T-Muurolol Sesquiterpenes from the Marine *Streptomyces* sp. M491 and Revision of the Configuration of Previously Reported Amorphanes[1]

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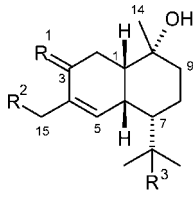
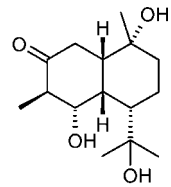
Two new sesquiterpenes, 15-hydroxy-T-muurolol (**3d**) and 11,15-dihydroxy-T-muurolol (**3e**), along with the plant cadinenes T-muurolol (**3f**) and 3α-hydroxy-T-muurolol (**3g**), were isolated from the marine-derived *Streptomyces* sp. M491. Their absolute configuration was established via NMR spectroscopy and X-ray crystallography of 3-oxo-T-muurolol (**3a**), which was reisolated from this strain. In addition, the absolute configuration of further sesquiterpenes previously reported from this strain was revised. These products were tested for their cytotoxicity against 37 human tumor cell lines using the MTT method. Only **3d** was cytotoxic against a range of human tumor cell lines with a mean IC_{50} of 6.7 μg/mL.

Of the more than 8000 known sesquiterpenes, most are derived from plants, around 500 are of fungal origin, and fewer than 50 are bacterial metabolites.[2] Nevertheless, in contrast to previous expectations, sesquiterpenes are not rare in bacteria. TLC analyses of streptomycete extracts regularly detect low-polarity compounds, which stain blue or violet with anisaldehyde/sulfuric acid, suggesting the presence of terpene derivatives. However, because unsaturated fatty acid derivatives with similar color reactions may mask them, their lipid-like NMR spectra are often not conspicuous, and their yields are regularly low; thus, only a few bacterial sesquiterpenes have been reported to date.[2]

In a previous investigation of metabolites from the marine-derived *Streptomyces* sp. M491 isolated from a sand sample from Qingdao coast (China), we reported four sesquiterpenes, which were stereochemically assigned as amorphanes.[3] In an attempt to explore further trace components from the same strain, we were able to reisolate the previous main products, crystallize one of them, and determine its absolute configuration by X-ray diffraction analysis. This led to a revision of the stereochemistry of these compounds from the amorphane series, as in **1/2**, to the muurolane configuration seen in **3/4**, and to the isolation and structure elucidation of an additional two new bacterial muurolane sesquiterpenes.



	R^1	R^2	R^3
1a	O	H	H
1b	H_2	H	OH
1c	O	OH	H



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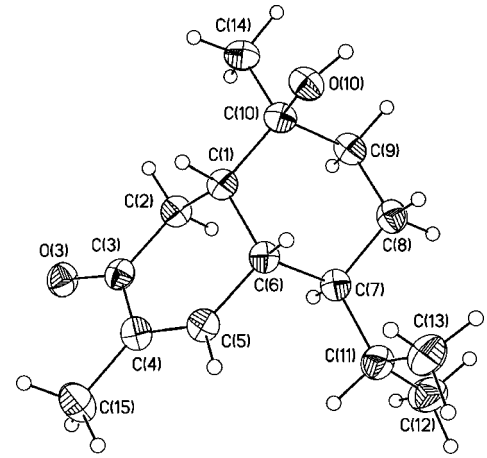

Figure 1. ORTEP drawing for 3-oxo-T-muurolol (**3a**).

Results and Discussion

Fermentation of strain M491 was performed on a 25 L scale in a shaker culture as previously described. The workup and separation were also done in a similar way.[3] By TLC, the terpenes appeared as colorless zones, which turned blue-violet using an anisaldehyde/sulfuric acid spray reagent.

