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Trefolane A, a Sesquiterpenoid with a New Skeleton from Cultures of the Basidiomycete *Tremella foliacea*

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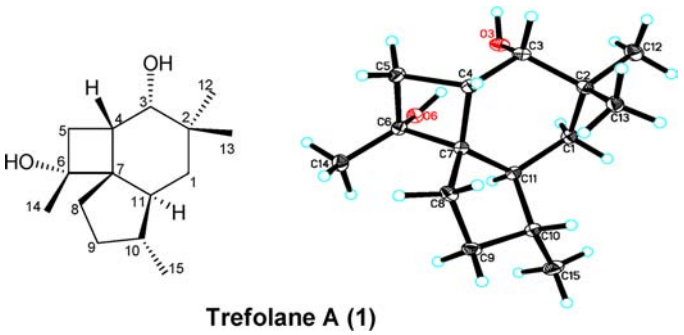
ABSTRACT



Trefolane A (1)

Trefolane A (1), an unprecedented skeleton with a 5/6/4 tricyclic ring system, was isolated from cultures of the basidiomycete *Tremella foliacea*. The structure was elucidated by means of spectroscopic methods and further confirmed by single crystal X-ray diffraction analysis. A possible biogenesis for trefolane A (1) was also proposed.

The fruiting bodies of higher fungi are under constant threat of other organisms feeding on them. As a consequence these organisms have developed a number of strategies for protection; one of them is the production of toxins. The basidimycetes produce a multitude of toxic sesquiterpenes derived from the humulane skeleton.[1] The rich structural variation of sesquiterpenes from basidiomycetes produces a rich variation of activities as well. HMAF based on illudane sesquiterpenes from the basidiomycete *Clitocybe illudens* is now in the clinical trial II phase and promises to become an antitumor drug derived from a fungal sesquiterpene.[2] Inspired by the potential biological activity of different sesquiterpenes from basidiomycetes, our group focused on sesquiterpene metabolites from high fungi, and also reported a number of sesquiterpenoids, which represented various types of carbon skeletons. For instance, mitissimols A−C are humulane sesquiterpenoids from mushroom *Lactarius mitissimus*.[3] Steperoxides A and B are two novel 3-nor-methyl-chamigrane sesquiterpene peroxides from *Steccherinum ochraceum*.[4] Agrocybone is an illudane−illudane bis-sesquiterpene from the basidiomycete *Agrocybe salicacola*, exhibiting weak antiviral activity against the respiratory syncytial virus.[5] Rufuslactone is

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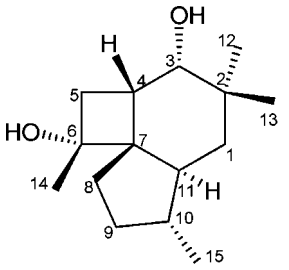
an antifungal sesquiterpene from the fruiting bodies of the basidiomycete *Lactarius rufus*.[6] Boledulins A−C are non-isoprenoid botryane sesquiterpenoids from basidiomycete *Boletus edulis*.[7] Currently, a novel sesquiterpenoid, trefolane A (**1**), has been obtained from cultures of the basidiomycete *Tremella foliacea*,[8] an edible fungus with gelatinous fruiting bodies. Trefolane A (**1**) possesses an unprecedented 5/6/4 system which was suggested to be transformed from the humulene skeleton. Its structure was established by extensive spectroscopic methods, and the absolute configuration was determined by single crystal X-ray diffraction analysis.



Trefolane A (1)

The culture broth (25 L) of *T. foliacea* was filtered, and the filtrate was extracted three times with EtOAc, while the mycelium was extracted three times with $CHCl_3$−MeOH (1:1). The EtOAc layer together with the mycelium extraction was concentrated under reduced pressure to give a crude extract (40 g). The extract was subjected to column chromatography over silica gel (200−300 mesh) eluted with a gradient of petroleum ether−acetone (1:0 → 0:1) to obtain 13 fractions (1−13). Fraction 5 was separated by RP-18 (MeOH−H_2O, 4:1−9:1), followed by Sephadex LH-20 (acetone) column chromatography to afford **1** (1.8 mg).

Table 1. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR Data of **1**[a] in $CDCl_3$ (δ in ppm, J in Hz)

entry	δ_H	δ_C
1a	1.60 (1H, overlapped)	40.3 (t)
1b	1.06 (1H, d, 6.5)	
2		35.7 (s)
3	3.43 (1H, d, 6.6)	74.5 (d)
4	2.12 (1H, m)	37.1 (d)
5	2.02 (2H, m)	35.6 (t)
6		74.4 (s)
7		55.7 (s)
8a	1.93 (1H, dd, 13.3, 5.7)	35.6 (t)
8b	1.47 (1H, ddd, 13.3, 5.7, 6.3)	
9a	1.73 (1H, m)	34.2 (t)
9b	0.88 (1H, m)	
10	1.58 (1H, overlapped)	41.8 (d)
11	1.83 (1H, dd, 6.5, 6.4)	39.9 (d)
12	1.04 (3H, s)	23.4 (q)
13	0.91 (3H, s)	29.4 (q)
14	1.25 (3H, s)	23.2 (q)
15	1.01 (3H, d, 6.8)	19.9 (q)

[a] Data were assigned by HSQC, HMBC, ^{1}H−^{1}H COSY, and ROESY spectra.

