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Bacterial Carotenoids. XLIV.* Zeaxanthin Mono- and Dirhamnoside

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The isolation of zeaxanthin (*1a*, 2 % of total carotenoid), zeaxanthin monorhamnoside (*3a*, 18 %) and zeaxanthin dirhamnoside (*2a*, 80 %) from a coryneform hydrogen bacterium strain 14 g (Institute of Microbiology, University of Göttingen) is reported.

The partial structures of these first, secondary, non-allylic, remarkably stable, carotenoid glycosides (*2a* and *3a*) were established by spectroscopic (visible light, IR, ^{1}H NMR and mass spectra) and chemical evidence. Acid hydrolysis provided rhamnose and strong alkaline hydrolysis zeaxanthin (*1a*) and modified dehydroaglycones.

The isolation and structure elucidation of carotenoid glycosides have recently been reviewed.[1] Also carotenoid glycosides subsequently isolated [2–5] belong to the same type of prim./sec. allylic or tert. glycosides.

We now report the first isolation of secondary, non-allylic carotenoid glycosides with remarkable stability.

The source of these rhamnosides is a yellow coryneform hydrogen bacterium strain 14 g under current investigation [6,7] at Institute of Microbiology, University of Göttingen.

RESULTS AND DISCUSSION

Strain 14 g contained *ca.* 0.074 % carotenoid per dry weight, comprising zeaxanthin (*1a*, 2 %), zeaxanthin dirhamnoside (*2a*, 80 %), and zeaxanthin monorhamnoside (*3a*, 18 %). Solubility properties, impurities and low recovery on acetylation of crude extracts complicated the isolation of the rhamnosides. Best results were obtained by purification of the free glycosides on acetylated polyamide columns.[3,4]

Zeaxanthin (*1a*, available 0.5 mg) exhibited visible spectrum and mass spectrum consistent with structure *1a*, was inseparable from authentic zeaxanthin (*1a*), and more polar than the 2,2′- or 4,4′-dihydroxy analogues.[8] Its diacetate *1b* was inseparable from authentic zeaxanthin diacetate (*1b*) and different from isozeaxanthin diacetate.

The major carotenoid (*2a*, available *ca.* 18 mg) had, according to the electronic spectrum, the same bicyclic chromophore as zeaxanthin (*1a*). Polarity and mass spectrometric data indicated its glycosidic character. The molecular ion of *2a* [*m/e* 860, consistent with $C_{40}H_{54}O_2(C_6H_{11}O_4)_2$] was shifted to *m/e* 1112 on peracetylation to *2b* and to *m/e* 944 on permethylation to *2c*, demonstrating the presence of six hydroxy groups. Molecular ions were generally supported by M − 79, M − 92, M − 106 and M − 158 ions caused by common in-chain fragmentations.[9]

At least one glycosidically bound methylpentose followed from the fragmentation pattern on electron impact. Thus *2a* itself had characteristic fragment ions M − 164, M − 163, M − 146 and *m/e* 147 and the hexaacetate *2b* diagnostically important M − 290, M − 289, M − 288, M − 272 and *m/e* 273 and 169 ions, typical of methylpentosides.[10,11] Corresponding fragment ions were also observed for the hexamethyl ether *2c*: M − 206, M − 188, *m/e* 189 and 167.[10] An *m/e* 331.1025 ($C_{14}H_{19}O_9$) ion of varying intensity in spectra of the hexaacetate *2b*, consistent with the tetraacetyl oxonium ion of a hexose,[10] could by other criteria (molecular ion

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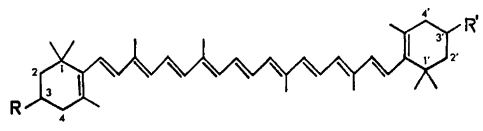
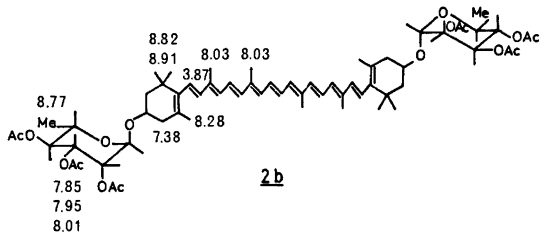


