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The First 12-Methylhopanoid: 12-Methylbacteriohopanetetrol from the Marine Sponge *Plakortis simplex*

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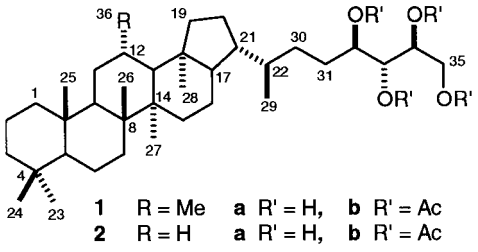
Abstract—Large amounts of bacteriohopanoids have been isolated from the marine sponge *Plakortis simplex*. In addition to the common bacteriohopanetetrol, the new 12-methylbacteriohopanetetrol, with an unprecedented methylation at C-12, was isolated and characterized by an extensive NMR analysis. While bacteriohopanetetrol is most likely of dietary origin, the unusual methylation at C-12 could be carried out by the sponge.

Introduction

Bacteriohopanoids are unique mixed-biogenesis compounds, in which a triterpenoid with the hopane skeleton is linked through the isopropyl group to a sugar-derived polyfunctionalized C_5 side chain. These compounds are characteristic of bacteria, where they play an important role in maintaining the stability of cellular membrane similar to that of sterols in higher organisms.[1] In addition to the simple tetrol **2a** (bacteriohopanetetrol),[2] several more complex bacteriohopanoids have been found to date, e.g. glycosylated derivatives[3] or compounds with additional hydroxyl groups.[4] In many cases, the primary hydroxyl group can be replaced by an amino group, which can be free,[5] or amide-linked to an aminoacid such as ornithine or triptophane.[6] Some of these compounds possess an additional methyl group at position 2 or 3, as well as a double bond at positions 6 and/or 11. During our continuing study of amphiphilic compounds from marine sponges, we have now isolated from the Caribbean sponge *Plakortis simplex* large amounts of two bacteriohopanoids, the well known bacteriohopanetetrol (**2a**) and 12-methylbacteriohopanetetrol (**1a**), a new bacteriohopanoid characterized by the methylation at position 12 of the hopane ring. This is an unprecedented feature not only among bacteriohopanoids, but also among all the natural terpenes based on the hopane skeleton.

Results

Specimens of *Plakortis simplex* were extracted, in sequence, with methanol and chloroform, and the extract partitioned between water and *n*-BuOH. A fraction enriched in bacteriohopanoids was obtained by subjecting the organic extract to subsequent RP-18 and SiO_2 column chromatographies. This fraction was acetylated with Ac_2O/Py and subjected to repeated direct-phase HPLC purification, yielding a fraction composed of peracetylated bacteriohopanetetrol (**2b**) and 12-methylbacteriohopanetetrol (**1b**). The two compounds were separated by reversed phase chromatography to give 18 mg of pure **1b** and 37 mg of pure **2b**. Natural tetrols **1a** and **2a** could be then obtained by methanolysis of the respective peracetates with 10% Et_3N in MeOH at 60°C for 12 h.



1 R = Me **a** R' = H, **b** R' = Ac
2 R = H **a** R' = H, **b** R' = Ac

Compound **2a** was identified as (32*R*,33*R*,34*S*)-32,33,34,35-bacteriohopanetetrol by comparison of the ^1H and ^{13}C NMR spectra of its peracetate **2b** with the data reported for the eight different side-chain diastereomers of bacteriohopanetetrol peracetate.[7] Because no ^{13}C NMR data of the natural (non-acetylated) bacteriohopanetetrol **2a** is reported in the literature, we also registered and assigned the ^{13}C NMR spectrum of compound **2a** (see Experimental).

The high resolution FAB mass spectrum of compound **1a** showed the peak of the pseudomolecular $[M+H]^+$ ion at *m/z* 561.4846 (calcd 561.4883), accounting for compound **1a** having a molecular formula $C_{36}H_{64}O_4$, i.e. one additional

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

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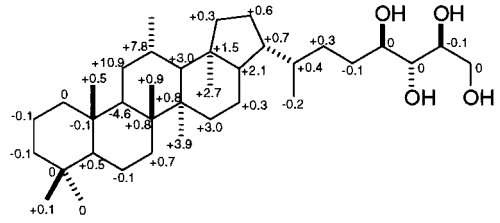

Figure 1. ^{13}C NMR shifts induced by the additional methyl group at C-12 [δ_C(**1a**)−δ_C(**2a**)].

