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NATURALLY OCCURRING DI-*CIS*-VIOLAXANTHINS FROM *VIOLA TRICOLOR*: ISOLATION AND IDENTIFICATION BY ^{1}H NMR SPECTROSCOPY OF FOUR DI-*CIS*-ISOMERS

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Key Word Index—*Viola tricolor*; Violaceae; 9,9'-, 9,13'-, 9,15- and 9,13-di-*cis*-violaxanthins.

Abstract—From blossoms of *Viola tricolor*, four new naturally occurring geometrical isomers of violaxanthin (5,6,5',6'-diepoxy-5,6,5',6'-tetrahydro-β,β-carotene-3,3'-diol) have been isolated in the crystalline state. By two-dimensional ^{1}H NMR methods, the new pigments are shown to be the 9,9'-, 9,13'-, 9,15- and 9,13-di-*cis* isomers. Thermal and iodine-catalysed photochemical stereomutations of the new natural pigments have also been studied.

INTRODUCTION

The majority of C_{40} carotenoids found in nature show an all-*trans* configuration about the conjugated polyene chromophore and only a few contain one or more *cis*-double bonds [1–11]. Most of our current knowledge about *cis*-carotenoids therefore has been inferred from studies on *cis*-isomers prepared artificially from the parent all-*trans*-molecules [1, 8–19]. Isolation and structure elucidation of naturally occurring *cis*-carotenoids is, however, of continuing interest because they may further our understanding of factors which regulate the occurrence of these isomers in nature.

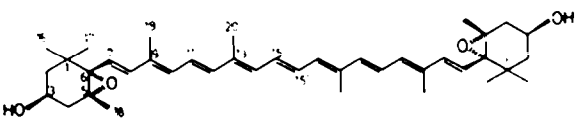
In an earlier paper describing the isolation and identification of the 13-mono-*cis* and the 15-mono-*cis* isomers of violaxanthin (3*S*,5*R*,6*S*,3'*S*,5'*R*,6'*S*-5,6,5',6'-diepoxy-5,6,5',6'-tetrahydro-β,β-carotene-3,3'-diol, **1**) from blossoms of *Viola tricolor* L. [5], we noted the occurrence of several isomeric congeners in the plant extract. UV/visible spectral analysis of the chromatographic fractions suggested that some of these contained di-*cis* isomers of **1** in quantities about 1 2% of the total carotenoid content. Subsequently, further amounts of di-*cis*-containing fractions were collected and, by means of careful column chromatography, four individual di-*cis*-violaxanthins were isolated and crystallized. The present paper describes their identification by ^{1}H NMR methods, UV/vis spectral characterization, and the results of stereomutation experiments carried out by thermal and iodine-catalysed photochemical methods [1, 5].

RESULTS AND DISCUSSION

In the final chromatographic step the di-*cis*-containing fraction was separated into four distinct zones denoted, in order of decreasing absorption affinities, as di-*cis*-neo A (**2**, mp 202°), di-*cis*-neo B (**3**, mp 135°), di-*cis*-neo C (**4**, mp 87°), and di-*cis*-neo D (**5**, mp 111°) violaxanthins. In a similar manner to our previous work [5], extreme care was taken in order to exclude secondary stereomutation of the isolated pigments during work-up and chromatographic procedures. Specially designed blank runs attested to the genuinely natural origin of the new isomeric carotenoids (see also Experimental).

The configuration of the double bonds in **2**–**5** were inferred from medium-field (200 and 300 MHz) ^{1}H NMR spectroscopy and, as usual, were based on the values of the observed interproton coupling constants and the chemical shift differences $\Delta = \delta_{cis} - \delta_{all\text{-}trans}$ measured for the olefinic protons in **2**–**5** and the all-*trans* reference compound (**1**) [20].

