30. Carotenoids of *Rhizobia*
I. New Carotenoids from *Rhizobium lupini*

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(6. 12. 76)

Summary

The main pigments of *Rhizobium lupini* were 2,3,2',3'-di-*trans*-tetrahydroxy-β,β-caroten-4-one and 2,3,2',3'-di-*trans*-tetrahydroxy-β,β-carotene. As minor components 7,8,7',8'-tetrahydro-ψ,ψ-carotene (ζ-carotene), β,β-carotene (β-carotene), and tentatively, a 2,3,2'(or 3')-trihydroxy-β,β-caroten-4-one and a 2,3,2'(or 3')-trihydroxy-β,β-carotene were identified.

Introduction. – Agar and suspension cultures of the soil and root nodule bacterium *Rhizobium lupini* are bright yellow to red in colour due to carotenoid pigments. This pigmentation character of the wild strain and several mutants thereof has been shown to be very useful for genetic experiments [1]. The present investigation was undertaken to examine the structures of these pigments which proved to be β,β-carotene derivatives.

Results. – The carotenoid composition of *Rhizobium lupini* 1–250 is shown in Table 1. 1.3 nmol carotenoid per mg dry weight was found for suspension cultures. For agar cultures this value may be somewhat higher.

β,β-Carotene and 7,8,7',8'-tetrahydro-ψ,ψ-carotene were identified on the basis of absorption spectra in visible light and chromatographical behaviour on adsorption and partition chromatography, as compared with standards.

The two main pigments, designated as pigment I and II, showed similar polarities than carotenoid glycosides which means that they were quite polar. They could not be quantitatively separated from each other in their natural forms. Peracetylation of the mixture, however, yielded two less polar derivatives which were separated on silica gel thin layers. Peracetylation using [^{14}C]-acetic anhydride revealed that four hydroxyl groups had been acetylated in both pigments (synthetic β,β-carotene-3,3'-diol (zeaxanthin) was used as a standard). The slightly more polar pigment I exhibited an absorption spectrum which was nearly identical with that of β,β-caroten-4-one (echinenone) and which changed to the spectrum of β,β-carotene after reduction with $NaBH_4$. Pigment II showed the spectrum of β,β-carotene. Upon alkaline treatment (1% KOH-solution in ethanol) pigment I was quantitatively transformed into a less polar derivative (the structure of which will be published later), whereas pigment II or the reduced form of pigment I did not change their chromatographic properties

when treated with alkali. Both pigments were relatively stable against 0.2 N methanolic HCl or 0.1 N H_2SO_4. There was no indication of allylic hydroxyl groups as revealed by negative HCl/$CHCl_3$ reactions. Attempts to form trimethylsilyl ethers from the tetra-acetates with trimethylchlorosilane were negative, so that tertiary hydroxyl groups could be excluded. From these data it was hypothesized that pigment I and pigment II are bicyclic carotenoids containing four hydroxyl groups plus a carbonyl function in the 4-position, and four hydroxyl groups, respectively.

Further information on the structures came from mass spectroscopy (MS.) and proton magnetic resonance spectroscopy (^{1}H-NMR.) of the peracetylated products.

The molecular ion in the mass spectrum of the peracetylated pigment I was only detectable as a minor peak at *m/e* 782. In the upper part of the mass spectrum the significant peaks were at *m/e* 722 (elimination of acetic acid), 680 (loss of acetic acid and ketene), 620 (loss of a further acetic acid molecule) and 578 (the loss of the fourth acetate in the form of ketene). The peak at *m/e* 528 corresponds to the combined loss of acetic acid and 194 mass units, the latter being a typical fragmentation step for astacenes [4].

In the middle of the mass-scale two peaks, *m/e* 245 and *m/e* 203, were dominant. They indicate the presence of an astacene-type end group [4], formed from the original end group by the elimination of acetic acid. The elimination of toluene, a typical fragmentation of the polyene chain found in less substituted carotenoids, occurred to a very small extent only.

The 270-MHz-FT ^{1}H-NMR. spectrum in $CDCl_3$ of the tetraacetate of pigment I showed all signals as expected for the proposed structure (see Table, experimental part and [5]). Thus, the presence of the 2′,3′-di-substituted β-end group was revealed by the observation of 3 methyl singlets (3 H each) at 1.70 ppm (CH_3 at C(5′)), 1.00 and 1.08 ppm (gem. methyl groups at C(1′)). The signals of the CH_2-group (C(4′)) were identified as an *AB*-part of an *ABXY*-type spectrum at 2.24 ppm (*d*×*d*, *J*(gem)=17.5 Hz, *J*(4a,3a)=9.5 Hz, 1 H, H(ax) at C(4′)) and 2.63 ppm (*d*×*d*, *J*(gem)=17.5 Hz, *J*(4e, 3a)=6.5 Hz, 1 H, H(eq) at C(4′)). The chemical shifts of these protons and the high value of the geminal coupling indicated that this CH_2 group should be located next to the Δ^5-double bond.

