Lipids (2011) 46:249–261
DOI 10.1007/s11745-010-3482-4

ORIGINAL ARTICLE

N-Acylated Bacteriohopanehexol-Mannosamides from the Thermophilic Bacterium *Alicyclobacillus acidoterrestris*

Tomáš Řezanka · Lucie Siristova · Karel Melzoch · Karel Sigler

Received: 20 July 2010 / Accepted: 23 September 2010 / Published online: 21 October 2010

Abstract Identification of molecular species of various *N*-acylated bacteriohopanehexol-mannosamides from the thermophilic bacterium *Alicyclobacillus acidoterrestris* by semipreparative HPLC and by RP-HPLC with ESI is described. We used triple-quadrupole type mass spectrometer, ^{1}H and ^{13}C NMR for analyzing this complex lipid. CD spectra of two compounds (model compound—7-deoxy-D-*glycero*-D-*allo*-heptitol obtained by stereospecific synthesis, and an isolated derivative of hopane) were also measured and the absolute configuration of both compounds was determined. On the basis of all the above methods, we identified the full structure of a new class of bacteriohopanes, represented by various *N*-acylated bacteriohopanehexol-mannosamides.

Keywords *Alicyclobacillus acidoterrestris* · Negative RP-HPLC–ESI–MS/MS · Circular dichroism · *N*-Acylated bacteriohopanehexol-mannosamides · ω-Cyclohexyl fatty acids

Abbreviations

BH	Bacteriohopane
BHpolyols	Bacteriohopanepolyols
CD	Circular dichroism
CID	Collision-induced dissociation
COSY	Correlation spectroscopy
DMAP	Dimethylaminopyridine
FAME	Fatty acid methyl ester(s)
GC–MS	Gas chromatography–mass spectrometry
HMBC	Heteronuclear multiple bond coherence
HMQC	Heteronuclear multiple quantum coherence
HR-FAB-MS	High resolution fast atom bombardment mass spectrometry
LC–MS	Liquid chromatography–mass spectrometry
LC–MS/ESI SIM	Selected ion monitoring
NOE	Nuclear Overhauser effect
ROESY	Rotating-frame Overhauser effect spectroscopy
RP-HPLC/MS-ESI	Reversed phase liquid chromatography–electrospray ionization mass spectrometry
RP-HPLC–ESI–MS/MS	Reversed phase liquid chromatography–electrospray ionization tandem mass spectrometry
SPE	Solid phase extraction
TFFH	Fluoro-*N,N,N′,N′*-tetramethylformamidinium hexafluorophosphate
TLC	Thin layer chromatography
VVM	Air volume per volume of culture medium per minute

T. Řezanka (✉) · K. Sigler
Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague, Czech Republic
e-mail: rezanka@biomed.cas.cz

L. Siristova · K. Melzoch
Department of Fermentation Chemistry and Bioengineering, Institute of Chemical Technology Prague, Technická 5, 166 28 Prague, Czech Republic

Introduction

Bacteriohopanepolyols (BHpolyols) are pentacyclic triterpenoids, which are present as membrane constituents in

many bacteria [1]. They perform a regulating and rigidifying function in membranes analogous to that of some sterols in eukaryotes [2–4]. BHpolyol side chains form many structures, which differ in terms of the number, position and nature of the functional groups [2, 5]. The side chain is responsible for the charge and the function of the molecule [6].

The most commonly occurring BHpolyols in bacteria contain four functional groups in the side chain, typically three hydroxyl groups at 32*R*, 33*R*, 34*S* [7, 8] with the C-35 position occupied by either another OH or an amino group [2].

In numerous strains belonging to various taxonomic groups (e.g. purple nonsulfur bacteria, methylotrophs, or cyanobacteria) the bacteriohopane (BH) derivatives are not present as free polyols but are linked to various polar moieties like glucosamine or *N*-acylglucosamine, anhydrogalacturonopyranosides, α-amino acids such as tryptophan and ornithine, adenosine, cyclitol ethers or other polar entities the structures of which are still unidentified [2, 9]. BHpolyols have been found in many other structural variations including double bonds in the ring system or substitution by methyl groups in ring A. Furthermore, BHpolyols with five and six functional groups in the side chain, i.e. BHpolyols with additional one or two OH group(s) at C-30 and/or C-31 position(s) were identified [10–14, 40]. These structures are restricted to a limited number of organisms or occur in an explicit group of organisms and can serve as specific bacterial marker information [2, 5].

To date there have been only a few hexafunctionalized side chain structures reported from nature. The basic structure has five hydroxyl groups and NH_2 functionality at C-35, and has only been observed in Type I methanotrophic bacteria [14, 15]. Further, BHhexol cyclitol ether was identified in sediments of two lakes from Antarctica [16], soils from Northern England [17], and BHhexol in sediments from the popular Loch Ness (UK) [2].

This report is part of our investigation of thermophilic bacteria, mainly of the genus *Alicyclobacillus*. We extended our analysis from glycophospholipids [18], unusual acylphosphatidylglycerols [19], and *O*-acyl glycosylated cardiolipins [20] to hopanoids. The structure of *N*-acylated BHhexol-mannosamides was confirmed not only by ESI–MS, but, after isolation, also by measurement of ^{1}H-, ^{13}C-NMR, and CD spectra, including synthesis of a model compound.

Experimental

Instrumentation

The liquid chromatograph was the semipreparative Gradient LC System G-1 (Shimadzu, Kyoto, Japan) with two LC-6A pumps (0.5 ml/min), an SCL-6A system controller, an SPD ultraviolet detector an SIL-1A sample injector and a C-R3A data processor, with a preparative normal phase HPLC column Supelcosil LC-Si HPLC column 5 µm particle size, length × I.D. 25 cm × 21.2 mm with mobile phase containing hexane–isopropanol-0.05% triethylamine in water (99.5:0:0.5, v/v/v) to hexane–isopropanol-0.05% triethylamine in water (20:78:2 v/v/v) for 40 min, a flow rate of 9.9 ml/min, and monitored by a variable wavelength detector at 210 nm (HP 1040M Diode Array Detector).

The HPLC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series, Hewlett Packard, USA) and two Ascentis® Express HILIC HPLC column 2.7 µm particle size, L × I.D. 15 cm × 2.1 mm (Supelco, Prague) in series were used. This setup provided us with a high-efficiency column—approximately 30,000 plates/30 cm. LC was performed at a flow rate of 300 µl/min with a linear gradient from the mobile phase containing methanol/acetonitrile/aqueous 1 mM ammonium acetate (60:20:20, v/v/v) to methanol/acetonitrile/aqueous 1 mM ammonium acetate (20:60:20, v/v/v) for 40 min. The whole HPLC flow (0.37 ml/min) was introduced into the ESI source without any splitting.

The detector was an Applied Biosystems Sciex API 4000 mass spectrometer (Applied Biosystems Sciex, Ontario, Canada) using electrospray mass spectra. The ionization mode was negative, the nebulizing gas (N_2) pressure was 345 kPa and the drying gas (N_2) flow and temperature were 9 l/min and 300 °C, respectively. The electrospray needle was at ground potential, whereas the capillary tension was held at 4,000 V. The cone voltage was kept at 250 V. The mass resolution was 0.1 Da and the peak width was set to 6 s. For an analysis, total ion currents (full scan) were acquired from 200 to 1,600 Da.

CID ions mass spectra were acquired by colliding the Q1 selected precursor ions with Ar gas at a collision target gas and applying collision energy of 50 eV in Q2. Scanning range of Q3 was *m/z* 200–1,600 with a step size of *m/z* 0.3 and a dwell time of 1 ms. A peak threshold of 0.3% intensity was applied to the mass spectra. The instrument was interfaced to a computer running Applied Biosystems Analyst version 1.4.1 software.

Gas chromatography–mass spectrometry of FAME was done on a GC–MS system consisting of Varian 450-GC, Varian 240-MS ion trap detector with electron impact ionization, and CombiPal autosampler (CTC, USA). The sample was injected onto a 25 m × 0.25 mm × 0.1 µm Ultra-1 capillary column (Supelco, Czech Republic) under a temperature program: 5 min at 50 °C, increasing at 10 °C/min to 320 °C and 15 min at 320 °C. Helium was the carrier gas at a flow of 0.52 ml/min. All spectra were scanned within the range *m/z* 50–600. The spectra was identified manually, se also Siristova et al. [18].

The oven temperature for identification of the acetylated alcohols BH (degradation products) was programmed from 50 to 275 °C at 10 °C/min and further at 5 °C/min to 350 °C and further with 8 min isothermal elution. The others conditions of GC–MS apparatus were identical.

Saccharides were separated on a single HILIC HPLC column (see above) by isocratic elution with acetonitrile and 0.1% acetic acid in water at a ratio of 40:60. They were identified by comparing their retention times with those of commercially obtained standards (galactosamine, glucosamine and mannosamine) and single ion monitoring at m/z 180 ($[M + H]^+$).

NMR spectra were recorded on a Bruker AMX 500 spectrometer (Bruker Analytik, Karlsruhe, Germany) at 500.1 MHz (^1H) and 125.7 MHz (^{13}C). Optical rotations were measured with a Perkin-Elmer 243 B polarimeter. The circular dichroism measurement was carried out under dry N_2 on a Jasco-500A spectropolarimeter at 24 °C. HR-FAB-MS (positive ion mode) were obtained with a PEG-400 matrix using a VG 7070E-HF spectrometer. All compounds were purchased from Sigma–Aldrich (Prague, Czech Republic).

