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NOTE

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1-Hydroxy-1-norresistomycin and Resistoflavin Methyl Ether: New Antibiotics from Marine-derived Streptomycetes[†,††]

Ines Kock, Rajendra P. Maskey, M. A. Farooq Biabani, Elisabeth Helmke, Hartmut Laatsch

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Abstract Cultivation of the marine-derived streptomycete isolate B8005 delivered three known antibiotics, resistomycin (**1**), resistoflavin (**3a**) and tetracenomycin (**4**), and a further member of the rare resistomycin class, the weakly antibiotically active 1-hydroxy-1-norresistomycin (**2**). From a related marine strain B4842, **1** and resistoflavin methyl ether (**3b**) have been isolated. The formation of **2** is of interest from a biosynthetic point of view.

Keywords resistomycin, 1-hydroxy-1-norresistomycin, marine streptomycetes, natural product, structure elucidation

In the course of our screening of streptomycetes for new bio-active secondary metabolites, extracts of the marine *Streptomyces* isolate B8005 showed inhibitory activity against *Escherichia coli*, *Staphylococcus aureus*, *Streptomyces viridochromogenes* (Tü 57), *Mucor miehei*, *Candida albicans*, and the microalga *Chlorella vulgaris*. Additionally it possessed cytotoxic activity against *Artemia salina*. In the TLC screening, the extract showed moderately polar yellow and red spots. Work-up delivered three known antibiotics, resistomycin (**1**) [1, 2], resistoflavin (**3a**) [1, 3] and tetracenomycin (**4**) [4], and a new resistomycin derivative, 1-hydroxy-1-norresistomycin (**2**), as a further active principle. From a second marine *Streptomyces* isolate B4842, resistoflavin methyl ether (**3b**) has been isolated, along with **1**. Resistomycin (**1**) is a quinone-related antibiotic with unique structure and possesses bactericidal and vasoconstrictive activity. It inhibits RNA and protein synthesis, but has no effect on DNA synthesis [5]. It was so far the only further member of this type of compounds.

The *Streptomyces* strain B8005 has been derived from sediment of the Laguna de Terminos at the Gulf of Mexico and was isolated on M3-medium [6]. The partial 16S rRNA gene sequence of the strain B8005 is identical with that of the strain *Streptomyces albogriseolus* (DSM 40003) and *Streptomyces viridodiastaticus* (DSM 40249). However, the strain B8005 differs from both strains in forming a chocolate brown substrate mycelium. The spore mass is gray, spore chains are spiral; the surface of spores is spiny. Melanin pigment is neither produced on peptone - yeast - extract iron agar [7] nor on tyrosine agar [7] but a chocolate brown pigment is found in media like yeast - extract malt - extract agar [8] and chitin agar [8]. The optimum growth temperature is at about 30°C. The strain does not grow at 10°C but shows growth at 45°C. Growth occurred in media from 0% up to 10% seawater salinity. Chitin, cellulose, starch, casein, gelatine, and esculin are degraded. The strain is catalase and nitrate reductase positive. The use of

H. Laatsch (Corresponding author), **I. Kock, R. P. Maskey, M. A. Farooq Biabani:** Department of Organic and Biomolecular Chemistry, University of Göttingen, Tammanstrasse 2, D-37077 Göttingen, Germany, E-mail: HLAATSC@gwdg.de
E. Helmke: Alfred-Wegener-Institute for Polar and Marine Research, Am Handelshafen 12, D-27570 Bremerhaven, Germany

[†]Art. No. XXIX on Marine Bacteria. Art. XXVIII: R. P. Maskey, E. Helmke, O. Kayser, A. Maier, H. H. Fiebig, A. Busche, H. Laatsch: Anti-cancer and antibacterial trioxacarcins with high anti-plasmodial activity from a marine *Streptomycete*. J Antibiot 57, 771–779 (2004).

[††]The same compound (1-hydroxy-1-norresistomycin) which was isolated independently from different strains appears in this issue (pages 526–529).

carbon sources was tested with SFN2-Biolog plates (Hayward, CA, USA) using BMS-N as basal medium [9]. Growth was obtained with: α-cyclodextrin, dextrin, glycogen, tween 40, tween 80, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, D-arabitol, cellobiose, D-fructose, D-galactose, gentiobiose, glucose, m-inositol, α-D-lactose, lactulose, maltose, D-mannitol, D-mannose, D-melibiose, β-methyl-D-glucoside, L-rhamnose, sucrose, D-trehalose, turanose, methylpyruvate, mono-methylsuccinate, citric acid, D-glucoronic acid, α-hydroxy butyric acid, β-hydroxy butyric acid, keto butyric acid, α-keto valeric acid, sebacic acid, succinic acid, bromosuccinic acid, glucuronamide, alaninamide, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, L-leucine, L-ornithine, L-phenylalanine, proline, L-pyroglutamic acid, serine, L-threonine, γ-amino butyric acide, urocanic acide, inosine, uridine, thymidine, 2-aminoethanol, glycerol, glucose-6-phosphate. The strain B4842 from mud sediment of a coastal site of Mauritius (Indian Ocean) was isolated on Olson medium containing 22 g actinomycete isolation agar (Difco) and 5 g glycerol in 1 litre of 50% natural sea water. The strain showed similar properties as B8005. Reference cultures of both strains are held in the Collection of Marine Actinomycetes at the Alfred-Wegener-Institute for Polar and Marine Research in Bremerhaven.

