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THREE DODECAENE C_{50}-CAROTENOIDS FROM HALOPHILIC BACTERIA*†

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Key Word Index—Halobacteriaceae; minor dodecaene C_{50}-carotenoids; haloxanthin; peroxide.

Abstract—The isolation by chromatography (CC, TLC, HPLC) and characterization by spectroscopic (UV-Vis, IR, MS, ^{1}H NMR, CD) and chemical (silylation, methylation, dehydration) methods of three new minor conjugated dodecaene C_{50}-carotenoids from *Haloferax volcanii* (each 2% of total carotenoids) and *Halobacterium salinarium* (one present) are reported. The data support the structures 3′,4′-dihydromonoanhydrobacterioruberin ($C_{50}H_{76}O_3$), 3′,4′-epoxymonoanhydrobacterioruberin ($C_{50}H_{74}O_4$) and a 3′,4′-dihydromonohydrobacterioruberin derivative named haloxanthin ($C_{50}H_{74}O_4$) with a novel peroxide end group.

INTRODUCTION

In the course of a recent study of the carotenoids of the moderately halophilic bacterium *Haloferax volcanii* NCMB 2012 [1] three new, minor C_{50}-carotenoids were encountered. In contrast to the tridecaene chromophore of the major C_{50}-carotenoids (**1**–**3**) of halophilic bacteria, these carotenoids exhibited dodecaene chromophores and in part unprecedented oxygen functions.

RESULTS AND DISCUSSION

Each of these minor carotenoids represented *ca* 2% of the total carotenoids and possessed aliphatic dodecaene chromophores according to their visible absorption spectra, including the location of the double *cis*-peak for *Z*-isomers [2].

A polar carotenoid had adsorptive properties similar to monoanhydrobacterioruberin (**1**), consistent with a triol. The mass spectrum showed $[M]^+$ at *m/z* 724, compatible with $C_{50}H_{76}O_3$. This was confirmed by fragment ions at $[M-18\ (H_2O)]^+$, $[M-58\ (Me_2CO)]^+$, $[M-92\ (\text{toluene})]^+$ and $[M-106\ (\text{xylene})]^+$ [3]. The ^{1}H NMR assignments were made by comparison with the chemical shifts for all-*E* bacterioruberin (**2**) and all-*E* bisanhydrobacterioruberion (**3**) [1, 4], thus demonstrating the localization of the unsymmetrical chromophore, and leading to the 3′, 4′-dihydromonoanhydrobacterioruberin structure (**4**, without chirality). In the absence of CD data the common 2*S*-chirality of aliphatic C_{50}-carotenoids [5] is assumed. Formally structure **4** is 2-(3-hydroxy-3-methylbutyl)-2′-(3-methyl-2-butenyl)-3,4-didehydro-1,2,1′,2′-tetrahydro-ψ-ψ-carotene-1,1′-diol.

Two additional minor C_{50}-carotenoids were previously referred to as carotenoids A and B [1]. Carotenoid A with a diagnostic $[M-100]^+$ ion in the mass spectrum was first obtained as a minor carotenoid from *Halobacterium salinarium* [6], whereas carotenoid B was encountered for the first time. Both carotenoids A and B had the same molecular formula $C_{50}H_{74}O_4$. However, they differed in polarity and mass spectral fragmentation.

The least polar carotenoid (A), here named haloxanthin, possessed two hydroxy groups accessible to silylation and to methylation with MeI and NaH [7], compatible with the relative polarity. The ^{1}H NMR spectra revealed the presence of an isopropylidene end group, accounting for one double bond. Since the molecular formula corresponded to 14 double bond equivalences and the dodecaene chromophore required 12, the presence of one ring containing two inert oxygen atoms was inferred. This conclusion was consistent with the diagnostic loss of 100 mu. ($C_5H_8O_2$ by exact mass measurements) in the mass spectrum. The mass spectrum exhibited other common fragmentations: $[M-18\ (H_2O)]^+$, $[M-58\ (Me_2CO)]^+$, $[M-92\ (\text{toluene})]^+$, $[M-106\ (\text{xylene})]^+$ and $[M-158\ (\text{dimethylcyclodecapentaene})]^+$ [3]. The intensity ratio $[M-92]^+$: $[M-106]^+$ was 0.22–0.31 in several mass spectra, compatible with an aliphatic dodecaene C-2 substituted carotenoid with a Δ^3-bond [8]. Structure **5** including ^{1}H NMR assignments is consistent with the available evidence.

