Bacterial Carotenoids. 52.* C_{50}-Carotenoids 22.† Naturally Occurring Geometrical Isomers of Bacterioruberin

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Bacterioruberin has been submitted to iodine-catalyzed stereomutation. The quantitative composition of the quasi-equilibrium mixture was established by separation of the geometrical isomers by a published HPLC procedure and identification by VIS and ^{1}H NMR spectroscopy: all-*E* (54 % of total), 5*Z* (17 %), 9*Z* (15 %), 13*Z* (9 %) and 5*Z*,9′*Z* (5 %). The di-*Z* isomer has not previously been characterized. These geometrical isomers served as HPLC and VIS spectral standards.

A fast and gentle procedure has been developed for the isolation of bacteriorubering from *Haloferax volcanii*, based on extraction at 3 °C and HPLC analysis within 10 min, showing the presence of all-*E* (57 % of total), 5*Z* (19 %), 9*Z* (11 %), 13*Z* (10 %) and 5*Z*,9′*Z* (3 %). Extrapolation to zero time gave slightly modified values. Isomerization after 10 min in solution at 3 °C and 20 °C was monitored by HPLC. The composition of the equilibrium mixtures at these temperatures are given.

This is the first report on the natural occurrence of a carotenoid as an equilibrium mixture of geometrical isomers.

(2*S*,2′*S*)-Bacterioruberin (**1**, Scheme 1) is the characteristic C_{50}-carotenoid of halophilic bacteria. The isolation and structure elucidation,[1–4] absolute configuration[5] and total syntheses of optically active C_{50}-carotenoids chemically derived from **1**[6] have been the subject of previous investigations in our laboratory.

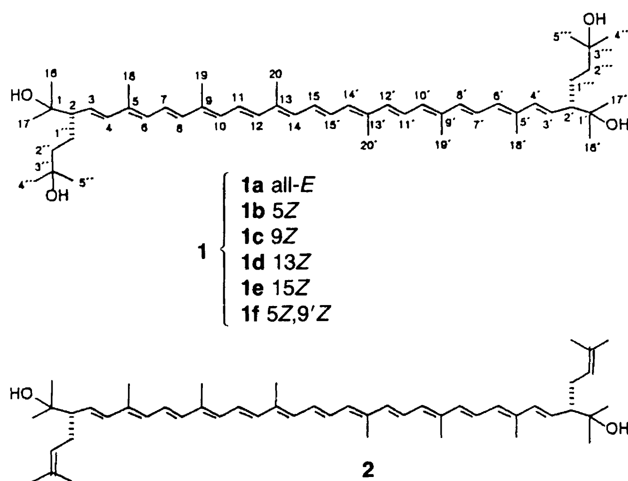
The great steric lability of bacterioruberin (**1**), possessing an aliphatic tridecaene chromophore, has long been noted.[2] Recently Riesen and Pfander[7] have briefly reported the separation of geometrical isomers of bacterioruberin (**1**) by HPLC and identification of the all-*E* (**1a**), 5-*Z* (**1b**), 9-*Z* (**1c**) 13-*Z* (**1d**) and 15-*Z* (**1e**) isomers by ^{1}H NMR spectroscopy. In the light of the steric instability of **1a** they suggested that only the all-*E* (**1a**) isomer was naturally occurring.

However, earlier work[2] already indicated that more than one geometrical isomer was present in *Halobacterium salinarium*. We now report further studies on naturally occurring geometrical isomers of bacterioruberin (**1**).

Results and discussion

Bacterioruberin (**1**) represented 81 % of the total carotenoid content of *Haloferax volcanii*.[8,9] All-*E* bacterioruberin was submitted to iodine-catalyzed stereomutation. The stereoisomeric mixture was separated in the reversed-phase HPLC system[7] and the individual isomers characterized by t_R-values and VIS and ^{1}H NMR (500 MHz) spectra. The ^{1}H NMR assignments were consistent with the isomerization shifts reported for the olefinic protons relative to the all-*E* (**1a**) isomer,[7,10] observed coupling patterns and coupling constants. ^{1}H NMR spectral assignments compiled in Table 1 are based on 1D and 2D (COSY) spectra of the all-*E* isomer (**1a**) and 1D spectra of the other isomers. The sp^3 methyl groups (Me-16,17,4″,5″, Scheme 1) were tentatively assigned for the **1a** isomer by comparison with data for bisanhydrobacterioruberin (**2**, Scheme 1)[8–10] and the 5-*Z* (**1b**) isomer, and these assignments for the *Z*-isomers are tentative.

