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Determination of Enantiomeric Composition of Partly Racemized Carotenols

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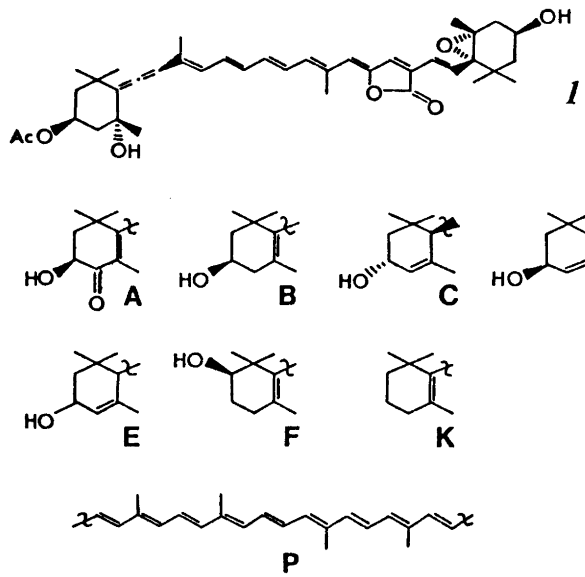
Methods for determining the enantiomeric composition of various racemized carotenols by converting them into diastereomeric esters with subsequent analysis have been studied.

Diastereomeric esters of (−)-camphanic acid with carotenols other than α-ketols could not be separated by HPLC. No separation was achieved for diastereomeric esters of methoxytrifluoromethylphenylacetic acid (MTPA).

^{1}H NMR analysis in the presence of Eu(fod)$_3$ of diastereomeric MTPA esters allowed quantitative determination of the enantiomeric composition of carotenols with 2-hydroxy-β- and 3-hydroxy-β-type end groups.

Naturally occurring carotenoids contain up to six chiral centres, as examplified by peridinin (*1*, Scheme 1).[1] For a long time it was assumed that chiral carotenoids occurred in nature as a single chiral isomer. More recently it was demonstrated that astaxanthin occurred in the (3*S*,3′*S*)- and (3*R*,3′*R*)-configuration (*2a* and *2b*) in different sources.[2,3] Furthermore, lutein (*3a*) and calthaxanthin (*3b*) are shown to be 3′-epimers[4-7] and six different configurations of ε,ε-carotene-3,3′-diol (*4*) have now been established.[8-11]

However, until very recently[10,12-14] only one configuration has been encountered for a carotenoid of given constitution within the same biological



2a A-P-A
2b ent A-P-ent A
2c A-P-ent A
3a B-P-C
3b B-P-D
4 E-P-E
5 F-P-K

Scheme 1.

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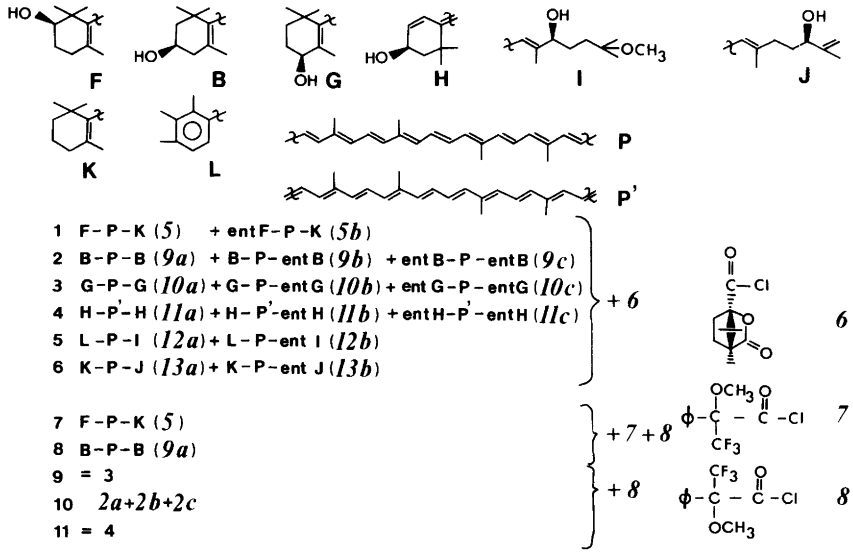

Scheme 2.

source. Lack of suitable methods for demonstrating enantiomeric composition may partly be responsible for this situation.

