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Novel Bacterial Triterpenoids of the Hopane Series from *Nitrosomonas europaea* and their significance for the formation of the C_{35} bacteriohopane skeleton

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Abstract. In addition to aminobacteriohopanetriol and adenosylhopane, three new hopanoids were isolated from the bacterium *Nitrosomonas europaea*: two *N*-acylaminobacteriohopanetriols, a hopanoid presenting a carbon/carbon bond between ribonolactone and hopane and related to a putative intermediate involved in the formation of the C_{35} bacteriohopane skeleton and finally a condensation product between aminobacteriohopanetriol and trinorbacteriohopan-32-al, an artifact resulting from the autoxidation of the aminotriol. © 1999 Published by Elsevier Science Ltd. All rights reserved.

Triterpenoids of the hopane series are widespread in bacteria[1] and apparently represent essential metabolites, most probably acting as membrane stabilizers.[2] The bacteriohopanepolyols, which are always the major hopanoids, are characterized by a C_{35} skeleton with an unusual carbon/carbon bond between the triterpenic hopane moiety and an additional polyhydroxylated C_5 unit derived from a D-pentose.[3-5] Neither intermediates, nor enzymes involved in the formation of the bacteriohopane skeleton were identified. In this contribution, the screening of *N. europaea* led to the detection of three new hopanoids, including the oxidized form of a putative intermediate involved in the formation of the C_{35} bacteriohopane skeleton.

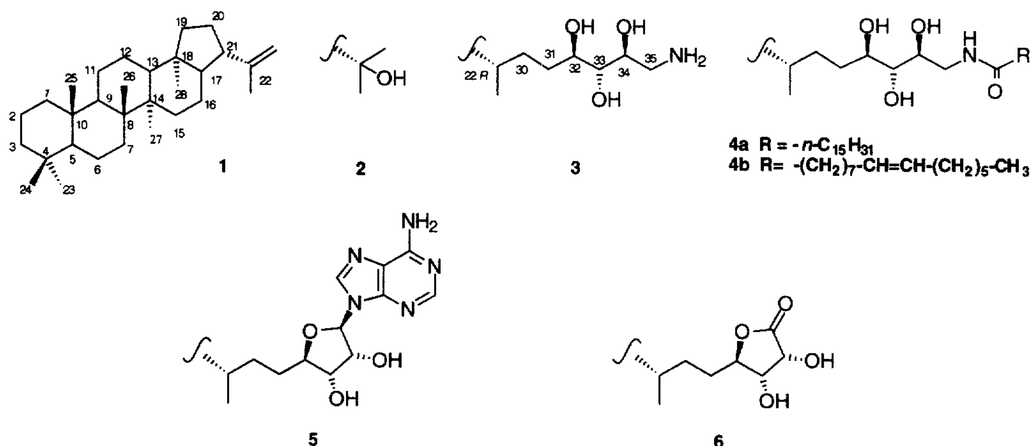

Fig. 1. Hopanoids of *Nitrosomonas europaea*.

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In a first attempt, the hopanoid content was determined after $H_5IO_6/NaBH_4$ treatment of the crude extract[1] obtained either from lyophilized bacterial cells or from an envelope-membrane fraction.[6] Results in both analyses were similar. GC and GC/MS indicated the presence of diploptene **1** (0.8 $\mu g \cdot g^{-1}$), diplopterol **2** (80 $\mu g \cdot g^{-1}$), (22*R*)-trinorbacteriohopan-32-ol (3.6 $mg \cdot g^{-1}$) and (22*R*)-tetranorbacteriohopan-31-ol (80 $\mu g \cdot g^{-1}$), qualitatively confirming former results obtained on another *N. europaea* strain.[1] The earlier procedure, well adapted to the isolation of bacteriohopanetetrols, involved a saponification followed by an ether extraction of the hopanoids prior to the $H_5IO_6/NaBH_4$ treatment and therefore seriously underestimated the hopanoid content in bacteria producing aminobacteriohopanepolyols or composite polar hopanoids which are poorly soluble in ether.[1] These observations were in accord with the structure of the intact hopanoids. Aminobacteriohopanetriol **3** (as major hopanoid) and adenosylhopane **5** (in much lower amounts) were identified by their ^{13}C- and/or ^{1}H-NMR spectra of their acetylated derivatives.[11,20] In addition to these already known hopanoids, three new compounds (**4a** and **4b**, **6** and **7**) were found (Fig. 1).

