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Note

High-performance liquid chromatography of *cis–trans* isomers of neurosporene: discrimination of *cis* and *trans* configurations at the end of an open conjugated chain

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Carotenoids in photosynthetic systems have dual functions of light-harvesting and photo-protection [1,2]. With purple photosynthetic bacteria, the natural selection of particular configurations of carotenoids, in relation to their functions, has been found; the all-*trans* configuration is selected by the light-harvesting complex for the light-harvesting function, whereas the 15-*cis* configuration is selected by the reaction centre for the photo-protective function [3–5]. In the thylakoid membranes of spinach and a cyanobacterium, 13-*cis* to all-*trans* isomerization of β-carotene on triplet excitation has been detected [6]. Carotenoids in animals are assumed to have a protective function against oxidation by quenching singlet oxygen and radicals [7]. In particular, mono-*cis* isomers of canthaxanthin have been found in the ovaries, eggs and haemolymph of the reproductively active female of the brine shrimp *Artemia* [8]. In order to reveal the protective functions of the *cis*-carotenoids in photosynthetic and non-photosynthetic organisms, a high-performance liquid chromatographic (HPLC) technique for determining and isolating each *cis–trans* isomer of carotenoids is of primary importance.

We have previously developed an HPLC technique using calcium hydroxide as adsorbent, and applied it to isomeric β-carotene [9,10], canthaxanthin [11], neurosporene [3] and spirilloxanthin [4]. The technique gave an excellent separation of the *cis–trans* configurations around double bonds in the inner part of the conjugated system. However, it was not established whether the technique can separate the *cis* and *trans* configurations at the end of an open conjugated chain.

In this investigation, the technique was applied to isomeric neurosporene; a set of chromatograms showed fourteen peaks of *cis–trans* isomers. Actually, the peak of the 5-*cis* (or 9′-*cis*) isomer was separated from that of the all-*trans* isomer, showing that the 5-*cis* and 5-*trans* (9′-*cis* and 9′-*trans*) configurations at the end of the conjugated chain can be distinguished. Further, the 5,13′-di-*cis* isomer was found in addition to the

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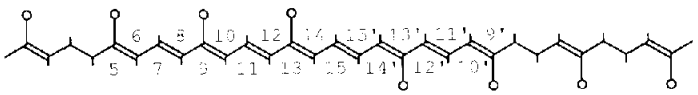
13′-mono-*cis* isomer. We could assign the configurations of eight peaks in the chromatogram: the configurations of the 5-*cis* (or 9′-*cis*), 9-*cis*, 13′-*cis*, 5,13′-*cis* and 5,9′-*cis* isomers were determined by using ^{1}H NMR spectroscopy after isolation of the isomers; the configuration of the 13-*cis* isomer was predicted by electronic absorption spectroscopy during the HPLC analysis; and the peak of the 15-*cis* isomer was identified by co-chromatography with an authentic sample [3]. In addition to the 7,9,9′-*cis* isomer found in tomato fruit *Tangerine* [12], the 5-*cis* isomer found in rose flower *Rosa pomifera* [13], the 15-*cis* isomer found in a photosynthetic bacterium *Rhodobacter spheroides* G1C [3] and the 5-*cis*, 9′-*cis* and 5′-*cis* isomers synthesized [14], we newly demonstrated the presence of the 9-*cis*, 13′-*cis*, 5,9′-*cis* and 5,13′-*cis* isomers among all the possible *cis–trans* isomers of neurosporene.



EXPERIMENTAL

All-*trans* neurosporene was obtained from the cells of *Rhodobacter spheroides* G1C. The cells were extracted with methanol and the extract was saponified with 6% potassium hydroxide solution. *n*-Hexane and saturated sodium chloride solution were added to the above solution and neurosporene was transferred to the hexane layer; the hexane layer was then washed with water and dried over anhydrous sodium sulphate. After the solution had been dried *in vacuo*, the residue was recrystallized with benzene and methanol. A mixture of isomeric neurosporene was obtained by iodine-catalysed photoisomerization of the above all-*trans* isomer in *n*-hexane.

The HPLC system consisted of a Jasco Trirotor-V (pump) and a Jasco Uvidec-100-IV (detector). HPLC analyses were performed at 25°C with calcium hydroxide columns (packed at 300 kg/cm^2 [9,10]), the detection wavelength being 435 nm. Fourteen peaks of isomeric neurosporene were identified by a combination of the following three different HPLC conditions (a.u.f.s. = 0.04): condition A (for an entire chromatogram with ordinary resolution), column 250 × 4 mm I.D. packed with calcium hydroxide (Nacalai, lot M6N0577, particle size 8.5 μm), eluent benzene–*n*-hexane (15:85) and flow-rate 2.0 ml/min; condition B (for resolution of peaks 3–7), column 300 × 7.5 mm I.D. packed with calcium hydroxide (Nacalai, lot M6E7143, particle size 8.7 μm), eluent benzene *n*-hexane (6:94) and flow-rate 6.0 ml/min; condition C (for resolution of peaks 12–14), column and flow-rate same as in B, eluent benzene–*n*-hexane (12:88).