In our collaboration with Kayser on insect carotenoids[15,16] it was observed that β,β-caroten-2-ol from insects had variable and smaller Cotton effect than earlier found for (2*R*)-β,β-caroten-2-ol (*5*) in our laboratory.[17] This prompted us to search for a method for quantitative analysis of partially racemized carotenols. A common principle for separation of enantiomers involves conversion into diastereomers and separation on the basis of different physical properties, including chromatography. Alternatively, the quantitative composition of a diastereomeric mixture may be estimated by NMR spectroscopy.

RESULTS AND DISCUSSION

From the work of Gerlach[18,19] and Müller *et al.*[20] the commercially available (−)-camphanic acid chloride (*6*, Scheme 2) appeared to be a favourable acylating agent for the preparation of diastereomeric carotenols for subsequent attempts of HPLC separation.[21] Meanwhile Müller and Vecchi[22] succeeded in separating (3*S*,3′*S*), (3*R*,3′*R*) and (3*R*, 3′*S*) astaxanthin (*2a,b,c*) by this principle.

A micro scale procedure for preparation of (−)-camphanic esters of selected carotenols (Scheme 2) and subsequent HPLC analysis was adapted. When testing our procedure with synthetic Roche standards (*2b,2c*)[23,24] and natural astaxanthin *ex Hommarus gammarus*, presumed to be (3*S*,3′*S*) (*2a*),[3] the latter unexpectedly turned out to be a mixture of all three optical isomers (*2a,b,c*). These results have been published.[12]

HPLC separation was attempted for esters of (−)-camphanic acid prepared from carotenol mixtures containing 2 – 3 optical isomers. The carotenol samples esterified with (−)-camphanoyl chloride were as specified in Scheme 2:

(1) Racemic natural β,β-caroten-2-ol (*5,5b*) *ex Cerura vinula;* racemic nature proved by the ^{1}H NMR method below.[16]

(2) Synthetic mixture of racemic (*9a,c*) and meso (*9b*) zeaxanthin.[25]

(3) Racemic (*10a,c*) and meso (*10b*) isozeaxanthin,[26] prepared by $LiAlH_4$ reduction of synthetic[27] canthaxanthin.

(4) Racemic (*11a,c*) and meso (*11b*) eschscholtzxanthin,[28] prepared by $LiAlH_4$ reduction of synthetic[29] rhodoxanthin.

(5) Racemic okenol (*12a,b*)[30] prepared by $LiAlH_4$ reduction of synthetic[30] okenone.

(6) Synthetic, racemic aleuriaxanthin (*13a,b*)[31].

In no case was reproducible separation achieved by HPLC at conditions where the camphanates of

the α-ketols *2a*+*2b*+*2c* were separated.

We now turned to the resolving agent methoxy-trifluoromethylphenylacetic acid chloride (MTPA chloride)[32] which offered two advantages. Both enantiomers of the acid chloride (*7* and *8*) could be synthesized,[30] and this enabled testing the separation of diastereomeric pairs even when only one enantiomer of the carotenol was available. Secondly this ester moiety offered the possibility of performing NMR-analysis[32-35] in addition to HPLC.

R(+)-MTPA chloride (*7*) and *S*(−)-MTPA chloride (*8*) were prepared in six steps from trifluoroacetic acid by published methods.[32,36]

Diastereomeric esters were prepared as follows (see Scheme 2):

(7) (2*R*)-β,β-Caroten-2-ol (*5*) *ex Trentepohlia iolithus*[17] was esterified with *R*(+)- and *S*(−)-MTPA chloride, respectively, and mixed, *cf.* Sample 1.

(8) Natural optically active (3*R*,3′*R*)-zeaxanthin (*9a*) *ex Flavobacterium* sp.[37] was esterified with *R*(+)- and *S*(−)-MTPA chloride and mixed, *cf.* Sample 2.

(9) Sample 3 = racemic and meso isozeaxanthin (*10a,c,b*)[26] was esterified with *S*(−)-MTPA chloride.

(10) Pure synthetic (*R,R*)-, (*S,S*)- and (*R,S*)-astaxanthin (*2a,c,b*)[23,24] were esterified with *S*(−)-MTPA chloride and mixed.

(11) Sample 4 = racemic and meso eschsholtzxanthin (*11a,c,b*)[28] was esterified with *S*(−)-MTPA chloride.

None of the diastereomeric mixtures (7−11) could be separated by HPLC at the conditions employed.

