Tubercullariols A – C, New Sesquiterpenes from the Mutant Strain M-741 of *Tubercularia* sp. TF 5

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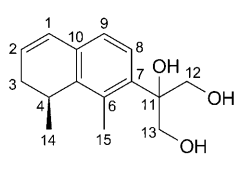
Three new sesquiterpenes, tubercullariols A – C (**1** – **3**, resp.), were isolated from the mutant strain M-741 of *Tubercularia* sp. TF 5, an endophytic fungus of *Taxus mairei*. Their structures were elucidated by spectroscopic analyses including 1D- and 2D-NMR experiments, and HR-Q-TOF-MS. Antitumor and antibacterial properties of these compounds were evaluated.

Introduction. – Endophytic fungi, particularly from higher plants, have proved to be a rich source of bioactive and chemically novel compounds with a huge medicinal and agricultural potential [1 – 3]. The fungal strain *Tubercularia* sp. TF 5 was isolated from the inner bark of *Taxus mairei* collected in Fujian Province, southeast China. Previous studies indicated that this strain has the potential to produce the anticancer compound taxol [4] and other compounds including diterpenoids and polyketides [5]. In order to further mine natural products from this strain, we mutated it by treating the protoplasts with UV and NTG at a dose of 70% lethal rate. A morphological and metabolic mutant strain M-741 was selected from 860 strains regenerated from fused-protoplasts. In our continuing search for taxol from M-741, three new sesquiterpenes were obtained from its fermentation culture. Here, we report the isolation and structural determination of these new sesquiterpenes, tubercullariols A – C (**1** – **3**), and their antitumor and antibacterial activities.[1])



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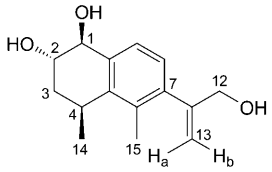

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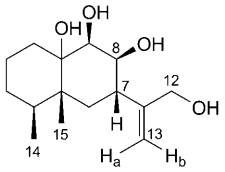


3[1])

Results and Discussion. – The mutant strain M-741 of *Tubercularia* sp. TF 5 was cultivated for 21 d on 5 l PDA media. The culture was extracted three times with an equal volume of AcOEt/MeOH/AcOH 80 : 15 : 5 (*v*/*v*/*v*) at room temperature. The

[1]) Eremophilane numbering. For systematic names, see *Exper. Part.*

crude extract was purified by repeated column chromatography (*RP-18*, *Sephadex LH-20*, and SiO_2) to afford three new sesquiterpenes.

Compound **1** was obtained as an optically active white powder. The molecular formula of **1** was determined to be $C_{15}H_{20}O_3$ according to the HR-Q-TOF-MS and NMR data. The IR absorption at 3433 cm^{-1} indicated the presence of OH groups.

The ^{13}C-NMR (DEPT) spectra of **1** (*Table 1*) exhibited 15 signals for two Me, three CH_2 (two of them O-bearing), and five CH groups, as well as five quaternary C-atoms, respectively. The ^{1}H-NMR spectrum demonstrated the presence of two Me groups at δ(H) 2.48 (*s*) and 1.07 (*d*, *J* = 7.0), four olefinic H-atoms at δ(H) 5.92 (*m*), 6.42 (*dd*, *J* = 9.0, 3.2), 6.89 (*d*, *J* = 8.4), and 7.21 (*d*, *J* = 8.4), and four OCH_2 H-atoms at δ(H) 4.00–4.07 (overlapped). The presence of a 1,2,3,4-tetrasubstituted benzene ring was indicated by a pair of *doublet*s with the equal *J* value of 8.4 Hz at δ(H) 6.89 and 7.21 in the ^{1}H-NMR, and six sp^2 C-atom signals at δ(C) 132.5 (C(10)[1]), 124.2 (C(9)), 124.4 (C(8)), 137.5 (C(7)), 133.4 (C(6)), and 140.9 (C(5)) in the ^{13}C-NMR spectra. A Me substituent at the benzene ring was indicated by the HMBC from the H-atoms of Me(15) to C(5), C(6), and C(7). The presence of a 1,4-disubstituted pent-1-ene moiety was revealed by the ^{1}H,^{1}H-COSY data, which was linked to the benzene ring at adjacent positions to form a dihydronaphthalene based on the HMBC correlations from Me(14) to C(5), and H–C(1) to C(9). The fourth substitutent was determined to be a 2-glyceryl residue on the basis of HMBC data from H–C(12) to C(7), C(11), and C(13), and from H–C(13) to C(7) and C(11). Therefore, a 1,5,7,9-eremophilatetraene-type sesquiterpene was identified [6][7]. The OH groups at C(11), C(12), and C(13) were confirmed by the C-atom signals shifted downfield at δ(C) 77.3, 67.4, and 67.5,

