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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION AND PROTON NUCLEAR MAGNETIC RESONANCE IDENTIFICATION OF THE 6-MONO-*cis* AND 6,6'-DI-*cis* ISOMERS OF RHODOXANTHIN

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SUMMARY

The separation by high-performance liquid chromatography of the all-*trans*, the 6-mono-*cis* and the 6,6'-di-*cis*-isomers from synthetic samples of rhodoxanthin is described. The structures of the *cis* isomers are deduced by ^1H nuclear magnetic resonance (NMR) at 400 MHz, including several homonuclear Overhauser ^1H NMR experiments and, in part, by ^{13}C NMR at 100 MHz.

INTRODUCTION

Rhodoxanthin, a natural C_{40} carotenoid with the retro diketone structure 1a (Fig. 1) constitutes the main pigment of the red fruit of the yew and also occurs in the fungus *Epicoccum nigrum* and in some bird feathers¹. As with other related compounds having retro and substituted retro end groups, there has long been some doubt as to whether these compounds should be formulated as all-*trans* according to 1a or as di-*cis* as in 1c¹. For rhodoxanthin, Mayer *et al.*² tentatively favoured the all-*trans* structure on the basis of ^1H nuclear magnetic resonance (NMR). By homonuclear Overhauser ^1H NMR experiments it was eventually conclusively shown³ that rhodoxanthin, eschscholtzanthin [all-*trans* (3S, 3'S)-4',5'-didehydro-4,5'-retro- β,β -carotene-3,3'diol] and other related retro compounds in fact possess the all-*trans* structure.

It was also found that the ^1H NMR spectra of such compounds frequently indicated the presence of at least one other minor isomer as was revealed by additional signals in the spectra⁴. This can be explained by the fact that these compounds are often not very stable. Thus, in chloroform solutions as used for the NMR measurements a slow isomerization takes place. A full spectroscopic characterization and hence an elucidation of the structure of these by-products was, however, impossible due to the superposition of the much stronger signals of the major all-*trans* component.

In this paper we report the high-performance liquid chromatographic (HPLC) separation and the NMR identification of two isomers of rhodoxanthin, namely the 6-mono-*cis* and the 6,6'-di-*cis* compounds.

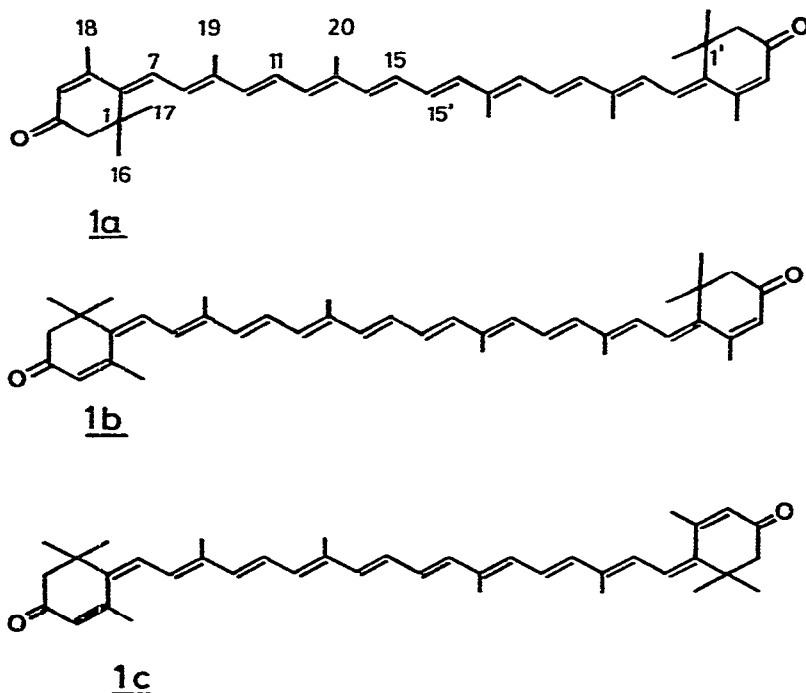


Fig. 1. Chemical structures of rhodoxanthin (1a) and its 6-*cis* (1b) and 6,6'-*di-cis* (1c) isomers.

