New C$_{30}$-Carotenoic Acid Glucosyl Esters from *Pseudomonas rhodos*

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Z. Naturforsch. 34c, 181 – 185 (1979); received December 14, 1978

C$_{30}$-Carotenoic Acid Glucosyl Ester, *Pseudomonas rhodos*

The carotenoid pigments of the Gram-negative bacterium *Pseudomonas rhodos* were identified as 4,4'-diapocarotene-4-oic acid, di(β-D-glucosyl) 4,4'-diapocarotene-4,4'-dioate, and β-D-glucosyl-4,4'-diapocarotene-4-oate-4'-oic acid. One hydroxy group of the glucose moieties, probably at C(6), was esterified with one of several fatty acids (12:0, 14:0, 14:1, 16:0, 16:1).

**Introduction**

*Pseudomonas rhodos* is a soil bacterium, isolated and first described by Heumann [1]. Colonies of this organism are coloured deep red by carotenoid pigments of unknown structures [2]. The present report describes the chemical structures of these pigments which proved to be apocarotenoic acids partly esterified with acylated glucose.

**Results**

Thin layer chromatography on silica gel of an acetone/methanol extract from *Pseudomonas rhodos* revealed two major very polar and one minor less polar red pigments. Due to the considerable differences in polarity the separation of these substances was easily accomplished by means of preparative column and thin layer chromatography.

**Pigment I** (4,4'-diapocarotene-4-oic acid). —
This component comprised about 8% of the total pigment content and was relatively nonpolar. The UV/VIS-absorption spectrum in ethanol showed two maxima at 477 and 508 nm and an inflection at 456 nm. Saponification, acetylation, and reduction tests (NaBH$_4$) were negative. The acidic nature of the pigment pointed to the presence of a carboxylic group. The pigment was easily methylated using diazomethane (Ia). Methylation was accompanied by a bathochromic shift of the absorption spectrum (481, 511 nm, inflexion at 458 nm).

The mass spectrum of the methyl ester showed the molecular ion (m/e 444) as the base peak and the two typical fragments M-92 (loss of toluene) and M-106 (loss of xylene) as prominent peaks in the upper part of the spectrum. The intensity ratio (M-92)/(M-106) of 1.0 found in the spectrum seems to be in agreement with the rules outlined by Francis [3] for various carotenoids.

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0341-0382 / 79 / 0300-0181 $ 01.00/0
The 270 MHz $^1$H-NMR spectrum of Ia revealed that the sample most likely consisted of a mixture of stereoisomers with the cis bonds close to the ester moiety. For the main component the configuration of $\Delta 5$ should be trans, however, since the broadened doublet of the proton at C(6) was found, as expected in this case, at very low field (7.30 ppm, $J(6,7) \approx 11.5$ Hz) due to the deshielding effect of the carboxylic group. The other olefinic protons gave rise to a complex unresolved pattern between 6 and 6.8 ppm with the exception of the broadened doublet at 5.94 ppm to be assigned to the proton at C($6'$) at the end of the conjugated chain. The ester methyl group of the main isomer presumed to have the all-trans structure was observed at 3.77 ppm.

More indicative for the all-trans structure were the signals of the "in-chain" methyls observed at 1.83 ppm (6 H, methyls at C(5')) , 1.98 and 2.00 ppm (ca. 15 H).

**Pigment II** (di(β-D-glucosyl)4,4'-diapocarotene-4,4'-dioate, fatty acid ester). This component accounted for about 32% of the total carotenoid content. Its absorption maxima in ethanol were observed at 499 and 530 nm with an inflexion at 475 nm. Under the assumption that this compound is also a C$_{30}$-derivative, two carboxylic groups should be postulated according to the absorption at relatively long wavelength compared with pigment I. The pigment, on the other hand, contained several hydroxy groups as revealed by acetylation. From these data it was hypothesized that sugar moieties may be esterified with the carboxylic groups similar to the crocetin glycosyl esters. Upon saponification with methanolic KOH containing some ethanol one less polar derivative appeared showing an altered absorption spectrum (488, 515 nm, inflexion at 463 nm). This derivative proved to be a mixture of methyl and ethyl esters of 4,4'-diapocarotene-4,4'-dioic acid (IIb) based on the results of MS and $^1$H-NMR.