The lactone **6** was identified by the ^{1}H- and ^{13}C-NMR spectra of its diacetate: they were identical with those of the same hopanoid previously obtained by chemical oxidation of an aminobacteriohopanetriol **3** derivative.[9] The unusual carbon/carbon bond between a terpenoid and a carbohydrate is clearly shown in adenosylhopane **5** and in the lactone **6**. An hypothetical biogenetic scheme for the formation of the bacteriohopane skeleton was proposed according to the structures of the known bacterial hopanoids.[5] The addition of a nucleophilic hopanoid onto a D-ribose derivative possessing a leaving group at C-5 (*e.g.* ribose 5-phosphate or 5-diphosphate, S-adenosyl methionine), would afford 30-(5'-ribosyl)hopane or adenosylhopane **5**. Ribosylhopane was never detected in a bacterium, but the identification of lactone **6**, which directly results from its oxidation, strengthens its probable key role in the formation of the C_{35} bacteriohopane skeleton.

The ^{1}H-NMR spectrum of the acetylated *N*-acyl aminobacteriohopanetriol **4a** and **4b** fraction was nearly identical (chemical shifts and coupling constants) with that of the tetra-acetate of aminotriol **3**. It only differed by the additional presence of the triplet (0.88 ppm) of a methyl group in terminal position on an aliphatic chain and, of a tall multiplet (1.25 ppm) from a saturated methylene chain and of a triplet (2.15 ppm) from a methylene group in α position of a carbonyl and by the absence of the most shielded methyl singlet (1.96 ppm) corresponding to the acetamido group of aminotriol tetra-acetate. This data was in accord with the structure of a fatty acid amide of aminotriol. The mass spectrum confirmed this hypothesis and indicated in addition that two fatty acids in a 7:3 ratio were linked to the aminotriol. Fragments of diagnostic values showed double peaks: molecular ions with *m/z* 909 and 907 as well as ions corresponding to the loss of acetic acid with *m/z* 949 and 947 and to ring C cleavage with *m/z* 688 and 686. This suggested that a major saturated fatty acid, palmitic acid, as well as a mono-unsaturated C_{16} fatty acid (*cis*-palmitoleic acid) were involved in the formation of the two amides **4a** and **4b**. The presence of the mono-unsaturated fatty acid was further corroborated by the presence of a multiplet of vinylic protons (5.35 ppm) in the ^{1}H-NMR spectrum. In order to confirm this identification, *N*-palmitoyl aminobacteriohopanetriol **4a** was synthesized by reacting aminotriol **3** with the thiazolidinethione derivative of palmitic acid.[10] The ^{1}H-NMR and the mass spectra of the acetylated synthetic hopanoid **4a** were identical with those of the natural major *N*-palmitoylaminobacteriohopanetriol. *Cis*-Δ^9-palmitoleic acid is the major unsaturated fatty acid *N. europaea.*[11] The position of the double-bond was confirmed on the free fatty acids by mass-spectrometry of their methyl esters after addition of dimethyldisulfide.[12] *N*-Palmitoleyl aminobacteriohopanetriol **4b** was therefore the most likely structure for the minor *N*-acylaminobacteriohopanetriol. Composite hopanoids such as **4a** and **4b** containing two hydrophobic moieties (the pentacyclic hopane skeleton and an *n*-acyl chain from a typical phospholipid fatty acid) were previously only recorded in *Alicyclobacillus* species.[13] The significance of such *N*-acylations is yet unknown. In order to check that the two *N*-acyl aminobacteriohopanetriols **4a** and **4b** did not represent artifacts resulting from the transfer of an acyl moiety of a phospholipid to aminotriol **3** during the extraction, aminotriol **3** and dipalmitoyl phosphatidylethanolamine, the major phospholipid of *N. europaea*,[11] were refluxed in $CHCl_3/CH_3OH$ (2:1) for 4 h. After acetylation, tetra-acetate of aminotriol **3** was recovered with 85% yield after TLC. It was accompanied by minor compounds, such as hopanoid **7**, but the formation of **4a** or lactone **6** was not observed.

