SYNTHESIS OF 3,7-DIMETHYL-9-(4'-(3H-DIAZIRINYL)-2',6'-DIMETHYLPHENYL)-2E, 4E,6E,8E-NONATETRAENAL-1-^{3}H, A PHOTOAFFINITY LABELING ANALOGUE OF ALL-TRANS-RETINAL†

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SUMMARY

The synthesis and spectroscopic properties of the title compound as well as its precursors are described.

Key words: retinal-photoaffinity label; diazirinyl retinal analogue; tritium

INTRODUCTION

Halobacteria, living in waters of very high salt concentrations, have purple membranes which they utilize as energy transducers. Absorption of light by the membrane causes protons to be pumped out of the cell thereby converting photoenergy to chemical energy. The purple membrane contains a single protein, bacteriorhodopsin, folded into its lipid bilayer. The color is due to the presence of one equivalent of retinal, 3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenal, covalently bound to the protein through a protonated Schiff base linkage. Although considerable information about the purple membrane has become available since the initial report by Oesterhelt and Stoeckenius, several important questions remain to be answered (1). Central is the question of the mechanism of the light-driven proton pumping activity. Several spectroscopic intermediates of its photocycle have been established and the structure of the chromophore in each intermediate is gradually being elucidated by resonance Raman, NMR, and other spectroscopic techniques. It is clear, however, that changes in the disposition of the protein and lipid

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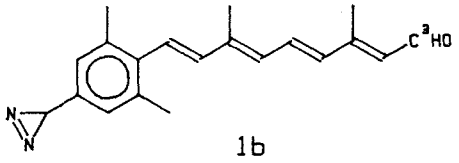
environment surrounding the bound retinal also contribute to producing the different spectroscopic intermediates of the photocycle (2). Unfortunately the purple membrane system has not been as amenable to high resolution three dimensional structure elucidation as other protein systems have and thus a full understanding of how these environmental effects operate will have to wait.

Another way to probe retinal's changing environment is to photoaffinity label its neighbors at particular stages of the photocycle for subsequent analyses. The synthesis of two retinal analogues: 3,7-dimethyl-9-(3'-(3H-diazirinyl)phenyl)-2E,4E,6E,8E-nonatetraenal-1-^{3}H (3) and 3-([1-^{14}C]-diazoacetoxy)-trans-retinal (4), for photoaffinity labeling, have been reported during the course of this study. We report here the synthesis, incorporation, and general photoaffinity labeling studies of a different analogue of retinal: 3,7-dimethyl-9-(4'-(3H-diazirinyl)-2',6'-dimethylphenyl)-2E,4E,6E,8E-nonatetraenal-1-^{3}H (1b). Photoaffinity labeling



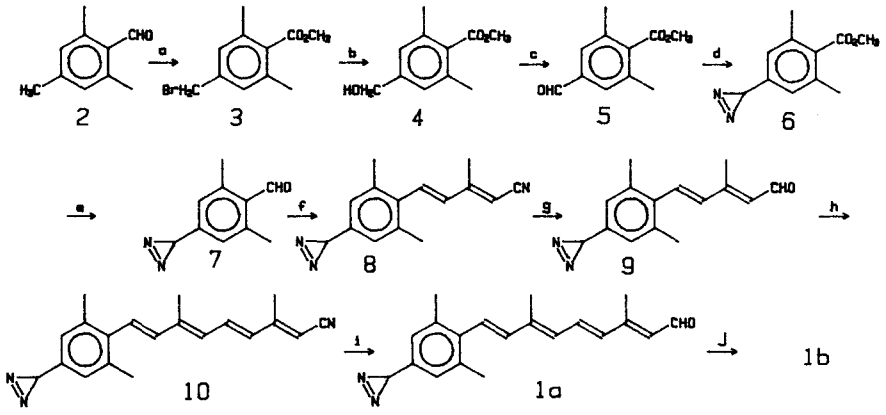
1b

studies using 1b have successfully been carried out to determine whether retinal bridges protein molecules in the purple membrane (5).