Table 1. *^{1}H- and ^{13}C-NMR Data of* **1** *and* **2**. At 600/150 MHz, resp., in $CDCl_3$ (**1**) and MeOD (**2**); δ in ppm, *J* in Hz.

Position	**1**		**2**	
	δ(C)	δ(H)	δ(C)	δ(H)
1	127.2 (*d*)	6.42 (*dd*, *J* = 3.2, 9.0)	75.6 (*d*)	4.40 (*d*, *J* = 8.4)
2	126.3 (*d*)	5.92 (*ddd*, *J* = 2.2, 6.4, 9.0)	68.7 (*d*)	4.01–4.05 (*m*)
3	30.9 (*t*)	2.55 (*ddt*, *J* = 2.7, 7.0, 17.4, H_α), 2.24 (*dd*, *J* = 6.4, 17.4, H_β)	37.2 (*t*)	1.91–1.99 (*m*, 2 H)
4	27.7 (*d*)	3.23 (*dq*, *J* = 7.0, 6.4)	30.8 (*d*)	3.28–3.30 (*m*)
5	140.9 (*s*)	–	140.1 (*s*)[a]	–
6	133.4 (*s*)	–	132.1 (*s*)	–
7	137.5 (*s*)	–	139.7 (*s*)[a]	–
8	124.4 (*d*)	7.21 (*d*, *J* = 8.4)	126.7 (*d*)	6.99 (*d*, *J* = 7.8)
9	124.2 (*d*)	6.89 (*d*, *J* = 8.4)	124.7 (*d*)	7.41 (*d*, *J* = 7.8)
10	132.5 (*s*)	–	136.7 (*s*)	–
11	77.3 (*s*)	–	150.8 (*s*)	–
12	67.4 (*t*)[a]	4.06, 4.07 (*AB*, *J* = 11.5)[a]	64.9 (*t*)	4.16, 4.19 (*AB*, *J* = 15.4)[a]
13	67.5 (*t*)[a]	4.00, 4.02 (*AB*, *J* = 11.5)[a]	111.2 (*t*)	4.93 (*d*, *J* = 1.8, H_a), 5.49 (*d*, *J* = 1.8, H_b)
14	18.4 (*q*)	1.07 (*d*, *J* = 7.0, 3 H)	14.4 (*q*)	1.31 (*d*, *J* = 7.2, 3 H)
15	16.2 (*q*)	2.48 (*s*)	20.4 (*q*)	2.28 (*s*)

[a]) Signals are interchangeable.

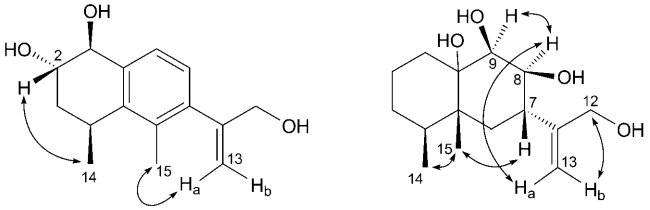
respectively. Thus, the structure of **1** was established to be 2-[(8*R**)-7,8-dihydro-1,8-dimethylnaphthalen-2-yl]propane-1,2,3-triol, and named tubercullariol A. The absolute configuration of this compound remains to be established.

Compound **2** was obtained as an optically active white powder. The molecular formula of **2** was determined to be $C_{15}H_{20}O_3$ according to the HR-Q-TOF-MS and NMR data. The IR absorption at 3410 cm^{-1} indicated the presence of OH groups.