The quantitative composition of the iodine-catalyzed quasi-equilibrium mixture is given in Table 2. Figures are based on identical extinction values for the all-*E* and



Scheme 1.

* No 51. *Acta Chem. Scand., Ser. B 38* (1984) 871.
† No 21. *Tetrahedron Lett. 25* (1984) 1191.

Table 1. Chemical shift assignments (δ, $CDCl_3$) for the geometrical isomers of bacterioruberin (**1**).[a]

Isomer	Me-16,17[b]	Me-4″,5″[c]	H-2	H-3	H-4	Me-19	H-6	H-7	H-8	Me-19	H-10	H-11	H-12	Me-20	H-14	H-15
All-*E* (**1a**)	1.23, 1.19	1.22, 1.21	2.02	5.46	6.21	1.94	6.15	6.61	6.39	1.99	6.26	6.66	6.40	2.00	6.29	6.66
5-*Z* (**1b**)	1.23, 1.20	1.23, 1.23	2.02	5.50	6.75	1.94	6.06	6.74	6.32	1.99	6.24	6.66	6.39	2.00	6.29	6.66
9-*Z* (**1c**)	1.23, 1.20	1.22, 1.22	2.02	5.49	6.22	1.95	6.23	6.60	6.92	2.00	6.11	6.82	6.33	2.00	6.29	6.66
13-*Z* (**1d**)	1.23, 1.19	1.22, 1.22	2.02	5.46	6.22	1.94	6.17	6.60	6.40	1.99	6.29	6.66	6.93	2.00	6.14	6.62
5*Z*,9′*Z* (**1f**)	1.24, 1.21	1.23, 1.23	2.02	5.50	6.75	1.94	6.06	6.74	6.32	1.99	6.23	6.66	6.39	2.00	6.28	6.66
[d]	1.24, 1.20	1.22, 1.22	2.02	5.49	6.22	1.95	6.23	6.61	6.92	2.00	6.11	6.61	6.33	2.00	6.27	6.66

[a] **1a** is symmetrical with identical values on the primed sides, see Scheme 1. The primed side is vertically unaltered for the mono-*Z* isomers. [b,c] See comments in the text. [d] The second set of values refers to the primed side for the di-*Z* isomer **1f**.

mono-*Z* isomers at 480–490 nm. Extinction coefficients for the *Z*-isomers are unknown, but are generally lower than for the all-*E* isomer.[11] The quasi-equilibrium reflects the thermodynamic stability of the isomers which is in the order all-*E* (**1a**) > 5*Z* (**1b**) > 9*Z* (**1c**) > 13*Z* (**1d**). The course of the reaction, shown in Fig. 1, demonstrates that the rate of conversion of all-*E* into the mono-*Z* isomers follows the sequence 5*Z* > 9*Z* > 13*Z*.

A minor di-*Z* isomer was assigned the 5*Z*,9′*Z*-configuration (**1f**) from VIS spectral properties and ^1H NMR data. Mono-*Z* isomers of carotenoids with conservative CD spectra are known to show an inverted Cotton effect, whereas di-*Z* isomers have similar CD properties relative to the all-*E* isomer.[12,13] Consistent with this generalization the mono-*Z* isomers of bacterioruberin (**1b**, **1c**, **1d**, **1e**) exhibited an inverted Cotton effect[7] and the di-*Z* isomer (**1f**) here isolated qualitatively showed little change in the CD spectrum relative to the all-*E* isomer (**1a**). The configuration for the di-*Z* isomer is compatible with the relative stability of Δ5 and Δ9 *Z*-bonds reflected by the composition of the iodine-catalyzed stereomutation mixture. A further minor, presumed di-*Z* isomer (9, 9′-di*Z*?) was not available in sufficient quantity.