The mass spectrum of IIb showed the three expected molecular ions, m/e 488 for the dimethyl ester, 502 for the monomethyl-monoethyl ester, and 516 for the diethyl ester.

The 270 MHz $^1$H-NMR-spectrum of IIb again revealed the presence of one main component presumably with all-trans structure and probably two further minor components assumed to be cis isomers. The spectrum of the main component showed a broad singlet at 2.00 ppm (18 H) assigned to all the "in-chain" methyl protons. The ester methyl group was detected at 3.79 ppm. From the complex pattern between approx. 6.3 and 6.8 ppm several protons could be tentatively assigned (see experimental part). The broadened doublet observed at 7.30 ppm (ca. 11.5 Hz) was assigned to the protons at C(6) and C(6'). $\Delta 5$(5'), therefore, must be trans.

Further information on the structure of the native pigment II was obtained from the MS and $^1$H-NMR of the peracetylated compound IIa.

In the mass spectrum of IIa the highest observable peak was m/e 984 due to the elimination of one sugar moiety from the molecular ion. A group of relatively intense peaks occurred between m/e 527 and 471. The atomic mass composition of the fragment corresponding to the most intense of these peaks, m/e 525,3047, was C$_{28}$H$_{45}$O$_{9}$ indicating a glucosyl residue esterified with three acetic acids and one palmitoleic acid. Corresponding fragments at m/e 527 (with palmitic acid), m/e 499 (with myristic acid), m/e 497 (with unsaturated C$_{14}$ acid), and m/e 471 (with lauric acid) revealed the non-uniformity of the fatty acid component of the native pigment. The presence of these acids was confirmed by gas-liquid chromatography (GLC) after saponification and methylation of the acids. Glucose was also identified by TLC and in the form of alditol acetate by GLC as the only sugar component present after hydrolysis.

The $^1$H-NMR-spectrum of IIa clearly indicated that its structure was symmetrical. From a comparison of the integrals it was concluded that two glucosyl moieties were present. The signals of the glucose protons at C(1) (5.79 ppm, $J_{1,2} \approx 8$ Hz), C(5) (3.90 ppm, $J_{4,5} \approx 9.5$ Hz) and of a further proton, absorbing at 5.17 ppm with two axial-axial couplings, are in agreement with the known conformation of this ring with all protons in axial conformation (β-D-conformation). The fatty acid chains are assumed to be attached to C(6) of the glucose moieties. Further assignments are given in the experimental part.

The hydroxy group at C(1) of the glucose moiety is proposed to be esterified with the carotenoid moiety. Indicative for this position is the absorption at considerably higher wavelength of the native pigment when compared with the saponified and methylated derivative (see above). Similar observa-
In the experiments, crocetin diglucosyl ester and crocetin dimethyl ester were isolated from Pseudomonas rhodos. The pigments were separated on silica gel columns using diapocarotene-4-oic acid. This could be concluded that pigment I contained coproporphyrazin (VIS, MS, NMR). From these data it was concluded that pigment III was a monoglucosyl 4,4'-diapocarotene-4-oic acid. This could be confirmed by MS and 1^H-NMR of the peracetylated pigment (III a).

The mass spectrum of pigment III a showed the molecular ion at m/e 984. The prominent peak at m/e 525 is due to the sugar moiety esterified with one palmitoleyl and three acetyl residues. Practically all other dominant peaks in the mass spectrum were fragment peaks of the esterified sugar moiety such as m/e 405 (elimination of two acetic acid molecules from m/e 525), m/e 345 (loss of a further acetic acid molecule), m/e 237 (palmitoleyl), m/e 169 (elimination of one acetic acid, one ketene, and the palmitoleic acid molecule from m/e 525), and m/e 109 (loss of a further acetic acid molecule).

According to the integral ratios of the corresponding signals in the 1^H-NMR spectrum, the peracetylated pigment III a possessed only one glucosyl moiety. The chemical shifts of the latter protons were found to be practically identical with those of II a. The olefinic portion was also very similar. However, the asymmetry of III a with only one ester group was evident from slightly different chemical shifts of most of the olefinic protons at both ends of the molecule (see experimental part).

Again, the C(1) and C(6) of the glucose moiety were tentatively assumed to be esterified with the carotenoid and fatty acid moiety, respectively (for discussion see above).