1a R=R'=OH
1b R=R'=OAc
1c R=R'=OMe

2a R=R'= Rhamnosyloxy
2b R=R'= Triacetylrhamnosyloxy
2c R=R'= Trimethylrhamnosyloxy

3a R = Rhamnosyloxy
R'=OH
3b R = Triacetylrhamnosyloxy
R'=OAc



2b

Scheme 1.

and glycoside hydrolysis) not be explained by a mixed hexoside, *cf.* Refs. 11 – 13. Contamination with an acetylated glucose polymer may offer an explanation, since this bacterium is known to produce polyglucose.[14] However, at this stage acetyl transfer within a disaccharide was also considered.

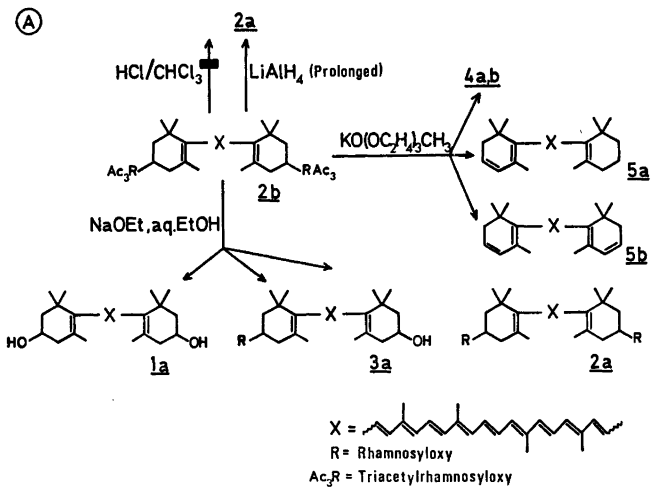
Methanolysis, followed by hydrolysis of the methyl glycosides thus formed gave no reducing sugars under conditions effective for secondary, allylic carotenoid glycosides.[11,12] Methanolysis under more vigorous conditions gave rhamnose as the only reducing sugar seen by paper chromatography. The identification was further confirmed by GLC of the derived rhamnose tetraacetate and rhamnitol pentaacetate in direct comparison with authentic standards. Evidence that zeaxanthin is the aglycone involved is given below, and it may therefore be concluded that the glycoside in question is a zeaxanthin dirhamnoside.

The possibility of the two rhamnose units being present in a disaccharide rather than separately bound to the diol aglycone *1a* was ruled out by ^{1}H NMR data: In the symmetrical zeaxanthin dimethyl ether (*1c*) the 18- and 18′-methyl groups cause one signal in deuteriopyridine at τ 8.24. In the monorhamnoside *3a* discussed below, two separate signals at τ 8.24 (18 – Me) and τ 8.17 (18′ – Me) are observed. The occurrence of only one signal at τ 8.24 (18,18′ – Me) in the dirhamnoside therefore demonstrated the symmetrical nature of *2a*.

Evidence leading to identification of the aglycone will now be considered. The ^{1}H NMR spectrum of the dirhamnoside hexaacetate (*2b*, Scheme 1, including assignments) suggested that the aglycone involved was a β,β-carotenediol derivative.[9] Contrary to secondary, allylic carotenoid glycosides [11,12] the present rhamnoside gave no modified aglycones on treatment with $HCl/CHCl_3$ or on prolonged $LiAlH_4$ reduction.

Others [15,16] have reported that the aglycone and dehydroaglycone may be obtained from tertiary carotenoid glycosides on base treatment. The hexaacetate *2b* gave on treatment with sodium ethoxide in aqueous ethanol zeaxanthin (*1a*, 5 %) and the monorhamnoside (*3a*, 45 %) in addition to the dirhamnoside (*2a*), thus proving that zeaxanthin (*1a*) is the aglycone involved, Scheme 2. The hexamethyl ether *2c* suffered no such hydrolysis, indicating that the free glycoside is required as an intermediate.

