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## Note

### Separation and determination of *cis/trans*- $\beta$ -carotenes by high-performance liquid chromatography

KIYOSHI TSUKIDA\* and KAYOKO SAIKI

Kobe Women's College of Pharmacy, Motoyama-kitamachi, Kobe 658 (Japan)

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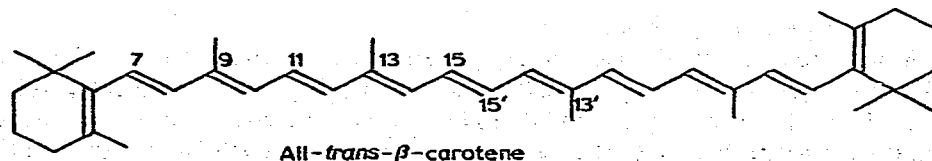
TOMOKO TAKII and YASUSHI KOYAMA

Kwansei Gakuin University, Uegahara, Nishinomiya 662 (Japan)

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It has long been recognized that all-*trans*- $\beta$ -carotene can be converted by various treatments into a quasi-equilibrium mixture of *cis/trans* isomers consisting mainly of mono-*cis* and di-*cis* forms<sup>1</sup>. However, owing to their lower vitamin A effects<sup>2</sup>, there have been no further studies on the stereochemistry of these *cis* compounds. Unfortunately, this applies even to the simplest mono-*cis* compounds, except for the central-*cis* isomer, 15-*cis*- $\beta$ -carotene, which has been synthesized<sup>3,4</sup>. Recent growing interest in the biochemical and biophysical roles of *cis*-carotenoids<sup>5</sup> led us to elucidate the structures of the two main geometrical isomers, neo-U and neo-B<sup>6</sup>, as 9-*cis*- and 13-*cis*- $\beta$ -carotene, respectively<sup>7</sup>. Vecchi *et al.*<sup>8</sup> independently succeeded in separating several *cis*- $\beta$ -carotenes by high-performance liquid chromatography (HPLC) on an alumina column using *n*-hexane with a controlled water content as the mobile phase. In spite of their great efforts, however, "hindered" mono-*cis* isomers<sup>1</sup> (7-*cis*- and 11-*cis*- $\beta$ -carotenes) were not detected in their experiments.

We present here our results on the isolation of the four mono-*cis*- and five di-*cis*- $\beta$ -carotenes by HPLC employing a lime column at ambient temperature. The thermal and photochemical stereoisomerization of all-*trans*- $\beta$ -carotene was also studied.



## EXPERIMENTAL

### Chemicals

All-*trans*- $\beta$ -carotene was kindly provided by Dr. Takahashi (Sumitomo Chemical Co., Osaka, Japan). Lime (calcium hydroxide, guaranteed reagent, particle size

1–10  $\mu\text{m}$ ) was purchased from Kishida Chemical Co. (Osaka, Japan). All solvents and reagents were of analytical-reagent grade and used without further purification.

### Spectra

Ultraviolet-visible (UV-VIS) spectra were recorded in *n*-hexane solution with a Shimadzu UV 200S instrument.

### HPLC

HPLC was performed on a Shimadzu LC2 liquid chromatograph, equipped with a UV-VIS detector, operating at either 450 or 335 nm. The column employed was made of stainless-steel tubing (250  $\times$  4 mm I.D.) and was slurry-packed using a high-pressure packing apparatus. Ultrasonic degassing (5 min) was utilized to obtain a stable suspension of lime (5 g) and tetrachloroethylene (37 ml). The slurry was then packed and rinsed with *n*-hexane (80 ml) from the solvent reservoir, care being taken not to exceed a pressure of 200 kg  $\text{cm}^{-2}$ . Injected samples were then eluted with 0.1–2% acetone in *n*-hexane at a flow rate of 1 ml  $\text{min}^{-1}$ . The acetone content in the *n*-hexane depended on the expected ingredients and the time of analysis required.

### Thermal isomerization

All-*trans*- $\beta$ -carotene was isomerized by immersing a thin-walled evacuated tube containing the crystals in a bath at 190–200°C for 15 min. The melt was rapidly solidified in ice-water and dissolved in *n*-hexane. HPLC was performed and individual peak areas were estimated after each recorded peak height using 450-nm light, being corrected by the *k* factors listed in Table I.

### Photoisomerization

An appropriate solution (10 ml) containing all-*trans*- $\beta$ -carotene (*ca.* 1.2 mg) and iodine (2% of the weight of the pigment, in *n*-hexane) was irradiated with a fluorescent lamp (20 W, 40 cm long) from a distance of 20 cm for 10 min at room temperature to produce a quasi-equilibrium mixture. After being isomerized, the solution was evaporated to dryness *in vacuo* and the residue was dissolved in *n*-hexane. HPLC was performed as described above.

