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Isolation, structural elucidation, and partial synthesis of lutein dehydration products in extracts from human plasma

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Abstract

All-*E*-(3*R*,6′*R*)-3-hydroxy-3′,4′-didehydro-β,γ-carotene (anhydrolutein I) and all-*E*-(3*R*,6′*R*)-3-hydroxy-2′,3′-didehydro-β,ε-carotene (2′,3′-anhydrolutein II) have been isolated and characterized from extracts of human plasma using semipreparative high-performance liquid chromatography (HPLC) on a C_{18} reversed-phase column. The identification of anhydroluteins was accomplished by comparison of the UV-Vis absorption and mass spectral data as well as HPLC–UV-Vis–mass spectrometry (MS) spiking experiments using fully characterized synthetic compounds. Partial synthesis of anhydroluteins from the reaction of lutein with 2% H_2SO_4 in acetone, in addition to anhydrolutein I (54%) and 2′,3′-anhydrolutein II (19%), also gave (3′*R*)-3′-hydroxy-3,4-dehydro-β-carotene (3′,4′-anhydrolutein III, 19%). While anhydrolutein I has been shown to be usually accompanied by minute quantities of 2′,3′-anhydrolutein II (ca. 7–10%) in human plasma, 3′,4′-anhydrolutein III has not been detected. The presence of anhydrolutein I and II in human plasma is postulated to be due to acid catalyzed dehydration of the dietary lutein as it passes through the stomach. These anhydroluteins have also been prepared by conversion of lutein diacetate to the corresponding anhydrolutein acetates followed by alkaline hydrolysis. However, under identical acidic conditions, loss of acetic acid from lutein diacetate proceeded at a much slower rate than dehydration of lutein. The structures of the synthetic anhydroluteins, including their absolute configuration at C(3) and C(6′) have been unambiguously established by ^1H NMR and in part by ^{13}C NMR, and circular dichroism.

1. Introduction

One class of micronutrients that has been extensively studied for their preventive effect against cancer is the carotenoids. To date, approximately 700 carotenoids have been isolated from natural sources and their chemical structures established [1]. During the past decade, we have demonstrated that fewer than 50 dietary carotenoids are absorbed, metabolized, and/or utilized by humans [2,3]. However, we have shown that only 14 dietary carotenoids from selected groups, i.e. mono- and dihydrox-

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ycarotenoids and hydrocarbon carotenoids, appear in the blood of healthy human subjects [2,4–6]. In recent years, lutein, a dihydroxycarotenoid, has been the major focus of study in our laboratories, because high concentrations of this carotenoid, abundant in most fruits and vegetables, can be detected and quantified in the extracts from human plasma by high-performance liquid chromatography (HPLC) [6]. Recent isolation and characterization of four oxidative metabolites of lutein from plasma as well as two human supplementation studies involving this compound, have revealed that in vivo oxidation is a key reaction during the metabolism of lutein [4,7]. In 1990, in addition to the oxidation products of lutein, we reported the isolation and tentative identification of a major dehydration product of lutein, namely, 2′,3′-anhydrolutein in the extracts from human plasma [4]. In earlier publications, we had reported the presence of this compound in a variety of squash and concluded that this compound was of a limited dietary source [8,9]. However, as this paper was being prepared, Deli et al. [10] reported the isolation and full characterization of two of the dehydration products of lutein (compounds I and II in Fig. 1) from extracts of ripened fruits of black paprika. Although the dietary source of the dehydration products of lutein are limited to squash and black paprika, we have routinely detected high concentrations of these compounds in plasma of healthy human subjects ingesting typical U.S. diets. Our original identification of 2′,3′-anhydrolutein (II) in squash [9] and human plasma [4], in the absence of ^{1}H NMR (nuclear magnetic resonance) data, was based solely on mass (MS) and UV-Vis absorption spectra of the isolated compound. In contrast to our earlier structural assignment, we present here unequivocal evidence to establish that the major dehydration product of lutein in human plasma is (3*R*,6′*R*)-3-hydroxy-3′,4′-didehydro-*β*,*γ*-carotene (compound I, Fig. 1) and that 2′,3′-anhydrolutein (compound II, Fig. 1) is present only at low concentration. This identification is based on comparison of the UV-Vis absorption and mass spectral data of the isolated compounds as well as HPLC–UV-Vis–MS spiking experiments with

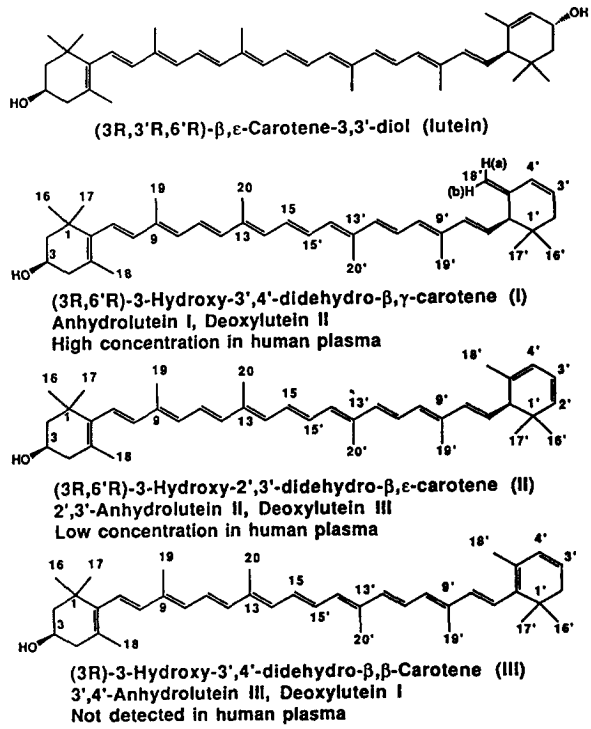

Fig. 1. Chemical structures of lutein and its dehydration products.

those of synthetic anhydroluteins I, II, and III (Fig. 1), which were fully characterized from their ^{1}H NMR, ^{13}C NMR (determined only for compound I), and circular dichroism (CD) spectra. A convenient method for partial synthesis of anhydroluteins from lutein in high yield is also described.

2. Experimental

2.1. Apparatus

A Beckman Model 114M ternary solvent delivery system equipped with a Beckman Model 421 controller was interfaced into a Hewlett-Packard (HP) 1040A rapid-scanning UV-Vis photodiode-array detector. The data were stored and processed by a HP 9000/Series 300 (ChemStation) computing system, in combination with a HP Model 9153B disk drive, color display monitor, Model 35741, and a Model 7470A

plotter. The absorption spectra of the carotenoids were recorded between 200 and 600 nm at the rate of twelve spectra per minute; spectra for examination and publication were selected from the apex of each peak in an attempt to minimize contamination in those cases where peaks were not completely resolved.

The mass spectra were obtained by interfacing the HPLC system into a Hewlett-Packard Model 5989A particle beam mass spectrometer (MS). The flow-rate with the HPLC–MS system was 0.7 ml/min. Eluate from the HPLC was divided with a ratio of 1:2 with the lesser amount entering the particle beam interface which was operated at a desolvation temperature of 45°C. Electron capture negative ionization (ECNI) was achieved using methane at a pressure of 160 Pa (1.2 Torr) and a source temperature of 250°C. Spectra were collected from m/z 100 to m/z 700 using a scan cycle time of 1.5 s.