Methyl groups were introduced at the 2' and 6' positions of the aromatic ring to mimic the 1,1 and 5-methyls present in retinal. The placement of the diazirine group on the aromatic ring permits the environment of retinal's cyclohexenyl ring to be probed for it is there where a large change of charge is expected when ground state bacteriorhodopsin is photolyzed to the first excited singlet. A proteinic Bronsted acid is suggested to be present in this region (6). Moreover, it may be possible to gather further information concerning the nature of one of the two point charges predicted to be near the cyclohexenyl ring and to be partially responsible for the opsin shift (2a). Being able to probe the environment at different photocycle stages might provide further information concerning the point charge model.

The synthesis of the title compound was carried out according to the reactions shown in Scheme I.

Scheme I



a) 1. NBS, hν/CCL_4, 2. CH_3OH; b) 1. NaOAc/HOAc, 2. $NaOH/H_2O$-CH_3OH;
c) MnO_2/CH_2Cl_2; d) 1. $NH_3/tBuOCl$, 2. HgO; e) $DIBAL/C_6H_6$;
f) $(C_2H_5O)_2P(O)\overline{C}HC(CH_3)$=CHCN/$C_6H_6$; g) $DIBAL/CH_2Cl_2$;
h) $(C_2H_5)_2P(O)\overline{C}HC(CH_3)$=CHCN/$C_6H_6$;
i) $DIBAL/C_6H_6$; j) 1. [^{3}H]-$NaBH_4$/EtOH), 2. MnO_2/CH_2Cl_2.

Synthesis of the diazirine ring (6) was carried out according to the method of Smith and Knowles (7) which requires the use of ammonia and t-BuOCl followed by oxidation with yellow mercuric oxide at 4°, in the dark. This necessitated prior generation of a formyl group in the 4-position of 5 which was accomplished by benzylic NBS-bromination (2 → 3), hydrolysis to the corresponding alcohol (3 → 4), followed by oxidation with active manganese dioxide. During NBS-bromination the aldehyde group suffers H-atom abstraction to generate the acyl bromide which upon treatment with methanol, yields the ester (3).

Elaboration of the polyene chain was accomplished in two stages through Emmons-Horner reactions of the precursor aldehydes with diethyl phosphonoacrylonitrile (8). The aldehydes as well as the final unlabeled aldehyde, were generated by DIBAL-reductions (9) of the ester (6), and nitriles (8 and 10), respectively. Structural assignments are supported by NMR, and in the case of the unlabeled target compound and its precursor nitrile, by high resolution mass spectrometry.

The retinal analogue (1) [λ_{max} (hexane) 364 nm] when incorporated into apomembrane exhibits a maximum at 450 nm. This is similar to that found for mesityl[retinal] (λ_{max} 460 nm) but shifted to shorter wavelength with respect to

native bR. Previous studies (10), with bacterioopsin combined with mesityl[retinal], a close analogue of 1, have, however, demonstrated the synthetic membrane's ability to photocycle, albeit slowly, and to form an M-type photocycle intermediate.

Specific peaks in the electronic spectrum of the reconstituted membrane due to the diazirine group was not evident. Irradiation at 365 nm, however, resulted in labeling the protein by approximately 30% of the diazirine groups present.

EXPERIMENTAL

Methyl 4-bromomethyl-2,6-dimethylbenzoate (3). Mesityl aldehyde,(10 mL, 67 mmol) dissolved in 500 mL of CCl, was allowed to react wih 25 g (140 mmol) of N-bromosuccinimide (3). The mixture was refluxed under light from a 200 w tungsten lamp for 1 hour. After the mixture cooled to room temperature, 20 ml of methanol was added and the mixture was filtered and concentrated. The residue was purified by flash chromatography (0.8 x 22 cm SiO, 5% THF/hexane, R_f 0.15). Yield 2.8 g (16%). ^{1}H NMR ($CDCl_3$, int TMS): δ 7.06 (s, 2H, aromatic-H's), 4.40 (s, 2H, CH_2) 3.90 (s, 3H, OCH_3), 2.30 (s, 6H, ring-methyls).

Methyl 4-hydroxymethyl-2,6-dimethylbenzoate (4). Methyl 4-bromomethyl-2,6-dimethylbenzoate (5 g, 19.5 mmol) in 50 mL of acetic acid containing 3.2 g (39 mmol) of sodium acetate was refluxed for 6 hours and the solvent removed under reduced pressure. The residue was used directly for the hyrolysis of the acetate. ^{1}H NMR ($CDCl_3$, int TMS): δ 7.01 (s, 2H, aromatic-H's), 5.03 (s, 2H, CH_2), 3.90 (s, 3H, OCH_3), 2.30 (s, 6H, ring methyl), 2.09 (s, 3H, CH_3CO).

