Animal Carotenoids 29* New (2*R*)-2-hydroxy-4-keto-β-type carotenoids from *Daphnia magna* (Crustaceae)

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Two new ketocarotenoids from *Daphnia magna* (Crustaceae) were assigned the constitutions 2-hydroxyechinenone (2-hydroxy-β,β-caroten-4-one) and 2-hydroxycanthaxanthin (2-hydroxy-β,β-carotene-4,4′-dione) on the basis of ^{1}H NMR, mass spectroscopy and base catalyzed dehydration. Carotenoid ketols with this particular substitution pattern have not previously been identified.
2*R*-Chiralities were supported by the CD spectra of the dione and of the lithium aluminum hydride reduced derivatives and by the Horeau method. The carbamate method could not be used for determination of the optical purity of β,β-caroten-2-ols.
The constitution of 3′-hydroxyechinenone (3′-hydroxy-β,β-caroten-4-one) *ex Arthrospira* sp. was confirmed by ^{1}H NMR and 3′*R*-chirality demonstrated by CD.

Carotenoids occurring in Crustaceae including *Daphnia magna* (Cladocera, Daphnideae) have recently been reviewed.[1] Whereas modern spectrometric methods were not employed in the early studies on the carotenoids of *D. magna*,[2–4] a detailed characterization of the individual carotenoids of known structure is being published elsewhere.[5]

We now report the structural elucidation of two new ketocarotenoids, shown to be (2*R*)-2-hydroxyechinenone(*1*) and (2*R*)-2-hydroxycanthaxanthin (*8*). Carotenoid ketols with this particular substitution pattern have not previously been identified.

Results and discussion

Natural 2-hydroxyechinenone (*1*, Scheme 1), 0.8 mg available, had molecular ion *m/z* 566, compatible with $C_{40}H_{54}O_2$. The visible spectra of natural *1* and its lithium aluminum hydride (LAH) reduced products *2a,b* with β,β-carotene chromophore were consistent with the presence of a 4-keto-β-type end group. The second oxygen function was shown to be a hydroxy group from an M-18 fragment ion in the mass spectrum and base catalyzed dehydration to product *3* (M=*m/z* 548) with new olefinic signals in the ^{1}H NMR spectrum (δ 6.22 d and δ 6.76 d, J = 10 Hz), as expected for a disubstituted *cis* cycloalkenone with end group *B*.[6] The smooth alkaline dehydration mechanistically required a β-hydroxyketone (*1*) as substrate. Spin decoupling at 400 MHz ^{1}H NMR identified the ABX system of H-2_{ax}-$3_{quasiax}$-$3_{quasieq}$ of ring *A* (Scheme 1) at δ 3.90, 2.62 and 2.80 respectively (J_{gem} = 18 Hz, $J_{ax,quasiax}$ = 9 Hz and $J_{ax,quasieq}$ = 5 Hz).

The LAH reduced products *2a* and *2b* could then be rationalized as the intramolecularly hydrogen bonded 2,4-*cis* (least strongly adsorbed) and 2,4-*trans* glycols respectively.

