FULL PAPER

Myrothecoside, a Novel Glycosylated Polyketide from the Terrestrial Fungus *Myrothecium* sp. GS-17

by **Songya Zhang**[a]), **Jing Zhu**[a]), **Tao Liu**[b]), **Suzan Samra**[a]), **Huaqi Pan**[c]), **Jiao Bai**[d]), **Huiming Hua**[d]), and **Andreas Bechthold**[a])

[a]) Institut für pharmazeutische Biologie und Biotechnologie, Albert-Ludwigs Universität, Freiburg 79104, Germany (phone: +49-761-2038371; e-mail: andreas.bechthold@pharmazie.uni-freiburg.de)
[b]) Department of Natural Products Chemistry, School of Pharmacy, China Medical University, Shenyang 110001, P. R. China
[c]) Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110016, P. R. China
[d]) Key Laboratory of Structure-Based Drug Design & Discovery, Ministry of Education, Shenyang Pharmaceutical University, Shenyang 110016, P. R. China

Based on the chemical analysis and targeted bioactivity screening, a new polyketide glycoside, myrothecoside, was isolated from a terrestrial halotolerant fungus, *Myrothecium* sp. GS-17. The structure of myrothecoside was elucidated on the basis of extensive spectroscopic analysis including 1D- and 2D-NMR (^{1}H,^{1}H-COSY, HSQC, HMBC, and NOESY) experiments, combined with mass spectroscopic data and physicochemical properties. This compound exhibited weak cytotoxicity against human leukemia (HL-60) cancer cell with an IG_{50} value of 63.61 μM, and also antifungal activities against plant pathogenic fungi *Rhizoctonia solani* and *Fusarium oxysporum* using standard agar diffusion tests at 20 μg/disk.

Keywords: Secondary metabolites, Fungi, *Myrothecium*, Glycosylated polyketides, Antibacterial activity

Introduction

Natural products derived from extreme-tolerant microbes, especially those originated from fungi, are becoming more and more rich sources for novel drug-like molecule with various kinds of bioactivity [1][2]. Glycosylation of natural product can dramatically influence the bioactivity and pharmacokinetic properties of its parental scaffold [3][4]. However, since 2000 there were not many (only fewer than 10) glycosylated secondary metabolites from fungi reported [5][6]. In the process of screening and investigation of bacteria inhabited in high salt environment, we found a halotolerant fungus, *Myrothecium* sp. GS-17, which was isolated from the soil sample of a saline in Gansu Province in P. R. China. The crude extract of this fungus was shown to exhibit strong growth inhibitory activity against human leukemia (HL-60) cell line. Many different bioactive secondary metabolites such as trichothecenes, amides, polyketides, sterols, and lactones, those compounds were isolated from this strain [7 – 9]. During the following research, we found a new glycosylated polyketide, named myrothecoside. This compound was isolated by various kinds of chromatography and its structure was elucidated by modern NMR spectroscopy subsequently. It represents a novel glycosylated polyketide with some chemical structural similarities to lovastatin, tanzawaic acids, and phomopsidin [10 – 12]. In this study, the internal transcribed spacer (ITS) sequence analysis of *Myrothecium* sp. GS-17 was performed. Our data indicate that *Myrothecium* sp. GS-17 is closely related to *Myrothecium verrucaria* Hmp-F73, and the new compound also can be detected in the extract of *Myrothecium verrucaria* Hmp-F73.

Results and Discussion

Genetic Characterization of Myrothecium sp. GS-17

The ITS analysis was performed using chromosomal DNA isolated from *Myrothecium* sp. GS-17. A phylogenetic tree was generated based on the complete ITS1-5.8S-ITS2, and partial 18S rDNA and 28S rDNA regions using the neighbor-joining clustering method [13]. The phylogenetic tree clearly revealed a close evolutionary relationship of this strain to other *Myrothecium* species (*Fig. 1*).