Treatment of the hexaacetate *2b* with potassium methoxytriethyleneglycolate [17] in dioxane gave unidentified modified aglycone products



Scheme 2.

(*4a,b*, *5a,b*) with electronic and mass spectra compatible with 3,4(3′,4′)-dehydrogenation, Scheme 2.

These alkaline degradations are not well understood, although possible analogies have been considered.[18–22]

Turning now to the stereochemistry of zeaxanthin dirhamnoside (*2a*), the all-*trans* isomer was the predominant geometrical isomer. The chirality of the aglycone was not established. Zeaxanthin from other sources is known to have 3*R*,3′*R*-configuration.[23,24] Rhamnose sufficient for rotation measurements could not be isolated; L-rhamnosides are most common.[25–28] Regarding the stereochemistry of the glycosidic linkage no definite conclusions can be made from the available evidence. The preference for α-L-rhamnopyranoside [22] (Scheme 1) is tentative.

Zeaxanthin monorhamnoside (*3a*, available 4 mg) exhibited the same electronic spectrum as *1a* and *2a* and had intermediate polarity. Its molecular ion at *m/e* 714 (consistent with $C_{40}H_{55}O_2(C_6H_{11}O_4)$ shifted to *m/e* 882 on acetylation, demonstrating the formation of a tetraacetate *3b*. The monorhamnoside *3a* and its tetraacetate *3b* exhibited the same prominent fragment ions as the dirhamnoside (*2a* and *2b*) on electron impact and showed an *m/e* 331 ion in agreement with the methylpentoside formulation. The ^{1}H NMR spectrum was consistent with structure *3a* and zeaxanthin (*1a*) was obtained as 13 % of the recovered carotenoid on alkaline hydrolysis with sodium ethoxide.

EXPERIMENTAL PART

Biological material. Strain 14 g of a yellow, coryneform hydrogen bacterium, Institute of Microbiology, University of Göttingen, was cultivated in Göttingen (Batch 1) and in the Department of Biochemistry of this University (Batch 2) in a Na-lactate medium as described in detail elsewhere;[22] available *ca.* 2.5 kg fresh cells.

General methods and instruments used were those generally employed.[29] When not otherwise stated R_F-values refer to Schleicher & Schüll No. 287 circular kieselguhr paper, acetone in petroleum ether (APE). TLC refers to kieselgel G.

Isolation. Several chromatographic systems and isolation procedures were investigated,[22] two being referred here: A. Most satisfactory was extraction of centrifuged cells with acetone at room temperature, followed by transfer of the concentrated pigment extract to ether with 5 % aqueous NaCl, drying of the pigment by azeotropic distillation with benzene and chromatography on acetylated polyamide.[3,4] The crude pigment, insoluble in petroleum ether, ether, acetone, chloroform, benzene, methanol, and CS_2, but soluble in pyridine or benzene-methanol (*ca.* 1:1) was dissolved in the latter system and diluted with benzene for chromatography. *1a* required benzene, *3a* 2 % methanol in benzene, and *2a* 6 % methanol in benzene for elution from acetylated polyamide; pigment recovery 79 %.

B. Partition of the crude pigment, dissolved in pyridine, between petroleum ether and 80 %

aqueous methanol resulted in precipitation of the pigments in the aqueous phase; 92 % recovery. Standard acetylation and TLC (40 % APE) gave the acetates *1b*, *3b*, and *2b*; 59 % recovery. If partition was omitted acetylation of the crude pigment gave only 5 – 30 % recovery.

Batch 1, *ca.* 2 kg wet weight, provided 325 mg [E(1 %, 1 cm) = 1900] extracted pigment; 0.074 % of the dry extracted residue, purified by procedure B. Batch 2 gave 27 mg pigment, purified by procedure A.