CH_2 compared to compound **2a**. The low-field region of the ^1H NMR spectrum of **1a** (pyridine-d_5) was identical with that of bacteriohopanetetrol **2a**, thus indicating that compound **1a** possesses a sugar-derived side chain with the same substitution pattern and stereochemistry as compound **2a**. In contrast, ^1H chemical shifts of most methyl groups were remarkably different, and one extra methyl doublet was present. This suggested that an additional methyl group could be linked to a former methylene of bacteriohopanetetrol. Examination of the ^{13}C NMR spectra of compounds **1a** and **2a** showed that most resonances were significantly shifted (the ^{13}C NMR shifts induced by the additional methyl group, as determined after structural elucidation was completed, are shown in Fig. 1). Therefore, not only location of the additional methyl group, but also demonstration that compound **1a** had indeed a hopane skeleton was impossible on the basis of simple spectral comparison, and structural elucidation of compound **1a** required extensive two-dimensional NMR analysis.

The polycyclic skeleton was devoid of functional groups, and most proton signals were severely overlapping. As a consequence, the most useful data came from the ^1H–^{13}C long-range couplings of the well-resolved methyl protons, evidenced by an HMBC experiment. All the theoretical cross peaks of these protons were clearly visible in the HMBC spectrum, and allowed unequivocal assignment of all the carbon atoms within 3 bonds from methyl protons. The HMQC spectrum was then used to identify the relevant protons.

The carbon framework deduced from the above arguments is depicted in Fig. 2 with bold-faced lines, and can be well accommodated in a hopane structure carrying a methyl group at C-12. The remaining connectivities needed to build up the hopane skeleton of compound **1a** are outlined with dashed lines; they were demonstrated on the basis of the following evidence.

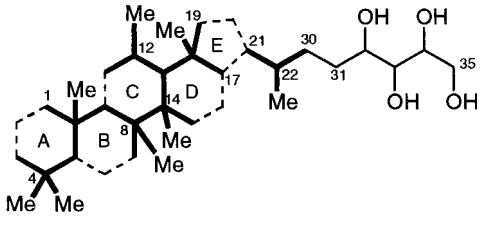

Figure 2. Partial structure of compound **1a** as determined by long-range proton-carbon coupling constants of methyl protons.

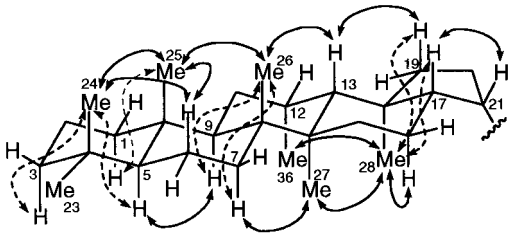

Figure 3. ROESY correlations (solid arrows) and ^1H–^1H long-range couplings (dashed arrows) observed for compound **1a**.

Protons at position 2 were identified from their COSY correlation peaks with the very shielded H-1ax (δ 0.73); the relevant carbon C-2 was coupled with both protons H-3ax and H-3eq, thus defining ring A. As for ring B, a cross peak between H-5 (δ 0.76) and two protons attributable to H-6ax and H-6eq was present in the COSY spectrum, while the same proton H-5 gave rise to a correlation peak with C-7 in the HMBC spectrum. The bond between C-9 and C-11 was deduced by the correlation peak of H-11ax (δ 1.50) with H-9 (δ 1.37) in the COSY spectrum. Several correlation peaks in the HMBC spectrum were used to define the five-membered ring E: H-19a was coupled with C-20 and C-21, and H-20a was coupled with C-17 and C-18. Finally, the remaining methylene group (C-16) could only be positioned between C-15 and C-17.