## RESULTS AND DISCUSSION

### HPLC separation of *cis*- $\beta$ -carotenes

A mixture of thermally isomerized products was analysed by HPLC and eighteen different peaks were well resolved on a recording chart. Typical results are shown in Table I and in Figs. 1 and 2. Each component was fractionated, accumulated and then identified by spectroscopic determination; each isomer gave satisfactory UV-vis and  $^1\text{H}$  nuclear magnetic resonance (NMR) (in part  $^{13}\text{C}$  NMR) data, which will be described in detail elsewhere. The components were found to be one irreversible product and seventeen  $\beta$ -carotenes, including unchanged all-*trans*, four mono-*cis*, five di-*cis* and seven unidentified isomers as shown in Table I. Although the 11-*cis* isomer has not been obtained yet, it is interesting that not only the ratio of  $\epsilon$  at the  $\lambda_{\text{max}}$  to  $\epsilon$  at the *cis*-peak but also the relative adsorption affinity of each mono-*cis* isomer seem to be a function of the molecular shape and hence of the spatial configu-

TABLE I  
HPLC OF  $\beta$ -CAROTENE ISOMERS

Mobile phase, 0.5% acetone in *n*-hexane.

Peak No.	$R_f$ (min)	Configuration of $\beta$ -carotene	Correction factor ( <i>k</i> ) for peak height at 450 nm
0*	2.2		
1	4.0		$\times 1.86$
2		13,15- <i>cis</i>	$\times 1.57$
3	5.0	15- <i>cis</i>	$\times 1.36$
4			$\times 1.43$
5			$\times 1.20$
6	6.0	9,13'- <i>cis</i>	$\times 1.50$
7	6.7	13- <i>cis</i>	$\times 1.39$
8	7.6	9,15- <i>cis</i>	$\times 1.38$
9	9.0	9,13- <i>cis</i>	$\times 1.38$
11	10.0		$\times 1.59$
12	11.9	7,13'- <i>cis</i>	$\times 1.45$
13	14.0	all- <i>trans</i> (unchanged)	$\times 1$
15	21.8	9- <i>cis</i>	$\times 1.09$
16			$\times 1.43$
17	32.0	7- <i>cis</i>	$\times 1.02$

\* Iodine-catalysed isomerization of the peak 0 gave no evidence of it being a member of the  $\beta$ -carotene set.

TABLE II  
UV-VIS CHARACTERISTICS OF MONO-*cis*- $\beta$ -CAROTENES IN *n*-HEXANE

Isomer	HPLC peak No.	UV-VIS			$\epsilon$ at $\lambda_{max}$	
		$\lambda_{max}$ (nm)	$\epsilon \times 10^{-4}$ *	<i>cis</i> peak $\lambda_{max}$ (nm)	$\epsilon \times 10^{-4}$ *	$\epsilon$ at <i>cis</i> peak
All- <i>trans</i>	13	450	13.90	337	0.80	17.4
Mono- <i>cis</i> :						
7- <i>cis</i>	17	446	13.87	337	0.86	16.1
9- <i>cis</i>	15	445	12.97**	338	1.13**	11.5
13- <i>cis</i>	7	442	10.73**	336	3.88**	2.8
15- <i>cis</i>	3	448	10.32	335	5.46	1.9
Di- <i>cis</i> :						
13,15- <i>cis</i>	2	436	10.43	339	1.49	7.0
9,13- <i>cis</i>	9	439	11.98	335	1.97	6.1
9,13'- <i>cis</i>	6	438	11.18	334	2.03	5.5
9,15- <i>cis</i>	8	441	10.95	334	2.20	5.0
7,13'- <i>cis</i>	12	440	11.17	334	2.81	4.0

\* Calculated from the values for all-*trans*- $\beta$ -carotene and the quasi-equilibrium mixture therefrom at a definite temperature.

\*\* Reported values<sup>7</sup> were revised.

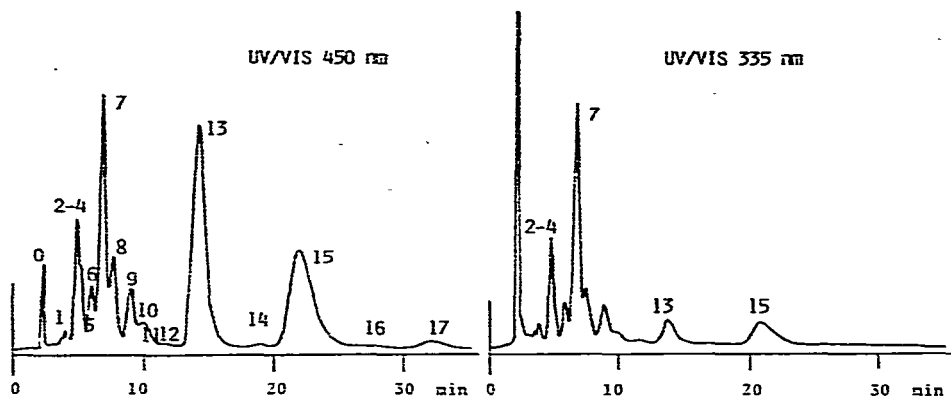


Fig. 1. HPLC of thermally isomerized products derived from all-*trans*- $\beta$ -carotene: simultaneous separation. Column (250  $\times$  4 mm I.D.), lime; mobile phase, 0.5% acetone in *n*-hexane; flow-rate, 1 ml min<sup>-1</sup>. Peak identification: see Table I.