Sodium hydroxide (22%) was added to the crude acetate in 20 mL of methanol until the solution was strongly basic. The solution was heated for 5 min at 70° and then stirred overnight at room temperature. The methanol was evaporated and the aqueous layer extracted with ether. The sodium sulfate-dried ether extract yielded 3 g (77%) of methyl 4-hydroxymethyl-2,6-dimethylbenzoate. Tlc: SiO_2, 10% THF/hexane, R_f 0.06. ^{1}H NMR ($CDCl_3$, int TMS): δ 7.01 (s, 2H, aromatic-H's), 4.61 (s, 2H, CH_2), 3.89 (s, 3H, OCH_3), 2.30 (s, 6H, ring-methyls).

4-Carbomethoxy-3,5-dimethylbenzaldehyde (5). Methyl 4-hydroxymethyl-2,6-dimethylbenzoate 3 g, 15.5 mmol) in 30 mL of methylene chloride was stirred with

1 g of active manganese dioxide for 8 hours at ambient temperature. The mixture was filtered through Celite and then flash chromatographed on silica gel (0.5 x 15 cm, 10% THF/hexane, R_f 0.19). Recovered starting material was recycled with fresh manganese dioxide. Yield 1.4 g (7.3 mmol, 47%). ^1H NMR ($CDCl_3$, int TMS): δ 9.94 (s, 1H, CHO), 7.52 (s, 2H, aromatic-H's), 3.93 (s, 3H, OCH_3), 2.35 (s, 6H, CH_3).

Methyl 4-(3H-diazirinyl)-2,6-dimethylbenzoate (6). Liquid ammonia (5 mL at -78° was mixed with 15 mL of methanol, previously cooled to -40°, to provide an appropriate 10 N solution. To this solution, at -40°, was added dropwise, a mixture of 1.3 mL of t-butyl alcohol and 1.3 ml of t-butyl hypochlorite over a period of 10 min. After 30 min at -40° the solution was poured over 1.1 g (5.7 mmol) of 4-carbomethoxy-3,5-dimethylbenzaldehyde. The mixture was kept at -40° for 1 hour and then allowed to warm to 0° over 5 hours during which time the aldehyde slowly dissolves. The mixture was then placed in the cold room (4°) where it was stirred vigorously in the dark. Portions of 500 mg of yellow mercuric oxide were added every 8-12 hours until there was no observable increase in the ultraviolet absorption in the 350-390 nm region of an aliquot in hexane. The mixture was filtered, the filtrate poured into 50 mL of 10% sodium bisulfate solution and the aqueous layer extracted with ethyl acetate (3 x 50 mL). The combined ethyl acetate extracts were washed with brine and dried over sodium sulfate. The product was isolated by preparative tlc (silica gel, 1 mm, two developments with 10% THF/hexane, R_f 0.31). Yield 81 mg (7%). ^1H NMR ($CDCl_3$, int TMS): δ 6.55 (s, 2H, aromatic-H's), 3.89 (s, 3H, OCH_3), 2.27 (s, 6H, ring CH_3), 1.97 (s, 1H, CHN_2); uv: λ_{max} (diazirine) 362 nm.

4-(3-diazirinyl)-2,6-dimethylbenzaldehyde (7). Methyl 4-(3H-diazirinyl)-2,6-dimethylbenzoate (50 mg, 0.24 mmol) in 10 mL of benzene was stirred at room temperature after the addition of 1 mL of a fresh 1 M solution of DIABAL in hexane. The progress of the reaction was monitored by tlc (silica gel, 10%, THF/hexane). The reaction was complete in 1 hour when it was treated sequentially with 2 g of silica gel containing 400 uL of water, 5 mL of ether and 5 mL of hexane. The mixture was stirred for 15 min and filtered. The silica gel was washed with methylene chloride. After drying, the solvent was evaporated and the

crude alcohol in 5 mL of CH_2Cl_2 was stirred overnight with 300 mg of activated MnO_2. The mixture was filtered through Celite and purified by tlc (silica gel, 1 mm, 10% THF/hexane, R_f 0.29). Yield 12 mg 28%. Uv (hexane) λ_{max} 360 nm. ^1H NMR ($CDCl_3$, int TMS): δ 10.56 (s, 1H, CHO), 6.60 (s, 2H, aromatic-H's), 2.57 (s, 6H, ring methyls), 2.01 (s, 1H, CHN_2).

