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OCCURRENCE OF 15-*CIS*-VIOLAXANTHIN IN *VIOLA TRICOLOR*

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Key Word Index—*Viola tricolor*; Violaceae; 15-*cis*-(= central-mono*cis*)-violaxanthin; 13-*cis*-violaxanthin; distribution of carotenoids.

Abstract—A new *cis* isomer in the violaxanthin series has been isolated from the blossoms of *Viola tricolor* and identified by MS, IR and UV as the central-mono*cis* form. It was converted to all-*trans*-violaxanthin by stereomutation. The CD correlation between 15-*cis*-violaxanthin and natural violaxanthin (5,6,5′,6′-diepoxy-5,6,5′,6′-tetrahydro-β,β-caroten-3,3′-diol) provided the basis for assignment of the absolute configurations 3*S*, 5*R*, 6*S*, 3′*S*, 5′*R*, 6′*S*. *Trans–cis* isomerization of all-*trans*-violaxanthin also resulted in 15-*cis*-violaxanthin. In addition a quantitative determination of the carotenoids was conducted.

INTRODUCTION

Central-mono*cis* carotenoids generally regarded as the most labile mono*cis* isomers, are often claimed not to occur in nature and not to appear in *trans–cis* equilibria [1–4]. It is known, however, that two central mono*cis* C_{40}-precursors (15-*cis*-phytoene and 15-*cis*-phytofluene) do occur in nature [5–10], and in the *trans-cis* equilibrium mixture of all-*trans*-canthaxanthin the central-mono*cis* form is also present [11]. Since many 15-*cis* carotenoids (lycopene, β-carotene, β-cryptoxanthin, zeaxanthin, canthaxanthin) have been synthesized, most of our knowledge on the properties of central-mono*cis* carotenoids comes from the synthetic field [2]. It should be noted that the natural occurrence of a central-mono*cis* carotenoid with the most common nonaen, decaen or undecaen chromophores has not been reported.

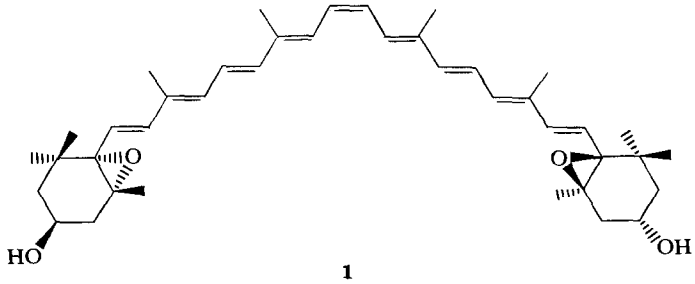
We now report the identification of 15-*cis*-violaxanthin (**1**) in the blossoms of *Viola tricolor* and the studies on the geometrical configuration of this new natural central-mono*cis*-C_{40} carotenoid.

RESULTS AND DISCUSSION

Although the overwhelming majority of plant carotenoids possess the all-*trans* configuration, several plants contain a large amount of mono*cis* carotenoids. For example the ratio between 9-*cis*-violaxanthin and all-*trans*-violaxantin present in the blossoms of *Viola tricolor* is 1.7:1, and that between 9-*cis*-antheraxanthin and all-*trans*-antheraxanthin (5,6-epoxy-5,6-dihydro-β,β-caroten-3,3′-diol) present in the pollen sacks of *Lilium candidum* is 8.1:1. These data demonstrate the great stability of the 9-*cis* type configuration of 5,6-epoxy-C_{40} carotenoids in nature [12]. To clarify the possible generality of this finding, a systematic investigation was undertaken to detect 13-*cis* and 15-*cis*-epoxy carotenoids in the blossoms of *Viola tricolor* in which plant they might be expected to occur along with 9-*cis*-violaxanthin and be somehow protected. *V. tricolor* was used because it readily yields large quantities of *cis*-epoxy carotenoids.

To avoid *trans–cis* rearrangement during the isolation procedure, all operations were carried out under very mild conditions and every step of the procedure (dehydration, extraction, saponification, washing with water, drying over dry Na_2SO_4, chromatography, elution, evaporation) was checked in control experiments. It was found that the pigments of *V. tricolor* are not converted into 15-*cis*-volaxanthin (**1**) during the work-up. It can, therefore, be stated that 15-*cis*-violaxanthin (**1**) is a genuine pigment in the blossoms of *V. tricolor*.