Geometrical isomers with sterically hindered *Z*-bonds (3*Z*, 7*Z* and 11*Z*) are generally not formed from the corresponding *E*-isomers,[11] nor was the 15*Z* isomer (**1e**) encountered here in the iodine-catalyzed stereoisomeric mixture.

The geometrical isomers **1a**, **1b**, **1c**, **1d**, **1f** obtained from the iodine-catalyzed stereomutation mixture served as HPLC and VIS spectral standards for investigations on the naturally occurring geometrical isomers of bacterioruberin (**1**).

A fast and gentle procedure for lysis of *Haloferax volcanii* cells, cold extraction at +3 °C with acetone in the dark followed by reversed-phase HPLC within 10 min, was developed. The stereoisomeric composition was monitored by HPLC over a period of 70 h under the same conditions at 3 °C in the dark. The results are shown in Fig. 2. Extrapolation to zero time suggests only a low degree of isomerization during the 10 min prior to analysis, and gives slightly corrected zero time values, see Table 2.

The reaction mixture, monitored by HPLC, was further kept at room temperature for 94 h, see Fig. 3. After 70 h an apparent equilibrium was reached at + 3 °C, whereas a new equilibrium at + 20 °C was reached after 140 h. The equilibrium at room temperature differed slightly from that at + 3 °C in the sense that the relative amount of the 5*Z* (**1b**) and 9*Z* (**1c**) isomers had increased at the expense of the all-*E* (**1a**) isomer.

In Table 2 figures are compared for the composition of the iodine-catalyzed equilibrium mixture and the equilibrium reached (Fig. 3) in solution at 20 °C in the absence of iodine. These data are very similar. Moreover, the data for the naturally occurring stereoisomeric mixture is closely similar to these equilibrium figures.

This is the first report on the natural occurrence of a carotenoid as a thermodynamic equilibrium mixture of geometrical isomers. Hitherto carotenoids have normally occurred as the single all-*E* or mono-*Z* isomers[14,15] in most carefully studied sources.

Table 2. The composition of stereoisomeric mixtures of bacterioruberin (**1**).

Geometrical isomer	% of total bacterioruberin (**1**)				
	I_2-cat. eq. mixture	Natural occurrence		Equilibrium in solution	
		Measured 10 min	Extrapolated 0 time	+ 3 °C	+ 20 °C
All-*E* (**1a**)	54	57	56	61	53
5-*Z* (**1b**)	17	19	19	16	19
9-*Z* (**1c**)	15	11	12	9	14
13-*Z* (**1d**)	9	10	10	10	9
5*Z*,9′*Z* (**1f**)	5	3	3	4	5

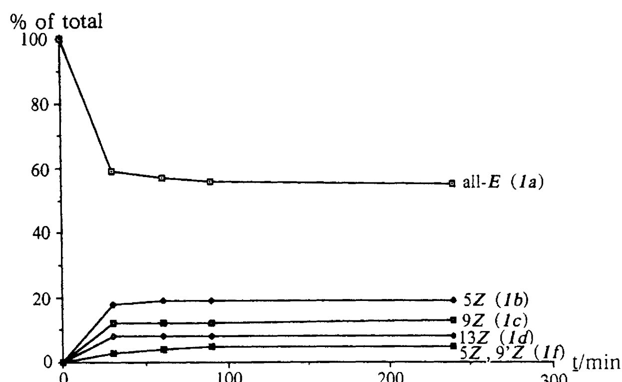


Fig. 1. Photochemical isomerization of all-*E* bacterioruberin (**1a**) in the presence of iodine.

