179. *trans/cis* Isomerization of Astaxanthin Diacetate/Isolation by HPLC. and Identification by ^{1}H-NMR. Spectroscopy of Three Mono-*cis*- and Six Di-*cis*-Isomers

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Dedicated to Dr. *O. Isler* on the occasion of his 70th birthday

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Summary

Thermal and iodine-catalyzed photochemical *trans/cis* isomerization of synthetic, racemic astaxanthin diacetate (3,3′-dihydroxy-β,β-carotene-4,4′-dione diacetate) yielded multi-component mixtures of *cis*-isomers. Separation and isolation of 10 different *cis*-isomers in quantities between 10 and 70 µg was achieved by HPLC. Investigation of their 270-MHz-FT-^{1}H-NMR. spectra led to the identification of 9 of these isomers, namely the 9-, 13-, and 15-mono-*cis*-, the 9,9′-, 9,13-, 9,13′-, 9,15-, 13,13′-, and 13,15-di-*cis*-astaxanthin diacetate.

Introduction. – Although *cis* isomeric retinoids and carotenoids are known to occur in nature only relatively rarely [1] [2], a growing interest has for many years been devoted to the 'artificial' preparation and spectroscopic investigation of such geometrical isomers. In his monograph [3] *Zechmeister* already reviewed the different experimental techniques available for the *trans/cis*-stereomutation and presented numerous examples for the preparation and isolation of *cis*-isomeric carotenoids. However, the structure elucidation of the geometrical isomers was considerably hampered by the fact that only UV./VIS. and IR. spectroscopy were available at that time.

In recent years, High Performance Liquid Chromatography (HPLC.) has greatly facilitated and accelerated the separation of complicated mixtures of *cis*-isomers. In addition, the availability of ^{1}H-NMR. spectrometers operating at higher magnetic fields and hence with greater resolution and the advent of *Fourier*-Transform (FT.)-^{1}H-NMR. spectroscopy with its strongly enhanced sensitivity considerably alleviated the elucidation of the structure of the isomers [4–6].

In the present paper we report on the isomerization of racemic, synthetic all-*trans* astaxanthin diacetate (**1**) and the isolation by HPLC. and identification by 270-MHz-FT-^{1}H-NMR. spectroscopy of three mono-*cis* and six di-*cis*-isomers. The reason for having used the diacetate for these experiments rather than the naturally

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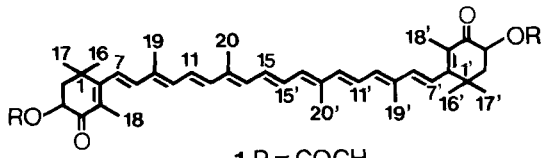
occurring astaxanthin (**2**) itself was the fact that, despite extensive efforts, we were unable to achieve a satisfactory separation of the *cis/trans*-mixtures of the underivatized compound although about 70 different chromatographic systems were tested.

Scheme. *Chemical structure of astaxanthin* (**2**) *and its diacetate* (**1**).



1 R = $COCH_3$
2 R = H

In the following, only the main features of the chromatographic work involved in this study can be reported (see Experimental Part). Instead we prefer to emphasize the discussion of the ^{1}H-NMR. spectroscopic data which, it is hoped, will be helpful for future applications to the elucidation of the structure of other geometrical isomers of retinoids and carotenoids. This should be especially valid for the hitherto (with the exception of the 15-*cis* [7]) unknown stereoisomers of astaxanthin itself since their spectra are expected to be very similar to those of the diacetates with the exception of chemical shift changes of protons at or near the endgroups.

Results. – 1. *Chromatography.* The preparation of the quasi-equilibrium mixtures of the stereoisomers of all-*trans*-astaxanthin diacetate obtained by thermal or iodine-catalyzed photoisomerization is briefly described in the Experimental Part. As was revealed by HPLC. the number of stereoisomers obtained by the two methods was identical, however, their relative concentrations were different. For illustration, two typical results were compiled in *Table 1* (see Experimental Part).

