c**. ^{1}H NMR (200 MHz): as for **4**, except for the signals at δ 6.92, 5.88 and 2.5–2.7 ppm which are 95–99% reduced in intensity.

3-(3,4,4a,5,6,7-Hexahydro-4a-methyl-2-naphthalenyl)-2-butenal (**5**)

Butyllithium (0.9 ml, 1.6M solution in hexane, 1.4 mmol) was added slowly using a syringe to a stirred solution of diethyl-(cyanomethyl)phosphonate (305 mg, 1.7 mmol)[19] in dry THF, cooled to 0 °C. The solution was then stirred for 30 min at room temperature. After cooling to 0 °C, a solution of **4** (182 mg, 0.96 mmol) in THF was added slowly and the stirred mixture was allowed to warm to room temperature. After 1½ h, the solution was poured into water and extracted three times with 20% ether/pet.-ether. The organic layers were washed with brine, dried over $MgSO_4$ and filtered through a layer of silica gel. After evaporation of solvents, the residue was purified by means of column chromatography (15% e/p) to give the yellow coloured 9*E*/*Z* C15 nitrile in 79% yield (161 mg, 0.76 mmol). The nitrile was dissolved in dry pet. ether and the stirred solution was cooled to −60 °C. Using a syringe, 1 ml diisobutylaluminumhydride (Dibal) (1M solution in hexane) was added slowly. The mixture was then allowed to warm up to −20 °C over 1 h, after which a suspension of 1/5 water/silica-gel in 50% ether/pet.-ether was added. The reaction mixture was stirred at 0 °C for 1 h. After drying over $MgSO_4$, the solids were filtered off and rinsed with dry ether. Evaporation of the solvents yielded a quantitative yield of vitually pure **5** as a 9*E*/*Z* 5:1 mixture (163 mg, 0.75 mmol). ^{1}H NMR* (200 MHz) (9-*E*)-**5**: δ 0.91 (s, 3H, 1-CH_3), 2.27 (d, J_{HH} 1 Hz, 9-CH_3), 1.1–1.9 (m, 6H, 16-H_2, 2-H_2 and 3-H_2), 2.0–2.5 (m, 4H, 16a-H_2 and 4-H_2), 5.69 (t, J_{HH} 4 Hz, 1H, 5-H), 6.05 (d, J_{HH} 8 Hz, 1H, 10-H), 6.53 (s, 1H, 7-H), 10.09 (d, J_{HH} 8 Hz, 1H, 11-H); (9-*Z*)-**5**: δ 1.02 (s, 3H, 1-CH_3), 2.07 (d, J_{HH} 0.7 Hz, 9-CH_3), 1.1-1.9 (m, 6H, 16-H_2, 2-H_2 and 3-H_2), 2.0–2.5 (m, 4H, 16a-H_2 and 4-H_2), 5.58 (t, J_{HH} 4 Hz, 1H, 5-H), 5.91 (d, J_{HH} 8 Hz, 1H, 10-H), 5.99 (s, 1H, 7-H), 9.63 (d, J_{HH} 8 Hz, 1H, 11-H).

(R)-**5** (**5a**). As above, **5a** (120 mg, 79%) was prepared from 129 mg **4a**. The ^{1}H NMR spectrum of **5a** is identical to that of **5**.

(S)-**5** (**5b**). As above, **5b** (129 mg, 79%) was prepared from 143 mg **4b**. The ^{1}H NMR spectrum of **5b** is identical to that of **5**.

*(1,3,3,8-$^2H_4$)***5** (**5c**). As above, **5c** (131 mg, 79%) was prepared from 147 mg **4c**. ^{1}H NMR (200 MHz): as for **5**, except for the signals of 5-H, 7-H and 16a-H_2 which are 95–99% reduced in intensity.

*(8-^2H)***5** (**5d**).As described above for **5**, **5d** (34 mg, 77%) was prepared from 44 mg **4d**, using 5 eq. C_2 phosphonate, 4.5 eq. *n*-butyllithium and 3 eq. Dibal. ^{1}H NMR (200 MHz): as for **5**, except for the absence of 5-H signal.