Isolation and Structure Characterization of Myrothecoside

For structure elucidation, 56 l of a liquid culture of *Myrothecium* sp. GS-17 were cultivated. Myrothecoside was isolated as described in the *Exper. Part*. It was obtained as colorless solid, and its molecular formula was determined as $C_{27}H_{45}NO_8$ by HR-ESI-MS (*m*/*z* 512.3221

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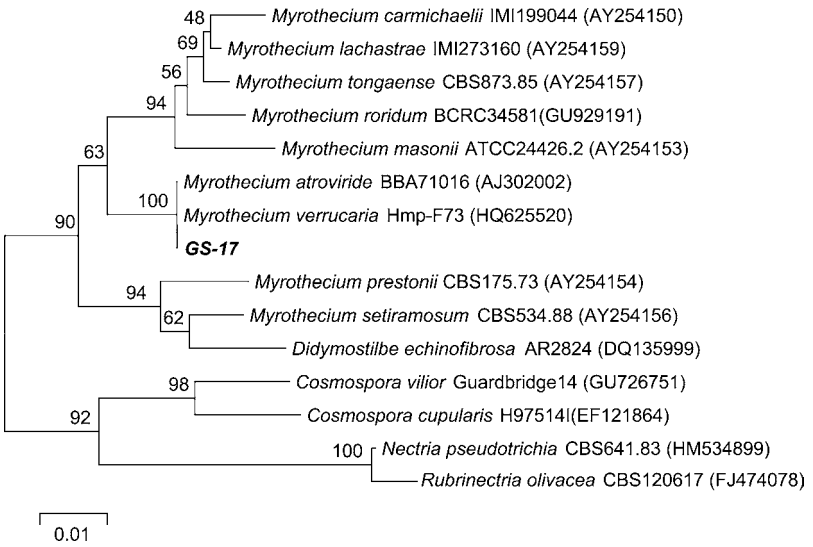

Fig. 1. Phylogenetic tree of the fungal ITS sequences of *Myrothecium* sp. GS-17 by the neighbor-joining method

$[M + H]^+$, calc. 512.3218), with six degrees of unsaturation. The IR spectrum indicated the presence of a OH or amino group (3380 cm^{-1}), Me groups (2948, 2905 cm^{-1}), and an amide C=O group (1665 cm^{-1}). The ^{1}H-NMR (600 MHz, (D_5)pyridine) spectrum of myrothecoside (*Table*) indicated the presence of a methoxy group at δ 3.77 (3 H, *s*), two oxymethines at δ 4.95 (1 H, *m*), 4.79 (1 H, *m*), two *cis*-olefinic H-atom at δ 5.59 (1 H, *dt*, *J* = 9.7 Hz, 3.4 Hz), 5.43 (1 H, *d*, *J* = 9.7 Hz), two *trans*-olefinic H-atom at δ 5.94 (1 H, *dd*, *J* = 15.2 Hz, 10.3 Hz), 5.39 (1 H, *dd*, *J* = 15.2 Hz, 8.0 Hz), and two amino H-atom δ 8.25 (1 H, *s*) and 7.86 (1 H, *s*). Twenty-seven C-atom signals were evident in the ^{13}C-NMR spectrum (150 MHz, (D_5)pyridine) of myrothecoside (*Table*) and deduced to be four olefinic CH carbons at δ(C) 128.5, 132.9, 133.0, 140.8, one C=O carbon at δ(C) 175.6, and six oxygenated carbons at δ(C) 98.5(C(1″)), 73.1(C(2″)), 76.1 (C(3″)), 79.1(C(4″)), 78.3(C(5″)), and 62.6(C(6″)) accounted for by a mannose residue [14].

Finally, the structure of myrothecoside was elucidated and confirmed by 2D-NMR (^{1}H,^{1}H-COSY, HSQC, and HMBC) (*Fig. 1*). The HMQC spectrum permitted the assignment of all H-atom to the corresponding C-atoms as shown in the *Table*. The ^{1}H,^{1}H-COSY spectrum of myrothecoside disclosed two spin-systems corresponding to parts *A* and *B* (*Fig. 2*). In the HMBC spectrum, the correlations from N–H (δ(H) 8.25, 7.86) to C(1) (δ(C) 175.6) and from CH_2(2) (δ(H) 3.01, 2.83), H–C(3) (δ(H) 4.79) to C(1) (δ(C) 175.6) indicated an amide located at C(2). The HMBC correlations observed from a MeO (δ(H) 3.77) to C(4″) (δ(C) 79.1) suggested that the MeO group was attached to C(4″) of a mannose. The cross-peak between H–C(5) (δ(H) 4.95) and C(1″) (δ(C) 98.5) suggested 4″-*O*-methylmannose located at C(5). The plane structure of myrothecoside was determined as shown. The geometries of the C(3′)=C(4′) and C(6)=C(7) bonds were assigned as (*Z*) and (*E*) on the basis of ^{1}H,^{1}H coupling constants (*J* = 9.7 and 15.2 Hz), respectively. The relative configuration of myrothecoside was elucidated by the analysis of the NOESY spectrum (*Fig. 2*). The NOE correlation of H–C(5′) (δ(H) 1.71) with H–C(10′) (δ(H) 0.70) indicated the *cis*-fused A/B ring system. The NOE correlations of H–C(5′) with H–C(1′), H–C(2′), H–C(9′), and H–C(10′) suggested these H-atom to be β orientated. The correlations of Me(2′a) with Me(9′a) and H–C(7′) suggested that they were α facial. The NOE correlations of H–C(1″) with H–C(3″) and H–C(5″) indicated mannose as the β-anomer in ^{4}C(1) conformation. After acid hydrolysis, the sugar moiety was identified as 4″-*O*-methyl-β-D-mannose by comparing the optical rotation with β-D-mannose. Thus, the structure of myrothecoside was determined as (3*R*,5*R*,6*E*)-3-hydroxy-5-[(4-*O*-methyl-β-D-mannopyranosyl)oxy]-7-[(1*R*,2*R*,4a*S*,6*S*,8*S*,8a*S*)-2,6,8-trimethyl-1,2,4a,5,6,7,8,8a-octahydronaphthalen-1-yl]hept-6-enamide.

