Prokaryotic triterpenoids: *O*- α -D-glucuronopyranosyl bacteriohopanetetrol, a novel hopanoid from the bacterium *Rhodospirillum rubrum**

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A novel hopanoid bearing a glucuronopyranosyl residue linked via an α -glycosidic bond to the hydroxyl group of C-35 in bacteriohopanetetrol was isolated, from the type strain of *Rhodospirillum rubrum* as well as from a mutant lacking blue carotenoid.

INTRODUCTION

Hopanoids are widely distributed prokaryotic triterpenoids [1]. Their role as membrane stabilizers is now well documented [2], but the significance of the diversity of the side-chain structures is not yet understood. Thus, to get more information about the structural variety of complex hopanoids, to investigate their distribution among eubacteria, and to estimate their possible chemotaxonomic interest, it is still necessary to analyse more strains. As several *Rhodospirillaceae* have been found to be good sources of complex bacteriohopane derivatives [3–5], this taxonomic group has been investigated further. In this paper, we report on the identification of a new bacteriohopanetetrol glycoside [Fig. 1; (I)] isolated from the type strain of *Rhodospirillum rubrum*, as well as from a blue mutant lacking carotenoids.

EXPERIMENTAL

General methods

G.l.c. was carried out on a Carlo Erba Fractovap 4160 apparatus fitted with a fused silica DB5 capillary column (0.25 mm $\times$ 30 m) and an on-column injector. The oven temperature was programmed to rise from 50 °C to 220 °C at a rate of 20 °C/min and from 220 °C to 310 °C at a rate of 6 °C/min. The flame-ionization detector was at 310 °C and hydrogen was used as the carrier gas. The quantity of each product was measured using g.l.c. by comparing the peak areas with that of an internal standard of *n*-dotriacontane and correcting these values by factors obtained with reference samples. Direct-inlet m.s. was done at 70 eV on a Finnigan TSQ70 apparatus.

G.l.c./m.s. on an LKB 9000S apparatus fitted with the same DB5 column as for g.l.c. was performed as described by Neunlist *et al.* [3]. N.m.r. spectra were recorded on a Bruker AC 250 spectrometer at 300 K. Chloroform was used as the internal reference ($\delta = 7.260$ p.p.m.) for ^1H spectra and $[\text{D}_6]\text{chloroform}$ ($\delta = 77.0$ p.p.m.) for ^{13}C spectra. Attributions of ^1H signals were confirmed by homonuclear $^1\text{H}/^1\text{H}$ correlation spectroscopy (COSY). Distortionless enhancement by polarization transfer (DEPT) was used for the attribution of ^{13}C signals. Heteronuclear $^1\text{H}/^{13}\text{C}$ correlations by 1J and long-range couplings were done on a Bruker W400 apparatus. The study of the non-decoupled spectrum led us to assign a value of 7 Hz to the 3J coupling constants between ^1H and ^{13}C , which was taken into account for this 3J -correlated $^1\text{H}/^{13}\text{C}$ spectrum.

Rh. rubrum DSM 467 (type strain) and DSM 468 (blue mutant) were grown at 30 °C for 8 days in medium DSM 27 [6]. The cells (650 mg/l for both strains, dry weight) were harvested by centrifugation (8000 *g*, 10 min) at 4 °C and freeze-dried.

Extraction and isolation of hopanoids

Freeze-dried cells were extracted three times for 1 h under reflux using chloroform/methanol (2:1, v/v), chloroform/ethanol (2:1, v/v) or dry tetrahydrofuran. After evaporation of the solvent, the crude extract was treated according to one of the following procedures: Procedure 1. Periodic acid treatment followed by sodium borohydride reduction, t.l.c. and acetylation of the alcohol fraction allowed determination of the hopanoid content by g.l.c. [1]. Procedure 2. To obtain the intact complex hopanoids, extracts were directly acetylated overnight at room temperature using acetic anhydride/pyridine (1:1, v/v) and separated by t.l.c. using chloroform/methanol/acetic acid (95:5:1, by vol) to yield the hexa-acetate of (I) (Fig. 1) ($R_f = 0.45$) which was still accompanied by some unidentified lipids (about