Zeaxanthin (*1a*)

Characterization. 1a available 0.5 mg, purified by standard saponification and TLC; λ_{max} (acetone) 429, 451 and 474 nm, % III/II [30] = 29; *m/e* 568 (M), 550 (M – 18), 489 (M – 79), 476 (M – 92), 462 (M – 106), 422 (M – 146), 410 (M – 158), was inseparable from authentic *1a* and more strongly adsorbed than isozeaxanthin in direct comparison.

Zeaxanthin diacetate (1b). Standard acetylation of *1a* (0.2 mg) gave *1b*; *m/e* 652 (M), 592 (M – 60), 560 (M – 92) 546 (M – 106), 532 (M – 60 – 60), 500 (M – 60 – 92), 494 (M – 158), 486 (M – 60 – 106), inseparable from authentic *2a*.

Zeaxanthin dirhamnoside (*2a*)

Characterization. 2a (Batch 2), repeatedly crystallized from petroleum ether-methanol and pyridine-petroleum ether-acetone gave a semi-crystalline material, m.p. 216 – 218 °C; R_F = 0.16, TLC 35 % APE; λ_{max} (acetone) 428, 452, and 480 nm, % III/II = 27, λ_{max} (benzene-methanol 1:1) 436, 461 (ε = 75 700, compared with ε = 130 000 for authentic *1a* in acetone) and 488 nm; *m/e* (230 °C) 860 (M), 842 (M – 18), 824 (M – 18 – 18), 781 (M – 79), 768 (M – 92), 754 (M – 106), 734 (M – 126), 714 (M – 146), 702 (M – 158), 697 (M – 163), 696 (M – 164), 678 (M – 18 – 18 – 146), 622 (M – 92 – 146), 608 (M – 106 – 146), 604 (M – 18 – 92 – 146), 602 (M – 258), 568 (M – 146 – 146), 565, 550, 508, 472, 339, 264, 147; τ (deuteriopyridine) 8.85 (*gem.* Me), 8.73 (lipid imp.), 8.34 (*d*, J = 5.5 Hz, Me in rhamnose), 8.24 (18,18′-Me), 7.96 (in-chain Me).

For comparison L-rhamnose had C-5 methyl signals at τ 8.36 (β, d, J = 6 Hz) and 8.40 (α, d, J = 6 Hz). Authentic zeaxanthin dimethyl ether *1c* had τ 8.85 (*gem.* Me), 8.22 (18,18′-Me), 8.01 and 7.97 (in-chain Me) and 6.62 (OMe).

Glycoside hydrolysis. Methanolysis [11] of *2a* in 0.15 N dry HCl for 20 h, 22 °C, was not effected; pigment recovery 75 % and no reducing sugars formed after hydrolysis.[11,31] Methanolysis of *2a* (15 mg) in 0.2 N HCl for 5 h, 65 °C, followed by hydrolysis [11,31] with aqueous polystyrene sulfonic acid and ascending chromatography,[22] followed by development with aniline-phthalic acid, showed presence of rhamnose ($R_{glucose}$ = 1.44) only in co-chromatography tests with saccarose (0.45), mannose (0.65), galactose (0.81), glucose (1.00) rhamnose (1.44), and fucose (2.02).

An aliquot of the hydrolysate was acetylated and submitted to GLC (Perkin Elmer F11, silicon XE-60/ethyleneglycol succinate, 157 – 218 °C, 1 °/min) provided two peaks with retention time 20.4 min (75 %) and 22.9 min (25 %). Peracetylated α,β-L-rhamnose showed the same retention times and a ratio of 90:10.

Remaining tetraacetyl rhamnose was saponified and reduced with $NaBH_4$ in water. The rhamnitol, thus prepared, was acetylated and submitted to GLC as above and found identical (retention time 24.5 min) in direct comparison with authentic L-rhamnitol pentaacetate prepared from L-rhamnose.

