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A biosynthetically significant new bacteriohopanoid present in large amounts in the Caribbean sponge *Plakortis simplex*

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Abstract—In addition to the previously reported bacteriohopanoids, the marine sponge *Plakortis simplex* was shown to contain large amounts of (32*R*,33*S*,34*S*)-32,35-anhydrobacteriohopanetetrol. The structure of this new bacteriohopanoid was determined by extensive NMR analysis, and further supports the hypothesized biosynthetic pathway to bacteriohopanoids. Altogether, the amounts of bacteriohopanoids in *P. simplex* is as high as 50% of sterols in weight, and these compounds could play a structural role in the sponge cell membranes. © 2001 Elsevier Science Ltd. All rights reserved.

1. Introduction

Bacteriohopanoids are bacterial metabolites composed of a pentacyclic triterpenoid with the hopane skeleton linked to a sugar-derived polyhydroxylated C_5 chain, that act as membrane stabilizers in bacteria as sterols do in eukaryotes. In spite of their widespread occurrence, their presence in bacteria was unsuspected until about 25 years ago, when bacteriohopanetetrol **1** was isolated from *Acetobacter xyllinum*.[1] Since then, over 40 different bacteriohopanoids have been isolated from several different species of bacteria.[2] Most structural variations involve modification of the polar C_5 chain, but methylation and/or unsaturation of the hopane nucleus is also observed. Very recently, our research group reported the isolation in remarkably large amounts of bacteriohopanetetrol **1** and the new 12-methyl derivative **2** from a sponge, namely *Plakortis simplex*.[3] While the primary origin of these compounds was considered bacterial, we suggested that the very unusual methylation at C-12 could be performed by the sponge. In addition, because of their concentration, we suggested that bacteriohopanoids could play a structural role in the sponge cell membranes.

A more detailed analysis of the lipophilic extract of *P. simplex* revealed the presence of even larger amounts of a further new bacteriohopanoid, 32,35-anhydrobacteriohopanetetrol (**3**). In this paper we describe the isolation and structure determination of the new bacteriohopanoid, which further supports the proposed biosynthetic pathway to bacteriohopanoids.

Keywords: marine metabolites; terpenes and terpenoids; natural products; polyols.

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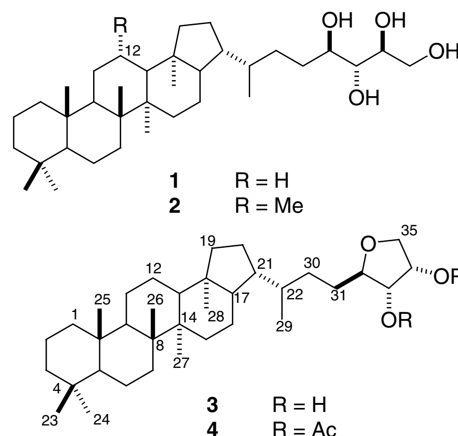

2. Results and discussion

Specimens of *P. simplex* (64 g of dry weight after extraction) were collected in the summer of 1998 along the coast of Berry Island (Bahamas), and extracted with methanol and chloroform. The organic extract was subjected, in sequence, to reversed- and direct-phase chromatography, giving a fraction mainly composed of the bacteriohopanoid **3**. Compound **3** could be isolated in the pure form as a white powder (121 mg) by crystallization from a mixture of *n*-hexane and chloroform.

The high resolution mass spectrum of compound **3** showed a molecular ion peak at *m/z* 529.4665 accounting for the molecular formula $C_{35}H_{60}O_3$. The ^1H and ^{13}C NMR spectra of compound **3** (pyridine-d_5) were very similar to those of compound **1**. In particular, all the high-field resonances in the ^{13}C NMR spectrum except those of C-22, C-30, and C-31 were within 0.1 ppm from the corresponding signals

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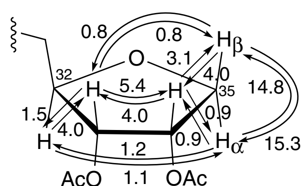


Figure 1. NOE enhancements (%) measured for compound **4** (saturated H→enhanced H).