The possibility of analyzing the enantiomeric composition of carotenols by ^{1}H NMR was now investigated. Sample 7 above gave only one methoxy signal. However, upon careful stepwise addition of the shift reagent Eu(fod)$_3$ the methoxy signal shifted downfield and was split into two signals. By using a 2:1 ratio of the two diastereomeric esters it could be demonstrated that the (*R,R*) isomer contained the methoxy group resonating at lowest field. This is in accordance with previous experience. Thus it has been concluded[38] that if the absolute configuration is defined according to the bulkiness of the substituents the "*R,R*" (or "*S,S*") isomer should give a larger shift for the methoxy signal than the "*R,S*" (or "*S,R*") isomer. In our case the chiral C-2 center of (2*R*)-β,β-caroten-2-ol (*5*, Scheme 2) has ("*R*")-configuration, also by using bulky group priority in the sequence rule.

The same experiment was carried out with (3*R*, 3′*R*)-zeaxanthin (*9a*, Sample 8, Scheme 2). In this case the methoxy group of the *R,S* configurated *S*(−)-MTPA ester showed the largest shift. The C-2 and C-4 substituents of the carotenoid moiety are less different in bulkiness.

CONCLUSION

In conclusion, the demonstration of enantiomeric composition by HPLC of esters of (−)-camphanic acid is not generally applicable to carotenols. Positive results are so far obtained for α-ketols such as astaxanthin (*2a,b,c*)[22] and adonirubin.[39]

Separation of diastereomeric MTPA esters of selected carotenols was not achieved in the present study.

^{1}H NMR analysis of MTPA esters in the presence of Eu(fod)$_3$ shift reagent of 2-hydroxy-β- and 3-hydroxy-β-type carotenols was successful and allows a quantitative determination of the relative percentage of *R*- and *S*-configurated carotenol. Application of this method for the demonstration of the racemic nature of β,β-caroten-2-ol (*5,5b*) from insects will be published elsewhere.[16] For dichiral carotenoids such as zeaxanthin (*9a,b,c*) the ^{1}H NMR method does not allow determination of the amount of meso compound present.

EXPERIMENTAL

General methods and instruments. All operations were carried out in inert atmosphere in subdued light and at temperatures not exceeding room temperature. HPLC was carried out on a Dupont 830 Liquid Chromatograph with a Spherisorb S5W column (250 × 4.6 mm) with acetone − hexane (0:100 − 40:60 v/v) containing 0.1 % methanol as mobile phase at a flow rate of 2 ml/min with gradient 1 %/min[21] at conditions where the camphanates of astaxanthin (*2a*+*2b*+*2c*) were separated into three peaks with near-to-base-line separation. Peak components were detected by a Varian 634 UV/vis. spectrometer. Otherwise vis. spectra were recorded on a Coleman-Hitachi 124 spectrometer and ^{1}H NMR spectra on a Jeol JNM-FX 100 Fourier-Transform NMR instrument.

Synthesis of resolving agents

1-Phenyl-2,2,2-trifluoroethan-1-one was prepared by the literature procedure[36] from phenylmagnesium bromide (9 mol) and trifluoroacetic acid (3 mol)

in 64 % yield (334 g); b.p. 150–153 °C, lit.[36] 150–152 °C.

3,3,3-Trifluoro-2-methoxy-2-phenylpropanenitrile was prepared from the above ketone (1 mol), sodium cyanide and dimethyl sulfate as described elsewhere[32] in 90 % yield (194 g); b.p. 68–72 °C/5–6 mm Hg, lit.[32] 85–89 °C/20 mmHg.

(±)-3,3,3-Trifluoro-2-methoxy-2-phenylpropanoic acid (MTPA) was prepared by hydrolysis of the above nitrile (0.90 mol) as described[32] in 61 % yield (128 g); b.p. 193–200 °C/40–55 mm Hg, lit.[32] 105–110 °C/1 mmHg.

(−)-3,3,3-Trifluoro-2-methoxy-2-phenylpropanoic acid [(−)-MTPA]. Racemic MTPA (45 g, 0.19 mol) was resolved by fractional crystallization of the salt with L(−)-1-phenylethanamine (23.25 g, 0.19 mol) and subsequent treatment with HCl as described in the literature[32] to give the (−)-acid (12.0 g, 0.05 mol); $[\alpha]_D^{20}$ −67.7° ± 1.1 (c = 1.13, ethanol), lit.[32] $[\alpha]_D^{24}$ −71.8° ± 0.6 (c = 3.28, methanol).