EXPERIMENTAL

Rhodoxanthin

Crystalline samples were kindly provided by Drs. R. Marbet and R. Zell of our company. The synthesis was previously described². In order to increase the concentration of the isomers, 1 g of 1a (95% pure) was dissolved in 50 ml chloroform and 0.1 ml triethylamine was added. The solution was refluxed while stirring for 18 h. After crystallization of *ca.* 100 mg of pure 1a the remaining mother-liquor was used for the separation of the *cis* isomers.

Stability

The stability of the three chromatographic fractions dissolved in deuteriochloroform was checked by comparing their HPLC chromatograms before and after running the ¹H NMR spectra. It was found that the all-*trans* isomer is significantly isomerized, mainly to the mono-*cis* compound. Thus, a sample of 1a with an initial purity of better than 95% was found after 16 h in C²HCl₃ to consist of 46% 1a, 41% 1b and 13% 1c. The two *cis* isomers, however, were relatively stable. A sample of 1c originally of 95% purity was later found to contain *ca.* 15% 1b and 1.5% 1a. Similarly, a sample of 1b of 95% purity contained 11% 1c and 12% 1a after several hours in C²HCl₃.

Chromatography

All analytical work was performed with an HPLC unit consisting of an Altex

100 solvent delivery system, septum injection port (Perkin-Elmer) and UV/VIS detector LCD-725 (Kontron). The separation columns (500 × 3.2 mm I.D.) were home-made. Spherisorb S 5-CN (mean particle diameter 5 μm) purchased from Phase Separations was used as column packing. The mobile phase consisted of *n*-hexane-isopropyl acetate-acetone (76:17:7). The flow-rate was kept at 1 ml/min. For the collection of the three chromatographic fractions the same mobile phase but a semi-preparative column (600 × 7.7 mm) and a flow-rate of 3 ml/min were used. UV-visible spectra from 290 to 600 nm were recorded with a Variscan spectrophotometer using the stopped-flow technique.

NMR

All spectra were measured on a Bruker WM-400 FT spectrometer with an ASPECT 2000 computer (32 K data) and disk unit. The ¹H NMR spectra at 400 MHz were run in 0.6 ml C²HCl₃ (100% ²H) for sample amounts of *ca.* 0.5 mg (1a and 1b) and *ca.* 0.1 mg (1c). The ¹³C NMR spectra were obtained at 100.6 MHz in C²HCl₃. The assignments given below for 1a and, in part, for 1b are mainly based on the spectra of a mixture of 1a and 1b (*ca.* 75% and 25%; *ca.* 30 mg in 0.6 ml) which were run with several increasing concentrations of the shift reagent Yb(dpm)₃ purchased from Stohler Isotope Chemicals (dpm = dipivaloylmethane). This method gave in most cases unambiguous assignments as was previously shown for other carotenoids⁵.

¹³C NMR data

All-trans 1a (31 mg in 0.6 ml C²HCl₃, 13°C). 198.95, C(3); 154.74, C(5); 142.70, C(6); 141.32, C(9); 138.04, C(10); 137.68, C(14); 137.51, C(13); 132.56, C(12); 129.85, C(15); 128.32, C(8); 128.15, C(7); 126.93, C(11); 126.05, C(4); 54.37, C(2); 38.56, C(1); 29.87, C(16,17); 22.31, C(18); 12.91, C(20); 12.41, C(19).