Table 1. UV./VIS.-data and relative concentrations of the isomers of astaxanthin diacetate, obtained by thermal isomerization (150°, 30 min) and iodine-catalyzed photoisomerization (in benzene, 60 min)[a])

Peak	Isomer	Concentration %		UV./VIS.	
		thermal	by light	λ_{max} (nm)	λ of *cis*-peak
I	9,9'-di-*cis*	2.6	1.7	460	n.o.
II	9,13'-di-*cis*	5.6	2.7	457	~360 (vw)
III	9,15-di-*cis*	2.3	0.9	457	~360 (w)
IV	9,13-di-*cis*	6.4	3.2	456	~358 (vw)
V	13,13'-di-*cis*	3.3	1.0	454	n.o.
VI	13,15-di-*cis*	4.6	0.6	454	n.o.
VII	?		1.1	–	–
VIII	all-*trans*	34.4	54.0	473	n.o.
IX	9-*cis*	21.6	18.4	465	~360 (w)
X	13-*cis*	19.0	16.3	464	364 (m)
XI	15-*cis*			465	363 (s)

[a]) n.o.: not observed; vw: very weak; w: weak; m: medium; s: strong.

In *Figure 1* we show the HPLC. chromatogram of a mixture obtained by iodine-catalyzed photoisomerization. As can be seen 10 peaks from 11 main components could be well resolved. The peaks are numbered and the chemical structures are given as derived below.

From each peak a UV./VIS. spectrum was recorded by the stopped-flow technique (see *Table 1*). By carrying out multiple chromatographic runs and collecting each peak at the outlet of the detector, between about 10 and 70 μg of each sample were isolated and used after an additional purification for ^{1}H-NMR. and, in part, for MS. investigation.

The stability of the isomers during the mostly longer accumulation times was in all cases checked by reinjection of part of the sample obtained after the ^{1}H-NMR. by evaporation of the solvent. With one exception (see below) the isomers were sufficiently stable, *i.e.* only minor isomerization was detected.

In the course of this study only geometrical isomers of **1** were detected. The extensive ^{1}H-NMR. investigation described below led to the identification of the

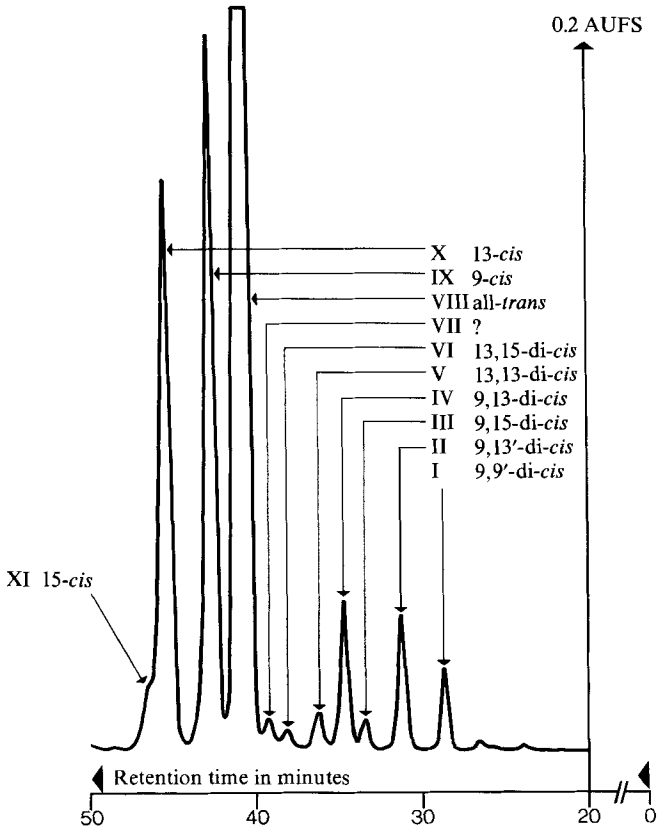


Fig. 1. *HPLC.-chromatogram of a mixture of* cis/trans-*isomers of astaxanthin diacetate,* obtained by iodine-catalyzed photoisomerization (exposure 1 h) of the all-*trans*-isomer in benzene (see Experimental Part). The identification of the components was accomplished by 270-MHz-FT-^{1}H-NMR.

unchanged all-*trans* and nine different *cis*-isomers. The structure of a further component (Peak VII) remains still unclear since its ^{1}H-NMR. spectrum was found to be identical to that of peak VI. As will be shown later this isomer obviously isomerized to VI during the procedure.