*(1-^2H)***5** (**5e**). As described above for **5**, **5e** (39 mg, 82%) was prepared from 42 mg **4e**, using 5 eq. C_2 phosphonate, 4.5 eq. *n*-butyllithium and 3 eq. Dibal. ^{1}H NMR (200 MHz): as for **5**, except for the absence of 7-H signal.

*(1,8-$^2H_2$)***5** (**5f**). As described above for **5**, **5f** (64 mg, 66%) was prepared from 86 mg **4f**, using 5 eq. C_2 phosphonate, 4.5 eq. *n*-butyllithium and 3 eq. Dibal. ^{1}H NMR (200 MHz): as for **5**, except for the absence of 5-H and 7-H signals.

5-Demethyl-8,16-methanoretinal (**1**)

According to the same procedure described above for **5** using diethyl (3-cyano-2-methyl-2-propenyl)phosphonate[20] instead of diethyl (cyanomethyl)phosphonate, the C15 aldehyde **5** (163 mg, 0.75 mmol) was converted into the crude retinal **1**. After purification by means of column chromatography (10% ether/pet.-ether), virtually pure (all-*E*)-**1** and a mixture of 9-*Z*, 13-*Z* and 9-*Z*,13-*Z* isomers of **1** were obtained (in total 162 mg, 0.57 mmol, 76%). The 9-*Z*, 13-*Z* and 9-*Z*,13-*Z* isomers were isolated in pure form by preparative HPLC. Additional amounts of the mono *cis* isomers were obtained by irradiating a dilute solution of (all-*E*)-**1** in acetonitrile (≈0.1 mg/ml) with a 100-W tungsten lamp for 1½ h under argon atmosphere. After irradiation, the solvent was evaporated and the mixture consisting of the all-*E*, 13-*Z*, 11-*Z* and 9-*Z* isomers was separated by preparative HPLC. In the 9-*Z* isomer fraction, the 11-*Z* isomer was also present in a 1:1 ratio. With analytical HPLC, this fraction could be resolved into two separate peaks.

(All-*E*)-**1**. Mass spectrometry: measured $M^{+\bullet}$ 282.1984 (calcd. for $C_{20}H_{26}O$: 282.1983); UV λ_{max}(ethanol) 397 nm, $\varepsilon\ 47 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$; λ_{max}(methanol) 395 nm, λ_{max}(hexaan) 382 nm. ^{1}H NMR: see Tables II and III. ^{13}C NMR: see Table IV.

(13-*Z*)-**1**. ^{1}H NMR: see Table II; UV λ_{max}(ethanol) 389 nm.

(11-*Z*)-**1**. ^{1}H NMR: see Table II.

(9-*Z*)-**1**. ^{1}H NMR: see Table II; UV λ_{max}(ethanol) 360 nm.

(9-*Z*,13-*Z*)-**1**. ^{1}H NMR: see Table II; UV λ_{max}(ethanol) 352 nm.

(R)-**1** (**1a**). As above, **1a** (113 mg, 76%) was prepared from 120 mg **5a**. Similarly as for **1**, the all-*E*, 13-*Z*, 9-*Z* and 9-*Z*,13-*Z* forms were obtained in pure form. For each of these isomers, the ^{1}H NMR, ^{13}C NMR and UV data were identical to those of the corresponding isomers of **1**.

(All-*E*)-**1a**:mass spectrometry: measured $M^{+\bullet}$ 282.1990 (calcd. for $C_{20}H_{26}O$: 282.1983). CD (ethanol) λ_{max} 383 nm, Δε positive 0.7 ± 0.2 $M^{-1} \cdot cm^{-1}$, $g\ 2 \cdot 10^{-5}$; λ_{max} 273 nm, Δε positive 3.6 ± 0.15 $M^{-1} \cdot cm^{-1}$, $g\ 5 \cdot 10^{-4}$.

(S)-**1** (**1b**). As above, **1b** (128 mg, 76%) was prepared from 129 mg **5b**. Similarly as for **1**, the all-*E*, 13-*Z*, 9-*Z* and 9-*Z*,13-*Z* forms were obtained in pure form. For each of these isomers the ^{1}H NMR, ^{13}C NMR and UV data were identical to those of the corresponding isomers of **1**.

