Tetrahedron Letters 51 (2010) 5493–5496

Contents lists available at ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

Structure elucidation of some highly unusual tricyclic *cis*-caryophyllane sesquiterpenes from *Marasmiellus troyanus*

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ARTICLE INFO

Article history:
Received 23 June 2010
Revised 11 August 2010
Accepted 12 August 2010
Available online 17 August 2010

Keywords:
Caryophyllane
Marasmiellus troyanus
Basidiomycetes
Sesquiterpene

ABSTRACT

Three new unusual sesquiterpenes (**1**–**3**) were isolated from the tropical rainforest basidiomycete, *Marasmiellus troyanus* and their structure elucidation was achieved by NMR spectroscopy, single-crystal X-ray structural analysis and a modified Mosher's ester method to determine the absolute stereochemistry of compound **1**. These unusual metabolites are probably derived from the caryophyllane class of sesquiterpenes and a possible biosynthetic route to these compounds is proposed. These small natural products represent the best possible features of chemical diversity, being chiral and exhibiting extensive functional group chemistry highlighting the value of natural products as a screening resource for therapeutics discovery programmes.

Basidiomycetes are prolific producers of natural products derived from the terpenoidal biosynthetic pathways. In particular, their ability to produce a wide array of sesquiterpene,[1] diterpene,[2] sesterterpene[3] and triterpene[4] natural products, makes them a highly attractive target for those interested in screening for biologically active compounds as these classes are chiral and produce an exceptional diversity of functional group chemistry. As part of our continuing efforts to isolate and characterise a novel chemistries from fungi[5] and plants,[6] we herein describe the isolation[7] and structure elucidation of three highly unusual *cis*-caryophyllane sesquiterpenes from *Marasmiellus troyanus* Murr., a pan-tropical decomposer agaric ('gill fungus'), which forms troops of small white fruiting bodies on woody debris in rainforests.[8]

The first of these compounds (**1**) (Fig. 1) was subjected to full NMR spectroscopic investigation and single-crystal X-ray structural analysis (Fig. 2). HRESI-MS confirmed the molecular formula of $C_{15}H_{24}O_4$ by accurate mass measurement of the protonated adduct $[M+H]^+$, 269.1755 indicating a sesquiterpene. Full ^1H and ^{13}C NMR spectral analysis (Tables 1 and 2) together with HMBC spectra allowed the full unambiguous assignment of all resonances. In the HMBC spectrum, two geminally coupled methyl groups (δ 1.32 and 1.39) exhibited couplings to a quaternary carbon (δ_C 34.7), to a methine (δ_C 50.0) and to a methylene carbon (δ_C 36.2). In the COSY spectrum, the hydrogens of this methylene coupled to a methine hydrogen (3.03, m) which in turn coupled to the hydrogen of the methine carbon which was coupled to by the geminal methyl pair. This indicated a dimethyl-substituted cyclobutane moiety which is common among sesquiterpenes such as β-caryophyllane (**4**).[9] Assuming a caryophyllane-type skeleton (Fig. 1), the methine carbon at δ_C 50.0 was C-1 and H-1 (δ 2.87) coupled to a deshielded oxymethine hydrogen (δ 4.43, br d, H-2), which in turn coupled to a further deshielded oxymethine (H-3, δ 4.48). In the HMBC spectrum, the C-3 carbon was coupled to by the hydrogens of a methyl doublet (δ 1.20) which in turn coupled to the methine carbon to which it was directly attached (C-4, 2.28) and to a further methine carbon (C-5). The COSY spectra revealed couplings between H-5 and a deshielded oxymethylene group (δ 4.20, 4.25). A methyl singlet (C-15) exhibited a ^3J coupling to C-5, to a quaternary carbon to which it was directly attached (C-8) and to a carbonyl carbon (δ_C 181.6, C-7) and back into the cyclobutane ring by a coupling to C-9. The carbonyl carbon was also coupled to by the oxymethylene (H_2-6) hydrogens indicating a lactonic bridge between C-6 and C-7. In the ^1H NMR spectrum, two broad singlets were present which did not display any connectivity to carbons in the HMQC spectra indicating that they were hydroxyl groups. From the accurate mass measurement, four oxygen atoms were present supporting the inclusion of the lactone and the two hydroxyl moieties which should be placed at C-2 and C-3 due to the downfield nature of the ^1H and ^{13}C resonances at these positions. Given the