Fig 1. Hopanoids of *Rh. rubrum* (I–IV) and synthetic derivatives of D-GlcA (V–VII)

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15%) according to the ^1H -n.m.r. spectrum. Further purification by either t.l.c. or h.p.l.c. of this hexa-acetate was not possible. However, as the hexa-acetate of the methyl or ethyl esters of (I), which were found in significant amounts after chloroform/methanol or chloroform/ethanol extraction, would be easily isolated by t.l.c. using cyclohexane/ethyl acetate (2:1, v/v; $R_F = 0.25$ for the methyl ester and $R_F = 0.30$ for the ethyl ester), the acetylated extracts as well as the hopanoid-containing t.l.c. fractions were treated with a diazomethane solution in diethyl ether to obtain the readily isolated hexa-acetate methyl ester of (I). The corresponding ethyl ester was obtained by refluxing (I) in ethanol in the presence of hydrochloric acid.

Hexa-acetate of the methyl ester of (I)

The ^1H -n.m.r. (250 MHz, ^2H]chloroform) characteristics were as follows: δ (p.p.m.) 0.682 (3H, s, $18\alpha\text{-CH}_3$), 0.785 (3H, s, $4\beta\text{-CH}_3$), 0.808 (3H, s, $4\alpha\text{-CH}_3$), 0.840 (3H, s, $10\beta\text{-CH}_3$), CH_3 , 0.908 (3H, d, J 6 Hz, 22R-CH_3), 0.940 (6H, s, $8\beta\text{-CH}_3$ and $14\alpha\text{-CH}_3$), 2.018 (3H, s, $\text{CH}_3\text{CO-}$), 2.034 (3H, s, $\text{CH}_3\text{CO-}$), 2.046 (3H, s, $\text{CH}_3\text{CO-}$), 2.070 (9H, s, 3 $\text{CH}_3\text{CO-}$), 3.67 (1H, dd, $J_{34,35a}$ 6 Hz, $J_{35a,35b}$ 12 Hz, 35-H_a), 3.75 (3H, s, $\text{CH}_3\text{O-}$), 3.95 (1H, dd, $J_{34,35b}$ 2.5 Hz, $J_{35a,35b}$ 12 Hz, 35-H_b), 4.31 (1H, d, $J_{4',5'}$ 10 Hz, $5'\text{-H}$), 4.84 (1H, dd, $J_{1',2'}$ 3.5 Hz, $J_{2',3'}$ 10 Hz, $2'\text{-H}$), 5.01 (1H, dt, $J_{31,32}$ 5 Hz, $J_{32,33}$ 4 Hz, 32-H), 5.16 (4H, m, $1'\text{-H}$, $4'\text{-H}$, 33-H and 34-H), 5.50 (1H, dd, $J_{2',3'} = J_{3',4'}$ 10 Hz, $3'\text{-H}$).

The ^{13}C -n.m.r. (62.9 MHz, ^2H]chloroform) characteristics were as follows: δ (p.p.m.) 15.8 and 15.9 (C-25 and C-28), 16.5 and 16.6 (C-26 and C-27), 18.7 (C-2 and C-6), 19.8 (C-29), between 20.5 and 20.9 (6 $\text{CH}_3\text{COO-}$), 20.9 (C-11), 21.6 (C-24), 22.8 (C-16), 24.0 (C-12), 26.5 (C-31), 27.5 (C-20), 30.9 (C-30), 33.2, 33.3 and 33.4 (C-4, C-7 and C-23), 33.7 (C-15), 36.1 (C-22), 37.4 (C-10), 40.3 (C-1), 41.6 (C-19), 41.7 (C-8), 41.8 (C-14), 42.1 (C-3), 44.3 (C-18), 45.9 (C-21), 49.3 (C-13), 50.4 (C-9), 52.9 ($\text{CH}_3\text{O-}$), 54.4 (C-17), 56.1 (C-5), 66.8 (C-35), 68.4 (C-5'), 69.1, (C-3'), 69.5 (C-34), 70.4 (C-2'), 70.9 (C-4'), 72.0 and 72.1 (C-32 and C-33), 96.4 (C-1'), 167.9 (-CO- of methyl ester), between 169.6 and 170.3 (6 -CO- of acetoxy groups).