In order to assign the spectra in terms of chemical shifts and proton–proton couplings, conventional double resonance methods were replaced by two-dimensional (2D) correlation spectroscopic (COSY) techniques [21]. Displayed as the contour map of the absolute value 2D data matrix, the relevant parts of the 200 MHz COSY spectrum of **5** are shown in Fig. 1. Chemical shift correlations mediated by interproton spin–spin couplings are manifested by the occurrence of off-diagonal ('cross') peaks. Evaluation of the chemical shift coordinates (δ_i, δ_j) of the cross peaks affords a consistent labelling of sequentially coupled protons which, in turn, leads to a straightforward assignment of the resonances to the individual proton sites of the molecules. In addition to the more intense cross peaks due to vicinal *J*-couplings, correlations mediated by longer range (^4J,^5J) spin–spin interactions were also observed under suitable experimental conditions [21]. This facilitated establishing



1 violaxanthin

2 9,9'-di-*cis*-**1** **4** 9,15-di-*cis*-**1**

3 9,13'-di-*cis*-**1** **5** 9,13-di-*cis*-**1**

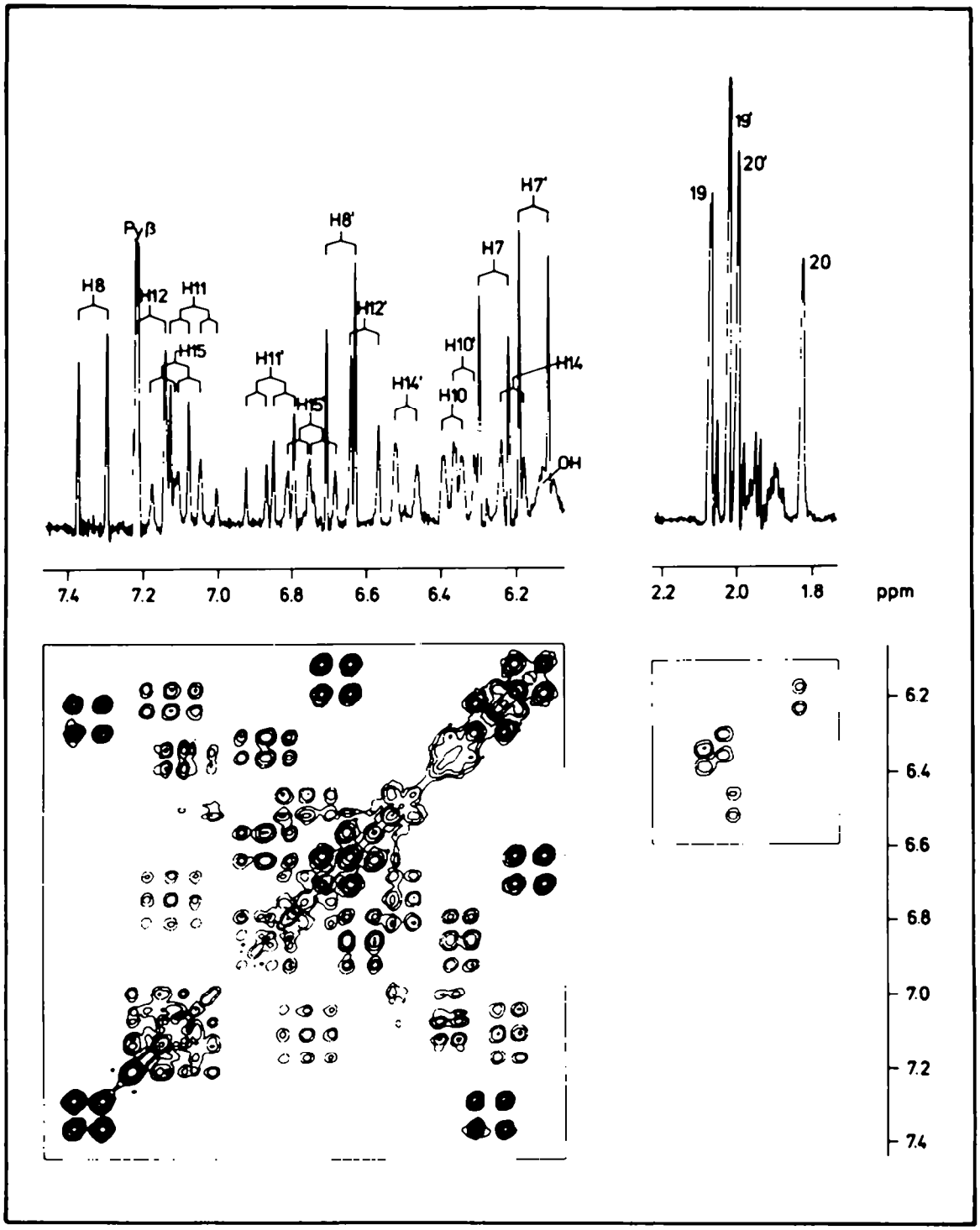


Fig. 1. Partial 200 MHz ^{1}H NMR spectrum of 9,13-di-*cis*-violaxanthin (**5**) in pyridine-d_5. Upper trace: conventional (1D) spectrum of the olefinic and 'in-chain' methyl protons; boxed regions: parts of the COSY contour map displaying chemical shift correlations *via* vicinal couplings between olefinic protons and *via* ^4J long range couplings between methyl and olefinic protons. Pyβ denotes solvent line.

proton–proton couplings across quaternary carbon atoms, an approach that proved to be of use in the assignment of 'in-chain' methyl resonances (see Fig. 1).

NOE difference spectra [22, 23] were also recorded for the identification of the head-group methyl signals and, occasionally, for the verification of assignments inferred from COSY experiments. The fully assigned ^{1}H NMR parameters for isomers **1**–**5**, together with relevant iso-

merization shift data, Δ, are collected in Table 1.

