The Substrate Specificity of β,β-Carotene 15,15′-Monooxygenase

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The synthesis of several substrate analogues of the enzyme β,β-carotene 15,15′-monooxygenase is reported. The substrate specificity of enriched enzyme fractions isolated from chicken intestinal mucosa was investigated. Regarding substrate binding/cleavage, these experiments demonstrate that *i*) any deviation from the 'rod-like' β,β-carotene structure is not tolerated, *ii*) one 'natural', unsubstituted β-ionone ring is required, *iii*) the position and presence of the Me groups attached to the polyene chain is significant. These results suggest a hydrophobic barrel-like substrate binding site in which the protein's amino acid residues through interaction with the Me groups, direct the central C=C bond in binding distance to the active site's metal-oxo center, supporting the unique regiospecificity of cleavage to retinal (provitamin A).

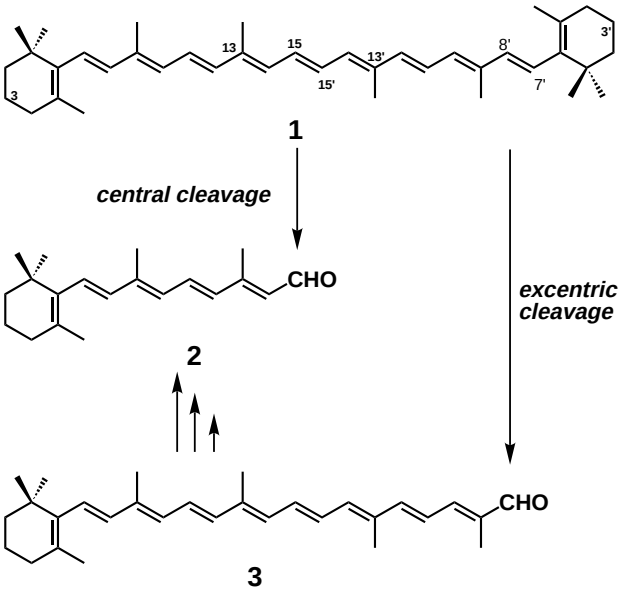
1. Introduction. – β,β-Carotene (**1**) is the parent structure and most important compound of the 'orange pigments of life' that comprise more than 650 carotenoids, which are abundant in photosynthetic bacteria, and in the plant and animal kingdoms [1][2]. Both plants and bacteria can biosynthesize carotenoids [3], but mammals rely on extraction from the diet [4]. The significance of β,β-carotene (**1**) in humans concerns its antioxidant activity and its enzymatic conversion to retinal (**2**) (pro-vitamin A). To date, two modes of cleavage of **1** have been proposed: the *central* cleavage of **1**, which provides two moles of **2** [5], and the more recently discovered *excentric* cleavage which yields first apocarotenals, such as 8-apocarotenal (**3**) that may be degraded to **2** by β-oxidation (*Scheme 1*) [6].

Central cleavage of **1** seems to be the most important metabolic pathway, and enzymatic activity has been detected in various tissues since its discovery in the mid-1950s. Since then many attempts to purify and characterize this enzyme have failed. Nevertheless, and despite the lack of solid information regarding the enzymatic reaction mechanism or the nature of the co-factor, the enzyme was termed β,β-carotene 15,15′-dioxygenase (EC 1.13.11.21).

Only very recently have we been able to establish a protocol for purification of the enzyme isolated from chicken intestinal mucosa, which led to the identification of the catalytically active protein [7]. The enzyme was enriched 226-fold to a specific activity of 2500 $pmol \cdot h^{-1} \cdot (mg\ protein)^{-1}$, and the band eluting from the final gel filtration that best correlated with β,β-carotene 15,15′-monooxygenase activity (*Fig. 1*) was sequenced, and complimentary oligonucleotides were designed.

With RT-PCR, a cDNA fragment was obtained, labelled, and subsequently used to screen a chicken duodenal expression library. The isolated full-length cDNA of β,β-carotene 15,15β-monooxygenase contains a sequence of 1578 bp that codes for a

Scheme 1. *Metabolism of β,β-Carotene* (**1**)



protein of 526 amino acids. Expression of the hexa-His-tailed protein in *E. coli* and BHK (baby hamster kidney) cells gave, after affinity chromatography, a catalytically active, cytosolic enzyme (60.3 kDa) that cleaves *β,β*-carotene (**1**) to retinal (**2**) as the only reaction product [8]. At about the same time, another research group published the expression of the *β,β*-carotene 15,15′-monooxygenase derived from *Drosophila melanogaster* [9].

Parallel to our efforts to purify the native protein [7] and to determine the reaction mechanism [10], we developed a synthetic supramolecular enzyme model that catalyzes carotenoid cleavage [11][12], and we also investigated the substrate specificity of *β,β*-carotene 15,15′-monooxygenase from chicken intestinal mucosa using enriched enzyme fractions. We wish to report here the synthesis of several potential substrates and incubation experiments with 14 substrate analogues.

2. Results and Discussion. – 2.1. *Synthesis of Substrates.* The surprisingly regioselective cleavage of *β,β*-carotene was often attributed to the central C=C bond of *β,β*-carotene being the sterically least-hindered compared to all other C=C bonds of **1** [13]. To investigate this point, we decided to synthesize the norcarotenoids **4** and **5**, such that the enzyme could cleave three double bonds of **4**, and five double bonds of **5** (*Fig. 2*).

The synthesis of 20,20′-dinor-*β,β*-carotene (**5**) by a $C_{18}+C_2+C_{18}=C_{38}$ approach has been reported earlier [14]. Synthesis of **4** and **5** by $C_{20}+C_{19}=C_{39}$ and $C_{19}+C_{19}=C_{38}$ strategies is claimed in an US patent [15], however, with low yields and rather scarce experimental data. The ready availability of the C_{15}-*Wittig* salt **6** [16][17] and the prospect to prepare additional analogues with different end groups prompted us to synthesize **4** and **5** by *Wittig* condensation reactions according to the building principle

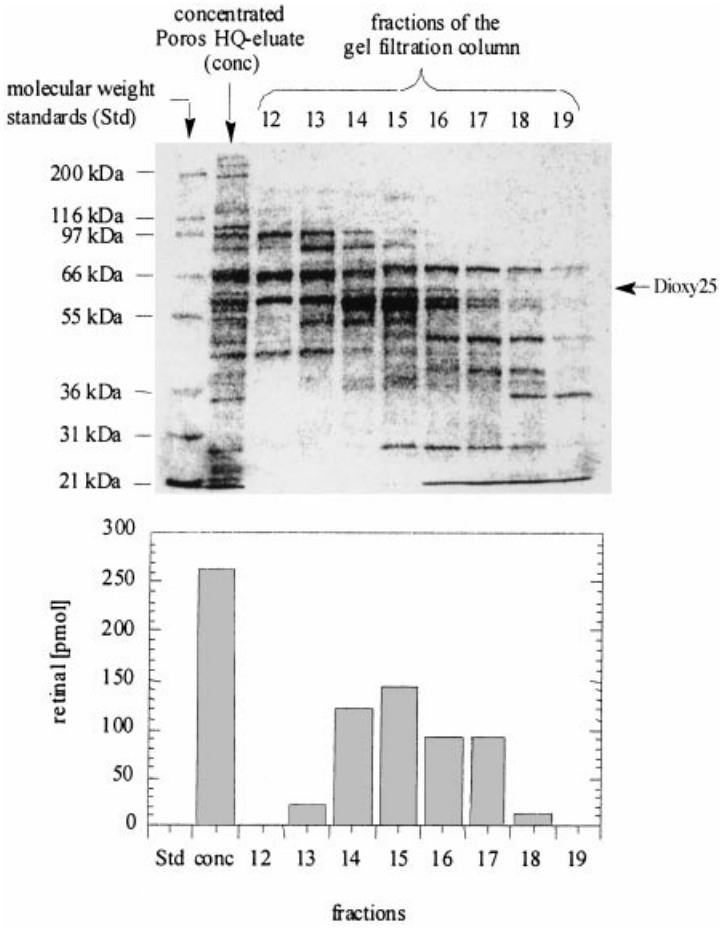
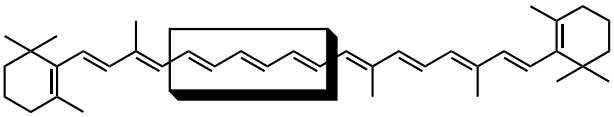


Fig. 1. *Identification of the enzyme* (see Dioxy25) *that catalyzes the* central *cleavage of* **1**. *Upper panel:* SDS gels of fractions of the final gel filtration chromatography. *Lower panel:* activity profile of the corresponding fractions.