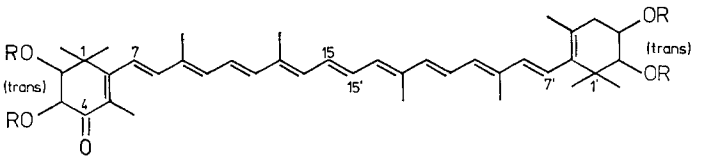
The signals of the coupled X-proton (H at C(3′)) and of the Y-proton (H at C(2′)) were clearly identified at 5.14 ppm (with splittings of 11, 9.5 and 6.5 Hz; see experimental part) and 5.04 ppm (11 Hz), respectively. The large coupling *J*(2′,3′) of 11 Hz showed that both protons were diaxial with respect to each other, *i.e.* the two acetoxy substituents are *trans*.

The structure of the other end group was derived from the observation that only 2 methyl signals (3 H each) remained to be assigned at δ-values < 1.9 ppm, namely the two gem. methyl groups at C(1) at 1.32 and 1.15 ppm. A further methyl singlet at 1.91 ppm (3 H), now being assigned to the methyl group at C(5), closely corresponds to the value of 1.87 ppm observed with β-4-oxo end groups as in β,β-carotene-4,4′-dione (canthaxanthin) [5]. No further signals to be attributed to the protons of the end group were detectable in the high-field part of the spectrum.

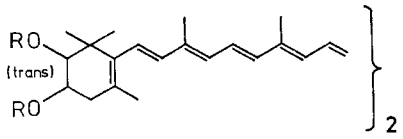
However, an *AB*-type spectrum (*J*(AB)=11.9 Hz, 2H) was observed at 5.32 and 5.53 ppm which was assigned to the protons at C(2) and C(3), respectively. The large coupling constant again points to a diaxial relationship of these two protons.

Table. *Carotenoids* of Rhizobium lupini

Compounds (listed with increasing polarity)	% of total
7,8,7',8'-Tetrahydo-ψ,ψ-carotene	1
β,β-Carotene	1
Pigment IV (2,3,2'(or 3')-trihydroxy-*β,β*-carotene)	2
Pigment III (2,3,2'(or 3')-trihydroxy-*β,β*-caroten-4-one)	3
Pigment II (2,3,2',3'-tetrahydroxy-*β,β*-carotene)	44
Pigment I (2,3,2',3'-tetrahydroxy-*β,β*-caroten-4-one)	48
Unidentified	1



Pigment I: R=H
Peracetylated: R=$COCH_3$



Pigment II: R=H
Peracetylated: R=$COCH_3$

The highly resolved olefinic part of the 270 MHz spectrum between 6.0 and 6.7 ppm was also largely assigned (see exper. part) since it can be additively built up, in good approximation, from the partial spectra of *e.g.* *β,β*-carotene (or of the pigment II; see below) and of *β,β*-carotene-4,4'-dione. The similarity of the chemical shifts is also in compliance with the assumed all-*trans* structure of the olefinic chain as was evidenced also from the UV. absorption.

The mass spectrum of the peracetylated pigment II showed the molecular ion at *m/e* 768, corresponding to *β,β*-carotene substituted by four acetoxy groups. The fragmentation pattern is very simple: after expulsion of toluene in the first fragmentation step (*m/e* 676), all four acetates are successively eliminated. Three of them are lost in the form of acetic acid and one as ketene leading to the peaks at *m/e* 616, 556, 514 and 454.

The ^{1}H-NMR. spectrum of the peracetylated pigment II clearly showed that the molecule is built up from two identical halves. Thus, only 3 signals (6H each) were observed at 1.00 and 1.08 ppm (geminal methyl groups at C(1) and C(1')) and at 1.70 ppm (methyl groups at C(5) and C(5')). Further relevant signals for the confirmation of the two identical 2,3-disubstituted *β*-end groups were those of the protons at C(2) and C(2'), C(3) and C(3') and C(4) and C(4'), which appeared practically at the same positions as before in the case of the pigment I.

All the remaining signals of the peracetylated pigment II could be assigned and most of the assignments were based on extensive decoupling experiments. The chemical shifts of the olefinic protons were found very similar to those of suitable reference carotenoids [5] and it seems reasonable, therefore, to assume an all-*trans* structure in agreement with the observed UV. spectrum.

The minor, less polar pigments III and IV exhibited the same absorption spectra as pigment I and II, respectively, and were shown by radioactive acetylation to contain one less hydroxyl group. The mass spectra of the peracetylated pigments III and IV were in agreement with this (*m/e* 724 and 710, respectively) and closely corresponded to those of pigments I and II. Especially for the peracetylated pigment III it was found that peaks at *m/e* 245 and 203 were present as in pigment I and hence the 2,3-disubstituted β-4-oxo end group was confirmed.

