Bacteriorhodopsin analogue from anthryl chromophores

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Received May 18, 1989

ANIL K. SINGH and MITA ROY. Can. J. Chem. **68**, 383 (1990).

Preparation and properties of the bacteriorhodopsin (bR) analogue having the 3,7-dimethyl-9-(9-anthryl)-2*E*,4*E*,6*E*,8*E*-nonatetraenal (**12**) chromophore is described. Synthesis of the chromophore has been achieved by successive introduction of C_5 units to 9-anthraldehyde (**3**) via the Horner reaction. The all-*trans* chromophore has been characterized by its ultraviolet–visible and ^1H nuclear magnetic resonance spectra. Incubation of **12** with bacterioopsin suspension (prepared by photobleaching of bR isolated from *Halobacterium halobium*) at ambient temperature in the dark gave the new bR analogue **15**, which showed an absorption band at 545 nm, and an opsin shift of 5575 cm^{-1}. The new pigment is stable to hydroxyl amine in the dark. It showed light–dark adaptation with the light-adapted form absorbing at a slightly red-shifted value of 550 nm. All-*trans*-retinal did not replace the anthryl chromophore in competitive bindings. Photolysis of the bR analogue **15**, followed by difference spectrophotometric analysis, indicated formation of a photoproduct with an absorption band near 400 nm. The results are discussed in terms of the stereoelectronic requirements of the bR reaction centre.

Key words: bacteriorhodopsin (bR), retinal analogue, reconstitution, opsin shift (OS), external point charge model (EPC).

ANIL K. SINGH et MITA ROY. Can. J. Chem. **68**, 383 (1990).

On décrit la préparation et les propriétés d'un analogue de la bactériorhodopsine (bR) possédant le chromophore 3,7-diméthyl-9-(9-anthryl)-2*E*,4*E*,6*E*,8*E*-nonatétraènal (**12**). On a réalisé la synthèse du chromophore en faisant appel à la réaction de Horner pour introduire des unités successives en C_5 sur le 9-anthraldéhyde (**3**). On a établi le caractère complètement *trans* du chromophore à l'aide de données d'UV–VIS et de RMN du ^{13}C. L'incubation, à la température ambiante et en l'absence de lumière, du composé **12** avec une suspension de bactérioopsine (préparée par photoblanchiment d'une bR isolée à partir de *Halobacterium halobium*) fournit le nouvel analogue **15** de la bR qui présente une bande d'absorption à 545 nm et un déplacement opsine de 5575 cm^{-1}. Le nouveau pigment ne réagit pas avec l'hydroxylamine, en l'absence de lumière. Il présente une adaptation de la lumière à la noirceur; la forme adaptée à la lumière absorbe à une valeur de 550 nm, qui est légèrement déplacée vers le rouge. Le rétinal complètement *trans* ne remplace pas le chromophore anthryle dans les sites où ils sont en compétition. La photolyse de la bR analogue **15**, suivie par une analyse spectrophotométrique différentielle, indique qu'il y a formation d'un photo-produit possédant une bande d'absorption près de 400 nm. On discute des résultats en fonction des facteurs stéréoélectroniques inhérents au centre réactionnel de la bR.

Mots clés : bactériorhodopsine (bR), analogue rétinal, reconstitution, déplacement opsine, modèle de point-charge externe.

[Traduit par la revue]

Introduction

Bacteriorhodopsin (bR), the sole protein component of the purple membrane (pm) of *Halobacterium halobium* (1, 2), is produced by the bacterial cell in response to oxygen starvation. The protein displays some characteristic features in terms of its colour and physiological functions. These aspects of bR have been the subject of intense investigations in recent years. The primary and secondary structures of bR are known (3, 4). The three-dimensional structure of bR has been elucidated to a resolution of 7–14 Å (5). The chromophore of bR is a retinal molecule attached to the ε-amino group of lysine-216 (4) of the apoprotein, bacterioopsin (bOP), via a protonated Schiff base (PSB) (6) linkage (Fig. 1). There are two forms of bR, the light-adapted bR (bR^{LA}, λ_{max} 570 nm) and the dark-adapted bR (bR^{DA}, λ_{max} 560 nm), the chromophores of which are respectively all-*trans*-retinal and a 1:1 mixture of all-*trans*- and 13-*cis*-retinal. Absorption of photons by bR leads to a photochemical cycle (7), which begins with the photochemical all-*trans* to 13-*cis* isomerization of the retinyl prosthetic group in the pigment. The pigment then decays through the K_{610} and L_{550} intermediates, and the Schiff base nitrogen deprotonates, producing the blue-shifted M_{412} species. The photocycle of bR drives the extrusion of protons from the cell to generate a proton gradient, which is then utilized by the Halobacteria to fuel active transport and ATP synthesis.

Specific active-site interactions (8, 9) between chromophore and apoprotein are believed to be responsible for the characteristic features of bR. An approach to the study of the nature of chromophore–apoprotein interactions has been to strip the natural pigment of its chromophore and then to incubate the apoprotein with suitably tailored retinal analogue to generate bioorganic models of bR (8). Such studies have greatly enhanced our understanding of the absorption behaviour of rhodopsins. However, several aspects of the structure, and mechanism of function, of bR are not yet fully understood.

