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Note

Isolation of *cis–trans* isomers of canthaxanthin by high-performance liquid chromatography using a calcium hydroxide column and identification of their configurations by ^{1}H NMR spectroscopy

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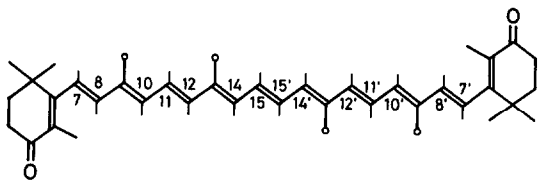
The roles of carotenoids in photosynthetic systems are well established[1–4], and the natural selection of certain types of *cis–trans* carotenoids in the specific sites of bacterial photosynthetic organella have been reported[5]. On the other hand, the roles and the configurations of these pigments in animals still remain obscure. Nelis *et al.*[6] found that massive amounts of mono-*cis* isomers of canthaxanthin (4,4′-diketo-β-carotene) were selectively localized in the ovaries, the eggs and the haemolymph of reproductively active females of the brine shrimp *Artemia*, which was the first observation of the *cis*-carotenoid in the animal organella, to the best of our knowledge. In order to reveal the physiological implications of the presence of *cis*-carotenoids in these organella, it is necessary to elucidate both the configurations and the biochemical and biophysical properties of these pigments both *in vitro* and *in vivo*. We have therefore attempted to establish a technique for the isolation and identification of the *cis* isomers of canthaxanthin as the first step in a series of investigations.

cis Isomers of canthaxanthin were first isolated by Gansser and Zechmeister[7] from a stereoisomeric mixture by means of column chromatography using calcium hydroxide as the stationary phase and benzene as the mobile phase. They found three mono-*cis* and three di-*cis* isomers, and tentatively assigned the mono-*cis* isomers to 9-*cis*, 13-*cis* and 15-*cis* on the basis of their electronic and infrared absorption spectra and of co-chromatography with synthetic 15-*cis* isomer. Nelis and co-workers[6,8] used non-aqueous reversed-phase and normal-phase chromatography for the separation of canthaxanthin isomers. They isolated the all-*trans*- and a mixture of *cis*-canthaxanthins by reversed-phase chromatography using a Zorbax ODS column and a ter-



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nary mobile phase consisting of acetonitrile, methanol and dichloromethane. Further, *cis* isomers separated by normal-phase chromatography on ROSIL silica were assigned to 9-*cis*, 13-*cis* and 15-*cis* on the basis of their absorption spectra.

Englert[9] reviewed high-field ^{1}H NMR spectroscopy for the determination of carotenoid configurations and reported a study of *cis* isomers of canthaxanthin, in which the presence of the 9-*cis*, 11-*cis*, 13-*cis*, 15-*cis*, 9,9′-*cis*, 9,11-*cis*, 9,13-*cis*, 9,15-*cis* and 13,13′-*cis* isomers was suggested. Complete publication, however, concerning the isolation and identification of these isomers has not appeared anywhere. In a previous investigation, we established a technique for isolating isomeric β-carotene by high-performance liquid chromatography (HPLC) using a calcium hydroxide column[10,11]. In this investigation, we applied this technique to isomeric canthaxanthin, and succeeded in separating thirteen different *cis–trans* isomers using *n*-hexane–benzene (5:95) as the mobile phase; seven *cis* isomers (9-*cis*, 13-*cis*, 15-*cis*, 9,9′-*cis*, 9,13-*cis*, 9,13′-*cis* and 13,13′-*cis*) were identified by 400 MHz ^{1}H NMR spectroscopy.

EXPERIMENTAL

All-*trans*-canthaxanthin was purchased from Roth (purity 98%) and used without further purification. A mixture of isomeric canthaxanthin was obtained by heating the crystals of the all-*trans* isomer sealed in an ampoule at 215°C for 5 min. The mixture was roughly separated into six fractions by the use of Lo-Bar column chromatography (50 × 3 cm I.D. column packed with calcium hydroxide, Kishida lot E48215T, at 6 kg/cm^2; development with benzene at 3 kg/cm^2). After the development, the calcium hydroxide was pushed out of the column and cut into six fractions. Each fraction was extracted with ethanol and each isomeric component was purified by HPLC.