As with pigment I (see above) alkaline treatment of pigment III yielded a less polar derivative. This also supports the conclusion that pigment III possesses the 2,3-disubstituted β-4-oxo end group as pigment I. The position of the remaining hydroxyl group in the other end group (2′ or 3′) in pigment III and IV could not be determined by ^{1}H-NMR. due to a lack of material.

Discussion. – The highly hydroxylated carotenoids of *Rhizobium lupini* contain unusual end groups which occur very seldom (2,3-dihydroxy) or have not been described before (2,3-dihydroxy-4-oxo). A hydroxyl group at the 2-position without functional groups, however, at 3- and/or 4-position is known from some carotenoids of the green alga *Trentepohlia* [2] [3]. The 2,3-dihydroxy end group has been reported recently for two carotenoids occurring in *Anacystis nidulans*, caloxanthin and nostoxanthin [6]. The identity of our pigments IV and II with caloxanthin and nostoxanthin, respectively, will further be investigated, especially by CD. measurements. The newly described 2,3-dihydroxy-4-oxo end group turned out to be very labile against alkali and, upon treatment with 1% ethanolic KOH-solution, yields a less polar derivative with an altered absorption spectrum in visible light the chemical structure of which is presently under investigation and will be described in a later publication.

We wish to thank Miss *Ch. Steinki* for technical assistance and Dr. *H. Thommen*, *F. Hoffmann-La Roche & Co.*, for valuable discussion. Supported by *Deutsche Forschungsgemeinschaft* (Kl 260/4 and He 130/24).

Experimental Part

Culture. – *Rhizobium lupini* strain 1–250 was grown in 0.8% nutrient broth (*Merck*) in 5 l fermentors.

Isolation of pigments. – Pigments were extracted with acetone and transferred into petroleum ether/ether 1:1 in a separatory funnel by dilution with water. Pigments were separated on silica gel columns using petroleum ether/ether/acetone with increasing polarity and subsequently by TLC. (silica gel H, *Merck*). Yield of pigments: about 5 mg of pigment I and pigment II and about 1 mg of pigment III and pigment IV.

Chemical methods. – Acetylation was performed using acetic anhydride in dry pyridine for 3 h at room temperature. Radioactive acetylation was carried out using diluted 1-[^{14}C]-acetic anhydride (25 mCi/mmol, *The Radiochemical Centre Amersham*), so that 200 to 400 cpm per hydroxyl group per sample were obtained. The acetates were purified by TLC. (silica gel and subsequently cellulose impregnated with triacylglycerols (10% triacylglycerols in petroleum ether) with the solvent system methanol/acetone/water 15:5:1) till radiochemical purity was obtained. Radioactivity was counted in a toluene-based scintillation fluid (5.0 g PPO, 0.5 g POPOP, in 1 l toluene). For the determination of specific activities ε values were used as mentioned below.

Alkaline treatment was performed with 1% KOH-solution in ethanol at room temperature for 5 min. Carbonyl groups were reduced using $NaBH_4$ in ethanol. Attempts for allylic elimination were performed in a 0.01 N hydrogen chloride/chloroform. For the formation of trimethylsilyl ethers trimethylchlorosilane in pyridine was used. For quantitative determination the following ε values

were adopted: 135,000 (β,β-carotene, β,β-carotene-4,4′-diol, pigment II, pigment IV), 119,000 (pigment I, pigment III), 123,000 (7,8,7′,8′-tetrahydro-ψ,ψ-carotene).

Instrumentation. – The mass spectra were taken on a MS-9 from *AEI* (Manchester) using a direct introduction probe. The ion source was held at 200°, the ionisation energy was 70 eV. After each fragment, in parentheses, the rel. intensity. The ^{1}H-NMR. spectra were run at 270 MHz on a HX-270-FT NMR. spectrometer (*Bruker-Spectrospin AG*) with superconducting magnet and BNC-80 computer (40K memory). Deuteriated chloroform was used as solvent. The sample temperature was 22°. Since the concentration of the solution was only *ca.* 3 mg dissolved in 0.4 ml solvent, pulsed *Fourier* Transform technique was applied to improve the signal to noise ratio. Between 20 and 60 FID's were accumulated into 32K memory in each run. Chemical shifts are given as δ-values in ppm, coupling constants J in Hz.

Spectroscopic data. – *β,β-Carotene.* Visible light (ethanol): 429, 449, 477 nm.

7,8,7′,8′-Tetrahydro-ψ,ψ-carotene. UV./VIS. (ethanol): 375, 397, 420 nm.