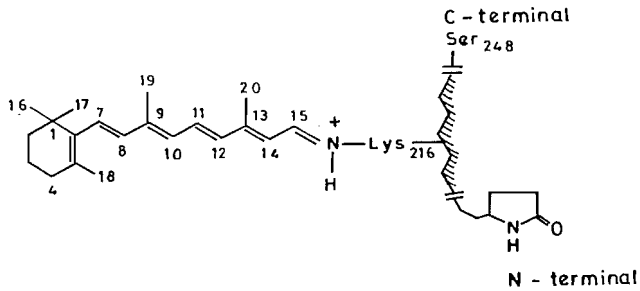

FIG. 1. All-*trans* retinal bound to bR apoprotein via a protonated Schiff base linkage.

The chromophore of bR has three distinct regions, the ring, the side chain, and the Schiff base region. To investigate the nature of the binding site in these regions, a bR analogue with a chromophore having modifications in a particular region can be prepared. The stereoelectronic properties of the ring binding site can thus be studied if bR analogues can be prepared from chromophores that have been suitably modified in the ring portion of the natural chromophore. In our earlier work (10) it

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was observed that the iodophenyl retinal analogue binds rapidly to bOP and shows a dramatically altered opsin shift (OS). An anthryl dienal chromophore (**6**) was also found to bind bOP readily, and showed an OS unexpected for a bR analogue having a chromophore with only two double bonds in its side chain. The results were explained in terms of the External Point Charge (EPC) model (8, 9) wherein opsin-bound charges are evoked to electrostatically interact with the chromophore's excited state positive charge near the β-ionyl ring. Thus, EPC attributes the OS to an interaction through space with a negative charge introduced by the protein, in addition to the Schiff base counterion. In bR (OS, 4870 cm^{-1}), it has been suggested that the negative charge is located in the vicinity of the β-ionyl moiety.

The present communication deals with the preparation and properties of the bR analogue from the anthryl chromophore (**12**) in which all four double bonds of the side chain are present, as in the natural chromophore, but the trimethyl cyclohexenyl ring has been replaced by an anthryl moiety.

Experimental

C_5-Phosphonate, methyl-4-(diethyl phosphono)-3-methyl-2-butenoate, was synthesized by following the procedures indicated in standard text (11). AR grade organic solvents were from Spectrochem, Bombay, and were purified before use. Other chemicals were either from Aldrich (USA) or Sigma (USA), and were used as received. Activated manganese dioxide (MnO_2) was prepared by following the published procedure (12). Sodium hydride (NaH) was used as a 50% oil dispersion, which was washed with two portions of anhydrous petroleum ether (40–60°C) prior to use. Horner reactions were carried out under an inert atmosphere of dry nitrogen gas, with anhydrous conditions. All the operations pertaining to retinal and related light-sensitive compounds were performed in a dark room having a dim red light. Column chromatography was done by using 100–200 mesh silica gel (SISCO Laboratories, Bombay). Flash column chromatography was performed on 230–400 mesh silica gel (Merck) according to the reported procedure (13).

Ultraviolet–visible spectra were recorded either on Beckman DU 6 or on Shimadzu UV 260 spectrophotometers. The ir spectra were recorded with a Perkin Elmer 681 IR spectrophotometer. Proton nmr spectra were taken either on Bruker 80 and 500 MHz or on Varian 100 MHz nmr spectrometers. Mass spectra were measured on a Shimadzu QP 1000 mass spectrometer. High pressure liquid chromatographic (hplc) analyses were carried out on a Beckman 110 A instrument using a Microporacil (10 μm, 4.5 × 250 mm, Si-60) column, and spectrophotometric detection. Ultracentrifugations were done on a Beckman L-8-55 M ultracentrifuge. Sonications were performed on a Branson B-12 sonifier. pH measurements were made on a Radiometer PHM-84 pH meter with GK 2401C electrodes.

Isolation of bR, from the cells of *Halobacterium halobium* grown under low oxygen tension and illumination conditions, was done according to the reported procedure (14). The apoprotein was prepared by photobleaching of bR in the presence of hydroxylamine at 4°C.

*9-Anthraldehyde (**3**)*

Anthracene (8.9 g, 0.05 mol), *N*,*N*-dimethyl formamide (DMF, 3.5 mL), phosphorous oxychloride (8.75 mL), and *ortho*-dichlorobenzene (20 mL) were placed in a round-bottomed flask fitted with a reflux condenser and mechanical stirrer. The mixture was heated at 90–95°C for 40 min with simultaneous stirring. Anthracene dissolved during this period to give a deep red solution. The reaction mixture was further refluxed for 3 h and then allowed to cool. The cold mixture was poured into an aqueous solution of sodium acetate (140 g/250 mL), and kept in a refrigerator overnight. Solid material deposited in the flask was recovered by filtration. The crude solid was further purified by column chromatography (5% ethyl acetate in petroleum ether, 40–60°C). Recrystallization of the column-purified material from glacial acetic acid, and further washing of the crystalline material with methanol, gave yellow needle-shaped crystals of 9-anthraldehyde; 6 g, 50%; mp 101–102°C; ir (KBr), ν: 2860, 1670, 1555, 1448, 900, 872, 851 cm^{-1}; uv (MeOH), λ_{max}, nm (ε): 390 (5748), 261 (68 210); ^1H nmr ($CDCl_3$, 100 MHz) δ: 7.60 (4H, m), 8.10 (2H, m), 8.80 (1H, s), 9.10 (2H, m), 11.70 (1H, s); ms, EI (*m*/*e*, % rel. int.): 206 (M^+, 86), 177 (M^+ − CHO, 100); hplc (5% ethyl acetate in hexane, 1.5 mL/min, 360 nm) R_t, 4.6 min.