6-cis 1b (1.4 mg in 0.24 ml C²HCl₃, 6°C). 199.25 and 198.97, C(3) and C(3'); 155.48, C(5); 154.99, C(5'); 143.80, C(6); 142.44, C(6'); 141.40, C(9'); 139.58, C(9); further peaks at 138.37, 137.92 (2 ×), 137.68, 137.51 and 137.48 due to C(10,10'), C(13,13') and C(14,14'); 132.56, C(12'); 132.43, C(12); 129.82 and 129.56, C(15,15'); 128.86, 128.75, 128.24, C(4'), C(7'), C(8) and C(8'); 126.91, 126.40, 125.91 and 125.48, C(11), C(11'), C(4) and C(7); 54.22, C(2'); 52.44, C(2); 41.26, C(1); 38.44, C(1'); 29.80, C(16',17'); 28.30, C(16,17); 25.46, C(18); 22.40, C(18'); 12.90, C(20,20'); 12.42, C(19,19').

6,6-Di-cis 1c (4 mg in 0.22 ml C²HCl₃, 13°C). 198.99, C(3); 155.46, C(5); 143.83, C(6); 139.58, C(9); 137.87, 137.54 and 137.05, C(10), C(13) and C(14); 132.47, C(12); 129.60, C(15); 128.83 and 128.79, C(4) and C(8); 126.40, C(11); 125.48, C(7); 52.50, C(2); 41.26, C(1); 28.30, C(16,17); 25.42, C(18); 12.88, C(20); 12.42, C(19).

RESULTS AND DISCUSSION

HPLC Separation

The chromatographic separation was accomplished by using a chemically bonded nitrile phase on silica gel as the stationary phase and *n*-hexane-isopropyl acetate-acetone (76:17:7) as the mobile phase. As is seen from Fig. 2 a complete separation of the *all-trans* and the two other isomers was achieved.

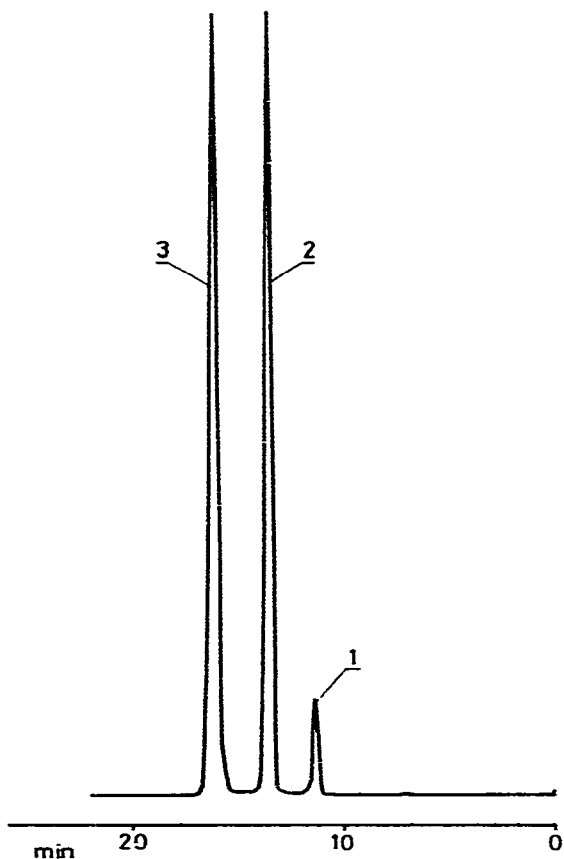


Fig. 2. Analytical high-performance liquid chromatogram of a mixture of 1a (peak 3), 1b (peak 2) and 1c (peak 1).

Identification

The 400-MHz ^1H NMR spectra including several homonuclear Overhauser experiments of the three chromatographic fractions (see Tables I and II) made possible the assignment of the isomeric structures all-*trans* (1a) to peak 3, of 6-*mono-cis* (1b) to peak 2 and 6,6'-*di-cis* (1c) to peak 1 as will readily be seen from the following arguments.