$C_{15} + C_9 + C_{15} = C_{39}$ and $C_{15} + C_8 + C_{15} = C_{38}$ with the dialdehydes **7** and **8** as central components (*Scheme 2*). The symmetrical C_8-dialdehyde **8** was readily prepared from 1,1,4,4-tetramethoxybut-2-ene and divinyl ether by published procedures [18].

The synthesis of a mixture of the (all-*E*)- and of (2*E*,4*Z*,6*E*)-stereoisomers of C_9-dialdehyde **7** (*Scheme 3*) could be achieved by *Wittig* condensation of the protected C_5-aldehyde *Wittig* salt **9** [19] with fumaraldehyde mono(dimethyl acetal) **10** [20][21] followed by *Amberlyst-15*-catalyzed deacetalization [22] of the intermediate diacetal. Repeated crystallization of (2*E*,4*E*/*Z*,6*E*)-**7** yielded the higher-melting (2*E*,4*Z*,6*E*)-**7** stereoisomer. The corresponding (all-*E*)-**7** isomer was obtained by an analogous route starting from the six-membered-ring acetal phosphonium salt **11** [23], taking advantage of the nicely crystalline intermediate (all-*E*)-**12** (*Scheme 3*). The reaction of (all-*E*)-**7** with the phosphonium salt **6** gave an isomeric mixture that isomerized in hot EtOH to



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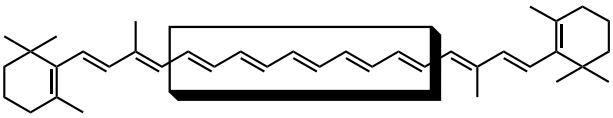

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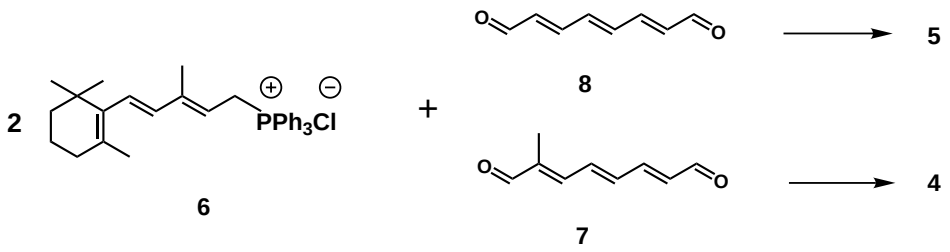
Fig. 2. *Two substrate analogues lacking one* (**4**) *or two* (**5**) *Me groups in the polyene chain*

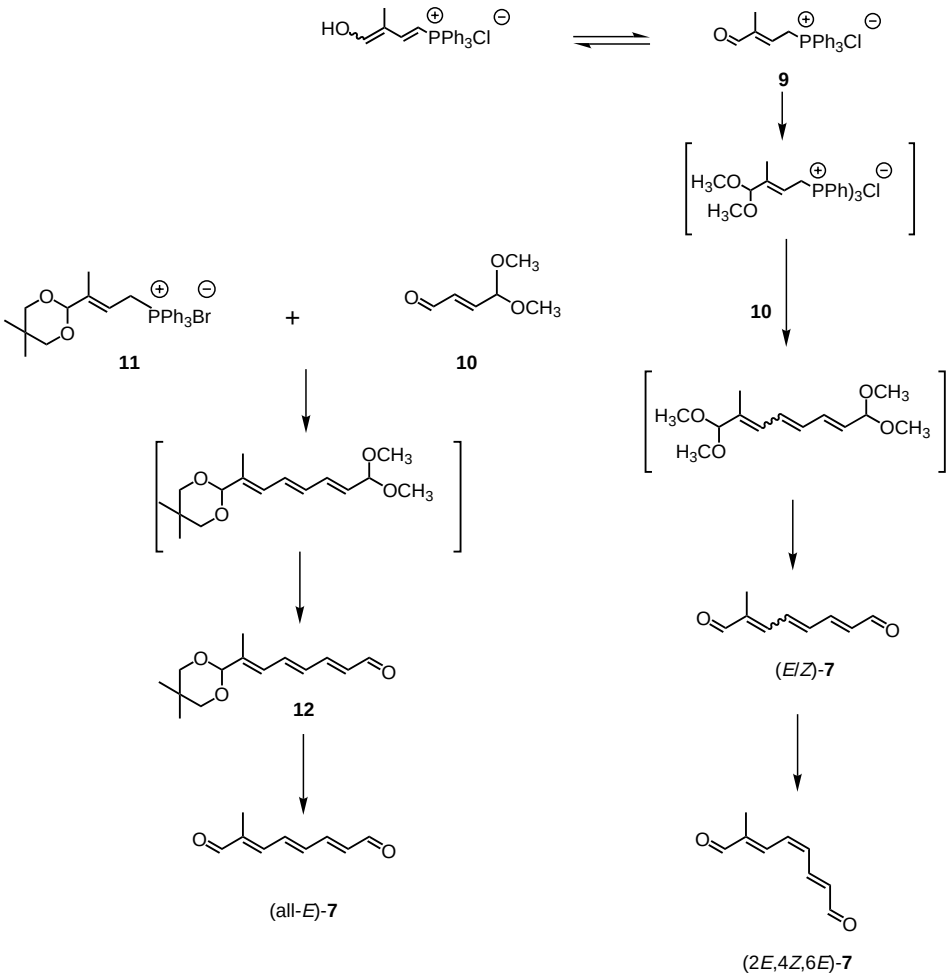
Scheme 2. *Synthesis of Norcarotenoids* **4** *and* **5** *with the* Wittig *Salt* **6** *as a Common Precursor*



furnish (all-*E*)-20-nor-*β*,*β*-carotene (**4**) (95% pure by HPLC). In contrast, isomerization of the (11*Z*)-, (11*Z*,11′*Z*)-, and (all-*E*)-isomeric mixture of bisnorcarotenoid **5**, obtained by condensation of **6** and **8**, proved to be difficult; in particular structure elucidation of the three isomers of **5** was not facile. Therefore, a protocol for the separation of this mixture by HPLC was developed (see *Exper. Part*), and the ^{1}H-NMR spectra of the isolated isomers were compared with those measured by direct LC-NMR analysis. Finally, a sample of (all-*E*)-**5** was obtained (97% pure by HPLC) for incubation experiments.