*Methyl 3-methyl-5-(9-anthryl)-2*E*,4*E*-pentadienoate (**4**)*

A solution of C_5-phosphonate (2.33 g, 8.9 mmol) in dry tetrahydrofuran (THF, 10 mL) was added dropwise to a suspension of NaH (0.43 g, 17.8 mmol) in 15 mL dry THF maintained at 0°C. The mixture was stirred (Teflon) for 1 h under N_2. The colour of the mixture turned gradually from colourless to pale yellow and then to dark yellow. A solution of 9-anthraldehyde (**3**, 1.83 g, 8.9 mmol) in 10 mL THF was added dropwise to the yellow reaction mixture at 0°C. The reaction mixture was further stirred for 1 h. The reaction was quenched with brine at 0°C, and the contents taken up in ether. The ethereal layer was separated, washed with brine, and dried over anhydrous sodium sulphate. Removal of ether under diminished pressure on a Rota-vapour gave a solid, which was purified by column chromatography (5% ethyl acetate in petroleum ether, 40°C–60°C). Crystallization of the column-purified material from a mixture of petroleum ether and ethyl acetate (5:1) yielded yellow crystals of ester **4**; 1.10 g, 50%; mp 90–92°C; ir (KBr), ν: 1721, 1170 (ester), 1621, 1525, 1451, 851 (aromatic) cm^{-1}; uv (MeOH), λ_{max}, nm (ε): 387 (18 287), 254 (216 286); ^1H nmr ($CDCl_3$, 80 MHz) δ: 2.76 (3H, s, 3-CH_3), 3.70 (3H, s, -$COOCH_3$), 6.04 (1H, s, H-2), 6.78 (1H, d, J = 16 Hz, H-4), 7.52 (4H, m, arom.), 7.94 (1H, d, J = 16 Hz, H-5), 8.20 (4H, m, arom.), 8.56 (1H, s, 10-H, arom.); ms, EI (*m*/*e*, % rel. int.): 302 (M^+, 71), 256 (100); hplc (5% ethyl acetate in petroleum ether, 1.5 mL/min) R_t, 4.2 min.

*3-Methyl 5-(9-anthryl)-2*E*,4*E*-pentadienal (**6**)*

An ethereal solution (15 mL) of pentadiene ester **4** (1 g, 3 mmol) was added dropwise to a suspension of lithium aluminum hydride (LAH) (0.22 g, 5.7 mmol) in 20 mL dry ether. The mixture was stirred (Teflon) for 1 h at −10°C. Reaction was quenched by dropwise addition of water at the same temperature. Ether was added to the reaction mixture, and the aqueous layer was separated. The ethereal layer was washed with brine, and dried over anhydrous sodium sulphate. Ether was then removed under reduced pressure. The resultant alcohol, 3-methyl 5-(9-anthryl)-2*E*,4*E*-pentadien-1-ol (**5**) was stirred with MnO_2 (2.03 g, 24 mmol) in 20 mL THF under N_2 at ambient temperature for 2.5 h. The dark brown mixture was filtered through a G-3 filter. THF was then removed by careful distillation on a Rota-vapour under reduced pressure. The resultant aldehyde **6** was purified by flash column chromatography (7% ethyl acetate in petroleum ether, 40°C–60°C). Further purification of the compound by crystallization from a mixture of ethyl acetate/petroleum ether (1:5) yielded dark-yellow-coloured crystals of **6**; 0.30 g, 40%; mp 70–72°C; ir (KBr) ν: 2860, 1668 (aldehyde), 1625, 1515, 1440, 850 (aromatic) cm^{-1}; uv (MeOH), λ_{max}, nm (ε): 387 (11 252), 252.6 (205 286); ^1H nmr ($CDCl_3$, 80 MHz) δ: 2.68 (3H, s, 3-CH_3), 6.18 (1H, d, J = 11.8 Hz, H-2), 6.80 (1H, d, J = 16.5 Hz, H-4), 7.35 (1H, d, J = 16.5 Hz, H-5), 7.53 (4H, m, arom.), 8.23 (4H, m, arom.), 8.44 (1H, s, 10-H, arom.), 10.32 (1H, d, J = 11.8 Hz, H-1); ms, EI, (*m*/*e*, % rel. int.): 272 (M^+, 84.7), 257 ($M^+ - CH_3$, 77), 243 (M^+ − CHO, 50.5), 228 ($M^+ - C_2H_4O$, 100); hplc (5% ethyl acetate in petroleum ether 40°C–60°C, 1.5 mL/min) R_t, 4.4 min.

*Methyl 3,7-dimethyl-9-(9-anthryl)-2*E*,4*E*,6*E*,8*E*-nonatetraenoate (**10**)*

A solution of C_5-phosphonate (0.562 g, 2.23 mmol) in 10 mL dry THF was added to a suspension of NaH (0.107 g, 4.46 mmol) in 20 mL dry THF. The mixture was stirred (Teflon) at 0°C in the dark under N_2 for 1 h. A solution of compound **6** (0.5 g, 1.84 mmol) in 15 mL dry THF was syringed in slowly over a period of 30 min while stirring was continued. After complete addition of the aldehyde, the reaction