Absorption spectra of the carotenoids in various solvents were recorded on a Beckman DU-7 UV-Vis spectrophotometer.

The ^{1}H NMR (400 MHz) and ^{13}C NMR (100.6 MHz) spectra were acquired on a Bruker ARX-400 spectrometer with ASPECTstation 1. A pulsed magnetic field z-gradient accessory kit with 10 A power amplifier (50 gauss/cm) was available for pulsed magnetic field gradient (PFG) experiments. All spectra were measured in $CDCl_3$ (100% D quality) at ca. 25°C with a 5-mm reverse probe head with optimum sensitivity for protons and reduced sensitivity for carbons. The 2D T-ROESY experiments [11] were performed with a $(180°_{x}–180°_{-x})_{n}$ spin lock of 0.6 s duration ($n = 2400$) as described in detail recently [12]. The ^{1}H-detected one-bond $^{1}H,^{13}C$-COSY was measured according to the PFG-HSQC pulse sequence of Wider and Wüthrich [13] with three half-sine-shaped gradients of amplitudes 30: − 18:6. In the 1D TOCSY [12,14] difference experiments we used a 180° DANTE pulse sequence to selectively invert the magnetization of a suitable proton. The subsequent MLEV-17 spin-lock provided magnetization transfer to neighboring spin-coupled protons resulting in a sub-spectrum of these protons [14].

The CD spectra were collected in EPA (diethyl ether–isopentane–ethanol, 5:5:2) solution between 230 and 400 nm with sensitivity of 50 mdeg/cm and a scan speed of 50 nm/min.

2.2. Chromatographic procedures

The analytical separations were carried out employing a combination of isocratic and gradient chromatography. An isocratic mixture of acetonitrile (85%), methanol (10%), and hexane–dichloromethane (1:1) (5%) (containing 0.1% diisobutylethylamine) at time 0 was followed by a linear gradient beginning at time 10 min and completed at time 40 min. The final composition of the gradient mixture was acetonitrile (45%), methanol (10%), hexane–dichloromethane (1:1) (45%). The semipreparative separations were conducted with an isocratic mixture of acetonitrile (85%), methanol (10%), and dichloromethane–hexane (1:1) (5%) (containing 0.1% diisobutylethylamine). The analytical separations were carried out on a Microsorb (Rainin Instrument, Woburn, MA, USA) stainless-steel (25 cm × 4.6 mm I.D.) C_{18} reversed-phase column (5-μm particles), which was protected with a Brownlee C_{18} reversed-phase guard cartridge (3 cm × 4.6 mm I.D.; 5-μm particles). For semipreparative separations the analytical column was replaced with a semipreparative column (25 cm × 10 mm I.D.; 5-μm particles), which was packed with the same adsorbent as above. Extracts from human plasma and synthetic compounds were dissolved in a mixture of acetonitrile (40%), methanol (20%), dichloromethane (20%), hexane (20%), containing 0.1% diisobutylethylamine and microfiltered through a 0.45-μm disposable filter assembly (Baxter, Scientific Product Division, McGaw Park, IL, USA) prior to HPLC analysis. The flow-rates with analytical and preparative columns were 0.70 ml/min and 2.5 ml/min, respectively. The monitoring wavelengths were 446 (λ_{max} of anhydrolutein I and II = 446 to 448) and 466 nm (λ_{max} of anhydrolutein III). The chromatograms were also monitored at 420, 400, 350 and 290 by the photodiode-array detector to determine the presence of impurities and carotenoid degradation products.

2.3. Reagents and materials

All-*E*-(3*R*,3′*R*,6′*R*)-lutein was isolated and recrystallized from a saponified extract of marigold flowers (*Tagetes erecta*, variety orangade) gratuitously provided by Kemin Industries (Des Moines, IA, USA) according to a procedure recently patented by one of the authors (FK) [15]. The ^{1}H NMR and the UV-Vis absorption spectra of the isolated all-*E*-(3*R*,3′*R*,6′*R*)-lutein were identical to our earlier published spectra of this compound [6]. The isolated lutein contained ca. 3% of an isomeric dihydroxycarotenoid, namely, β,β-carotene-3,3′-diol or zeaxanthin. Since zeaxanthin and lutein could not be separated by HPLC on a C_{18} reversed-phase column, the accurate distribution of these carotenoids in various samples was determined by HPLC on a silica-based nitrile bonded column as previously published [6]. The presence of low concentrations of zeaxanthin in lutein was not of concern since this compound was unreactive in the dehydration experiments. Lutein diacetate was prepared from lutein according to the original method of Karrer and Solmssen [16].

2.4. Partial synthesis of anhydroluteins

Preparation of acidic solvents

The acidic reagents were prepared by dropwise addition of 2 ml of concentrated sulfuric acid to 100 ml of the solvent, i.e. acetone or tetrahydrofuran (THF) cooled at 0°C. After the addition was completed, the mixtures were allowed to warm to room temperature. In the case of chloroform (ethanol-free), 0.2 ml of concentrated H_2SO_4 was directly added to a solution of lutein (1.76 mmol) in 200 ml of this solvent at 0°C, and the mixture was allowed to come to room temperature.

Dehydration of lutein in acidic solvents

A solution of all-*E*-(3*R*,3′*R*,6′*R*)-lutein (1.0 g, 1.76 mmol) in acetone or THF (200 ml) was treated with 30 ml of 2% solution of sulfuric acid in acetone or THF (v/v), and the mixture was stirred at room temperature for 5 h under an atmosphere of nitrogen. The product was partitioned between dichloromethane (200 ml) and water (200 ml); the organic layer was separated and washed with a 5% solution of sodium bicarbonate (200 ml). The dichloromethane layer was removed, treated with triethylamine (1 ml), dried over sodium sulfate, and evaporated to dryness to yield ca. 1.2 g of a crude product. The products were tentatively identified by HPLC–UV-Vis photodiode-array–mass spectrometry (HPLC–UV-Vis–MS) in the order of elution as: unreacted lutein and/or zeaxanthin as well as their *Z*-isomers, anhydrolutein I, 2′,3′-anhydrolutein II, 3′,4′-anhydrolutein III, *Z*-3′,4′-anhydrolutein III, and several unidentified side products (Fig. 2a). The yields of the dehydration products of lutein under various reaction conditions are summarized in Table 1.