Another aim of this study was to investigate whether the enzyme *β*,*β*-carotene 15,15′-monooxygenase tolerates deviations from the 'rod-like' structure of the standard substrate. This can be achieved by replacing the central (*E*)-C=C bond of **1** with, *e.g.*, a (*Z*)-configured C=C bond, a triple bond, or a single bond. These modifications (see *Fig. 3*) result in bent carotenoids, which, if not cleaved, could be inhibitors of the enzyme. The (15*Z*)-*β*,*β*-carotene (**13**) as well as 15,15′-dehydro-*β*,*β*-carotene (**14**) were available, while for 15,15′-dihydro-*β*,*β*-carotene (**15**), a synthetic procedure had to be developed. First attempts to employ *McMurry* conditions [24] for the reductive coupling of retinol failed, thus we decided on a double-*Wittig* reaction of **6** [25][26] with the C_{10}-dialdehyde **16**, which was prepared from **17** *via* **18** and **19** according to [27] in 15% overall yield (*Scheme 4*).

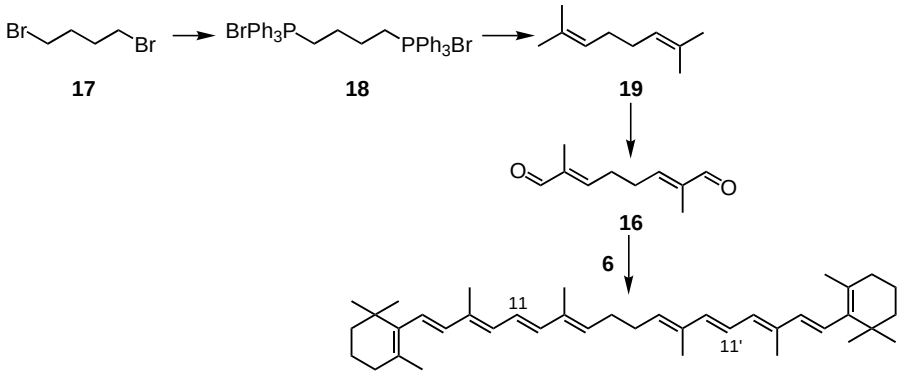
Scheme 3. *Synthesis of (all-*E*)-***7***, a Building Block for the Preparation of 20-Nor-β,β-carotene* (**4**)



The *Wittig* reaction furnished a mixture of (all-*E*)-**15**, (11*Z*)-**15**, and (11*Z*,11′*Z*)-**15**, which could be separated by semiprep. HPLC to yield 95.8% pure (all-*E*)-**15** distinguishable by UV, MS, and ^{1}H-NMR analysis from the two isomers. Isomerization of (11*Z*)-**15**, and (11*Z*,11′*Z*)-**15** → (all-*E*)-**15** under various conditions (I_2 in heptane, or photochemically) proved to be impossible.

The third aim of this work was to investigate whether modifications of the β-ionone substructure of β,β-carotene are tolerated by the enzyme β,β-carotene 15,15′-monooxygenase. For this purpose, we chosed easily available carotenoids (see below). This aspect is of particular interest not only because we gain information on the active site, but also because we expected to identify a nonsymmetric substrate analogue that could be used to determine the enzymatic reaction mechanism [10]. It is important to note

Scheme 4. *Synthesis of 15,15′-Dihydro-β,β-carotene* ((all-*E*)-**15**)



that $^{18}O_2$ experiments with *β,β*-carotene (**1**) as a substrate are not suitable to distinguish between a monooxygenase and a dioxygenase mechanism since two identical molecules are produced.

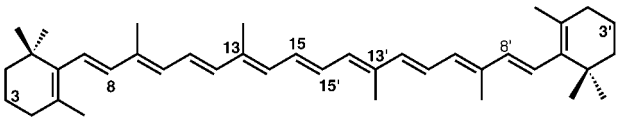
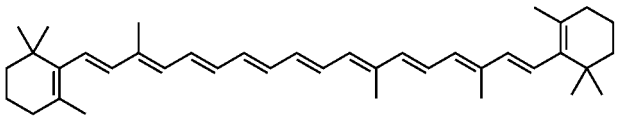
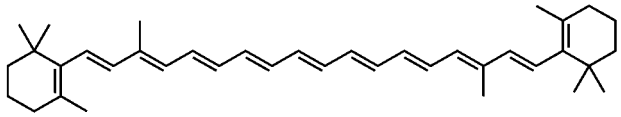
2.2. *Incubations with Substrate Analogues of β,β-Carotene 15,15′-Monooxygenase.* Incubations were performed with enzyme fractions obtained from chicken intestinal mucosa. The pellet from the 20–45° $(NH_4)_2SO_4$ fractionation was used to cleave *β,β*-carotene (**1**) to retinal (**2**) in 30% yield [7] (37°, pH 7.8, 3 h). Apo-*β*-carotenals that could be derived from *excentric* cleavage were not observed. The HPLC system (diode-array detection, internal standard) was optimized for clear separation of every possible apocarotenyl metabolite and retinoic acid. Retinoic acid was detected only in the 45–60% $(NH_4)_2SO_4$ fraction, and it was shown in control experiments that this metabolite derives from retinal [7]. The yield given for retinal does not include the fraction that is converted to retinoic acid, hence the figures represent the lowest limit of enzymatic carotenoid cleavage.

The incubation of (all-*E*)-20-nor-*β,β*-carotene (**4**) gave 45% yield (relative to **1**) of retinal and equal amounts of nor-retinal while, under the same conditions, the bis-nor-carotenoid **5** was metabolized only 2% (*Table 1*). These results indicate the significance of the Me groups of the polyene chain for substrate binding. Further, these experiments show that the enzyme *β,β*-carotene 15,15′-monooxygenase is regiospecific with respect to the central double bond even with substrate **4**, which has three C=C bonds of similar reactivity and steric environment to the active site.

On incubation of the dihydro-*β,β*-carotene (all-*E*)-**15**, slow oxidation to *β,β*-carotene (**1**) was observed followed by cleavage to retinal (**2**) (*Table 2*); **13** and **14** were not substrates for *β,β*-carotene 15,15′-monooxygenase.

The three compounds (see *Table 2*) were also not competitive inhibitors of the enzyme, in contrast to earlier reports for **14** [28]. *Fig. 3* shows the structures of the three substrates calculated with the TITAN force-field package; it is obvious that (all-*E*)-**15** in its preferred diaxial conformation at the central C–C bond displays 'a step' within the carotenoid structure that leads to two separated chromophores.

Table 1. *Enzymatic Formation of Retinal* (**2**) *from* **1** *and Substrate Analogues* **4** *and* **5**

Substrate	Yield [%] of retinal (**2**)
 **1**	100[a])
 **4**	45[b])
 **5**	2[b])

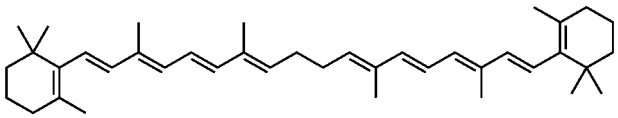
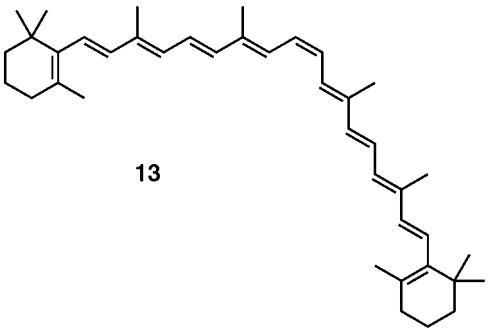
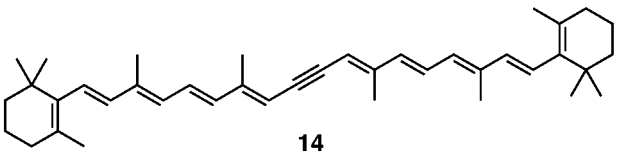
[a]) Absolute yield 30%. [b]) Relative to yield from **1**, set at 100% for convenient comparison.