ration (Tables I and II); thus, the 15-*cis* isomer has the most bent shape and the least adsorptivity, and the 7-*cis* isomer *vice versa*. These isomers were sufficiently stable for recording the spectra in spite of the longer accumulation times. Although five di-*cis* isomers could be identified in a similar way, seven isomers remained uncertain.

Recently Vecchi *et al.*<sup>8</sup> achieved the separation of thirteen *cis*- $\beta$ -carotenes (unchanged all-*trans*, three mono-*cis*, four di-*cis*, one tri-*cis* and four unidentified isomers) by HPLC employing an alumina column (Spherisorb A 5Y) under a "moisture control system". Compared with our procedure, their method takes a longer time for one run (*ca.* 80 min at 20°C) and each retention time ( $R_T$ ) seems to depend markedly upon the temperature and the moisture content of the mobile phase. The lime-packed column that we used is inexpensive and is durable enough for the present purpose; we have had no serious troubles with this column throughout our experiments. As minor

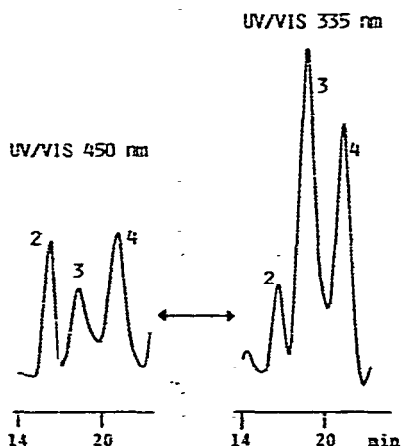


Fig. 2. HPLC of thermally isomerized products derived from all-*trans*- $\beta$ -carotene: separation of peaks 2, 3 and 4. Column (250  $\times$  4 mm I.D.), lime; mobile phase, 0.1% acetone in *n*-hexane; flow-rate, 1 ml min<sup>-1</sup>. Estimated ratio (corrected) of peaks 2:3:4 = 2.3:2.8:3.1.

isomers indicating high  $R_f$  values might be completely obscured under a tailing background in the Vecchi *et al.* method, it is apparently a great advantage of our method that good resolution can also be obtained even in such a high  $R_f$  region. Previously unknown "sterically hindered" isomers possessing the 7-*cis* geometry, *viz.*, 7-*cis*- and 7,13'-*cis*- $\beta$ -carotenes, were thus identified for the first time in this work.

#### *Trans*→*cis* isomerization

Typical results for the photochemical and thermal isomerization of all-*trans*- $\beta$ -carotene are given in Table III. The compositions of the mixtures were determined by estimating the peak area of each isomer recorded on an HPLC chart. 'Hindered' *cis* isomers (7-*cis*- and 7,13'-*cis*- $\beta$ -carotenes) were found only in the thermal isomerization products, whereas "unhindered" mono-*cis* isomers ["methylated *cis*" (9- and 13-*cis*) and "central *cis*" (15-*cis*)]<sup>9</sup> were obtained in both the photochemical and thermal isomerization mixtures. Therefore, the thermal isomerization technique may be useful for obtaining some otherwise unavailable steric forms of "sterically hindered" *cis* isomers, although the 11-*cis* isomer has not yet been observed in our experiments.

TABLE III

PERCENTAGE COMPOSITION OF *cis/trans* ISOMERIZED MIXTURES FROM ALL-*trans*- $\beta$ -CAROTENE

$\beta$ -Carotene	Content (%)	
	Thermal isomerization*	$I_2$ -catalysed photoisomerization in <i>n</i> -hexane**
All- <i>trans</i> (unchanged)	26.7	47
Mono- <i>cis</i>	46.4	40
7- <i>cis</i>	1.7	
9- <i>cis</i>	22.4	20
13- <i>cis</i>	19.5	18.5
15- <i>cis</i>	2.8	1.5
Di- <i>cis</i>	19.3	9.8
13,15- <i>cis</i>	2.3	1.3
9,13- <i>cis</i>	6.2	4.0
9,13'- <i>cis</i>	3.3	
9,15- <i>cis</i>	7.5	4.5
7,13'- <i>cis</i>	Trace	
Unidentified	7.6	3.2

\* The crystals were heated at 190–200°C for 15 min in an evacuated tube.

\*\* The solution was irradiated with a fluorescent lamp (20 W) from a distance of 20 cm for 10 min at room temperature.

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