Columns for HPLC were packed at 300 kg/cm^2 with calcium hydroxide (Nakarai, lot M6E7143, 200–300 mesh) by a method described elsewhere[11]. A 300 × 4 mm I.D. column with *n*-hexane–benzene (5:95) as eluent was used to analyse components having longer retention times (peaks 5, 10 and 11 in Fig. 1a; see below), and

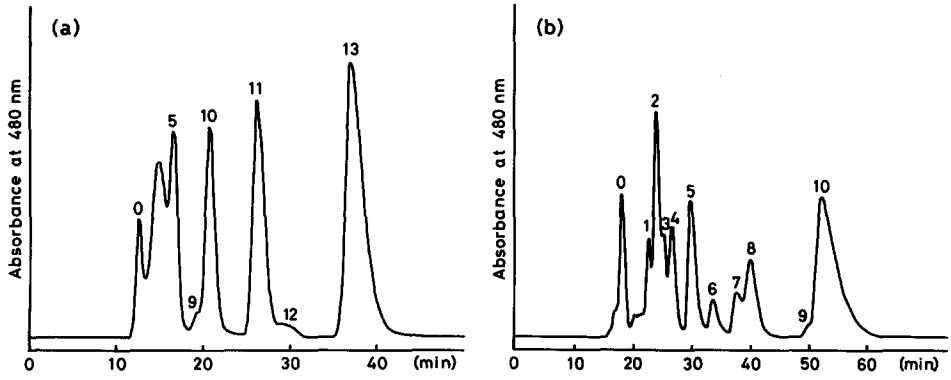

Fig. 1. Elution profiles for a mixture of isomers obtained by melting crystalline all-*trans*-canthaxanthin. Column (4 mm I.D.) packed with calcium hydroxide; eluent, *n*-hexane–benzene (5:95); detection at 480 nm. (a) Column length 300 mm and flow-rate 0.2 ml/min; (b) column length 600 mm and flow-rate 0.3 ml/min. The assignment of the peaks is given in Table II.

TABLE I

CHEMICAL SHIFTS (ISOMERIZATION SHIFTS) OF ISOMERIC CANTHAXANTHIN IN DEUTEROBENZENE (ppm)

	Peak 13, all-trans*	*Peak 11, 9-cis*	*Peak 10, 13-cis*	*Peak 3, 15-cis*	*Peak 4, 9,9'-cis*	*Peak 5, 9,13-cis*	*Peak 2, 9,13'-cis*	*Peak 1, 13,13'-cis*
7H	6.15	6.18 (+0.03)	6.16 (+0.01)	6.14 (−0.01)	6.18 (+0.03)	6.18 (+0.03)	6.17 (+0.02)	6.16 (+0.01)
7'H		6.15 (+0.00)	6.15 (+0.00)			6.15 (+0.00)	6.16 (+0.01)	
8H	6.38	7.13 (+0.75)	6.41 (+0.03)	6.35 (−0.03)	–	7.11 (+0.73)	–	6.42 (+0.04)
8'H		6.38 (+0.00)	6.39 (+0.01)			6.38 (+0.00)	6.41 (+0.03)	
10H	6.29	6.22 (−0.07)	6.27 (−0.02)	6.29 (+0.00)	6.21 (−0.08)	6.23 (−0.06)	6.19 (−0.10)	6.30 (+0.01)
10'H		6.28 (−0.01)	6.29 (+0.00)			6.27 (−0.02)	6.28 (−0.01)	
11H	6.73	6.93 (+0.20)	6.72 (−0.01)	6.74 (+0.01)	6.92 (+0.19)	6.91 (+0.18)	6.93 (+0.20)	6.72 (−0.01)
11'H		6.72 (−0.01)				6.72 (−0.01)	6.71 (−0.02)	
12H	6.50	6.42 (−0.08)	7.05 (+0.55)	6.51 (+0.01)	6.42 (−0.08)	6.99 (+0.49)	6.41 (−0.09)	7.06 (+0.56)
12'H		6.50 (+0.00)	6.50 (+0.00)			6.52 (+0.02)	7.05 (+0.55)	
14H	6.36	6.33 (−0.03)	6.16 (−0.20)	6.90 (+0.54)	6.29 (−0.07)	6.10 (−0.26)	6.35 (−0.01)	6.19 (−0.17)
14'H			6.40 (+0.04)			6.38 (+0.02)	6.12 (−0.24)	
15H	6.70	6.66 (−0.04)	6.94 (+0.24)	6.47 (−0.23)	6.62 (−0.08)	–	6.58 (−0.12)	6.90 (+0.20)
15'H			6.63 (−0.07)			6.59 (−0.11)	6.94 (+0.24)	