The (*Z*)-configuration of the central C=C bond yields a completely bent carotenoid (see **13**), while the 15,15′-dehydrocarotene **14** is less bent, but the distance between the Me groups at C(13) → C(13′) is larger by 1.5 Å than in **1**. These results suggest that the substrate binding pocket of the enzyme does not tolerate substrates displaying any deviation from the 'rod-like' structure of β,β-carotene (**1**).

Results from incubations of substrates that have end groups other than those found in **1** are shown in *Table 3*. It is interesting to note that substrates with only one β-ionone ring substituted with a polar group like cryptoxanthin (**20**) or 3-oxoechinenone (**21**) are readily cleaved to retinal, whereas zeaxanthin (**22**) is not a substrate, and α-carotene (**23**), having a distorted end group, is only a poor substrate. When one β-ionone ring is replaced by a more polar head, as in 2′-dehydroplectariaxanthin **24**, only traces of retinal were detected. These experiments suggest that the substrate binding site is not uniform for both end groups, such that a substrate having polarity in one β-ionone ring and the overall shape of the native substrate β,β-carotene is tolerated. In agreement with these results are the results of incubations of the enzyme with canthaxanthin (4,4′-dioxo-**1**), astaxanthin (3,3-dihydroxy-4,4′-dioxo-**1**), and lutein (3*R*,3′*R*-dihydroxy-**23**); in all cases no cleavage to form retinal was detected.

In summary, the enzyme β,β-carotene 15,15′-monooxygenase has a rather rigid binding site in which the substrate-protein interaction determines the absolute preference to cleave the central C(15)=C(15′) bond of those carotenoids that are at least 'half-identical' to the native substrate **1**.

Table 2. *Enzymatic Formation of Retinal* (**2**) *from the Structurally Diverse Carotenoids (all-*E*)-***15**, **13**, *and* **14**

Substrate	Yield[a]) [%] of retinal (**2**)
 (all-E)-**15**	15[b])
 **13**	0
 **14**	0

[a]) Relative to yield from **1**, set at 100%. [b]) Cleavage observed only subsequent to oxidation to **1**.

Experimental Part

General. All reactions were performed under Ar. Column chromatography (flash chromatography, FC) was performed with silica gel (SiO_2, 0.040–0.063 mm, 230–400 mesh). HPLC: *Hewlett-Packard* HPLC system *Series 1050* with diode-array detector and *Hewlett-Packard Chemstation*. *Method 1: Supelcosil LC-18* column, 250 × 4.6 mm, 5 μm (*Supelco*), MeCN/H_2O 25:75 (*v*/*v*), flow 1.5 ml/min, 340 nm. *Method 2: YMC Carotenoid C30* column, 250 × 4.6 mm, 5 μm (*Stagroma*), acetone, flow 1 ml/min, monitoring wavelength 450 nm; anal. isolation of several isomers were carried out by this method (10-μl injection of a 60-mg/ml carotenoid soln.). *Method 3: YMC Silica 200A* column, 250 × 4.0 mm, 3 μm (*Stagroma*), hexane/0.02% (*v*/*v*) Et_3N, flow 2 ml/min, monitoring wavelength 400 nm. *Method 4:* for LC-NMR coupling: *Prontosil* C_{30} column, 250 × 4.0 mm, 3 μm (*Bischoff*), acetone/D_2O 96:4, flow 1 ml/min, monitoring wavelength 450 nm. UV: λ in nm (ε). IR: $\tilde{\nu}$ in cm^{-1}. ^{1}H-NMR: Chemical shifts (δ) in ppm, coupling constants *J* in Hz. EI-MS: *Finnigan MAT SSQ-7000*, direct introduction, 70 eV, ion-source temp. 240°.

Enzymatic Reactions. All operation were carried out on ice, and refrigerated centrifuges (4°) were used. All chemicals were purchased in highest available purity from either *Fluka AG* (CH-Buchs), *Sigma*, *Aldrich*, or *Bio-Rad*. Standards of carotenoids and retinoids were generous gifts from *F. Hoffmann-La Roche*, Basel.

Animals. Roughly four-week-old fattened chickens (*Vedette*) or 22-week-old laying hens (*LSL Lohmann*) fed on a diet low in pigments (*Kliba 556 SP18* feed) for two weeks were killed by decapitation. The first third of

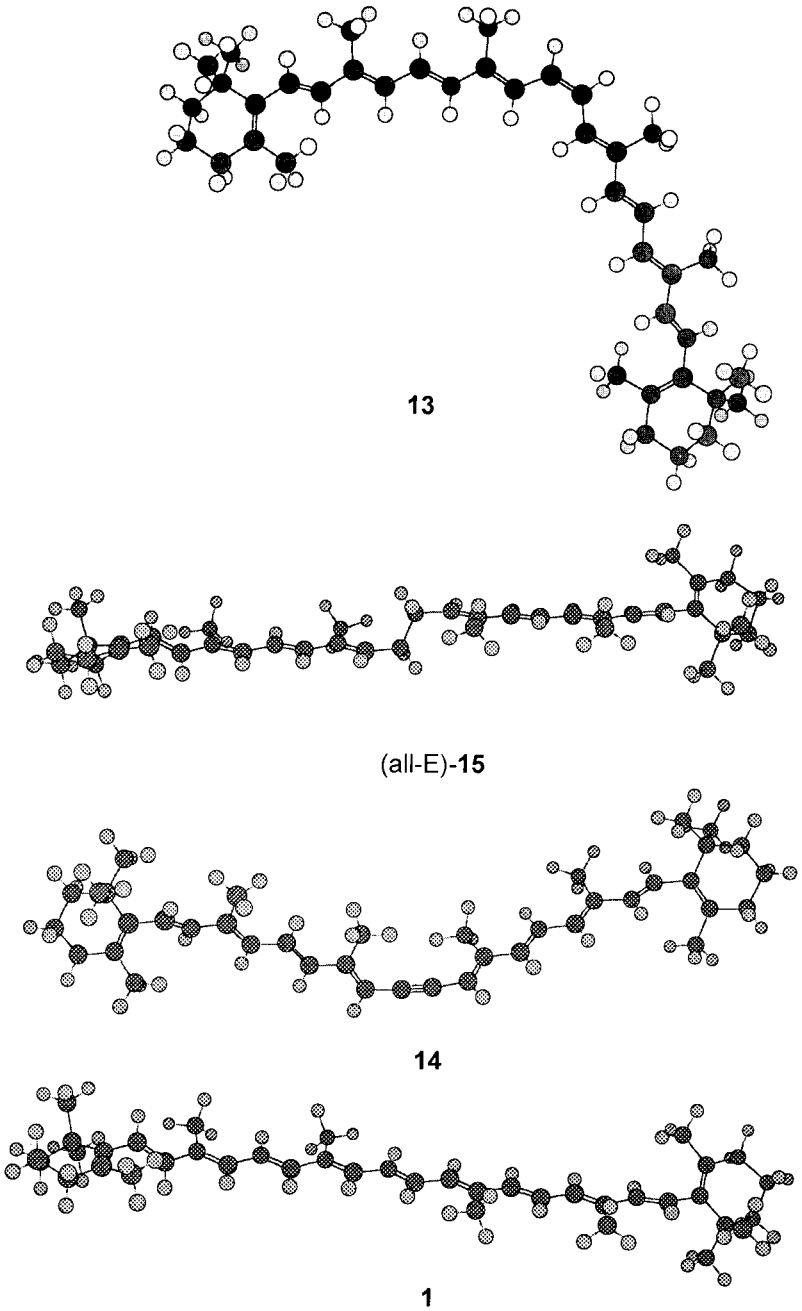
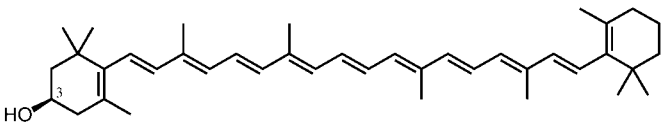
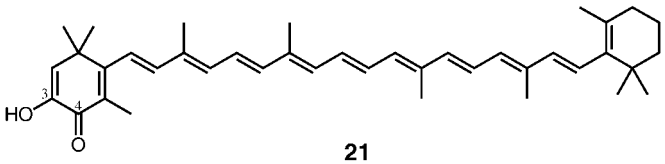
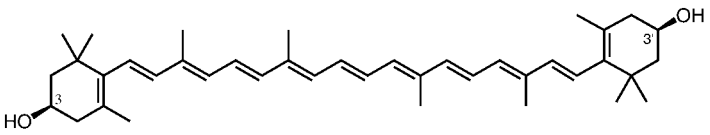
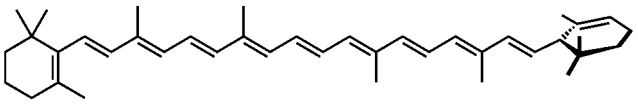
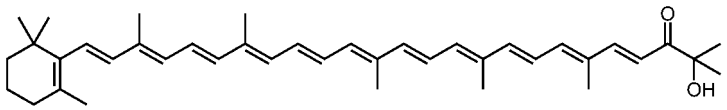