(+)-3,3,3-Trifluoro-2-methoxy-2-phenylpropanoic acid [(+)-*MTPA*]. The remaining MTPA salts in the mother liquor above was converted to the acid by HCl treatment, and the regenerated MTPA treated with D(+)-1-phenylethanamine (9.3 g, 0.076 mol). The resulting salt was fractionally crystallized as described[32] to give the (+) acid (4.7 g, 0.02 mol); $[\alpha]_D^{20}$ +69.7° ± 1.8 (c = 0.95, ethanol), lit.[32] $[\alpha]_D^{25}$ +68.5° ± 1.3 (c = 1.49, methanol).

(−)-MTPA chloride. (−)-MTPA (12.0 g, 0.051 mol) was treated with thionyl chloride[32] to give the acid chloride (10.5 g, 0.039 mol); b.p. 97.5–99 °C/12 mmHg; $[\alpha]_D^{20}$ −125.7° ± 1.3 (c = 0.9, CCl_4).

(+)-MTPA chloride. (+)-MTPA (4.7 g, 0.02 mol) was reacted as above to give the (+) acid chloride (3.8 g, 0.016 mol); b.p. 96.5–99 °C/11–12 mmHg, lit.[32] 54–56 °C/1 mmHg; $[\alpha]_D^{24}$ +129° ± 2 (c = 5.17, CCl_4).

(±)-MTPA chloride. (±)-MTPA (20.5 g, 0.087 mol) treated as above gave (±)-MTPA chloride (20 g, 0.074 mol); b.p. 96–100 °C/13 mmHg.

HPLC analysis of diastereomeric esters

Standard procedure for the reduction of oxocarotenoids. To the appropriate oxocarotenoid (0.5–5 mg) in anhydrous ether (5–25 ml) at 0 °C was added an excess of a filtered solution of $LiAlH_4$ in dry ether. After 5 min at 0 °C the reaction mixture was poured into a half saturated solution of Rochelle salt, the organic layer was separated, washed with water and the solvent evaporated. Water was removed by azeotropic distillation with benzene and the carotenoids isolated by TLC (SiO_2) and characterized by R_F-values and vis.spectra.

Standard procedure for preparation of carotenol esters. A solution of the appropriate acid chloride (25–100 mg) in anhydrous pyridine (1–3 ml) was added to a solution of the appropriate carotenols (0.1–10 mg) in pyridine (1 ml) at 0 °C. After 45 min at this temperature hexane (10 ml) and water were added. The organic phase was washed 5–7 times with water. Solvent and water were removed by azeotropic distillation with benzene and the carotenoids isolated by TLC (SiO_2) and characterized by R_F-values and vis. spectra.

Camphanic esters, *cf.* Scheme 2, were prepared by esterification with (−)-camphanoyl chloride.

1. β,β-Caroten-2-ol (*5,5b*, 0.1 mg) *ex Cerura vinula*[40] was esterified by the standard procedure. HPLC analysis gave one peak.

2. Synthetic zeaxanthin[25] (*9a,b,c*, 0.5 mg) was esterified. HPLC gave one major peak.

3. Synthetic canthaxanthin[27] (2 mg) was reduced by the standard procedure and of the resulting isozeaxanthin (*10a,b,c*) 0.7 mg was esterified. HPLC gave one peak.

4. Synthetic rhodoxanthin[29] (5 mg) was reduced. Of the resulting eschscholtzxanthin (*11a,b,c*)[28] 3 mg was esterified. HPLC showed one major peak.

5. Okenone[30] (0.7 mg) was reduced. Of the resulting racemic okenol (*12a,b*)[30] 0.25 mg was esterified. HPLC gave one major peak.

6. Synthetic racemic aleuriaxanthin[31] (*13a,b*, 2 mg) was esterified. HPLC analysis resulted in one main peak. MTPA esters, *cf.* Scheme 2, were prepared by esterification with the appropriate acid chlorides.

7. (2*R*)-β,β-Caroten-2-ol (*5*, 3 mg) *ex Trentepohlia iolithus*[17] was esterified with *S*(−)-MTPA chloride, providing the *R,S*-ester.

Similarly (2*R*)-β,β-caroten-2-ol (*5*, 2 mg) was esterified with *R*(+)-MTPA chloride to give the *R,R*-ester. The two diastereomeric esters were mixed 1:1 (7a). HPLC of the mixture showed one peak. A 63:37 mixture (7b) was used for ^{1}H NMR.