1-Cyano-2-methyl-4-(4'-(3H-diazirinyl)-2',6'-dimethylphenyl)-1E, 3E-butadiene (8). In a typical run 325 mg (1.5 mmol) of (E)-3-methyl-4-(diethylphosphono)acrylonitrile (8,11) was dissolved in 5 mL of benzene and 72 mg (1.5 mmol) of sodium hydride (in mineral oil) was added at ambient temperature. The reaction mixture turned red and was stirred at 35° for 30 min. 4-(3H-Diazirinyl)-2,6-dimethylbenzaldehyde (~ 0.5 mmol) was added in 2 mL of benzene and the mixture stirred overnight. The reaction was quenched with brine and the mixture extracted with ethyl acetate. After dryng the extract over sodium sulfate, the residue, after solvent evaporation, was chromatographed on 1 mm-thick silica gel plates (10% THF/hexane, R_f 0.21). NMR ($CDCl_3$, int TMS): δ 6.96 (d, 1H, J 16.7, vinyl), 6.58 (s, 2H, aromatic-H's), 6.32 (d, 1H, J 16.7 vinyl), 5.26 (s, 1H, H-1), 2.27 (s, 6H, ring-methyls), 1.97 (s, 1H, CHN_2), 1.54 (s, 3H, 2-CH_3). Uv (hexane) λ_{max} (diazirine) 367 nm.

3-Methyl-5-(4'-(3H-diazirinyl)-2',6'-dimethylphenyl)- 2E,4E-pentadienal (9). 1-Cyano-2-methyl-4-(4'-(3H-diazirinyl)-2', 6'-dimethylphenyl)-1E,3E-butadiene (3.9 mg) dissolved in 5 mL of methylene chloride and cooled in ice, was stirred and treated every 30 min with 100 uL portions of a fresh 1 M solution of DIBAL in toluene until tlc indicated the absence of starting material. The reaction mixture was quenched by the addition of moist silica gel, followed by THF, and then water. It was then stirred for 30 min, filtered and dried to provide the aldehyde. NMR ($CDCl_3$, int TMS): δ 10.17 (d, 1H, J 7.9, CHO), 6.59 (s, 2H, aromatic-H's), 6.00 (d, 1H, J 7.7, H-2), 6.39 (d, 1H, J 16.3, H-4), 2.40 (s, 3H, 3-CH_3), 2.29 (s, 6H, ring methyls), 1.97 (s, 1H, CHN_2). Uv (hexane): λ_{max} 370 and 390 nm (shoulders). Tlc: SiO_2, 10% THF/hexane, R_f 0.12.

1-Cyano-2,6-dimethyl-8-(4'-(3H-diazirinyl)-2',6'-dimethylphenyl)-1E, 3E,5E,7E-octatetraene (10). (E)-3-methyl-4-(diethylphosphono)-acrylonitrile (8,11) (200 mg) in 5 mL of benzene was treated with 44 mg of sodium hydride (in

mineral oil) at room temperature. The red reaction mixture was stirred at 35° for 30 min after which 0.2 to 0.5 mmol of 3-methyl-5-(4'-(3H-diazirinyl)-2',6'-dimethylphenyl)-2E,4E-pentadienal was added. The mixture was stirred overnight, treated with brine, and the aqueous layer extracted with ethyl acetate. The residue obtained from the sodium sulfate-dried solution was chromatographed on 1 mm-thick silica plates using 10% THF/hexane as solvent, R_f 0.15. NMR ($CDCl_3$, 300 MHz): δ 6.97 (dd, 1H, J 14.7, 11.4, H-4), 6.69 (d, 1H, J 16.4, H-8), 6.58 (s, 2H, aromatic-H's), 6.35 (d, 1H, J 16.4, H-7), 6.34 (d, J 14.9, H-3), 6.19 (d, 1H, J 11.4, H-5), 5.22 (s, 1H, H-1), 2.29 (s, 6H, aromatic methyl), 2.24 (s, 3H, 2-CH_3), 2.10 (s, 3H, 6-CH_3), 1.98 (s, 1H, CHN_2). MS: M^+ 303.1732; calc for $C_{20}H_{21}N_3$: 303.1735. Uv (hexane): λ_{max} 356 nm.