Fig. 3. *Structures of carotenoids* **13**, **15, 14**, *and* **1** *generated by molecular-modelling studies* (TITAN force-field calculations)

Table 3. *Enzymatic Formation of Retinal* (2) *from Carotenoids* **21**–**25** *with One or Both End Groups Modified*

Substrate	Yield[a]) [%] of retinal (**2**)
 **20**	40
 **21**	45
 **22**	0
 **23**	8
 **24**	2

[a]) Relative to yield from **1**, set at 100%.

the small intestine was removed, separated from the pancreas with scissors, and flushed with 0.9% NaCl soln. The intestines were immediately placed on dry ice and stored at $-80°$ until further use.

Enzyme Preparation. The intestine was thawed on ice and cut lengthwise. The mucosa was scraped off with a glass slide and homogenized with a *Teflon* pestle (*Heidolph RZR 2020*, 6 passes at 200 rpm) in 4 vol of 0.1M K_3PO_4 phosphate buffer, pH 7.8, containing 4 mM $MgCl_2$ and 30 mM nicotinamide, and 5 μM soybean trypsin inhibitor, 10 mM 6-aminocaproic acid, and 5 mM benzamidine as a protease inhibitor. After centrifugation for 1 h at 62000 g (*Heraeus sepatech Suprafuge 22*), the supernatant was subjected to 20–45% $(NH_4)_2SO_4$ fractionation (20–45% AS). The pellet obtained by centrifugation (10 min at 5000 g (*IEC Centra-7R*)) was stored in aliquots at $-80°$ until further use.

Incubation Procedure. In a standard incubation, the 20–45% AS precipitate was redissolved in a small volume (15 ml) of 0.1M phosphate buffer, pH 7.8, containing 4 mM $MgCl_2$ and 30 mM nicotinamide to give a protein concentration of *ca.* 5 mg/ml (by *Bradford* assay), and 20 mM glutathione (reduced, GSH) in a total volume of 10 ml. The incubation was started by adding 250 µl of a soln. containing 8.8 nmol carotenoids as mixed micelles, which were prepared in the following manner. A stock soln. of 80 µg/ml β,β-carotene in $CHCl_3$ (235 µl) and of 10 mg/ml α-tocopherol in hexane (500 µl) were placed in a small glass vial wrapped in aluminum foil. After removing the solvent under a faint stream of N_2, the mixture was solubilized in 1 ml of micellar soln. (prepared from glycocholic acid and lecithin: glycocholic acid (1.16 g) was dissolved in 5 ml of H_2O under stirring and by dropwise addition of 5N NaOH. After the pH was adjusted to 6.8–7.2 with AcOH and the volume brought to 10 ml with H_2O, 80 mg of acolectin was added and dissolved under stirring). This β,β-carotene micellar soln. was agitated on a vortex mixer and kept in the dark until use. Incubations were carried out in reaction vials in a shaking water bath at 37° in the dark for 3 h. Control incubations without added carotinoid or without added enzyme preparation were performed regularly.

Extraction and HPLC. The incubation was stopped by cooling on ice for 5 min and quenching with 6.5 ml of MeCN, followed by vortexing. Extraction (3×) with $CHCl_3$ was performed by vortexing and centrifuging for 10 min at 5000 g at 4°. The $CHCl_3$ layer was removed, concentrated *in vacuo*, and the residue was redissolved in 200 µl of hexane/$CHCl_3$ 9:1 containing 10 ng/µl retinyl acetate as internal standard. The final extract was passed through a 0.45-µm mini-filter (*Nylon*, *Schleicher & Schuell*), and a 20-µl aliquot was analyzed by HPLC (*Hewlett-Packard 1100* system; *Lichrospher 100-RP-18*, 5 µm, 12.5 cm × 4.6 mm (*Bischoff*); 25°). The mobile phase was MeCN/THF/H_2O (+1% ammonium acetate) (solvent *A*, 50:20:30 (*v*/*v*/*v*), or solvent *B*, 50:44:6 (*v*/*v*/*v*)) at a flow rate of 1 ml/min; the elution program was 3 min at 100% A, followed by a 6-min linear gradient to 60% *B*, 12 min at 60% *B*, a 1-min linear gradient to 100% *B*, 9 min hold at 100% *B*, then a 7-min gradient back to 100% *A*. This program allowed separation of apocarotenals, which could have been formed after excentric cleavage. Retinyl acetate was used as internal standard.

For analysis of cryptoxanthin, the gradient was changed to delay elution of the 3-hydroxyretinal by having an initial 8 min of mobile phase *C* (solvent *C*, MeCN/THF/(H_2O + 1% ammonium acetate) 40:10:50 *v*/*v*/*v*) before changing to 100% *A* in 3 min; the same gradient as described above was used for the remainder of the elution. Detection with the photo-diode-array detector allowed collection of UV spectra (300–550 nm). The detector was set at 340 and 380 nm for retinoids, and 450 nm for carotenoids. Peaks were identified by coelution with standard retinoids and quantified relative to the internal standard, with determination of peak areas calibrated against known amounts of standards.