2. *^{1}H-NMR. data.* – The ^{1}H-NMR. spectra of carotenoids, even their highly crowded olefinic parts, can be largely interpreted if they are recorded at sufficiently high magnetic fields [5]. The ^{1}H-NMR. spectra of astaxanthin, its 15-*cis*-isomer, and of all-*trans*-astaxanthin diacetate were recently discussed in detail [7]. They served as a basis for the assignment of the signals of the stereoisomers isolated in the course of this study.

The chemical shifts and assignments for the all-*trans* diacetate and nine stereoisomers are compiled in *Table 2*. Not given are the values of the individual coupling

Table 2. *Chemical shifts* [δ in ppm ($CDCl_3$)] *and assignment of the 270-MHz-^{1}H-NMR. signals of all*-trans

Protons		VIII: all-*trans* δ	IX: 9-*cis* δ	IX: 9-*cis* $\Delta\delta$	I: 9,9′-di-*cis* δ	I: 9,9′-di-*cis* $\Delta\delta$	X: 13-*cis* δ	X: 13-*cis* $\Delta\delta$	V: 13,13′-di-*cis* δ	V: 13,13′-di-*cis* $\Delta\delta$
H–C(2)	ax	~2.08	2.10	0.02	2.10	0.02	2.08	–	2.08	–
	eq	~2.00	n.i.	–	2.02	0.02	n.i.	–	~2.01	–
H–C(2′)	ax	~2.08	2.08	–	2.10	0.02	2.08	–	2.08	–
	eq	~2.00	n.i.	–	2.02	0.02	n.i.	–	~2.01	–
H–C(3)		5.53	5.55	0.02	5.55	0.02	5.54	–	5.53	–
H–C(3′)			5.53	–			5.53	–		
H–C(7)		6.21	6.22	–	6.21	–	6.22	–	6.22	–
H–C(7′)			6.20	–			6.20	–		
H–C(8)		6.41	6.95	0.54	6.94	0.53	6.41	–	6.40	–
H–C(8′)			6.41	–			6.41	–		
H–C(10)		6.30	6.24	−0.06	6.24	−0.06	(6.35)	(0.05)	6.34	0.0
H–C(10′)			6.30	–			~6.30	–		
H–C(11)		6.66	6.71	0.05	6.70	0.04	~6.65	–	6.64	−0.0
H–C(11′)			6.65	–			~6.65	–		
H–C(12)		6.46	6.36	−0.10	6.37	−0.09	6.99	0.53	6.98	0.5
H–C(12′)			6.45	–			6.45	–		
H–C(14)		~6.30	~6.30	–	~6.28	−0.02	6.18	−0.12	6.16	−0.1
H–C(14′)			~6.30	–			(6.35)	(0.05)		
H–C(15)		~6.68	~6.67	–	6.65	−0.03	6.84	0.16	6.76	0.0
H–C(15′)			~6.67	–			6.60	−0.08		
H_3C(16)		1.227	~1.230	–	1.231	–	1.229	–	1.229	–
H_3C(17)		1.350	~1.350	–	1.354	–	1.355	–	1.352	–
H_3C(16′)		1.227	~1.230	–	1.231	–	1.229	–	1.229	–
H_3C(17′)		1.350	~1.350	–	1.354	–	1.355	–	1.352	–
H_3C(18)		1.906	1.939	0.03	1.939	0.03	1.906	–	1.907	–
H_3C(18′)			1.904	–			1.906	–		
H_3C(19)		2.001	~2.003	–	2.006	–	~2.01 br.[b])	–	~2.007 br.	–
H_3C(19′)			~2.003	–			~2.01 br.[b])	–		
H_3C(20)		1.995	1.974	−0.02	1.996	−0.03	~2.00 br.[b])	–	~2.007 br.	–
H_3C(20′)			1.990	–			1.985[b])	–		
AcO	C(3)	2.192	2.199	–	2.200	–	2.197	–	2.196	–
	C(3′)		2.192	–			2.197	–		