From the region of intermediate polarity, a terpene with NMR data identical to those of the previously reported 10α-hydroxyamorph-4-en-3-one (**1a**)[3] was isolated. Serendipitously, it crystallized from dichloromethane as small tetragonal prisms, allowing its crystallographic structure analysis and the determination of its absolute configuration. From these data, the *cis*-fused cyclohexane rings and the *trans* orientation of *i*Pr-7 and Me-10 were confirmed, but it was necessary to change the previously assigned 7α,10α-configuration to a 7β,10β-orientation. The previous incorrect assignment was based on the observation of a substantial Overhauser effect between H-1/Me-14, as expected for **1a**. These new crystallographic data established the structure of the terpene as (1*S*,6*R*,7*S*,10*S*)-(−)-3-oxo-T-muurolol (**3a**), not the amorphane **1a** as previously thought. The crystal structure is shown in Figure 1. (−)-3-Oxo-T-muurolol was previously reported as a plant constituent from *Taiwania cryptomerioides*[4] and also as a fungal metabolite. The reported ^{13}C NMR data were identical with our values within a range of $\Delta\delta \pm 1$.[5]

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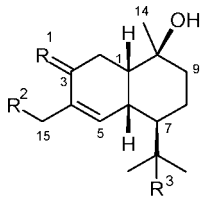
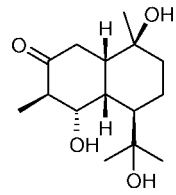


	R^1	R^2	R^3
3a	O	H	H
3b	H_2	H	OH
3c	O	OH	H
3d	H_2	OH	H
3e	H_2	OH	OH
3f	H_2	H	H
3g	αOH,βH	H	H



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The proton spectra of all other sesquiterpenes isolated here were comparable. In particular, the bridgehead protons H-6 showed similar coupling patterns, with the exception of **4**, and selective NOE experiments further supported the muurolane skeletons. Irradiation of H-6 caused enhancement of H-1, and vice versa, indicating their *cis* relationship. Me-14 showed correlations with CH_2-2, CH_2-9, and/or CH_2-8, but not with either H-7 or *i*Pr-7. The latter gave, however, a correlation with H-6. All the sesquiterpenes isolated from *Streptomyces* sp. M491 can be expected to belong to the same stereochemical series. Correspondingly, the previously described compounds[3] are not 10α,11-dihydroxyamorph-4-ene, 10α,15-dihydroxyamorph-4-en-3-one, and 5α,10α,11-trihydroxyamorphan-3-one, but should be derived from 1*S*,6*R*,7*S*,10*S*-muurolene, and have structures **3b**, **3c**, and **4**, respectively.

(−)-T-Muurolol (**3f**) was isolated as colorless oil from fraction A. The ^{1}H NMR spectrum showed an olefinic proton at δ 5.63 (H-5) and an olefinic methyl at δ 1.59 (Me-15). Another three methyl signals, appearing as two doublets (δ 0.86, 0.85) and one singlet (δ 0.99), were observed. The remaining proton signals appeared in the aliphatic region between δ 2.5 and 1.0. The ^{13}C NMR spectrum revealed 15 carbon signals, including one oxygen-connected carbon at δ 72.4 (C-10). (+)-HRESIMS established the molecular formula as $C_{15}H_{26}O$ (*m*/*z* 245.18773 $[M + Na]^+$). Our data agreed very well with literature values for (−)-T-muurolol (**3f**),[6] a frequently reported plant metabolite, e.g., from *Pinus sylvestris*,[6] with potential use as a termite-controlling agent.[7] This is, however, the first report of (−)-T-muurolol (**3f**) as a microbial metabolite.

Compound **3d** was isolated as a colorless solid, with the molecular formula $C_{15}H_{26}O_2$ (by (+)-HRESIMS). The ^{1}H NMR spectrum was similar to that of T-muurolol (**3f**), showing an olefinic proton at δ 5.85. However, a methylene group was apparent in **3d**, with an AB signal observed at δ 3.99 and 4.01 instead of the C-15 methyl group. In the HMBC spectrum, correlations were observed between this methylene group and the double-bond carbons, confirming that Me-15 was oxidized. The structure of the new 15-hydroxy-T-muurolol (**3d**) was confirmed by further HMBC correlations: H_3-14 showed couplings to C-1, C-9, and C-10; H_2-15 showed correlations to C-3, C-4, and C-5; and oppositely, H-5 correlated with C-3 and C-15. Correlations between H_3-12/13 and both C-7 and C-11 were also observed.