*(2*E*,4*E*/*Z*,6*E*)-2-Methylocta-2,4,6-trienedial* ((2*E*,4*E*/*Z*,6*E*)-**7**). To a stirred mixture of *Wittig* salt **9** (11.4 g, 30 mmol), methyl orthoformate (4.13 g, 4.28 ml, 39 mmol), and MeOH (11 ml) was added TsOH (70 mg, 0.366 mmol) at r.t. The mixture became a red-brown soln. and was stirred for 40 min; i-PrOH (75 ml), a few drops (*ca.* 0.1 ml) of 5.27N MeONa in MeOH, fumaraldehyde mono(dimethyl acetal) (**10**; 4.15 g, 30 mmol) were added, and, finally, over a period of 30 min, 5.27N MeONa in MeOH (2.3 ml, 12.1 mmol) was added. After 75 min, the mixture was poured on ice and conc. NaCl soln. and extracted with AcOEt (2 × 200 ml). The combined org. layers were washed with NaCl soln. (3 × 50 ml), dried (Na_2SO_4), and concentrated *in vacuo* to give 18.4 g of an orange semisolid. This crude product was taken up in 150 ml of hexane/AcOEt/Et_3N 500:500:1 (*v*/*v*/*v*) and filtered through 300 g of silica gel, eluting with the same solvent mixture (1700 ml) to give crude (*E*/*Z*)-condensation product as a yellow-orange oil (7.6 g). Acetone (160 ml), H_2O (9.5 ml) and *Amberlyst-15* (6.2 g) were added, and the mixture was stirred for 20 min at r.t. After 90 min, the soln. was decanted (the residual *Amberlyst-15* was washed with total 200 ml of CH_2Cl_2) and poured on ice/$NaHCO_3$ soln. The CH_2Cl_2 layers were washed with $NaHCO_3$ soln., dried (Na_2SO_4), and concentrated *in vacuo* to yield (*E*/*Z*)-**7** (4.7 g). Orange solid. HPLC (*Method 1*): 46.3% (all-*E*)-**7**, 46.3% (2*E*,4*Z*,6*E*)-**7**.

*(all-*E*)-7-(5,5-Dimethyl[1,3]dioxan-2-yl)octa-2,4,6-trienal* (**12**). Obtained from *[3-(5,5-dimethyl[1,3]dioxan-2-yl)but-2-enyl]triphenylphosphonium bromide* (**11**) and fumaraldehyde mono(dimethyl acetal) (**10**) according to the procedure described for the synthesis of (*E*/*Z*)-**7**. Compounds **12** crystallized from hexane as light yellow needles. M.p. 88–90°. UV (EtOH) 320 (337). IR (KBr): 1675, 1657, 1609, 1152, 1098. ^{1}H-NMR (400 MHz, $CDCl_3$): 9.57 (*s*, CHO); 7.18 (*dd*, *J* = 15.2, 11.2, H–C(3)); 6.93 (*dd*, *J* = 14.8, 10.9, H–C(5)); 6.49 (*dd*, *J* = 11.2, 14.8, H–C(4)); 6.36 (*d*, *J* = 10.9, H–C(6)); 6.16 (*dd*, *J* = 7.9, 15.2, H–C(2)); 1.93 (*s*, Me–C(7)); 4.8 (*s*, H–C(8)); 3.52 (*s*, H–C(9), H–C(11)); 3.68 (*d*, *J* = 10.9); 1.23 (*s*, Me–C(5′)); 0.76 (*s*, Me–C(5′)). MS: 236 (100, $M^{+\bullet}$), 221 (16), 207 (57), 168 (21), 150 (21), 142 (66), 212 (65). Anal. calc. for $C_{14}H_{20}O_3$: C 71.16, H 8.53; found: C 71.05, H 8.48.

*(all-*E*)-2-Methylocta-2,4,6-trienedial* ((all-*E*)-**7**). Obtained by deacetalization of (all-*E*)-**12** (2M H_2SO_4, MeOH, 50°). Crystallization from Et_2O/hexane at 0°. Light yellow powder. M.p. 84–87°. IR (KBr): 1676, 1619,

1412, 1383, 1289, 1158, 1146, 1106, 1012, 995, 838, 617. ^{1}H-NMR (400 MHz, $CDCl_3$): 9.67 (*d*, *J* = 7.8, H–C(8)); 9.54 (*s*, H–C(1)); 7.26 (*dd*, *J* = 11.2, 15.3, H–C(6)); 7.11 (*dd*, *J* = 11.6, 14.8, H–C(4)); 6.95 (*d*, *J* = 11.6, H–C(3)); 6.85 (*dd*, *J* = 14.8, 11.2, H–C(5)); 6.33 (*dd*, *J* = 15.3, 7.8, H–C(7)); 1.95 (*s*, Me). MS: 150 (75, $M^{+\bullet}$), 121 (100, $[M - CHO]^+$), 107 (13), 93 (25), 91 (48), 82 (20), 79 (25), 77 (60).

*(2*E*,4Z,6*E*)-2-Methylocta-2,4,6-trienedial* ((2*E*,4*Z*,6*E*)-**7**). Obtained from (2*E*,4*E*/*Z*,6*E*)-**7** by repeated cystallization from *t*-BuOMe/hexane. Light yellow crystals. M.p. 95–97°. HPLC (*Method 1*): 99.4%; 0.6% of (all-*E*)-**7**. UV (2% CH_2Cl_2, cyclohexane): 328 (2320), 313 (2703), 207 (5283). IR (KBr): 1682, 1669, 1610, 1234, 1197, 1157, 1124, 1002, 986. ^{1}H-NMR (400 MHz, $CDCl_3$): 9.74 (*d*, *J* = 7.6, H–C(8)); 9.62 (*s*, H–C(1)); 7.7 (*dd*, *J* = 11.4, 15.2, H–C(6)); 7.4 (*d*, *J* = 10.9, H–C(3)); 6.89 (*dd*, *J* = 10.9, 11.4, H–C(4)); 6.63 (*dd*, *J* = 11.4, 11.4, H–C(5)); 6.34 (*dd*, *J* = 15.2, 7.6, H–C(7)); 1.95 (*s*, Me–C(2)). MS: Identical with the spectrum of (all-*E*)-**7**. Anal. calc. for $C_9H_{10}O_2$: C 71.98, H 6.71; found: C 71.99, H 6.61.

20-Nor-β,β-carotene (**4**). To the soln. of *[(2*E*,4*E*)-3-methyl-5-(2,6,6-trimethylcyclohex-1-enyl)penta-2,4-dienyl]triphenylphosphonium chloride* (**6**; 6.013 g, 12.0 mmol), **7** (0.8 g of (2*E*,4*E*/*Z*,6*E*)-**7** as described above, content 94% by HPLC, 5.0 mmol), CH_2Cl_2 (40 ml), and H_2O (5 ml) was added, at 37°, 5.27N MeONa in MeOH (2.3 ml, 12.1 mmol, 1 ml over 1 h, 1.3 ml over 2 h) with a *Metrohm 665 Dosimat*. The mixture was poured on ice and extracted with CH_2Cl_2 (150 ml). The CH_2Cl_2 layer was washed with H_2O, dried (Na_2SO_4), and evaporated to yield 6.5 g of dark red crystals, which were taken up in EtOH (300 ml) and heated under reflux for 7 h. The heating was removed, and crystallization was effected by stirring overnight, followed by cooling to 0°. The crystals were separated by filtration, washed with EtOH (250 ml, 0°) and dried (r.t., pressure *ca*. 0.1 mm) to give 1.22 g (47%) of crude **4**. Red solid. M.p. 133–134°. HPLC (*Method 3*): 98.5%. Another sample of crude **4** (0.22 g, 96.7% by HPLC) was chromatographed on 300 g of silica gel (hexane/CH_2Cl_2 9:1) to yield 0.16 g of red oil, homogeneous by TLC. Crystallization from 20 ml of Et_2O/EtOH 1:1 at 4° gave 0.12 g of (all-*E*)-**4**. Red shiny plates. M.p. 154-155°. HPLC (*Method 3*): 95.2% (all-*E*)-**4**. UV/VIS (2% CH_2Cl_2, hexane): 473 (2495), 445 (2809), 269 (352). MS: 522 (100, M), 444 (2, $[M - C_6H_6]^+$), 430 (9, $[M - C_7H_8]^+$). Anal. calc. for $C_{39}H_{54}$: C 89.59, H 10.41; found: C 89.73, H 10.37.