Cultivation, Isolation and Identification

Alicyclobacillus acidoterrestris CCM 4660 (Czech Collection of Microorganisms, Brno, Czech Republic) was cultivated in an alicyclobacillus medium containing (g/l): yeast extract 6.0, glucose 5.0, $CaCl_2 \cdot 2H_2O$ 0.25, $MgSO_4 \cdot 7H_2O$ 0.5, $(NH_4)_2SO_4$ 0.2, KH_2PO_4 3.0 and 1 ml/l of trace element solution ($ZnSO_4 \cdot 7H_2O$ 0.1 g/l, $MnCl_2 \cdot 4H_2O$ 0.03 g/l, H_3BO_3 0.3 g/l, $CuCl_2 \cdot 6H_2O$ 0.2 g/l, $CaCl_2 \cdot 2H_2O$ 0.01 g/l, $NiCl_2 \cdot 6H_2O$ 0.02 g/l, $Na_2MoO_4 \cdot 2H_2O$ 0.03 g/l), pH was adjusted to 4. A volume of 600 ml of inoculum was prepared in 500-ml round-bottom flasks on a temperature controlled shaker at 45 °C and 200 rpm. The biomass was cultivated in a 5-l mechanically stirred fermentor with the aim of obtaining inoculum for a 50-l fermentor. Inoculum was cultivated for 12 h under aerobic conditions with aeration rate 0.8 VVM. The stirrer frequency was 300–400 rpm and the cultivation temperature was 45 °C.

The strain main cultivation was carried out in a 50-l mechanically stirred fermentor for 15 h, under aerobic conditions with an aeration rate of 1 VVM. The stirrer frequency was 350–400 rpm and the cultivation temperature was 45 °C. Cells were harvested in the log phase when the optical density of the culture was maximal. The dry biomass was 1.49 g/l media.

The extraction procedure was based on the method of Bligh and Dyer [21]. The alcohol-water mixture was cooled and one part chloroform was added and the lipids were extracted for 30 min. Insoluble material was sedimented by centrifugation and the supernatant was separated into two phases. The aqueous phase was aspirated off and the chloroform phase was washed three times with two parts 1 M KCl each. The resulting chloroform phase was evaporated to dryness under reduced pressure.

First, total lipid extracts were applied to Sep-Pak Cartridge Vac 35 cc (Waters; with 10 g of aminopropyl-silica-based polar bonded phase), and from the cartridge were subsequently eluted chloroform–methanol mixture (98:2) [22] for removing of non-polar lipids, and polar lipids, but not phospholipids were further eluted by an acetone. The eluate was reduced in volume and subjected to normal phase HPLC.

The aliquot of total BHs (5 mg) was stirred for l h at room temperature with periodic acid (H_5IO_6; 3 mg) in tetrahydrofuran/water (3 ml; 8:1 v/v). The vicinal diols were oxidized to yield aldehyde products. After addition of water (10 ml) the mixture was extracted with petroleum ether (3 × 5 ml). The combined extracts were evaporated and further stirred for l h at room temperature with sodium borohydride ($NaBH_4$; 1 mg) in ethanol (3 ml) to produce alcohols. The excess of $NaBH_4$ was destroyed by water and the mixture was extracted with petroleum ether (3 × 5 ml). The extracts were evaporated and acetylated, see below.

The total extract was acetylated overnight at room temperature with acetic anhydride/pyridine (30 ml; 1:1 v/v). The peracetylated BHs were evaporated and further analyzed.

The fraction of *N*-acylated BHhexol-glycoside after semipreparative HPLC was treated for 16 h at 100 °C with a 10% solution of dry HCl in CH_3OH. The reaction mixture was taken to dryness, suspended in water, the pH was regulated by 5% NaOH to 9, BHhexol was extracted by diethyl ether, the water mixture was acidified by 5% HCl to pH 4, free fatty acids were extracted by $CHCl_3$ and water phase was after lyophilization was silylated, see above. The fatty acid methyl esters were prepared by reaction of the free fatty acids with methanol, see Rezanka et al. [23].

Preparation of 7-Deoxyheptitol

D-*glycero*-D-*allo*-Heptose (**8a**) and D-*glycero*-D-*altro*-heptose (**8b**) were prepared from KCN and D-allose (**6**) according to procedures described previously [24, 25]. Briefly, the pH of an aqueous solution (15 ml) of potassium cyanide (269 mg, 4.14 mmol) was lowered to 7.2 by dropwise addition of aqueous acetic acid (3 M) with rapid stirring at room temperature. An aqueous solution (10 ml) of D-allose **6** (248 mg, 1.38 mmol) was slowly added, with addition of acetic acid (3 M) or sodium hydroxide (1 M) as appropriate to maintain a pH of 7.2–7.5 during addition of D-allose and subsequent reaction, which was allowed to proceed for 1.5 h. The reaction mixture was analyzed by TLC (cellulose sheets, 90:10 acetone–water) to reveal a

complete conversion of D-allose (**6**) (R_f 0.67) into the aldonitriles (**7a** and **7b**) (R_f 0.8–0.9). The pH of the aldonitrile solution was lowered to 4.7 by dropwise addition of acetic acid (3 M) and the solution was stored at 4 °C for ca. 1 h. Further, the pH of the solution was again lowered to 4.2 by dropwise addition of acetic acid (3 M). Following addition of prereduced 5% palladium on barium sulfate (86 mg = 4.3 mg, 0.04 mmol Pd), the flask was evacuated three times and flushed with hydrogen. Vigorous stirring under an atmosphere of hydrogen was then allowed to proceed at room temperature for ca. 15 h. The reaction mixture was analyzed by TLC (90:10 acetone–water) to reveal a complete reduction of the aldonitriles (**7a** and **7b**) (R_f 0.8–0.9) into the imines (R_f 0.4–0.6). The catalyst was removed over Celite and the pH of the resultant filtrate was lowered from pH 4.4 to ca. pH 3.0 by repeated batch-wise treatment with Dowex® 50WX2 hydrogen form 200–400 mesh (4 × 4 g). The final filtrate was analyzed by TLC (95:5 acetone–water) and no imine was detected. The water was removed by lyophilization to leave the epimeric mixture of **8a** and **8b** as a pale yellow oil; the yield of epimeric sugars was 243 mg (84%) and they were separated by semi-preparative TLC.

Aldrich Analtech TLC Uniplates™ with spherical particles (size 50 μm) of α-cellulose matrix, size 20 cm × 20 cm, layer thickness 250 μm, fluorescent indicator were used. The mobile phase was acetone–water (95:5, v/v), D-glucose being used as standard. Identification under UV light gave the following R_f values for the compounds: D-*glycero*-D-*allo*-heptose (**8a**) (0.35), D-glucose (0.51) and D-*glycero*-D-*altro*-heptose (**8b**) (0.63). The yield of D-*glycero*-D-*allo*-heptose (**8a**) was 106 mg, with $[\alpha]_D^{23}$ +12.8 (1 day, c = 0.15), lit. data: $[\alpha]_D^{21}$ +14.1 [26], $[a]_D$ +10.7 (24 h) (c 1.6, H_2O) [27], $[a]_D$ +7.2 (3 min) → +12.7 (24 h) (c 4.6, H_2O) [28]; ^{13}C NMR (125 MHz, D_2O) δ major: 93.8 (C-1), 73.8, 72.1, 71.3, 71.2, 67.7, 61.8; minor: 92.7 (C-1), 72.0, 71.7, 67.6, 67.1, 66.9, 62.1 [27, 28]; ^{13}C NMR δ 94.4, 72.9, 72.0, 71.9, 74.6, 68.5, 62.6 (the values for C-1 → C-7 of β-pyranose), the values for C-1 were 93.4 (α-pyranose), 94.4 (β-pyranose), 96.8 (α-furanose), and 101.4 (β-furanose) [29]. Our data measured for mixture of four forms at C-1 were 93.2 (α-pyranose), 94.5 (β-pyranose), 97.0 (α-furanose), and 101.5 (β-furanose) and the following values are for the major form: ^{13}C NMR (125 MHz, D_2O) δ 94.5 (C-1), 73.3 (C-2), 72.5 (C-3), 71.6 (C-4), 73.8 (C-5), 68.4 (C-6), and 62.2 (C-7); HR-FAB-MS (m/z): 211.0823 $[M + H]^+$, calc. for $[C_7H_{14}O_7 + H]^+$ 211.0818.