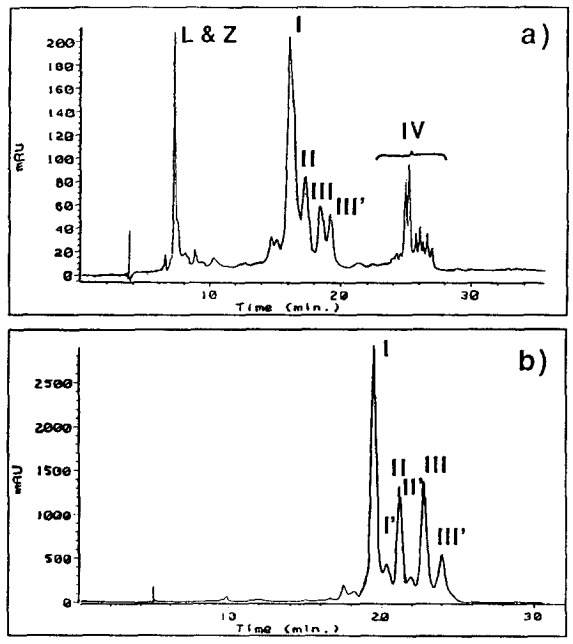

Fig. 2. HPLC separation of anhydroluteins (conditions described in text). (a) Analytical separation of the crude product from the reaction of lutein with dilute H_2SO_4. (b) Semipreparative separation of a mixture of anhydroluteins after purification by flash column chromatography. L & Z = mixture of unreacted lutein and zeaxanthin; I = all-*E*-anhydrolutein I; I′ = *Z*-anhydrolutein I; II = 2′,3′-anhydrolutein II; II′ = *Z*-2′,3′-anhydrolutein II; III = 3′,4′-anhydrolutein III; III′ = *Z*-3′,4′-anhydrolutein III; IV = mixture of unidentified carotenoid side-products.

Table 1
Yield (%) of the dehydration products of lutein under three different reaction conditions[a,b]

Reaction conditions	Unreacted lutein and zeaxanthin	I	II	III + Z-isomer	Unidentified (side-products)
Lutein (1.76 mol) + 2% H_2SO_4–acetone (30 ml) in acetone, 5 h	4[c]	54	19	19	4
Lutein (1.76 mmol) + 2% H_2SO_4–THF (30 ml) in THF, 5 h	16[d]	35	12	15	22
Lutein (1.76 mmol) + 0.2 ml H_2SO_4 (conc.) in $CHCl_3$ (EtOH free), 1 h	0	16	12	8	64

[a] Yields are based on the HPLC peak area of each compound in the crude product at the wavelength of its main absorption maximum.
[b] The general procedure for dehydration of lutein is described in the text.
[c] Only unreacted zeaxanthin (no lutein) was recovered under these conditions.
[d] Approximately 12% lutein and 4% zeaxanthin.

2.5. Partial purification of anhydroluteins by recrystallization

The crude product was dissolved in minimum amounts of diethyl ether and was treated with petroleum ether (b.p. = 30 to 60°C) until the solution became cloudy. The mixture was kept at −10°C overnight and the orange crystals were removed either by filtration or centrifugation. The HPLC analysis of the recrystallized product showed the presence of a mixture of anhydroluteins (total of 90–95% based on HPLC peak area) and unreacted lutein and/or zeaxanthin (5–10%).

2.6. Partial purification of anhydroluteins by flash column chromatography

The crude product was partially purified by flash column chromatography [17] according to the following procedure. Although this technique did not separate the various isomers of synthetic anhydroluteins, it removed the unreacted lutein as well as all the unidentified carotenoid impurities from the mixture. The fractions which consisted of a purified mixture of anhydroluteins, were combined and subjected to semipreparative HPLC for complete separation. The crude products obtained under different reaction conditions were dissolved in dichloromethane (8 ml) and chromatographed under a stream of nitrogen on a flash column (30 cm × 4 cm I.D.) employing silica gel (150 g, 60–200 mesh, J.T. Baker, Phillipsburg, NJ, USA) as adsorbent and petroleum ether (b.p. = 30 to 60°C)–acetone (95:5) as eluent. At the flow-rate of 1 in./min (2.54 cm/min), 35 fractions were collected. The volume of each fraction was ca. 100 ml. Based on HPLC analysis, appropriate fractions (16–33, about 0.7 g) were combined and applied to semipreparative HPLC (Fig. 2b). The following anhydroluteins were separated and fully characterized.

All-E-(3R,6'R)-3-hydroxy-3',4'-didehydro-β,γ-carotene (I)

UV-Vis absorption: dichloromethane, λ_{max} = 454 ($E^{1\%}$ = 2657), 484 ($E^{1\%}$ = 2393); benzene, λ_{max} = 433, 457, 487 (lit. λ_{max} = 432, 456, 486 [18]) also see Fig. 3, Tables 2 and 3. Mass spectrum (ECNI, methane): molecular anion peak at m/z = 550 (100%). CD in EPA solution, λ nm (mdeg): 236 (0), 245 (+9.66), 262 (0), 281 (−16.24), 312 (0), and 334 (+1.46)(Fig. 4) [lit. in methanol, λ nm (mdeg): 235 (0), 245 (+9.1), 260 (0), 278 (−13.4), 306 (0), and 330 (+2.3)] [18].

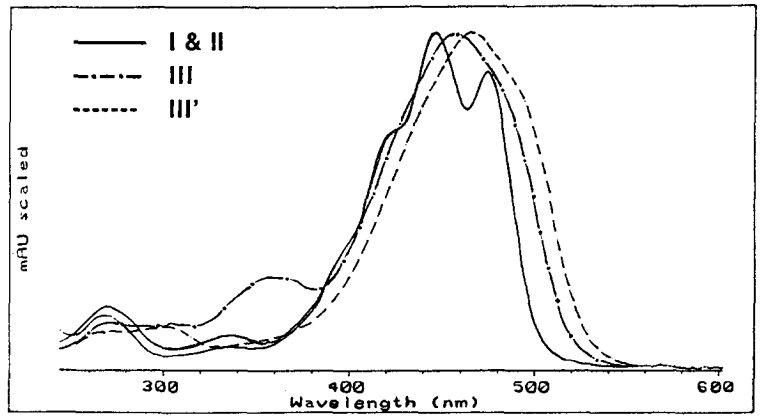

Fig. 3. UV-Vis absorption spectra of anhydrolutein I, 2′,3′-anhydrolutein (II), 3′,4′- anhydrolutein (III) and its *Z*-isomer obtained by a photodiode-array detector in the HPLC solvents (described in text). For values of the absorption maxima see Table 2.