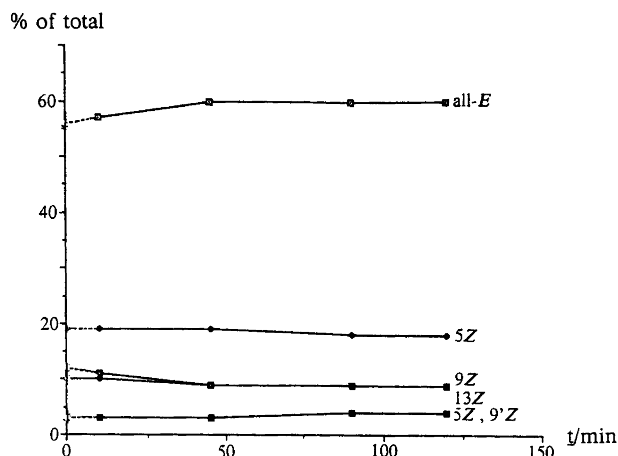


Fig. 2. Further isomerization of the extracted mixture of bacterioruberin (**1**) isomers at + 3 °C in darkness and extrapolation to zero time.

Experimental

Biological material. Deep-frozen cells of *Haloferax volcanii* strain DS2, cultivated in the dark at 33 °C were used.[8,9]

General methods. General precautions for work with carotenoids were taken.

Semipreparative HPLC (system 1) was carried out on a Perkin Elmer Series 2 instrument with a Pye-Unicam PU 4021 detector and a Merck Hitachi D-200 integrator using two coupled V18-103 (3.2×110 mm) and Spheri-5 ODS (4.6×220 mm) columns. VIS spectra were recorded by the stopped-flow technique. Eluted fractions were collected in an ice bath. Analytical HPLC (system 2) was carried out on a Hewlett Packard Series 1050 instrument with an HP 1040A diode array detector and an HP 9153C integrator with on-line recording of VIS spectra, detector set-point 480–490 nm, using a Spheri-5RP-18 (4.6×220 mm) column. For both semipreparative and analytical work the reversed-phase system by Riesen and Pfander[7] was best, using methanol–ethyl acetate–water–triethyl amine 85.5:9:5.5:1. Quantitative calculations are based on HPLC integrals without correction for different, unknown extinction coefficients for the *Z*-isomers.

VIS absorption spectra were recorded in the HPLC eluent (mainly methanol) if not otherwise stated. Spectral fine structure is expressed as % III/II and *cis*-peak intensity as % D_B/D_{II}.[16]

^{1}H NMR spectra (500 MHz) were recorded on a Bruker FT instrument in $CDCl_3$, CD spectra on a Jobin Yvon Auto Dicrograph Mark IV and mass spectra on an AEI MS 902 spectrometer.

(2S,2'S)-Bacterioruberin (**1**). **1** was isolated from lysed cells of *Haloferax volcanii* by acetone extraction, column chromatography on silica and repeated TLC on silica and was crystallized three times from acetone–heptane,[8,9] yield 3 mg enriched in **1a**; MS *m/z* (rel. % to *M*−106), 740 (*M*, 92 %), 738 (*M*−2, 7 %), 722 (*M*−18, 30 %), 704 (*M*−18−18, 21 %), 686 (*M*−18−18−18, 12 %), 682 (*M*−58, 30 %), 664 (*M*−58−18, 23 %), 648 (*M*−92, 21 %), 634 (*M*−106, 100 %), 582 (*M*−158, 23 %) cf. Ref. 4.

*All-*E*-Bacterioruberin* (**1a**) was obtained by semipreparative HPLC (system 1) t_R = 5.72 min, by analytical HPLC (system 2) t_R = 3.11 min; VIS λ_{max} nm 368, 386, 468, 494, 527, of % III/II = 57, % D_B/D_{II} = 11; CD nm ($\Delta\varepsilon$, ethanol) 224 (−5.4), 245 (−2.7), 276 (−5.8), 301 (0), 321 (6, 3), 337 (0), 394 (−3.5), 421 (−2.4); ^{1}H NMR see Table 1. Assignments of the olefinic protons were based on a 2D COSY spectrum.