Central-mono*cis*-violaxanthin (**1**) isolated in a crystalline state (mp 107°) from *V. tricolor* had the same



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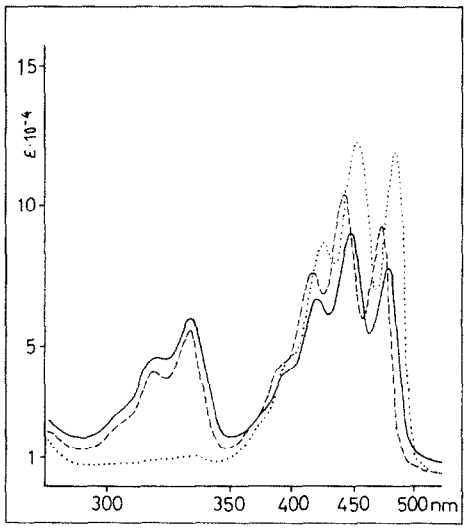


Fig. 1. UV and visible light absorption spectra of 15-*cis*-violaxanthin (——), 13-*cis*-violaxanthin (- - - - -) and all-*trans*-violaxanthin (.....) in benzene.

physical and chemical properites as that obtained by stereomutation of natural all-*trans*-violaxanthin. The MS confirmed the formula $C_{40}H_{56}O_4$, and indicated the presence of two hydroxyl groups and two epoxy groups: (rel. int.) *m/e* 600.4163 (22; M), 584 (2; M-16), 508 (3; M-92), 493 (2; M-107), 221 (100), 181 (35). The visible absorption spectrum of 15-*cis*-violaxanthin (**1**) exhibited a well-defined, fine structure and had less intense absorption in the region of the principal maxima than the other unhindered mono*cis* forms of violaxanthin (Fig. 1). The shift (5 nm) in λ_{max} of 15-*cis*-violaxanthin (**1**) [$\lambda_{max}^{C_6H_6}$ nm (ε_{max}): 478 (81 000), 448 (95 000), 423 (68 000) and 337 (59 000)], compared with that of all-*trans*-violaxanthin (Fig. 1) indicates that the form was a mono*cis* isomer with a sterically unhindered *cis* bond. The very strong *cis* peak (Q = 1.61), which is higher than that of 13-*cis*-violaxanthin (**2**) [$\lambda_{max}^{C_6H_6}$ nm (ε_{max}): 475 (99 700), 445 (115 200), 419 (81 200) and 337 (58 700); Q = 2.00] suggested that the *cis* double bond was central in position. Furthermore, at 780 cm^{-1} central-mono*cis*-violaxanthin (**1**) exhibited a strong band which was absent from the IR spectra of 9-*cis*, 13-*cis*- and all-*trans*-violaxanthin. As is known [13] this peak at 780 cm^{-1} is characteristic of C—H out of plane deformation vibration of a *cis*-disubstituted double bond.

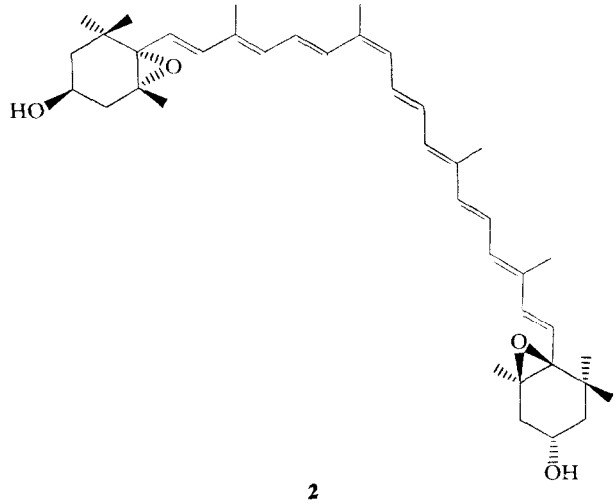
The CD spectrum of all-*trans*-violaxanthin formed by iodine-catalysed stereomutation of central-mono*cis*-violaxanthin (**1**) and 13-*cis*-violaxanthin (**2**) was the same as that of natural violaxanthin. The absolute configuration of the chiral centres of central-mono*cis*-violaxanthin (**1**) and 13-*cis*-violaxanthin (**2**) can therefore, be described as 3*S*, 5*R*, 6*S*, 3′*S*, 5′*R*, 6′*S*. The CD spectra of central-mono*cis*-violaxanthin (**1**) and 13-*cis*-violaxanthin (**2**) resembled each other [14], but were the inverse of that of all-*trans*-violaxanthin.

15-*cis*-Violaxanthin (**1**) is photolabile and in diffuse daylight, at room temperature, it rearranged almost completely into a mixture of all-*trans*-violaxanthin and 9-*cis*-violaxanthin, in which 95–97% of the total pigments was all-*trans*-violaxanthin. The effect of illumination on the Q values of 15-*cis*-vioaxanthin (**1**) and 13-*cis*-violaxanthin (**2**) is shown in Fig. 2. As is seen, there was a slight difference in photosensitivity to scattered daylight between 15-*cis*-violaxanthin (**1**) and 13-*cis*-violaxanthin (**2**). Since wavelengths above 550 nm do not act on them, the isomers can be handled safely in red light.