(All-*E*)-**1b**. Mass spectrometry: measured $M^{+\bullet}$ 282.1992 (calcd. for $C_{20}H_{26}O$: 282.1983). CD (ethanol) λ_{max} 383 nm, Δε negative 0.7 ± 0.2 $M^{-1} \cdot cm^{-1}$, $g\ 2 \cdot 10^{-5}$; λ_{max} 271 nm, Δε negative 3.4 ± 0.15 $M^{-1} \cdot cm^{-1}$, $g\ 5 \cdot 10^{-4}$.

*(5,7,16a,16a-$^2H_4$)***-1** (**1c**). As above, **1c** (130 mg, 76%) was prepared from 131 mg **5c**. The all-*E* isomer was isolated in pure form.

(All-*E*)-**1c**. Mass spectrometry: measured $M^{+\bullet}$ 286.2232 (calcd. for $C_{20}H_{22}D_4$: 286.2234). ^{1}H NMR: the same parameters as for (all-*E*)-**1** except that the intensity of the signals of 5-H, 7-H, 16a-H and 16a-H′ are reduced to less than 10% of those in all-*E*-**1**. The signals of 16-H and 16-H′ form a AB pattern [δ 16-H 1.62, δ 16-H′ 1.36, J(16-H–16-H′) 13 Hz] and J(4-H–5-^{2}H) ≈ 1 Hz. ^{13}C NMR: as for (all-*E*)-**1**, except that the singlets at δ 128.6 (7-C) and 23.0 (16a-C) are absent and the singlet at δ 127.3 (5-C) is reduced to about 5% of the intensity. Low-intensity signals are at δ 126.9 [t, J(C–^{2}H) 23 Hz, 5-C], 128.3 [t, J(C–^{2}H) 23 Hz, 7-C] and 22.7 [m, J(C–^{2}H) 18 Hz, 16a-C].

*(5-^2H)***1** (**1d**). As described above for **1**, **1d** (38 mg, 75%) was prepared from 34 mg **5d**, using 5 eq. C_5 phosphonate, 4.5 eq. *n*-butyllithium and 3 eq. Dibal. Similarly as for **1**, the all-*E*, 13-*Z*, 9-*Z* and 9-*Z*,13-*Z* forms were isolated in pure form.

(All-*E*)-**1d**. Mass spectrometry: measured $M^{+\bullet}$ 283.2043 (calcd.

* For the sake of comparison, the IUPAC retinoid numbering system[40] is used.

for $C_{20}H_{25}DO$: 283.2046), ^2H incorporation: 93.9% ^2H. ^1H NMR: the same parameters as for (all-*E*)-**1** except that the 5.63 ppm signal (5-H) is almost reduced to zero and J(4-H–5-^{2}H) ≈ 1 Hz. ^{13}C NMR: as for (all-*E*)-**1** except for the absence of the 127.3 ppm signal and the presence of a triplet at 127.0 ppm [J(C–^{2}H) 23 Hz, 5-C].

The ^1H NMR data of the *cis*-**1d** isomers are identical to those of the corresponding isomers of **1**, except that the 5-H signal is almost reduced to zero.

*(7-^2H)***1** (**1e**). As described above for **1**, **1e** (35 mg, 69%) was prepared from 39 mg **5e**, using 5 eq. C_5 phosphonate, 4.5 eq. *n*-butyllithium and 3 eq. Dibal. Similarly as for **1**, the all-*E*, 13-*Z*, 9-*Z* and 9-*Z*,13-*Z* forms were isolated in pure form.

(All-*E*)-**1e**. Mass spectrometry: measured $M^{+\bullet}$ 283.2040 (calcd. for $C_{20}H_{25}DO$: 283.2046), ^2H incorporation: 97.0% ^2H. ^1H NMR: the same parameters as for (all-*E*)-**1** except that the signal at 6.33 ppm (7-H) is absent. ^{13}C NMR: as for (all-*E*)-**1** except for the absence of 7-C signal at 128.6 ppm and the presence of a triplet at 128.3 ppm [J(C–^{2}H) 23 Hz, 7-C].

Then ^1H NMR data of the *cis*-**1e** isomers are identical to those of the corresponding isomers of **1**, except for the absence of 7-H signal.