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mixture was further stirred at 0°C for 1 h, and then allowed to warm up to ambient temperature over a period of 3 h. At the end of the 3 h period, the reaction mixture was again brought to the low temperature (−10°C), and worked up by addition of brine and ether. The ethereal layer was washed throughly with brine and water, and further dried over anhydrous sodium sulphate in the dark. Removal of ether gave a viscous material, which was subjected to multiple flash column chromatography (7% ethyl acetate in petroleum ether, 40°C–60°C). Repetitive flash column separations gave ester **10** (0.029 g) as a yellowish gummy material; ir (KBr) ν: 1721, 1170 (ester), 1621, 1525, 1451, 851 (aromatic) cm^{-1}; ^1H nmr ($CDCl_3$, 500 MHz) δ: 2.19 (3H, s, 3-CH_3), 2.30 (3H, s, 7-CH_3), 3.75 (3H, s, -OCH_3), 5.80 (1H, s, H-2), 5.82 (1H, d, J = 15 Hz, H-4), 6.29 (1H, d, J = 11 Hz, H-6), 6.74 (1H, d, J = 16 Hz, H-8), 6.79 (1H, d, J = 16 Hz, H-9), 7.10 (1H, dd, J = 11 Hz, 15 Hz, H-5), 7.14–8.42 (9H, m, arom.); ms, EI, (m/e, % rel. int.): 367 (M^+, 67); hplc (7% ethyl acetate in petroleum ether, 40°C–60°C, 1.5 mL/min) R_t, 4.3 min.

*3,7-Dimethyl-9-(9-anthryl)-2*E*,4*E*,6*E*,8*E*-nonatetraenal (12)*

An ethereal solution (15 mL) of nonatetraenoate **10** (0.22 g, 0.76 mmol) was added to a suspension of LAH (0.057 g, 1.5 mmol) in 10 mL dry ether at −10°C. The reaction mixture was stirred (Teflon) at that temperature in the dark for 30 min. Reaction was terminated by careful addition of about 2 mL of brine to the reaction mixture maintained at −10°C. Ether was added, and the aqueous layer was removed. The ethereal layer was washed with brine and water, and dried over anhydrous sodium sulphate. About two thirds of the ether was removed from the yellow solution on a Rota-vapour under reduced pressure, and the remaining ether was evaporated by gentle N_2 jet. The dark yellowish viscous alcohol **11** obtained was immediately treated with a 20 mL THF suspension of MnO_2 (0.61 g, 6.75 mmol) in the dark under N_2. After 3 h of stirring (Teflon) at ambient temperature, the mixture was filtered through a G-3 filter, and finally through a Celite column (2 cm, in a 5-mm pipette). Most of the THF was removed on a Rota-vapour, and the residue was subjected to flash column chromatography (5% ethyl acetate in petroleum ether, 40°C–60°C), when an orange viscous material (0.1 g) was recovered from the effluent. High pressure liquid chromatographic analysis (7% ethyl acetate in petroleum ether, 40°C–60°C, 1.5 mL/min) showed it to contain approximately 60% of the desired aldehyde **12**; ir (KBr), ν: 2770, 1668 (CHO), 1621, 1528, 1451, 851 (aromatic) cm^{-1}; uv (MeOH), λ_{max}: 410 nm; ^1H nmr ($CDCl_3$, 500 MHz) δ: 2.34 (3H, s, 3-CH_3), 2.37 (3H, s, 7-CH_3), 6.00 (1H, d, J = 8.00 Hz, H-2), 6.35 (1H, d, J = 11 Hz, H-6), 6.43 (1H, d, J = 15.20 Hz, H-4), 6.75 (1H, d, J = 16.30 Hz, H-9), 6.76 (1H, d, J = 16.30 Hz, H-8), 7.26 (1H, d, J = 15.20 and 11.00 Hz, H-5), 7.40–8.42 (9H, m, arom.), 10.13 (1H, d, J = 8.00 Hz, H-1); hplc, R_t, 4.5 min.

Protonated Schiff bases (PSB, 8 and 14)

The PSBs were prepared by addition of dry, freshly distilled *n*-butyl amine to methanolic solutions of the respective aldehydes. The reaction was carried out over 4 Å molecular sieves at 0°C under N_2 in the dark. After 12 h, the methanol and excess *n*-butyl amine were removed under vacuum, and the semisolid residue was redissolved in anhydrous methanol. Protonation of the Schiff bases thus prepared was accomplished by addition of methanol saturated with dry hydrogen chloride gas. Protonation of the Schiff bases was indicated by a red shift in their absorption spectra.

The bR analogue (15)

An ethanolic solution of anthryl retinal analogue **12** was incubated with freshly prepared bOP at ambient temperature in the dark over a period of 24 h. The final ethanol concentration was ≤1% v/v. The growth of the pigment was followed by periodic recording of the absorption spectrum of the mixture. A new peak at 545 nm appeared as a result of binding of anthryl analogue **12** with bOP. The competitive displacement experiments were done by adding an aliquot of ethanolic solution of all-*trans*-retinal to the synthetic pigment. The synthetic pigment was purified by washing of excess chromophore by ultracentrifugation (*n*-hexane, 50 000 g, 10 min).

Preparation of light- and dark-adapted pigments

The dark-adapted bR analogue (bR^{DA}_{545}-**15**) suspensions were irradiated with a 500 W tungsten lamp at a distance of 5 cm using 1% $CuSO_4$–H_2O solution as filter at 4°C. The sample was continuously stirred with a small Teflon bar. The photolysed sample showed an absorption band at 550 nm corresponding to the light-adapted form of **15** (bR^{LA}_{550}-**15**). Incubation of bR^{LA}-**15** in the dark over a period of 24 h regenerated bR^{DA}-**15**.

Photolysis of bR analogue 15 at low temperature

bR-Analogue **15** was suspended in a solution containing 3 mL of 25% NaCl and 75% glycerol. The pH of the sample was adjusted to 9.0 by adding 0.5 M NaOH solution. The sample was cooled to 0°C, and light adapted as described in the previous section. The light-adapted sample was further cooled to −60°C, and irradiation was continued. The difference uv–vis spectrum was obtained by recording the absorption spectra of photostationary and initial-stage samples. A new species with absorption near 400 nm was detected from the difference spectrum. A control experiment was performed by observing changes in the cooled non-irradiated sample.