Trefolane A (**1**),[9] a colorless crystal, was detected to possess the molecular fomula $C_{15}H_{26}O_2$ (HREIMS: m/z = 238.1932 $[M]^+$), indicating three degrees of unsaturation. The ^{13}C and DEPT NMR spectra revealed three sp^3 quaternary carbons (one oxygenated at δ_C 74.4), four sp^3 methines (one oxygenated at δ_C 74.5), four sp^3 methylenes, and four methyls (Table 1). These data suggested that **1** was a sesquiterpenoid with a three-ring system.

The gross structure of **1** was initially deduced by comprehensive analysis on its 1D and 2D NMR spectra. According to the ^{1}H−^{1}H COSY spectrum, two fragments [**a**: −CH_2-(8)−CH_2(9)−CH(10)−CH(11)−CH_2(1)−; **b**: −CH(3)−CH(4)−CH_2(5)−] were established as shown in Figure 1. In **a**, protons of CH_2-8 and CH-11 showed significant HMBC correlations to an sp^3 quaternary carbon at δ_C 55.7 (s, C-7), which suggested that C-8, C-9, C-10, C-11, and C-7 constructed a five-membered carbon ring A (Figure 1). In the HMBC spectrum, two singlets, assigned to two methyls, showed correlations to an sp^3 quaternary carbon at δ_C 35.7 (s, C-2). Meanwhile, H-1 and H-3 also showed critical HMBC correlations to this quaternary carbon (C-2), which allowed the connection between **a** and **b** by C-2. Furthermore, H-4 gave the key correlation to C-7. These HMBC correlations indicated that a six-membered carbon ring B was established by C-1, C-2, C-3, C-4, C-7, and C-11 (Figure 1). In **b**, protons of CH_2-5 and a methyl (C-14) showed HMBC correlations to an oxygenated sp^3 quaternary carbon at δ_C 74.4 (s, C-6); in addition, the weak HMBC correlation from H-5 to C-7 was observed. These HMBC correlations, as well as the requirement of degrees of unstaturation, revealed that C-4, C-5, C-6, and C-7 formed a four-membered carbon ring C (Figure 1). Therefore, the planar structure of **1** was established to possess a 5/6/4 tricyclic backbone with hydroxy substituents at C-3 and C-6, respectively.

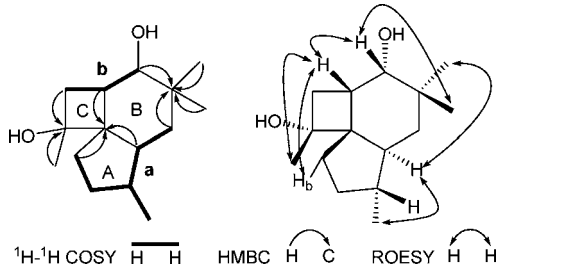


Figure 1. Key 2D NMR correlations of **1**.

ROESY was used to reveal the stereoconfiguration of **1**, in which the cross peaks of H-3/Me-13, H-3/H-4,

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(8) *Tremella foliacea* was collected in Xiang city, Sichuan province, People's Republic of China. A voucher specimen (No. 45869) was deposited at Kunming Institute of Botany, Chinese Academy of Sciences. The culture medium consisted of glucose (5%), peptone from porcine meat (0.15%), yeast powder (0.5%), KH_2PO_4 (0.05%), and $MgSO_4$ (0.05%). The pH was adjusted to 6.5 before autoclaving. Fermentation was carried out on a shaker at 25 °C and 160 rpm for 25 days.

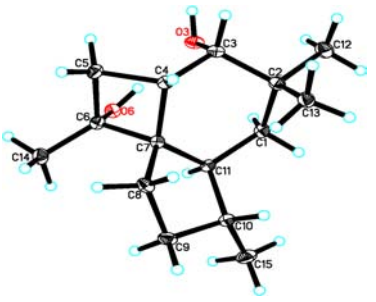


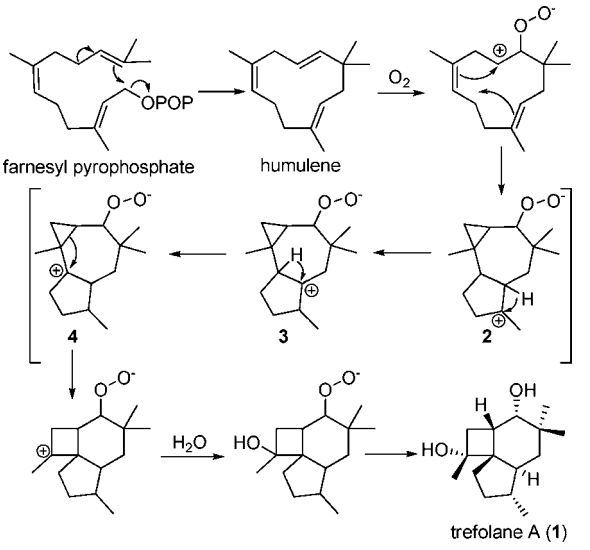
Figure 2. X-ray structure of **1**.