The *Streptomyces* strain B8005 was fermented on a 15 litre scale. After defatting with cyclohexane, the crude ethyl acetate extract was pre-separated on a medium pressure silica gel column with a chloroform/methanol gradient into four fractions. Further purification of the fractions on Sephadex LH-20 delivered a new antibiotic, 1-hydroxy-1-norresistomycin [10] (**2**). In addition, three known quinones, resistomycin (**1**), resistoflavin (**3a**) and tetracenomycin D (**4**) were obtained as yellow (**1**, **3a**) and red (**4**) solids, respectively, which showed colour reactions with dilute sodium hydroxide characteristic of peri-hydroxy quinones. In a similar manner, a 20 litre batch fermentation of *S.* sp. B4842 delivered after usual work-up 44 mg of **1** and 2.4 mg of **3b**. The known compounds were identified by a search in AntiBase [11] using the molecular weight and the ^{1}H NMR data, and by comparison with reference

Table 1 ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data of resistomycin (**1**), 1-hydroxy-1-norresistomycin (**2**) and resistoflavin methyl ether (**3b**)

	^{1}H NMR			^{13}C NMR		
C No.	**1**[a)]	**2**[a)]	**3b**[b)]	**1**[a)]	**2**[a)]	**3b**[a)]
1	—	—		46.0	74.1	46.3
2	—	—		204.7	204.3	203.3
2a	—	—		102.5	102.5	108.5
2b	—	—		139.3	139.2	140.4
3	14.42 (s, OH)	14.55 (s, OH)	13.60 (s, OH)	170.1	169.9	170.1
4	6.29 (s)	6.39 (s)	6.54 (s)	100.1	100.3	103.2
5	14.30 (s, OH)	14.00 (s, OH)	12.85 (s, OH)	169.5	169.7	168.0
5a	—	—	—	105.6	106.3	106.9
6	—	—	—	184.2	184.3	184.4
6a	—	—	—	106.2	107.0	108.5
6b	—	—	—	128.3	129.0	149.3
7	13.92 (s, OH)	13.61 (s, OH)	12.72 (s, OH)	167.7	168.2	168.0
8	6.93 (s)	7.08 (s)	6.95 (s)	119.1	119.4	121.0
9	—	—	—	151.5	152.5	150.1
9a	—	—	—	114.0	115.0	120.2
10	—	6.32 (s, OH)	—	162.3	160.4	179.8
11	7.21 (s)	7.48 (s)	6.60 (s)	109.6	110.2	127.0
11a	—	—	—	152.1	152.3	156.7
11b	—	—	—	107.2	106.9	68.2
1-Me	1.56 (s)	1.48 (s)	1.56 (s)	25.5	25.8	24.9
9-Me	2.87 (s)	2.94 (s)	2.78 (s)	28.4	33.3	24.2
OMe	—	—	3.40 (s)	—	—	58.0

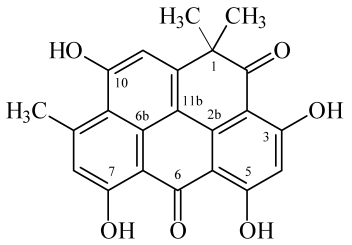
a) [D_6]DMSO, b) $CDCl_3$;

data.