The dehydration product *3* was reduced with

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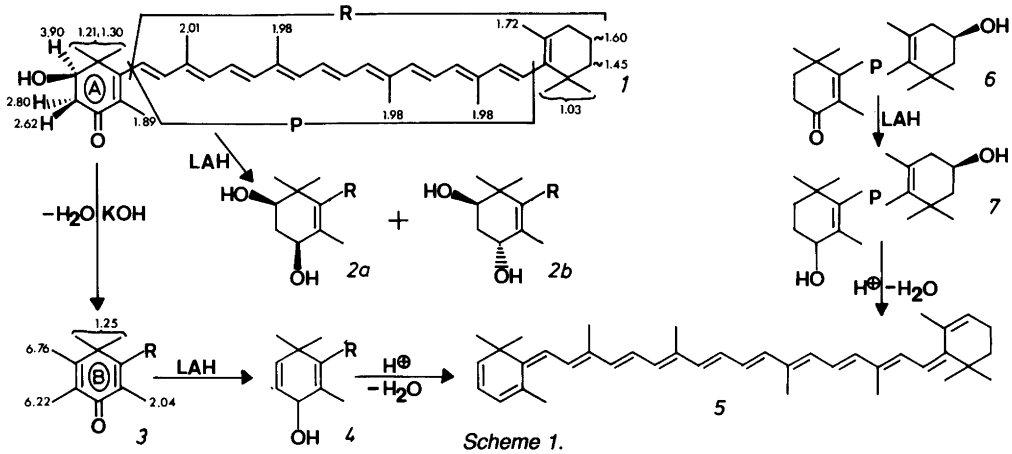
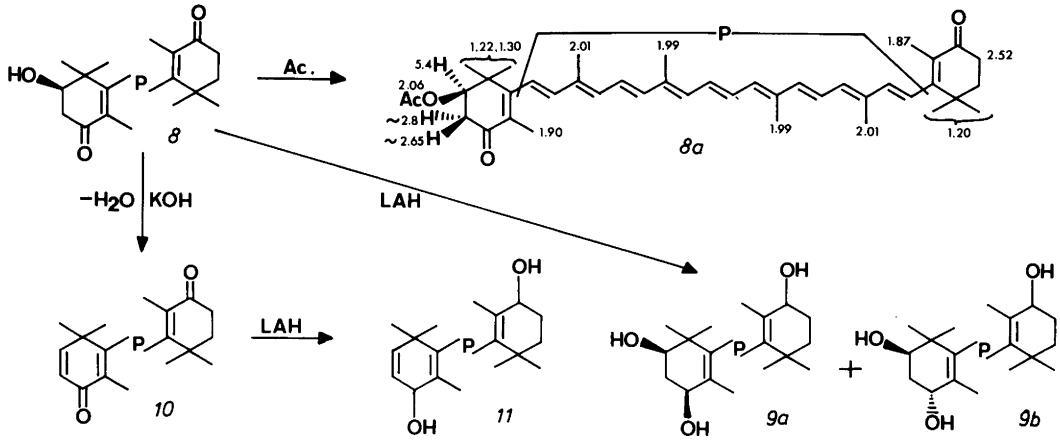


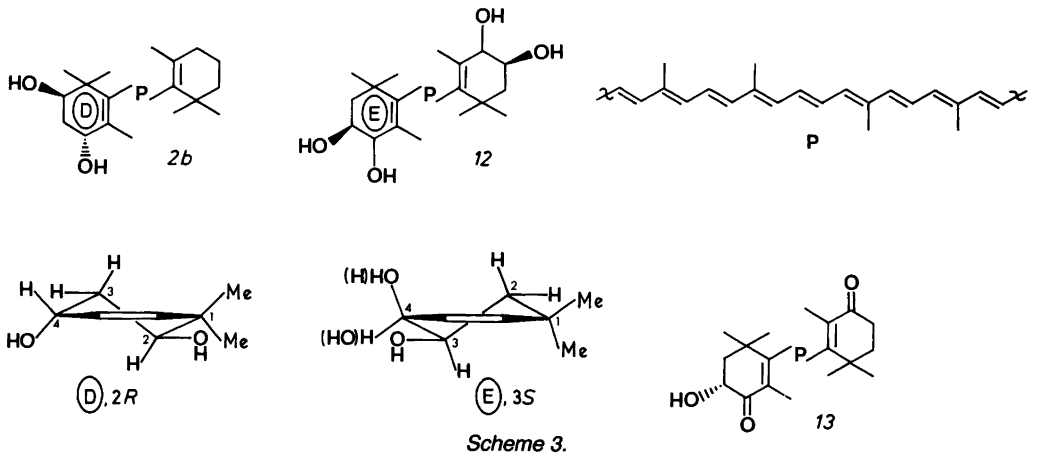
LAH to the bisallylic alcohol *4*, which upon treatment with weak acid at conditions for allylic dehydration[7] afforded *retro*-bisdehydrocarotene (*5*).[8] The latter product was prepared for comparison from 3′-hydroxyechinenone (*6*) *ex Arthrospira* sp. via the diol *7* as previously described.[9] The constitution previously assigned to 3′-hydroxyechinenone (*6*) was confirmed by ^{1}H NMR and 3′*R*-chirality demonstrated by CD.

2-Hydroxycanthaxanthin (*8*, Scheme 2), 0.3 mg available, exhibited its molecular ion at *m/z* 580, compatible with $C_{40}H_{52}O_3$. LAH-reduction afforded the triols *9a,b* with β,β-carotene chromophore. Like natural *1*, natural *8* underwent diagnostic base catalyzed dehydration to the dione *10*. Product *10* upon LAH reduction provided the diol *11* with β,β-carotene chromophore. On preparative scale natural *8* was separated from lutein after acetylation to the monoacetate *8a*, which exhibited ^{1}H NMR signals compatible with the constitution assigned (Scheme 2).