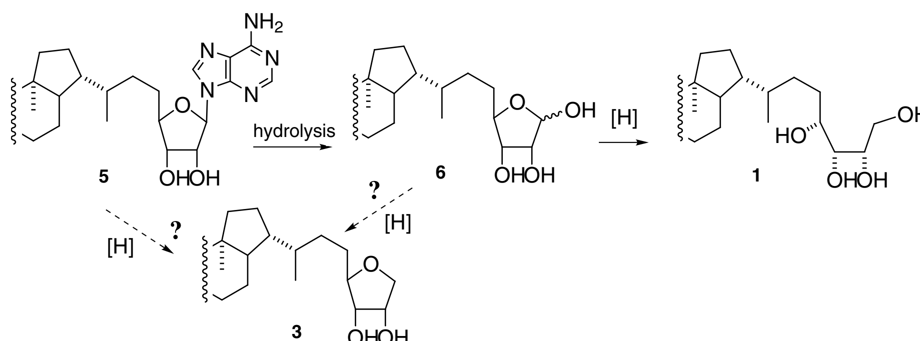
of compound **1**. On the other hand, the chemical shifts of oxygen-bearing carbon atoms were considerably shifted, even though one CH_2 and three CH were still present. These data clearly indicated that compound **3** is closely related to compound **1**, any difference being confined at the polyoxygenated C_5 side chain. In particular, on account of the molecular formula, compound **3** must be an anhydro derivative of bacteriohopanetetrol **1**.

The structure of the side chain of compound **3** was established on the basis of two-dimensional NMR data. For these experiments we switched to $CDCl_3$ as the solvent, because of the overlapping of two key side-chain protons (H-33 and one of the protons at C-35) in the ^1H NMR spectrum recorded in pyridine-d_5. The COSY spectrum was used to assign the side-chain proton signals. Starting from the oxymethylene protons at δ 4.11 and 3.69 (H-35a and H-35b) sequential couplings allowed us to identify the oxymethine protons at δ 4.24 (H-34), δ 3.76 (H-33), and δ 3.61 (H-32), and finally the methylene protons at δ 1.52 (H_2-31). Further assignments based on the COSY spectrum were not possible for the very close chemical shift of the subsequent protons; however, the HOHAHA spectrum showed a distinct correlation peak between H-32 and the methyl doublet H_3-29 (the only methyl doublet in the spectrum), confirming that the carbon skeleton of compounds **3** and **1** are in fact identical. An HMQC experiment allowed the assignment of the relevant carbon atoms (see Experimental).

The ether linkage between C-32 and C-35 was demonstrated with an HMBC experiment, displaying distinct correlation peaks of both protons at C-32 with C-35. These results were confirmed by peracetylation of compound **3** with Ac_2O in pyridine. The ^1H NMR (C_6D_6) spectrum of the peracetyl derivative **4** displayed two acetyl methyl singlets and, in the mid-field region, five signals, which were assigned to the relevant protons in the side chain on the basis of the COSY spectrum in a similar way as described for compound **3**. The presence of the ether linkage between C-32 and C-35 was apparent due to the shielded chemical field of the protons at C-35 (δ 3.70, H-35α and δ 3.89, H-35β) and C-32 (δ 4.04) compared with those of the acetoxymethine protons H-33 (δ 5.04) and H-34 (δ 5.32).