Compound **3e** was also isolated as a colorless solid and has the formula $C_{15}H_{26}O_3$ (by (+)-HRESIMS). The ^{1}H spectrum was similar to that of **3d**, with an olefinic proton and the AB signals of an oxygenated methylene group. However, the two methyl doublets of the isopropyl group in **3d** were replaced by two methyl singlets, which gave HMBC correlations to the signal of an oxygenated carbon atom not present in **3d**. Correspondingly, the isopropyl methine signal of C-11 was missing in the ^{13}C NMR spectrum. The terpene was therefore identified as 11,15-dihydroxy-T-muurolol (**3e**).

The colorless solid obtained from fraction B was identified in a similar way as 3α-hydroxy-T-muurolol (**3g**). The configuration at C-3 was determined from the observance of a NOESY correlation between H-1 and H-3. 3α-Hydroxy-T-muurolol (**3g**) was previously isolated from *Taiwania cryptomerioides*[4] and obtained by synthesis,[8] but is reported here for the first time as a microbial metabolite. The ^{1}H values agree very well with published values,[8] but are clearly distinguishable from those of the 3β-hydroxy isomer.[5,8]

For the highly polar sesquiterpene $C_{15}H_{26}O_4$ previously described as **2**, irradiation of H-5 showed an Overhauser effect on Me-15 and H-6, and irradiation of H-4 caused a signal enhancement of H-7. On the basis of **3a**, this further confirmed the structure as (1*S*,4*R*,5*S*,6*S*,7*R*,10*S*)-3-oxo-5,11-dihydroxy-T-muurolol (**4**).

The terpenes described here were tested for their cytotoxicity.[9] Except for **3d**, which was weakly cytotoxic (IC_{50} = 6.7 μg/mL), the other compounds **3a**, **3b**, **3c**, **3e**, **3f**, and **4** showed no activity (IC_{50} >10 μg/mL) against 37 human tumor cell lines.

Experimental Section

General Experimental Procedures. The optical rotations were measured on a Perkin-Elmer polarimeter (model 241). UV/vis spectra were recorded on a Perkin-Elmer Lambda 15 UV/vis spectrometer. IR spectra were recorded on a Perkin-Elmer 1600 Series FT-IR spectrometer with KBr pellets. NMR spectra were measured on a Varian Unity 300 (300.145 MHz), a Varian Unity 500 (499.876 MHz), and a Varian Inova 600 (599.740 MHz) spectrometer. ESI mass spectra were recorded on a Quattro Triple Quadrupol mass spectrometer, with a Finnigan TSQ 7000 with nano-ESI API ion source. HRESI mass spectra were measured on a Micromass LCT mass spectrometer coupled with a HP 1100 HPLC with a diode array detector. EIMS was performed on a Finnigan MAT95 (70 eV), and perfluorokerosene was used as reference substance in HREIMS. Flash chromatography was carried out on silica gel (230−400 mesh). Thin-layer chromatography (TLC) was performed on Polygram SIL G/UV_{254} (Macherey-Nagel & Co.). Size exclusion chromatography was done on Sephadex LH-20 (Pharmacia). XAD-16 resin was obtained from Rohm and Haas, France.

Cytotoxicity Test. Cytotoxicity of selected compounds against cell lines 1218L, T24, 498NL, SF268, HCT116, HT29, 251L, 536L, 1121L, 289L, 526L, 529L, 629L, H460, 401NL, MCF7, DA231, 276L, 394NL, 462NL, 514L, 520L, 1619L, 899L, OVCAR3, 1657L, PANC1, 22RV1, DU145, LNCAP, PC3M, 1752L, 1781L, 393NL, 486L, 944L, and 1138L was carried out using the MTT method as described previously.[9] Human tumor cell lines tested were derived from patient tumors engrafted as a subcutaneously growing tumor in NMRI nu/nu mice, or obtained from American Type Culture Collection, Rockville, MD, National Cancer Institute, Bethesda, MD, or Deutsche Sammlung von Mikroorganismen and Zellkulturen, Braunschweig, Germany.