8. (3*R*,3′*R*)-β,β-Carotene-diol (zeaxanthin, *9a*, 10 mg) *ex Flavobacterium* sp.[37] was esterified with *S*(−)-MTPA chloride. The same diol (5 mg) was esterified with *R*(+)-MTPA chloride. The crude esters were purified separately providing 11.2 mg (14.8 μmol) and 5.6 mg (7.4 μmol), respectively, of the two diastereomeric esters. In 1:1 mixture (7a) HPLC showed one main peak. A 2:1 mixture (8b) was used for ^{1}H NMR.

9. Racemic and meso isozeaxanthin (*10a,c,b*, 0.7 mg) prepared as for Sample 3, was esterified with *S*(−)-MTPA chloride. HPLC of the esters showed one peak.

10. (*R,R*),(*S,S*)- and (*R,S*,meso)-astaxanthin[23,24] (*2a,c,b*, 0.5 mg of each) were esterified separately with *S*(−)-MTPA chloride. The resulting esters were

Table 1. LIS of the $-OCH_3$ signal in $CDCl_3$ of the (*R,S*)-MTPA esters of sample 7b: (2*R*)-β,β-caroten-2-ol (63 % *R,S* and 37 % *R,R* monoesters) and of sample 8b: (3*R*)-β-β-carotene-3,3'-diol (67 % *R,S* and 33 % *R,R* diesters).

Sample	$Eu(fod)_3$ μmol/300 μl	δ-OCH_3 *R,R*	δ-OCH_3 *R,S*
7b	0	3.53	3.53
	1.00	3.53	3.53
	1.20	3.70	3.70
	1.35	3.89	3.80
	1.50	4.06 (*ca.* 62 %)[a]	3.92 (*ca.* 38 %)[a]
8b	0	3.53	3.53
	0.55	3.70	3.70
	0.70	4.45	4.20
	0.95	4.67	4.53
	1.30	5.02 (*ca.* 67 %)[a]	4.82 (*ca.* 33 %)[a]

[a] Relative integrals.

mixed in 1:1:1 ratio. HPLC analysis showed one major peak.

11. Racemic and meso eschscholtzxanthin (*11a, c,b*, 2 mg) prepared as for sample 4 above was esterified with *S*(−)-MTPA chloride. HPLC showed one main peak.

^{1}H NMR analysis of diastereomeric esters

General procedure. The diastereomeric mixture of the carotenol esters (3−8 mg) was dissolved in $CDCl_3$ (300 μl), a few drops of 1 % TMS in $CDCl_3$ was added and the ^{1}H NMR spectrum recorded.

A freshly prepared 0.005 N solution of $Eu(fod)_3d_{30}$ in $CDCl_3$ was then added in 10,50 or 100 μl portions. Each time the volume was adjusted to 300 μl and the shifts recorded.

β,β-Caroten-2-ol MTPA ester (Sample 7b), δ($CDCl_3$), 0.97 s (3H, 16-CH_3), 1.03 s (6H, 16',17'-CH_3), 1.27 s (3H, 17-CH_3), 1.50 s (CH_2), 1.70 s (6H, 18,18'-CH_3), 1.97 s (12H, in-chain-CH_3), 3.53 s (3H, -OCH_3), 4.85−5.15 (1H, H-2), 5.9−7 m (14H, olefinic), 7.3−7.5 m (5H, aromatic).

Addition of $Eu(fod)_3$ caused no significant shifts except for the $-OCH_3$ signal, see Table 1.

When (2*R*)-β,β-caroten-2-ol esterified with (*S*)-MTPA alone was tested, this pure diastereomer gave no splitting of the $-OCH_3$ signal upon stepwise addition of 0−1.5 μmol $Eu(fod)_3$/300 μl.

β,β-Carotene-3,3'-diol (zeaxanthin) di-MTPA ester (Sample 8b). δ($CDCl_3$) 1.03 s and 1.13 s (6H, 16,17,16',17'-CH_3), 1.70 s (6H, 18.18'-CH_3), 1.95 s (12H, in-chain-CH_3), 3.53 s (3H, $-OCH_3$), 6.0−6.8 m (14H, olefinic), 7.3−7.5 m (5 H, aromatic).

The LIS of the $-OCH_3$ signal are given in Table 1.

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