*(5,7-$^2H_2$)***1** (**1f**). As described above for **1**, **1f** (70 mg, 84%) was prepared from 64 mg **5f**, using 5 eq. C_5 phosphonate, 4.5 eq. *n*-butyllithium and 3 eq. Dibal. Similarly as for **1**, the all-*E*, 13-*Z*, 9-*Z* and 9-*Z*,13-*Z* forms were isolated in pure form.

(All-*E*)-**1f**. Mass spectrometry: measured $M^{+\bullet}$ 283.2104 (calcd. for $C_{20}H_{24}D_2O$: 284.2109), ^2H incorporation: 92.0% $^2H_2$ and 7.1% ^2H. ^1H NMR: the same parameters as for (all-*E*)-**1** except that the signal at 6.33 ppm (7-H) is absent and at 5.63 ppm (5-H) is almost reduced to zero; J(4-H–5-^{2}H) ≈ 1 Hz. ^{13}C NMR: as for (all-*E*)-**1**, except for the absence of signals at 127.3 ppm (5-C) and 128.6 ppm (7-C). Two triplets are present: at δ 127.0 ppm [J(C–^{2}H) 23 Hz, 5-C], and 128.3 ppm [J(C–^{2}H) 23 Hz, 7-C].

The ^1H NMR data of the *cis*-**1f** isomers are identical to those of the corresponding isomers of **1**, except that the 7-H signal is absent and the 5-H signal almost reduced to zero.

5-Iodo-2-methyl-1-pentene

n-Butyllithium (47.5 ml 1.6M solution in hexane, 76 mmol) was added slowly, to a stirred, cooled (–30°C), suspension of triphenylmethylphosphonium iodide (32 g, 79 mmol) in THF (100 ml). The temperature was then raised to 0°C. After stirring for 1 h at this temperature, the reaction mixture was cooled to –20°C. A solution of freshly distilled 5-chloro-2-pentanone (7.4 gr, 61.4 mmol) in THF was then added slowly. The reaction temperature was then allowed to rise to 0°C. After stirring for 2 h at this temperature, the mixture was worked-up: 80 ml of saturated aqueous NH_4Cl solution was added, the mixture was diluted with pet. ether and then filtered over a silica gel layer. The filtrate was extracted three times with pet. ether. The organic layers were washed with brine and dried over $MgSO_4$. After partial concentration *in vacuo* and subsequent distillation, 5-chloro-2-methyl--1-pentene (b.p. 124–128°C) was obtained in 84% yield (6.1 g, 51.5 mmol). ^1H NMR (200 MHz): δ 1.72 (s, 3H, 2-CH_3), 1.93 (m, 2H, 4-H_2), 2.17 (t, J_{HH} 7.2 Hz, 2H, 3-H_2), 3.54 (t, J_{HH} 6.3, 2H, 5-H_2), 4.72 (d, $^2J_{HH}$ 0.8 Hz, 1H, 1-H), 4.76 (bs, 1H, 1-H). IR (film): 1649 cm^{-1} (C=C stretch). GC/MS: *m/z* 118:120 = 3:1 (calcd. for $C_6H_{11}Cl$: 118:120 = 3:1).

A solution of 5-chloro-2-methyl-1-pentene (3.35 g, 28.3 mmol) and NaI (14 g, 93 mmol), in dry acetone p.a. (60 ml) was refluxed for 16 h, after which time the acetone was removed by evaporation. After addition of water to the residue, the mixture was extracted three times with pet. ether. The organic layers were washed with brine and dried over $MgSO_4$. After removal of solvent, the yield of virtually pure 5-iodo-2-methyl-1-pentene was 4.95 g (24 mmol, 83%). ^1H NMR (200 MHz): δ 1.72 (s, 3H, 2-CH_3), 1.97 (m, 2H, 4-H_2), 2.12 (t, J_{HH} 7.7 Hz, 2H, 3-H_2), 3.18 (t, J_{HH} 6.7 Hz, 2H, 5-H_2), 4.73 (d, $^2J_{HH}$ 0.8 Hz, 1H, 1-H), 4.74 (d, 4 Hz, 1H, 1-H). GC/MS: *m/z* 210 (calcd. for $C_6H_{11}I$: 210).