**Discussion**

A number of C_{30} carotenoids have been found recently in bacteria. The triterpenoid "bacterial phytoene" has been reported for *Staphylococcus aureus* [6] and *Halobacterium cutirubrum* [7]. This compound has also been shown to be present in *Streptomyces faecium* together with a series of other C_{30} carotenes [8], 4-hydroxy-7',8'-dihydro-4,4'-diapocarotene and its monoglucosylated derivative [9], 7',8'-didehydro-4,4'-diapocarotene-4-oic acid [10], and 7',8'-didehydro-4,4'-diapocarotene-4-al and 4,4'-dipropocarotene-4-al [11]. Another C_{30} carotenoid glycoside, 1-mannosyloxy-3,4-didehydro-1,2-dihydro-8'-apocarotene-8'-oate, has been isolated from a halophilic coccus [12]. The glucosyl esters of 4,4'-diapocarotene-4,4'-dioic acid occurring in *Pseudomonas rhodos* as described in this investigation represent a new group of bacterial carotenoids. The diapo-structure of these compounds was derived from the 1^H-NMR spectrum of pigment II a which clearly showed a symmetrical structure. They resemble in a certain manner the glycosyl esters of the C_{30} carotenoid crocetin (8,8'-diapocarotene-8,8'-dioic acid) from *Crocus sativus* [4, 5] (see these references for the older literature on crocetin esters). The pigment composition of *Pseudomonas rhodos* seems to be rather unique, since related species such as *Pseudomonas echinoides* [13] or *Rhizobium lupini* [14] contain derivatives of β,β-carotene.

We wish to thank Miss Ch. Steinki for technical assistance. Supported by Deutsche Forschungsgemeinschaft (K1 260/5).

**Experimental Part**

**Culture**

*Pseudomonas rhodos* strain B9 (R. Schmitt, Regensburg, GFR), a derivative of wild-type strain 9-6 [1], was grown in 0.8% nutrient broth (Difco) in 100 ml shaken cultures at 30°C. The cultures were illuminated with three fluorescent tubes (Osram, 40 W/25-1).

**Isolation of the pigments**

The pigments were extracted with acetone and methanol and transferred into diethyl ether in a separatory funnel by dilution with water. The pigments were separated on silica gel columns using
petroleum ether/acetone/methanol mixtures and further purified by TLC.

Chemical methods

Acetylation was performed using acetic anhydride in dry pyridine for 2 h at room temperature. Diazomethane was used as methylation agent and 1% KOH in ethanol for saponification. The fatty acids were determined after saponification and methylation by GLC on 10% EGSSX on 100 – 120 mesh GasChrom P (column 1.8 m x 3 mm) isothermally at 170 °C in a Varian Aerograph equipped with a flame ionization detector. The identification of the acids was based on a comparison with reference standards. Glucose was identified after hydrolysis by TLC on cellulose layers (solvent: pyridine/acetic acid/water = 3:2:1) and by GLC as the alditol acetate derivative [15].

Instrumentation

Spectrometers used and experimental details were as recently described [14]. All the 270 MHz 1H-NMR spectra were run in the Fourier transform mode with CDCl₃ as the solvent.

Spectroscopic data

Pigment I (4',4'-diapocarotene-4-oic acid). — Pigment yield about 0.8 mg. VIS (ethanol): native form, 456, 477, 508 nm; methyleylated form, 458, 481, 511 nm. — MS of methylated form (Ia): m/e 444 (45, M); 352 (7); 338 (8); 157 (45); 119 (45); 105 (53); 91 (95); 43 (100). — 1H-NMR data of Ia: 1.83 ppm (s, 6H, H₃C-C(5')); 1.98 and 2.00 (ca. 15H together, "in-chain" methyl groups); 3.77 (s, ca. 3H, OCH₃); 5.94 (d, broad, J ≈ 11.5, H-C(11)); ca. 6 – 6.86 (m, olefinic protons); 7.30 (d, J ≈ 11.5, H-C(6')).