D-*glycero*-D-*allo*-Heptose diethyl thioacetal = (2*S*,3*R*,4*S*,5*S*)-7,7-bis(ethylthio)heptane-1,2,3,4,5,6-hexaol. A mixture of D-*glycero*-D-*allo*-heptose (**8a**) (105 mg) and concentrated HCl (5 ml) was shaken for about 10 min to dissolve most of the sugar and then, as the solution began to assume a reddish color, 5 ml of ethylthiol was added and shaking was continued for 2.5 h. The product was allowed to stand overnight in the refrigerator. About 5 ml of cold water was added, the dithioacetal filtered, and washed with cold ethanol. The yield, after one recrystallization from ethanol, was 100 mg (63.3%). Very surprisingly, this compound (**9**) has not yet been described. $[a]_D$ +10.7 (c 0.16, MeOH); ^{1}H-NMR (500 MHz, CD_3OD) δ 4.92 (1H, *d*, J = 1.6, H-7), 4.93 (1H, *dd*, J = 1.6, 5.5, H-6), 5.06 (1H, t, J = 5.5, H-5), 5.19 (1H, t, J = 5.5, H-4), 4.71 (1H, t, J = 5.5, H-3), 4.65 (1H, *ddd*, J = 5.5, 3.2, 8.2, H-2), 4.52 (1H, *dd*, H = 11.0, 3.2, H-1a), 4.42 (1H, *dd*, J = 11.0, 8.2, H-1b), 2.89 (2H, m, $SC\underline{H}_2$), 2.89 (2H, m, $SC\underline{H}_2$), 1.22 (6H, m, $CH_2C\underline{H}_3$); ^{13}C NMR (125 MHz, CD_3OD) 56.1 (C-7), 74.2 (C-6), 73.3 (C-5), 71.2 (C-4), 74.6 (C-3), 72.8 (C-2), 64.4 (C-1), 64.8 ($S\underline{C}H_2$), 15.0 ($CH_2\underline{C}H_3$); HR-FAB-MS (m/z): 317.1099 $[M + H]^+$, calc. for $[C_{11}H_{24}O_6S_2 + H]^+$ 317.1092.

7-Deoxy-D-*glycero*-D-*allo*-Heptitol, i.e. (2*R*,3*R*,4*S*,5*S*,6*S*)-heptane-1,2,3,4,5,6-hexaol (**10**). Reductive desulfurization of similar dithioacetals has already been described [30]. To a solution of the derivative **9** (95 mg, 0.30 mmol) in dioxane (5 ml) was added Raney Ni (300 mg). The suspension was heated at 80 °C for 1 h with stirring to give the derivative **9**. Crystallization from absolute ethanol yielded 43.5 mg (74%) of the desired desoxyheptitol as clusters of needles, with an m.p. at 124–125 °C and $[\alpha]_D$ +1.7 (c 0.16, MeOH); ^{1}H-NMR (500 MHz, CD_3OD) δ 3.65 (1 H, *dd*, J = 10.1, 11.5, 1a-H) and 3.84 (1 H, *dd*, J = 3.4, 11.5, 1b-H), 3.77 (1 H, *ddd*, J = 2.2, 3.4, 10.1, 2-H), 3.70 (1 H, *dd*, J = 3.9, 2.2, 3-H), 3.92 (1H, *dd*, J = 3.9, 3.4, 4-H), 3.62 (1 H, *dd*, J = 3.4, 5.2, 5-H), 3.94 (1 H, *dq*, J = 5.2, 6.5, 6-H), 1.21 (3 H, *d*, J = 6.5, 7-H); ^{13}C NMR (125 MHz, CD_3OD) 19.1 (C-7), 70.4 (C-6), 73.2 (C-5), 71.7 (C-4), 74.2 (C-3), 72.7 (C-2), 64.1 (C-1); HR-FAB-MS (m/z): 197.1025 $[M + H]^+$, calc. for $[C_7H_{16}O_6 + H]^+$ 197.1025. Again, though it is hard to believe, this compound has not yet been described.

General Procedure for Bichromophoric Derivatization

Anthroylation

To an ice cooled suspension of the 9-anthracenecarboxylic acid (0.1 mmol, 22.2 mg) and fluoro-*N,N,N′,N′*-tetramethylformamidinium hexafluorophosphate (TFFH, 0.1 mmol, 26.4 mg) in a minimum of CH_2Cl_2 (ca. 5 ml) was added Et_3N (0.5 mmol, 50.5 mg, 70 μl) resulting in a clear solution with some heat evolution. After stirring for 30 min at 20 °C the alcohol (**10**) (0.1 mmol, 19.6 mg) and a catalytic amount of DMAP (0.01 mmol, 1.22 mg) were added and the reaction mixture was stirred at 20 °C overnight. Water (5 ml) was added and the mixture extracted with CH_2Cl_2 (3 × 3 ml). The combined organic extracts were dried ($MgSO_4$),

filtered and concentrated in vacuo. The resulting crude residue was purified by SPE with silica gel (MeOH/CH_2Cl_2, 1:9) to yield the desired ester (**11**) [yield 33.6 mg (84%)]. HR-FAB-MS (*m/z*): 401.1606 $[M + H]^+$, calc. for $[C_{22}H_{24}O_7 + H]^+$ 401.1600. The eluate was concentrated, the mixture was separated by normal phase HPLC (Supelcosil™ LC-Si HPLC Column 5 µm particle size, L × I.D. 25 cm × 10 mm, EtOAc-hexane, 3:7; 2 ml/min; 311 nm UV detection), and used for the next step, cinnamoylation.

The preparation of the anthroyl derivative of BHhexol (**13**) was performed similarly as with (**11**). From the 9-anthracenecarboxylic acid (1 µmol, 222 µg), TFFH (1 µmol, 264 µg) in CH_2Cl_2 (200 µl), Et_3N (5 µmol, 505 µg, 0.7 µl) and the alcohol (**3**) (1 µmol, 578 µg) with catalytic amount of DMAP (1 µmol, 122 µg) compound **13** was obtained after purification by SPE with silica gel (MeOH-CH_2Cl_2, 2:98) in a yield of 610 µg (78%). HR-FAB-MS (*m/z*): 783.5199 $[M + H]^+$, calc. for $[C_{50}H_{70}O_7 + H]^+$ 783.5204.

Cinnamoylation

To an ice cooled suspension of the p-methoxycinnamic acid (712 mg, 4 mmol) and fluoro-*N,N,N′,N′*-tetramethylformamidinium hexafluorophosphate (TFFH, 5 mmol, 1.32 g) in a CH_2Cl_2 (35 ml) was added Et_3N (25 mmol, 2.52 g, 3.48 ml) resulting in a clear solution with some heat evolution. After stirring for 30 min at 20 °C the anthroyl derivative (**11**) (30 mg, 75 µmol) and a catalytic amount of DMAP (0.1 mmol, 12.2 mg) were added and the reaction mixture was stirred at 20 °C overnight. H_2O (10 ml) was added and the mixture extracted with CH_2Cl_2 (3 × 5 ml). The combined organic extracts were dried ($MgSO_4$), filtered and concentrated in vacuo. The resulting crude residue was purified by SPE with silica gel (MeOH/CH_2Cl_2, 1:9) to yield (66.6 mg, i.e. 74%) the desired ester (**12**). HR-FAB-MS (*m/z*): 1,201.4221 $[M + H]^+$, calc. for $[C_{72}H_{64}O_{17} + H]^+$ 1,201.4226.

The preparation of cinnamoyl derivative of BHhexol (**14**) was performed similarly as with the derivative (**12**). From the p-methoxycinnamic acid (712 µg, 4 µmol), TFFH (5 µmol, 1.32 mg) in CH_2Cl_2 (0.1 ml), Et_3N (25 µmol, 2.52 mg, 3.5 µl) and the alcohol (**13**) (0.77 µmol, 603 µg) with catalytic amount of DMAP (0.1 µmol, 12 µg), 915 µg (75%) of compound (**14**) was obtained after purification by SPE with silica gel (MeOH-CH_2Cl_2, 2:98). HR-FAB-MS (*m/z*): 1,583.7816 $[M + H]^+$, calc. for $[C_{100}H_{110}O_{17} + H]^+$ 1,583.7820.

Results

A. acidoterrestris was cultivated in a 50-l fermentor as described previously [20]. The total lipids were obtained by Bligh-Dyer extraction [21] of lyophilized cells and a part of the total lipids was cleaved by H_5IO_6 followed by $NaBH_4$ reduction. This releases mainly a mixture of alcohols arising from the BHpolyol derivatives, identical in GC retention times and mass spectra with the previously described alcohols which were used for the identification of BHpolyols from sediments [31]. Three alcohols were identified by GC–MS, i.e. bishomohopanol representing BHtetrols, homohopanol belonging to BHpentols, and the BHhexol hopanol. The detection of hopanol suggests the presence of a hopanoid with a hexafunctionalized side-chain in the total lipid extract.

Further, part of the total lipids was fractionated by means of cartridges with aminopropyl silica-based polar bonded phase, which were rinsed in a chloroform–methanol mixture (98:2) [22] for removing non-polar lipids. Polar lipids, but not phospholipids, were further eluted by acetone. Polar non-phospholipids (25% of total lipids, i.e. 385 mg) were further separated by normal phase HPLC, as described by Moreau et al. [22]. Briefly, non-phospholipids were subjected to semi-preparative normal phase HPLC with a linear gradient in for 40 min and with detection at 210 nm, as described by Fox et al. [1]. Table 1 gives the data obtained after normal phase semipreparative HPLC. The relative amounts of the C32, C31 and C30 alcohols, i.e. the relative contributions of tetra-, penta-, and hexafunctionalized side-chains were in a 2:1:1 ratio, which is in agreement with data from Table 1. This table also summarizes appropriate classes of hopanoids, i.e. tetrols, pentols, and hexols, whereas minority derivatives of BHs (up to 2% of total lipids) were omitted.