By considering the well-established trends in ^{1}H chemical shifts upon *trans*-to-*cis* isomerization of the polyene chain in carotenoid systems [20], the sites of the *cis*-double bonds in the newly isolated violaxanthin isomers could be readily identified on the basis of pertinent Δ values. Thus, for example, the significant downfield shift (Δ > 0) of resonances due to H-8 and H-11 protons and the increased shielding (Δ < 0) of H-10 and H-12 with respect to their chemical shifts in the all-*trans* **1** have been shown to be typical for *trans*-to-*cis* isomerization about the 9(10)-double bond [20] and to reflect variations in the resulting steric interactions (and diamagnetic anisotropy effects) experienced by these protons in the 9(10)-*cis* and 9(10)-*trans* environments. From inspection of the differential shieldings Δ in Table 1 it follows that the 9-*cis* configuration is a stereochemical property common to each of the new violaxanthin isomers **2**–**5**. Symmetry considerations furthermore indicate that this very isomerism occurs twice in **2** which settles the geometrical configuration of this molecule as 9,9′-di-*cis*. Analoguous differential shieldings of protons of neighbouring positions (and the characteristic change in the ^3J(H-15, H-15′) coupling value for isomer **4**) attest to the occurrence of a second *cis*-configuration about the 13′(14′) (**3**), 15(15′) (**4**) and 13(14) (**5**) double bonds in the other natural di-*cis*-violaxanthin isomers and define the stereochemistry of the polyene chains as 9,13′-di-*cis* (neo B, **3**), 9,15-di-*cis* (neo C, **4**) and 9,13-di-*cis* (neo D, **5**), respectively.

Displayed in Figs 2 and 3 are the pertinent UV/VIS absorption curves; the relevant spectral data, including λ_{max} and Q ($= E_{max}/E_{cis\text{-}peak}$) values, are compared with those of the all-*trans*- and mono-*cis*-violaxanthins in Table 2. It can be seen that, as compared to their values in the all-*trans* form (**1**), the absorption maxima in the new isomers exhibit hypsochromic shifts that are roughly