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doi:10.1016/j.tetlet.2010.08.036

Table 1
^{1}H (500 MHz, mult, *J* in Hz) NMR assignments for **1**[a], **2**[b] and **3**[c]

No.	**1** δ_H	**2** δ_H	**3** δ_H
1	2.87, bt, 9.0	2.30 m	2.29, dt, 12, 2.5
2	4.43, br d, 10.5	5.19, d, 10.4	3.93, bt, 11.0
3	4.48, t, 5	—	3.66, dd, 9.0, 1.5
4	2.28, m	3.05, m	—
5	2.98, m	3.65, m	2.82, m 1.58, ddd, 11.0. 6.5, 4.5
6	4.20, bt, 9.0 4.25, bt, 8.0	4.46, t, 8.3 4.19, dd, 11.4, 8.6	3.98, m
7	—	—	—
8	—	—	—
9	3.03, m	2.89, m	3.37 m
10	1.75, m 1.95, m	1.93, t, 11.4 1.82, ddd, 10.6, 7.1, 3.5	1.94, ddd, 11.0. 9.0, 3.0 1.72, t, 10.5
11	—	—	—
12	1.39, s	1.36, s	1.25, s
13	1.32, s	1.26, s	1.12, s
14	1.20, d, 7.5	1.19, d, 7.3	0.88, s
15	1.77, s	1.05, s	5.08, dd, 3.0, 2.5 4.70, t, 2.5
OH-2	5.74, br s	6.51, br s	2.91, d, 9.0
OH-3	6.72, br s	—	4.32, d, 9.0
OH-7	—	—	5.65, s

[a] In $CDCl_3$.
[b] In C_5D_5N.
[c] In acetone-d_6.

Table 2
^{13}C NMR assignments (125 MHz) for **1**[a], **2**[b] and **3**[c]

Carbon	**1**	**2**	**3**
1	50.0	52.6	49.8
2	72.2	74.2	70.2
3	79.2	213.3	79.8
4	34.6	43.8	48.7
5	41.0	37.6	34.0
6	68.5	67.6	65.3
7	181.6	179.5	109.5
8	47.9	47.6	151.4
9	38.1	38.3	34.1
10	36.2	35.0	39.5
11	34.7	36.0	34.9
12	26.4	26.1	32.0
13	30.6	29.7	27.3
14	14.5	11.4	23.7
15	19.7	18.8	109.6

[a] In $CDCl_3$.
[b] In C_5D_5N.
[c] In acetone-d_6.

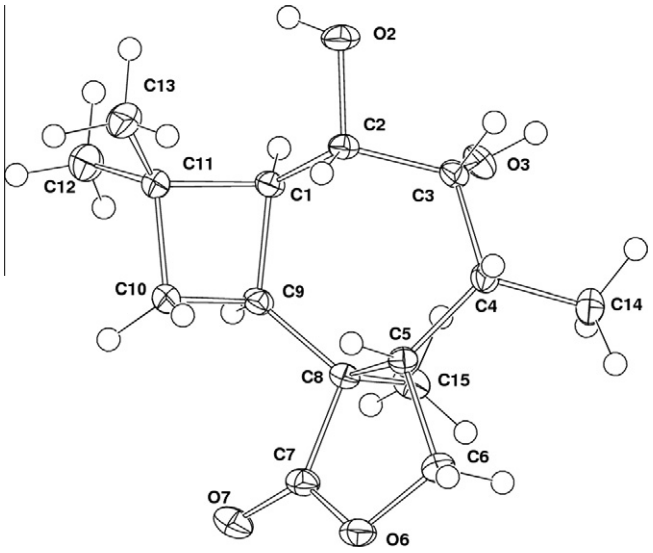

Figure 2. Molecular projection of **1** (50% probability amplitude displacement ellipsoids for C, O; arbitrary radii of 0.1 Å for H).