The electronic absorption spectra (300–520 nm) of isomeric components were recorded during HPLC analysis by using a Shimadzu SPD-M6A diode-array detector connected to the above Jasco Trirotor-V. The HPLC conditions were as follows: column, 500 × 7.5 mm I.D. with lot M6E7143 as above; eluent, benzene–*n*-hexane (6:94) (for peaks 2–7) or benzene *n*-hexane (12:88) (for peaks 8–14); flow-rate, 6.0 ml/min; and a.u.f.s. = 0.04.

Isolation of peaks 9–13 was performed by using the same kind of column as in condition B and eluents of benzene–*n*-hexane in the range 15:85 to 40:60. For rough

fractionation of peaks 12 and 13, a 250 × 7.5 mm I.D. column packed with alumina (Woelm basic for thin-layer chromatography, particle size not specified) and diethyl ether–*n*-hexane (2:98) as eluent were used, the flow-rate being 2.0 ml/min (a.u.f.s. = 2.56). The purity of each component was examined by HPLC by using the above conditions for analyses; peak 9 could not be purified completely. ^{1}H NMR spectra (400 MHz) were recorded on a Jeol GX-400 spectrometer for the isolated sample (*ca.* 0.1 mg) dissolved in benzene-d_6 (CEA, 99.93%); detailed conditions for the measurements were as described elsewhere [15].

RESULTS AND DISCUSSION

Fig. 1 shows the entire elution profile with ordinary resolution and also those (insets) with higher resolution for peaks 3–7 and 12–14. First, peaks 5 and 14 were assigned to the 15-*cis* and all-*trans* isomers, respectively, by co-chromatography with authentic samples [3]. Second, peaks 13, 12, 9, 11 and 10 were assigned to the 5-*cis* (or 9′-*cis*), 5,9′-*cis*, 9-*cis*, 13′-*cis* and 5,13′-*cis* isomers, respectively, based on their ^{1}H NMR spectra as described below. The olefinic H signals were assigned by using the correlation peaks in the ^{1}H–^{1}H COSY spectra and the vicinal coupling constants through single (10–12 Hz) and double (15–16 Hz) bonds. Configurational assignments were based on the values of chemical shifts and of "isomerization shifts" (changes in the values of chemical shifts with reference to those of the all-*trans* isomer), which are listed in Table I. The patterns of the isomerization shifts for the 9-*cis* and 13-*cis* (13′-*cis*) configurations have been established [15,16].

Peak 13 is assigned to the 5-*cis* (or 9′-*cis*) isomer based on the high-field shift (hfs) of the 6H (10′H) signal and the low-field shift (lfs) of the 7H (11′H) signal; the chemical shifts of the signals of 8H–10′H (6H–12′H) of this isomer are the same as those of the all-*trans* isomer within the limit of 0.02 ppm.

Peak 12 is assigned to the 5,9′-*cis* isomer based on the hfs of both 6H and 10′H signals and the lfs of both 7H and 11′H signals; the chemical shifts in the central part of the polyene chain of this isomer are approximately the same as those of the all-*trans* isomer, indicating that this is a peripheral di-*cis* isomer.

Peak 9 is assigned to the 9-*cis* isomer based on the hfs of the 10H signal and the lfs

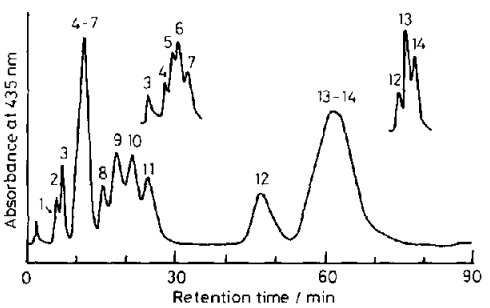


Fig. 1. Elution profiles of a mixture of isomeric neurosporene obtained by iodine-catalysed photo-isomerization. The entire elution profile with ordinary resolution (condition A, see Experimental) and elution profiles with higher resolution for peaks 3–7 (condition B) and for peaks 12–14 (condition C) are shown. See Table II for the assignment of the peaks.

TABLE I

CHEMICAL SHIFTS AND ISOMERIZATION SHIFTS OF ISOMERIC NEUROSPORENE (ppm)

Isomerization shifts with absolute values larger than 0.02 ppm are given in parentheses.