As to the chiralities of the two new *sec.* carotenols a CD approach was attempted. *A priori* a CD correlation of the diol *2b*, Scheme 1, derived from 2-hydroxyechinenone (*1*) with the tetrol *12* obtained by LAH-reduction of (3*S*,3′*S*)-astaxanthin was considered feasable, see Scheme 3. Three pieces of evidence are relevant to this approach.

i) It is known that the Cotton effect of the tetrol *12* is mainly governed by the chirality at C-3(3′), whereas the chirality at C-4(4′) has little in-





Scheme 3.

fluence on the preferred conformation of the chiral β-ring,[10] see Scheme 3.

ii) According to the conformational rule the Cotton effects of a 2-hydroxylated and a 3-hydroxylated β,β-carotene with the same chirality are opposite, because the β-rings prefer opposite conformations with the hydroxy substituents equatorial.[11]

iii) Recently it was pointed out that Δε for a carotenoid with a 2-substituted β-ring is about 2.4 times lower than with a 3-substituted β-ring,[12] rationalized by better stabilization of the preferred conformation in the latter case.

The CD spectrum of the *trans* diol *2b* (end group *D*) in the 260–400 nm region was opposite in sign and reduced in intensity relative to that of the mixed tetrol *12* (end group *E*) with mirror image conformation to that of end group *D*, Fig. 1, Scheme 3. The same chiralities at C–2 and C–3 for *2b* and *12* were consequently inferred. However, due to the *R/S* priority rules diol *2b* possesses 2*R*-chirality and *12* 3*S*-configuration. 2*R*-Chirality for 2-hydroxyechinenone (*1*) is consequently inferred. The predicted reduction of Δε for the diol *2b* versus the tetrol *12* is around 4.8 times. Correlation with the *trans* diol *2b* was preferred to the *cis* diol *2a*, since internal hydrogen bonding might change the chiral conformation in the latter case.

The same 2*R*-chirality for 2-hydroxycanthaxanthin (*8*) was biogenetically expected and supported by a CD correlation between natural *8* and (3*R*)-adonirubin (*13*)[13] of opposite chirality. Thus the Cotton effect was qualitatively the same, Fig. 2, with reduced Δε values for the 2-substituted carotenol *8*. In this case the predicted reduction of Δε for *8* versus *13* is around 2.4 times. Since the CD spectrum of the natural ketone *1*, was similar to that of the natural dione *8*, allowing for *ca* 10 nm bathochromic shift in *8* due to chromophoric differences in these monochiral carotenoids, 2*R*-configuration for *8* was also inferred by this alternative approach.

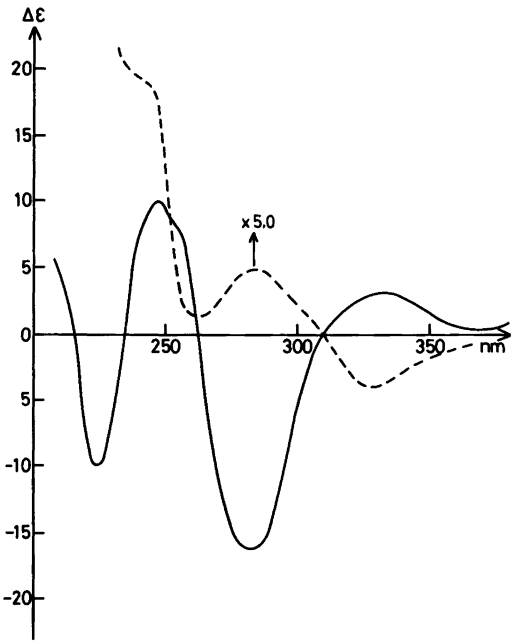


Fig. 1. CD spectra at room temperature in EPA solution of —— tetrol *12* and --- the *trans* diol *2b*.