A series of one-dimensional NOE difference experiments were used to establish the relative configurations at the five-membered tetrahydrofuran ring of compound **3**. The experiments were best performed on the peracetyl derivative **4**, because in its ^1H NMR spectrum all signals of interest were at least 0.1 ppm apart; the results are shown in Fig. 1. As a general trend, in five membered rings NOEs can be observed between vicinal protons both *cis* and *trans* oriented, while a NOE between protons in the 1,3 relationship is indicative of their *cis* orientation. The NOE enhancement observed between H-33 and one of the protons at C-35 (H-35β) was indicative of their 1,3-*cis* relationship; saturation of H-34 caused the intensity of both vicinal protons at C-35 to increase, but the effect was far more intense for the *cis* proton H-35β (3.1% vs 0.9%); finally, the NOE enhancement detected for H-32 upon irradiation on H-35α indicated that these two protons laid on the same side of the ring. Therefore, the relative configurations of the stereogenic centers at the side chain of compound **4** (and consequently of compound **3**) were the same[†] as in compound **1**. As for the absolute configuration of the side chain, we assume it to be the same as in **1** because of the close relationship between the two compounds. This assumption is strengthened by biogenetic considerations.

Although the mechanism by which hopanes are converted into bacteriohopanoids in bacteria is not known in detail, it has been demonstrated that the C_5 side chain of most bacteriohopanoids derives from ribose, whose stereochemistry is kept in bacteriohopanetetrol.[4] The C-adenosyl derivative **5**, which has been isolated from *Rhodopseudomonas acidophila*,[5] and the C-ribosyl derivative **6**, never isolated from a natural source, have been proposed to be biosynthetic



Scheme 1. Proposed biosynthetic pathway to compound **3**.

[†] Even though the configuration at C-33 is the same in all compounds **2-4**, it is marked as *R* in compounds **2** and **4** and as *S* in compound **3**, because in the latter compound the alkylated oxygen atom at C-32 gives a higher rank to the relevant substituent.

intermediates[6] (Scheme 1). Compound **3** fits well into this biogenetic hypothesis, because it could be produced, rather than by cyclization of bacteriohopanetetrol **1**, through a branch of the above biogenetic sequence involving the reductive removal of the adenine of compound **5** (Scheme 1), or, alternatively, the reduction of the acetal function of compound **6**.

Like compounds **1** and **2**, 32,35-anhydrobacteriohopanetetrol **3** is present in large amounts in *P. simplex* and the total amount of the three bacteriohopanoids is as high as 50% in weight compared to steroids. This data strengthens our previous considerations of a possible structural role of bacteriohopanoids in the cell membranes of *P. simplex*.[3]

On the other hand, even if bacteriohopanoids are typical bacterial metabolites, and as such dietary metabolites of *P. simplex*, two out of the three compounds isolated from this sponge have never been reported from bacteria, in spite of the over 300 species of bacteria screened.[7] This can be explained (a) assuming that bacteria present in the marine environment of *P. simplex* carry on a unique biogenetic pathway, or (b) assuming that *P. simplex* is capable of further elaboration of dietary bacteriohopanoids (i.e. methylation at C-12 of dietary **1** to give compound **2**, and reduction of dietary **5** or **6** to give compound **3**). A study of bacterial strains isolated from *P. simplex* is in progress in our laboratory, and could allow us to clarify this point.

3. Experimental

3.1. General methods

FAB-MS spectra were performed in a glycerol matrix on a VG Prospec-Autospec (Fisons) mass spectrometer. Optical rotations were measured at 589 nm on a Perkin–Elmer 192 polarimeter using a 10-cm microcell. ^{1}H and ^{13}C NMR spectra were determined on a Bruker AMX-500 spectrometer at 500.13 and 125.77 MHz, respectively; chemical shifts were referenced to the residual solvent signal ($CDCl_3$: δ_H=7.26, δ_C=77.0; C_6D_6: δ_H=7.15, δ_C=128.0; pyridine-d$_5$: δ_H=8.71, 7.56, and 7.19, δ_C=149.8, 135.3, and 123.4). Homonuclear ^{1}H connectivities were determined by the COSY experiment. The reverse multiple-quantum heteronuclear correlation (HMQC) spectra were recorded by using a pulse sequence with a BIRD pulse 0.5 s before each scan to suppress the signal originating from protons not directly bound to ^{13}C; the interpulse delays were adjusted for an average $^{1}J_{CH}$ of 142 Hz. The gradient-enhanced multiple-bond heteronuclear correlation (HMBC) experiment was optimized for a $^{3}J_{CH}$ of 8.3 Hz. High performance liquid chromatographies (HPLC) were achieved on a Varian 2510 apparatus equipped with an Varian Star 9040 refractive index detector.

