New Butenolides from Two Marine Streptomycetes[1]

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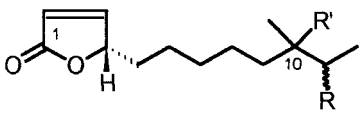
Chemical examination of two marine Streptomycetes has resulted in the isolation of four new butenolides, namely 4,10-dihydroxy-10-methyl-dodec-2-en-1,4-olide (**1**), two diastereomeric 4,11-dihydroxy-10-methyl-dodec-2-en-1,4-olides (**2**/**3**), and 4-hydroxy-10-methyl-11-oxo-dodec-2-en-1,4-olide (**4**). The structures were identified by interpretation of the 2D NMR and mass spectral data.

Butenolides, a family of α,*β*-unsaturated lactones, are often encountered among fungi,[2] bacteria,[3] and gorgonians,[4] to name a few. Their saturated analogues act as signaling substances in bacteria[5] and enhance spore formation of Streptomycetes or induce metabolite production.[6] In a continuing search for bioactive constituents from marine microorganisms, we found that extracts from the *Streptomycete* strains B 5632 and B 3497 from marine sediments formed several new butenolides.

The isolate B 5632 was fermented in a 20-L scale on YMG medium with artificial seawater for 3 days. The fermentation broth was filtered over Celite and exhaustively extracted with ethyl acetate. This extract was partitioned between methanol and cyclohexane for defatting, and the methanol layer, after concentration, was chromatographed on a flash column. Bioassay-guided fractionation led to the localization of the activity from which four known antimycins and three new butenolides (**1**−**3**) were obtained. In a similar way, strain B 3497 delivered a new keto butenolide (**4**) in addition to antimycin A. The antimycins were responsible for the strong antifungal activity of the extracts against *Mucor miehei* (Tü284).



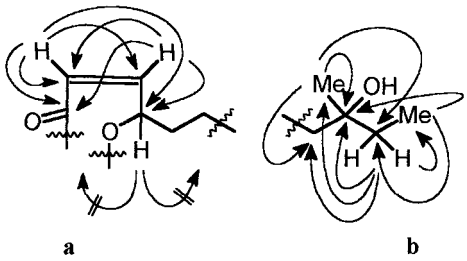
1: R = H; R' = OH

2, 3: R = OH; R' = H

4: R = O; R' = H

Analytical HPLC indicated that the butenolide fraction (localized by a strong blue-violet color on spraying with anisaldehyde) contained two related compounds. Compound **1** was obtained as an oil by preparative HPLC. Under EIMS conditions, no molecular ion was visible; however, its molecular mass was fixed as 226 Da by pseudomolecular ions at *m*/*z* 244 [$M^+ + NH_4$] and 226 [$M^+ + NH_4 - H_2O$] on CIMS. An APT spectrum showed that the compound contained two methyl, six methylene, three methine, and two quaternary carbons, according to a formula $C_{13}H_{22}O_3$, in agreement with chemical shifts. The proton signals at *δ* 7.44 and 6.11 and a carbon signal at *δ* 173.2 indicated that the compound had an α,*β*-unsaturated lactone, ester, or acid moiety. The molecular formula demands three double-bond equivalents. As two are accounted for by an ester or lactone carbonyl and a double bond, the molecule must be monocyclic. The H−H COSY spectrum showed couplings between the two olefinic protons and a multiplet at *δ* 5.01, indicating an oxygenated carbon next to the double bond. This methine showed further coupling to two methylene protons, which, in turn, were coupled to another methylene group. An HMQC spectrum correlated the proton signal at *δ* 5.01 to a methine signal at *δ* 83.4. This sequence resulted in fragment **a**.