Pigment I. (2,3,2′,3′-di-*trans*-tetrahydroxy-β,β-caroten-4-one). VIS. (ethanol): 457, 475 nm; in reduced form 429, 449, 477 nm. – MS. of the peracetylated form of pigment I: *m/e* 782 (1, *M*); 768 (6, imp.); 722 (7); 680 (20); 620 (3); 578 (4); 528 (4); 245 (15); 203 (100); 43 (90). – ^{1}H-NMR. data of the peracetylated form of pigment I in $CDCl_3$: 1.00 and 1.08 (*s*, 3H each; gem. methyl groups at C(1′)); 1,15 and 1.32 (*s*, 3H each; gem. methyl groups at C(1)); 1.70 (*s*, 3H; H_3C–C(5′)); 1.91 (*s*, 3H; H_3C–C(5)); 1.97 (*s*, 3H), 1.99 (*s*, 6H) and 2.01 (*s*, 3H), 'in-chain' methyl groups; 2.04 and 2.10 (2*s*, 3H each; O-acetyl groups at C(2′) and C(3′)); 2.12 and 2.17 (2*s*, 3H each; O-acetyl groups at C(2) and C(3)); 2.24 (*d*×*d*, J=17.5 and 9.5, 1H, H(ax)–C(4′)); 2.63 (*d*×*d*, J=17.5 and 6.5, 1H, H(eq)–C(4′)); 5.04 (*d*, J=11, 1H, H(ax)–C(2′)); 5.14 (*m*, 1H, H(ax)–C(3′)); 5.32 (*d*, J=12, 1H, H(ax)–C(2)); 5.53 (*d*, J=12, 1H, H(ax)–C(3)); 6.01 (*d*, J=16, 1H, H–C(7′)); 6.11 (*d*, J=16, 1H, H–C(8′)); *ca.* 6.18 (*2d*, J=16 and 12, 2H, H–C(7) and H–C(10′)). Other signals were only tentatively assigned: 6.27 (*d* ?; H–C(14′)); 6.31 (2×*d* ?; H–C(10) and H–C(14)); 6.38 (*d*, J=15, H–C(12′)); 6.40 (*d*, J=16, H–C(8)); 6.46 (*d*, J=15, H–C(12)); *ca.* 6.65±0.02 (*m*, 4H, H at C(11), C(11′), C(15) and C(15′)).

Pigment II. (2,3,2′,3′-di-*trans*-tetrahydroxy-β,β-carotene). VIS. (ethanol): 429, 449, 477 nm. – MS. of the peracetylated form: *m/e* 768 (100, *M*); 708 (10); 616 (7); 556 (3); 514 (2); 454 (3); 311 (12); 264 (25); 43 (>100). – ^{1}H-NMR. data of the peracetylated form in $CDCl_3$: 1.00 and 1.08 (2*s*, 6H each; gem. methyl groups at C(1) and C(1′)); 1.70 (*s*, 6H; methyl groups at C(5) and C(5′)); 1.97 and 1.98 (2*s*, 6H each; methyl groups at C(9), C(9′), C(13), C(13′)); 2.04 and 2.10 (2*s*, 6H each; O-acetyl groups at C(2), C(2′), C(3), C(3′)); 2.24 (*d*×*d*, J=17 and 9, 2H, H(ax)–C(4), H(ax)–C(4′)); 2.63 (*d*×*d*, J=17 and 6.5, 2H, H(eq)–C(4) and H(eq)–C(4′)); 5.04 (*d*, J=11, 2H, H(ax)–C(2) and H(ax)–C(2′)); 5.14 (*m*, 2H, H(ax)–C(3) and H(ax)–C(3′)); 6.01 (*d*, J=16, 2H, H–C(7) and H–C(7′)); 6.11 (*d*, J=16, 2H, H–C(8) and H–C(8′)); 6.18 (*d*, J=11.5, 2H, H–C(10) and H–C(10′)); 6.27 (*AA′*-part of *AA′BB′*-spectrum, 2H, H–C(14) and H–C(14′)); 6.38 (*d*, J=15, 2H, H–C(12) and H–C(12′)); *ca.* 6.64 (*d*×*d*, J=15 and 11, 2H, H–C(11) and H–C(11′); and *BB′*-part of *AA′BB′*-spectrum, 2H; H–C(15) and H–C(15′)).

Pigment III. (2,3,2′(or 3′)-Trihydroxy-β,β-caroten-4-one). VIS. (ethanol): 457, 475 nm; in reduced form 429, 449, 477 nm. – MS. of peracetylated form: *m/e* 724 (2, *M*); 706 (3); 664 (15); 622 (25); 562 (8); 254 (15); 203 (100); 43 (63).

Pigment IV. (2,3,2′(or 3′)-Trihydroxy-β,β-carotene). VIS. (ethanol): 429, 449, 477 nm. – MS. of peracetylated form: *m/e* 710 (35, *M*); 650 (5); 43 (100).

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