2-Methyl-2-(4-methyl-4-pentenyl)cyclohexanone (**7**)

2-Methylcyclohexanone was converted into sodio-2-formyl-6--methylcyclohexanone according to published procedures[21]. *n*-Butyllithium (8 ml 1.6M in hexane, 12.8 mmol) was added slowly, to a stirred and cooled (0°C) solution of diisopropylamine (1.29 g, 12.8 mmol) in 50 ml THF, using a syringe, to give a yellow solution which was then stirred for 20 min. Through a powder funnel, sodio-2-formyl-6-methylcyclohexanone (1.59 g, 9.8 mmol) was then added over a period of 30 min, followed by the addition of 30 ml THF. The resulting yellow suspension was stirred at room temperature for $1\frac{1}{2}$ h. A solution of 5-iodo-2-methyl-1-pentene (2.5 g, 11.9 mmol) in 10 ml THF was then added slowly to the cooled (0°C) reaction mixture and the mixture was stirred for 5 h at room temperature. The reaction was quenched with ice-cold water and ether. The organic layer was further extracted with 3 × 50 ml cold water and then washed with saturated aqueous NH_4Cl solution and brine, successively, then dried over $MgSO_4$ and the solvents evaporated off. After purification by means of column chromatography (2% e/p), unreacted 5-iodo-2-methyl-1-pentene was collected (1.06 g, 5.0 mmol). To the combined aqueous extracts, NaOH (400 mg, 10 mmol) was added and the mixture was refluxed for 14 h. The reaction mixture was cooled and extracted three times with ether. The ether layers were washed with saturated aqueous NH_4Cl solution and brine, successively. After drying over $MgSO_4$ and removal of solvent, the product was purified by means of column chromatography to give **7** as a colourless oil (586 mg, 3.0 mmol, 44% at 70% conversion). ^1H NMR* (200 MHz): δ 1.05 (s, 3H, 2-CH_3), 1.0–1.9 (m, 10H), 1.69 (s, 3H, 4′-CH_3), 1.99 (t, J_{HH} 7 Hz, 2H, 3′-H_2), 2.2–2.5 (m, 2H, 6-H_2), 4.66 (s, 1H, 5′-H), 4.70 (s, 1H, 5′-H). ^{13}C NMR (50 MHz): δ 22 (s, methyl 2-C), 22 (s, methyl 4′-C), 38.5 (s, 6-C), 48 (s, 2-C), 110 (s, 5′-C), 145 (s, 4′-C), 215 (s, 1-C), 21, 21, 27, 39, 38, 37 (s, 3-, 4-, 5-, 1′-, 2′- and 3′-C).

*(6,6-$^2H_2$)***7** (**7d**). According to the same procedure as described for **2c**, compound **7** (1.24 g, 6.4 mmol) was treated three times with CH_3OD in the presence of methanolate to give **7d** (990 mg, 5.1 mmol, 79%). ^1H NMR (200 MHz): as for **7**, except for the absence of 6-H signal. ^{13}C NMR (50 MHz): as for **7**, except for the signal at 38.5 ppm which is reduced to a small multiplet.

6-Methyl-6-(4-methyl-4-pentenyl)-1-cyclohexenecarbonitrile (**8**)

A mixture of **7** (586 mg, 3.0 mmol), trimethylsilyl cyanide[15] (444 mg, 4.5 mmol), zinc iodide (24 mg, 0.08 mmol) and dry benzene (1 ml) was refluxed for 5 h. After cooling, water was added and the reaction mixture was extracted three times with pet. ether. The organic layers were washed with brine, dried over $MgSO_4$ and the solvents evaporated off. After purification by means of column chromatography (8% e/p), the trimethylsilated cyanohydrin was obtained (870 mg, 3.0 mmol, 98%). To this was added $POCl_3$ (1.4 ml, 15 mmol), pyridine (4 ml) and benzene (0.3 ml). The reaction mixture was refluxed for 46 h, after which time it was cooled to 0°C and cautiously treated with ice-cold 1.3N HCl solution. The mixture was extracted six times with ether. The organic layers were washed with saturated aqueous $NaHCO_3$ solution and brine, successively, dried over $MgSO_4$ and evaporated. Purification of the product by means of column chromatography (8% e/p) yielded **8** (388 mg, 1.9 mmol, 63% based on **7**).