Additionally, the ^{1}H NMR spectrum showed a clear quartet and a triplet, indicating the presence of an isolated ethyl group in the molecule. A signal for an isolated methyl group and the HMBC correlations showed that the molecule must contain fragment **b**. Because the molecule contains one ring as discussed above, the open ends of the partial structures **a** and **b** need to be closed while incorporating three methylene units.



a **b**

Three structures are in accordance with these data, the butenolide **1** and two macrocyclic lactones. The negligible H−H coupling of the α- and *β*-protons with the adjacent methine proton indicated a nearly 90° orientation and a flat system; a strained ring is also consistent with the small cis coupling constant and the IR_{CO} signal at $\nu = 1750$ cm^{-1}. The shift values and the carbonyl frequency are in very good agreement with data reported for maritolide (**5**) from *Diospyros maritima*[7] (^{13}C NMR data, see Table 1; $\nu_{CO} = 1755$ cm^{-1}) and related compounds. Although, no HMBC coupling of 4-H with the carbonyl group or between ring and chain is visible, as in **5**, there is no doubt that this compound is 4,10-dihydroxy-10-methyl-dodec-2-en-1,4-olide (**1**).

The absolute configuration at C-4 has been fixed by CD measurements and comparison with a butenolide (**6**) of

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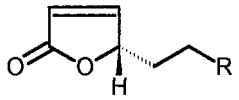
Table 1. ^{13}C NMR Data for Butenolides **1**−**5** in $CDCl_3$

atom	1	2	3	4	5[a]
1 CO	173.2	173.2	173.2	173.0	173.8
2 CH	121.6	121.6	121.6	121.4	121.6
3 CH	156.2	156.3	156.3	156.2	156.2
4 CH	83.4	83.4	83.4	83.2	83.4
5 CH_2	33.1	32.4	33.2	32.9	33.2
6 CH_2	25.0	25.0	25.0	24.7	
7 CH_2	29.9	29.6	29.7	29.2	
8 CH_2	23.6	27.0	27.1	26.8	
9 CH_2	41.1	32.4	32.4	32.5	
10	72.9 C_q	39.6 CH	40.0 CH	47.0 CH	
11	34.3 CH_2	71.3 CH	71.7 CH	212.6 CO	
12 CH_3	8.2	19.5	20.3	27.9	
13 CH_3	26.4	14.2	14.6	16.1	

[a] Only relevant values are listed.

known absolute stereochemistry.[8] As butenolide **1** shows a negative n−π* and a positive $\pi - \pi$* Cotton effect, the configuration at C-4 must be *S*, as in **6**.



5: R = $(CH_2)_6$-COOEt

6: R = OH

The doubling of signals in the ^{13}C and the ^1H NMR spectra indicated that the oily fraction 2 was a mixture of two related compounds in a ratio close to 1:1. They did not deliver an M^+ ion in the EIMS, but showed the same mass in the CI spectrum and gave a butenolide carbonyl signal at ν = 1758 cm^{-1} and a similar NMR pattern as **1**. However, the terminal ethyl signal was substituted by two overlapping CH−CH_3 doublets, and two additional methine signals indicated that oxygen in **1** had shifted from C-10 to C-11, resulting in the diastereomeric structures **2** and **3**. The CD spectrum with a positive $\pi - \pi$* transition at 208 nm indicated a 4*S* configuration.

Compound **4** was obtained from another *Streptomycete* strain B 3497. Its molecular formula was fixed as $C_{13}H_{20}O_3$ by the pseudomolecular ion at *m/z* 242 [$M^+ + NH_4$] in its CIMS and the NMR data. The ^1H NMR pattern was very similar to those of compounds **1**−**3**, and the presence of an extra carbonyl absorption at 1708 cm^{-1} in addition to the α,β-unsaturated lactone carbonyl absorption (1754 cm^{-1}) suggested that compound **4** contains a ketone carbonyl. The presence of a methyl ketone singlet and the absence of a terminal methyl triplet in the proton NMR spectrum indicated structure **4**, which was finally confirmed by COSY and HMQC spectra similarly as for **1**. Due to the positive $\pi - \pi$* Cotton effect, the configuration at C-4 must be the same as in **1** and **2**/**3**.

The presence of two positional isomers **1** and **2**/**3** suggests an epoxide precursor in their biosynthesis, as it also may be involved in the biosynthesis of the phytotoxins seiridin, isoseiridin,[9] hydroxyseiridin, hydroxyisoseiridin,[10] and *iso*-cladospolide B.[2] Corresponding epoxides have not yet been reported; however, the epoxyrollins[11] A and B with epoxides in C-2 side chains are related structures.