Table 1. ^{1}H NMR data of isomeric violaxanthins*

H	all-*trans* (**1**) δ_H	9,9′-di-*cis* (**2**) δ_H	Δ	9,13′-di-*cis* (**3**) δ_H	Δ	9,15-di-*cis* (**4**) δ_H	Δ	9,13-di-*cis* (**5**) δ_H	Δ
7	6.170	6.230	0.06	6.230	0.06	6.236	0.08	6.254	0.08
8	6.665	7.338	0.67	7.339	0.67	7.337	0.67	7.332	0.67
10	6.369	6.277	−0.09	6.277	−0.09	6.313	−0.06	6.331	−0.04
11	6.860	7.069	0.21	7.068	0.21	7.101	0.24	7.070	0.21
12	6.635	6.552	−0.08	6.546	−0.09	6.571	−0.06	7.167	0.53
14	6.484	6.430	−0.05	6.486	0.00	6.931	0.45	6.208	−0.27
15	6.849	6.744	−0.11	6.705	−0.14	6.514	−0.34	7.107	0.62
Me-16	1.262	1.253	−0.01	1.259	0.00	1.259	0.00	1.260	0.00
Me-17	1.156	1.137	−0.02	1.140	−0.02	1.139	−0.02	1.137	−0.02
Me-18	1.288	1.273	−0.01	1.274	−0.01	1.278	−0.01	1.275	−0.01
Me-19	2.036	2.052	0.02	2.056	0.02	2.056	0.02	2.075	0.04
Me-20	2.024	1.796	−0.23	1.813	−0.21	1.808	−0.21	1.827	0.19
7′	6.170	6.023	0.06	6.170	0.00	6.165	0.00	6.150	−0.02
8′	6.665	7.338	0.67	6.689	0.02	6.685	0.02	6.663	0.00
10′	6.369	6.277	−0.09	6.391	0.02	6.406	0.04	6.367	0.00
11′	6.860	7.069	0.21	6.862	0.00	6.906	0.04	6.852	0.01
12′	6.635	6.552	−0.08	7.221	0.59	6.675	0.04	6.623	−0.01
14′	6.484	6.430	−0.05	6.270	−0.21	7.015	0.53	6.489	0.00
15′	6.849	6.744	−0.11	7.140	0.29	6.554	−0.30	6.740	−0.11
Me-16′	1.262	1.253	−0.01	1.259	0.00	1.259	0.00	1.260	0.00
Me-17′	1.156	1.137	−0.02	1.151	0.00	1.148	0.00	1.154	0.00
Me-18′	1.288	1.273	−0.01	1.288	0.00	1.288	0.00	1.288	0.00
Me-19′	2.036	2.052	0.02	2.035	0.00	2.035	0.00	2.024	−0.01
Me-20′	2.024	1.796	−0.23	2.016	0.00	1.988	−0.02	2.002	−0.02
$J_{7,8}$	15.5	15.4		15.4		15.5		15.5	
$J_{10,11}$	11.2	11.4		11.4		11.4		10.6	
$J_{11,12}$	14.8	14.9		14.9		14.8		14.9	
$J_{14,15}$	10.9	11.0		11.0		11.0		12.0	
$J_{15,15'}$	14.1	14.1		14.1		12.3		14.1	
$J_{7',8'}$	15.5	15.4		15.4		15.5		15.5	
$J_{10',11'}$	11.2	11.4		11.3		11.4		11.2	
$J_{11',12'}$	14.8	14.9		14.8		15.1		14.8	
$J_{14',15'}$	10.9	11.0		11.0		11.0		12.0	

Head-group protons:† H-2$_{ax}$ 1.605, H-2$_{eq}$ 1.932, H-3 4.313, H-4$_{ax}$ 2.016, H-4$_{eq}$ 2.672; $J_{2_{ax},2_{eq}} = -12.7$, $J_{2_{ax},3} = 10.4$, $J_{2_{eq},3} = 3.6$, $J_{2_{eq},4_{eq}} = 1.5$, $J_{3,4_{ax}} = 8.8$, $J_{3,4_{eq}} = 4.9$, $J_{4_{ax},4_{eq}} = -14.2$.