^1H NMR (200 MHz): δ 1.14 (s, 3H, 6-CH_3), 1.1–2.8 (m, 8H), 1.71 (s, 3H, 4′-CH_3), 2.02 (t, J_{HH} 8 Hz, 2H, 3′-H_2), 2.05–2.2 (m, 2H, 3-H_2), 4.68 (s, 1H, 5′-H), 4.71 (s, 1H, 5′-H), 6.57 (t, J_{HH} 4 Hz, 1H, 2-H).

*(2-^2H)***8** (**8d**). As above, **8d** (649 mg, 63%) was prepared from 990 mg **7d**. ^1H NMR (200 MHz): as for **8**, except for the absence of 2-H signal at 6.57 ppm.

6-Methyl-6-(4-methyl-4-pentenyl)-1-cyclohexenecarboxaldehyde (**9**)

Using a syringe, diisobutylaluminum hydride (1.8 ml 1M Dibal solution in hexane, 1.8 mmol) was added slowly to a stirred solution of **8** (273 mg, 1.3 mmol) in dry pet. ether (14 ml) at –60°C. The mixture was allowed to warm up to 0°C. After $\frac{3}{4}$ h stirring at 0°C, the reaction mixture was cooled to –30°C. A suspension of silica gel (4 g) in 50% ether/pet.-ether (15 ml) and water (0.8 ml) was added and the mixture was then stirred for 2 h at 0°C. After drying over $MgSO_4$, the solids were filtered off and rinsed with ether. After removal of solvents, the product was purified by means of column chromatography (8% e/p) yielding **9** (252 mg, 1.2

* Side-chain locants have been primed.

mmol, 91%). ^{1}H NMR (200 MHz): δ 1.18 (s, 3H, 6-CH_3), 1.0–2.0 (m, 8H), 1.68 (s, 3H, 4'-CH_3), 1.95 (t, J_{HH} 8 Hz, 2H, 3'-H_2), 2.3 (m, 2H, 3-H), 4.64 (s, 1H, 5'-H), 4.67 (s, 1H, 5'-H), 6.75 (t, J_{HH} 4 Hz, 1H, 2-H), 9.33 (s, 1H, aldehyde-H).

*(2-^{2}H)***9** (**9d**). As above, **9d** (119 mg, 88%) was prepared from 133 mg **8d**. ^{1}H NMR (200 MHz): as for **9**, except for the absence of 2-H signal at 6.75 ppm.

*(Aldehyde-^{2}H)***9** (**9e**). Reduction of **8** (139 mg) with diisobutylaluminum deuteride (Dibal-^{2}H), which was prepared according to published procedures[23], yielded **9e** (109 mg, 77%). ^{1}H NMR (200 MHz): as for **9**, except for the absence of signal at 9.33 ppm.

*(2-^{2}H, aldehyde-^{2}H)***9** (**9f**). Reduction of **8d** (322 mg) with Dibal-^{2}H gave **9f** (252 mg, 77%)[23]. ^{1}H NMR (200 MHz): as for **9**, except for the absence of signals at 6.75 and 9.33 ppm.

1-(3,4,4a,5,6,7-Hexahydro-4a-methyl-2-naphthalenyl)ethanone (**4**)

A mixture of **9** (252 mg, 1.2 mmol), OsO_4 (17 mg, 0.07 mmol), acetone *p.a.* (5 ml) and water (5 ml) was stirred for 5 min at 25°C. Powdered $NaIO_4$ (796 mg, 3.7 mmol) was then added in portions over a period of 30 min. The temperature was then raised to 35°C. The suspension was stirred for 4 h, after which water was added. The mixture was extracted three times with ether. The organic layers were washed with brine and dried over $MgSO_4$. After removal of solvent, the crude oil was used directly for subsequent cyclization to **4**: it was dissolved in 2% KOH/methanol (8 ml). The mixture was refluxed for 2 h after which and after cooling, saturated aqueous NH_4Cl solution was added. The reaction mixture was extracted six times with ether. The organic layers were washed with brine and dried over $MgSO_4$. After removal of solvents and purification by means of column chromatography (20% e/p), the yield of **4** was 102 mg (0.5 mmol, 44%). The ^{1}H NMR spectrum of this compound is identical to that of **4**, prepared from **3**.