* Numbering of peaks as in the chromatogram (Fig. 1).

a 600 × 4 mm I.D. column with the same eluent to analyse components having shorter retention times (peaks 1–4 in Fig. 1b). For the collection of the former components, an automatic collecting system (a Jasco Uvidec 100-IV HPLC apparatus combined with a Jasco AS-L350 intelligent sample processor and a Gilson 210 programmable fraction collector) and a semi-preparative column (300 × 7.5 mm I.D.) with benzene as eluent were used. The latter components were collected manually by using a 500 × 7.5 mm I.D. column with *n*-hexane–benzene (1:1) as eluent.

The 400 MHz ^{1}H NMR spectra of the isomers in deuterobenzene (CEA, 99.93%) solution were recorded on a Jeol JNM GX-400 spectrometer (with a digital resolution of 0.24 Hz); the measuring conditions were as described elsewhere[12].

RESULTS AND DISCUSSION

Fig. 1 shows the elution profiles of isomeric canthaxanthin analysed by using (a) the 300 and (b) the 600 × 4 mm I.D. column and an *n*-hexane–benzene (5:95) as eluent with detection at 480 nm. The all-*trans* isomer gave peak 13. The broad peak which is not numbered in Fig. 1a is resolved into seven peaks (peaks 1–4 and 6–8) in Fig. 1b.

Table I lists the chemical shifts and the "isomerization shifts" (in parentheses) for the isomers which gave peaks 13, 11, 10, 3, 4, 5, 2 and 1. The assignment of the ^{1}H signals for the all-*trans* isomer (peak 13) was made from the ^{1}H–^{1}H COSY spectrum. Where the assignments of the ^{1}H signals for the mono-*cis* isomers are given, their configurations were assigned by using the empirical rule of "isomerization shifts" (changes in chemical shifts of the ^{1}H signals relative to those of the all-*trans* isomer); a high-field shift (hfs) is expected for the ^{1}Hs on the convex side of the *cis* bend, whereas a low-field shift (lfs) is expected for the ^{1}Hs in the concave side of the *cis* bend. The assignment of the ^{1}H signals for the isomer which gave peak 11 was made by using a ^{1}H–^{1}H COSY spectrum, and was assigned to the 9-*cis* isomer (the hfs of the 10H and 12H signals and the lfs of the 8H and 11H signals). The assignments of both the ^{1}H signals and the configurations which gave peaks 10 and 3 were obtained by referring to the previously reported ^{1}H NMR spectra of β-carotene isomers[12], and were assigned to the 13-*cis* and 15-*cis* isomers, respectively.

Once the assignments of the ^{1}H signals for the mono-*cis* isomers had been established, the di-*cis* configurations could be easily identified by referring to the chemical shift of each mono-*cis* configuration. Although only three among five mono-*cis* isomers were found at this stage, considering the majority of the 9-*cis* and 13-*cis* isomers, the major di-*cis* isomers supposedly possess these configurations. We could isolate these isomers, which gave peaks 4, 5, 2 and 1; they were assigned to the 9,9′-*cis*, 9,13-*cis*, 9,13′-*cis* and 13,13′-*cis* isomers, respectively. The basis of the configurational assignments is described below.

Peak 4 is assigned to the 9,9′-*cis* isomer. This isomer shows "isomerization shifts" characteristic of the 9-*cis* configuration (the hfs of the 10H and 12H signals and the lfs of the 11H signal), and the number of signals observed shows that the structure of this isomer has a centre of symmetry. The 8H signal is expected to be shifted towards lower field and masked by the signal of ^{1}H in deuterobenzene.