Hexa-acetate of the ethyl ester of (I)

The ^1H -n.m.r. (400 MHz ^2H]chloroform) characteristics were as follows: δ (p.p.m.) 0.682 (3H, s, $18\alpha\text{-CH}_3$), 0.786 (3H, s, $4\beta\text{-CH}_3$), 0.808 (3H, s, $4\alpha\text{-CH}_3$), 0.841 (3H, s, $10\beta\text{-CH}_3$), 0.910 (3H, d, J 6.5 Hz, 22R-CH_3), 0.940 (6H, s, $8\beta\text{-}$ and $14\alpha\text{-CH}_3$), 2.018 (3H, s, $\text{CH}_3\text{COO-}$), 2.034 (3H, s, $\text{CH}_3\text{COO-}$), 2.046 (3H, s, $\text{CH}_3\text{COO-}$), 2.070 (9H, s, 3 $\text{CH}_3\text{COO-}$), 3.67 (1H, dd, $J_{34,35a}$ 7 Hz, $J_{35a,35b}$ 12 Hz, 35-H_a), 3.95 (1H, dd, $J_{34,35b}$ 2.5 Hz, $J_{35a,35b}$ 12 Hz, 35-H_b), 4.20 (2H, q, J 6.5 Hz, $\text{CH}_3\text{CH}_2\text{O-}$), 4.29 (1H, d, $J_{4',5'}$ 10 Hz, $5'\text{-H}$), 4.85 (1H, dd, $J_{1',2'}$ 3.5 Hz, $J_{2',3'}$ 10 Hz, $2'\text{-H}$), 5.01 (1H, dt, $J_{31,32}$ 5 Hz, $J_{32,33}$ 4 Hz, 32-H), 5.16 (4H, m, $1'\text{-H}$, $4'\text{-H}$, 33-H and 34-H), 5.50 (1H, t, $J_{2',3'} = J_{3',4'}$ 10 Hz, $3'\text{-H}$).

The ^{13}C -n.m.r. (62.9 MHz; ^2H]chloroform) characteristics were the same as for the methyl ester of (I) except for the following signals: δ (p.p.m.) 13.9 ($\text{CH}_3\text{CH}_2\text{O-}$), 62.1 ($\text{CH}_3\text{CH}_2\text{O-}$), 68.5 (C-5'), 69.3 (C-3'), 167.4 (-CO- of ethyl ester). The signal at 52.9 p.p.m. corresponded to the compound with the ester methyl group missing.

The mass-spectrum (direct-inlet, electron impact, 70 eV) characteristics were as follows: m/z 1002 (M^+ , 0.2% calculated for $\text{C}_{56}\text{H}_{86}\text{O}_{16}$: 1002.6), 987 ($M^+ - \text{CH}_3$, 0.1%), 942 ($M^+ - \text{AcOH}$, 0.9%), 882 ($M^+ - 2 \text{ AcOH}$, 0.3%), 822 ($M^+ - 3 \text{ AcOH}$, 0.2%), 781 (ring C cleavage [7], 5%), 721 (781 - AcOH, 1%), 671 (cleavage of glycosidic bond between C-1' and oxygen atom at C-35, bacteriohopane moiety, 4%), 655 (cleavage of glycosidic bond between C-35 and oxygen atom, bacteriohopane

moiety, 12%), 369 ($M^+ - \text{side-chain}$, 15%), 331 (cleavage of glycosidic bond between C-1' and oxygen atom at C-35, sugar moiety, 56%), 271 (331 - AcOH, 67%), 211 (331 - 2 AcOH, 18%), 191 (ring C cleavage [7], 57%), 169 (100%).