[a]) n.i.: not identified; br.: broad signal.

constants since they were found in their usual range as known from related compounds [5–7].

The resonances were assigned on the analogy of the expected signal patterns known from the all-*trans*-compound. In several cases the assignments were confirmed by decoupling experiments. Some chemical shifts are given in parentheses in order to indicate that these signals could not be unambigously recognized due to other strongly overlapping signals. In a few cases the approximate location of such signals could only be obtained by decoupling experiments.

Readily detectable were the *AB*-type pattern of H–C(7,7′) and H–C(8,8′) with $J(7,8) \sim 16$ Hz and the former signal additionally broadened mainly by long-range coupling to $H_3C(18,18')$. This large value of $J(7,8)$ simplified the differentiation with the other doublets present, namely of H–C(12,12′) with $J(11,12) \sim 14.8$ Hz,

astaxanthin-diacetate and its cis-*isomers*. Relevant values of the isomerization shift $\Delta\delta = \delta_{cis} - \delta_{trans}$ in ppm[a])

XI: 15-*cis*		IV: 9,13-di-*cis*		II: 9,13′-di-*cis*		III: 9,15-di-*cis*		VI: 13,15-di-*cis*	
δ	$\Delta\delta$	δ	$\Delta\delta$	δ	$\Delta\delta$	δ	$\Delta\delta$	δ	$\Delta\delta$
2.08	–	2.10	–	2.09	–	2.10	0.02	2.08	–
n.i.	–	n.i.	–	n.i.	–	n.i.	–	n.i.	–
2.08	–	2.08	–	2.08	–	2.08	–	2.08	–
n.i.	–	n.i.	–	n.i.	–	n.i.	–	n.i.	–
5.53	–	5.54	–	5.54	–	5.54	–	5.53	–
		5.53	–	5.53	–	5.53	–	5.53	–
6.21	–	6.23	–	~6.21 br.	–	6.22	–	6.22[1])	–
		6.20	–	~6.21 br.	–	6.21	–	6.21[1])	–
6.41	–	6.93	0.52	6.94	0.53	6.94	0.53	6.41	–
		6.41	–	6.41	–	6.41	–	6.41	–
6.30	–	~6.29	–	(6.23)	(–0.07)	(6.24)	(–0.06)	6.34	0.04
		~6.29	–	6.34	0.04	6.30	–	6.29	–
6.69	0.03	6.70	0.04	6.69	0.03	6.74	0.08	6.69[1])	0.03
		6.64	–	6.64	–	6.69	0.03	6.68[1])	–
6.51	0.05	6.90	0.44	6.36	–0.10	(6.44)	(–)	7.00	0.54
		6.44	–0.02	6.98	0.52	6.50	0.04	(6.56)	(0.10)
~6.69	0.39	6.17	–0.13	6.26	–0.04	~6.74	0.44	(6.61)	(0.31)
		~6.29	–	6.17	–0.13	~6.74	0.44	(6.71)	(0.41)
~6.45	–0.23	6.83	0.15	6.59	–0.09	~6.43	–0.25	(6.43)	(–0.25)
		6.59	–0.09	6.82	0.14	~6.43	–0.25	n.i.	–
1.227	–	1.228	–	1.231	–	1.230	–	1.227	–
1.355	–	1.355	–	1.355	–	1.355	–	1.351	–
1.227	–	1.228	–	1.231	–	1.230	–	1.227	–
1.355	–	1.350	–	1.355	–	1.355	–	1.351	–
1.908	–	1.933	0.03	1.939	0.03	1.939	0.03	~1.907	–
		1.906	–	1.911	–	1.912	–	~1.907	–
2.007	–	1.981[b])	–	~2.01[b])	–	2.015	–	~2.01 br.[b])	–
		~1.997[b])	–	~2.01[b])	–	2.008	–	~2.01 br.[b])	–
1.993	–	2.024[b])	–	~2.01[b])	–	1.973	–0.02	2.062[b])	–
		~1.997[b])	–	~1.96[b])	–	1.990	–	1.988[b])	–
2.193	–	2.198	–	2.199	–	2.198	–	2.195	–
		2.194	–	2.199	–	2.198	–	2.195	–