Table 2 gives the data obtained after RP-HPLC from the fraction of per-acetylated *N*-acylated BHhexol-glycosides,

Table 1 BH derivatives from *A. acidoterrestris* after normal phase semipreparative HPLC

Compound	%[a]
N-acyls glucosamine BHtetrol	29.3
N-acyls-mannosamine-BHpentol	5.9
N-acyls-mannosamine-BHhexol	11.2
BHtetrol	12.5
BHpentol	8.6
BHhexol	6.7
BHtetrol-glucosamine	4.5
BHpentol-mannosamine	9.4
BHhexol-mannosamine	9.8
BHtetrol-ether	2.1
∑ tetrols	48.4
∑ pentols	23.9
∑ hexols	27.7

[a] Minority derivatives of BHs (up to 2% of total lipids) were omitted

Table 2 Per-acetylated *N*-acylated BHhexol-glycosides separated by RP-HPLC and FAs content by GC–MS

Compound	%[a]	%[b,c]
N-C15-mannosamine-BHhexol	2.7	2.94
N-C17-mannosamine-BHhexol	30.8	31.13
N-C19-mannosamine-BHhexol	54.7	54.41
N-C21-mannosamine-BHhexol	5.8	6.11
N-C23-mannosamine-BHhexol	3.2	2.98
N-C25-mannosamine-BHhexol	1.7	1.52
N-C27-mannosamine-BHhexol	0.9	0.76
N-C29-mannosamine-BHhexol	0.2	0.15

[a] Determined by ESI

[b] Minority fatty acids up to 0.1% of total fatty acids were omitted

[c] Determined by GC–MS

which had the broadest interval of molecular weights. A total of 8 peaks were separated by LC–MS/ESI on narrow-bore columns connected in series. The chromatogram is given in Fig. 1.

Diagnostic fragment ions for hexafunctionalized bacteriohopane structures include the ion at *m/z* 771 resulting from the loss of the terminal group at C35 (either OH or any other moiety linked to C35 via oxygen [32]) and *m/z* 711, 651, 591, 531 and 471 from sequential loss of the remaining 5 acetylated OH groups from C30 to C34. The ESI/MS spectrum (Fig. 2) of a proposed acetylated *N*-acyls glycoside of BHhexol (**2**, $n = 3$) includes all these diagnostic ions as well as a further series *m/z* 579, 519, 459, 399, 339 and 279 from neutral loss of the A and B rings (i.e. loss of 192 Da) after a loss of one to six of the acetylated hydroxyls [32]. The composite hexafunctionalized structure **2** is proposed on the basis of observation of the diagnostic ions *m/z* 771 (etc.) and ions at *m/z* 554 (etc.), indicating the mass of the terminal group, and by comparison with spectra reported for the penta- and hexafunctionalized cyclitol ethers, see also Table 3.

The structure of pentacyclic nucleus including the side chain was essentially determined by ^{1}H-NMR spectroscopy using COSY, ROESY, HMQC, and HMBC experiments. The ^{1}H-NMR spectrum (data see Table 4) of acetylated hopanoids (**2**) showed signals in the methyl region typical of bacteriohopane derivatives. Both signals, including the ^{13}C NMR, are in agreement with previously published papers [7, 14, 33].

The ROESY spectrum displayed a number of correlation peaks between methyl groups and ring protons in the 1,3 diaxial relationship. Thus, 24-Me, H-6ax, 25-Me, 26-Me, and 13-H were shown to be all located on the upper face of

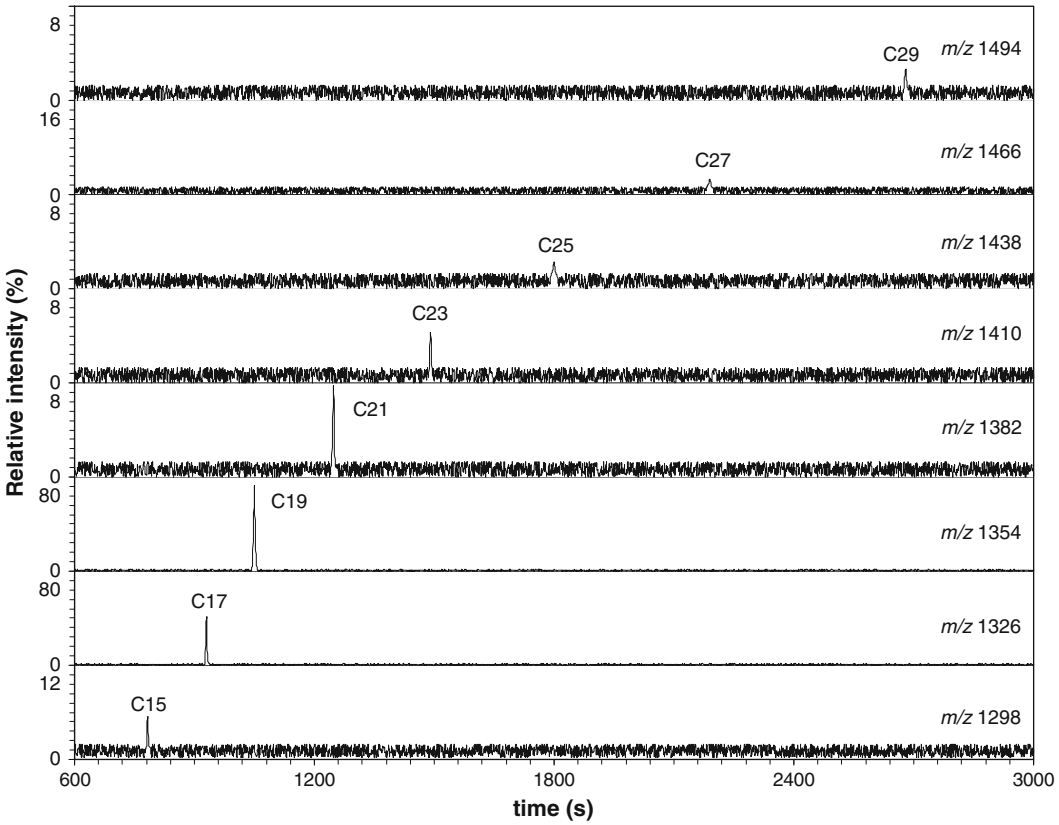

Fig. 1 LC–MS/ESI SIM chromatograms of *N*-acylated BHhexol glycosides from total lipids of *A. acidoterrestris* after normal phase HPLC; fraction of *N*-acyls-mannosamine-BHhexol is shown. The SIM traces depict $[M + H]^{+}$ masses, see *m/z* values

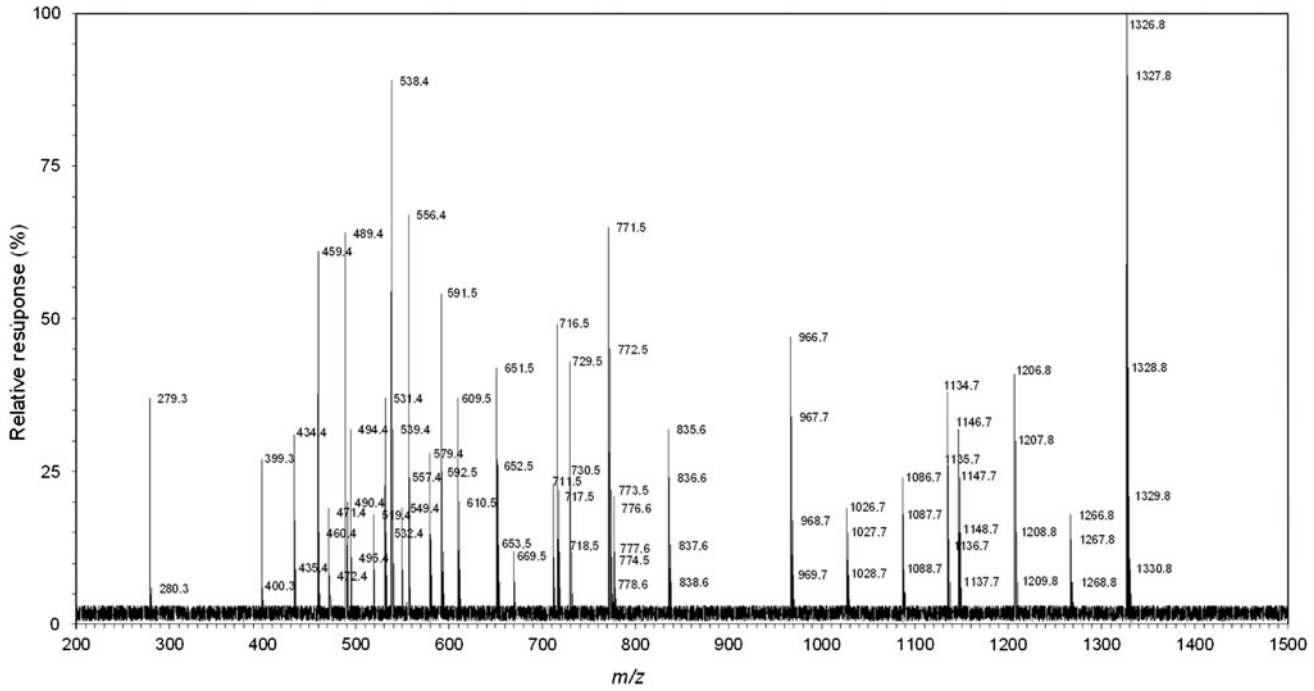

Fig. 2 The tandem quadrupole ESI product-ion spectrum of the $[M + H]^+$ ion of *N*-C17-mannosamine-BHhexol at *m/z* 1,326

the molecule, whereas 27-Me, 28-Me, H-5ax, and H-9ax were all on its lower face. Also the four-bond couplings (W coupling) of an angular methyl group with a ring proton in the COSY were observed. These couplings are only possible when the dihedral angle between the methyl group and the ring proton is near 180° and these couplings confirmed a trans connection of all the rings, hence the stereochemistry of compound **2** was the same as in previously isolated bacteriohopanoids. This was confirmed by HMBC correlations between Me groups and carbons of the ring system. Thanks to this fact the ^{13}C-resonance (see δ_C values in Table 5) of the five cycle moiety could be determined [7, 14, 33].