Despite spore formation-promoting activity, some other butenolides[3] are active against *Pseudomonas aeruginosa* and inhibit the chitinase from *Serratia marcescens*. Compound **4** did not show activity against *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, *Mucor miehei* (Tü284), or *Streptomyces viridochromogenes* (Tü57), and **1**−**4** showed no influence on spore formation of *S. viridochromogenes* (Tü57) or of some marine Streptomycetes.

Experimental Section

General Experimental Procedures. The CD spectra and optical rotations were run on a JASCO J-500 Aa spectropolarimeter. NMR spectra were measured on a Varian Unity 300 (300.145 MHz) and a Varian Inova 500 (499.876 MHz) spectrometer in $CDCl_3$ with TMS as internal standard. CIMS was recorded on a Finnigan MAT 95 A instrument using NH_3. Preparative HPLC was performed using a RP_{18} column with a diode array detector. Flash chromatography was carried out on Si gel (230−400 mesh).

Strain Description. The Actinomycete stain B3497 was collected from a North Atlantic Ocean sediment (58°19′N, 14°57′W at 680 m depth) and isolated on a casein/peptone medium (50% seawater) at 18 °C.[12] The strain B5632 has been derived from muddy sediment of a mangrove site near Auckland, New Zealand, and was isolated on Olson medium containing 22 g of actinomycete isolation agar (Difco) and 5 g of glycerol in 1 L of 50% natural seawater. The reference cultures of B5632 are kept on yeast extract−malt extract agar[12] in the collection of marine Actinomycetes at the Alfred-Wegener-Institute for Polar and Marine Research in Bremerhaven. According to the partial 16S rRNA gene sequence, the strains B5632 and B3497 are representatives of the *Streptomyces coelicolor* group.

The strains produce a yellow-brown substrate mycelium and a yellow aerial mycelium with straight to flexuous (rectiflexibiles) spore chains. The surface of the spores is smooth. The strains do not produce melanin on either peptone/yeast extract/iron agar or on tyrosine agar.[13]

The optimum temperature for strain growth is 30 °C. The strains grow slowly at 10 °C but show no growth at 45 °C. Chitin, starch, casein, gelatine, and esculin are degraded. Cellulose is not cleaved. The strains are catalase positive. Only strain B5632 forms nitrate reductase. The strains deviate further in the use of the following carbon sources (SFN-Biolog plates): turanose, D-glucosaminic acid, α-keto butyric acid, α-keto valeric acid, propionic acid, L-leucine, L-threonine, 2-aminoethanol with positive growth response for B5632 and acetic acid, bromosuccinic acid, L-histidine, thymidine with positive growth results for B3497.

Fermentation. Fermentation was performed using standard conditions[1] on YMG medium with artificial seawater. The bacterial cultures B 5632 and B 3497 were grown on agar plates at 28 °C for 3 days and then transferred to shaker cultures (15 × 200 mL). These seed cultures were used to inoculate the fermenter, which was harvested after 3 days.

Extraction and Isolation. Fermentation broth of B 5632 was filtered through a press filter using Celite, and the filtrate and bacterial cells were extracted with ethyl acetate. The combined ethyl acetate extracts were concentrated in vacuo at 37 °C and the residue (1.5 g) partitioned between methanol and cyclohexane. The defatted methanol layer (0.8 g) was concentrated and loaded on a flash column (30 × 600 mm), which was eluted with chloroform and a stepwise increasing gradient of methanol. The residue from the chloroform−methanol (8:2) fraction (50 mg) gave, respectively, yellow and blue-violet spots on spraying with anisaldehyde/sulfuric acid and was further purified on a Sephadex LH-20 column eluting with chloroform−methanol (6:4) into two main fractions. The first fraction (20 mg) was rich in antimycins (R_f = 0.43, $CHCl_3$−MeOH, 95:5, yellow with anisaldehyde), while the second (15 mg) contained butenolides. HPLC and MS analysis showed that the antimycin mixture contained only known compounds. Preparative HPLC (C_{18}, Eurosphere, 80A/5 μm, 16 × 250 mm, CH_3CN-H_2O, 1:1) of the butenolide fraction resulted in the isolation of pure compound **1** (2 mg) and a mixture of **2** and **3** (2 mg). In the same way, cultivation and workup of a 20-L culture from B 3497 gave 12 mg of antimycin A (R_f = 0.43, $CHCl_3$−MeOH, 95:5) and 4 mg of **4**.