Zeaxanthin dirhamnoside hexaacetate. (2b). Standard acetylation of *2a* (12 mg) gave *2b* (90 % yield), purified by TLC: λ_{max} as for *2a*; *m/e* 1112 (M), 1070 (M – 42), 1068 (M – 44), 1033 (M – 79), 1020 (M – 92), 1006 (M – 106), 992 (M – 60 – 60), 980 (M – 132), 976 (M – 44 – 92), 968 (M – 144), 956 (M – 156), 954 (M – 158), 868 (M – 244), 840 (M – 272), 824 (M – 288), 823 (M – 289), 822 (M – 290), 731 (M – 92 – 289), 717 (M – 106 – 289), 687 (M – 44 – 92 – 289), 568, 381, 351, 331.1025 ($C_{14}H_{19}O_9$), 273, 169, ν_{max} (KBr) 2925, 2855, 1752, 1445, 1370, 1225, 1180, 1150, 1135, 1050, 977, 910, 890, 840, 790 and 685 cm^{-1}; τ ($CDCl_3$), *cf. 2b*, Scheme 1, 8.91, 8.82 (*gem.* Me, *ca.* 12 H); 8.77 (*Me*–CH?); 8.28 (Me – 18,18′, *ca.* 6 H); 8.03 (in-chain Me, *ca.* 12 H); 8.01, 7.95, 7.85 (acetate Me, *ca.* 18 H); 7.38 (allylic CH_2); 4.0 – 6.0 (CH in rhamnose moiety); 3.0 – 4.0 (olefinic H), stepwise addition of Eu(dpm)$_3$ [32] until 3.1 mol/mol *2b* caused no shift of the aglycone methyl groups, 55 Hz downfield shift of allylic CH_2 and 30 – 19 Hz downfield shifts of CH_3 and $CH_3C{=}O$ of the rhamnose moiety; R_F = 0.65 in 10 % APE.

An acetylated compound more polar than *2b* by TLC and representing 4 % of *2b* was, from electronic spectrum (% III/II = 0), mass spectrum (as *2b*), and reversibility tests, shown to be a *cis* isomer of *2b*.

$LiAlH_4$-reduction of the hexaacetate 2b. Treatment of *2b* (0.5 mg) with $LiAlH_4$ in dry ether for 5 min – 24 h [11,12] gave no modified aglycone but only the free rhamnoside *2a*; pigment recovery 30 – 50 %.

Attempted allylic elimination of the hexaacetate 2b. 2b (0.3 mg) treated with 0.03 – 0.2 N dry HCl in ethanol-free chloroform for 2 – 15 min gave no new, coloured products.

Alkaline hydrolysis of the hexaacetate 2b. 2b (0.75 mg), in 2.5 N NaOEt in 96 % ethanol (10 ml) was kept for 3 h at 50 – 60 °C. The pigments were transferred to ether on dilution with water after acidification to pH 4; pigment recovery 42 %. Paper chromatography revealed the formation of zeaxanthin (*1a*). Acetylation of the products gave zeaxanthin diacetate (*1b*, 5 %, *m/e* 652 = M), zeaxanthin monorhamnoside tetraacetate (*3b*, 45 %, *m/e* 882 = M) and zeaxanthin dirhamnoside hexaacetate (*2b*, 50 %) with electronic spectra and R_F-values as authentic samples.