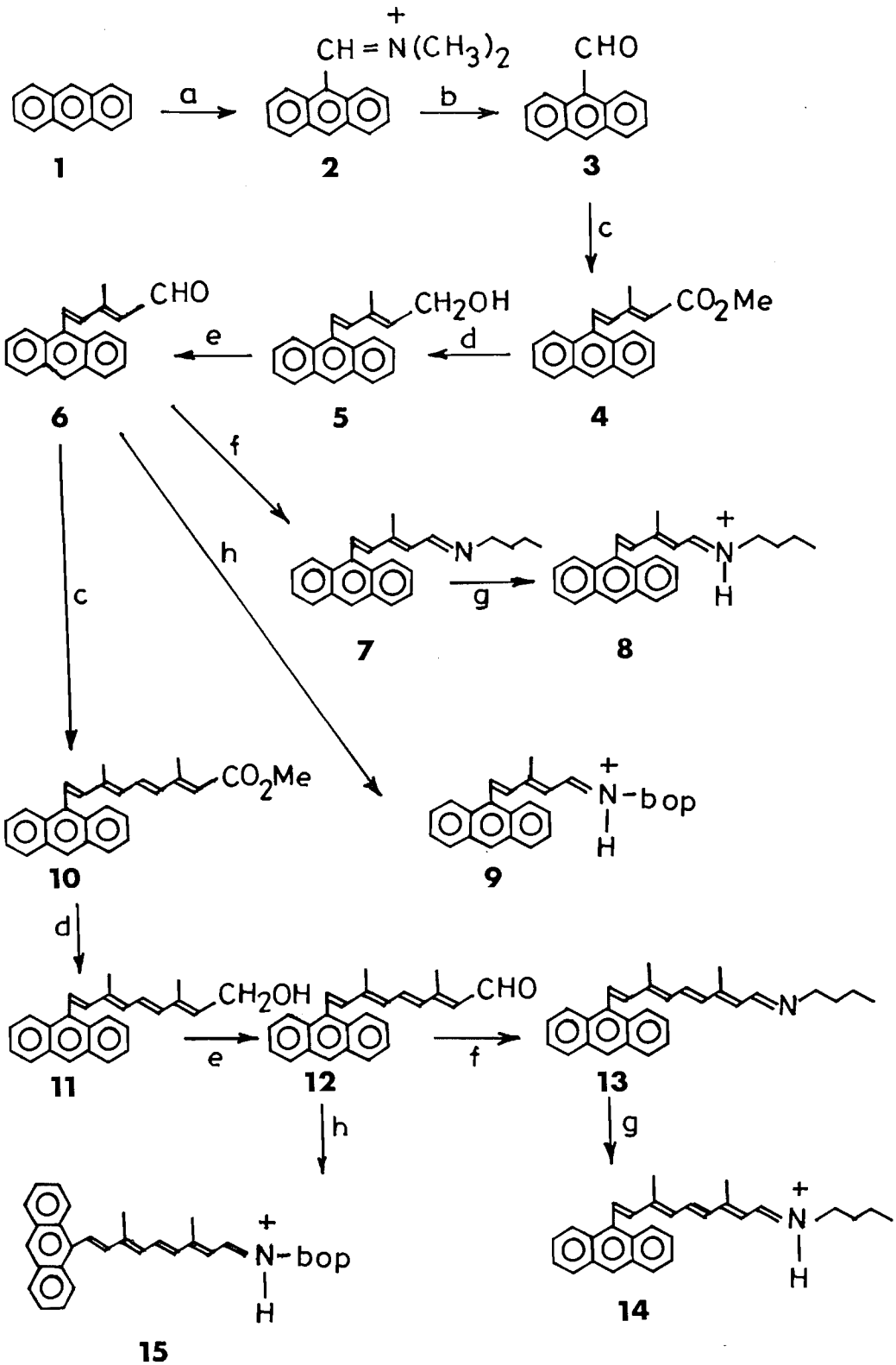
Results and discussion

Synthesis of retinal analogue 12

The procedure adapted for the synthesis of analogue **12** is outlined in Scheme 1. 9-Anthraldehyde (**3**) was prepared (15) by formylation of anthracene in 50% yield. The adduct **2**, formed as a dark red liquid, was hydrolysed with aqueous sodium acetate solution, and the mixture was allowed to stand overnight at 4°C. This resulted in the solidification of aldehyde **3** as a yellow solid. *ortho*-Dichlorobenzene did not hinder solidification; rather, it helped in partially purifying the desired compound by dissolution of the products in the last step. Thus the purity of the aldehyde was improved by allowing it to solidify in the presence of dichlorobenzene, such that it retained the unreacted anthracene. Long needle-shaped crystals of **3** gave satisfactory physicochemical analysis.

The desired C_5-phosphonate was synthesized starting from 3,3-dimethyl acrylic acid (11). Esterification of the acid involved the usual preparation of its acyl chloride followed by its *in situ* reaction with dry methanol. Allylic bromination of the acrylate with *N*-bromosuccinimide in carbon tetrachloride under reflux conditions, followed by fractional distillation of the reaction residue, afforded the extremely lachrymatic bromoester, methyl 4-bromo-3-methyl-2-butenoate. Reaction of the bromoester with triethyl phosphite at 180°C for 1 h gave the desired phosphonate, which was isolated from the reaction mixture by fractional distillation. The ^1H nmr analysis of the phosphonate showed it to be a 2:3 mixture of 2-*cis*- and 2-*trans* butenoate. The identity of the isomers was established by the appearance of two singlets, in the nmr spectrum, at δ 2.30 and 2.05, corresponding to 3-CH_3 protons of 2-*cis* and 2-*trans* isomers, respectively. The allylic methylene protons at C-4 adjacent to the phosphonate group appeared at δ 3.25 and δ 2.90 for the two isomers. All other intermediate compounds also showed satisfactory data conforming to their structure.