The ^1H-NMR spectrum of acetylated hopanoid **7** (Fig. 2) isolated from *N. europaea* displayed the usual methyl singlets of the hopane skeleton and two methyl singlets at 2.03 (two methyls) and 2.07 ppm (one methyl) from three acetoxy groups and brought little additional information. Formation of hopanoid **7** by simply refluxing aminotriol **3** in $CHCl_3/CH_3OH$ yielded an interesting clue for its identification. Aminotriol **3** is readily degraded into trinorbacteriohopan-32-al **8** in the presence of oxygen.[14] Condensation of this aldehyde with aminotriol **3** would produce an aminal which was shown to correspond to hopanoid **7**. The FAB mass spectrum of acetylated **7** was characterized by an *m/z* 1108 pseudo-molecular ion $(M+H)^+$. The presence of an acetoxy group on a nitrogen atom was verified by solvolysis of the triacetate of **7** with basic Amberlyst A-26 (OH^- form) in THF/CH_3OH. This method easily cleaves ester acetoxy groups, leaving unchanged the acetamides.[15] Reacetylation with $(CD_3CO)_2O$ again afforded the triacetate of **7**, but in its ^1H-NMR spectrum, the signals of two acetoxy groups (2.03 and 2.07 ppm) were missing, whereas a singlet (2.03 ppm) corresponding to an acetamide was still present. The absolute configuration of the asymmetric C-32' aminal carbon atom was not determined. Both diastereomers at C-32' might be present as the 32'-H signal (5.52 ppm) was broad and unresolved. Hopanoid **7** was not present in the aminotriol utilized as starting material in the simulation experiments. The compound found in trace amounts in *N. europaea* was therefore most likely formed by autoxidation of aminotriol either during the storage of the cell material or during the extraction.

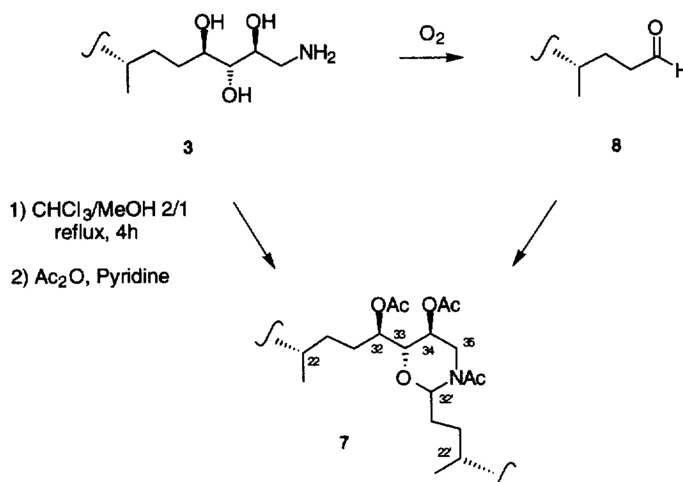

Fig. 2. Formation of the aminal **7** from aminobacteriohopanetriol **3** and from aminotriol autoxidation aldehyde **8**.