Peak 5 is assigned to the 9,13-*cis* isomer. This isomer gives "isomerization shifts" coprresponding to the 9-*cis* configuration (the hfs of the 10H signal and the

TABLE II

ASSIGNMENT OF THE PEAKS IN THE CHROMATOGRAM

Peak No.	*Configuration*	*Peak No.*	*Configuration*
1	13,13'-*cis*	8	–
2	9,13'-*cis*	9	–
3	15-*cis*	10	13-*cis*
4	9,9'-*cis*	11	9-*cis*
5	9,13-*cis*	12	–
6	–	13	All-trans
7	–		

lfs of the 8H and 11H signals) and the 13-*cis* configuration (the hfs of the 14H and 15'H signals and the lfs of the 12H signal). It gives also chemical shifts characteristic of the all-*trans* configuration (the chemical shifts of the 7'H–14'H signals). The 15H signal was supposedly masked by the 11H signal.

Peak 2 is assigned to the 9,13'-*cis* isomer. The "isomerization shifts" of this isomer are simply an addition of those of the 9-*cis* configuration (the 10H, 11H and 12H signals) and those of the 13'-*cis* configuration (the 12'H, 14'H, 15'H and 15H signals; see the values for the 12H, 14H, 15H and 15'H signals of the 13-*cis* isomer). The chemical shifts characteristic of the all-*trans* configuration are not retained. The 8H signal must be masked by the signal of ^{1}H in deuterobenzene.

Peak 1 is assigned to the 13,13'-*cis* isomer. This isomer shows "isomerization shifts" characteristic of the 13-*cis* configuration (the hfs of the 14H signal and the lfs of the 12H and 15H signals), and the number of signals observed indicates the centre of symmetry in the molecular structure.

Table II summarizes the configurational assignment of the peaks. The presence of seven *cis*-isomers is evidenced by ^{1}H NMR spectroscopy. Englert[9] suggested the presence of additional 11-*cis*, 9,11-*cis* and 9,15-*cis* isomers. The assignment of peaks 6, 7, 8, 9 and 12 is in progress.

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REFERENCES

1 P. Mathis and C. C. Shenck, in G. Britton and T. W. Goodwin (Editors), *Carotenoid Chemistry and Biochemistry*, Pergamon Press, Oxford, 1982, p. 339.
2 R. J. Cogdell, *Pure Appl. Chem.*, 57 (1985) 723.
3 D. Siefermann-Harms, *Biochim. Biophys. Acta*, 811 (1985) 325.
4 D. Siefermann-Harms, *Physiol. Plant.*, 69 (1987) 561.
5 Y. Koyama, in N. I. Krinsky, M. M. Mathews-Roth and R. F. Taylor (Editors), *Carotenoids '87*, Plenum Press, New York, in press.
6 H. J. C. F. Nelis, P. Lavens, L. Moens, P. Sorgeloos, J. A. Jonckheere, G. R. Criel and A. P. De Leenheer, *J. Biol. Chem.*, 259 (1984) 6063.
7 Ch. Gansser and L. Zechmeister, *Helv. Chim. Acta*, 40 (1957) 1757.

8 H. J. C. F. Nelis, M. M. Z. Van Steenberge, M. F. Lefevere and A. P. De Leenheer, *J. Chromatogr.*, 353 (1986) 295.
9 G. Englert, in G. Britton and T. W. Goodwin (Editors), *Carotenoid Chemistry and Biochemistry*, Pergamon Press, Oxford, 1982, p. 107.
10 K. Tsukida, K. Saiki, T. Takii and Y. Koyama, *J. Chromatogr.*, 245 (1982) 359.
11 Y. Koyama, M. Hosomi, A. Miyata, H. Hashimoto, S. A. Reames, K. Nagayama, T. Kato-Jippo and T. Shimamura, *J. Chromatogr.*, 439 (1988) 417.
12 Y. Koyama, M. Hosomi, H. Hashimoto and T. Shimamura, *J. Mol. Struct.*, in press.