Pigment II (di(β,δ-glucosyl)4',4'-diapocarotene-4,4'-dioate, occurring as fatty acid ester, probably at the C(6) of the glucose moieties). — Pigment yield about 2.5 mg. VIS (ethanol): native form, 475, 499, 530 nm; as dimethyl 4,4'-diapocarotene-4,4'-dioate 463, 488, 515 nm. — MS of peracetylated form (IIa): m/e 984 (0.2); 966 (0.2); 527 (4); 525 (12); 499 (5); 497 (2); 485 (6); 471 (14); 469 (3); 405 (3); 288 (13); 239 (9); 237 (17); 235 (5); 211 (14); 209 (7); 197 (18); 183 (31); 169 (100); 126 (21); 109 (60); 98 (40); 97 (40); 43 (100). — 1H-NMR data of IIa: apocarotene: 1.973 (s, 6H, CH₃ at C(5)) and C(5')); 1.996 (s, ca. 12H, CH₃ at C(9), C(9'), C(13), C(13')); 6.35 (m, H-C(14) and H-C(14')); 6.40 (d, H-C(10) and H-C(10')); 6.48 (dxd, J ≈ 11.5 and 15; H-C(7) and H-C(7')); 0.48 (d, J ≈ 14.8, H-C(12) and H-C(12')); 6.66 (dxd?, H-C(11) and H-C(11')); 6.69 (d, J ≈ 15, H-C(8) and H-C(8')); 7.34 (d, J ≈ 11.5, H-C(6) and H-C(6')). Glucosyl group: 2.026, 2.046 and 2.095 (s, 6H each, O-acetyl); 3.90 (dxdm, 2H, H-C(5'')); 4.12 (d, 2H, J gem ≈ 12, H-C(6'')); 4.33 (dxd, J ≈ 12 and 4, H-C(6'')); 5.17 (tr, J ≈ 9.5, 2H); ca. 5.30 ± 0.03 (m, ca. 4H, axial protons at C(2''), C(3'') and C(4'')); 5.79 (d, J ≈ 8, 2H, H-C(1'')). Fatty acid chains: 0.87 (tr, ca. 6H, "end-of-chain" methyl groups) ca. 1.2 – 1.25 (m, ca. 40H, "in-chain" methylene groups); 2.25 (tr, J ≈ 7, ca. 4H, CO-CH₂) and 1.51 (m, ca. 4H, CO-CH₂CH₂ groups).

MS of dimethyl, methyl-ethyl and diethyl 4,4'-diapocarotene-4,4'-dioate (IIb): m/e 516 (45, M); 502 (100, M₂); 488 (33, M₃). — 1H-NMR data of IIb: 2.002 (s, 18H, "in-chain" methyl groups); 3.771 (s, 2H, OCH₃); 4.22 (q, ca. 3H, OCH₂); ca. 6.32 (m, H-C(14) and H-C(14')); 6.35 (d, J ≈ 11, H-C(10) and H-C(10')); 6.46 (d, J ≈ 15, H-C(12) and H-C(12')); 6.51 (dxd, J ≈ 15 and 11, H-C(7) and H-C(7')); 6.63 (d, J ≈ 15, H-C(8) and H-C(8')); ca. 6.65 and 6.68 (m, H-C(11), H-C(11'), H-C(15), H-C(15')); 7.30 (d, J ≈ 11.5, H-C(6) and H-C(6')).

Pigment III (β,δ-glucosyl-4,4'-diapocarotene-4-oate-4'-oic acid, occurring as fatty acid ester, probably at the C(6') of the glucose). — Pigment yield about 3.5 mg. VIS (ethanol): native form, 471, 494, 525 nm; as dimethyl 4,4'-diapocarotene-4,4'-dioate, 463, 488, 515 nm. — MS of the peracetylated form (IIIa): m/e 984 (1, M); 525 (14); 405 (8); 345 (10); 288 (7); 239 (11); 237 (35); 169 (100); 109 (63); 98 (29); 97 (43); 43 (100). — 1H-NMR data of IIIa: apocarotene: 1.970, 1.996 and 2.013 ("in-chain" methyl groups); ca. 6.36 – 6.69, mostly unresolved, complex pattern with part of the chemical shifts as with pigment IIa, others slightly shifted due to the unsymmetric structure; 7.34 (d, J ≈ 12, H-C(6)); 7.39 (d, J ≈ 12, H-C(6')). Glucosyl group and fatty acid chain: all shifts identical with those given for IIa (see above); integrals correspondingly smaller according to structure IIIa.