chiral nature of **1** and those seven-membered rings have a degree of flexibility, single-crystal X-ray structural analysis was conducted[10] to confirm the structure of **1** and to attempt to resolve the relative stereochemistry of this unusual compound. This showed that **1** was a cis-fused caryophyllane type sesquiterpene, confirmed the NMR spectral interpretation, and resolved the relative chemistry as depicted in Figures 1 and 2. For the assignment of the absolute configuration, Mosher's ester methodology[11] was considered but we were concerned that the presence of two vicinal diols at positions 2 and 3 could complicate the analysis. Fortunately the reaction of **1** with the *S* and *R* enantiomers of MPA gave only the 2-esters, presumably because the 3-carbinol is more sterically hindered, having hydroxyl and methyl groups as neighbours (Fig. 3). Shifts of H-1 and H-3 were diagnostic for an *S* chiral centre at C-2. The beauty of this methodology was that by resolving the absolute stereochemistry of one chiral centre, the relative configuration provided by the X-ray data then permitted unambiguous

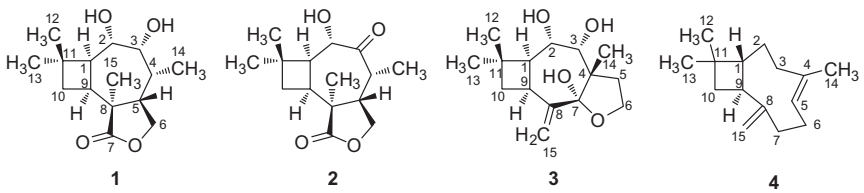

Figure 1. Structures of **1**–**4**.

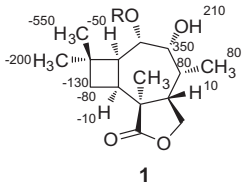

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Figure 3. $\Delta\delta$ values [$\delta_R - \delta_S$] in ppb for *R*- and *S*-MPA esters of **1**.

assignment of the stereochemistry of all stereogenic centres as 1*S*, 2*S*, 3*R*, 4*R*, 5*S*, 8*S* and 9*S*. Compound **1** was therefore assigned as 2*S*,3*R*-dihydroxy-carophyllan-[5,8]-6,7-olide[12] and is described herein for the first time.

Compound **2** was submitted to full ^{1}H, ^{13}C, and 2-dimensional NMR analysis again permitting the unambiguous assignment of all resonances. Accurate mass measurement of **2** revealed a molecular formula of $C_{15}H_{22}O_4$ being two hydrogens less than that of **1**. The NMR data for **2** were highly similar to those for **1** (Tables 1 and 2) with the exception of a 3-keto group whose presence was confirmed by a ^{3}J HMBC correlation from methyl-14 to C-3 (δ 213.3) which would explain the difference in molecular formula compared to **1**. Compound **2** was therefore assigned as the 3-oxo derivative of **1**, 2*S*-hydroxy-3-oxo-carophyllan-[5,8]-6,7-olide[13] and is reported here for the first time.

Resonances for the final compound (**3**) were distinctly different from those for **1** and **2**, with the ester functional group being replaced with a hemiketal, methyl 15 being an exo-cyclic methylene and, from analysis of the HMBC spectra (Fig. 4), ring C of the tricycle was now found to be a [4,7] rather than a [5,8] linkage as seen in **1** and **2**.