The ^{13}C-NMR (DEPT) spectra of **2** (*Table 1*) exhibited 15 signals for two Me, three CH_2 (one oxygenated), and five CH groups (two of them O-bearing), as well as five quaternary C-atoms, respectively. The ^{1}H-NMR spectrum demonstrated the presence of two Me groups at δ(H) 2.28 (*s*) and 1.31 (*d*, *J* = 7.2), four olefinic H-atoms at δ(H) 4.93 (*d*, *J* = 1.8), 5.49 (*d*, *J* = 1.8), 6.99 (*d*, *J* = 7.8), and 7.41 (*d*, *J* = 7.8), respectively. As for compound **1**, the presence of a tetrasubstituted benzene ring was indicated by a pair of *doublet*s with the equal *J* value of 7.8 Hz at δ(H) 6.99 and 7.41 in the ^{1}H-NMR, and six sp^2-C-atom signals at δ(C) 136.7 (C(10)[1])), 124.7 (C(9)), 126.7 (C(8)), 139.7 (C(7)), 132.1 (C(6)), and 140.1 (C(5)) in the ^{13}C-NMR spectra. A Me substitutent at the benzene ring was indicated by the HMBC from the H-atoms of Me(15) to C(5), C(6), and C(7). The presence of a 1,4-disubstituted 1,2-pentanediol moiety was revealed by the ^{1}H,^{1}H-COSY data, which was linked to the benzene ring at adjacent positions to form a tetrahydronaphthalene based on the HMBC from Me(14) to C(5), and H–C(1) to C(10). The fourth substitutent of the benzene moiety was determined to be a (1-hydroxymethyl)ethenyl residue on the basis of HMBC data from H–C(12) to C(7), C(11), and C(13), and from H–C(13) to C(7) and C(11). Therefore, a 5,7,9,11-eremophilatetraene-type sesquiterpene was identified [6][7]. The OH groups at C(1), C(2), and C(12) were confirmed by the downfield shifted signals at δ(C) 75.6, 68.7, and 64.9, respectively. The relative configuration of **2** was revealed by the NOE correlations between H–C(2) and H–C(14) as shown in the *Figure*. The *trans*-orientation of HO–C(1) and HO–C(2) was supported by the *J* value 8.4 Hz of H–C(1). Therefore, the structure of **2** was elucidated as (1*R**,2*R**,4*R**)-1,2,3,4-tetrahydro-6-(3-hydroxyprop-1-en-2-yl)-4,5-dimethylnaphthalene-1,2-diol, and named tubercullariol B.

Compound **3** was obtained as an optically active white powder. The molecular formula was determined to be $C_{15}H_{26}O_4$ on the basis of the HR-Q-TOF-MS (positive-ion mode) and NMR data. The IR absorption at 3531 cm^{-1} indicated the presence of OH groups.

The ^{13}C-NMR (DEPT) spectra (*Table 2*) and HMQC experiments revealed the signals for two Me, six CH_2 (one of them O-bearing), and four CH groups (two of them



2[1]) **3**[1])

Figure. *Selected NOE correlations for compounds* **2** *and* **3**

Table 2. *^1H- (600 MHz) and ^{13}C-NMR* (DEPT) (150 MHz) *Spectral Data of* **3** (MeOD).

Position	δ(H)	δ(C)
1	1.23–1.26 (*m*), 2.16 (*dt*, *J* = 4.9, 13.8)	30.7 (*t*)
2	1.52–1.55 (*m*), 1.80–1.89 (*m*)	19.9 (*t*)
3	1.35–1.37 (*m*, 2 H)	29.6 (*t*)
4	1.96 (*dq*, *J* = 7.1, 6.6)	34.8 (*d*)
5		39.3 (*s*)
6	1.35–1.37 (*m*), 1.65 (*t*, *J* = 12.0)	38.5 (*t*)
7	2.53 (*ddd*, *J* = 2.4, 3.8, 11.5)	39.0 (*d*)
8	4.08 (*dd*, *J* = 3.8, 11.3)	71.2 (*d*)
9	3.53 (*d*, *J* = 3.8)	78.4 (*d*)
10		75.1 (*s*)
11		151.8 (*s*)
12	4.10, 4.14 (*AB*, *J* = 14.1)	64.6 (*t*)
13	5.06 (*d*, *J* = 1.5, H_a), 5.19 (*dd*, *J* = 1.5, 3.1, H_b)	108.9 (*t*)
14	0.73 (*d*, *J* = 6.6, 3 H)	13.4 (*q*)[a])
15	1.13 (*s*)	13.8 (*q*)[a])

[a]) Assignments may be interchanged.