Condensation of 9-anthraldehyde (**3**) with C_5-phosphonate in the presence of NaH gave the diene ester **4**. It could be purified by flash column chromatography, and gave the expected physicochemical analyses. It showed an intense, sharp uv band at 254 nm due to ^1B transition, and weak but structured bands centered at 387 nm due to transversely polarized ^1L_a transitions. The ir spectrum of **4** exhibited an intense band at 1721 cm^{-1} featuring an ester carbonyl. Mass spectroscopic analysis of **4** revealed the molecular ion peak at m/e 302, and the base peak at m/e 256 corresponding to $M^+ - C_2H_6O$. Another prominent



SCHEME 1. (a) DMF–$POCl_3$, *o*-dichlorobenzene; (b) NaOAc; (c) $(EtO)_2POCH_2.(CH_3)CH{=}CH.COOMe$, THF, NaH; (d) $LiAlH_4$–Et_2O; (e) MnO_2–THF; (f) *n*-butyl amine, MeOH; (g) MeOH–HCl, dry; (h) bOP, HEPES, dark.

mass peak was observed at *m/e* 271 due to the $M^+ - OMe$ fragment. The proton nmr spectrum of **4** showed the expected singlets at δ 3.70 (-COOMe), δ 2.76 (-3-CH_3), and δ 6.04 (H-2). The coupling constant of 16 Hz between protons at C-4 and C-5 was indicative of a *trans* configuration between the C_4—C_5 double bond. The anthryl ring showed three signals. The two multiplets at δ 7.52 and 8.20 corresponded to four protons each. The C-10 proton of the anthryl ring was observed as a singlet at δ 8.56.

Reduction of the ester function in **4** by LAH required low temperature and early termination of the reaction. Reactions at ambient temperature resulted in the formation of several side

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TABLE 1. Ultraviolet–visible absorption data for anthryl chromophores in methanol

Compound	λ_{max}, nm (ε)
1	380 (9000), 254 (180 000)
6	387 (11 252), 254 (205 285)
7	386, 254
8*	454, 254
12	410 (24 794), 261 (80 561)
13	393, 260
14*	418, 261

*Chloride salts.

products that did not analyse for the desired dienol group. Prolonged reaction even at low temperatures gave similar undesired results. The alcohol **5** obtained from the careful reduction of **4** was oxidised by MnO_2 to aldehyde **6**. Dienal **6** was purified by a combination of flash column chromatography and crystallization. Both the reduction of ester **4** and oxidation of the resulting alcohol proceeded with no detectable stereochemical change as evidenced by homogeneous hplc patterns and by spectral analyses. The ir spectrum of **6** showed characteristic bands for an aldehyde group at 1668 and 2860 cm^{-1}. Significant features in the ^1H nmr spectrum of **6** were the appearance of a doublet at δ 6.18 with a coupling constant of 11.80 Hz for the C-2 proton (which was a singlet at 6.04 in the ester **4**) that arose due to coupling of the adjacent proton at C-1. As expected, the configuration at the C_4—C_5 double bond was *trans*, with a coupling constant of 16 Hz between protons at C-5 and C-4. Mass spectrometric analysis showed a fragmentation pattern characteristic of a dienal.

Further elongation of the chain of **6** was done by adding one more C_5 unit to it. The resulting ester **10** could be purified by flash column chromatography. While its intense ir band at 1721 cm^{-1} was very characteristic of an ester carbonyl group, the 500 MHz ^1H nmr spectrum provided confirmation of its configurational structure. The tetraenyl chain showed the expected signals for its *trans*-coupled protons. LAH reduction of **10**, followed by MnO_2 oxidation of the resulting alcohol **11**, gave tetraenal **12**, which could be satisfactorily characterized by its uv–vis and ^1H nmr analyses. As expected, the tetraenyl compound was obtained as a mixture of several configurational isomers. The all-*trans* analogue showed characteristic nmr signals for olefinic and aldehydic protons. The aldehyde proton for the all-*trans* compound resonated at δ 10.13 as a doublet with a coupling constant of 8 Hz. The corresponding H-2 proton is seen at δ 6.00, also as a doublet with a *J* value of 8.00 Hz. The *trans* coupling between the H-4 and H-5 protons and *s-trans* coupling between H-5 and H-6 were observed to be 15.2 and 11.0 Hz, respectively. Compound **12** showed the characteristic aldehyde peak at 1668 cm^{-1}. Expected uv–vis absorption bands at 410 and 261 nm due to ^1L_a and ^1B transitions, respectively, confirmed the conjugated nature of the elongated side chain. The absorption properties of the anthryl chromophores are discussed in the following section.