Haloxanthin was isolated as a mixture of geometrical isomers (all-*E*,5*Z*,9*Z*,5′*Z*- and 9′*Z* isomers) according to the ^{1}H NMR data and complicating the ^{1}H NMR assignments. The tert. diol structure assigned to haloxanthin (**5**)

*Part 54 in the series 'Bacterial Carotenoids'. For Part 53 see ref. [1].

†Part 24 in the series 'C-50 carotenoids'. For Part 23 see ref. [1].

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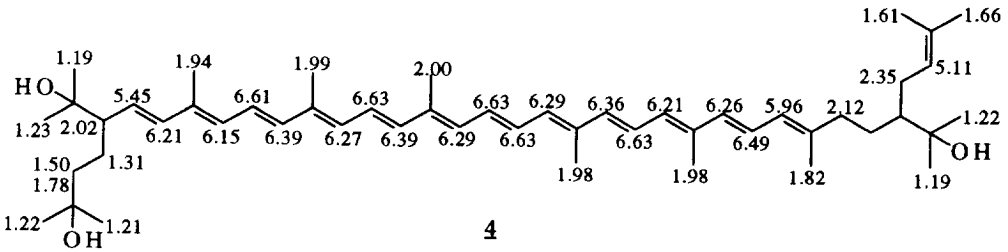

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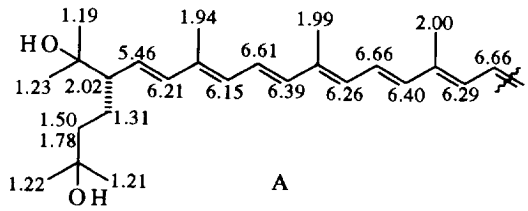
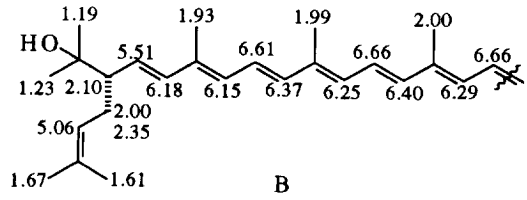
1 A,B
2 A,A
3 B,B



A



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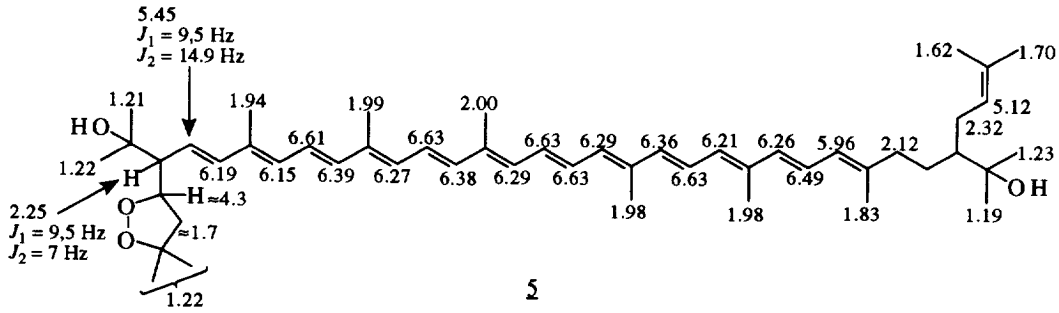