The ^{1}H-NMR spectrum of the octaacetate of compound (**2**) shows that the signals of the protons at C30 to C34 are nearly identical to those of the corresponding protons of the synthetic tetraacetate glycoside [33] and/or isolated pentaacetate of aminoBHtetrol [14] and are easily identifiable. Decoupling experiments performed on the C34 proton permitted an assignment of the signals of the two C35 protons which are nearly equivalent and appear as two doublets at 3.68 and 3.98 ppm. The fact that the signals of the C35 protons as well as the signal of the C35 carbon atom were shifted in comparison to the signals of the corresponding atoms of acetylated BHpolyol [9] indicated that a sugar moiety was located on the oxygen atom at C35. From the ^{1}H-NMR spectrum eight acetoxy groups are present: five at C30 to C34 and three on the residue linked at C35. Decoupling experiments initiated on the exchangeable NH proton of the acylamide group as well as dimensional proton/proton correlation allowed the whole pattern of the substituent to be established as compatible with the structure of an acylated sugar.

The large vicinal coupling constants, $^3J_{3',4'} = 9.5$ Hz and $^3J_{4',5'} = 9.5$ Hz, and the NOE between H-3′ and H-5′ indicated that H-3′, H-4′ and H-5′ protons were located at an axial position. The observation of NOE between H-4′ and 2′-NH proton suggested that 2′-NH was oriented in an axial position. These data indicate that the monosaccharide is a mannosamine in pyranose form; α configuration was determined by a large coupling constant, $^1J_{C\text{-}1',H\text{-}1'} = 166.1$ Hz [34] and small $^3J_{H\text{-}1'\text{-}H\text{-}2'} = 1.8$ Hz [35]. This implies also that the linkage between the BHhexol and the mannosamine moiety is an *O*-glycosidic bond.

HMBC analysis indicated that 2-NH′ of the mannosamine was esterified by a fatty acyl group by the correlation peak between H-2′ (δ 4.63) and C-1′′ (δ 172.8) of the fatty group and that 3-OH′, 4-OH′, and 6-OH′ of the mannosamine moiety were esterified by acetates by the correlation peaks between H-3′ (δ 5.38), H-4′ (δ 5.06), and H-6′ (δ 4.30, 4.14) and the other three carbonyl carbons (δ 170.7, 170.6, 170.2). The key HMBC are showed in Fig. 3.

The components of *N*-acylated BHhexol-mannosamide were also analyzed after hydrolysis (Fig. 4). The hydrolyzate contained BHhexol as the aglycone moiety and mannosamine was the only sugar which was detected by GC–MS in this fraction. Mannosamine had $[\alpha]_D^{25}$-4.1, compared to the literature data, $[\alpha]_D^{25}$ -4.2 for D-mannosamine

Table 3 The main molecular species of *N*-acylated BHhexol glycoside (**2**) from *A. acidoterrestris*

Ions	*m/z*	Abundance
M + H	1,326.8	100
M + H-AcOH	1,266.8	18
M + H-2AcOH	1,206.8	41
M + H-3AcOH	1,146.7	32
M + H-192	1,134.7	38
M + H-4AcOH	1,086.7	24
M + H-5AcOH	1,026.7	19
M + H-6AcOH	966.7	47
M + H-4AcOH-Acyl	836.6	32
M + H-5AcOH-Acyl	776.6	21
M + H-SC	771.5	65
M + H-SC-42	729.9	43
M + H-6AcOH-Acyl	716.5	49
M + H-SC-AcOH	711.5	23
M + H-SC-AcOH-42	669.5	12
M + H-SC-2AcOH	651.5	42
M + H-SC-2AcOH-42	609.5	37
M + H-SC-3AcOH	591.5	54
M + H-SC-192	579.4	28
SC + 18	556.4	67
SC	538.4	89
M + H-SC-3AcOH-42	549.4	19
M + H-SC-4AcOH	531.4	37
M + H-SC-AcOH-192	519.4	18
SC-AcOH	494.4	32
M + H-SC-4AcOH-42	489.4	64
M + H-SC-5AcOH	471.4	19
M + H-SC-2AcOH-192	459.4	61
SC-2AcOH	434.4	31
M + H-SC-3AcOH-192	399.3	27
SC-3AcOH	374.3	42
M + H-SC-4AcOH-192	339.3	27
M + H-SC-5AcOH-192	279.3	37

(2-amino-2-deoxy-D-mannopyranose) [36]. The mixture of homologous ω-cyclohexyl fatty acids, isolated from the hydrolyzate, was also determined by GC–MS. The fatty acid composition obtained by GC–MS was nearly identical with the results obtained from ESI analysis (see Table 2).

Bisseret and Rohmer [37], who synthesized the eight possible diastereomers of BHtetrols, established the absolute stereochemistry of the side chain on the basis of their ^{1}H-NMR spectra. Since the 32 diastereomers for BHhexols have not yet been synthesized, we had to use the CD exciton chirality method [38] that has been applied to sugar polyols [39] and/or different acyclic polyols such as BHpolyols [40] with up to five contiguous OH groups, or aminoBHpentols [41, 42]. These CD studies clearly showed that with flexible

Table 4 ^{1}H NMR of compound **2** (500 MHz, $CDCl_3$)

No.	^{1}H
22	2.23 (1H, *m*)
23	0.80 (3H, *s*)
24	0.83 (3H, *s*)
25	0.84 (3H, *s*)
26	0.94 (3H, *s*)
27	0.96 (3H, *s*)
28	0.69 (3H, *s*)
29	0.92 (3H, *d*, $J_{22,29} = 6.5$)
30	5.24 (1H, *dd*, $J_{22,30} = 2.1$; $J_{30,31} = 9.0$)
31	5.23 (1H, *dd*, $J_{30,31} = 9.0$; $J_{31,32} = 3.5$)
32	5.26 (1H, *dd*, $J_{31,32} = 3.5$; $J_{32,33} = 6.5$)
33	5.33 (1H, *dd*, $J_{32,33} = 6.5$; $J_{33,34} = 4.2$)
34	5.10 (1H, *ddd*, $J_{33,34} = 4.2$; $J_{34,35a} = 7.2$; $J_{34,35b} = 3.8$)
35a	3.68 (1H, *dd*, $J_{34,35a} = 7.2$; $J_{35a,35b} = 11.0$)
35b	3.98 (1H, *dd*, $J_{34,35b} = 3.8$; $J_{35a,35b} = 11.0$)
1′	4.78 (1H, *d*, $J_{1',2'} = 1.8$)
2′	4.63 (1H, *ddd*, $J_{1',2'} = 1.8$; $J_{2'3'} = 4.8$; $J_{2'NH} = 9.0$)
3′	5.38 (1H, *dd*, $J_{2',3'} = 4.8$; $J_{3'4'} = 9.5$)
4′	5.06 (1H, *t*, $J_{3'4'=4',5'} = 9.5$)
5′	3.96 (1H, *ddd*, $J_{4'5'} = 9.5$; $J_{5'6a'} = 4.5$; $J_{5'6b'} = 2.5$)
6a′	4.14 (1H, *dd*, $J_{5'6a'} = 4.5$; $J_{6a'6b'} = 12.5$)
6b′	4.30 (1H, *dd*, $J_{5'6a'} = 2.5$; $J_{6a'6b'} = 12.5$)
NH	6.45 (1H, *d*, $J_{2'NH} = 9.0$)
2‴	2.14 (2H, *t*, $J = 7.5$)
3‴	1.66 (2H, *m*)
4‴-ω8	1.23–1.45 (14H, *m*)
ω7	1.14 (2H, *m*)
Cyclohexyl's CH	1.36 (1H, *m*)
Cyclohexyl's CH_2	1.08–1.68 (*m*)
CH_3CO	2.018 (3H, *s*)
CH_3CO	2.023 (3H, *s*)
CH_3CO	2.029 (3H, *s*)
CH_3CO	2.036 (3H, *s*)
CH_3CO	2.036 (3H, *s*)
CH_3CO	2.049 (3H, *s*)
CH_3CO	2.054 (3H, *s*)
CH_3CO	2.054 (3H, *s*)

compounds it is preferable to explore the exciton coupling between two different chromophores such as anthroyl and cinnamoyl.