Fermentation of *Streptomyces* sp. Isolate M491 and Workup. The fermentation and extraction procedure used has been described in previous work.[3] The extract from a 25 L fermentation was separated on silica gel (column 50 × 3 cm, CH_2Cl_2/0−10% CH_3OH gradient), yielding four fractions, A−D. T-Muurolol (**3f**) (12.5 mg) was obtained from fraction A by further separation on Sephadex LH-20 (MeOH). Fraction B was purified on silica gel (CH_2Cl_2) to afford 15-hydroxy-T-muurolol (**3d**) (17.6 mg), 3-oxo-T-muurolol (**3a**) (78.5 mg), 3α-hydroxy-T-muurolol (**3g**) (4.3 mg), 3-oxo-5,15-dihydroxy-T-muurolol (**4**) (7.3 mg), and 11-hydroxy-T-muurolol (**3b**) (57 mg). Fraction C was first separated by Sephadex (CH_2Cl_2/MeOH, 1:1). A subfraction staining blue with ansisaldehyde/H_2SO_4 was further purified by RP-18 column chromatography (CH_3OH/H_2O) to afford 11,15-dihydroxy-T-muurolol (**3e**) (1.5 mg).

(−)-15-Hydroxy-T-muurolol (3d): colorless solid, R_f 0.39 (CH_2Cl_2/3% CH_3OH); $[\alpha]_D^{25}$ −103 (*c* 3.00, MeOH); IR (KBr) ν_{max} 3326, 2962, 1670, 1453, 1374, 1300, 1238, 1191, 1144, 1028 894, 840 cm^{-1}; NMR data, see Tables 1 and 2; EIMS *m*/*z* (%) 43 (37), 55 (8), 71 (26), 79 (31), 91 (33), 93 (59), 109 (42), 119 (95), 135 (25), 147 (36), 150 (58), 162 (94), 177 (36), 190 (100), 202 (22), 208 (7), 220 (26), 238 (6), 177 (36); (+)-HRESIMS *m*/*z* 261.18258 $[M + Na]^+$ (calcd for $C_{15}H_{26}O_2Na$, 261.18250).

Table 1. ^{1}H NMR Data (δ values in $CDCl_3$) of 15-Hydroxy-T-muurolol (**3d**) (300 MHz) and 11,15-Dihydroxy-T-muurolol (**3e**) (600 MHz)

position	**3d** δ (J in Hz)	**3e** δ (J in Hz)	position	**3d** δ (J in Hz)	**3e** δ (J in Hz)
1	1.57, m	1.52, m	9	1.53, m	1.50, m
2	1.65, m	1.65, m	10		
	1.55, m	1.58, m			
3	2.09, m	2.10, m	11	1.96, m	
4			12	0.88, d (6.9)	1.20, s
5	5.85, d (5.6)	6.08, d (6.3)	13	0.84, d (6.9)	1.25, s
6	2.36, m	2.56, m	14	1.21, s	1.23, s
7	1.27, m	1.45, m	15	4.01, 3.99, AB	3.97, 3.99 AB
8	1.37, m	1.25, m		(11.8)	(14.2)

Table 2. ^{13}C NMR Data (δ values in $CDCl_3$) of 15-Hydroxy-T-muurolol (**3d**) (125 MHz), 11,15-Dihydroxy-T-muurolol (**3e**) (150 MHz), T-Muurolol (**3f**) (75 MHz), and 3α-Hydroxy-T-muurolol (**3g**) (150 MHz)

position	**3d**	**3e**	**3f**	**3g**
1	46.2	47.0	46.1	46.2
2	20.5	20.3	20.9	32.1
3	26.8	26.6	31.3	72.2
4	137.0	137.5	133.5	137.8
5	126.3	127.1	124.8	129.5
6	34.2	33.9	34.5	36.1
7	43.8	50.1	43.9	45.4
8	19.3	29.7	19.4	20.4
9	34.6	34.6	34.6	35.4
10	72.3	72.0	72.4	72.6
11	26.7	74.5	26.7	28.1
12	21.5	30.5	21.6	22.0
13	15.4	24.2	15.4	15.8
14	29.3	29.2	29.3	29.4
15	67.3	67.0	23.6	19.8

(−)-11,15-Dihydroxy-T-muurolol (3e): colorless solid, R_f 0.10 (CH_2Cl_2/7% CH_3OH); $[\alpha]_D^{25}$ −48 (*c* 3.58, MeOH); IR (KBr) ν_{max} 3422, 2955, 2926, 2853, 1758, 1459, 1377, 1146, 904, 582 cm^{-1}; NMR data, see Tables 1 and 2; DCIMS (NH_3) *m/z* (%) 272 $[M + NH_4]^+$ (100), 254 $[M - H_2O + NH_4]^+$ (71), 526 $[2\ M + NH_4]^+$ (2); (+)-HRESIMS *m/z* 277.17752 $[M + Na]^+$ (calcd $C_{15}H_{26}O_3Na$, 277.17741).