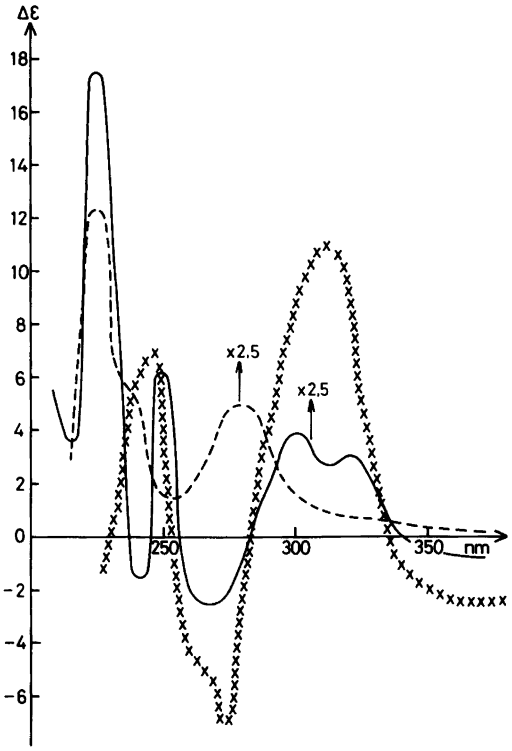


Fig. 2. CD spectra at room temperature in EPA solution of -- 2-hydroxyechinenone (*1*), —— 2-hydroxycanthaxanthin (*8*) and of xxx (3*R*)-adonirubin.[13]

Confirmation of the stereochemical assignments for the natural secondary carotenols *1* and *8* were ultimately sought by the modified Horeau method,[14] previously employed for confirmation of the 2*R*-assignment of natural β,β-caroten-2-ol.[15] The Horeau experiment confirmed that the chirality at C–2 of *1* was dominantly 2*R*. The reduced "chiral effect" in the experiment compared to that of (2*R*)-β,β-caroten-2-ol[15] may be ascribed to the small sample (0.4 mg) available relative to the reagent.

Regarding the optical purity of the carotenols *1* and *8*, produced metabolically by *D. magna*,[4,5] neither the CD nor Horeau method provides evidence except for the excess enantiomer. Whereas diastereomeric camphanates[16] prepared from racemized β,β-caroten-2-ol could not be separated by HPLC,[17] diastereomeric carbamates of racemized β,β-carotene-3,3′-diol (zeaxanthin) have recently been successfully separated.[18]

The carbamate of ketol *1* appeared optically homogeneous by HPLC. However, attempts to separate the carbamates of (2*R*)-β,β-caroten-2-ol *ex Trentepohlia iolithus* and of partly racemized β,β-caroten-2-ol *ex Ectatosoma* sp.[19] failed. The carbamate method may thus not be used for the determination of optical purity of β,β-caroten-2-ol.

In conclusion, the two new carotenoid ketols synthesized from echinenone and canthaxanthin by *D. magna*[5] are shown to be (2*R*)-2-hydroxy-β,β-caroten-4-one (*1*) and (2*R*)-2-hydroxy-β,β-carotene-4,4′-dione (*8*) of unestablished optical purity,

The ketol *1* is considered identical with an unidentified ketocarotenoid partly characterized from *D. magna* by Herring[4] including its reaction with base. Ketol *8* probably represents his "second ketocarotenoid".

As to other naturally occurring 2-hydroxy-β-type carotenoids algal β,β-caroten-2-ol, β,β-carotene-2,2′-diol and β,ε-caroten-2-ol are considered as optically pure 2*R* (2′*R*) isomers,[11,12,15] whereas the algal *trans* 2,3-glycols caloxanthin and nostoxanthin have the opposite chirality at C-2.[20] Insect β,β-caroten-2-ol, β,β-carotene-2,2′-diol and 2′-hydroxy-β,β-caroten-2-one are partly racemized.[19]

Experimental

Biological material. Several batches of *Daphnia magna* were cultivated using the green alga *Scenedesmus* sp. as the sole source of nutrient as described elsewhere.[5,21]

Isolation of the carotenoids. The carotenoids were isolated as described elsewhere;[5] yield of total carotenoids around 0.5 mg/g dry wt.[1] Natural *1* and *8* comprised 17 % and 8 % respectively of the total carotenoid.