^{1}H NMR (Table 4) and ^{13}C NMR in $CDCl_3$ were in agreement with literature values [10]. The assignment of the proton signals was supported by a 2D ^{1}H,^{1}H-COSY and some 1D TOCSY experiments. The ^{13}C assignments were based on a one-bond ^{1}H,^{13}C 2D COSY linking all protons and their directly attached ^{13}C nuclei. Despite this, some of the signals were too close to each other to be separately assigned. ^{13}C NMR: 12.69, 12.76, 12.82 (C-19, C-20, C-20′), 13.14 (C-19′), 21.62 (C-18), 25.33, 28.44 (C-16′, C-17′), 28.75 (C_{ax}-16), 30.28 (C_{eq}-17), 33.48 (C-1′), 37.14 (C-1), 38.55 (C-2′), 42.59 (C-4), 48.48 (C-2), 55.52 (C-6′), 65.12 (C-3), 112.98 (C-18′), 124.89 (C-11), 125.01 (C-11′), 125.57 (C-7), 126.18 (C-5), 127.83 (C-3′), 128.33 (C-4′), 129.26 (C-7′), 129.97, 130.13 (C-15, C-15′), 130.67 (C-10′), 131.34 (C10), 132.41 (C-14′), 132.63 (C-14), 135.50, 135.66 (C-9, C-9′), 136.41, 136.53 (C-13, C-13′), 136.62 (C-8′), 137.31 (C-12′), 137.61 (C-

Table 2
UV-Vis absorption maxima (nm) of synthetic anhydroluteins and their geometrical isomers determined by a photodiode-array detector in semipreparative HPLC separations (conditions described in text)

Anhydrolutein[a]	Absorption maxima[b,c] (nm)			
	Overtone	*Z*-peak	Fine structure	$[\epsilon_2/\epsilon_1]$[d]
all-*E*-(I)	268	334	(446) 476	[0.036]
Z-(I)	270	332	(442) 468	[0.264]
all-*E*-(II)	268	336	(448) 476	[0.027]
Z-(II)	272	334	(440) 468	[0.427]
all-*E*-(III)			(466)	
Z-(III)		356	(456)	[0.391]

[a] Complete chemical names and structures of the all-*E*-compounds are shown in Fig. 1.
[b] Values in parentheses indicate main absorption maxima.
[c] Points of inflection in the absorption spectra of carotenoids are not reported.
[d] Ratio of absorption intensities (ϵ_2) at *Z*-peak in the near-UV region (332–358 nm) to absorption intensities (ϵ_1) at main absorption maxima.

Table 3
UV-Vis absorption maxima of isomeric anhydroluteins isolated and purified by semipreparative HPLC in various solvents

Anhydrolutein[a,b]	Absorption maxima[c,d] (nm), $[\epsilon_2/\epsilon_1]^e$		
	Dichloromethane	Ethanol	Hexane
all-*E*-(I)	273,337,(454),484,[0.188]	332,(446),474,[0.141]	266,332,421,(444),473,[0.065]
all-*E*-(II)	273,339,(455),484,[0.231]	334,(447),475,[0.142]	267,333,422,(445),474,[0.118]
all-*E*-(III)	(474)	(464)	(460)
Z-(III)	367,(464),[0.31]	361,(455),[0.31]	350,(452),[0.33]

[a] Complete chemical names and structures of the all-*E*-compounds are shown in Fig. 1 and Table 5.
[b] For the value of the extinction coefficients see the text.
[c] Values in parentheses represent main absorption maxima.
[d] Points of inflection in the absorption spectra of carotenoids are not reported.
[e] Ratio of absorption intensities (ϵ_2) at *Z*-peak in the near-UV region (331–339 nm) to absorption intensities (ϵ_1) at main absorption maxima.

12), 137.80 (C-6), 138.53 (C-8), and 145.71 (C-5′).

All-E-(3R,6′R)-3-hydroxy-2′,3′-didehydro-β,ε-carotene (II)

UV-Vis absorption: dichloromethane, λ_{max} = 455 ($E^{1\%}$ = 2727), 484 ($E^{1\%}$ = 2417); benzene, λ_{max} = 433, 458, 488 (lit. λ_{max} = 434, 459, 489 [18]) also see Fig. 3, Tables 2 and 3. Mass spectrum (ECNI, methane): molecular anion peak at m/z = 550 (100%). CD in EPA solution, λ nm (mdeg): 239 (+8.31), 248 (0), 263 (−19.59), 274 (0), 287 (+21.96), and 336 (0) (Fig. 4) [lit. in methanol, λ nm (mdeg): 235 (0), 243 (+9.3), 260 (0), 278 (−14.4), 305 (0), and 333 (+1.9)] (18). ^{1}H NMR (Table 4) in agreement with literature values [10].

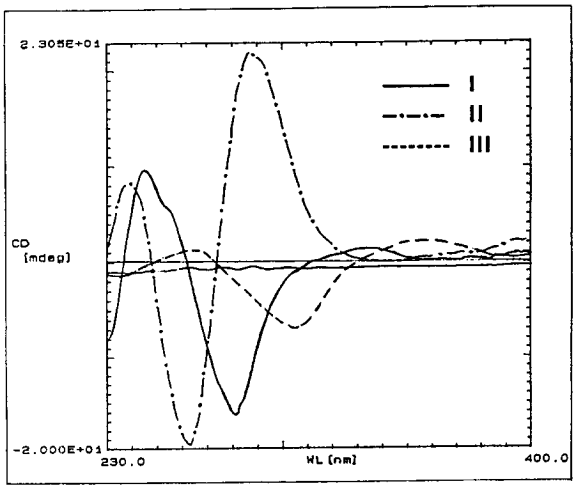

Fig. 4. Circular dichroism (CD) spectra of synthetic anhydrolutein I, 2′,3′-anhydrolutein (II), and 3′,4′-anhydrolutein (III) in EPA solution. Spectral values and conditions described in text.

All-E-3R-3-hydroxy-3′,4′-didehydro-β,β-carotene (III)

UV-Vis absorption: dichloromethane, λ_{max} = 474 ($E^{1\%}$ = 2310); benzene, λ_{max} = 476 (lit. λ_{max} = 478 [18]) also see Fig. 3, Tables 2 and 3. Mass spectrum (ECNI, methane): molecular anion peak at m/z = 550 (100%). CD in EPA solution λ nm (mdeg): 236 (−1.42), 248 (0), 261 (+1.16), 274 (0), 304 (−6.74), 330 (0), and 358 (+2.15) (Fig. 4) [lit. in methanol, λ nm (mdeg): 231 (+0.2), 255 (+0.9), 274 (0), 305 (−3.4), 325 (0), 355 (+1.6)] (18). ^{1}H NMR (Table 4) in agreement with literature values [10].

Z-3R-3-hydroxy-3′,4′-didehydro-β,β-carotene (III′)

This *Z*-isomer was tentatively identified from its UV-Vis absorption (Fig. 3, Tables 2 and 3) and mass spectra (ECNI, methane): molecular anion peak at m/z = 550 (100%).