Bioactivity Screening

The cancer cell cytotoxicity and the antibacterial activity of myrothecoside were evaluated. The cytotoxic potential of the isolated compound was investigated by determining its concentrations required for 50% growth inhibition (IG_{50} value) for three human cancer cell lines (HL-60, MCF-7, PC-3). Myrothecoside shows a weak activity against human leukemia (HL-60) cancer cell with an IG_{50} value of 63.61 μM. The notable growth inhibitory activity of the original extract against HL-60 cell line was attributed to the presence of the trichothecenes, which was reported to show various remarkable cytotoxic activities [15]. Moreover, myrothecoside was active against plant pathogenic fungi *Rhizoctonia solani* and

Table. D- and 2D-NMR Data (600 and 150 MHz, resp., in (D_5)pyridine) of myrothecoside. δ in ppm, J in Hz[a]).

Position	δ(H)	δ(C)	HMBC	NOESY
NH_2	8.25, 7.86 (2*s*)		C(1)	
1		175.6		
2	3.01 (*dd*, *J* = 14.2, 4.2), 2.83 (*dd*, *J* = 14.3, 8.0)	44.0	C(1), C(3), C(4)	
3	4.79 (*m*, overlapping)	67.0	C(1), C(2), C(40	
4	2.34 (*dd*, *J* = 14.0, 8.2), 2.04 (*dt*, *J* = 14.0, 5.5)	43.7	C(2), C(3), C(5)	
5	4.95 (*dd*, *J* = 8.0, 5.5)	76.7	C(1″), C(3), C(4)	
6	5.39 (*dd*, *J* = 15.2, 8.0)	128.5	C(4), C(5), C(7)	
7	5.94 (*dd*, *J* = 15.2, 10.3)	140.8	C(1′), C(6)	
1′	2.30 (*m*, overlapping)	49.3	C(3′), C(2′), C(10′), C(7), C(2′a)	H(9′), H(5′), H(1′), H(10′)
2′	2.10 (*m*, overlapping)	37.8	C(5′), C(3′)	H(9′), H(5′), H(1′)
3′	5.59 (*dt*, *J* = 9.7, 3.4)	133.0	C(5′), C(2′), C(1′), C(2′a)	
4′	5.43 (*d*, *J* = 9.7, 3.4)	132.9	C(5′), C(3′), C(2′), C(10′)	
5′	1.71 (*t*-like, *J* = 10.9)	43.2	C(6′)	H(9′), H(2′), H(1′), H(10′)
6′	1.56 (*d*, *J* = 10.9), 0.64 (*q*-like, *J* = 12.3)	42.2	C(7′), C(5′), C(10′), C(7′a)	H(7′), H(7′a)
7′	1.36 (*m*, overlapping)	32.9	C(9′)	H(6′), H(5′), H(9′a)
8′	1.44 (*d*, *J* = 13.2), 0.57 (*q*-like, *J* = 12.3)	47.1	C(9′), C(7′), C(6′), C(10′)	H(10′), H(9′a)
9′	1.22 (*m*, overlapping)	37.0	C(10′)	H(5′), H(2′), H(1′), H(10′)
10′	0.76 (*q*-like, *J* = 9.7)	47.1	C(9′), C(7′), C(6′), C(1′)	H(9′), H(8′), H(5′)
2′a	0.98 (*d*, *J* = 7.1)	17.1	C(1′), C(2′), C(3′)	H(9′a)
7′a	0.82 (*d*, *J* = 6.4)	22.9	C(8′)	H(6′)
9′a	0.95 (*d*, *J* = 6.3)	23.3	C(8′), C(10′)	H(8′), H(7′), H(2′a)
5-*O*-4-Methyl-*β*-D-mannose				
1″	4.99 (*s*)	98.5	C(2″), C(3″), C(5″)	H(2″), H(3″), H(5″)
2″	4.44 (*d*, *J* = 3.0)	73.1	C(3″), C(4″)	H(1″), H(3″)
3″	4.15 (*dd*, *J* = 9.3, 3.0)	76.1	C(5″)	H(1″), H(2″), H(5″)
4″	4.07 (*t*-like, *J* = 9.3)	79.1	C(3″), C(5″), C(6″), C(7″)	
5″	3.70 (*dt*, *J* = 9.3, 5.5)	78.3	C(4″)	H(1″), H(3″), H(6″)
6″	4.34 (*d*, *J* = 11.9), 4.20 (*dd*, *J* = 11.9, 5.5)	62.6	C(4″)	H(5″)
7″	3.77 (*s*)	60.9	C(4″)	