As for the stereochemistry of the hopane nucleus of compound **1a**, useful information was provided by the ROESY spectrum, that displayed a number of correlation peaks between methyl groups and ring protons in the 1,3 diaxial relationship (see Fig. 3). Thus, H_3-24, H-6ax, H_3-25, H_3-26, and H-13 were shown to be all located on the upper face of the molecule, whereas H_3-27, H_3-28, H_3-36, and H-7ax were all on its lower face. In addition, several correlation peaks were present in the COSY spectrum indicative of the four-bond coupling (W coupling) of an angular methyl group with a ring proton. Such couplings (see Fig. 3) are only possible when the dihedral angle between the methyl group and the ring proton is near 180°, and were therefore used to prove the *trans* junction between ring B and ring C (coupling H_3-27/H-9), between ring D and ring E (coupling H_3-28/H-17) and between ring A and ring B (indirectly, coupling H_3-24/H-5), as well as for stereochemical assignment of methylene protons as shown in Table 1.

All these data confirmed that the stereochemistry of compound **1a** was the same as in previously isolated bacteriohopanoids; in addition, the orientation of the additional methyl group at C-12 could be defined as α.

Discussion

To the best of our knowledge, this is the first report of bacteriohopanoids from a sponge, and more generally from an eukaryote. Since all the bacteriohopanoids reported so far have been isolated from bacteria, these compounds are very probably obtained by the *P. simplex* from dietary or symbiotic bacteria. In fact, compounds **1a** and **2a** have been isolated in relatively large amounts: this on the one hand

Table 1. ^{1}H and ^{13}C NMR data of compound **1a** (pyridine-d_5) (assignment based on COSY, ROESY, HMQC, and HMBC experiments)

Position		δ_H (mult., J in Hz)	δ_C (mult.)
1	ax	0.73 (ddd, 12.7, 12.7, 3.0)	40.5 (CH_2)
	eq	1.63[a]	
2	ax	1.56[a]	18.9 (CH_2)
	eq	1.35[a]	
3	ax	1.14[a]	42.2 (CH_2)
	eq	1.34[a]	
4		–	33.4 (C)
5		0.76 (br. d, 11.7)	56.9 (CH)
	ax	1.23[a]	
6	ax	1.46[a]	18.9 (CH_2)
	eq	1.51[a]	
7	ax	1.51	34.9 (CH_2)
	eq	1.28[a]	
8		–	42.7 (C)
9		1.37[a]	46.1 (CH)
10		–	37.5 (C)
11	ax	1.50[a]	32.1 (CH_2)
	eq	1.14[a]	
12		2.12 (m)	32.0 (CH)
13		1.33[a]	52.6 (CH)
14		–	42.7 (C)
15	ax	1.36[a]	36.9 (CH_2)
	eq	1.16[a]	
16	ax	1.47[a]	23.4 (CH_2)
	eq	1.65[a]	
17		1.16[a]	56.8 (CH)
18		–	46.0 (C)
19	a	1.57[a]	42.2 (CH_2)
	b	0.93 (m)	
20	a	1.65[a]	28.6 (CH_2)
	b	1.88 (br. ddd, 13.3, 8.9, 8.9)	
21		1.77 (dddd, 9.3, 9.3, 9.3, 5.7)	46.7 (CH)
22		1.66[a]	37.7 (CH)
23		0.88 (s)	33.6 (CH_3)
24		0.80 (s)	21.9 (CH_3)
25		0.83 (s)	16.6 (CH_3)
26		1.00 (s)	17.6 (CH_3)
27		1.16 (s)	20.6 (CH_3)
28		0.98 (s)	18.8 (CH_3)
29		1.05 (d, 5.8)	20.4 (CH_3)
30	a	1.94 (br. ddd, 11.0, 11.0, 6.0)	32.7 (CH_2)
	b	1.64[a]	
31	a	2.15 (m)	30.3 (CH_2)
	b	2.08 (m)	
32		5.41 (m)	74.3 (CH)
33		4.35 (t, 6.1)	76.3 (CH)
34		4.57[a]	75.1 (CH)
35	a	4.58[a]	65.2 (CH_2)
	b	4.42 (dd, 12.2, 7.1)	
36		1.17 (d, 7.3)	19.9 (CH_3)

[a] Overlapping signal.

seems to rule out the possibility of simple contamination of bacteria, and on the other hand suggests that *P. simplex* is capable of actively incorporating and accumulating the bacteriohopanoids, possibly in order to take some advantage from their presence. Bacteriohopanoids are involved in the modulation of the fluidity of the cellular membrane of prokariotes in the same way as sterols in eukaryotes. While a well-grounded hypothesis about the biological significance, if any, of bacteriohopanoids in *P. simplex* is not possible at the moment, it can be noted that a structural role of these compounds in the sponge cell membrane would not be impossible on account of their large amounts, about 20% compared to steroids.