[b]) Assignments of corresponding signals may be interchanged.

proving the Δ^{11}-*trans*-configuration in all compounds. Easily identified, in general, were also the signals of H–C(11,11′), which appeared as doublets of doublets (14.8 and *ca.* 11 Hz) indicating again that Δ^{11} and $\Delta^{11'}$ were *trans*.

In symmetric compounds the *AA′BB′*-type partial spectrum of H–C(14,14′) and H–C(15,15′) was also readily detected with the half spectrum of the former protons additionally broadened by coupling to $H_3C(20,20')$.

In the spectra of the unsymmetrical compounds 9-*cis* (IX) and 9,15-di-*cis* (III) the *AA′BB′*-symmetry is practically undisturbed, whereas with 13-*cis*- (X) and corresponding di-*cis*-compounds H–C(14) and H–C(14′) as well as H–C(15) and H–C(15′) are no longer equivalent as expected.

When the assignment of most of the signals was completed the elucidation of the different structures was straightforward. A relevant parameter for this purpose is known to be the isomerization shift $\Delta\delta = \delta_{cis} - \delta_{trans}$ (in ppm), *i.e.* the chemical shift difference of the different proton signals between the *cis*- and the all-*trans*-compound [5] [6]. These $\Delta\delta$-values are therefore also given in *Table 2*.

It is known that isomerization of Δ^9 is clearly evidenced by a strong downfield shift of the signal of H–C(8). The same is true for the signal of H–C(12) in compounds with Δ^{13}-*cis* [5]. Similarly, a Δ^{15}-*cis*-configuration is recognizable from a downfield shift of the signals of H–C(14) and H–C(14′) and an upfield shift of the signals of H–C(15) and H–C(15′). Thus, in 15-*cis*-carotenoids the relative positions of these signals are reversed compared to the all-*trans*, as was first reported for β-carotene [5] and astaxanthin [7]. In a few cases the location of the signals of H–C(14) and H–C(14′) was confirmed by decoupling experiments, *i.e.* by the observation of a sharpening of these signals upon irradiation of the signals of $H_3C(20,20')$ or, conversely, by the sharpening of the latter signals upon irradiation at the assumed position of the former. These experiments were important in the case of the 15-*cis*- and 9,15-di-*cis*-isomers to locate and distinguish the signals of H–C(14,14′) and H–C(15,15′) unambiguously. The structure of the 15-*cis*-isomer is further corroborated by the close resemblance of the olefinic part of the spectrum to that of 15-*cis*-astaxanthin [7].

A further important help in the assignment of the signals was the known fact that in most cases the complex olefinic portion of the spectra could be surprisingly well predicted, at least in part, by the additive superposition of suitable partial spectra. Thus, as an example, the relatively simple spectra of the 9,9′-di-*cis*- and the all-*trans*-isomer can be added together to predict the spectrum of the 9-*cis*-isomer. Correspondingly, the spectra of the 9,9′-di-*cis* and of the 13,13′-di-*cis*-isomer can be added giving, to a good approximation, the experimentally observed spectrum of the 9,13′-di-*cis*-isomer. However, this additivity breaks down, as expected, if the *cis*-bonds were too close together *e.g.* in the 9,13-di-*cis*. Here part of the spectrum closely resembles that of the all-*trans*-compound in contrast to the case of the 9,13′-di-*cis*-isomer.