[a]) Assignments based on HSQC, COSY, HMBC, and NOESY experiments.

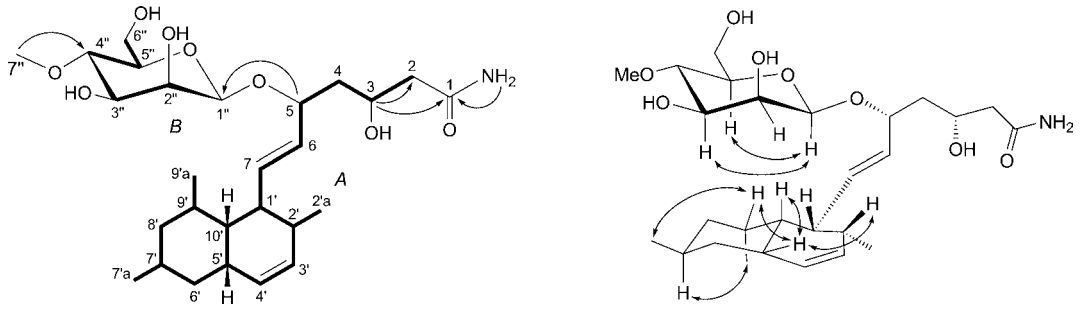

Fig. 2. Key HMBC (H → C), ^{1}H–^{1}H-COSY (▬), and NOESY (H ↔ H) correlations of myrothecoside

Fusarium oxysporum using standard agar diffusion tests at 20 μg/disk [16].

Proposed Biosynthetic Pathway for Myrothecoside

Based on the chemical similarity with lovastatin, tanzawaic acids, and phomopsidin (*Fig. 3*), a proposed biosynthetic pathway for this compound is postulated as shown in *Fig. 4*.

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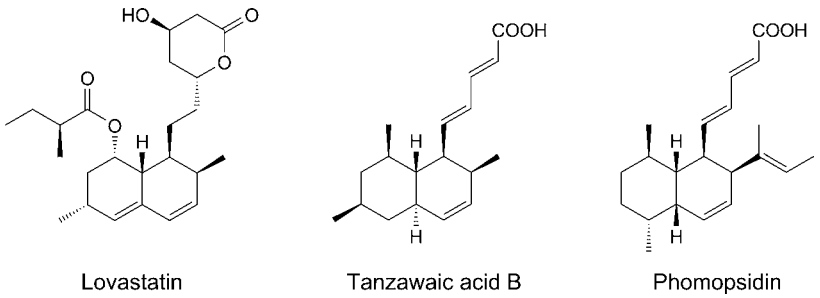