Based on the similarity of CD spectra of our compound (BHhexol, **14**) and the previously published aminoBHpentol derivative [41], we concluded that our compound is also a homolog of *allo* configuration. To verify this hypothesis, we consequently prepared from a commercially

Table 5 ^{13}C NMR of compound **2** (125 MHz, $CDCl_3$)

No.	^{13}C	No.	^{13}C
1	40.4t	33	71.6d
2	18.7t	34	71.0d
3	42.2t	35	64.5d
4	33.3s	1′	100.3d
5	56.2d	2′	55.4d
6	18.7t	3′	69.3d
7	33.2t	4′	71.4d
8	41.5s	5′	68.5d
9	50.5d	6′	62.1t
10	37.5s	1″	172.8s
11	20.8t	2″	34.1t
12	24.0t	3″	24.8t
13	49.3d	4″-ω8	27.4–30.0t
14	41.8s	ω7	38.5t
15	33.7t	Cyclohexyl's CH	29.1d
16	22.6t	Cyclohexyl's CH_2	26.6–34.6t
17	54.2d	C=O	172.8s
18	44.5s	C=O	170.7s
19	41.6t	C=O	170.6s
20	27.9t	C=O	170.5s
21	42.9d	C=O	170.2s
22	38.1d	C=O	169.7s
23	21.5q	C=O	169.5s
24	33.3q	C=O	169.4s
25	15.8q	$\underline{C}H_3CO$	21.1q
26	16.4q	$\underline{C}H_3CO$	21.1q
27	16.5q	$\underline{C}H_3CO$	20.9q
28	15.9q	$\underline{C}H_3CO$	20.8q
29	16.4q	$\underline{C}H_3CO$	20.7q
30	69.8d	$\underline{C}H_3CO$	20.6q
31	70.3d	$\underline{C}H_3CO$	20.5q
32	71.0d	$\underline{C}H_3CO$	20.4q

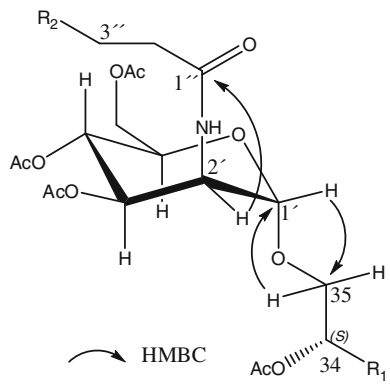

Fig. 3 Partial structure of a new compound (**2**). Only important HMBC correlations are shown

available saccharide D-allose (**6**) appropriate 7-deoxy-D-*glycero*-D-*allo*-heptitol (**10**) via the D-*glycero*-D-*allo*-heptose (**8a**) according to Fig. 5. This helped us remove the obstacle mentioned by Zhou et al. [41], viz. that configurational determination of aminoBHpentol is not straightforward due to the lack of reference hexols.

The original version of the Kiliani-Fischer synthesis was modified according to several authors [24, 25]. Instead of conversion of the cyanohydrin (aldonitrile) to a lactone, the cyanohydrin is reduced with hydrogen (palladium on barium sulfate) in water to an imine that quickly hydrolyzes at pH 4.2 to an aldehyde; thus the final sugars are produced in just two steps rather than three and, moreover, the final sugars are separated instead of the lactones. We used semipreparative TLC on cellulose plates and employed a previously described solvent system (acetone–water 95:5, v/v) with D-glucose as standard. The compound with an R_f lower than glucose was D-*glycero*-D-*allo*-heptose (**8a**) while that with an R_f higher than glucose was D-*glycero*-D-*altro*-heptose (**8b**), see also paper [25]. The measurement of ^{13}C-NMR spectra of (**8a**) showed that, in keeping with literature data [27], this compound is present as a mixture of four forms, i.e. α and β pyranoses and α and β furanoses in a ratio of 15:75:5:5. Experimental part gives only the values for the most copious form, i.e. β-pyranose.

Heptose (**8a**) was converted first to the diethyl thioacetal (**9**), and then, by reductive desulfurization with Raney nickel, to 7-deoxy-D-*glycero*-D-*allo*-heptitol (**10**). This desoxyheptitol was esterified by selective anthroylation of primary hydroxyl by anthracene-9-carboxylic acid to (2*R*, 3*R*, 4*S*, 5*S*, 6*S*)-2,3,4,5,6-pentahydroxyheptyl anthracene-9-carboxylate (**11**). To make sure that we had indeed the appropriate and pure epimer (viz. the separation of heptoses by TLC after reduction) we used a separation of this compound by means of normal phase HPLC, see also Wiesler and Nakanishi [43]. They reported that mixtures of saccharides (after cyanohydrin synthesis) could be directly subjected to anthroylation, after which separation of the fluorescent monoesters was greatly simplified. The secondary hydroxyls of anthroyl derivative (**11**) were per-p-methoxycinnamoylated to an appropriate "bichromophoric" derivative (**12**) and CD spectrum of this compound was measured, see Fig. 6. The curve for L-*allo* (**12**) is plotted as a mirror image of the D-series, see also Zhou et al. [39].

On the basis of all above mentioned spectra (including CD spectrum of synthetic compound **12**), we confirmed that the configuration of the side chain is 30*R*, 31*R*, 32*R*, 33*S*, 34*S* (D-*glycero*-D-*allo*) and the components of this fraction are *N*-acylated-mannosamine-BHhexols which contain—α-D-2-amino-2-deoxymannopyranose esterified by ω-cyclohexyl fatty acids from C-15 to C-29. The correct name of the glycoside with C-17 fatty acids is 11-cyclohexyl-*N*-((2*S*,3*S*,4*R*,5*S*,6*R*)-2-((2*S*,3*S*,4*R*,5*R*,6*R*,7*S*)-7-((3*S*,3a*S*,5a*R*,

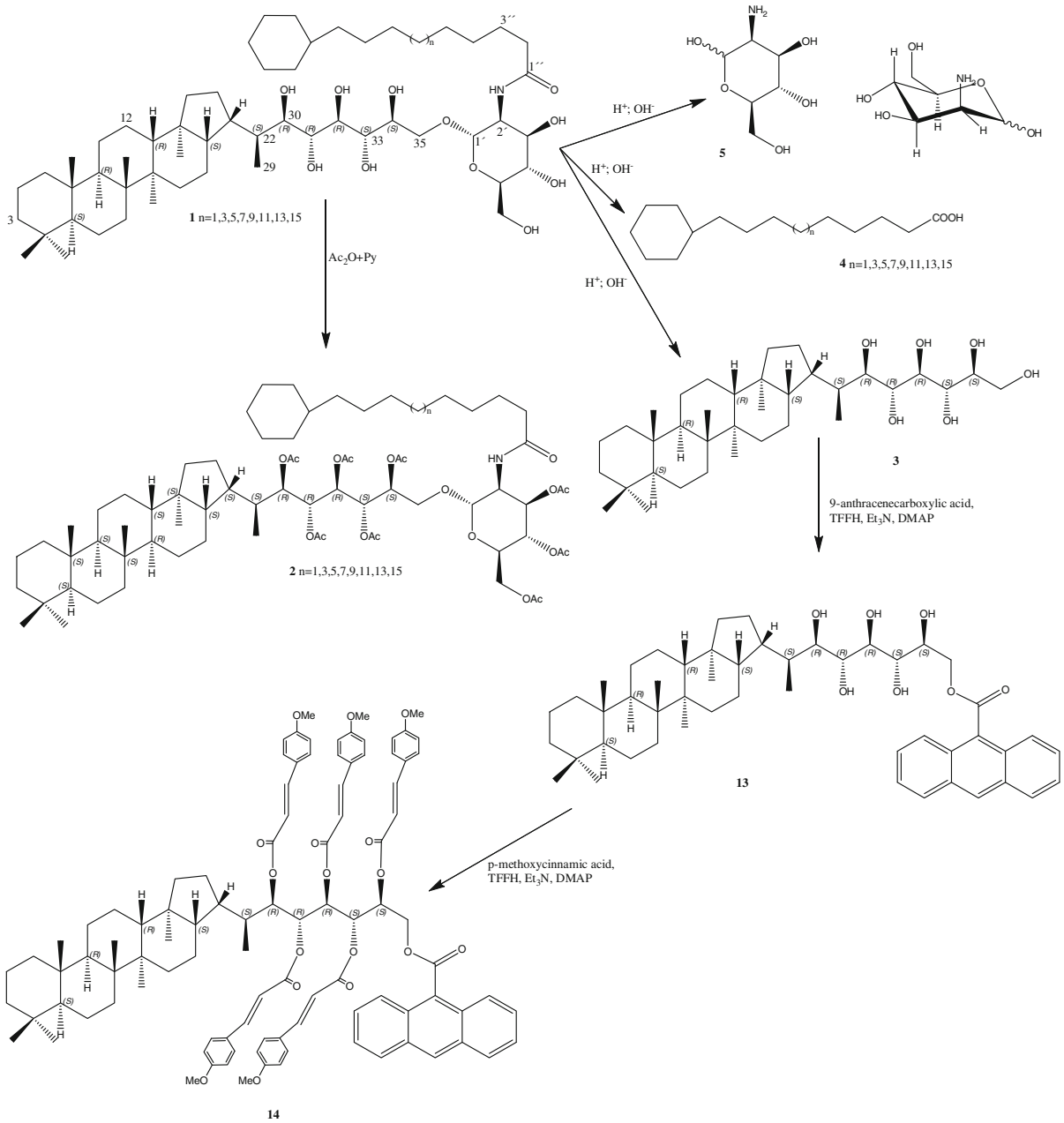

Fig. 4 Structure of **1** and synthesis of appropriate derivatives

5b*R*,7a*S*,11a*S*,11b*R*,13a*R*,13b*S*)-5a,5b,8,8,11a,13b-hexamethylicosahydro-1*H*-cyclopenta[a]chrysen-3-yl)-2,3,4,5,6-pentahydroxyoctyloxy)-4,5-dihydroxy-6-(hydroxymethyl) tetrahydro-2*H*-pyran-3-yl)undecanamide (**1**).