Methanolysis of glycoside (I) hexa-acetate and synthesis of glucuronic acid derivatives

The hexa-acetate of (I) was heated at 100 °C for 16 h in a saturated solution of hydrogen chloride in methanol. After evaporation of the reagent, the residue was re-acetylated and the acetylation products separated by t.l.c. (cyclohexane/ethyl acetate, 4:1, v/v) to yield tetra-acetoxybacteriohopane ($R_F = 0.35$) and the D-GlcA derivatives remaining on the baseline. These were further separated using chloroform/methanol as eluent (98:2, v/v) yielding, next to unidentified compounds, ester (V) (Fig. 1; $R_F = 0.55$, 28% of the mixture from g.l.c.), ester (VI) (Fig. 1; $R_F = 0.50$, 3%) and lactone (VII) (Fig. 1; $R_F = 0.45$, 61%). Identical treatment of D-GlcA afforded derivatives (V), (VI) and (VII) in the same ratios.

GlcA derivative (V)

The ^1H -n.m.r. (250 MHz, ^2H]chloroform) characteristics were as follows: δ (p.p.m.) 2.017 (3H, s, $\text{CH}_3\text{CO-}$), 2.023 (3H, s, $\text{CH}_3\text{CO-}$), 2.068 (3H, s, $\text{CH}_3\text{CO-}$), 3.443 (3H, s, $\text{CH}_3\text{O-}$), 3.775 (3H, s, $\text{CH}_3\text{O-}$), 4.30 (1H, d, $J_{4',5'}$ 10 Hz, $5'\text{-H}$), 4.90 (1H, dd, $J_{1',2'}$ 3.5 Hz, $J_{2',3'}$ 10 Hz, $2'\text{-H}$), 5.04 (1H, d, $J_{1',2'}$ 3.5 Hz, $1'\text{-H}$), 5.18 (1H, dd, $J_{3',4'}$ 10 Hz, $J_{4',5'}$ 10 Hz, $4'\text{-H}$), 5.52 (1H, dd, $J_{2',3'} = J_{3',4'}$ 10 Hz, $3'\text{-H}$).

The ^{13}C -n.m.r. (62.9 MHz, ^2H]chloroform) characteristics were as follows: δ (p.p.m.) 20.5 ($\text{CH}_3\text{CO-}$), 20.7 (2 $\text{CH}_3\text{CO-}$), 52.9 ($\text{CH}_3\text{OCO-}$), 56.0 ($\text{CH}_3\text{O-}$), 68.2 (C-5'), 69.3 (C-3'), 69.6 (C-4'), 70.5 (C-2'), 97.1 (C-1'), 168.1 (-CO- of ester), between 169.6 and 170.1 (3 $\text{CH}_3\text{CO-}$).

The mass spectrum (g.l.c./m.s.) characteristics were as follows: m/z 289 ($M^+ - \text{CO}_2\text{CH}_3$, 1%), 257 (289 - $\text{C}_2\text{H}_2\text{O}$ [8], 0.5%), 229 (289 - AcOH, 3%), 215 (257 - $\text{C}_2\text{H}_2\text{O}$, 1%), 186 (6%), 169 (229 - AcOH, 8%), 157 ($\text{AcOCH} = \text{CH-CHOAc}^+$ [8], 10%), 145 (triacyetyl [8], 5%), 127 (169 - $\text{C}_2\text{H}_2\text{O}$, 22%), 103 (diacyetyl [8], 7%), 43 (CH_3CO^+ [8] 100%).

GlcA derivative (VI)

The ^1H -n.m.r. (250 MHz, ^2H]chloroform) characteristics were as follows: δ (p.p.m.) 2.016 (3H, s, $\text{CH}_3\text{CO-}$), 2.022 (3H, s, $\text{CH}_3\text{CO-}$), 2.050 (3H, s, $\text{CH}_3\text{CO-}$), 3.522 (3H, s, $\text{CH}_3\text{O-}$), 3.764 (3H, s, $\text{CH}_3\text{O-}$), 4.04 (1H, m, $5'\text{-H}$), 4.48 (1H, d, $J_{1',2'}$ 7.5 Hz, $1'\text{-H}$), 5.01 (1H, m, $2'\text{-H}$), 5.24 (2H, m, $3'\text{-H}$ and $4'\text{-H}$).