Table 4
^{1}H NMR data (400 MHz in $CDCl_3$) for all-*E*-anhydrolutein I, all-*E*-2′,3′-anhydrolutein II, and all-*E*-3′,4′-anhydrolutein III

Position	I	II	III
H_{ax}-C(2)	1.48 (t, $J_{gem} \sim 12$, $J_{2a,3a} \sim 12$)	1.48 (t, $J_{gem} \sim 12$, $J_{2a,3a} \sim 12$)	1.48 (t, $J_{gem} \sim 12$, $J_{3a,4a} \sim 12$)
H_{eq}-C(2)	1.77 (ddd, $J_{gem} \sim 12$, $J_{2e,3a} \sim 3.5$, $J_{2e,4e} \sim 2$)	1.77 (ddd, $J_{gem} = 12$, $J_{2e,3a} = 3.5$, $J_{2e,4e} \sim 2$)	1.77 (ddd, $J_{gem} = 12$, $J_{2e,3a} = 3.5$, $J_{2e,4e} \sim 2$)
H-C(2′)	~1.91 (AB, br, $J_{gem} \sim 17$) ~2.01	5.32 (d, $J_{2',3'} = 9.6$)	2.08 (m)
H_{ax}-C(3)	4.00 (m, br, $\Sigma J = 31$)	4.00 (m, br, $\Sigma J \sim 32$)	4.00 (m, br, $\Sigma J \sim 38$)
H-C(3′)	5.71 (d, br, $J_{3',4'} = 9.5$; $J_{2'a,3'} \sim J_{2'e,3'} \sim 4$)	5.75 (dd, $J_{2',3'} = 9.6$; $J_{3',4'} = 5.2$)	5.73 (dt, $J_{2',3'} \sim 4.5$, $J_{3',4'} = 9.7$)
HO-C(3)	1.33 (d, 4.9)	1.34 (d, 4.6)	1.34 (d, ~5)
H_{ax}-C(4)	2.05 (dd, br, $J_{3a,4a} = 9.7$, $J_{gem} \sim 16.5$)	2.05 (dd, br, $J_{3a,4a} \sim 9.5$, $J_{gem} \sim 17$)	2.05 (dd, br, $J_{3a,4a} = 9$, $J_{gem} \sim 17$)
H_{eq}-C(4)	2.39 (ddd, $J_{gem} \sim 16.5$, $J_{3a,4e} = 6$, $J_{2e,4e} \sim 1.5$)	2.39 (ddd, $J_{gem} \sim 16.5$, $J_{3a,4e} \sim 6$, $J_{2e,4e} \sim 1.5$)	2.39 (ddd, $J_{gem} \sim 17$, $J_{3a,4e} = 5.5$, $J_{2e,4e} \sim 1.5$)
H-C(4′)	6.15 (d, $J_{3',4'} \sim 9.5$)	5.60 (m, br)	5.85 (d, br, $J_{3',4'} = 9.5$)
H-C(6′)	2.65 (d, $J_{6',7'} = 9.2$)	2.21 (d, 9.8)	–
H-C(7)	6.09 (d, $J_{7,8} = 16.2$)	6.09 (d, $J_{7,8} = 16.6$)	6.10 (d, $J_{7,8} = 16.5$)
H-C(7′)	5.64 (dd, $J_{7',8'} = 15.5$, $J_{6',7'} = 9.2$)	5.57 (dd, $J_{6',7'} = 9.7$, $J_{7',8'} = 15.4$)	6.19 (d, $J_{7',8'} \sim 16.5$)
H-C(8)	6.14 (d, $J_{7,8} \sim 16$)	6.13 (dd, $J_{7,8} \sim 16$)	6.13 (d, $J_{7,8} \sim 16.5$)
H-C(8′)	6.16 (d, $J_{7',8'} \sim 15.5$)	6.14 (d, $J_{7',8'} \sim 15.5$)	6.29 (d, $J_{7',8'} \sim 16.5$)
H-C(10)	6.15 (d, $J_{10,11} \sim 11.5$)	6.15 (d, $J_{10,11} \sim 10.7$)	6.16 (d?)
H-C(10′)	6.13 (d, $J_{10',11'} = 12$)	6.12 (d, $J_{10',11'} = 11.5$)	6.19 (d?)
H-C(11)	6.64 (dd, $J_{10,11} = 11.2$, $J_{11,12} \sim 14.5$)	6.64 (dd, $J_{10,11} = 11.5$, $J_{11,12} \sim 14.7$)	6.66 (dd?)
H-C(11′)	6.61 (dd, $J_{10',11'} \sim 11.5$ $J_{11',12'} \sim 15$)	6.61 (dd, $J_{10',11'} \sim 11.4$ $J_{11',12'} \sim 14.7$)	6.64 (dd?)
H-C(12)	6.36 (d, $J_{11,12} = 14.9$)	6.36 (d, $J_{11,12} = 14.8$)	6.37[a](d, $J_{11,12} = 14.7$)
H-C(12′)	6.34 (d, $J_{11',12'} = 14.9$)	6.34 (d, $J_{11',12'} = 14.7$)	6.36[a](d, $J_{11',12'} = 14.7$)
H-C(14) } H-C(14′) }	~6.25 (m, br)	~6.24 (m, br)	~6.25 (m)
H-C(15) } H-C(15′) }	~6.63 (m)	~6.63 (m)	~6.63 (m)
CH_3(16)	1.074 (s)	1.074 (s)	1.075 (s)
CH_3(17)	1.074 (s)	1.074 (s)	1.075 (s)
CH_3(16′)	0.877[a] (s)	0.937[a] (s)	1.040 (s)
CH_3(17′)	0.901[a] (s)	1.006[a] (s)	1.040 (s)
CH_3(18)	1.735 (s)	1.736 (s)	1.736 (s)
CH_3(18′)	H_a: 4.88 (s) H_b: 4.82 (s)	1.717 (s)	1.878 (s)
CH_3(19)	~1.966 (s)	1.957 (s)	1.968[a] (s)
CH_3(19′)	~1.919	1.885 (s)	1.976[a] (s)
CH_3(20)	~1.966 (s)	~1.968 (s)	1.976[a] (s)
CH_3(20′)	~1.966 (s)	~1.968 (s)	1.984[a] (s)

[a] These assignments may be interchanged.
δ in ppm, in brackets coupling constants in Hz; s = singlet, d = doublet, t = triplet, br = broad.

2.7. Isolation of anhydroluteins from human plasma

A large volume (880 ml) of human plasma (American Red Cross, Washington, DC, USA) was extracted and chromatographed on C_{18} reversed-phase plates according to our published procedure [4]. The band consisting of anhydroluteins was subjected to semipreparative HPLC on a C_{18} reversed-phase column. In the order of chromatographic elution, two anhydroluteins were collected and identified as (3*R*,6′*R*)-3-hydroxy-3′,4′-didehydro-β,γ-carotene (major component) and (3*R*,6′*R*)-3-hydroxy-2′,3′-didehydro-β,ε-carotene (minor component). Their identification was based on the UV-Vis absorption and mass spectral data as well as HPLC–UV-Vis–MS spiking experiments using the corresponding synthetic compounds described above.

2.8. Partial synthesis of anhydroluteins from lutein diacetate

(a) Synthesis of anhydrolutein acetates

A solution of lutein diacetate (1 g, 1.53 mmol) [containing 3–5% zeaxanthin diacetate] in tetrahydrofuran (200 ml) was treated with 30 ml of 2% solution of sulfuric acid in THF (v/v) and the mixture was stirred at room temperature under an atmosphere of nitrogen for 48 h. The product was worked-up as described earlier. Examination of the product by HPLC–UV-Vis photodiode-array–MS revealed the presence of the following compounds in the order of elution on C_{18} reversed-phase column.

Unreacted lutein diacetate and zeaxanthin diacetate (9%).

UV-Vis (nm) absorption in the HPLC solvents, $\lambda_{max} = 446$, 476 (lutein, 4%); 450, 480 (zeaxanthin, 5%). Mass spectrum (ECNI, methane): molecular anion peak at m/z 652 (100%) as well as anion peak at $m/z = 592$ (50%, $[M\text{-}AcOH]^-$). In addition to the unreacted lutein, a total of 13% of a mixture of unidentified side products was also obtained.