3.2. Collection, extraction and isolation

Specimens of *Plakortis simplex* were collected in the summer of 1998 near the coast of Berry Island (Bahamas), and identified by Professor M. Pansini (University of Genoa, Italy). They were frozen immediately after collection and kept frozen until extraction. Reference specimens were deposited at the Istituto di Zoologia, University of Genoa, Italy (voucher number 98-41). The sponge (64 g dry weight after extraction) was homogenized and extracted with methanol (3×1 L) and then with chloroform (2×1 L); the combined extracts were partitioned between H_2O and *n*-BuOH. The organic layer was concentrated in vacuo and afforded 20.4 g of a dark brown oil, which was chromatographed on a column packed with RP-18 silica gel. A fraction eluted with $CHCl_3$ (2.7 g) was further chromatographed on a SiO_2 column with solvent of increasing polarity. The sterol fraction (384 mg) was eluted with *n*-hexane/EtOAc (6:4), and was composed almost exclusively of common Δ^{5} and $\Delta^{5,7}$ sterols.[3] The subsequent fraction (164 mg), eluted with *n*-hexane/EtOAc (2:8), was mainly composed of the bacteriohopanoid **3**, that could be isolated in the pure form as white powder (121 mg) by crystallization from a mixture of *n*-hexane and chloroform.