I_2-catalyzed stereomutation. (i) All-*E* **1** (3.2 mg) was dissolved in benzene (79 ml) and I_2 (0.05 mg) added in benzene (1 ml). The mixture was exposed to weak sunlight for 3 h. The reaction was monitored by VIS spectroscopy and the quasi-equilibrium mixture analyzed by HPLC, see Table 1. (ii) HPLC-pure **1a** (0.2 mg) dissolved in benzene

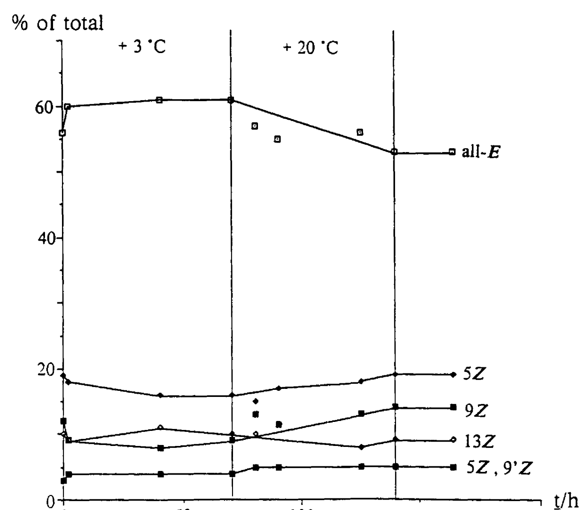


Fig. 3. Further isomerization of the extracted mixture of bacterioruberin (**1**) in the dark at + 3 °C for 70 h, then at + 20 °C.

(5 ml), containing I_2 (0.004 mg) was exposed to weak sunlight for 4 h at 20 °C. The reaction was monitored by HPLC, see Fig. 1.

(5Z)-Bacterioruberin (**1b**). Available 0.3 mg, t_R = 7.16 min (system 1) and t_R = 3.93 min (system 2); VIS λ_{max} nm 383, 462, 492, 525, % III/II = 57, % D_B/D_{II} = 13; ^{1}H NMR see Table 1.

(9Z)-Bacterioruberin (**1c**). Available 0.2 mg, t_R = 8.87 min (system 1) and t_r = 4.79 min (system 2); VIS λ_{max} nm 370, 386, 462, 488, 523, % III/II = 57, % D_B/D_{II} = 25; ^{1}H NMR see Table 1.

(13Z)-Bacterioruberin (**1d**). Available 0.2 mg, t_R = 12.26 min (system 1) and 5.30 min (system 2); VIS λ_{max} nm 368, 385, 461, 488, 521, % III/II = 46, % D_B/D_{II} = 85; ^{1}H NMR see Table 1.

(5Z,9'Z)-Bacterioruberin (**1f**). Available 0.1 mg, t_R = 6.39 min (system 1) and 3.26 min (system 2); VIS λ_{max} nm 368, 385, 460, 486, 518, % III/II = 33, % D_B/D_{II} = 14; ^{1}H NMR see Table 1; CD nm ($\Delta\varepsilon$) ethanol 236 (−0.1) 287 (0), 255 (0.9), 268 (0), 283 (−0.9), 295 (0), 339 (1.6), 347 (0), 392 (−3.2), 424 (0.3).

Minor, unidentified bacterioruberin isomer. (9Z,9'Z?), available 20 µg, t_R = 13.10 min (system 1), VIS λ_{max} nm 370, 384, 460, 484, 516, % III/II = 44, % D_B/D_{II} = 36.

Naturally occurring stereoisomers. Cells (2 g wet weight) were lyzed with cold, distilled H_2O (2 ml) at 3 °C in a cold room in the dark for 15 min. The residue was extracted with cold acetone for 10 min and ether (2 ml) added. The ether extract was filtered and submitted to immediate HPLC analysis. The extract was kept in the dark at 3 °C for 70 h and the isomerization was monitored by HPLC, Fig. 2. Thereafter the extract was kept in the dark at room temperature for 94 h, and the isomerization was monitored by HPLC, Fig. 3.

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