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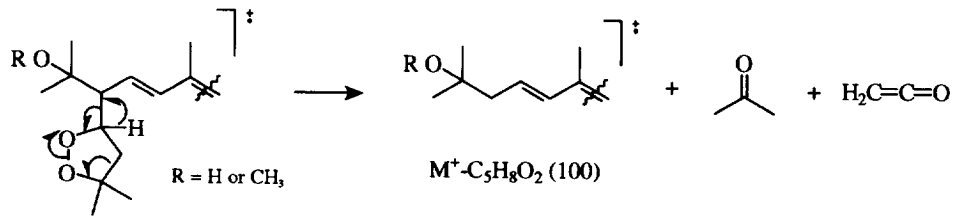
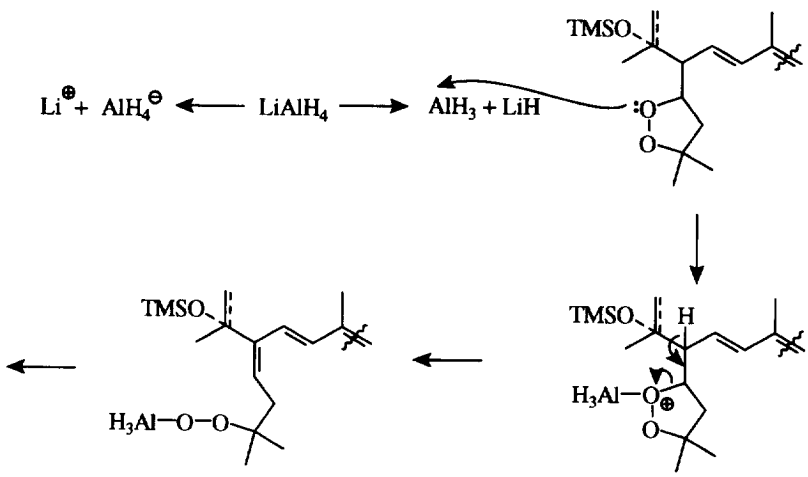




is compatible with the observed formation of two monoanhydro and a bisanhydro derivative of unchanged visible absorption upon dehydration with phosphorous oxychloride in pyridine. This dehydration reaction is known to occur in accordance with Saytzeff's rule and does not result in conjugated carotenoid products [6, 9].

The peroxide structure is novel in the carotenoid context and is indirectly supported by the observed spin patterns and coupling constants for H-2 (double doublet, $J_{2,3} = 9.5$ Hz, $J_{2,1'} = 7.0$ Hz) and H-3 (double doublet, $J_{2,3} = 9.5$ Hz, $J_{3,4} = 14.9$ Hz). A general stability towards reductive opening of the cyclic peroxide with $LiAlH_4$ was noted. The low yield of tridecaene products on $LiAlH_4$ treatment of haloxanthin ditrimethylsilyl ether and bisanhydrohaloxanthin may be rationalized by structure **5** (cf. ref. [10] for alternative modes of dissociation of $LiAlH_4$). Haloxanthin, obtained by three different isolations from two different bacteria, is not considered an isolation artifact. Its biosynthetic formation may be rationalized from an isopropylidene precursor via an allylic peroxide. The semirational name for structure **5** is 2-(1,3-epidioxy-3-methylbutyl)-2'-(3-methyl-2-butenyl)-3,4-didehydro-1,2,1',2'-tetrahydro-ψ,ψ-carotene-1,1'-diol, cf. ref. [11]. 2*S*,2'*S* chirality is tentatively assumed by analogy with other bacterioruberin (**2**) derivatives, (see ref. [5]).

The evidence for carotenoid **B**, including relative polarity, Vis, MS, CD and ^{1}H NMR data points towards structure **6**. No ^{1}H NMR signal at δ5.96 (H-6' in haloxanthin, **5**) was present, but direct evidence for the epoxy function was lacking. The CD spectrum for all-*E* carotenoid **B** resembled that of (2*S*,2'*S*)-bacterioruberin (**2**), 10 nm hypsochromically shifted owing to the difference in chromophoric length. The 5*Z* isomer gave an inverted Cotton effect, cf. [12]. Epoxidic carotenoids are not expected as part of the general carotenogenesis in halophilic bacteria [1], and **6** could represent an oxidative isolation artifact of monoanhydrobacterioruberin (**1**). The formal structure **6** is (2'*S*)-2-(3-hydroxy-3-methylbutyl)-2'-(3-methyl-2-butenyl)-3',4'-epoxy-3,4,3',4'-tetradehydro-1,2,1',2'-hydro-ψ,ψ-carotene-1,1'-diol.