Discussion

To the best of our knowledge, this is the first report of BHhexol from a specific bacterium. Bacteriohopanoids having hexol in the side chain, including their composite derivatives, have so far been identified only in sediments but their configuration has been unknown [2, 16, 17]. Based on the comparison with the synthesized model compound (**12**), we determined its absolute configuration by using CD. Still, the identification of a novel hopanoid from *A. acidoterrestris* is not too surprising since only several tens of bacterial and cyanobacterial genera have been tested for the presence of hopanoids [16]. Although the biological significance of our compound is not yet clear; it is probable that in *A. acidoterrestris* the long chain ω-cyclohexyl FAs, which are bound by an amidic bond to

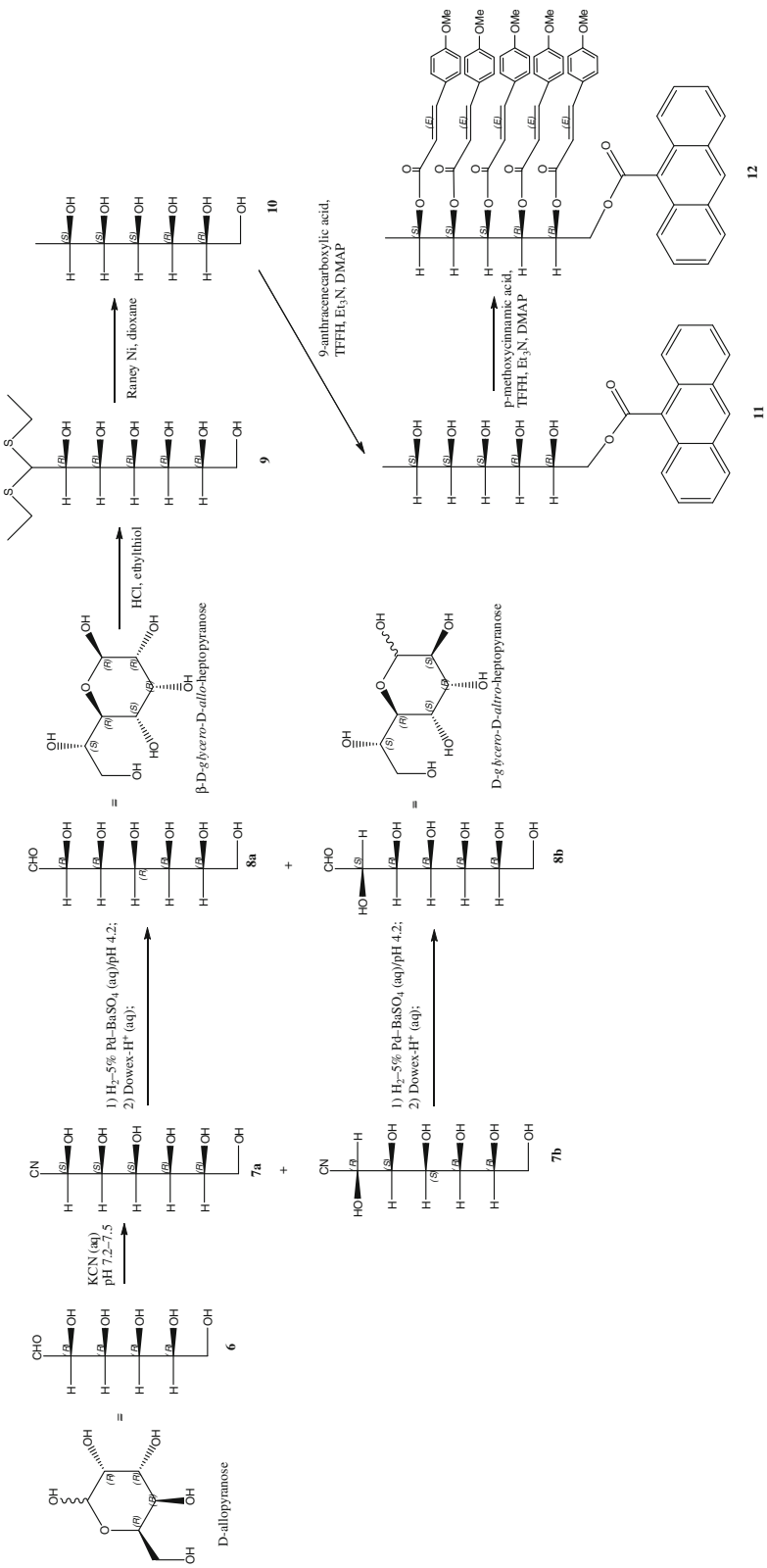

Fig. 5 Enantioselective synthesis of 7-deoxy-D-*glycero*-D-*allo*-heptitol (**10**) and its derivatives used for CD

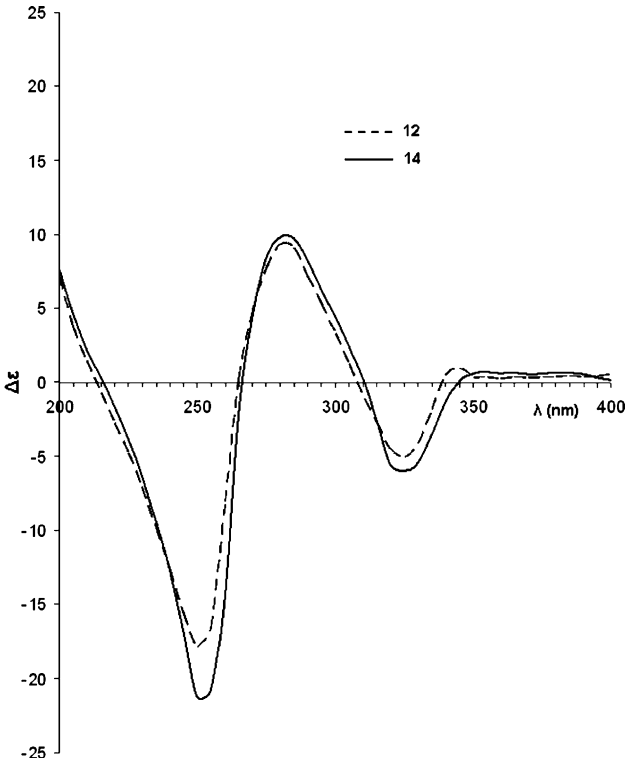

Fig. 6 CD spectra of compounds **12** (synthesized standard) and **14** (BHhexol)

the amino group in position 2 of the appropriate monosaccharide, play an important role in the thermophilicity of this bacterium.

Different monosaccharide derivatives have been found to be bound glycosidically to the primary C35 hydroxyl: Simonin et al. [44] described galacturonic and altruronic acids while all other studies describe 2-amino-2-deoxyglucose and one study reports on 6-amino-6-deoxyglucose [44]. We assume that the change in the configuration of the amino group on C2 (mannose is a 2-epimer of glucose), i.e. axial to equatorial, alters the planarity of the whole molecule.

The last result of our study is the heretofore undescribed large variability of chain lengths. Only ω-cyclohexyl FA with 17 and 19 carbon atoms have so far been described [18]. Solely in *N*-acyl glycoside of BHtetrol did Talbot et al. [5] succeeded in identifying by ESI also other homologues, one shorter (C15) and one longer (C21). We have extended the range of homologues by other longer-chain members—odd-numbered C23 to C29.

In conclusion we can state that, by using modern analytical methods, we succeeded in identifying one of the possible sources of BHhexol in sediments, i.e. *A. acidoterrestris*, and determined the absolute configuration of the side chain of BHhexol by CD and by its comparison with the synthetic model compound 7-deoxy-D-*glycero*-D-*allo*-heptitol, i.e. (2*R*,3*R*,4*S*,5*S*,6*S*)-heptane-1,2,3,4,5,6-hexaol (**10**), which has not yet been described.

Acknowledgments The research was supported by projects of the *Ministry of Education, Youth and Sports of the Czech Republic* (no. 1M06011; GACR P503/10/P182), and by *Institutional Research Concepts* AV 0Z 502 0910 and MSM6046137305.