O-bearing), as well as three quaternary C-atoms including an olefinic C-atom at δ(C) 151.8. Elucidation of the ^{1}H,^{1}H-COSY and HMQC spectra of **3** enabled the deduction of the fragments $-CH_2(1)-CH_2(2)-CH_2(3)-CH(4)-Me(14)^1)$ and $-CH(9)-CH(8)-CH(7)-CH_2(6)-$, which was further supported by the HMBC from Me(14) to C(3) and C(4), from H–C(6) to C(7) and C(8), and from H–C(9) to C(8) and C(7). The linkage of these two fragments was established by the HMBC from Me(15) to C(4), C(5), C(6), and C(10), and from C(10) to H–C(9) and H–C(6). The presence of an exo-C=C bond was indicated by the ^{1}H-NMR signals for exomethylene H-atoms at δ(H) 5.06 (*d*, *J* = 1.5, H_a) and 5.19 (*dd*, *J* = 1.5, 3.1, H_b), as in compound **2**, and a (1-hydroxymethyl)ethenyl residue was determined by the HMBC data from H–C(12) to C(11) and C(13), and from H–C(13) to C(7) and C(11), which was linked to C(7), indicated by the HMBC data from C(7) to H–C(12), H_a–C(13), and H_b–C(13). The relative configuration of **3** was determined by the NOE correlations between H–C(14) and H–C(15), H–C(15) and H–C(7), H_a–C(13), and H–C(8), and between H–C(8) and H–C(9) as shown in the *Figure*. Thus, the structure of **3** was established to be (1*R**,2*R**,3*R**,4a*S**,5*R**)-octahydro-3-(3-hydroxyprop-1-en-2-yl)-4a,5-dimethylnaphthalene-1,2,8a(1*H*)-triol [8][9], and named tuberculariol C.

Compounds **1**–**3** were tested in antitumor and antimicrobial assays *in vitro*. The antibacterial activities of **1**–**3** were tested at a concentration of 50 μg/ml against *Saccharomyces cerevisiae* ATCC9763, *Escherichia coli* CMCC44103, and C*andida albicans* AS2.538 using the MIC (= minimum inhibitory concentration) method with 96-well microplates. No inhibitory activities were observed for all three compounds. The cytotoxicities against HeLa cell line were measured at 72 h post treatment by the MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method. The results showed that compounds **1**–**3** exhibited only weak inhibitory activity against HeLa cells.

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Experimental Part

General. Column chromatography (CC): silica gel (SiO_2; 200–300 and 80–100 mesh; *Qingdao Marine Chemical Factory*, Qingdao, P. R. China), or SiO_2 *GF*$_{254}$ (*Merck*), or *RP-18* gel (*Merck*), or *Sephadex LH-20* gel (*Amersham Biosciences*) were used. TLC: precoated SiO_2 *GF*$_{254}$ plates (0.20–0.25 mm, *Qingdao Marine Chemical Factory*). Optical rotations: *AUTOPOL®IV*. IR Spectra: *Thermo Nicolet 380* FT-IR spectrophotometer, with KBr cells; in cm^{-1}. NMR Spectra: *Bruker ARX 600* spectrometer, operating at 600/150 MHz, δ in ppm rel. to Me_4Si; *J* in Hz. HR-Q-TOF-MS: *Bio TOF™-Q* mass spectrometer (*Bruker*); in *m*/*z*.

Isolation and Fermentation of the Mutant Strain M-741. The wild-type strain of *Tubercularia* sp. TF 5 was maintained on potato-dextrose-agar (PDA) slants. Stock culture was transferred onto fresh PDA slant and allowed to develop for 5 d at 28°. Then it was inoculated into a 250 ml *Erlenmeyer* flask containing 50 ml of liquid PD medium and statically cultured at 28° for 4 d. The mycelium was collected and washed by distilled H_2O, and then suspended in 10 ml lytic buffer containing 1% lywallzyme (*Guangdong Institute of Microbiology*, Guangzhou, P. R. China), 0.25% cellulase (*Sinopharm Chemical Reagent CO., LTD.*, Shanghai, P. R. China), 0.5M NaCl, and 0.05M K_3PO_4, pH 6.0. After incubation for 3 to 4 h under gentle shaking (50 rpm) at 30°, the protoplasts were collected and purified by filtering through a cheese cloth, and harvested by centrifugation for 5 min at 3500 rpm. The protoplast pellets were washed with and resuspended in 0.5M NaCl, and irradiated for 30 min by 254 nm UV under the dark. The irradiated protoplast suspension was further treated for 60 min by 40 μg/ml NTG (= *N*-methyl-*N*-nitro-*N*-nitrosoguanidine), and inoculated onto PDA regeneration plates containing 0.5M NaCl as stabilizer. After cultivation for 6 d at 28°, 860 colonies of the protoplast regeneration plates were transferred on slants, and inoculated on new PDA plates and cultivated for 21 d at 25°. These cultures were screened on the basis of colony morphology and TLC analysis of their MeOH extracts. The mutant strain M-741 was selected due to its morphological and TLC difference from the initial strain *T.* sp. TF 5, and was inoculated onto *Petri* dishes containing 5 l PDA medium and cultivated for 21 d at 25°.