Thus, the ^1H NMR data of peak 3 were identical with those of all-*trans* rhodoxanthin³. The previous Overhauser experiments were fully confirmed by application of the improved technique of Overhauser difference spectroscopy. In addition to the signal being irradiated, in the difference spectrum (free induction decay with irradiation on-resonance minus off-resonance) only those signals appear which have altered intensities caused by the nuclear Overhauser effect⁶. This technique is especially useful in cases like this where many of the signals overlap. As previously, the relevant result for 1a (peak 3) was the observation of an enhancement of the intensity of the signal of the protons at C(8,8') of 14% upon irradiation of the protons at C(16,17,17',16') (1.387 ppm, 12 H), showing their close spatial proximity and hence

TABLE I

400-MHz ^1H NMR DATA (C^2HCl_3 , CHEMICAL SHIFTS IN ppm, COUPLING CONSTANTS IN Hz IN PARENTHESES) OF RHODOXANTHIN (1a) AND ITS 6-*cis* (1b) AND 6,6'-*Di-cis* ISOMERS (1c)

n.o. = Not observed (due to superimposed signals).

Protons	All-trans (1a)	6- <i>cis</i> (1b)	6,6'- <i>di-cis</i> (1c)
H ₃ C(16,17)		1.251	
H ₃ C(16',17')	1.387	1.386	1.251
H-C(2)		2.340	
H-C(2')	2.395	2.394	2.341
H-C(4)		≈ 5.937	
H-C(4')	5.936	≈ 5.937	5.940
H-C(7)		6.667 (12.2)	
H-C(7')	6.904 (12.7)	6.903 (12.3)	6.668 (11.9)
H-C(8)		6.551 (12.2)	
H-C(8')	6.802 (n.o.)	6.800 (n.o.)	6.551 (12)
H-C(10)		6.404 (≈ 14.5)	
H-C(10')	6.462 (≈ 15)	6.456 (14.5)	6.402 (≈ 15)
H-C(11)		6.739 (15. 11.5)	
H-C(11')	6.785 (14.5. 12)	6.781 (n.o.)	6.739 (14.5. 11.5)
H-C(12)		6.242 (11.7)	
H-C(12')	6.260 (11.5)	6.255 (11.7)	6.238 (10.9)
H-C(14,14')		≈ 6.43*	
H-C(15,15')	≈ 6.43*	≈ 6.43*	≈ 6.43*
H ₃ C(18)		2.304 (1.2)	
H ₃ C(18')	2.155 (≈ 1)	2.154 (≈ 1)	2.305
H ₃ C(19)		2.016	
H ₃ C(19')	2.029	2.029	2.017
H ₃ C(20)		1.987	
H ₃ C(20')	1.995	1.993	1.985

* Centre of gravity of the corresponding AA'BB'-type spectrum.

$\Delta^{6,6'}$ *trans*. In this context it is worth mentioning that the differentiation between the two doublets of protons at C(7,7') and C(8,8') (AB-type spectrum) is possible by the observation of an additional broadening of the latter doublet by coupling to the methyl protons at C(19,19'). $\Delta^{8,8'}$ *trans* follows from the observed effect on the protons at C(7,7') upon irradiation of the methyl protons at C(19,19'). In the same experiment the enhancement of the protons at C(11, 11') is unexpectedly small. However, $\Delta^{10,10'}$ *trans* clearly follows from the observed coupling constants $J_{10,11} = J_{10',11'}$ of ca. 15 Hz. In another experiment, namely saturation of the signal at 1.995 ppm [protons at C(20,20')], the full AA'BB'-multiplet of the strongly coupled protons at C(14,15,15',14') is enhanced. Although $\Delta^{14,14'}$ *trans* could not be derived from these experiments this will be assumed to be the case for 1a, 1b and 1c. For all three compounds the 100-MHz ^{13}C NMR spectra gave additional evidence for $\Delta^{8,8'}$ and $\Delta^{12,12'}$ *trans* from the chemical shifts of C(19,19') and C(20,20'), as is shown in the Experimental section.