Further structural studies were hampered by the low abundance and unavailability of these minor, sterically labile C_{50}-carotenoids.

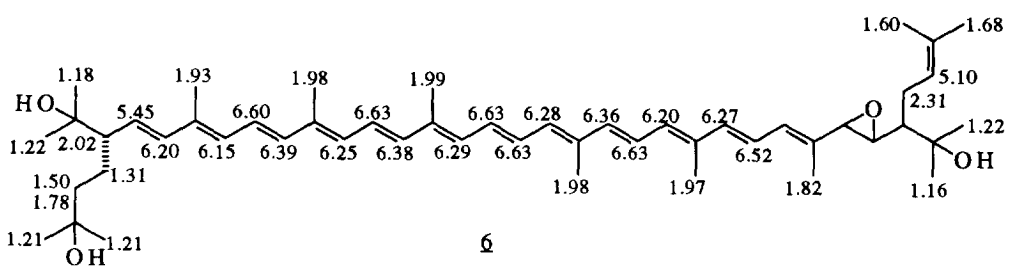
EXPERIMENTAL

Biological material and general methods. See refs. [1,6]. Several chromatographic systems were employed including CC, TLC, PC [13] and HPLC. The R_f values cited and systems here referred to were: TLC-1: alkaline plates [14] Me_2CO–1.1.1-trichloroethane (2:3); TLC-2: SiO_2, Me_2CO–hexane, (1:1); TLC-3: SiO_2, Me_2CO–hexane (2:3); TLC-4: SiO_2, Me_2CO–hexane (3:7); TLC-5: SiO_2, Me_2CO–propanol–$CHCl_3$ (5:1:9); TLC-6: Merck 5553, Me_2CO–hexane (3:7); TLC-7: Merck 5553, Me_2CO–hexane (2:3); PC-1: Schleicher & Schüll 287, Me_2CO–hexane (1:9). R_t values refer to HPLC-1: rev. phase Spheri-5RP-18 MeOH–EtOAc–H_2O–Et_3N (76:19:4:1) and HPLC-2: norm. phase Spheri-3-cyano hexane–ipropOAc–Me_2CO–MeOH (760:70:170:1).

Vis spectra: Me_2CO. Spectral fine structure is expressed as % III/II and % D_B/D_{II} [15]. Only diagnostic peaks are cited for the mass spectra. Intensities are given relative to the highest peak in the *m/z* region referred to.

3',4'-Dihydromonoanhydrobacterioruberin (**4**). Obtained, 0.3 mg, slightly more polar then monoanhydrobacterioruberin (**1**) by TLC-1 (R_f 0.50); Vis λ_{max} nm: 360, 373, 455, 481, 512, % III/II = 23, % $D_B/D_{II} = 25$ (stereoisomeric mixture); MS *m/z* (rel. int.): 724 $[M]^+$ (17), 722 $[M-2]^+$ (4), 706 $[M-18]^+$ (1), 666 $[M-58]^+$ (3), 664 $[M-60]^+$ (1), 632 $[M-92]^+$ (3), 620 $[M-106]^+$ (4), 105 (100); ^{1}H NMR (500 MHz, $CDCl_3$): see structure **4** ($J_{2,3} = 9$ Hz, $J_{3,4} = 15$ Hz, $J_{6,7} = J_{6',7'} = 11$ Hz, $J_{7,8} = J_{7',8'} = 15$ Hz, $J_{10,11} = J_{10',11'} = 11$ Hz, $J_{11,12} = J_{11',12'} = 15$ Hz). Assignments were facilitated by comparison with **5** and **6**, possessing the same chromophore.