H-4/Me-14, H-4/H-8b, H-11/Me-12, and H-11/H-15 demonstrated that H-3, H-4, H-10, Me-13 C-8, and Me-14 possessed the same orientation, while H-11, Me-12, and Me-15 were in the opposite orientation (Figure 1).

Compound **1** represented a new type of carbon skeleton in the family of sesquiterpenoids. Thus, more solid evidence is necessary for the verification of this structure. Fortunately, a single crystal X-ray diffraction experiment not only confirmed the 5/6/4 tricyclic system as elucidated above but also determined the absolute stereochemistry to be 3*S*,4*S*,6*R*,7*S*,10*R*,11*S* (Figure 2).[10] Thus, compound **1** was established as depicted and named as trefolane A.

Humulene is formed from farnesyl pyrophosphate by an enzymatic cyclization reaction. Biogenetically, it is suggested that most sesquiterpenoids derived from higher

Scheme 1. Plausible Biogenetic Pathway to **1**



fungi, subdivision Basidiomycotina, were started from humulene with three pathways.[1] One route leads to caryophyllane, and another pathway ends in the irregular sesquiterpenes of the tremulane type. The third pathway is the most important one. It produces the tricyclic sesquiterpene protoilludane which is at the biosynthetical crossroad for many sesquiterpene classes. As shown in Scheme 1, we suggest that trefolane A (**1**) started from humulene with the fourth pathway, in which two new carbon bonds were formed between C-7 and C-4, and C-7 and C-11. Considering the present pathway of **1**, a new type of sesquiterpene with a 3/7/5 ring system may be discovered in future.

Trefolane A (**1**) was evaluated for its cytotoxicity against five human cancer cell lines, SK-BR-3 breast, SMMC-7721 hepatocellular carcinoma, HL-60 myeloid leukemia, PANC-1 pancreatic cancer, and A-549 lung cancer, using the MTT method reported previously[11] with minor revision.[12] Unfortunately, no significant activity was detected (IC_{50} > 40 μM). In order to perform more biological assays for this unusual sesquiterpenoid, further scale-up isolation is in progress.

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Supporting Information Available. NMR, MS, and IR spectra and the X-ray crystallographic data (CIF file) of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

(9) Trefolane A (**1**): colorless crystals (CH_2Cl_2/acetone = 10/1); mp 156 °C; $[\alpha]^{22}_D$ + 3.5 (*c* 0.18, MeOH); IR (KBr) ν_{max}: 3426, 3440, 2928, 1729, 1452, 1383, 1287, 1120 cm^{-1}; ^1H (600 MHz) and ^{13}C NMR (150 MHz) data ($CDCl_3$), see Table 1; HREIMS *m*/*z* 238.1932 [M] $^+$ (calcd for $C_{15}H_{26}O_2$, 238.1933).

(10) Crystal data for trefolane A (**1**): $C_{15}H_{26}O_2$, MW = 238.36; monoclinic, space group $P2_12_12_1$; *a* = 8.2769(3) Å, *b* = 12.3605(4) Å, *c* = 13.4157(4) Å, α = *β* = *γ* = 90.00, *V* = 1372.51(8) $Å^3$, *Z* = 4, *d* = 1.154 g/cm^3, crystal dimensions 0.20 × 0.33 × 0.56 mm^3 was used for measurement on a Bruker APEX DUO with a graphite monochromater, Cu Kα radiation. The total number of reflections measured was 8057, of which 2443 were observed, $I > 2\sigma(I)$. Final indices: R_1 = 0.0342, wR_2 = 0.0888. The crystal structure of **1** was solved by direct method SHLXS-97 (Sheldrick, 1990) and expanded using difference Fourier technique, refined by the program SHLXL-97 (Sheldrick, 1997) and the full-matrix least-squares calculations. Crystallographic data for the structure of **1** have been deposited in the Cambridge Crystallographic Data Centre (deposition number: CCDC 894183). Copies of these data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB21EZ, UK.; fax: (+44) 1223-336-033; or desposit@ccdc.cam.ac.uk).

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(12) Cytotoxicity assay. *All the cells were cultured in RPMI-1640 or DMEM medium (Hyclone, USA), supplemented with 10% fetal bovine serum (Hyclone, USA) in 5% CO_2 at 37 °C.* The cytotoxicity assay was performed according to the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method in 96-well microplates. Briefly, 100 μL of adherent cells were seeded into each well of 96-well cell culture plates and allowed to adhere for 12 h before drug addition, while suspended cells were seeded just before drug addition with an initial density of 1 × 10^5 cells/mL. Each tumor cell line was exposed to the test compound at concentrations of 0.0625, 0.32, 1.6, 8, and 40 μm in triplicates for 48 h, with cisplatin (sigma, USA) as a positive control. After compound treatment, cell viability was detected and cell growth curve was graphed.