Another interesting point is the methylation at position 12 of the hopane skeleton. In spite of the large number of species studied and the variety of compounds isolated, a natural 12-methylbacteriohopanoid has never been found. Marine sponges have been shown to be able to further elaborate dietary metabolites (such as fatty acids[8] and sterols[9]). Therefore, while the bacterial origin of *P. simplex* hopanoids appears unquestionable, it seems not unreasonable to hypothesize that the unusual methylation at C-12 could be a structural modification carried out by the sponge.

Experimental

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 (C_6D_6: δ_H=7.15, δ_C=128.0; CD_5N: 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. Through-space ^{1}H connectivities were evidenced using a ROESY experiment with a mixing time of 500 ms. 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 a Varian Star 9040 refractive index detector.

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 Prof. 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. The sponge (64 g of 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, as judged by the ^{1}H NMR spectrum; it was not further analyzed. The fraction (448 mg) eluted with EtOAc/MeOH (9:1) was mainly composed of glycolipids, but contained also compounds **1a** and **2a**, which possess comparable amphiphilic properties. This fraction was peracetylated with Ac_2O in pyridine for 12 h and separated by repeated normal-phase

HPLC [eluents: *n*-hexane/EtOAc (6:4) and *n*-hexane/EtOAc (8:2)] to give a mixture of **1b** and **2b** (75 mg). Pure compounds **1b** (18 mg) and **2b** (37 mg) could be obtained from this mixture by reversed-phase HPLC on an RP-18 column, using MeOH as eluent. Natural (non-acetylated) compounds **1a** and **2a** were obtained in quantitative yield by keeping the respective peracetates in a mixture of 800 μL of MeOH and 200 μL of Et_3N for 24 h at 60°C.

(32*R*,33*R*,34*S*)-12α-Methyl-32,33,34,35-bacteriohopanetetrol (1a). White powder, $[\alpha]_D=+7$ ($CHCl_3$, $c=0.5$); HRFAMBS (positive ions): *m/z* 561.4846 ($[M+H]^+$, $C_{36}H_{65}O_4$ gives 561.4883); ^1H and ^{13}C NMR: see Table 1.