Ultraviolet–visible absorption properties of anthryl chromophores

The uv–vis absorption data for the anthryl compounds are given in Table 1. The spectrum of parent anthracene, a *cata*-condensed nucleus, shows an intense peak at 254 nm (ε, 180 000) due to ^1B transitions. The other major band in the spectrum of anthracene is due to ^1L_a transitions, and consists of a series of five vibrational sub-bands, the longest of which occurs at about 380 nm with ε of 9000. Introduction of an olefinic chain at C-9 in anthracene did not result in any major change in the shape of the spectrum. With the introduction of a chain at the C-9 position, an increase in the resonance stabilization of the vertically polarized excited states could be anticipated and the absorption band associated with this transition (^1L_a) should be shifted to longer wavelength. However, only a small shift of 7 nm was observed. The failure to observe a significant red shift may be due to less resonance coupling between the chain and the nucleus. This could be a result of increased size of the substituent, and accordingly enhanced potentialities for steric interference with resonance. As the steric strain in aldehyde **6** gets relieved due to twisting of the essentially single bond between the anthracene ring and the diene chain, departure from coplanarity is experienced, and thus the absence of a significant red shift in the absorption spectrum of **6**. On further extension of the chain, as in compound **12**, a bathochromic shift of the ^1L_a band to 410 nm was observed, probably due to an increased conjugation in the chain.

The Schiff base (SB) of aldehyde **6** and *n*-butyl amine in MeOH showed a ^1L_a band only at 386 nm. On the other hand, the SB of aldehyde **12** and *n*-butyl amine showed a 17-nm blue-shifted absorption band as compared to the parent aldehyde. On protonation, however, a red shift of 68 nm for the transversely polarized band was observed for the protonated Schiff base (PSB) **8** of dienal **6**. However, the PSB **14** of tetraenal **12** showed a red shift of only 25 nm for the transversely polarized band. The additional resonance energy acquired by the conjugation with the powerful chromophore ($=\overset{+}{N}HR$) acts to rotate the substituent in the PSB of dienal **6** into the plane of the anthracene ring, and thus accounts for the marked bathochromic shift. In the PSB of tetraenal, however, the resonance effect acting to bring the chain to coplanarity is opposed by the steric overlap of the methyl group with the hydrogens in the anthryl ring.

The bR analogue

Synthesis of the new pigment is carried out by combining a concentrated ethanolic solution of the retinal analogue with freshly bleached protein. Apoprotein, when incubated with retinal analogue **12**, resulted in the formation of a new bR analogue, which showed an absorption band at 545 nm in its dark-adapted form. Most of the binding occurred in the first 30 min of the incubation (Fig. 2). Further characterization of the pigment was done by determining the opsin shift (OS) value.

OS is expressed by the λ_{max} of PSB in cm^{-1} minus the λ_{max} of the pigment in cm^{-1} (8). The PSB is usually prepared by reacting the retinal or its analogues with *n*-butyl amine, followed by its production with HCl. The bR regenerated from all-*trans* retinal shows an OS of 4870 cm^{-1}. Following a similar procedure, the OS of bR analogue **15** has been determined to be 5575 cm^{-1}. The bR analogue prepared from dienal **6** exhibited an OS of 3911 cm^{-1} (Table 2). It is interesting to note that bR analogues can be prepared by a chromophore having only two double bonds in the side chain. The bR active site is thus found to be quite flexible and nonspecific, at least in the ring binding site. The bR analogue prepared from 11,12-dihydro retinal, essentially a dienal, has no measurable OS in the uv–vis region (8). The 9,10-dihydro retinal, a trienal, has an OS of only 300 cm^{-1} (8). In view of these results, the OS shown by anthryl dienal-based bR is quite interesting. The anthryl analogue may be considered as having some of the required double bonds in the anthryl ring. This imparts a more planar conformation to the

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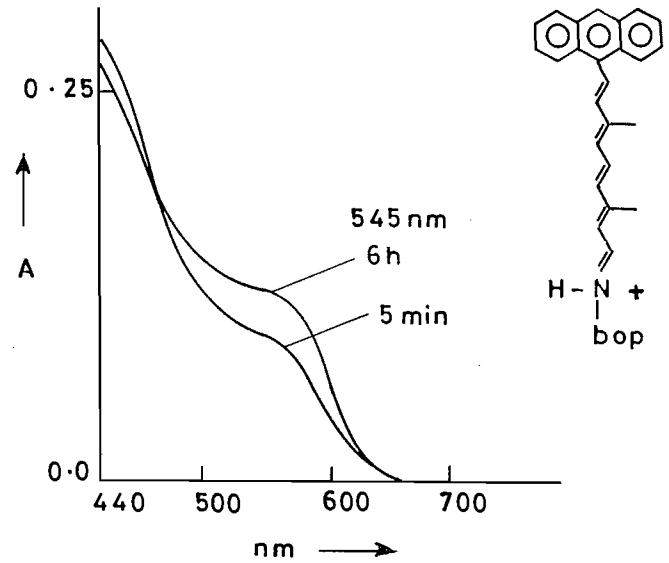

FIG. 2. Formation of bR analogue **15** from anthryl retinal analogue **12**.

TABLE 2. Opsin shifts (cm^{-1}) for bR analogues

Compound	λ_{max}, nm			Opsin shift
	CHO	PSB	bR	
Anthryl pentadienyl retinal analogue	387	454	550	3911*
Anthryl tetraenyl retinal analogue	410	418	545	5575
Retinal	380	440	560	4870

*Reference 10.