Treatment with potassium methoxytriethyleneglycolate [17] at room temperature was carried out with the hexaacetate (*2b*, 1.5 mg) in dioxane (1.4 ml, chromatographed on alumina and distilled under N_2) and potassium methoxytriethyleneglycolate (0.6 ml, *ca.* 0.5 N) added. The mixture was stirred mechanically for 1.5 h and worked up as above; pigment recovery 30 %. TLC gave two major zones, presumably comprising four products: *4a, b* (70 %, $R_F = 0.18$, TLC, 5 % APE), λ_{max} (acetone) 460 nm (round), *m/e* 548 (M_a, consistent with $C_{40}H_{52}O$), 546 (M_b, $C_{40}H_{50}O$?, 80 % of *m/e* 548), 456 ($M_a - 92$), 454 ($M_b - 92$), 442 ($M_a - 106$), 440 ($M_b - 106$) and *5a, b* (25 %, $R_F = 0.60$ in above system), λ_{max} (acetone) 442, 461, and (483) nm, *m/e* 534 (M_a, $C_{40}H_{54}$), 532 (M_b, $C_{40}H_{52}$, 50 % of *m/e* 534), 442 ($M_a - 92$,) 440 ($M_b - 92$), 428 ($M_a - 106$).

Zeaxanthin dirhamnoside hexamethyl ether (2c). Methylation of *2a* (9 mg) by the modified Kuhn procedure [33] gave *2c*; 40 % pigment recovery, quantitative conversion. Compound *2c* had λ_{max} as *2a*; *m/e* 944 (M), 942 (M − 2), 912 (M − 32), 883 (M − 61), 852 (M − 92), 850 (M − 2 − 92), 838 (M − 106), 830 (M − 114), 814 (M − 130), 812 (M − 132), 800 (M − 144), 786 (M − 158), 784 (M − 160), 770 (M − 174), 762 (M − 182), 756 (M − 188), 738 (M − 206), 694 (M − 92 − 158), 692 (M − 92 − 160), 664 (M − 92 − 188), 189, 157; $R_F = 0.90$, 10 % APE.

Alkali treatment with 2.5 % NaOEt as for *2b* caused no reaction.

Zeaxanthin monorhamnoside (*3a*)

Characterization. 3a, precipitated from methanol-benzene-petroleum ether, available 4 mg, m.p. 149 − 150 °C had μ_{max} (methanol-benzene 1:1) 436, 460 and 487 nm, % III/II = 23; *m/e* (220 °C) 714 (M), 696 (M − 18), 680 (M − 34), 672 (M − 42), 635 (M − 79), 622 (M − 92), 608 (M − 106), 602 (M − 112), 596 (M − 118), 588 (M − 34 − 92), 582 (M − 132), 568 (M − 146), 558 (M − 156), 556 (M − 158), 551 (M − 163), 550 (M − 164), 516 (M − 92 − 106), 508 (M − 206), 504 (M − 210), 476 (M − 92 − 146), 462 (M − 106 − 146), 339, 264, 163; τ (deuteriopyridine) 8.82 (*gem.* Me), 8.70 (lipid imp.), 8.34 (d, $J = 5.5$ Hz, Me in rhamnose), 8.24 (18 − Me), 8.17 (18′ − Me) and 7.95 (in-chain Me); $R_F = 0.50$, 35 % APE.

Zeaxanthin monorhamnoside tetraacetate (3b). 3a (0.4 mg) was quantitatively acetylated to *3b* λ_{max} as *3a*; *m/e* 882 (M), 840 (M − 42), 838 (M − 44), 822 (M − 60), 803 (M − 79), 790 (M − 92), 776 (M − 106), 748 (M − 42 − 92), 730 (M − 60 − 92), 726 (M − 156), 724 (M − 158), 716 (M − 60 − 106), 666 (M − 60 − 156), 664 (M − 60 − 158), 638 (M − 244), 610 (M − 272), 594 (M − 288), 593 (M − 289), 331 (15 % of *m/e* 273), 273, 153, (100 %); $R_F = 0.54$, 5 % APE.

Alkaline hydrolysis of tetraacetate 3b. 3b (0.5 mg) was treated with 2.5 % NaOEt as described above for *2b*; pigment recovery 40 %. Zeaxanthin (*1a*) was identified by paper chromatography. Acetylation of the product, followed by TLC gave zeaxanthin diacetate (*1b*, 13 %, inseparable from authentic *1b*) and *3b* (87 %, inseparable from the starting material *3b*).

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