20,20′-Dinor-β,β-carotene (**5**). To a soln. of **6** (6.013 g, 12.0 mmol) and *(all-*E*)-octa-2,4,6-trienedial* (**8**; 0.68 g, 5.0 mmol) in i-PrOH (60 ml) at 40° was added 5.27N MeONa in MeOH (2.3 ml, 12.1 mmol) over 3 h with a *Metrohm 665 Dosimat*. After 1 h, the mixture was slowly cooled to 6°, filtered, and dried to give crude **5** as red-orange crystals (3.26 g, according to HPLC (*Method 3*) mainly a mixture of (11*Z*)-, (11*Z*,11′*Z*)-, and (all-*E*)-isomers 24:50:24). FC of 0.22 g of the above mixture of stereoisomers on 300 g silica gel (hexane/CH_2Cl_2 9:1) yielded 120 mg of a viscous oil (R_f (silica gel; hexane/CH_2Cl_2 9:1, 0.30), which was crystallized from 20 ml of Et_2O/EtOH 1:1 (overnight at 4°) to give 42 mg of (all-*E*)-**5**. Shiny red-brown crystals. M.p. 183–184°. Purity 97%. UV/VIS (hexane) 468 (2656), 442 (2994), 269 (328). MS: 508 (100, $M^{+\bullet}$), 430 (4, $[M - C_6H_6]^+$).

*(11*Z*)-20,20′-Dinor-β,β-carotene* ((11*Z*)-**5**) *and (11*Z*,11′*Z*)-20,20′-Dinor-β,β-carotene* ((11*Z*,11′*Z*)-**5**). Structure elucidation of the isomers (11*Z*)-**5** and (11*Z*,11′*Z*)-**5** could be achieved either after repeated chromatographic fractionation of crude **5** by HPLC (*Method 2*) and subsequent analysis by off-line ^{2}D-NMR experiments or by on-line stopped-flow HPLC/NMR coupling (HPLC, *Method 4*). Chromatography on C_{30}-columns yielded an excellent separation with very good selectivity for all occurring isomers (*Fig. 4*). UV/VIS Absorption maxima recorded by a photodiode-array detector (*Table 4*) were also in agreement with the proposed structures.

Interpretation of the ^{1}H-NMR spectra of **5** was accomplished on the basis of the observed δ and *J* values as well as the ^{1}H,^{1}H connectivities in 2D-COSY spectra (not shown here). The resulting δ values for the (all-*E*)- and the (*Z*)-isomers are collected in *Tables 4* and *5*. Since the LC-NMR measurements were performed with acetone/D_2O as solvent, the δ values differ from those measured in $CDCl_3$.

(Butane-1,4-diyl)bis[triphenylphosphonium Bromide] (**18**). The salt **18** was synthesized according to [29]. ^{1}H-NMR (250 MHz, $CDCl_3$): 7.85–8.00 (*m*, 12 arom. H); 7.60–7.80 (*m*, 18 arom. H); 3.90–4.15 (*m*, 2 H–C(1), 2 H–C(4)); 2.15–2.35 (*m*, 2 H–C(2), 2 H–C(3)). EI-MS: 580 (2), 579 (5), 578 (2, $[M - HBr - Br]^+$), 291 (15), 290 (100), 289 (45, $[M - 2Br]^{2-}$).

2,7-Dimethylocta-2,6-diene (**19**). The salt **18** (23.7 g, 32 mmol) was dissolved in 150 ml of DMSO. Then, 73 ml (116 mmol, 2.2 equivalent) of 1.6M BuLi soln. in hexane was added under cooling with an ice bath so that the reaction temp. did not exceed 10°. The deep red-orange suspension was stirred for an additional 15 min. Then, 5.17 ml (4.09 g, 70.4 mmol, 2.2 equiv.) acetone was added dropwise, and the mixture was stirred for another 2 h, and an additional 5.17 ml (4.09 g, 70.4 mmol, 2.2 equiv.) acetone was added. Then, a 10-cm *Vigreux* column was placed on the flask, and acetone and hexane were distilled off. Subsequently, the *Vigreux* column was removed and the mixture distilled at 1 atm. A fraction that distilled in the range of 100–130° was collected to which 70 ml of hexane was added, the org. layer was washed with 10 ml of H_2O and dried (Na_2SO_4). After

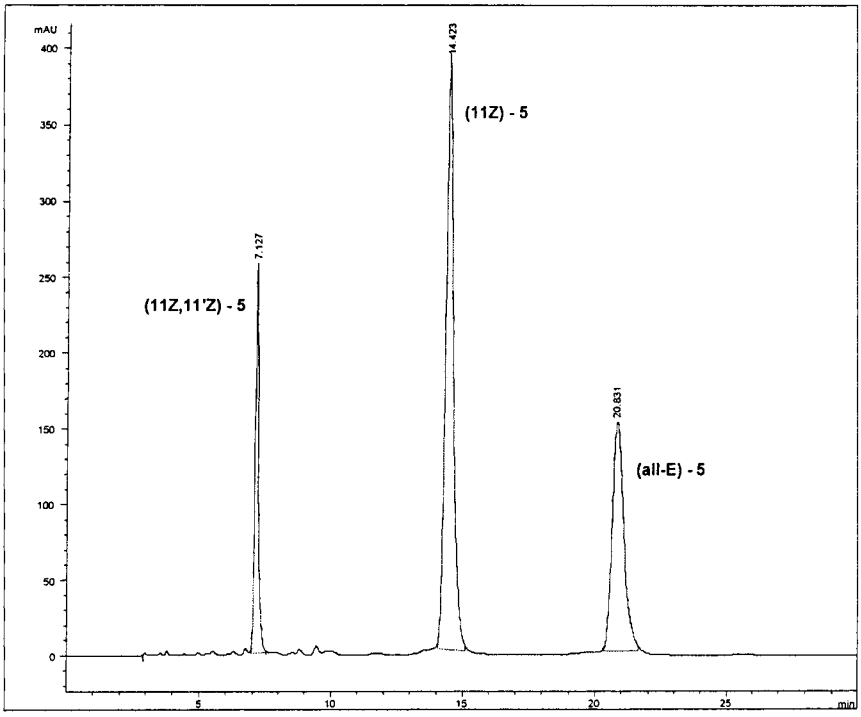


Fig. 4. *HPLC Separation of the stereoisomers of 20,20′-dinor-β,β-carotene* (**5**) *on a* C_{30}*-bonded column* (*Method 4*)

Table 4. *UV/VIS Absorption Maxima of Stereoisomers of* **5** (in acetone/D_2O 96 : 4)

Stereoisomer	λ_{max} [nm] (relative intensity)		
(all-*E*)-**5**		445 (100)	471 (89)
(11*Z*)-**5**	341 (20)	443 (100)	467 (86)
(11*Z*,11′*Z*)-**5**		441 (100)	462 (84)

removal of the solvent, **19** (1.11 g, 25%) was isolated. Colorless oil. IR (film): 2966v*s*, 2923v*s*, 2855v*s*, 1660v*w*, 1451*s*, 1378*s*. ^{1}H-NMR (250 MHz, $CDCl_3$): 5.10–5.20 (*m*, 2 olef. H); 1.95–2.05 (*m*, 2 CH_2); 1.69 (*s*, 2 Me), 1.61 (*s*, 2 Me). EI-MS: 138 (6, M^+), 123 (6), 95 (10), 82 (18), 70 (17), 69 (100), 67 (8), 41 (60).