Analysis of the HMBC and COSY spectra again allowed full unambiguous assignment of all ^{1}H and ^{13}C resonances (Tables 1 and 2 and Fig. 4). The geminal methyl pair of the cyclobutane again gave correlations to C-11, C-1 and C-10 and in the COSY spectrum H-1 coupled to H-2 which in turn coupled to H-3. In the HMBC spectrum, methyl-14 coupled to C-3, C-4, C-5 and C-7 which was a deshielded carbon (δ_C 109.5). In the COSY spectrum, the hydrogens of methylene C-5 coupled to a deshielded oxymethylene pair (C-6) which in the HMBC spectrum coupled through oxygen to C-7 indicating that a hemiketal group should be placed at C-7. For compound **3**, C-15 was an *exo*-methylene (rather than a methyl as seen in **1** and **2**) and in the HMBC spectrum the hydrogens of this group coupled to C-7, C-8 and C-9, completing all the resonances for **3**. Stereochemistries at carbons 1, 2, 3 and 9 were identical to those for **1** and **2**. In the NOESY spectrum, methyl-14 gave NOE correlations to H-3 and CH_2-15, suggesting that they were on the same face. A key NOE between the hydroxyl of the hemiketal at C-7 and the C-3 hydroxyl indicated an alpha orientation for the hemiketal hydroxyl and a trans B/C ring junction, which was also seen for compounds **1** and **2**. Compound **3** is therefore assigned as 2*S*,3*R*,7*S*-trihydroxy-carophyllan-[4,7]-6,8-oxide.[14]

These compounds are new members of the rare cis-fused caryophyllane class of sesquiterpene and the novel tricyclic nature has not been recorded previously. The nearest related natural products

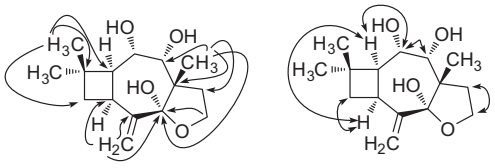
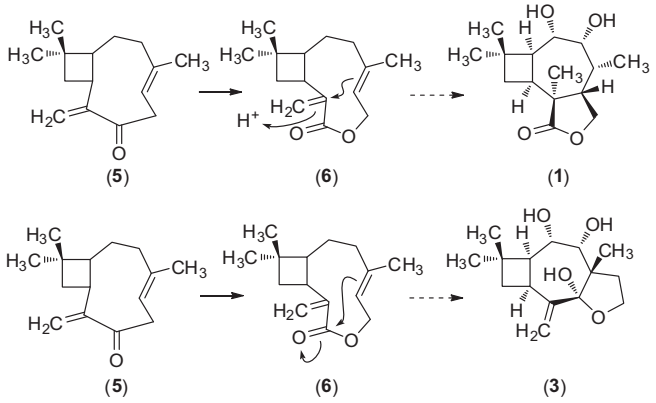


Figure 4. HMBC (single headed arrows) and COSY correlations (double headed arrows) for **3**.



Scheme 1. Possible biosynthetic pathways of **1** and **3**.

are cis-fused caryophyllanes from the ectomycorrhizal fungus *Hebeloma longicaudum*.[15]

The biosynthesis of compounds **1** and **2** (Scheme 1) is likely to follow from a caryophyll-4,8(15)-diene precursor (**5**), which could undergo a Baeyer–Villiger type oxidation to afford the lactone (**6**). Subsequent attack of the exo-cyclic methylene by the 4,5 double bond would give [5,8] ring closure and would result in a stable tertiary carbocation at C-4. Oxidation at positions 2 and 3 would lead to metabolites **1** and **2**.

The biosynthesis of compound **3** is more problematic. Starting from the same lactone intermediate (**6**) as **1** and **2**, attack of the lactone carbonyl by the 4,5 double bond would result in a hemiketal at C-7 and a [4,7] B/C ring junction architecture. This would be less favourable, as in the formation of this 4–7 bond a carbocation would be generated on C-5 (rather than on C-4 above) and this would be a secondary and less stable carbocationic centre.