(4*S*)-4,10-Dihydroxy-10-methyl-dodec-2-en-1,4-olide (1): R_f 0.53 ($CHCl_3$−MeOH 95:5), blue-violet with anisaldehyde−

sulfuric acid; UV (MeOH) λ_{max} (log ϵ) 207 (3.63) nm; $[\alpha]^{22}_D$ +44° (*c* 0.072, CH_3OH); CD $[\theta]_{205}$ (MeOH) +24 800; IR (KBr) ν_{max} 3436, 2928, 2856, 1750, 1568, 1459, 1379, 1166, 1103, 819 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 7.44 (1 H, dd, J = 5.6, 1.5 Hz, H-3), 6.11 (1 H, dd, J = 6.1, 2.3 Hz, H-2), 5.01 (1 H, dddd, J = 7.5, 5.3, 1.9, 1.9 Hz, H-4), 1.78 (1 H, m, H-5a), 1.62 (1 H, m, H-5b), 1.42 (2 H, q, J = 7. 3 Hz, H_2-11), 1.40 (m), 1.32 (m), 1.22 (m), 1.12 (3 H, s, CH_3-13), 0.84 (3 H, t, J = 7.6 Hz, CH_3-12); ^{13}C NMR, see Table 1; CIMS *m*/*z* 244 $[M + NH_4]^+$ and 226 $[M + NH_4 - H_2O]^+$.

(4*S*)-4,11-Dihydroxy-10-methyl-dodec-2-en-1,4-olides (2/3): inseparable mixture of two diastereomers, R_f 0.53 ($CHCl_3$−MeOH 95:5), blue-violet with anisaldehyde−sulfuric acid; UV (MeCN) λ_{max} (log ϵ) 200 (3.80) nm; $[\alpha]^{22}_D$ +84.5° (*c* 0.119, CH_3-OH); CD $[\theta]_{208}$ (MeOH) +23 700; IR (KBr) ν_{max} 2949, 2882, 1758, 1475, 1400, 1182, 1121, 838 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.44 (1 H, dd, J = 5.7, 1.5, H-3), 6.11 (1 H, dd, J = 5.7, 1.9, H-2), 5.03 (1 H, m, H-4), 3.72, 3.65 (each 1 H, m, H-11 of 2 and 3), 1.92−1.25 (11 H, m), 1.14, 1.16 (each 3 H, 2 d, J = 6.4, CH_3-12 of 2 and 3), 0.88, 0.86 (each 3 H, 2 d, J = 6.8, CH_3-13 of **2** and **3**); ^{13}C NMR, see Table 1; CIMS *m*/*z* 244 $[M + NH_4]^+$ and 226 $[M + NH_4 - H_2O]^+$.

(4*S*)-4-Hydroxy-10-methyl-11-oxo-dodec-2-en-1,4-olide (4): R_f 0.37 ($CHCl_3$−MeOH 95:5), violet with anisaldehyde−sulfuric acid; UV (MeCN) λ_{max} (log ϵ) 200 (4.13), 250 (3.47) nm; $[\alpha]^{22}_D$ +45° (*c* 0.119, CH_3OH); CD $[\theta]_{204}$ (MeCN) +25 400; IR (film) ν_{max} 2935, 2860, 1754, 1708, 1600 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.44 (1 H, dd, J = 5.9, 1.5 Hz, H-3), 6.12 (1 H, dd, J = 5.9, 2.0 Hz, H-2), 5.04 (1 H, m, H-4), 2.50 (1 H, m, J = 5 Hz, H-10), 2.12 (3 H, s, CH_3-12), 1.70 (1 H, m), 1.60 (2 H, m), 1.40 (2H, m), 1.35−1.15 (5 H, m), 1.09 (3 H, d, J = 7 Hz, CH_3-13); ^{13}C NMR, see Table 1; EIMS *m*/*z* 224 $[M]^+$ (14), 182 (20), 153 (100); DCIMS *m*/*z* 242 $[M + NH_4]^+$ (100), 225 $[M + H]^+$ (0.01); *anal.* C 69.66%, H 9.07%, calcd for $C_{13}H_{20}O_3$, C 69.61%, H 8.99%.

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