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16. Culture of *Nitrosomonas europaea* (Schmidt strain),[17] preparation of the fraction containing the cell wall and all membranes (including the notable invaginations of the cell membrane),[6] analytical procedures and spectroscopic identifications[8] were as described previously. Freeze-dried intact cells (250 mg) or the envelope-membrane fraction (250 mg) were extracted with $CHCl_3/CH_3OH$ (2:1, v/v) and treated using the $H_5IO_6/NaBH_4$ derivatisation method for the cleavage of the bacteriohopanepolyol side chains in order to determine their hopanoid content.[1] Intact hopanoids from lyophilized envelope-membrane fraction (8 g and 32 g) were extracted three times with $CHCl_3/CH_3OH$ (2:1, v/v). The extract was evaporated to dryness and directly acetylated. After removal of the reagent, TLC ($CHCl_3/CH_3OH$, 98:2, v/v) yielded **1** (R_f=0.90), the mixture of the triacetates of **4a** and **4b**, the diacetate of **6** and the triacetate **7** (0.85>R_f>0.60), the free fatty acid fraction (0.60>R_f>0.30) and finally the mixture of the tetra-acetate of **3** and the diacetate of **5** (0.30>R_f>0.20). The mixture (0.85>R_f>0.60) resulting from the first TLC was further separated by TLC ($CHCl_3$) yielding the diacetate of **6** (R_f=0.38, 0.2 $mg{\cdot}g^{-1}$), the mixture of the triacetates of **4a**, **4b** and the triacetate of hopanoid **7** (R_f=0.15). An additional TLC separation ($CHCl_3$/hexane, 2:1, v/v) afforded the triacetates of **4a** and **4b** (R_f=0.06, 0.1 $mg{\cdot}g^{-1}$) and the triacetate of **7** (R_f=0.12, 0.05$mg{\cdot}g^{-1}$). TLC ($CHCl_3/CH_3OH$, 95:5 v/v) allowed also the separation of the tetra-acetate of **3** (R_f=0.60, 3.5 $mg{\cdot}g^{-1}$) from the diacetate of **5** (R_f=0.50, 0.5 $mg{\cdot}g^{-1}$).
Triacetate of **4a** obtained by chemical synthesis. ^{1}H-NMR (250 MHz, $CDCl_3$): δ = 0.68 (3H, s, 18α-CH_3); 0.79 (3H, s, 4β-CH_3); 0.81 (3H, s, 10β-CH_3); 0.85 (3H, s, 4α-CH_3); 0.88 (3H, t, J= 6Hz, terminal CH3 of palmitoyl chain); 0.90 (3H, d, J=6.2Hz, 22*R*-CH3); 0.94 (3H, s, 14α- and 8β-CH3); 1.25 (nH, m, -CH_2-); 2.07 (6H, s, 2xCH_3COO-); 2.10 (3H, s, CH_3COO-); 2.15 (2H, t, J= 7.6Hz, -CH_2-CONH-); 3.35 (1H, dt, J= 6.0Hz and 14.7Hz, 35-H_b); 3.71 (1H, ddd, J= 3.2 Hz, 6.0 Hz and 14.7 Hz, 35-H_a); 5.02 (1H, dt, J= 3.9Hz and 9.5 Hz, 32-H); 5.06 (1H, dt, J= 3.2Hz and 6.0 Hz, 34-H); 5.15 (1H, dd, J= 3.9Hz and 6.0Hz, 33-H); 5.72 (1H, t, J= 6.0Hz, -NH-). ^{13}C-NMR (63 MHz, $CDCl_3$): δ/ppm = 14.1 (terminal CH_3); 15.86 and 15.92 (C-25 and C-28); 16.5 and 16.6 (C-26 and C-27); 18.7 (C-2 and C-6); 18.8 (C-29); 20.9 (CH_3COO-); 20.97 (C-11 and CH_3COO-); 21.02 (CH_3COO-); 21.6 (C-24), 22.7 (-CH_2-); 22.8 (C-16); 24.0 (C-12); 25.7 (-CH_2-); 25.9 (C-31); 27.6 (C-20); 29.3 (-CH_2-); 29.4 (-CH_2-); 29.5 (-CH_2-); 29.7 (-CH_2-); 29.7 (-CH_2-); 31.0 (C-30); 32.0 (-CH_2-); 33.3 (C-7); 33.3 (C-4); 33.4 (C-23); 33.7 (C-15); 36.1 (C-22); 36.8 (-CH_2-); 37.4 (C-10); 39.0 (C-35); 40.4 (C-1); 41.6 (C-19); 41.7 and 41.8 (C-14 and C-8); 42.1 (C-3); 44.4 (C-18); 46.0 (C-21); 49.3 (C-13); 50.5 (C-9); 54.5 (C-17); 56.2 (C-5); 70.7 (C-34); 71.9 (C-33); 72.2 (C-32); 170.2 (C=O); 170.2 (C=O); 170.6 (C=O); 173.3 (C=O). Mass spectrum (direct