References

1. Fox PA, Carter J, Farrimond P (1998) Analysis of bacteriohopanepolyols in sediment and bacterial extracts by high performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry. Rapid Commun Mass Spectrom 12:609–612
2. Talbot HM, Farrimond P (2007) Bacterial populations recorded in diverse sedimentary biohopanoid distributions. Org Geochem 38:1212–1225
3. Ourisson G, Rohmer M (1992) Hopanoids. 2. Biohopanoids: a novel class of bacterial lipids. Acc Chem Res 25:403–408
4. Kannenberg EL, Poralla K (1999) Hopanoid biosynthesis and function in bacteria. Naturwissenschaften 86:168–176
5. Talbot HM, Rohmer M, Farrimond P (2007) Rapid structural elucidation of composite bacterial hopanoids by atmospheric pressure chemical ionisation liquid chromatography/ion trap mass spectrometry. Rapid Commun Mass Spectrom 21:880–892
6. Stroeve P, Pettit CD, Vasquez V, Kim I, Berry AM (1998) Surface active behavior of hopanoid lipids: bacteriohopanetetrol and phenylacetate monoester bacteriohopanetetrol. Langmuir 14:4261–4265
7. Shatz M, Yosief T, Kashman Y (2000) Bacteriohopanehexol, a new triterpene from the marine sponge *Petrosia* species. J Nat Prod 63:1554–1556
8. Rohmer M (1993) The biosynthesis of triterpenoids of the hopane series in the eubacteria: a mine of new enzyme reactions. Pure Appl Chem 65:1293–1298
9. Costantino V, Fattorusso E, Imperatore C, Mangoni A (2000) The first 12-methylhopanoid: 12-methylbacteriohopanetetrol from the marine sponge *Plakortis simplex*. Tetrahedron 56:3781–3784
10. Pan W, Zhang Y, Liang G, Rohmer M, Sinay P, Vincent SP (2007) Diastereoselective synthesis of aminobacteriohopanetetrol, a biomarker for methanotrophic bacteria: confirmation of the absolute configuration. Chem Biodivers 4:2182–2189
11. Simonin P, Jurgens UJ, Rohmer M (1992) 35-*O*-β-6-amino-6-deoxyglucopyranosyl bacteriohopanetetrol, a novel triterpenoid of the hopane series from the cyanobacterium *Synechocystis* SP PCC-6714. Tetrahedron Lett 33:3629–3632
12. Herrmann D, Bisseret P, Connan J, Rohmer M (1996) A non-extractable triterpenoid of the hopane series in *Acetobacter xylinum*. FEMS Microbiol Lett 135:323–326
13. Joyeux C, Fouchard S, Llopiz P, Neunlist S (2004) Influence of the temperature and the growth phase on the hopanoids and fatty acids content of Frateuria aurantia (DSMZ 6220). FEMS Microbiol Ecol 47:371–379
14. Neunlist S, Rohmer M (1985) Novel hopanoids from the methylotrophic bacteria *Methylococcus capsulatus* and *Methylomonas methanica*. (22S)-35-aminobacteriophane-30, 31, 32, 33, 34-pentol and (22S)-35-amino-3β-methylbacteriophane-30, 31, 32, 33, 34-pentol. Biochem J 231:635–639
15. Cvejic JH, Bodrossy L, Kovacs KL, Rohmer M (2000) Bacterial triterpenoids of the hopane series from the methanotrophic bacteria *Methylocaldum* spp.: phylogenetic implications and first evidence for an unsaturated aminobacteriohopanepolyol. FEMS Microbiol Lett 182:361–365
16. Talbot HM, Summons RE, Jahnke LL, Cockell CS, Rohmer M, Farrimond P (2008) Cyanobacterial bacteriohopanepolyol signatures from cultures and natural environmental settings. Org Geochem 39:232–263

17. Cooke MP, Talbot HM, Farrimond P (2008) Bacterial populations recorded in bacteriohopanepolyol distributions in soils from Northern England. Org Geochem 39:1347–1358
18. Siristova L, Melzoch K, Rezanka T (2009) Fatty acids, unusual glycophospholipids and DNA analyses of thermophilic bacteria isolated from hot springs. Extremophiles 13:101–109
19. Rezanka T, Siristova L, Melzoch K, Sigler K (2009) Identification of (*S*)-11-cycloheptyl-4-methylundecanoic acid in acylphosphatidylglycerol from *Alicyclobacillus acidoterrestris*. Chem Phys Lipids 158:104–113
20. Rezanka T, Siristova L, Melzoch K, Sigler K (2009) Direct ESI–MS analysis of *O*-acyl glycosylated cardiolipins from the thermophilic bacterium *Alicyclobacillus acidoterrestris*. Chem Phys Lipids 161:115–121
21. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917
22. Moreau RA, Powell MJ, Osman SF, Whitaker BD, Fett WF, Roth L, O'Brien DJ (1995) Analysis of intact hopanoids and other lipids from the bacterium *Zymomonas mobilis* by high-performance liquid chromatography. Anal Biochem 224:293–301
23. Rezanka T (1990) Identification of very long polyenoic acids as picolinyl esters by Ag ion-exchange high-performance liquid chromatography, reversed-phase high-performance liquid chromatography. J Chromatogr 513:344–348
24. Patching SG, Middleton DA, Henderson PJF, Herbert RB (2003) The economical synthesis of [2′-13C, 1,3–15N2]uridine; preliminary conformational studies by solid state NMR. Org Biomol Chem 1:2057–2062
25. Snyder JR, Johnston ER, Serianni AS (1989) D-talose anomerization: NMR methods to evaluate the reaction kinetics. J Amer Chem Soc 111:2681–2687
26. Dmitriev BA, Chernyak AYa, Chizhov OS, Kochetkov NK (1972) Monosaccharides—communication 26. β-methyl-D-*allo*-heptopyranoside-4-ulose and syntheses based on it. Russ Chem B 21:2230–2235
27. Kim M, Grzeszczyk B, Zamojski A (2000) Homologation of protected hexoses with Grignard C1 reagents. Tetrahedron 56:9319–9337
28. Pratt JW, Richtmyer NK (1955) D-*glycero*-D-*allo*-Heptose, L-*allo*-Heptulose, D-*talo*-Heptulose and related substances derived from the addition of cyanide to D-allose. J Amer Chem Soc 77:6326–6328
29. Angyal SJ, Tran TQ (1983) Equilibria between pyranoses and furanoses. 5. The composition in solution and the C-13 NMR-spectra of the heptoses and the heptuloses. Aust J Chem 36:937–946
30. Fukase H, Horii S (1992) Synthesis of a branched-chain inosose derivative, a versatile synthon of *N*-substituted valiolamine derivatives from D-glucose. J Org Chem 57:3642–3650
31. Bravo J-M, Perzl M, Hartner T, Kannenberg EL, Rohmer M (2001) Novel methylated triterpenoids of the gammacerane series from the nitrogen-fixing bacterium *Bradyrhizobium japonicum* USDA 110. Eur J Biochem 268:1323–1331
32. Talbot HM, Squier AH, Keely BJ, Farrimond P (2003) Atmospheric pressure chemical ionisation reversed-phase liquid chromatography/ion trap mass spectrometry of intact bacteriohopanepolyols. Rapid Commun Mass Spectrom 17:728–737
33. Pan W, Zhang Y, Liang G, Vincent SP, Sinay P (2005) Concise syntheses of bacteriohopanetetrol and its glucosamine derivative. Chem Commun 27:3445–3447
34. Kasai R, Okihara M, Asakawa J, Mizutani K, Tanaka O (1979) Carbon-13 NMR study of α- and β-anomeric pairs of D-mannopyranosides and L-rhamnopyranosides. Tetrahedron 35:1427–1432
35. Lin C-H, Sugai T, Halcomb RL, Ichikawa Y, Wong C-H (1992) Unusual stereoselectivity in sialic acid aldolase-catalyzed aldol condensations: synthesis of both enantiomers of high-carbon monosaccharides. J Amer Chem Soc 114:10138–10145
36. Kaji E, Lichtenthaler FW, Nishino T, Yamane A, Zen S (1988) Practical syntheses of immunologically relevant β-glycosides of 2-acetamido-2-deoxy-D-mannopyranose. Methyl *N*-acetyl-β-D-mannosaminide, *N*-acetyl-β-D-mannosaminyl-(1 → 6)-D-galactose, and methyl *N*-acetyl-β-D-mannosaminyl-(1 → 4)-α-D-glucopyranoside. Bull Chem Soc Jpn 61:1291–1297
37. Bisseret P, Rohmer M (1989) Bacterial sterol surrogates. Determination of the absolute configuration of bacteriohopanetetrol side chain by hemisynthesis of its diastereoisomers. J Org Chem 54:2958–2964
38. Berova N, Nakanishi K, Woody RW (2000) Circular dichroism: principles and applications, 2nd edn. Wiley Blackwell, New York
39. Zhou P, Berova N, Wiesler WT, Nakanishi K (1993) Assignment of relative and absolute configuration of acyclic polyols and aminopolyols by circular dichroism-trends follow Fischer's sugar family tree. Tetrahedron 49:9343–9352
40. Zhao N, Berova N, Nakanishi K, Rohmer M, Mougenot P, Jurgens UJ (1996) Structures of two bacteriohopanoids with acyclic pentol side-chains from the cyanobacterium *Nostoc* PCC 6720. Tetrahedron 52:2777–2788
41. Zhou P, Berova N, Nakanishi K, Knani M, Rohmer M (1991) Microscale CD method for determining absolute configurations of acyclic amino tetrols and amino pentols. Structures of aminobacteriohopanepolyols from the methylotrophic bacterium *Methylococcus luteus*. J Amer Chem Soc 113:4040–4041
42. Zhou P, Berova N, Nakanishi K, Rohmer M (1991) Assignment of absolute stereochemistry of aminopolyols by the bichromophoric exciton chirality method. J Chem Soc Chem Commun, pp 256–258
43. Wiesler WT, Nakanishi K (1989) A simple spectroscopic method for assigning relative and absolute configuration in acyclic 1, 2, 3-triols. J Amer Chem Soc 111:3446–3447
44. Simonin P, Jurgens UJ, Rohmer M (1996) Bacterial triterpenoids of the hopane series from the prochlorophyte *Prochlorothrix hollandica* and their intracellular localization. Eur J Biochem 241:865–871