*(all-*E*)-2,7-Dimethylocta-2,6-dienedial* (**16**) [27]. Diene **19** (497.3 mg, 3.60 mmol) was dissolved in 14 ml of 97% aq. EtOH and heated under Ar to 55°. To this soln., 2.16 g (19.5 mmol, 5.4 equiv.) of SeO_2 dissolved in 120 ml of 97% aq. EtOH was slowly added at such a rate that the reaction temp. was between 50 and 55°. Then the mixture was heated to reflux for 16 h under an inert atmosphere. After cooling to r.t., the mixture was filtered through a plug of *Celite*, and the solvent was removed *in vacuo* at 25°. The residue was dissolved in 250 ml of Et_2O and washed with sat. aq. $NaHCO_3$ and NaCl, then dried (Na_2SO_4), and the solvent was removed to provide 626 mg of a brown oil. This oil was dissolved in 30 ml of AcOEt, and 2.0 g of MgO was added. The suspension was stirred for 2 h at r.t. under Ar. The suspension was filtered through a plug of *Celite*. The filtrate was concentrated on a rotary evaporator and the residue distilled at 0.15 mbar at 130–150° to provide 368 mg of **16**. Light brown distillate. ^{1}H-NMR (250 MHz, $CDCl_3$): 9.43 (*s*, 2 CHO); 6.45–6.55 (*m*, 2 olef. H); 2.59 (*dd*, *J* = 3.6, 3.6, 2 CH_2); 1.78 (*s*, 2 Me).

*(all-*E*)-15,15′-Dihydro-β,β-carotene* (**15**). *Wittig* salt **6** (1.76 g, 3.51 mmol, 2.2 equiv.) was dissolved in 80 ml of EtOH, and a soln. of 264 mg (1.59 mmol) of **16** dissolved in 2.0 ml of EtOH was added. To this soln., 710 µl

Table 5. *^{1}H-NMR* (acetone/D_2O 96 : 4) *Chemical Shifts* (δ [ppm]) *of Olefinic Protons of Stereoisomers of* **5**

H-Atom	(all-*E*)-**5**	(11*Z*)-**5**	(11*Z*,11′*Z*)-**5**
H–C(7)	6.12	6.16	6.16
H–C(7′)	6.12	6.16	6.16
H–C(8)	6.04	6.12	6.12
H–C(8′)	6.04	6.12	6.12
H–C(10)	6.08	6.6	6.6
H–C(10′)	6.08	6.10	6.6
H–C(11)	6.62	6.31	6.31
H–C(11′)	6.62	6.62	6.31
H–C(12)	6.29	6.04	6.05
H–C(12′)	6.29	6.29	6.05
H–C(13)	6.34	6.85	6.85
H–C(13′)	6.34	6.34	6.85
H–C(14)	6.30	6.31	6.31
H–C(14′)	6.30	6.31	6.31
H–C(15)	6.30	6.37	6.37
H–C(15′)	6.30	6.30	6.37

Table 6. *^{1}H-NMR Data* (δ [ppm], $CDCl_3$) *of Stereoisomers of* **5**

H-Atom	(all-*E*)-**5**	(11*Z*)-**5**	(11*Z*,11′*Z*)-**5**
Me–C(1)	1.02	1.03	1.03
Me–C(1′)	1.02	1.02	1.03
CH_2(2), CH_2(2′)	1.47	1.47	1.47
CH_2(3), CH_2(3′)	1.61	1.60	1.62
CH_2(4), CH_2(4′)	2.02	2.02	2.02
Me–C(5)	1.71	1.72	1.72
Me–C(5′)	1.71	1.71	1.72
Me–C(9), Me–C(9′)	1.94	1.94	1.94
H–C(7)	6.19	6.18	6.23
H–C(7′)	6.19	6.21	6.23
H–C(8)	6.10	6.11	6.18
H–C(8′)	6.10	6.17	6.18
H–C(10)	6.10	6.53	6.53
H–C(10′)	6.10	6.11	6.53
H–C(11)	6.61	6.35	6.37
H–C(11′)	6.61	6.61	6.37
H–C(12)	6.31	6.09	6.10
H–C(12′)	6.31	6.31	6.10
H–C(13)	6.32	6.78	6.79
H–C(13′)	6.32	6.32	6.79
H–C(14)	6.32	6.32	6.37
H–C(14′)	6.32	6.32	6.37
H–C(15)	6.32	6.32	6.38
H–C(15′)	6.32	6.32	6.38

5.4M MeONa in MeOH was slowly added under an inert atmosphere and cooling with a water bath (15°). This soln. was then stirred at r.t. for 2 h and subsequently heated to reflux for an additional 60 min. After cooling to r.t., 300 ml of hexane were added. The org. layer was washed three times with a total of 240 ml 85% aq. MeOH and dried (Na_2SO_4). After concentration, the residue was redissolved in 35 ml of hexane containing some Et_2O. The solution was then reduced to a total volume of *ca.* 15 ml with a rotary evaporator, cooled to 0° for 30 min and filtered to remove precipitated Ph_3PO. The filtrate was concentrated to give 823 mg of a red oil. This residue

was chromatographed on an *Alox II N* column (hexane/benzene 4:1) to give, after evaporation, 569 mg of an orange oil. It was rechromatographed on an *Alox II N* column (hexane/benzene 5:1) to yield, after evaporation, a yellow oil (377 mg, 44%) of a mixture of three isomers of **15**[1]). Final purification was accomplished by semi-prep. HPLC (*Chromasil* C_8, 10 μm, MeOH/hexane 9:1) to yield 5.6 mg (95.8% pure by HPLC) of **15**. Slightly yellow, amorphous solid. UV (cyclohexane), 332 ($\varepsilon = 68700$, $A^{1}_{1} = 1280$). ^{1}H-NMR (400 MHz, $CDCl_3$): 6.50 (*dd*, *J* = 11.2, 15.2, H–C(11), H–C(11′)); 6.27 (*d*, *J* = 15.2, H–C(12), H–C(12′)); 6.11 (*s*, H–C(7), H–C(7′), H–C(8), H–C(8′)); 6.08 (*d*, *J* = 11.2, H–C(10), H–C(10′)); 5.50–5.55 (*m*, H–C(14), H–C(14′)); 2.26 (2*d*, *J* = 3.6, 3.6, H–C(15), H–C(15′)); 1.99–2.03 (*m*, 2 H–C(4), 2 H–C(4′)); 1.94 (*s*, 3 H–C(19), 3 H–C(19′)); 1.81 (*s*, 3 H–C(20), 3 H–C(20′)); 1.71 (*s*, 3 H–C(18), 3 H–C(18′)); 1.60–1.62 (*m*, 2 H–C(3), 2 H–C(3′)); 1.47 (*dd*, *J* = 6.0, 2.8, 2 H–C(2), 2 H–C(2′)); 1.02 (*s*, 3 H–C(16), 3 H–C(16′), 3 H–C(17), 3 H–C(17′)). EI-MS: 538.6 (6, M^+), 378.4 (6), 270.2 (20), 269.2 (100, M^{2+}), 145.0 (8), 119.0 (15), 95.1 (16), 93.1 (13), 82.1 (15), 69.2 (10).

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[1]) The λ_{max} of the different isomers, determined by a diode-array UV detector, were 334 for the (all-*E*)-, 328 for the (11*Z*)-, and 321 nm for the (11*Z*,11′*Z*)-15,15′-dihydro-β,β-carotene.