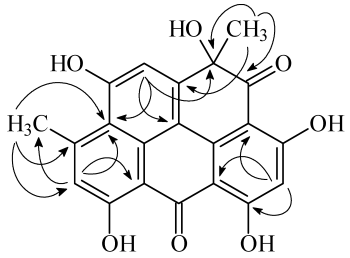
Compound **2** was obtained as an orange solid with the molecular weight of 378 Dalton and the molecular formula $C_{21}H_{14}O_7$ (EI HRMS, *m*/*z* 378.0739). The ^1H and ^{13}C NMR data of this compound were very similar to those of resistomycin (**1**). The ^1H NMR showed three deep-field signals of chelated hydroxy groups at δ 14.55, 14.00 and 13.61, three aromatic ^1H singlets at δ 7.48, 7.08 and 6.39 and a signal of an aromatic methyl group at δ 2.94. The aliphatic methyl singlet at δ 1.48 indicated the intensity of only three protons, in contrast to six in **1**, and there was an additional H/D exchangeable signal at δ 6.32 in **2**. The ^{13}C NMR spectrum delivered signals for two carbonyl groups, four aromatic carbons bearing oxygen, twelve additional aromatic carbons, one aromatic methyl and one aliphatic methyl group with similar chemical shift as of resistomycin (**1**). The only major shift difference was displayed between the aliphatic quaternary carbon at δ 46.0 (C-1) in **1** and the aliphatic hydroxylated carbon atom at δ 74.1 (C-1) in **2**. The detailed evaluation of the ^1H, ^{13}C and the HMBC data (see arrows in formula **2**) in comparison with the molecular formulae of **1** and of the new resistomycin derivative showed the latter to be the new 1-hydroxy-1-nor derivative **2**, where one of the aliphatic methyl group at C-1 in **1** was replaced by an OH group. As a consequence, **2** is optically active, where **1** is not. Resistomycin (**1**) is a decaketide, where the geminal C-1 methyl groups are introduced as C1 units by means of adenosyl-methionine [12]. It seems plausible that the biosyntheses of **1** and **2** differ in this final step.

In addition to **1**, compound **3b** was obtained from extracts of *S.* sp. B4842 as a yellow solid with the molecular formula $C_{23}H_{18}O_7$, as inferred from EI-HRMS, *m*/*z* 406.1051. Its IR spectrum showed a broadened hydroxyl absorption at 3400, and signals at 1660, 1640 and 1600 cm^{-1} which indicated the presence of two or more conjugated carbonyls. In addition to three chelated OH groups and three ^1H singlets of aromatic protons, the ^1H NMR spectrum showed a methoxyl signal at δ 3.40, an aromatic methyl, and geminal methyl groups at δ 1.56. These data were comparable with the resistoflavin methyl ether **3b**, which was further confirmed by a ^{13}C NMR signal at δ 58.0. Indeed, an identical product was obtained, when **3a** was refluxed in methanol in the presence of *p*-toluene sulfonic acid. When we re-cultivated the strain B4842 and strictly avoided methanol during work-up, **3b** was not detectable by TLC or HPLC diode array screening; resistoflavin (**3a**) was isolated instead. It cannot be excluded therefore that **3b** was generated as an artifact during the isolation procedure.

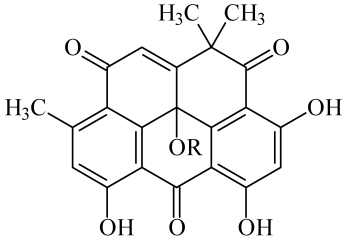
Resistomycin (**1**) [1, 2], resistoflavin (**3a**) [2, 3] and tetracenomycin D (**4**) [4] showed antibacterial and antitumor activity [13, 14] as reported in the literature. Compounds **1** and **3a** were mentioned to have also antiviral [15] and antiprotozoan activity [3]. The new 1-hydroxy-1-norresistomycin (**2**) showed antibacterial activity against



1



2



3a: R = H
3b: R = Me

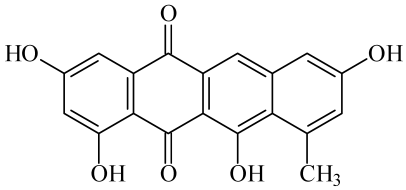


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Table 2 Physico-chemical properties of 1-hydroxy-1-norresistomycin (**2**) and resistoflavin methyl ether (**3b**)

	2	**3b**
Nature	Orange solid	Yellow solid
Molecular formula	$C_{21}H_{14}O_7$	$C_{23}H_{18}O_7$
Rf	0.57 ($CHCl_3$/10% MeOH)	0.42 ($CHCl_3$/30% MeOH)
m.p.		244–246°C
EI MS (70 eV)	378.1 ([M^+], 40), 363.1 (55), 268.0 (50).	406.3 ([M^+]), 391 ([M^+]–Me)
EI HRMS	378.0739 (calcd. 378.07395)	406.1051 (calcd. 406.10525)
UV/VIS λ_{max} [nm]	204, 288, 464, 516 nm (MeOH)	303, 379, 539, 547 nm (MeCN)
CD (MeOH) λ_{max} (Θ)	203.0 (+9003), 240.4 (−943), 338.8 (+1220), 367.0 (−138), 417.4 (+540) nm	—
IR (KBr) ν (cm^{-1})	3422 (OH), 2924, 2854, 1639 (C=O), 1594 (C=C), 1145 (C–O), 1085 (C–O), 629	3300–3540 (br), 1650, 1640, 1600, 1300, 1290, 780

Escherichia coli, *Staphylococcus aureus* and *Streptomyces viridochromogenes* (Tü 57), the activity (MIC >40 µg/ml) was, however, weaker than that of **1**. Resistoflavin methyl ether (**3b**) showed MIC values of 3.1 µg/ml against *Bacillus subtilis* (**1**: 6.2 µg/ml). Activity against *Escherichia coli*, *Streptococcus aureus*, and *Candida albicans* using the agar diffusion method was found at 10 µg/ml. Resistomycin (**1**) and resistoflavin methyl ether (**3b**) showed also antioxidative properties using the DPPH method [16].