General methods. These were as commonly employed.[22] TLC was carried out on SiO_2. R_f values refer to mixtures of acetone in hexane (% AH). VIS spectra were recorded in acetone. Spectral fine-structure is expressed as % III/II.[23] For mass spectra of derivatives diagnostically useful ions only are cited. ^1H NMR spectra were recorded in $CDCl_3$ and CD spectra in EPA (diethyl ether:isopentane:ethanol 5:5:2). Acetylation with acetic anhydride in pyridine and reduction with LAH were carried out by standard procedures.[24]

(2R)-2-Hydroxy-β,β-caroten-4-one (1)

Natural 1. Available in total *ca* 0.8 mg; R_f = 0.44 (30% AH); VIS λ_{max} nm 458, (480); ^{1}H NMR (400 MHz) δ 1.03 (6H, s, Me-16′, 17′), 1.21 (3H, s, Me-16/17), 1.30 (3H, s, Me-16/17), *ca* 1.45 (2H, m, H-2′$_{ax}$, 2′$_{eq}$.), *ca* 1.60 (2H, m, H-3′$_{ax}$, 3′$_{eq}$), 1.72 (3H, s, Me-18′), 1.89 (3H, s, Me-18), 1.98 (9H, s, Me-20, 19′, 20′), 2.01 (3H, s, Me-19), *ca* 2.62 (1H, dd; J_{gem} = 18 Hz, $J_{ax,quasiax}$ = 9 Hz, H-3$_{quasiax}$), 2.80 (1H, dd, J_{gem} = 18 Hz $J_{ax,quasieq}$ = 5 Hz, H-3$_{quasieq}$), *ca* 3.90 (1H, m, H-2), 6.15–6.70 (*ca* 14H, olefinic). Irradiation of the δ 3.9 multiplet resulted in doublets at δ 2.6 and δ 2.8, *J* = 18 Hz. Irradiation of the double doublet at δ 2.6 resulted in change of the spin patterns at δ 2.8 and 3.9. MS *m/z* 566 (M, 100%), 548 (M-18, 40%), 474 (M-92, 10%), 460 (M-106, 5%), 400 (5%), 269 (5%), 91 (5%). CD nm (Δε) 225 (+5), 250 (+0.5), 285 (+2), see Fig. 2.

Modified Horeau experiments: The procedure used elsewhere[14,15] was modified for smaller scale. 2-Hydroxyechinenone (*1*) (0.4 mg) and α-phenyl butyric anhydride (3 μl) in dry pyridine (20 μl) were kept in a sealed vial at 40°C for 2 h. (*R*)-α-phenyl ethyl amine (6 μl) was added and the reaction mixture was shaken for 15 min. A parallel experiment with cyclohexanol (1 μl) was performed. To the reaction mixtures ethyl acetate (15 ml) was added and GLC analysis was performed (OV-17 (5%), 1.5 m, 210°C). The GLC analysis showed repeatedly after the correction found from cyclohexanol a ratio of *R,S*-amide:*R,R*-amide of 1:0.97.

Carbamate of *1* (0.1 mg) was prepared by the published procedure.[18] Carbamates of (2*R*)-β,β-caroten-2-o1[15] and of racemized β,β-caroten-2-o1[19] were prepared for comparison. HPLC analyses gave no separation for the two diastereomeric carbamates of β,β-caroten-2-o1. The carbamate of *1* could not be separated.

β,β-Carotene-2,4-diol (2a,b). Reduction of *1* (0.1 mg) with LAH in dry ether gave *2a,b,* separated by TLC. *2a* (*cis* diol) had R_f = 0.38 (30% AH); VIS λ_{max} nm (424), 449, 475; % III/II = 17. *2b* (*trans* diol) had R_f = 0.29 (30% AH); VIS λ_{max} nm (424), 450, 475; % III/II = 17; CD nm (Δε) 215 (+7.5), 230 (+9), 275 (0), 280 (+1), 305 (0), 325 (−0.5), Fig. 1.

2,3-Didehydro-β,β-caroten-4-one (3). Treatment of *1* (0.2 mg) with 5% KOH in methanol for 7 h provided *3* in quantitative yield; R_f = 0.73 (30% AH); VIS λ_{max} nm 458, (480); ^{1}H NMR (400 MHz) δ 1.03 (6H, s, Me-16′, 17′), 1.28 (6H, s, Me-16, 17), 1.73 (3H, s, Me-18′), 1.98 (6H, br.s., Me-19′, 20′), 2.04 (9H, br.s., Me-18, 19, 20), *ca* 6.1–6.8 (*ca* 14H, Olefinic), 6.23 (1H, d, *J* = 10 Hz, H-3), 6.76 (1H, d, *J* = 10 Hz, H-2); MS m/z 548 (M^+, 100%), 456 (M-92, 3%), 424 (5%), 414 (5%), 410 (10%), 274 (M^{++}, 5%), 105 (5%).

2,3-Didehydro-β,β-caroten-4-o1 (4). Reduction of *3* (0.1 mg) with LAH in dry ether provided *4*; R_f = 0.65 (30% AH); VIS λ_{max} nm (425), 451, 477, % III/II = 14.