chromophore. Besides, as has been observed, the protonated anthryl dienyl chromophore shows more of planar characteristics. In addition, the dienyl chromophore may be undergoing interactions with opsin-bound charges present in the vicinity of the flattened anthryl moiety at the active site. The anthryl ring could be disposed in the region of the active site, which is occupied by C-8 to C-10 of the natural chromophore, retinal. According to the EPC model, electronic and steric perturbations at the active site are expected to strongly influence the absorption spectra of the protein. The PSB of dienal **6** and tetraenal **12** showed absorption bands at 454 and 418 nm respectively. However, when these aldehydes were attached to lysine-216 of the protein in the form of a protonated Schiff base, they exhibited absorption bands at 550 (10) and 545 nm respectively. The longer chromophore **12** shows absorption that is now only 5 nm blue-shifted as compared to the shorter chromophore. The source of the 96-nm shift in dienyl anthryl-based bR (as compared to the absorption of *n*-butyl amine PSB of dienal) and the 127-nm shift in tetraenal anthryl-based bR (as compared to the absorption of *n*-butyl amine PSB of tetraenal) has to be partly due to additional interactions between the anthryl chromophore and opsin-bound charges. In this context, it is important to note the recent suggestion (16) that the bR active site has opsin-bound charges in the form of ion pairs. A comparison of the absorption data of native bR and the bR analogues prepared from anthryl retinal analogues **6** and **12** indicates that there is less conjugation in the polyene chain and

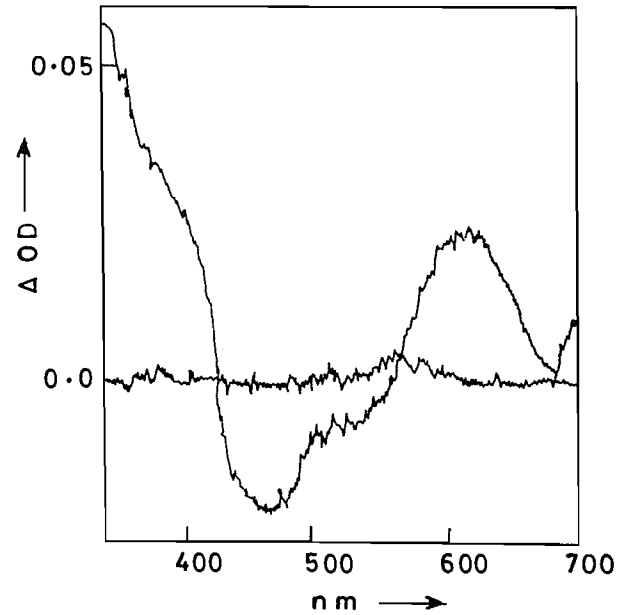

FIG. 3. Difference uv–vis absorption spectrum of the photolysed bR analogue **15** at −60°C.

the anthryl ring in the case of the chromophore in bR analogue **15**. Similar observations were made in the absorption behaviour of chromophore **14** in solution. However, decreased interactions between the twisted chromophore and the now more distant opsin-bound charges can also be partly responsible for such behaviour of the chromophore in the protein.

It has been found that the ring binding site readily accepts acyclic polyenes (17). The ring binding site appears to be quite unrestrictive, as large groups such as aromatic derivatives and 4-substituted analogues (18) can be easily accommodated by bR. Bioorganic models of bR from retinal analogues with a locked 6-*s*-*trans* conformation (9), the phenyl (10, 19) and naphthyl (18, 20, 21) compounds, have suggested a view of the binding site in which the bR protein appears to bind the chromophore in its planar conformation.

Stability of the bR analogue

Stability of the pigment can be inferred from competitive binding with all-*trans* retinal, and with the behaviour of the bR analogue towards hydroxylamine. It was found that addition of all-*trans* retinal did not displace the anthryl chromophore, as the appearance of the bR band at 560 nm was not more than 5%. Similarly, addition of hydroxylamine to bR analogue and incubation in the dark did not significantly alter the 545 nm absorption of the pigment. However, it was found that the bR analogue with the anthryl tetraenyl chromophore was relatively more stable (>24 h) than the bR analogue with anthryl dienal chromophore (~8 h). It can thus be inferred that a complete longitudinal fitting of the chromophore at the active site is necessary for the stability of bR. This also suggests that the longitudinal chain binding site is more important than the ring binding site.

The anthryl bR analogue **15** also showed expected light and dark adaptation. Thus, on illumination, dark-adapted **15** showed a red-shifted absorption band at 550 nm.

The M intermediate generation

During the photocycle of bR a blue-shifted M intermediate with absorption band at 412 nm is formed. Concomitantly a

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proton is released in the medium. This proton is later taken up in the photocycle with the decay of the M intermediate. To see whether such an intermediate is generated for the anthryl bR analogue, photolysis of analogue **15** was carried out at low temperature. For measuring the absorption spectra of the blue-shifted intermediate a temperature of −60°C, high salt concentration, and a pH of 9.0 was maintained. Difference uv–vis analysis (Fig. 3) of the photomixture showed the appearance of a band near 400 nm, which could be due to the unprotonated Schiff base chromophore of the M anthryl bR formed during the photolysis of bR-**15**. Thus it is apparent that the presence of the trimethyl cyclohexenyl moiety may not be essential for bR to undergo the photocycle.

Conclusions

Thus, synthetic retinal analogue **12** binds to bacterioopsin to give a new bR analogue. It is found that the ring binding site is unrestrictive and nonspecific. The binding site of bR prefers to bind the chromophore in a planar conformation. The longitudinal chain binding site appears to play a more important role than the ring binding site. Both resonance and steric compatibility of the chromophore seem to play important roles in the regulation of the absorption behaviour of bR. The presence of the trimethyl cyclohexenyl ring does not seem to have much effect on the functional properties of the protein. However, a proper fit of the chromophore at the active site seems to be important for both the structural and functional features of bR.

Acknowledgements

Financial assistance from the Department of Science and Technology, Government of India, is gratefully acknowledged. Our thanks are due to the Tata Institute of Fundamental Research, Bombay, for providing the 500 MHz nmr facility. We are also grateful to the project advisory committee of the DST for useful suggestions.

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