3.2.1. (32*R*,33*S*,34*S*)-32,35-Anhydrobacteriohopanetetrol (3). White powder, mp 266-267°C (dec.); $[\alpha]_D$=+17 (pyridine, *c*=2.2); HRFABMS (positive ions): *m/z* 529.4665 ($[M+H]^+$, $C_{35}H_{61}O_3$ gives 529.4621); ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.24.(1H, q, *J*=4.9 Hz, H-34), 4.11 (1H, dd, *J*=10.0 and 5.4 Hz, H-35a), 3.76 (1H, t, *J*=6.1 Hz, H-33), 3.69 (1H, dd, *J*=10.0 and 3.9 Hz, H-35b), 3.61 (1H, m, H-32), 1.77 (H-20b), 1.73 (H-21), 1.70 (H-11eq), 1.63 (H-1eq), 1.63 (H-12), 1.52 (H-2ax), 1.52 (H-30b), 1.52 (H_2-31), 1.51 (H-11ax), 1.50 (H-16ax), 1.50 (H-20a), 1.48 (H-19a), 1.47 (H-22), 1.44 (H_2-7), 1.33 (H-3eq), 1.33 (H-15ax), 1.32 (H-2eq), 1.32 (H-6ax), 1.32 (H-6eq), 1.28 (H-13), 1.25 (H-16eq), 1.24 (H-17), 1.23 (H-9), 1.20 (H-15eq), 1.16 (H-30a), 1.11 (1H, ddd, *J*=13.2, 13.2, and 3.8 Hz, H-3ax), 0.93 (3H, s, H_3-26), 0.93 (3H, s, H_3-27), 0.92 (3H, d, *J*=6 Hz, H_3-29), 0.87 (H-19b), 0.84 (3H, s, H_3-23), 0.79 (3H, s, H_3-24), 0.79 (3H, s, H_3-28), 0.76 (H-1ax), 0.71 (H-5), 0.67 (3H, s, H_3-25); ^{13}C NMR (125 MHz, $CDCl_3$): δ 83.1 (CH, C-32), 75.4 (CH, C-33), 72.8 (CH_2, C-35), 70.5 (CH, C-34), 56.1 (CH, C-5), 54.4 (CH, C-17), 50.4 (CH, C-9), 49.2 (CH, C-13), 46.0 (CH, C-21), 44.3 (C, C-18), 42.1 (CH_2, C-3), 41.7 (C, C-8), 41.6 (C, C-14), 41.5 (CH_2, C-19), 40.3 (CH_2, C-1), 37.3 (C, C-10), 36.7 (CH, C-22), 33.6 (CH_2, C-15), 33.3 (CH_3, C-23), 33.2 (CH_2, C-7), 33.2 (C, C-4), 31.7 (CH_2, C-30), 30.1 (CH_2, C-31), 27.6 (CH_2, C-20), 23.9 (CH_2, C-12), 22.7 (CH_2, C-11), 21.5 (CH_3, C-24), 20.9 (CH_2, C-16), 20.0 (CH_3, C-29), 18.6 (CH_2, C-2), 18.6 (CH_2, C-6), 16.5 (CH_3, C-26), 16.5 (CH_3, C-27), 15.8 (CH_3, C-25), 15.8 (CH_3, C-28); ^{13}C NMR (125 MHz, pyridine-d$_5$): δ 83.0 (CH, C-32), 76.9 (CH, C-33), 73.5 (CH_2, C-35), 71.7 (CH, C-34), 56.4 (CH, C-5), 54.6 (CH, C-17), 50.7 (CH, C-9), 49.6 (CH, C-13), 46.4 (CH, C-21), 44.6 (C, C-18), 42.3 (CH_2, C-3), 42.0 (CH_2, C-19), 41.9 (C, C-8), 41.9 (C, C-14), 40.5 (CH_2, C-1), 37.6 (C, C-10), 37.0 (CH, C-22), 34.0 (CH_2, C-15), 33.6 (CH_3, C-23), 33.6 (CH_2, C-7), 33.4 (C, C-4), 32.4 (CH_2, C-30), 30.8 (CH_2, C-31), 27.9 (CH_2, C-20), 24.2 (CH_2, C-12), 23.1 (CH_2, C-16), 21.8 (CH_3, C-24), 21.2 (CH_2, C-11), 20.5 (CH_3, C-29), 19.0 (CH_2, C-2), 19.0 (CH_2, C-6), 16.7 (CH_3, C-26), 16.7 (CH_3, C-27), 16.1 (CH_3, C-25), 16.1 (CH_3, C-28).

3.2.2. (32*R*,33*R*,34*S*)-32,35-Anhydrobacteriohopanetetrol diacetate (4). Compound **3** (6 mg) was dissolved in 500 μl of dry pyridine, and acetic anhydride (100 μl) was added.

After 12 h, the reaction mixture was dried under vacuum, giving 6.9 mg of compound **4** as a colorless glass: $[\alpha]_D=+54$ ($CHCl_3$, $c=0.7$); HREIMS: *m/z* 612.4788 ($[M]^+$, $C_{39}H_{64}O_5$ gives 612.4754); ^1H NMR (500 MHz, C_6D_6): δ 5.32 (1H, ddd, $J=5.4$, 5.4, and 4.2 Hz, H-34), 5.04 (1H, dd, $J=6.8$ and 5.7 Hz, H-33), 4.04 (1H, ddd, $J=7.3$, 7.3, and 4.2 Hz, H-32), 3.89 (1H, dd, $J=10.2$ and 5.5 Hz, H-35β), 3.70 (1H, dd, $J=10.2$ and 4.0 Hz, H-35α), 1.71 (3H, s, Ac), 1.65 (3H, s, Ac), 0.99 (3H, d, $J=7$ Hz, H_3-29), 0.98 (3H, s), 0.97 (3H, s), 0.92 (3H, s), 0.87 (3H, s), 0.86 (3H, s), 0.73 (3H, s).

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