Haloxanthin (**5**). On CC on alumina activity grade 3 [16] eluted with 1% MeOH in C_6H_6 and on silica columns eluted just ahead of monoanhydrobacterioruberin (**1**) [cf. 1], as a mixture of geometrical isomers, *ca* 2 mg from three different isolations, Vis λ_{max} nm: 357, 377, (456), 481, 512, % III/II = 31, % $D_B/D_{II} = 28$. After rechromatography and crystallization from Me_2CO–hexane; Vis λ_{max} nm: all-*E* 362, 377, 460, 486 ($E^{1\%}_{1cm} = 2170$, $\epsilon = 160\,000$) and 519, % III/II = 63, % $D_B/D_{II} = 8$; R_f 0.37 (PC-1), R_F 0.70 (TLC-1), R_F 0.49 (TLC-2), R_t 7.01 min (HPLC-1); IR ν^{KBr}_{max} cm^{-1}: 3400 m (OH), 1160 w (*tert.* OH), 970 s (disubst. *trans* C = C), 910 m (C–C–O); MS *m/z* (rel. int.): 738.561 $[M]^+$ (20), calc. 738.559 for $C_{50}H_{74}O_4$, 736 $[M-2]^+$ (8), 720 $[M-18]^+$ (2), 680 $[M-58]^+$ (4), 646 $[M-92]^+$ (4), 638.5073 $[M-C_5H_8O_2]$ (34), calc. 638.5062 for $C_{45}H_{66}O_2$, 632 $[M-106]^+$ (16), 580 $[M-158]$ (6), 119 (100); ^{1}H NMR (400 MHz, $CDCl_3$) of a stereoisomeric mixture containing apparently all-*E*, 5*Z*, 5'*Z*, 9*Z* and 9'*Z* configurations, led to the assignments shown on structure **5** for the major



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all-*E* isomer with the following coupling constants: $J_{2,3}$ = 9.5 Hz (cf. 8.6 Hz for **2** [1]), $J_{2,1'}$ = 7.0 Hz, $J_{3,4}$ = 14.9 Hz, $J_{6,7} = J_{6',7'}$ = 11 Hz, $J_{7,8} = J_{7',8'}$ = 15.6 Hz, $J_{10,11}$ = 11.5 Hz, $J_{10',11'}$ = 12.3 Hz, $J_{11,12} = J_{11',12'}$ = 15.6 Hz. *Z* isomers were present prior to HPLC purification as judged by ^{1}H NMR: 5*Z* 6.04 *d* (*J* = 15.0 Hz, H-6), 6.73 *m* (H-7), 6.74 *d* (*J* = 15.0 Hz, H-4); 9*Z* 6.80 *m* (H-11), 6.92 *d* (*J* = 15.0 Hz, H-8); 5′*Z* 5.94 *d* (*J* = 11.0 Hz, H-6′) and 9′*Z* 6.04 *d* (H-6′), 6.05 *d* (H-10′), 6.79 *d* (*J* = 15 Hz, H-8′).

Compound **5** was resistant towards treatment with $LiAlH_4$ in dry Et_2O and towards standard acetylation [17] with Ac_2O in pyridine.

Dehydration with $POCl_3$. Standard dehydration of **5** with $POCl_3$ in pyridine [5] gave 61% pigment recovery. The recovered carotenoid consisted of the bisanhydro derivative (20%), two monoanhydro derivatives (1 + 1%) and unreacted **5** (78%), separated by TLC-4.

The mixed monoanhydro derivatives had unchanged Vis absorption; MS *m/z* (rel. int.): 720 $[M]^+$ (6), 702 $[M-18]^+$ (9), 662 $[M-58]^+$ (1), 663 $[M-87]^+$ (1), 618 $[M-92]^+$ (1), 614 $[M-106]^+$ (5–11), 69 (100).