(32*R*,33*R*,34*S*)-12α-Methyl-32,33,34,35-bacteriohopanetetrol tetraacetate (1b). White powder $[\alpha]_D=+32$ ($CHCl_3$, $c=0.5$); HREIMS: *m/z* 728.5262 (M^+, $C_{44}H_{72}O_8$ gives 728.5227); ^1H NMR (500 MHz, C_6D_6): δ 5.66 (1H, dd, $J=5.8$ and 4.3 Hz, H-33), δ 5.60 (1H, ddd, $J=6.6$, 5.8 and 2.5 Hz, H-34), δ 5.41 (1H, ddd, $J=9.6$, 4.3, and 4.3 Hz, H-32), δ 4.55 (1H, dd, $J=12.5$ and 2.5 Hz, H-35a), δ 4.28 (1H, dd, $J=12.2$ and 6.6 Hz, H-35b), 2.17 (1H, m, H-12), 1.87 (1H, br. ddd, $J=13.5$, 9.0 and 9.0 Hz, H-20a), 1.83–0.96 (many overlapping signals), 1.23 (3H, d, $J=7.5$ Hz, H_3-36), 1.21 (3H, s, H_3-27), 1.07 (3H, s, H_3-28), 1.04 (3H, s, H_3-26), 1.00 (3H, d, $J=6.5$ Hz, H_3-29), 0.93 (3H, s, H_3-23), 0.90 (3H, s, H_3-25), 0.88 (3H, s, H_3-24), 0.81 (1H, br. d, $J=12.2$ Hz, H-5), 0.78 (overlapping, H-1ax); ^{13}C NMR (125 MHz, C_6D_6): δ 72.0 (CH, C-32), 72.0 (CH, C-33), 70.0 (CH, C-34), 62.3 (CH_2, C-35), 57.0 (CH, C-5), 56.8 (CH, C-17), 52.8 (CH, C-13), 46.3 (CH, C-21), 46.2 (CH, C-9), 46.1 (C, C-18), 43.4 (C, C-14), 42.7 (C, C-8), 42.3 (CH_2, C-3), 42.3 (CH_2, C-19), 40.7 (CH_2, C-1), 37.6 (C, C-10), 37.0 (CH_2, C-15), 36.7 (CH, C-22), 35.0 (CH_2, C-7), 33.6 (CH_3, C-23), 33.5 (C, C-4), 32.2 (CH_2, C-11), 32.1 (CH, C-12), 31.6 (CH_2, C-30), 28.4 (CH_2, C-20), 26.3 (CH_2, C-31), 23.5 (CH_2, C-16), 21.9 (CH_3, C-24), 20.7 (CH_3, C-27), 19.9 (CH_3, C-29), 19.0 (CH_2, C-6), 19.0 (CH_2, C-2), 18.8 (CH_3, C-28), 17.7 (CH_3, C-26), 16.6 (CH_3, C-25).

(32*R*,33*R*,34*S*)-32,33,34,35-Bacteriohopanetetrol (2a). FABMS (positive ions): *m/z* 547 ($[M+H]^+$); ^1H NMR (500 MHz, pyridine-d_5): 1.05 (3H, d, $J=5.5$ Hz, H_3-29), 0.94 (3H, s), 0.92 (3H, s), 0.86 (3H, s), 0.79 (6H, s), 0.67 (3H, s); ^{13}C NMR (125 MHz, pyridine-d_5): δ 76.3 (CH, C-33), 75.2 (CH, C-34), 74.3 (CH, C-32), 65.2 (CH_2, C-35), 56.4 (CH, C-5), 54.7 (CH, C-17), 50.7 (CH, C-9), 49.6 (CH, C-13), 46.0 (CH, C-21), 44.5 (C, C-18), 42.3 (CH_2, C-3), 41.9 (C, C-8), 41.9 (C, C-14), 41.9 (CH_2, C-19), 40.5 (CH_2, C-1), 37.6 (C, C-10), 37.3 (CH, C-22), 33.9 (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), 30.4 (CH_2, C-31), 28.0 (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), 20.6 (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).

(32*R*,33*R*,34*S*)-32,33,34,35-Bacteriohopanetetrol tetraacetate (2b). EIMS, ^1H-, and ^{13}C NMR spectra ($CDCl_3$) matched those reported.[6,7]

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References

1. Ourisson, G.; Rohmer, M. *Acc. Chem. Res.* **1992**, *25*, 403–408.
2. Rohmer, M., et al., *Tetrahedron Lett.* **1976**, 3633–3636.
3. Simonin, P., et al., *Tetrahedron Lett.* **1992**, *33*, 3629–3632.
4. Zhao, N.; Berova, N.; Nakanishi, K.; Rohmer, M.; Mougenot, P.; Jürgens, U. J. *Tetrahedron* **1996**, *52*, 2777–2788.
5. Neunlist, S.; Holst, O.; Rohmer, M. *Biochem. J.* **1985**, 635–639.
6. Neunlist, S.; Holst, O.; Rohmer, M. *Eur. J. Biochem.* **1985**, *147*, 561–568.
7. Bisseret, P.; Rohmer, M. *J. Org. Chem.* **1988**, *54*, 2958–2964.
8. Djerassi, C.; Lam, W. *Acc. Chem. Res.* **1991**, *24*, 69–75.
9. (a) Kerr, H. G.; Baker B. *J. Nat. Prod. Rep.* **1991**, *8*, 465–497; (b) Aiello, A.; Fattorusso, E.; Menna, M. *Steroids* **1999**, *64*, 687–714.