3-Acetoxy-3′,4′-didehydro-β,γ-carotene (48%, anhydrolutein acetate I).

UV-Vis (nm) absorption in the HPLC solvents, $\lambda_{max} = 446$, 476. Mass spectrum (ECNI, methane): molecular anion peak at m/z 592 (100%).

3-Acetoxy-2′,3′-didehydro-β,ε-carotene (16%, 2′,3′-anhydrolutein acetate II).

UV-Vis (nm) absorption in the HPLC solvents, $\lambda_{max} = 448$, 476. Mass spectrum (ECNI, methane): molecular anion peak at m/z 592 (100%).

All-E-3-acetoxy-3′,4′-didehydro-β,β-carotene and its mono-Z-isomer (14%, 3′,4′-anhydrolutein acetate III).

According to the HPLC profile, all-*E*-3′,4′-anhydrolutein (9%) was shown to be followed by its mono-*Z*-isomer (5%). UV-Vis (nm) absorption in the HPLC solvents, all-*E* compound, $\lambda_{max} = 466$; mono-*Z* compound, $\lambda_{max} = 456$, *Z*-peak at 356. Mass spectra (ECNI, methane): both compounds showed molecular anion peaks at m/z 592 (100%).

(b) Saponification of anhydrolutein acetates

The above crude mixture of synthetic anhydrolutein acetates (ca. 1 g) in tetrahydrofuran (100 ml) was treated with 50 ml of 10% methanolic KOH under an atmosphere of nitrogen at room temperature for 2 h. The product was worked-up according to the usual procedure [4] to give 0.7 g of a mixture of unreacted lutein–zeaxanthin and anhydrolutein I, 2′,3′-anhydrolutein II, 3′,4′-anhydrolutein III and its *Z*-isomer, which were identified by comparison of their HPLC–UV-Vis–MS spectra with those of synthetic compounds fully characterized earlier.

3. Results and discussion

In an earlier publication, we had tentatively identified 2′,3′-anhydrolutein (II) as the major dehydration product of lutein in the extracts from human plasma [4]. Due to the insufficient amount of sample for NMR analysis, this identifi-

cation was based solely on the UV-Vis and mass spectral data of the isolated compound. In an attempt to validate our earlier structural assignment, the dehydration products of lutein were prepared by partial synthesis and were fully characterized. Comparison of the HPLC–UV-Vis–MS data of the isolated anhydroluteins from human plasma as well as HPLC spiking experiments using synthetic anhydroluteins were employed to confirm the structures. These experiments definitively reveal that the major dehydration product of lutein in extracts from human plasma is (3*R*,6′*R*)-3-hydroxy-3′,4′-didehydro-β,γ-carotene (anhydrolutein I, Fig. 1), not 2′,3′-anhydrolutein (II). In our HPLC profile of human plasma on a C_{18} reversed-phase column [4], the HPLC peak due to anhydrolutein I was followed by a minor component (7–10% of anhydrolutein I) which has now been identified as 2′,3′-anhydrolutein (II, Fig. 1). Based on these studies, we have also confirmed our earlier observation that 3′,4′-anhydrolutein (III), one of the minor dehydration products of lutein formed by partial synthesis, is not detected in the extracts from human plasma. The mechanism of acid catalyzed dehydration of lutein which leads to the formation of three isomeric anhydroluteins is shown in Fig. 5. The first step involves the protonation of the allylic hydroxyl group of lutein followed by the loss of water to form a secondary allylic carbonium ion. Proton elimination from the resonance hybrid intermediates of these allylic carbonium ions results in the formation of anhydrolutein I, II and III. These anhydroluteins have been known for over 50 years and were first synthesized by Zechmeister and Sease [19] from conversion of lutein in a boric acid–naphthalene melt. However, under the conditions employed, the yields based on lutein were: anhydrolutein I (10%), 2′,3′-anhydrolutein (II, 4%), and 3′,4′-anhydrolutein (III, 3–4%). Anhydrolutein I has also been synthesized by Buchecker et al. [20] from allylic reduction of lutein using $AlCl_3/LiAlH_4 = 3/1$

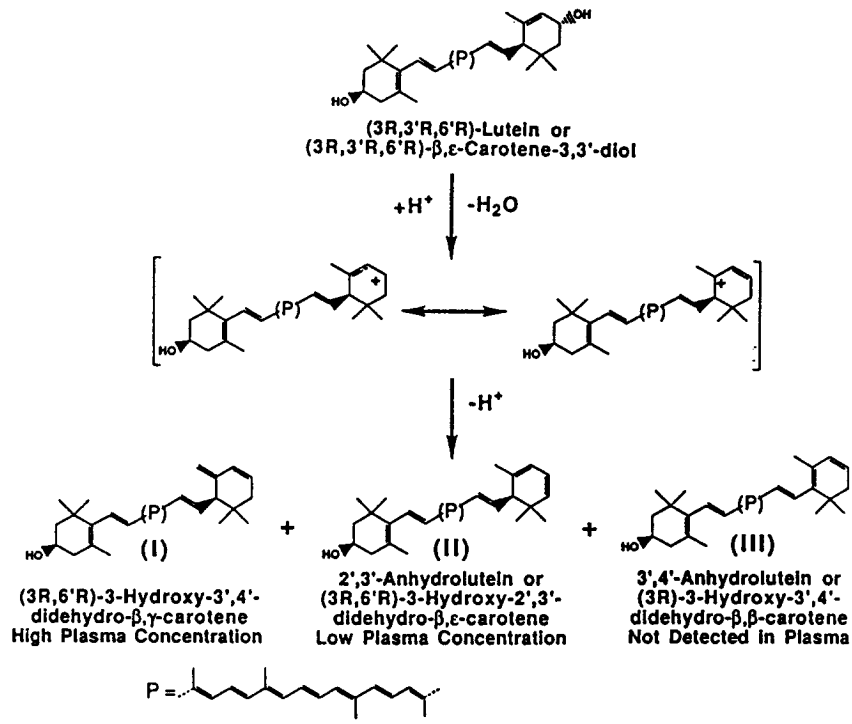

Fig. 5. Formation of three isomeric anhydroluteins from acid catalyzed dehydration of lutein.