Inspection of the chemical shift data of *Table 2* furthermore reveals small but relevant shift changes at some of the signals of the cyclic endgroups in all five isomers with Δ^9-*cis* partial structure, above all at H–C(2) and $H_3C(18)$. This also served as a further hint as to the presence of a Δ^9 (and $\Delta^{9'}$) *cis*-configuration.

A few concluding remarks must be made to the spectra of isomers VI and of VII.

It was found that both isomers gave identical ^{1}H-NMR. spectra. Since only very small sample quantities were available and correspondingly longer accumulation times had to be applied, this means that one of the two isomers rearranged. Reinjection of the isomer VII after obtaining the spectrum showed that it was this compound which isomerized to VI. Since it is shown below that isomer VI possesses a 13,15-di-*cis*-structure it seems possible that the unstable isomer VII has one more *cis*-bond, possibly an unhindered one, *e.g.* at C(9) or C(9′).

Concerning the structure of VI the following observations seem relevant:

From the signals of H–C(7,7′) and H–C(8,8′) it follows that the double bonds at C(7,7′) and C(9,9′) must be *trans*. A doublet with $J \sim 15$ Hz at 7.00 ppm (1 H) points to Δ^{13}-*cis* since this signal can only be assigned from its splitting to H–C(12) with a Δ^{13}-*cis* partial structure. The other doublet of H–C(12′) can only tentatively be assigned at 6.56 ppm. In addition, from the signal patterns of H–C(11) and H–C(11′) it follows that Δ^{11} and $\Delta^{11'}$ must be *trans*. The only remaining possibility for isomer VI is therefore a 13,15-di-*cis*-structure.

Conclusion. – The results presented here clearly demonstrate again the power of HPLC. in separating complex mixtures of stereoisomeric carotenoids and of high-field FT.-^{1}H-NMR. spectroscopy in providing largely interpretable spectra even if only very small sample quantities are available. By these techniques it was possible to isolate ten different *cis* isomeric astaxanthin diacetates and to elucidate their structures with the exception of one unstable isomer.

It is interesting to note that all *cis*-isomers isolated so far possess so-called unhindered *cis*-structures which are known to be relatively stable (see [3], p. 13). It is plausible, however, that additional isomers with further *cis*-configurations might also be present albeit in very much smaller concentrations. In this context it seems worth noting that in the course of a related study on the photoisomerization of an aromatic analogue of retinoic acid several strongly hindered *cis*-isomers even with three consecutive *cis* double-bonds were isolated and found sufficiently stable to be fully characterized by ^{1}H-NMR. spectroscopy [6]. Although in this related study stereomutation was achieved without the application of iodine as a catalyst, the different results cannot be ascribed to this fact. Preliminary photoisomerization studies with all-*trans*-astaxanthin diacetate without catalyst namely showed that a very similar HPLC. diagram was obtained, however, the equilibrium was reached only after about 2 to 3 weeks compared to less than one hour in our experiments with catalyst.

The authors are indebted to Messrs. *W. Grunauer* and *E. Glinz* for their skilful technical assistance, to Drs. *F. Kienzle* and *R. Zell* for a generous gift of racemic all-*trans*-astaxanthin, and to Mr. *W. Meister* for the mass spectra.

Experimental Part

Compounds. Crystalline, racemic all-*trans*-astaxanthin was kindly provided by Drs. *F. Kienzle* and *R. Zell* of our firm. Its synthesis has been described recently [8].

Solvents. All solvents were of analytical grade *(Merck)* and used without further purification.

Acetylation. About 50 mg of astaxanthin were dissolved in pyridine/dichlormethane/acetic anhydride 1:1:1. After degassing with argon the solution was stored in darkness and at ambient temperature for at least 12 h. The solvents were evaporated i.V. at 25–30°. Finally the residue was dried i.HV. and at ambient temperature for 1 h.