*In pyridine-d_5 soln, at 25°. Chemical shifts are in ppm relative to internal TMS, coupling constants in Hz. For strongly coupled spin systems, chemical shift and coupling values were obtained by means of computer-aided iterative analyses using frequency lists at 300 MHz. $\Delta = \delta_{cis} - \delta_{all\text{-}trans}$

†These resonances show minor (≤ 0.01 ppm) upfield shifts upon *trans*/*cis* isomerization at the 9(9′)-double bond.

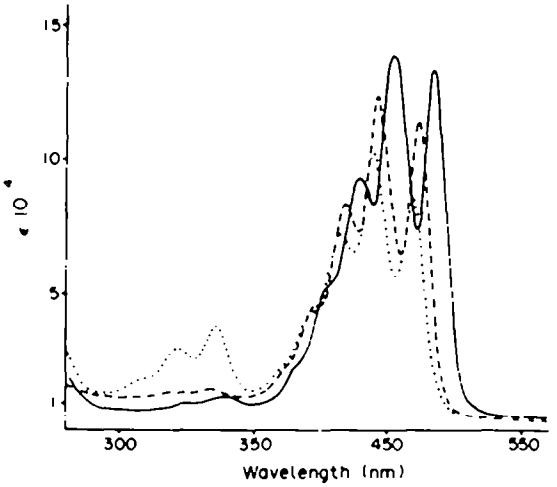


Fig. 2. UV and visible light absorption spectra of all-*trans*-violaxanthin (——), 9,9'-di-*cis*-violaxanthin (- - - - -) and 9,13'-di-*cis*-violaxanthin (.....) in benzene.

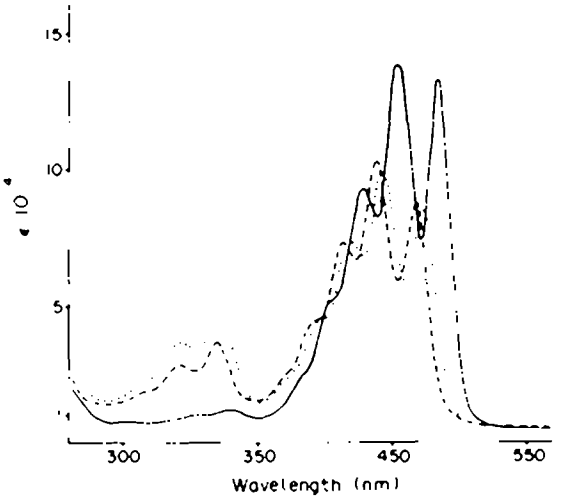


Fig. 3. UV and visible light absorption spectra of all-*trans*-violaxanthin (——), 9,13-di-*cis*-violaxanthin (- - - - -) and 9,15-di-*cis*-violaxanthin (.....) in benzene.

twice as large as those measured for the mono-*cis* isomers, a characteristic feature of di-*cis* C_{40} carotenoids [2] which served for the initial detection of **2**–**5** in our previous work [5]. More specifically, if the actual, NMR-based, configurations are also taken into account, the shifts in the λ_{max} values for di-*cis* **2**–**5** are seen to be the sums of the shifts measured for the pertinent mono-*cis* isomers. In a similar, retrospective, manner the Q values of the di-*cis* isomers can also be accounted for by noting the absence of a *cis*-peak in the 9-(or 9')-mono-*cis* isomer and the characteristic variations in the Q values according to the position of the *cis*-configured double bonds in the polyene chain.

The newly isolated pigments were subjected to stereomutations induced by iodine-catalysed photoisomerization and thermal effects [1, 5]. The isomeric compositions of the pertinent product mixtures are given in Table 3. It can be seen that, while the composition of the product mixtures obtained by catalytic photoisomerization is largely independent of the actual geometric

Table 2. UV/visible spectral data of di-*cis* violaxanthins in benzene compared with all-*trans* and mono-*cis* forms