Experimental

Materials and Methods

IR spectra were recorded on a Perkin-Elmer 1600 Series FT-IR spectrometer as KBr pellets. UV-VIS-spectrum were recorded on Perkin-Elmer Lamda 15 UV/VIS spectrometer. NMR spectra were measured on Varian VXR 200, VXR 300, VXR 500 (tetramethylsilane as internal standard). Mass spectra were recorded on a Finnigan MAT 95 instrument (70 eV, high resolution with perfluorokerosene as reference). Rf values were measured on Polygram SIL G/UV254 (Macherey-Nagel & Co.). Column chromatography: Silica gel 60 (0.05~0.2 mm and 0.04~0.063 mm/230~400 mesh, Macherey-Nagel & CO). Assay discs: i.d. 9 mm, Schleicher & Schuell, Dassel, Germany. Size exclusion chromatography was done on Sephadex LH-20 (Pharmacia). High speed counter current chromatography was done on a HSCCC Chromatograph (P. C. Inc., model HSCCC).

Fermentation of *Streptomyces* sp. B8005

Agar cultures of *Streptomyces* sp. isolate B8005 incubated for 72 hours at 28°C were used for the inoculation of ninety 1 litre Erlenmeyer flasks each containing 165 ml of malt - extract yeast - extract medium [8]. After an incubation of 4 days at 28°C on a horizontal shaker at 110 rpm, the culture broth was mixed with Celite and filtered through a press filter to separate the mycelium from the water phase. Both phases were separately extracted with ethyl acetate. Due to the similarity shown on TLC, both organic phases were combined and evaporated to dryness. The crude extract was dissolved in methanol (150 ml) and extracted with cyclohexane (150 ml) for defatting. The methanol phase (7.4 g of an orange red extract) was then separated by medium pressure column chromatography on silica gel with a chloroform/methanol-gradient (1 l $CHCl_3$, 1 l $CHCl_3$/1% MeOH, 1 l $CHCl_3$/2% MeOH, 1 l $CHCl_3$/5% MeOH, 1 l $CHCl_3$/10% MeOH) into four fractions. After crystallisation from chloroform followed by purification of the solid by PTLC ($CHCl_3$/12% MeOH), fraction I yielded resistoflavin (**3a**, 6 mg, R_f=0.72). Similarly, fraction III yielded 36 mg of resistomycin (**1**) as a yellow solid with R_f=0.66. From fraction IV, 7.5 mg of 1-hydroxy-1-norresistomycin (**2**, R_f=0.66) were obtained as an orange solid, which was further purified by PTLC ($CHCl_3$/10% MeOH) followed by column chromatography on Sephadex LH-20 ($CHCl_3$/10% MeOH). Fraction V was separated by HSCCC (ethyl acetate/cyclohexane/methanol/water=3 : 2 : 2 : 3) with the upper phase as mobile phase. All red fractions were combined and purified by HPLC (MeCN/30% H_2O→MeCN in 30 minutes) to get 5 mg of red tetracenomycin D (4, R_f=0.54).

Fermentation of *Streptomyces* sp. B4842

The fermentation of strain B 4842 was performed in a 20 litre jar fermentor containing 18 litres of malt extract medium [8] in synthetic sea water/tap water 1 : 1. The medium was adjusted to pH 7.8 and sterilised for 1 hour at 120°C, incubation was carried out at 28°C for 3 days with

automatic addition of 2 N NaOH to maintain the pH at 5~6, and polypropylene glycol to control foaming. Sterile air was supplied (5 l/minute) and agitation was at 120 rpm. The entire culture broth was filtered over Celite, and the culture filtrate was extracted three times with each 5 litres of ethyl acetate. The combined organic layers were then evaporated to dryness to yield 1.6 g of crude extract. The latter was defatted with cyclohexane and the resulting polar residue was subjected to Sephadex LH-20 column chromatography with methanol. Antibiotically active fractions were chromatographed on silica gel ($CHCl_3$/MeOH 70 : 30) to yield resistomycin (**1**, 44 mg) and the ether **3b** (2.4 mg).

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