inlet, electron impact): *m/z* = 909 (M^+, 0.05%); 849 (M^+-AcOH, 0.1%); 688 (ring C cleavage, 100%); 628 (688-AcOH, 28%); 568 (688-2AcOH, 7%); 508 (688-3AcOH, 17%); 369 (M^+- side-chain, 15%); 239 ($C_{15}H_{31}CO^+$, 9%); 191 (ring C cleavage, 32%). All signals observed in the ^{1}H-NMR and mass spectra of the synthetic triacetate of **4a** were found as major signals in those of the acetylated mixture of *N*-acylaminobacteriohopanetriols **4a** and **4b** isolated from *N. europaea*, indicating the presence of *N*-palmitoylaminobacteriohopanetriol in this bacterium. Additional signals corresponded to the signature of a minor analog with an unsaturated palmitoleyl chain.
Diacetate of **6**. ^{1}H-NMR: δ = 0.70 (3H, s, 18α-CH_3); 0.79 (3H, s, 4β-CH3); 0.82 (3H, s, 10β-CH_3); 0.85 (3H, s, 4α-CH_3); 0.94 (9H, 2s and d, J=6.5Hz, 8β- and 14α-CH_3, 22*R*-CH_3); 2.12 (3H, s, CH_3COO-); 2.16 (3H, s, CH_3COO-); 4.46 (1H, dd, J= 8.0Hz and 5.0Hz, H-32); 5.29 and 5.62 (2H, 2d, J=5.6Hz and J=5.6Hz, H-34 and H-33). ^{13}C-NMR: δ/ppm = 15.9 (C-28 and C-25); 16.55 and 16.63 (C-27 and C-27); 18.7 (C-2 and C-6); 19.9 (C-29); 20.2 (CH_3COO-); 20.6 (CH_3COO-); 21.0 (C-11); 21.6 (C-24), 22.8 (C-16); 24.0 (C-12); 27.6 (C-20); 29.1 (C-31); 31.1 (C-30); 33.3 (C-4); 33.3 (C-7); 33.4 (C-23); 33.7 (C-15); 36.2 (C-22); 37.5 (C-10); 40.4 (C-1); 41.6 (C-19); 41.7 and 41.8 (C-14 and C-8); 42.2 (C-3); 44.4 (C-18); 45.9 (C-21); 49.4 (C-13); 50.5 (C-9); 54.4 (C-17); 56.2 (C-5); 66.5 and 71.8 (C-33 and C-34); 83.6 (C-32); 169.3 (C=O); 169.8 (C=O); 170.2 (C=O). Mass spectrum (direct inlet, electron impact): *m/z* = 626 (M^+, 8%); 611 (M^+-Me, 9%); 405 (ring C cleavage, 100%); 369 (M^+- side-chain, 29%); 345 (405-AcOH, 5%); 285 (405-2AcOH, 3%); 191 (ring C cleavage, 78%).
Triacetate of hopanoid **7**. A solution of aminotriol **3** (100 mg, 0.18 mmol) and dipalmitoyl phosphatidylethanolamine (250 mg, 0.36 mmol) in $CHCl_3/CH_3OH$ (2:1, v/v, 700 ml) was refluxed for 4 h in the presence of air. After evaporation of the solvent and addition of pyridine/acetic acid anhydride (1:1, v/v, 5 ml), the reaction mixture was stirred overnight at room temperature. After removal of the reagents, the residue was separated by TLC ($CHCl_3/CH_3OH$, 98:2, v/v) yielding the tetra-acetate of aminobacteriohopanetriol **3** (R_f=0.21, 130 mg) and a fraction (R_f>0.40) containing the triacetate of hopanoid **7**. The latter fraction was further purified by TLC ($CHCl_3/CH_3OH$, 95.5:0.5, v/v, 3 migrations) to give a fraction enriched in the triacetate of **7** (R_f=0.75). An additional TLC purification (ethyl acetate/cyclohexane, 1:1, v/v, 3 migrations) afforded the triacetate of **7** (R_f=0.57) which was obtained pure after a last TLC ($CHCl_3$, 4 migrations, R_f=0.40, 1.5 mg). ^{1}H-NMR: δ = 0.69 (6H, s, 18α- and 18'α-CH_3); 0.79 (6H, s, 4β- and 4'β-CH_3); 0.81 (6H, s, 10β- and 10'β-CH_3); 0.85 (6H, s, 4α- and 4'α-CH_3); 0.91 (6H, d, J=6.3Hz, 22*R*- and 22'*R*-CH_3); 0.946 (12H, s 14α-, 14'α-, 8β- and 8'β-CH_3); 2.03 (6H, s, 2xCH_3CO-); 2.07 (3H, s, CH_3CO-); 3.40 (1H, m, 33-H); 3.56 (2H, m, 35-H); 4.99 (1H, m, 32-H); 5.13 (1H, m, 34-H); 5.51 (1H, m, 32'-H). FAB-MS: 1108 ($M+H^+$, 1.3%); 698 (92%); 656 (100%).
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