These novel and unusual natural products represent the best features of natural chemical diversity, being chiral and possessing extensive functional group chemistry. It is surprising that natural products of this type still remain un-tapped as a screening resource for pharmaceutical drug discovery. The lack of success of combinatorial chemistry to afford new drug entities[16] indicates that it is only a matter of time before pharmaceutical companies return to Basidiomycetes to exploit their chemical diversity for drug discovery.

Acknowledgement

S.G. thanks Emmanuel Samuel for running high-resolution mass spectrometry.

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7. A suspension of disrupted mycelium (2 ml) in sterilised water was added to each of twenty 1000 ml conical flasks, containing 200 ml of sterilised 2% potato dextrose broth (Difco). The flasks were shaken at 200 rpm for two weeks at 27 °C. The harvested filtrate was then extracted with Diaion HP20 resin (400 ml; Mitsubishi) which had previously been washed with HPLC grade methanol (Merck) and thoroughly conditioned with distilled water. The resin was removed, washed with distilled water (2 × 1000 ml) and eluted with HPLC grade methanol (2 × 1000 ml). The methanolic eluant was evaporated to dryness. Vacuum/liquid chromatography was used to purify the three products, **1**, **2** and **3**. Column eluant (4.2 g) was dissolved in chloroform (15 ml), to which was added silica gel (flash chromatography grade; BDH; 6 g) and evaporated. The slurry was applied to the top of a 60 mm diameter silica gel column. The column was eluted with 100 ml volumes of *n*-hexane/ethyl acetate mixtures, beginning with 100% *n*-hexane followed by sequential stepwise 10% increase in ethyl acetate, with a final elution of 10% methanol/ethyl acetate. On evaporating the column fractions, three were noticed to contain white needles within a yellow oil. Millilitre quantities of methanol, ethyl acetate, diethyl ether and chloroform were used to remove the oil and afford pure white needles of **1**. Compound **2** was eluted from the silica column with 80% ethyl acetate/*n*-hexane, **3** in 90% ethyl acetate/*n*-hexane and **1** with 10% methanol/ethyl acetate.
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10. $C_{15}H_{24}O_4$, $M = 268.3$ Orthorhombic, space group $P2_12_12_1$, $a = 5.8060(8)$, $b = 14.4068(10)$, $c = 16.7080(10)$ Å, $V = 1398$ Å^3. $D_c = 1.27_5$ cm^{-3}. $\mu_{Mo} = 0.091$ mm^{-1}; specimen: $0.43 \times 0.15 \times 0.12$ mm (no correction). Monochromatic MoKα radiation, $\lambda = 0.7107_3$ Å; T ca. 153 K; CCD instrument, ω-scans; 31882 reflections, merging to 2940 unique, $2840 > 2\sigma(I)$ ($2\theta_{max} = 65°$). $R_1 = 0.033$, $wR_2 = 0.087$ (weights $(\sigma^2(F_o^2) + (0.0538P)^2 + 0.1274P)^{-1}$ ($P = (F_o^2 + 2F_c^2)/3$)) x_{abs} not refined. $|\Delta\rho_{max}| = 0.33$ e Å^{-3}. O,H(2)···O(7) ($1/2 - x$, $1 - y$, $z - 1/2$) are 2.886(1), 2.2 (est.) Å and O, H(3)···O(2) ($1/2 + x$, $11/2 - y$, $1 - z$) 2.760(1), 2.0 (est.) Å. CCDC 638780.
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13. HR-ESI-MS $[M+H]^+$, 267.1593 $C_{15}H_{23}O_4$ requires 267.1591; $[\alpha]_D^{24}$ 102.2 (*c* 0.084 in methanol); IR ν_{max} (thin film) cm^{-1} = 3474, 2935, 1736, 1706, 1226, 1120, 1063, 1007, 991.
14. HR-ESI-MS $[M+H]^+$ 269.1750 $C_{15}H_{25}O_4$ requires 269.1753; $[\alpha]_D^{24}$ 23.7 (*c* 0.077 in methanol); IR ν_{max} (thin film) cm^{-1} = 3244, 2948, 1541, 1457, 1073, 997.
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