3,7-Dimethyl-9-(4'-(3H-diazirinyl)-2',6'-dimethylphenyl)-2E,4E,6E,8E-nonatetraenal (1a). The corresponding nitrile was reduced in methylene chloride with a 1 M DIBAL in hexane solution by the same method as described above for the reduction of 1-cyano-2-methyl-4-(4'-(3H-diazirinyl)-2',6'-dimethylphenyl)-1E,3E,-butadiene. Yield: ~ 3 mg. NMR($CDCl_3$, 300 MHz): δ 10.12 (d, 1H, J 8.3, CHO), 7.15 (dd, 1H, J 11.2, 15.0,H-5),6.70 (d, 1H, J 16.3,H-9), 6.58 (s, 2H, aromatic-H's), 6.42 (d, 1H, J ~ 14.4, H-4), 6.37 (d, 1H, J 16.3, H-8), 6.26 (d, 1H, J 10.9,H-6) 5.99 (d, 1H, J 8.1, H-2), 2.33 (s, 3H, 3-CH_3), 2.29 (s, 6H, ring methyl), 2.11 (s, 3H, 7-CH_3),1.97 (s, 1H, CHN_2). Uv (hexane): λ_{max} 364 nm (ε = 64000). MS: M^+ 306.1687; calc for $C_{20}H_{22}N_2O$: 306.1732. Tlc: SiO_2, 10% THF/hexane, R_f 0.07.

3,7-Dimethyl-9-(4'-(3H-diazirinyl)-2',6'-dimethylphenyl)-2E,4E,6E,8E-nonatetaenal-1-^{3}H (1b). The unlabeled aldehyde (~ 3 mg) was transferred to a vial and to this was added a solution of 25 mCi (273 mCi/mmol) of $NaBH_4$ (Amersham) in 500 uL of redistilled ethanol. The solution was stirred in the dark for 30 min after which 50 uL of acetone was added and the mixture stirred for another 30 min. Ethanol and 2-propanol were removed under vacuum. Five hundred uL of ethanol was added and the solvent removed again. The labeled retinol, in 500 uL of methylene chloride was stirred with 30 mg of activated manganese dioxide for 30 min. An additional 30 mg of manganese dioxide was added and the stirring repeated for another 30 min. The mixture was filtered through Celite and the

major amount of solvent was removed in a stream of argon. The product was isolated by preparative tlc (0.25 mm silica gel). Yield 612 ug; specific activity 38.3 mCi/mmol.

Reconstitution of bacteriorhodopsin. Native bacteriorhodopsin was bleached in the usual way with hydroxylamine under orange light (12). The apomembrane was washed thoroughly to remove hydroxylamine and an aliquot was titrated spectrophotometrically with retinal to determine the concentration of active protein. The tritated diazirine retinal analogue (612 ug), in a small volume of ethanol, was added to 53.7 mg (2.02 umol) of the apomembrane in 44 ml of 0.1 M sodium N-2-hydroxyethylpiperazine-N'-2-ethane sulfonate (HEPES) buffer solution (pH 7.2). The solution was kept at 4° in the dark overnight. The membrane was then washed with 2% of delipidated bovine serum albumin (Sigma) in 0.1 M HEPES (4 x 50 mL) and then with 0.1 M HEPES (4 x 50 mL) according to the method of Katre et al (13).

Photoaffinity labeling. In a typical experiment 38 ug of labeled membrane (0.69 uCi/mL) and 76 ug of unlabeled diazirinyl membrane were suspended in 120 uL of 0.05 M HEPES. Ten uL aliquots were irradiated with a Bausch and Lomb high pressure mercury lamp (SP-200) using a Bausch and Lomb monochromator (# 33-86-07). Non-covalently bonded retinal analogue was removed by the method of Huang et al. (3) which involves membrane denaturation and retinal dissociation from the protein in 10% SDS solution, protein precipitation with ethanol, and washing. Protein residues were dissolved in sodium hydroxide and counted in Aquasol (New England Nuclear).

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