($AlHCl_2$) in good yield. The partial synthesis of anhydroluteins from lutein according to our method in acidic acetone employs mild reaction conditions and results in yields greater than 90%. Lutein dehydration products are rare in nature and have only been isolated from the extracts of a variety of squash [8,9] and fruits of black paprika [10]. Despite the limited dietary sources, we routinely detected anhydrolutein I and II by HPLC in the plasma extracts from subjects living in the United States whose diets do not include squash or black paprika. One explanation may be that the ingested dietary lutein, abundant in most fruits and vegetables, may serve as a precursor of anhydroluteins I and II. The formation of anhydroluteins in the human gastrointestinal tract (GI) could result from acid catalyzed dehydration of lutein in the acidic stomach similar to the reaction pathway described earlier. Indeed the dehydration of lutein in organic solvents likewise yields anhydrolutein I as the major product and 2′,3′-anhydrolutein (II) as a minor product. Although the dehydration of lutein in an aqueous medium in a biochemical environment, e.g. human GI tract, would be expected to be somewhat different than the dehydration of this compound in organic solvents, the qualitative and quantitative distribution of the products suggest that there are indeed similarities between the two processes. For example, while the average plasma concentration of lutein is 10–30 μg/dl (180–530 nmol/l), the average plasma concentration of anhydroluteins is only 2–5 μg/dl (40–90 nmol/l). This suggests that if anhydroluteins are formed from dietary lutein in the human digestive tract, less than 20% (based on molar equivalence) of lutein is converted to anhydroluteins. This conversion most probably occurs within the first 1–2 h following ingestion, when dietary lutein is subjected to the stomach acids. Although it may be only coincidental, these results are remarkably similar to the data obtained in the kinetic studies of acid catalyzed dehydration of lutein in organic solvents under various conditions. The kinetics experiments, conducted by monitoring the quantitative and qualitative formation of the synthetic products by HPLC at various intervals, have also indicated that after 1–2 h a total of only about 15–20% of anhydroluteins were formed and 80–85% of lutein remains unreacted.

The synthetic anhydroluteins were also prepared by the loss of acetic acid from lutein diacetate in acidic solvents to give the corresponding anhydrolutein acetate I (48%), 2′,3′-anhydrolutein acetate II (16%), and 3′,4′-anhydrolutein acetate III (14%). The alkaline hydrolysis of these anhydrolutein acetates resulted in the formation of anhydroluteins. However, loss of acetic acid from lutein diacetate in acidic media proceeded at a much slower rate than the dehydration of lutein under identical conditions. In certain fruits and vegetables lutein is usually esterified with fatty acids such as palmitic and myristic acids [2,3,8]. Therefore, the dietary lutein bispalmitate or lutein bismyristate may also lose palmitic or myristic acid in the acidic stomach to form the corresponding fatty acid esters of anhydroluteins. Dietary carotenol fatty acid esters, in general, have not been detected in human plasma. However, upon ingestion, this class of carotenoids are believed to undergo hydrolysis in the presence of pancreatic secretions in the small intestine, to cleave off their fatty acid moiety and to regenerate their parent hydroxycarotenoids [4,7]. Consequently, if the fatty acid esters of anhydroluteins are formed in the stomach, they would similarly be converted to anhydroluteins which can then be absorbed and utilized by the body. In acidic organic solvents under similar conditions described earlier in this text, loss of long-chain fatty acids (palmitic or myristic acid) from lutein bispalmitate and lutein bismyristate proceeds much more readily than the loss of acetic acid from lutein diacetate. Therefore, it is quite likely that the presence of anhydroluteins in human plasma may be due to metabolic conversion of both the dietary lutein and lutein bisfatty acid esters.

3.1. Partial synthesis of anhydroluteins

The dehydration of lutein in dilute H_2SO_4 solution in THF, in addition to anhydroluteins (62%), results in the formation of a number of

unidentified carotenoid side products (22%) as shown in Table 1 and the HPLC profile in Fig. 2a. The total yield of this reaction can be increased to about 92% if the reaction is conducted in acetone. The presence of about 3% zeaxanthin in the isolated all-*E*-(3*R*,3′*R*,6′*R*)-lutein used in these reactions did not interfere with the formation of anhydroluteins. This is because neither of the two hydroxyl groups of zeaxanthin are allylic; therefore these functional groups are not activated to promote the acid catalyzed dehydration of this compound. The crude products from dehydration of lutein were purified by recrystallization or flash column chromatography to remove the zeaxanthin and the unidentified carotenoid side products. However, both methods resulted in a purified mixture of anhydroluteins which were subjected to semipreparative HPLC for isolation of individual compounds. It must be pointed out that the purification of the products by flash column chromatography using normal-phase silica gel as adsorbent, resulted in partial isomerization of anhydrolutein I to 2′,3′-anhydrolutein (II) and 3′,4′-anhydrolutein (III). This is clearly shown in the semipreparative HPLC profile (Fig. 2b) of a mixture of anhydroluteins after purification by flash column chromatography in which the relative abundance of the anhydroluteins (based on HPLC peak area) is significantly different than the relative abundance of these compounds in the crude mixture (Fig. 2a). On the other hand, the initial relative distribution of the anhydroluteins in the crude mixture of these compounds was not altered by recrystallization from ether and petroleum ether.

3.2. Discussion of the NMR data

The ^{1}H NMR spectra of the anhydroluteins were consistent with the recently reported spectra of these compounds by Deli et al. [10]. The chemical shifts and in certain cases the coupling constants of the various protons for anhydroluteins are shown in Table 4. Since these values for the β-end groups and the in-chain protons of anhydroluteins were in agreement with previously published values [10,14,21], only the relevant proton NMR signals due to the dehydrated end-groups of these dehydration products of lutein are discussed.

Anhydrolutein I

In the ^{1}H NMR spectrum of this compound, the olefinic protons H-C(3′) and H-C(4′) of the γ-end group absorb at δ 5.71 and 6.16, respectively. While the former signal appears as a doublet ($J_{3',4'}$ = 9.5 Hz) of triplets (ca. 4 Hz), it is difficult to detect the latter signal in the ^{1}H spectrum due to severe overlap of the proton signals (6 protons near δ 6.15). However, this is clearly revealed by a 1D TOCSY experiment as a doublet at δ 6.15 ($J_{3',4'}$ ca. 9.5 Hz). In this experiment, the magnetization of H-C(3′) was inverted. After a spin-lock of 120 ms duration the difference spectrum showed the additional signals of H_a-C(18′) (very weak), H_b-C(18′) (medium) and of a broad AB-type pattern at δ 1.91 and 2.01 (strong) with a geminal coupling of ca. 16.5 Hz that must be assigned to the protons at C(2′). The large line width of the individual components is quite obviously caused by several unresolved couplings with the protons at C-(3′), C-(4′), and C-(18′). As before with the signal of H-C(4′), the signals of the two protons at C-(2′) cannot be identified unambiguously in the regular 1D spectrum due to the overlap of the strong methyl signals absorbing near δ 2.0. Further assignments, such as H_a-C(18′) and H_b-C(18′), were based on corresponding cross peaks in the 2D T-ROESY spectrum. It is interesting to note that the signal of H-C(6′) at δ 2.65 in anhydrolutein I is shifted further downfield compared to lutein (δ 2.41, d) due to the deshielding effect of the neighboring exocyclic double bond. The ^{13}C chemical shifts of anhydrolutein I are also found in agreement with those published previously [10].