	Peak 14[a], all-*trans*	Peak 13		Peak 12, 5,9′-*cis*	Peak 9, 9-*cis*	Peak 11, 13′-*cis*	Peak 10, 5,13′-*cis*	Peak 5[a], 15-*cis*
		5-*cis*	(9′-*cis*)					
6H	6.14	6.09(−0.05)	6.15	6.09(−0.05)	6.16[b]	6.15	6.10(−0.04)	6.17(+0.03)
7H	6.66	6.73(+0.07)	6.65	6.73(+0.07)	6.68[b]	6.65	6.72(+0.06)	6.68
8H	6.42	6.41	6.42	6.40	7.08[b](+0.66)	6.43	6.42	6.43
10H	6.34	6.34	6.34	6.33	6.16[b](−0.18)	6.33	6.33	6.37(+0.03)
11H	6.75	6.74	6.74	6.74	7.01[b](+0.26)	6.74	6.73	6.79(+0.04)
12H	6.48	6.47	6.47	6.47	6.42[b](−0.06)	6.48	6.48	6.50
14H	6.33[b]	6.31	6.31	6.31	6.32[b]	6.33	6.33	6.90[b](+0.57)
15H	6.69[b]	6.70	6.70	6.70[b]	6.68[b]	6.59[b](−0.10)	6.59(−0.10)	6.47[b](−0.22)
15′H	6.69[b]	6.70	6.70	6.70[b]	6.68[b]	6.95[b](+0.26)	6.95[b](+0.26)	6.47[b](−0.22)
14′H	6.33[b]	6.31	6.31	6.31	6.31[b]	6.10(−0.23)	6.10(−0.23)	6.90[b](+0.57)
12′H	6.42	6.42	6.41	6.40	6.42[b]	6.98(+0.56)	6.98(+0.56)	6.47(+0.05)
11′H	6.66	6.65	6.73(+0.07)	6.73(+0.07)	6.65[b]	6.65	6.65	6.70(+0.04)
10′H	6.14	6.15	6.09(−0.05)	6.09(−0.05)	6.16[b]	6.16	6.16	6.17(−0.03)

[a] Values from ref. 3.

[b] Approximate values.

TABLE II

ASSIGNMENT OF PEAKS AND ELECTRONIC ABSORPTION OF ISOMERIC NEUROSPORENE

Peak No.	Configuration	Wavelength (nm) (isomerization shift)	Relative intensity of *cis*-peak
2		451(17)	–
3		454(14)	0.11
4		461(7)	0.33
5	15-*cis*	464(4)	0.48
6		461(7)	0.07
7	(13-*cis*)	462(6)	0.57
8		461(7)	0.11
9	9-*cis*	461(7)	0.20
10	5,13′-*cis*	461(7)	0.21
11	13′-*cis*	462(6)	0.25
12	5,9′-*cis*	468(0)	0.03
13	5-*cis* (or 9′-*cis*)	468(0)	0.03
14	all-*trans*	468	0.03

of the 8H and 11H signals; the values of the isomerization shifts of 10H, 8H and 11H in Table I, *i.e.*, −0.18, +0.66 and +0.26, parallel those of 9-*cis*-β-carotene [15], *i.e.*, −0.14, +0.69 and +0.26, respectively. The values of the chemical shifts for 6H and 7H and for 14H–10′H are approximately the same as those of the all-*trans* isomer, which indicates that this is the 9-mono-*cis* isomer.

Peak 11 is assigned to the 13′-*cis* isomer based on the hfs of the 14′H and 15H signals and the lfs of the 12′H and 15′H signals; the values of the isomerization shifts of 14′H, 15H, 12′H and 15′H, *i.e.*, −0.23, −0.10, +0.56 and +0.26, respectively, parallel those of 13′-*cis*-β-carotene, *i.e.*, −0.19, −0.07, +0.60 and +0.25, respectively. The values of chemical shifts of other Hs are approximately the same as those of the all-*trans* isomer, indicating that this is the 13′-mono-*cis* isomer.

Peak 10 is assigned to the 5,13′-di-*cis* isomer. It shows isomerization shifts characteristic of both 5-*cis* and 13′-*cis* configurations; the values of the chemical shifts of 8H–14H and of 11′H and 10′H are the same as those of the all-*trans* isomer.