Isomer	λ_{max} (nm)	λ_{max} shift (nm)	Q
all-*trans* (**1**)	483, 453, 426		
9-*cis*	478, 448, 422	5	> 10
13-*cis*	475, 445, 419	8	2.00
15-*cis*	478, 448, 423	5	1.61
9,9'-di-*cis* (**2**)	472, 442, 418	11	> 10
9,13'-di-*cis* (**3**)	470, 440, 415	13	2.92
9,15-di-*cis* (**4**)	474, 443, 419	9	2.46
9,13-di-*cis* (**5**)	469, 440, 415	14	3.28

configuration of the starting molecule, substantial variations in the composition are obtained when the stereomutation is carried out by means of thermal isomerization. The observed lower conversions, the configuration-dependent extent of stereomutation and the formation of the 9-mono-*cis* isomer as the major stereomutation product are all in accordance with the known high resistance against isomerization of the 9(9')-*cis* double bond of 5,6-epoxy carotenoids [8–11].

It may be noted that preparation of di-*cis*-violaxanthins from all-*trans* **1** by means of iodine-catalysed photoisomerization was found to be impractical. This is due partly to the relatively low yields of the di-*cis*-isomeric products (see Table 3) and partly to technical difficulties encountered in their chromatographic separation from epimeric luteoxanthins (5,6,5',8'-diepoxy-5,6,5',8'-tetrahydro-β,β-carotene-3,3'-diol) and auroxanthins (5,8,5',8'-diepoxy-5,8,5',8'-tetrahydro-β,β-carotene-3,3'-diol), by-products of the photoisomerization reaction.

Our attempts to isolate the 13,13'- and 13,15-di-*cis*-violaxanthins, the remaining two di-*cis* isomers of **1** with 'unhindered' *cis* double bonds, either from the natural pigment or from photoisomerization product mixtures were unsuccessful.

Table 3. Composition of mixtures obtained from di-*cis*-violaxanthins A, B, C and D

	Percentage of isomers in the pigments recovered			
Starting material	All-*trans*	9-*cis*	Di-*cis*	Unidentified (mainly monofuranoids)
(a) By refluxing in benzene solution in the dark				
2	—	6.2	84.5	9.3
3	1.3	43.7	55.0	—
4	2.0	43.7	54.3	in traces
5	1.6	31.7	65.2	1.5

(b) By iodine-catalysed stereomutation in light			
	All-*trans*	9-*cis* + 13-*cis*	Di-*cis*
2	51.7	34.5	13.7
3	51.2	36.2	12.6
4	46.9	39.2	13.9
5	50.3	36.6	13.1
Average value	50.0	36.6	13.3
1	51.8	36.1	12.1

EXPERIMENTAL

Biological materials and methods. Yellow blossoms of *Viola tricolor* (fr. wt 2.9 kg) were collected near Pécs (southern Hungary) in May 1979. General handling methods, chromatography and quantitative determination of the carotenoid content have been described elsewhere [5, 24]. All operations were performed in the dark. Mps were determined with a Boetius hot stage apparatus and are uncorrected. UV/VIS spectra were recorded with a Perkin Elmer 402 instrument.

A Bruker AM-300 NMR instrument was used to obtain the conventional (1D) ^{1}H NMR spectra which served for computer-aided spectral analyses. Homonuclear (^{1}H) chemical shift correlated 2D spectra were obtained on a Bruker WP-200/SY spectrometer by incrementing in a regular manner the length of the period t_1 in the 90-t_1-90-t_2 COSY sequence and accumulating the NMR response during t_2 [21]. Time-domain data matrix size was 1024* 256, this was transformed after sine-bell multiplication in both dimensions to give a symmetrized, absolute value correlation map of digital resolution 2 Hz/data point. The difference method was used to obtain the 1D NOE spectra and low power selective gated preirradiation technique was employed to induce the NOE effects [22, 23]. In order to prevent cleavage of the highly sensitive epoxide rings during measurements, all NMR spectra were obtained on pyridine-d_5 solns (3 mg in 0.5 ml) at ambient temp, using TMS as the internal reference.