Stereomutation of 15-*cis*-violaxanthin (**1**) in refluxed solution [1] and in darkness resulted in an equilibrium mixture (Table 1). The Q value was only moderately affected by the direction of the stereomutation, i.e. it did not make much difference whether it was started from the all-*trans*, or the 15-*cis* form. The amount of the 15-*cis* form in the equilibrium was about 6.0%. For comparison the composition of an iodine-catalysed equilibrium mixture which was prepared [1] from all-*trans*-violaxanthin is also shown in Table 1.

Acid treatment of 15-*cis*-violaxanthin (**1**) gave a furanoid derivative with the λ_{max} characteristic of all-*trans*-auroxanthin; central-mono*cis*-auroxanthin was never detected.

The distribution of carotenoids and additional information on the identification of the pigments are presented in Table 2.



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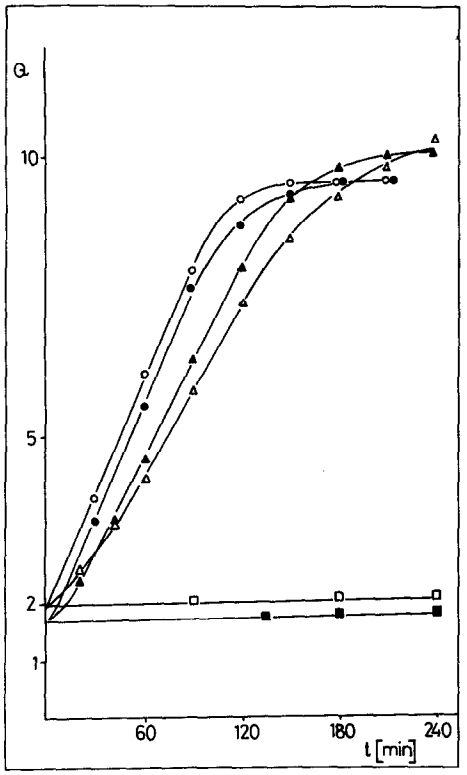

Fig. 2. The effect of illumination on the Q values of 15-*cis*-violaxanthin (●—● irradiation at 366 nm, ▲—▲ diffuse daylight, ■—■ red light) and 13-*cis*-violaxanthin (○—○ irradiation at 366 nm, △—△ diffuse daylight, □—□ red light). AU solutions in benzene.

EXPERIMENTAL

Biological material and methods. Yellow blossoms of *Viola tricolor* were collected at Pécs in April 1979. General methods, including instrumentation, chromatography and quantitative determination of carotenoids have been described elsewhere [15]. All operations were performed in darkness.

Pigment isolation. Immediately after collection 30 g of the blossoms were placed into 300 ml cold MeOH (2–4°) and allowed to stand for dehydration. After 2 hr, the blossoms were filtered by suction, and were kept for extraction in 150 ml MeOH at 2–4° for 30 min. The yellow methanolic soln was sucked through a Buchner funnel and extraction was completed with 50 ml MeOH and 250 ml Et_2O. The methanolic and ethereal extracts were combined, washed with cold H_2O, dried ($NaSO_4$), and saponified with 30% KOH–MeOH at room temp. for 14 hr. After saponification, the ethereal soln was washed free of alkali with H_2O, dried (Na_2SO_4), evapd to dryness *in vacuo*, dissolved in C_6H_6, and chromatographed on $CaCO_3$ (Biogal, Hungary) with C_6H_6 (Table 2). After usual work-up, the zone at the top of the column (a mixture of 13-*cis*- and 15-*cis*-violaxanthin) was subjected to re-chromatography on $CaCO_3$ with C_6H_6 containing increasing amounts of Me_2CO up to 4%. Two zones developed: 13-*cis*- and 15-*cis*-violaxanthin, the latter being absorbed below the former. After chromatography, all zones but the top zone (mixture of 13-*cis*- and 15-*cis*-vioxanthin) were combined and the combined pigments were treated exactly in the same way as the above-mentioned methanolic and ethereal extracts. No formation of 15-*cis*-violaxanthin was observed. Isolation of 15-*cis*-violaxanthin (**1**) and 13-*cis*-violaxanthin (**2**) in a solid state followed the above procedures starting with 500 g of blossoms.