2′,3′-Anhydrolutein (II)

The ^{1}H NMR spectrum of this compound shows the olefinic protons H-C(2′) and H-C(3′) of the 2′,3′-didehydro-ϵ-end group at δ 5.32 (d, $J_{2',3'}$ = 9.6 Hz) and δ 5.75 (dd, $J_{3',4'}$ = 5.2 Hz), respectively. The signal of H-C(4′) is located as a broad singlet at δ 5.60 and is partly overlapped by H-C(7′). A 1D TOCSY experiment, starting

by inversion of the magnetization of H-C(6′) at δ 2.21 (d, $J_{6',7'} = 9.8$ Hz), helped to identify the signal of H-C(8′) as a doublet ($J_{7',8'} = 15.5$ Hz) at δ 6.14. Again, this is a very crowded region with several other closely spaced protons. Further assignments were corroborated by a 2D T-ROESY spectrum which also confirmed the all-*E* geometry of the olefinic chain. All of our results are consistent with previously published data.

3′,4′-Anhydrolutein (III)

While the olefinic H-C(3′) proton appears at δ 5.73 as a doublet ($J_{3',4'} = 9.4$ Hz) of triplets ($J_{2',3'}$ ca. 4.5 Hz), the H-C(4′) proton gives rise to doublet (ca. 9.4 Hz) at δ 5.85 with additional unresolved triplet structure (ca. 1.4 Hz) due to coupling with H_2-C(2′). The latter is seen as a multiplet at δ 2.08 which is in agreement with published values [10,14].

3.3. Discussion of UV-Vis and CD data

The UV-Vis absorption maxima of the anhydroluteins obtained by a photodiode-array detector (HPLC solvents) and by a conventional spectrophotometer in single solvents are shown in Tables 2 and 3, respectively. The separation of anhydroluteins by semipreparative HPLC revealed the presence of at least one *Z*-isomer for each of these compounds (Fig. 2b). The hypsochromic shift of 8–10 nm from the absorption maxima of the all-*E* compounds as well as the presence of a strong *Z*-peak in the near-UV region (332–358) in the absorption spectra of these *Z*-anhydroluteins suggests that these compounds may possess a mono-*Z* geometry in which the *Z*-double bond is located at a more central position, i.e. 13, 13′, 15, or 15′. Interestingly, the *Z*-isomer of 3′,4′-anhydrolutein (III) was described by Zechmeister and Sease in 1943 [19]. Unfortunately, at the present time in the absence of NMR data, the stereochemistry of these *Z*-isomers cannot be determined with certainty. Since in anhydroluteins I and II the length of the chromophore remains unchanged and is identical to that of lutein, the absorption spectra of these three compounds are almost superimposable. The sole difference appears to be a 2 nm bathochromic shift in the absorption maximum of 2′,3′-anhydrolutein (II) as determined by a photodiode-array detector in the HPLC solvents (see Table 2). This 2 nm difference between the absorption maximum of anhydrolutein I ($\lambda_{max} = 446$ nm) and 2′,3′-anhydrolutein (II, $\lambda_{max} = 448$ nm) can be effectively used to differentiate between these two compounds in extracts from human plasma by using an HPLC–photodiode-array detection system. In the case of 3′,4′-anhydrolutein (III), the conjugated system in comparison to anhydrolutein I and II is extended and as a result a 20 nm bathochromic shift in the absorption maximum of this compound is clearly evident. This is shown in the UV-Vis absorption spectra of the all-*E*-anhydroluteins I, II, III and the mono-*Z* isomer of 3′,4′-anhydrolutein (III′) in Fig. 3.

The CD spectra of anhydroluteins I, II, and III in methanol have been previously reported by Baranyai et al. [18]. We have measured the CD spectra of the anhydroluteins in a solution of diethyl ether–isopentane–ethanol (5:5:2) (EPA) as shown in Fig. 4, since most of the current CD data on carotenoids have been accumulated in this solvent system. The CD spectrum of anhydrolutein I in EPA is almost identical to the CD spectrum of this compound in methanol (see Section 2). However, comparison of the prominent signals in the CD spectrum of 2′,3′-anhydrolutein (II) in EPA [263 nm (−19.6 mdeg), 287 nm (+22 mdeg)] with that in methanol [278 nm (−14.4 mdeg), 243 nm (+9.3 mdeg)] reveals substantial hypsochromic shifts in the CD spectrum of this compound in EPA. However, both anhydroluteins I and II exhibit similar spectra to that of lutein and zeaxanthin indicating that the *R*-configurations at C-3 and C-6′ positions in these compounds as a result of dehydration of lutein are not altered. The CD spectrum of 3′,4′-anhydrolutein (III) in EPA and methanol are almost identical (see Section 2) and in comparison with anhydroluteins I and II exhibit lower intensities as well as bathochromic shift of about 23–24 nm (in EPA), which is in accordance with the UV-Vis spectra of these compounds. Similarly the sign pattern in the CD spectrum of 3′,4′-anhydrolutein (III) is also identical to that

Table 5
Systematic and trivial names of the dehydration products of lutein

Structure[a]	Systematic names	Trivial names	References
I	3-Hydroxy-3′,4′-didehydro-β,γ-carotene (high plasma concentration)	Anhydrolutein I Deoxylutein II	[7,10,18,19]
II	3-Hydroxy-2′,3′-didehydro-β,ε-carotene (low plasma concentration)	2′,3′-Anhydrolutein II Deoxylutein III	[2,4,7,8,10,18,19]
III	3-Hydroxy-3′,4′-didehydro-β,β-carotene also known as 3′-hydroxy-3,4-dehydro-β-carotene (not detected in plasma)	3′,4′-Anhydrolutein III Deoxylutein I	[1,2,4,8,10,18,19]

[a] The chemical structures are shown in Fig. 1.

of zeaxanthin in EPA [6], indicating that the chiral center at C-3 in this compound must also be of (*R*) absolute configuration. Therefore, based on the ^{1}H NMR and CD data as well as the mechanism of dehydration of lutein discussed earlier (Fig. 5) we conclude that the dehydration of lutein is accompanied by retention of configuration at C-3 and C-6′ for anhydroluteins I and II and at C-3 for 3′,4′-anhydrolutein. These results are also in agreement with the conclusion of Baranyai et al. in their chiroptical studies of these anhydroluteins [18]. The isolated anhydroluteins I and II from human plasma have not been subjected to CD studies to determine their absolute configuration. However, it is reasonable to assume that if these compounds are formed from acid catalyzed dehydration of lutein, their absolute configuration should be identical to that of synthetic anhydroluteins I and II (3*R*, 6′*R*).

3.4. Nomenclature

The correct systematic and the trivial names of synthetic anhydroluteins are shown in Table 5. In the literature, the original synthetic compounds which were first prepared by Zechmeister and Sease [19] and later by Baranyai et al. [18], have been referred to as deoxylutein I, II, and III (Fig. 1). Throughout this text, we have referred to 3-hydroxy-3′,4′-didehydro-β,γ-carotene as anhydrolutein I, since this compound is the major dehydration product of lutein found in human plasma and is also the major product from dehydration of lutein by partial synthesis. Similarly, the trivial names 2′,3′-anhydrolutein II and 3′,4′-anhydrolutein III have been designated to 3-hydroxy-2′,3′-didehydro-β,ε-carotene (minor component in human plasma) and 3-hydroxy-3′,4′-didehydro-β,β-carotene (not detected in human plasma), respectively.

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