The ^{13}C -n.m.r. (62.9 MHz, ^2H]chloroform) characteristics were as follows: δ (p.p.m.) 20.5 ($\text{CH}_3\text{CO-}$), 20.6 ($\text{CH}_3\text{CO-}$), 20.7 ($\text{CH}_3\text{CO-}$), 52.9 ($\text{CH}_3\text{OCO-}$), 57.3 ($\text{CH}_3\text{O-}$), 69.5 (C-4'), 71.2 (C-2'), 72.1 (C-3'), 72.7 (C-5'), 101.7 (C-1'), 167.3 (-CO- of ester), between 169.3 and 170.2 (3 $\text{CH}_3\text{CO-}$).

The mass spectrum (g.l.c./m.s.) was identical with that of compound (V).

GlcA derivative (VII)

The ^1H -n.m.r. (250 MHz, ^2H]chloroform) characteristics were as follows: δ (p.p.m.) 2.115 (3H, s, $\text{CH}_3\text{CO-}$), 2.248 (3H, s, $\text{CH}_3\text{CO-}$), 3.394 (3H, s, $\text{CH}_3\text{O-}$), 4.99 (2H, m, $1'\text{-H}$ and $4'\text{-H}$), 5.16 (1H, dd, $J_{1',2'}$ 5 Hz, $J_{2',3'}$ 7 Hz, $2'\text{-H}$), 5.24 (2H, m, $3'\text{-H}$ and $5'\text{-H}$).

The mass-spectrum (g.l.c./m.s.) characteristics were as follows: m/z 243 ($M^+ - \text{CH}_3\text{O}$, 1%), 232 ($M^+ - \text{C}_2\text{H}_2\text{O}$ [8], 7%), 183 (243 - AcOH, 1%), 157 (cleavage of lactone, 1%), 141 (183 - $\text{C}_2\text{H}_2\text{O}$, 2%), 128 (cleavage of lactone, 11%), 115 (157 - $\text{C}_2\text{H}_2\text{O}$, 10%), 103 (diacyetyl [8], 1%), 43 (CH_3CO^+ [8], 100%).

RESULTS AND DISCUSSION

Diploptene (II) (40 $\mu\text{g/g}$ of freeze-dried cells), diplopterol (III) (5 $\mu\text{g/g}$ of freeze-dried cells) as well as the two diastereoisomers of bacteriohopanepolyol derivatives (IVa) (25 $\mu\text{g/g}$ in type strain and 50 $\mu\text{g/g}$ in blue mutant) and (IVb) (950 $\mu\text{g/g}$ in type strain and 820 $\mu\text{g/g}$ in blue mutant) were identified by g.l.c. using procedure 1 which allows the detection of nearly all known complex bacteriohopanepolyols, whatever the structure of their side-chain.

Hopanoid contents were nearly identical in both strains, in good agreement with those previously determined for other *Rh. rubrum* strains and similar to those found for most hopanoid producers [1]. Further, the hopanoid concentrations measured for the hexa-acetate methyl ester of glycoside (I) (1.6 mg/g in type strain and 1.8 mg/g in blue mutant) obtained by procedure 2 (direct isolation of an intact glycoside derivative) and those obtained by procedure 1 (side-chain cleavage) were of the same order of magnitude with regard to the differences of side-chain structures, indicating that most probably no other bacteriohopanepolyol than glycoside (I) was present in significant amounts.

Methyl ester or ethyl ester of the hexa-acetate of hopanoid (I) were readily formed by treatment with diazomethane or acidic ethanol, indicating the presence of a free carboxylic group. These esters, also isolated after chloroform/methanol or chloroform/ethanol extractions, were side-products resulting from reaction with the solvent, since they could not be detected in the tetrahydrofuran extracts. As these derivatives were much easier to purify than the hexa-acetate of (I) (especially from the blue mutant, as the absence of carotenoids facilitates the detection and purification of the lipids), they were utilized to characterize the new bacteriohopanetetrol glycoside (I).