Pigment isolation. After extraction, saponification, chromatography and the usual work-up [5, 24] the zones between 9-*cis* violaxanthin and 1 were combined and after the usual procedures (89 mg) the soln was subjected to re-chromatography on $CaCO_3$ (Biogal, Hungary) with 20% petrol in C_6H_6 (O_2-free). The following zones were obtained: band 1 (9-*cis*-violaxanthin), band 2 (di-*cis*-neoviolaxanthin A, 2; 15.4%), band 3 (di-*cis*-neoviolaxanthin B, 3; 41.7%), band 4 (di-*cis*-neoviolaxanthin C, 4); band 5 (di-*cis*-neoviolaxanthin D, 5; 28.9%) and band 6 (violaxanthin). After the usual work-up, the C_6H_6 solns were evaporated to dryness *in vacuo* at 30°, and the residues were crystallized from C_6H_6 by addition of petrol at −20°.

Di-cis-neoviolaxanthin A (2). Orange-coloured prisms (3.4 mg) were obtained, mp 202°. VIS $\lambda_{max}^{C_6H_6}$ nm (log ε): 472 (5.06), 442 (5.09) and 417 (4.91); $Q > 10$; λ_{max}^{petrol} nm: 460, 432 and 408; λ_{max}^{EtOH} nm: 461, 432 and 409. $\lambda_{max}^{C_6H_6}$ nm (after acid treatment): 435, 408 and 387. The UV spectrum is presented in Fig. 2.

Di-cis-neoviolaxanthin B (3). Dark red, irregular forms (12.8 mg) were precipitated, mp 135°, which, on friction formed needles and prisms on a microscopic slide. VIS $\lambda_{max}^{C_6H_6}$ nm (log ε): 470 (4.92), 440 (4.95) and 416 (4.84); $Q = 3.00$; λ_{max}^{petrol} nm: 458, 429 and 406; λ_{max}^{EtOH} nm: 459, 430 and 407. $\lambda_{max}^{C_6H_6}$ nm (after acid treatment): 432, 407 and 385. The UV spectrum is shown in Fig. 2.

Di-cis-neoviolaxanthin C (4). Red, amorphous forms (3.3 mg) were obtained, mp 87°, which, on friction, resulted in needles on a microscopic slide. VIS $\lambda_{max}^{C_6H_6}$ nm (log ε): 474 (4.91), 443 (4.98) and 419 (4.85); $Q = 2.29$; λ_{max}^{petrol} nm: 461, 433 and 410; λ_{max}^{EtOH} nm: 462, 434 and 411. $\lambda_{max}^{C_6H_6}$ nm (after acid treatment): 434, 408 and 386. The UV spectrum is presented in Fig. 3.

Di-cis-neoviolaxanthin D (5). Orange-coloured, amorphous material (5.8 mg) was obtained, mp 111°, which, on friction, formed prisms on a microscopic slide. VIS $\lambda_{max}^{C_6H_6}$ nm (log ε): 469 (4.81), 440 (5.00) and 416 (4.74); $Q = 3.25$; λ_{max}^{petrol} nm: 461, 433 and 410; λ_{max}^{EtOH} nm: 462, 434 and 411. $\lambda_{max}^{C_6H_6}$ nm (after acid treatment): 432, 406 and 385. The UV spectrum is presented in Fig. 3.

Reference compound. Violaxanthin (1). Mp 189°; VIS $\lambda_{max}^{C_6H_6}$ nm (log ε): 483 (5.12), 453 (5.13) and 426 (4.95); λ_{max}^{petrol} nm: 470, 439 and 417; λ_{max}^{EtOH} nm: 471, 441 and 417. $\lambda_{max}^{C_6H_6}$ nm (after acid treatment): 436, 410 and 387. The UV spectra are shown in Figs 2 and 3.

Stereomutation. (a) Thermal: the di-*cis* isomer (0.3 mg) in 30 ml C_6H_6 was refluxed in darkness for 2 hr. (b) I_2 catalysis in light: the di-*cis*-isomer (0.3 mg) in 50 ml C_6H_6 was left to stand with I_2 (6×10^{-3} mg) in diffuse daylight, at room temp. for 40 min. The extent of the stereomutation was monitored by UV spectroscopy.

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