Finally, peak 7 is temporarily assigned to the 13-*cis* isomer by using its electronic absorption spectrum. Table II lists the wavelength of the $^1Bu^+ \leftarrow {}^1Ag^-$ transition at λ_{max} for the isomeric neurosporene; "the isomerization shift" (the shift of the wavelength of the particular transition for each *cis* isomer with reference to that of the all-*trans* isomer) is shown in parentheses. It lists also the relative intensity of the *cis*-peak (the intensity ratio of the absorption of the $^1Ag^+ \leftarrow {}^1Ag^-$ *vs.* $^1Bu^+ \leftarrow {}^1Ag^-$ transition). Both the wavelength of the $^1Bu^+ \leftarrow {}^1Ag^-$ transition and the relative intensity of the $^1Ag^+ \leftarrow {}^1Ag^-$ transition are known to be configuration-sensitive [3,4,17,18]; the isomerization shift should be less than 10 nm for a central and a peripheral mono-*cis* isomer, and the relative intensity of the *cis*-peak is the highest for the central-bent isomer concerning the conjugated chain. Thus, peak 7, which shows the highest *cis*-peak (relative intensity 0.57) and an isomerization shift of 6 nm, is predicted to be a 13-*cis* isomer. (A terminal *cis* or *trans* configuration around the $C_5 = C_6$ and $C_{9'} = C_{10'}$ bonds is left as an open question.) All the above configurational assignments are also listed in Table II.

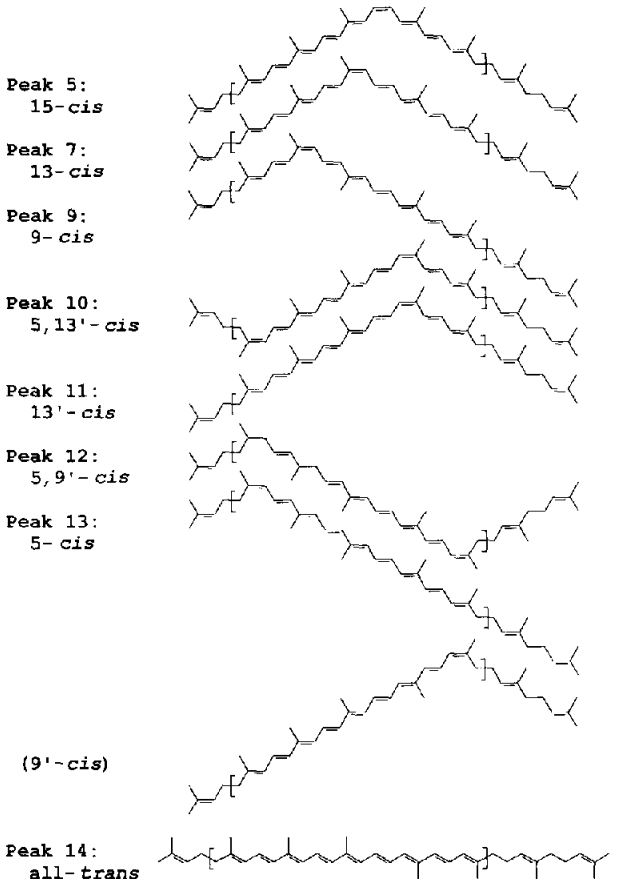


Fig. 2. Configurations of the isomers of neurosporene identified. The configurations are shown in order of elution (from top to bottom). A pair of square brackets indicates the conjugated chain.

Fig. 2 shows the configurations of the isomers identified in this investigation in order of elution. The following correlations between the configuration and the retention time are seen: (1) for the conjugated chain shown by a pair of square bracket, a central-*cis* isomer (15-*cis* or 13-*cis*) tends to elute earlier than a peripheral-*cis* isomer (9-*cis* or 13′-*cis*); (2) a di-*cis* isomer tends to elute earlier than the constituent mono-*cis* isomers; 5,13′-*cis* elutes earlier than 5-*cis* or 13′-*cis*, while 5,9′-*cis* elutes earlier than 5-*cis* or 9′-*cis*; (3) the terminal-*cis* (5-*cis* or 9′-*cis*) isomer elutes earlier than the all-*trans* isomer. The results strongly suggest that intermolecular interaction between the conjugated chain of an isomer molecule and a flat surface of calcium hydroxide (in the molecular dimension) plays an important role in the mechanism of the present adsorption chromatography.

This investigation shows that the 15-*cis* isomer found in the reaction centre of *Rhb. spheroides* G1C [3] takes an extended *trans* configuration around the $C_5 = C_6$ and $C_{9'} = C_{10'}$ bonds. No indication of the combination of the hfs of 6H (10′H) and

the lfs of 7H (11'H), which is characteristic of the 5-*cis* (9'-*cis*) configuration, is seen in the values of the chemical shifts of the 6H, 7H, 11'H and 10'H signals of the particular isomer (Table I). This is important information from the viewpoint of intermolecular interaction between the carotenoid and the apo-protein and of the mechanism of energy dissipation in the reaction centre.

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