15-cis-*Violaxanthin* (**1**). The C_6H_6 soln was evapd to dryness *in vacuo* at 30°, and the residue was crystallized from C_6H_6 by addition of petrol at −20°. Irregular yellow plates (3.5 mg) were obtained, mp 109°, which, on friction, formed orange-coloured prisms on a microscope slide. **1** had *m/e* 600.4163 (M^+) and the predicted fragmentation pattern [16]. $\lambda_{max}^{C_6H_6}$ nm (log ε): 479 (4.91), 448 (4.98), 423 (4.83) and 337 (4.77); Q = 1.61; λ_{max}^{hexane} nm: 465, 436 and 412; $\lambda_{max}^{C_6H_6}$ nm (after acid treatment): 435, 408 and 387; ν_{max}^{KBr} cm^{-1}: 780. The UV spectrum is presented in Fig. 1.

13-cis-*Violaxanthin* (**2**) was crystallized as for 15-*cis*-violaxanthin; yield 12.4 mg of bright yellow plates, mp 108°.

Table 1. Composition of equilibrium mixtures obtained from all-*trans*-, 15-*cis*- and 13-*cis*-violaxanthin

Starting material	$E_{max}/E_{cis\text{-}peak}$ Starting material	In equilibrium	Proportion (%) of isomers in the pigments recovered All-*trans*	15-*cis*	13-*cis*	9-*cis*	Di*cis* and furanoid
(1) By refluxing the benzene solution in darkness							
All-*trans*	>10	5.07	71.9	4.7	18.6	—	4.8
15-*cis*	1.65	4.55	68.2	8.8	18.5	—	4.5
13-*cis*	1.98	4.57	66.7	4.5	26.1	—	2.7
Average value		4.73	68.9	6.0	21.1		4.0
(2) By iodine-catalysed stereomutation in light							
All-*trans*	>10	5.8	51.8	3.4	8.9	23.8	12.1
15-*cis*	1.65	5.50	50.0	3.1	12.3	23.3	11.3
13-*cis*	1.98	5.80	49.4	3.6	12.8	24.4	9.8
Average value		5.70	50.4	3.4	11.3	23.8	11.0

Table 2. Quantitative distribution of carotenoids in the blossoms of *Viola tricolor*

Carotenoid*	(mg/g) Dry wt	Percentage of total caroteniods	Abs. maxima (nm) C_6H_6	Abs. maxima (nm) $HCl–C_6H_6$
13-*cis*-			475	434
Violaxanthin	0.16	1.7	445	408
			420	386
15-*cis*-			478	435
Violaxanthin	0.06	0.6	448	408
			423	387
9-*cis*-			478	435
Violaxanthin	4.97	51.3	448	408
			424	387
9,9′-*cis*-			483	464
Antheraxanthin	0.14	1.4	453	436
			430	412
			458	436
Luteoxanthin	0.05	0.5	432	410
			408	387
Di*cis*-			472	434
violaxanthin	0.09	0.9	442	408
			415	387
Di*cis*-			470	433
violaxanthin	0.17	1.8	441	408
			415	386
Di*cis*-			468	433
violaxanthin	0.15	1.5	439	408
			414	385
			483	436
Violaxanthin	2.87	29.6	452	409
			426	387
			488	464
Antheraxanthin	0.50	5.2	458	437
			433	413
			487	
Lutein	0.39	4.0	457	
			432	
			494	
β,β-Carotene	0.14	1.4	464	
Total	9.69			

*Carotenoids in order of decreasing adsorption on column chromatography (see Experimental).

It had *m/e* 600.4163 (M^+) and the expected fragments [16]. $\lambda_{max}^{C_6H_6}$ nm (log ε): 475 (5.00), 445 (5.06), 419 (4.91) and 337 (4.77); Q = 2.00; λ_{max}^{hexane} nm: 462, 433 and 409; $\lambda_{max}^{C_6H_6}$ nm (after acid treatment): 434, 408 and 386. The UV spectrum is shown in Fig. 1.

Violaxanthin, violeoxanthin, antheraxanthin, lutein and β-carotene were isolated in a crystalline state and showed the expected properties. Luteoxanthin (5,6,5′,8′-diepoxy-5,6,5′,8′-tetrahydro-β,β-carotene-3,3′-diol), di*cis*-violaxanthins and 9-*cis*-antheraxanthin were identified in solution (Table 2).

Preparation of 15 cis- *and* 13-cis-*violaxanthin by stereomutation in refluxed solution.* All-*trans*-violaxanthin (110 mg) in 550 ml C_6H_6 was refluxed in darkness for 2 hr. The rate of stereomutation was monitored by UV spectroscopy at 337 nm; Q (at equilibrium) = 5.70. After usual work-up described above, 4 mg 15-*cis*-violaxanthin (mp 109°), 15.4 mg 13-*cis*-violaxanthin (mp 108°) and 65 mg of all-*trans*-violaxanthin were obtained. The physical and chemical properties of 15-*cis*- and 13-*cis*-violaxanthin were identical with those for the compounds originating from natural pansy blossoms. The composition of the stereoisomeric mixture is presented in Table 1.

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