Extraction and Isolation. The cultivated PD agar was chopped, diced, and extracted with AcOEt/MeOH/AcOH (80:15:5) exhaustively. The org. soln. was collected through filtration, and the combined filtrate, upon evaporation, yielded the crude extract as a brown syrup, which was partitioned successively between H_2O and AcOEt (1:1), until the AcOEt layer became colorless. The combined org. layers were concentrated *in vacuo* to yield an AcOEt extract (3.17 g) which was subjected to MPLC over *RP-18* SiO_2 (80 g) using a stepwise gradient of 30, 50, 70, and 100% (*v*/*v*) MeOH in H_2O (1 l for each gradient) to yield 8 fractions (*Fr. 1–8*). *Fr. 4* (153 mg) was separated by CC over *Sephadex LH-20* eluted with MeOH to give four fractions *Fr. 4.1–Fr. 4.4*. Further separations of *Fr. 4.4* were carried out by repeated CC over SiO_2 (200–300 mesh) eluted with $CHCl_3$/MeOH (500:1) and *Sephadex LH-20* eluted with acetone, resp., to yield **1** (5 mg). *Fr. 3* (206 mg) was separated by CC over *Sephadex LH-20* eluted with MeOH to give four fractions (*Fr. 3.1–Fr. 3.4*). *Fr. 3.3* (56 mg) was subjected to repeated MPLC over *RP-18* SiO_2, CC over *Sephadex LH-20* and SiO_2, resp., to afford compounds **2** (2 mg) and **3** (3 mg).

Tuberculariol A (=*2-[(8*R**)-7,8-Dihydro-1,8-dimethylnaphthalen-2-yl]propane-1,2,3-triol*; **1**). White powder. $[\alpha]_D^{20} = -46.0$ ($c = 0.5$, $CHCl_3$). IR (KBr): 3433. ^{1}H- and ^{13}C-NMR: *Table 1*. ESI-MS (pos.): 271 ($[M+Na]^+$). HR-ESI-Q-TOF-MS: 271.1414 ($[M+Na]^+$, $C_{15}H_{20}NaO_3^+$; calc. 271.1305).

Tuberculariol B (=*(1*R**,2*R**,4*R**)-1,2,3,4-Tetrahydro-6-(3-hydroxyprop-1-en-2-yl)-4,5-dimethylnaphthalene-1,2-diol*; **2**). White powder. $[\alpha]_D^{20} = -75.0$ ($c = 0.2$, MeOH). IR (KBr): 3410. ^{1}H- and ^{13}C-NMR: *Table 1*. ESI-MS (pos.): 271 ($[M+Na]^+$). HR-ESI-Q-TOF-MS: 271.1711 ($[M+Na]^+$, $C_{15}H_{20}NaO_3^+$; calc. 271.1305).

Tuberculariol C (=*(1*R**,2*R**,3*R**,4a*S**,5*R**)-Octahydro-3-(3-hydroxyprop-1-en-2-yl)-4a,5-dimethylnaphthalene-1,2,8a(1*H*)-triol* (**3**). White powder. $[\alpha]_D^{20} = -93.0$ ($c = 0.3$, MeOH). IR (KBr): 3531. ^{1}H-

and ^{13}C-NMR: *Table 2*. ESI-MS (pos.): 293 ($[M+Na]^+$). HR-ESI-Q-TOF-MS: 293.1911 ($[M+Na]^+$, $C_{15}H_{20}NaO_4^+$; calc. 293.1723).

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