Isomerization. – *Thermal isomerization* of 200 mg of racemic, crystalline **1** dissolved in 20 ml dimethylformamide, degassed with argon, was accomplished by keeping the solution at 150° for 30 min. Subsequently the solution was evaporated i.V. at 30–35° to dryness.

Iodine-catalyzed photoisomerization: containing 1 mg of iodine in 1 ml of benzene was added to a solution of 100 mg of **1** in 100 ml of benzene and thoroughly mixed. The solution was degassed with argon and exposed to the light of daylight fluorescence tubes for 60 min. Further handling of the samples was performed as described above.

Preliminary studies on the rate of this isomerization reaction showed equilibration to take place within 15 to 20 min.

Chromatographic equipment. All analytical work has been performed with a HPLC. unit consisting of an *Altex* 100 delivery solvent system, septum injection port *(Perkin Elmer)* and UV./VIS. detector LCD-725 *(Kontron)*. The separation columns were home-made and had the dimensions 500 × 3.2 mm (length, i.d.). Stationary phase: *LiChrosorb* SI 60, particle size 5 μm. Mobile phase hexane/ethyl acetate/acetonitrile 88:10:2; flow rate 0.8 ml/min. The reaction mixture was injected directly on to the column. No influence of the iodine on the separation characteristics or the lifetime of the column was observed.

Isolation of the fractions: By carrying out multiple chromatographic runs and collecting each peak at the outlet of the detector 11 different fractions I to XI were obtained in quantities between *ca.* 10 and 70 μg. Immediately before the ^{1}H-NMR. and the mass spectroscopic measurements the fractions were further purified by the same chromatographic system in one further run in order to get a purity of at least 95%. Simultaneously from each of these fractions a UV./VIS. spectrum was recorded between 290 and 600 nm by the stopped-flow method with a *Variscan* spectrophotometer. The results are compiled in *Table 1* together with the concentrations of the isomers as measured from the absorbance at $\lambda = 473$ nm. Since the molar absorptivities of the *cis*-isomers are unknown and probably smaller than that of the all-*trans*, the concentrations of the *cis*-isomers are probably slightly underestimated.

It is interesting to note that the relative strength of the *cis*-peak (see *Table 1*) decreases from 15-*cis* to 13-*cis* and 9-*cis* as expected [3]. However, the *cis*-peak remains smaller or even unobservable in all other cases with two *cis*-configurations. This shows that the *cis*-peak is of minor significance for the elucidation of the structure of di-*cis* isomeric carotenoids.

MS.: The spectra (*m/z*, rel. intensity, interpretation) were obtained with an *AEI*-MS 9 mass spectrometer using electron impact ionization (70 eV). The samples were dissolved in 10 μl of methanol/dichloromethane 1:1 and transferred to the tip of a glass rod. After evaporation of the solvent by a N_2-stream the samples were introduced directly into the preheated (approx. 200°) ion source of the mass spectrometer.

all-*trans*-**1**: 680 (100%, *M*), 622 (10%), 620 (10%, $M - AcOH$), 592 (10%), 588 ($M - C_7H_8$), 119 (20%), 105 (20%), 91 (20%), 43 (Ac, 50%).

The peak intensities were not well reproducible due to partial decomposition during the introduction. The mass spectra of isomers IV, VI and IX which were recorded for control, showed the peak at *m/z* 680 plus the further relevant peaks as given above with comparable intensities.

^{1}H-NMR.-spectra. All spectra were run on a *Bruker* HX-270 FT.-spectrometer equipped with an ASPECT 2000 computer (32 K data) and disk unit. The samples were dissolved in *ca.* 0.2 ml of $CDCl_3$ (so-called 100% D quality) obtained from *CEA*-France or *Stohler Isotope Chemicals.* Cylindrical NMR. micro tubes (type 508-CP, *Wilmad Glass Company*) were used. Accumulation times needed were between 1 and 63 h (week-end).

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