*(8,8-^{2}H)***4** (**4d**). As above, **4d** (44 mg, 40%) was prepared from 119 mg **9d**. ^{1}H NMR (200 MHz): as for **4**, except for the absence of 8-H signal at 5.88 ppm.

*(1-^{2}H)***4** (**4e**). As above, **4e** (42 mg, 42%) was prepared from 109 mg **9e**. ^{1}H NMR (200 MHz): as for **4**, except for the absence of 1-H signal at 6.92 ppm.

*(1,8-$^2H_2$)***4** (**4f**). As above, **4f** (86 mg, 37%) was prepared from 252 mg **9f**. ^{1}H NMR (200 MHz): as for **4**, except for the absence of signals at 6.92 (1-H) and 5.88 (8-H) ppm.

*Preparation of the Schiff base and protonated Schiff base of (all-*E*)-***1**

The Schiff base was prepared by addition of an excess of *n*-butylamine to a dilute solution (optical density 0.8–1.2) of (all-*E*)-**1** in 4 ml methanol. Addition of a few drops of concentrated hydrochloric acid to this methanol solution completely converted the Schiff base into the protonated form. λ_{max}(SB) 373 nm, λ_{max}(PSB) 467 nm.

Isolation of purple membrane and preparation of bacterioopsin

Halobacterium halobium (low-carotenoid strain R1 S9) was cultured and purple membrane isolated according to published procedures[41,42]. Bacterioopsin was prepared as described by *Oesterhelt* et al.[43].

Binding and kinetic experiments

Binding and kinetic experiments were performed as described earlier at room temperature and at 4°C[43,44,10]. Regeneration was followed in 0.2-cm and 1-cm path-length cuvettes.

Light–dark adaptation

Light–dark adaptation was performed as described earlier[10]. λ_{max} [bR(**1**), bR(**1a**), bR(**1b**)], light-adapted forms: 552 nm, λ_{max} [bR(**1**), bR(**1a**), bR(**1b**)], dark-adapted forms: 547 nm.

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

Soybean phospholipid (asolectin) was purified using the method of *Darley–Usmar* et al.[45]. bR, bR(**1**), bR(**1a**) and bR(**1b**) were prepared from $4.1 \cdot 10^{-5}$ M bO in distilled water with a two-fold molar excess of (modified) retinal. The regeneration of the pigment was followed spectroscopically until regeneration was complete. To 40 mg of purified soybean phospholipids, solvent in $CHCl_3$, and then dried in a round-bottom flask on a rotatory film evaporator, 1 mg bR (analogue) (conc 1 mg/ml) in 150mM KCl and 2mM EDTA (pH 7) was added. The mixture was shaken with glass beads until the lipids were removed from the glass wall of the flask. The suspension was then sonified using a MSE probe-type ultrasonifier (probe diameter 9 mm, freq. 21 kHz, ampl. 4 μm) for 900 s according to *Hellingwerf*[46]. The mixture was kept under nitrogen and cooled in ice during sonication. According to this procedure, liposomes containing bO, bR, bR(**1**), bR(**1a**) and bR(**1b**) were prepared.

The light-dependent pH changes were measured in a 2-ml temperature-controlled multi-purpose cuvette (20°C) equipped with a stirring device and containing 200 μl freshly prepared (modified) bR liposomes and 1.8 ml 150mM KCl, 0.2mM EDTA (pH 7). The pH of the medium was measured continuously using an Ingold pH electrode (10-420-3522) connected to an amplifier (Radiometer PHM 63), and recorded on a Pantos U-228 unicorder. The cuvette was illuminated with a cold light source (100 W 12V halogen lamp, Bellaphot, Osram). The pH changes upon illumination were calibrated by the addition of small amounts of 0.6mM oxalic acid. For each reconstitution, three photocycles were measured. Valinomycin (2 μl 0.1 mg/ml) was then added and, finally, nigericin (2 μl 0.1 mg/ml).

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