TABLE II
400-MHz ¹H NUCLEAR OVERHAUSER RESULTS FOR RHODOXANTHIN AND ITS TWO *cis* ISOMERS

Compound	Irrad. signals	Enhancements	Conclusion
1a all- <i>trans</i>	1.387 ppm } H ₃ C(16,17)	H-C(2,2) 6%; H-C(8,8') 14%	A ^{6,6'} <i>trans</i>
	} H ₃ C(16',17')	H-C(7,7') ≈ 2%; H-C(4,4') ≈ 2%	
	2.395 ppm H-C(2,2')	H ₃ C(16,16',17,17') ≈ 2%; H-C(4,4') ≈ 2%	A ^{6,6'} <i>trans</i> A ^{8,8'} <i>trans</i>
	2.155 ppm H ₃ C(18,18')	H-C(4,4') 25%; H-C(7,7') 8%	
	2.029 ppm H ₃ C(19,19')	H-C(7,7') 7%; H-C(11,11') 4%	
1.995 ppm H ₃ C(20,20')	H-C(11,11') ≈ 2%; H-C(14,15,15',14') > 0**		
1b 6- <i>cis</i>	1.251 ppm H ₃ C(16,17)	H-C(7) 13%; H-C(4) ≈ 3%; H-C(2) 8%	A ⁶ <i>cis</i> A ^{6'} <i>trans</i>
	1.386 ppm H ₃ C(16',17')	H-C(8') 14%; H-C(4') ≈ 3%; H-C(2') 13%	
	2.394 ppm H-C(2')	H-C(4') ≈ 3%; H ₃ C(16',17') ≈ 2%	A ⁶ <i>cis</i> A ^{6'} <i>trans</i>
	2.340 ppm H-C(2)	H-C(4) ≈ 3%; H ₃ C(16,17) ≈ 1%	
	2.304 ppm H ₃ C(18)	H-C(4) 24%; H-C(8) 8%	
	2.154 ppm H ₃ C(18')	H-C(4') 26%; H-C(7') 9%	
1c di- <i>cis</i>	1.251 ppm H ₃ C(16,17,16',17')	H-C(7,7') 11%; H-C(2,2') 3%	A ^{6,6'} <i>cis</i> A ^{6,6'} <i>cis</i>
	2.341 ppm H-C(2,2')	H ₃ C(16,17,16',17') ≈ 1%; H-C(4,4') ≈ 2%	
	2.305 ppm H ₃ C(18,18')	H-C(4,4') 9%; H-C(8,8') 5%	

* Enhancement unexpectedly small.

** The full AA'BB'-multiplet of the four strongly coupled protons is enhanced ca. 4% per proton.

The ^1H NMR of peak 1 exhibited the same number of signals, indicating the same symmetry as 1a. Despite the small amount of sample available, several Overhauser experiments (of reduced signal-to-noise ratio) were performed which provided support for $\Delta^{6,6'}$ *cis* (see Table II).

The number of signals in the ^1H NMR of fraction 2 pointed to the lower symmetry of the molecule. The fact that the spectrum can be fully interpreted to a good approximation by a superposition of the individual spectra of fractions 3 and 1 is seen by inspection of the data of Table I. This result is quite reasonable since the influence of an end group with either Δ^6 *cis* or *trans* on the chemical shifts of the "in-chain" olefinic protons is expected to become small near the centre of the molecule.

CONCLUSIONS

By HPLC a full separation of the two major geometrical isomers from all-*trans* rhodoxanthin was successfully achieved. With the aid of the ^1H NMR spectrum, even that obtained at 400 MHz, it is practically impossible to measure quantitatively the concentration of all three components since the chemical shifts of either end group are not sufficiently influenced by the configuration of the other end of the molecule. This might explain why for retro- and substituted retro-compounds the presence of only one isomer besides the all-*trans* (1a) was discussed in the literature^{3,4}.

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