(−)-T-Muurolol (3f): colorless oil, R_f 0.62 (CH_2Cl_2); $[\alpha]_D^{20}$ −43 (*c* 0.115, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 5.63 (d, 5.6, H-5), 2.41 (m, H-6), 2.03 (m, H-11), 1.82 (m, H-3), 1.59 (s, H-15), 1.50−1.45 (m, H-8b), 1.50−1.40 (m, H-2), 1.45−1.35 (m, H-9b), 1.50−1.40 (m, H-1), 1.30−1.20 (m, H-7), 1.30−1.25 (m, H-9a), 1.30−1.20 (m, H-8a), 0.99 (s, H-14), 0.86 (d, 7.0, H-12), 0.85 (d, 7.0, H-13); ^{13}C NMR data, see Table 2; (+)-DCIMS *m/z* (%) 222 $[M - H_2O + NH_4]^+$ (100), 240 $[M + NH_4]^+$ (22); (+)-HRESIMS *m/z* 245.18773 $[M + Na]^+$ (calcd $C_{15}H_{26}ONa$, 261.18807).

3α-Hydroxy-T-muurolol (3g): colorless solid, R_f 0.34 (CH_2Cl_2/3% CH_3OH); ^{1}H ($CDCl_3$) δ 5.66 (d, 5.7, H-5), 4.03 (dd, 8.6, 8.6, H-3), 2.34 (m, H-6), 1.97 (m, H-11), 1.88 (m, H-2a), 1.72 (d, 1.1, H-15), 1.67 (m, H-1), 1.55−1.50 (m, H-9a), 1.45−1.40 (m, H-9b), 1.55 (m, H-2b), 1.42 (m, H-7), 1.40 (m, H-8a), 1.35 (m, H-8b), 1.17 (s, H-14), 0.90 (d, 6.9, H-12), 0.85 (d, 6.9, H-13); ^{13}C NMR data, see Table 2; (+)-DCIMS *m/z* (%) 255 $[M + NH_3 + NH_4]^+$ (22), 238 $[M + NH_4]^+$ (100), 221 $[M + H]^+$ (9); (+)-HRESIMS *m/z* 261.18264 $[M + Na]^+$ (calcd $C_{15}H_{26}O_2Na$, 261.18250).

X-ray Crystallography Analysis of 3-Oxo-T-muurolol (3a). Crystals suitable for X-ray structure analysis were obtained by slow evaporation of a **3a** solution in CH_2Cl_2/CH_3OH. The X-ray data were collected on a SMART CCD 6000 diffractometer using Cu Kα radiation (λ = 1.54178 Å). The structure was solved by direct methods with SHELXS and refined by full-matrix least-squares on F^2 for all data with SHELXL.[10] Hydrogen atoms were placed on calculated positions, except the ones on the water molecule, which were located in the difference Fourier map and refined isotropically. The absolute structure could be determined by using the anomalous scattering of oxygen. Using the method proposed by Simon Parsons, the absolute structure parameter is 0.07(2). Diffraction data and refinement statistics are given in Table 3. CCDC-675029 contains the supplementary crystallographic data for this paper.[11]

Table 3. Crystal Data and Structure Refinement for 3-Oxo-T-muurolol (**3a**)

formula (asymmetric unit)	$(C_{15}H_{24}O_2)_2 \cdot H_2O$
fw	490.70
cryst syst	tetragonal
space group	$I4_1$
cell dimens (Å): $a = b$, c	23.510(3), 10.433(2)
volume (Å^3)	5766.5(16)
Z	8
calcd density (Mg/m^3)	1.130
completeness (%)	99.2
no. of indep reflns	4514
$R_1(I > 2\sigma)$	2.24
wR_2(all data)	5.77
Flack x param	0.07(10)

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Supporting Information Available: ^{1}H NMR spectra of muurolol derivatives **3a**−**g** and **4**; table of bond angles and lengths of **3a**. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

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(11) Crystallographic data for structure **3a** have been deposited with the Cambridge Crystallographic Data Centre. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

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