The methyl signal region of the ^1H -n.m.r. spectra of hexa-acetylated methyl or ethyl ester of (I), as well as the high-field part of their ^{13}C -n.m.r. spectra, was characteristic of a hopane skeleton [9,10]. This structural feature was confirmed by the mass spectrum of hexa-acetylated ethyl ester of (I), showing the characteristic ring C cleavage of m/z 191 [7] and the loss of the side-chain at m/z 369.

The full structure of the side-chain could be determined by spectroscopic methods and chemical degradation. Selective decoupling experiments and $^1\text{H}/^1\text{H}$ COSY allowed assignment of all the signals and identification of the structure of the side-chain. With regard to the bacteriohopanetetrol moiety, chemical shifts of the C-35 protons ($\delta = 3.65$ p.p.m. and 3.95 p.p.m.) and C-35 carbon were similar to those observed for the corresponding atoms in another bacteriohopanetetrol glycoside [11] and clearly indicated that an oxygen atom, rather than a nitrogen atom, was linked to C-35 [4]. The results (^1H - and ^{13}C -n.m.r.) concerning the carbohydrate part were consistent with those of the synthetic D-GlcA derivative (V). The 3J $^1\text{H}/^1\text{H}$ coupling constants, of the order of magnitude of 10 Hz, corresponded to *trans* diaxial values, suggesting the chair conformation of a pyranose ring with equatorial substituents in all positions, except for the anomeric carbon atom. The lower value of the anomeric proton coupling constant ($J_{1,2}$, 3.5 Hz) is characteristic of an α -glycosidic bond. Heteronuclear $^1\text{H}/^{13}\text{C}$ correlation by long-range coupling allowed detection of the coupling between the C-1' carbon of the GlcA moiety and the two protons at C-35 of the bacteriohopanetetrol part, and determined unambiguously which hydroxyl group of the tetrol was involved in the glycosidic bond.

Electron-impact m.s. of hexa-acetylated ethyl ester supported the structure of a GlcA tetrol glycoside showing a molecular ion at m/z 1002, a ring C cleavage fragment possessing the side-chain at m/z 781 as well as the typical fragmentation around the glycosidic bond at m/z 671 and 655 already observed in other acetylated bacteriohopanetetrol glycosides [11].

Methanolysis of the glycosidic bond of (I) in methanol/hydrogen chloride followed by acetylation allowed separate identification of the triterpenic and the carbohydrate moieties and comparison of them directly with reference compounds. Comparison of the ^1H -n.m.r. spectrum of the tetra-acetoxyl-bacteriohopane obtained from *Rh. rubrum* with those of the eight synthetic side-chain diastereoisomers allowed determination of the side-chain configuration of (I) as 32*R*, 33*R* and 34*S* [12].

The three GlcA derivatives (V), (VI) and (VII), obtained by methanolysis of the hexa-acetate of (I), were identical (t.l.c., g.l.c. co-elution, g.l.c./m.s. and ^1H -n.m.r.) with the corresponding derivatives prepared from D-GlcA.

Hopanoid (I) isolated from *Rh. rubrum* is the first bacteriohopane glycoside possessing an α -glycosidic bond between a bacteriohopanetetrol and a carbohydrate. All complex tetrol glycosides isolated until now from either *Bacillus acidocaldarius* [13], *Methylobacterium organophilum* [11] or *Zymomonas mobilis* [14] are β -glycosides. Furthermore, complex hopanoids with a basic amino or guanidinium group, i.e. bearing a positive charge when protonated, have been found quite often until now. Glycoside (I) is on the other hand the only one bearing, with its carboxylic acid group, a potential negative charge. Many hopanoids differing by their side-chain structures have been found in the few eubacteria strains thoroughly investigated [2]. All of them are amphiphilic lipids possessing all the features necessary to act as membrane stabilizers [15]. It has, however, still to be determined whether this structural diversity hides other physiological significances.

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