Bisanhydrohaloxanthin exhibited an unchanged Vis spectrum; MS *m/z* (rel. int.): 702 $[M]^+$ (4), 633 $[M-69]^+$ (1), 618 $[M-84]^+$ (1), 615 $[M-87]^+$ (1), 610 $[M-92]^+$ (1), 596 $[M-106]^+$ (7), 69 (100). Treatment with $LiAlH_4$ in dry Et_2O for 1 hr gave 33% recovery, after TLC-3, Vis λ_{max} nm: 369, 388, 467, 494 and 528.

Silylation [5] at − 35° of **5**, monitored by PC, revealed one intermediate, pigment recovery 94%. TLC-3 provided haloxanthin ditrimethylsilyl ether with unchanged Vis absorption; MS *m/z* (rel. int.): 882 $[M]^+$ (3), 792 $[M-90]$ (1), 790 $[M-92]^+$ (1), 776 $[M-106]^+$ (2), 131 (100). Treatment with $LiAlH_4$ in dry Et_2O for 1 hr gave 54% pigment recovery. Besides unreacted material, one product (25%) was isolated by TLC-3; Vis λ_{max} nm: 370, 388, 468, 494 and 528.

Methylation of **5** with MeI and NaH [6] gave haloxanthin dimethyl ether, purified by TLC-6, with unchanged Vis spectrum; MS *m/z* (rel. int.): 766 $[M]^+$ (7), 684 $[M-84]^+$ (7), 674 $[M-92]^+$ (5), 666 $[M-100]^+$ (100), 660 $[M-106]^+$ (10). No new product was isolated after treatment with $LiAlH_4$ in Et_2O.

3,4-Epoxymonoanhydrobacterioruberin (**6**). Isolated (0.5 mg) from the pre-bacterioruberin CC fraction [cf. 5] as a mixture of geometrical isomers; Vis λ_{max} nm: 373, (455), 482, 516;% III/II = 57,% D_B/D_{II} = 16; MS *m/z* (rel. int.): 738 $[M]^+$ (100), 736 $[M-2]^+$ (15), 720 $[M-18]^+$ (17), 680 $[M-58]^+$ (12), 662 $[M-58-18]^+$ (4), 646 $[M-92]^+$ (15), 632 $[M-106]^+$ (58), 580 $[M-158]^+$ (3).

By coincidence **5** and **6** were not separated by TLC-7 (R_f 0.34). Compound **6** was purified by TLC-5 (R_f 0.70) and HPLC-2 (R_t 3.29 min for all-*E*). HPLC purified all-*E* had Vis λ_{max} nm: 356, 372, 455, 483, 514;% III/II = 75, % D_B/D_{II} = 6; ^{1}H NMR (400 MHz) $CDCl_3$: for assignments see structure **6**. $J_{2,3}$ = 10 Hz, $J_{3,4}$ = 15.3 Hz, $J_{6,7} = J_{6',7'}$ = 11.5 Hz, $J_{7,8} = J_{7',8'}$ = 15 Hz, $J_{10',11}$ = 12.5 Hz, $J_{11,12} = J_{11',12'}$ = 15 Hz, $J_{14,15}$ = 12.5 Hz; CD nm (Δε) EtOH:221 (− 1.5), 236 (0), 242 (1.1), 252 (0), 268 (− 2.7), 281 (0), 311 (8.1), 345 (0), 384 (− 2.1), 416 (− 0.2).

HPLC-1 purified 5*Z* (R_t 3.77 min) had Vis λ_{max} nm: 356, 373 (453), 480, 511, % III/II = 52, % D_B/D_{II} = 10; ^{1}H NMR (400 MHz, $CDCl_3$): consistent with 5*Z*-configuration δ6.10 *d* (*J* = 12.9 Hz, H-6), and 6.74 *d* (*J* = 15.5 Hz, H-4); CD nm (Δε): 213 (− 2.5), 218 (− 1.6), 240 (− 3.6), 257 (0), 270 (3.7), 284 (0), 373 (− 4.0), 347 (0), 366 (0.8), 384 (1.8), 395 (0).

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