Retro-bisdehydrocarotene (5). Treatment of *4* (0.1 mg) with 0.03 N HCl in $CHCl_3$ provided *5*; R_f = 0.80 (30% AH), inseparable from *5* prepared below from *7*; VIS λ_{max} nm 460, 484, 515, % III/II = 1.

(3′R)-3′-Hydroxy-echinenone (6), ex Arthrospira sp., was left from a previous study;[9] R_f 0.44 (30% AH), not separable from natural *1*; VIS λ_{max} 458 (480); ^{1}H NMR (100 MHz) δ 1.07 (6H, s, Me-16′, 17′), 1.19 (6H, s, Me-16, 17), 1.72 (3H, s, Me-18′), 1.85 (3H, s, Me-18), 1.97 (12H, s, Me-19, 20, 19′, 20′), 2.51 (*ca* 2H, m, H-4), 6.11 s (2H, s, H-7′, 8′), 6.15–6.8 m (*ca* 12H, olefinic); MS *m/z* 566 (M^+, 40%), 550 (M-16, 3%), 548 (M-18, 5%), 474 (M-92, 2%), 460 (M-106, 2%), 95 (100%). CD nm (Δε) 235 (0), 258 (+4.5), 272 (0), 295 (−7.5), 315 (0), 345 (2.5).

4,3′-β,β-Carotene-diol (7). Natural *6* (0.1 mg) was reduced with LAH in dry ether providing *7*; R_f = 0.42 (30% AH); VIS λ_{max} nm 424, 449, 479, % III/II = 17. Treatment of *7* (0.1 mg) with 0.03 N HCl in $CHCl_3$ gave amongst other products *retro*-bisdehydrocarotene (*5*) with properties as described for *5* above.

(2R)-2-Hydroxy-β,β-carotene-4,4′-dione (8)

Natural 8. Available in total *ca* 0.3 mg; R_f 0.36 (30% AH); VIS λ_{max} nm 470; MS *m/z* 580 (M^+, 15%), 562 (M-18, 15%), 522 (15%), 488 (M-92, 2%), 219 (100%), 159 (40%), 97 (40%). CD nm (Δε) 225 (+7), 240 (−0.5), 250 (+3.5), 270 (−1.0), 300 (+1.5), 345 (0), Fig. 2.

2-Hydroxy-β,β-carotene-4,4′-dione 2-acetate (8a). Acetylation of *8* (0.1 mg) in mixture with lutein afforded lutein diacetate and *8a*, separated by TLC. *8a* had R_f = 0.44 (30% AH); VIS λ_{max} nm 470; ^{1}H NMR (400 MHz) δ 1.20 (6H, s, Me-16′, 17′), 1.22 (3H, s, Me-16/17), 1.30 (3H, s,

Me-16/17), 1.87 (3H, s, Me-18'), 1.90 (3H, s, Me-18), 1.99 (6H, s, Me-20, 20'), 2.01 (6H, s, Me-19, 19'), 2.06 (3H, s, Ac), 2.52 (2H, m, H-3'), 2.65 (1H, m, H-$3_{quasiax}$), *ca* 2.8 (m, H-3_{qasieq} + imp.), *ca* 5.4 (1H, m, H-2), 6.2–6.75 (*ca* 14H, olefinic). MS *m/z* 622 (M, 2 %), 606 (M-16, 2 %), 562 (M-60, 50 %), 470 (M-92-60, 2 %), 213 (90 %), 149 (100 %).

β,β-Carotene-2,4,4'-triol (*9a, b*). Reduction of *8* (0.05 mg) with LAH in dry ether provided *9a, b*. VIS λ_{max} nm (420), 449, 475, % III/II = 20. Separation by TLC gave three zones R_f = 0.25, 0.18 and 0.12 (30 % AH) which presumably represented *9a, 9b* and geometrical isomer(s).

2,3-Didehydro-β,β-carotene-4,4'-dione (*10*). Treatment of *8* (0.1 mg) with 5 % KOH in methanol for 7 h gave *10* in quantitative yield, R_f = 0.55 (30 % AH), VIS λ_{max} nm 470.

2,3-Didehydro-β,β-carotene-4,4'-diol (*11*). Reduction of *10* (0.05 mg) with LAH in dry ether provided *11* in quantitative yield; R_f = 0.30 (30 % AH); VIS λ_{max} nm (420), 448, 478.

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