Fig. 3. Structures of lovastatin, tanzawaic acid B, and phomopsidin

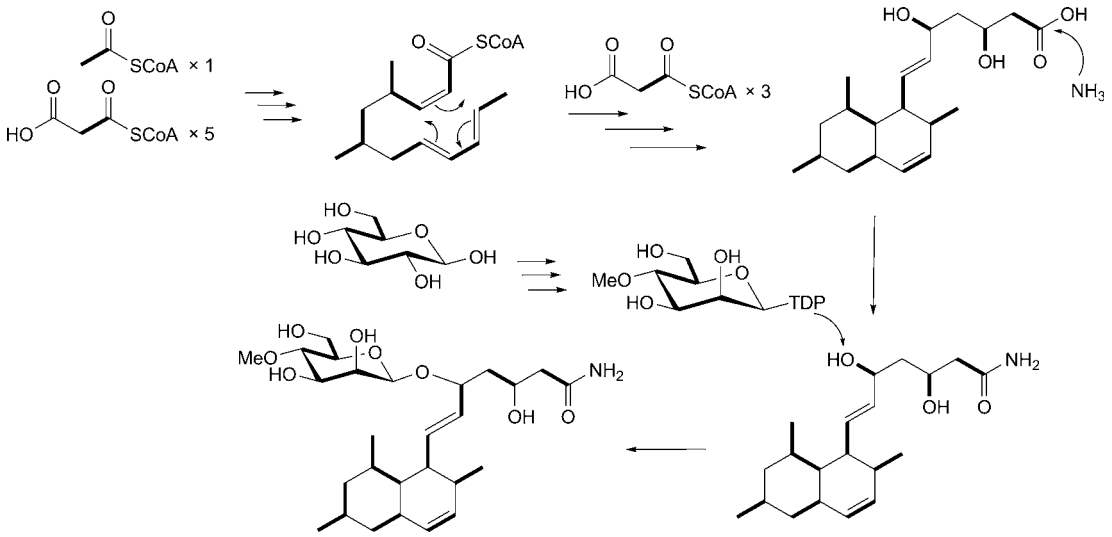

Fig. 4. Proposed biosynthetic pathway of myrothecoside

Experimental Part

General

Column chromatography (CC): silica gel (SiO_2; 200 – 300 mesh; *Qingdao Ocean Chemical Factory*, Qingdao, P. R. China) and ODS (50 μm, *YMC Co., LTD.*, Kyoto, Japan); *Sephadex LH-20* (*GE Healthcare*, Waukesha, WI, USA). RP-HPLC analysis and semipreparation: *HITACHI L2130* (Kyoto, Japan) series pumping system equipped with a *HITACHI L2400* UV detector and performed with a C_{18} column (10 mm × 250 mm, 10 μm; *YMC Co., LTD.*). Optical rotations: *PerkinElmer 341* polarimeter (Waltham, MA, USA). UV Spectra: *Shimadzu UV-2201* (Kyoto, Japan) spectrometer; λ_{max} in nm. FT-IR Spectra: *Bruker IFS-55* spectrometer (Ettlingen, Germany), KBr disk; $\tilde{\nu}$ in cm^{-1}. ^1H- and ^{13}C-NMR spectra: *Bruker ARX-300* and *AV-600* NMR spectrometers; δ in ppm rel. to Me_4Si as internal standard, J in Hz. ESI-MS: *Agilent 1100* ion trap spectrometer (Palo Alto, CA, USA); in m/z. HR-ESI-MS: *Varian QFT-ESI* mass spectrometer; in m/z. A PCR was performed on a *Applied Biosystems-Thermal Cycler* following standard procedures [17] using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The phylogenetic tree was constructed using the neighbor-joining method of MEGA (Version 5.0) [13]. For comparing extracts of *Myrothecium verrucaria* Hmp-F73 and *Myrothecium* sp. GS-17, an HPLC/ESI-MS (*Agilent 1100* LC/MS system) with a *Waters* (Milford, MA, USA) *X-Bridge* C_{18} column (4.6 × 100 mm, 3.5 μm) was used.

Fungal Material

The halotolerant fungal strain GS-17 was separated from the soil sample of a salina in Gansu Province. The fungus was identified as *Myrothecium* sp. based on morphological and molecular taxonomic methods, and it was deposited in Department of Natural Products Chemistry, Shenyang Pharmaceutical University, P. R. China. The initial cultures were maintained on the potato dextrose agar (PDA) solid medium. A small loop of spores growing on a PDA slant was inoculated into a 250 ml *Erlenmeyer* flask containing 75 ml of seawater based culture medium (maltose 2%, monosodium glutamate 1%, KH_2PO_4 0.05%, $MgSO_4{\cdot}7H_2O$ 0.03%, glucose 1%, yeast extract 0.3%, corn steep liquor 0.1%, mannitol 2%, seawater, pH

6.5) and cultured at 28 °C for 2 days on a rotary shaker at 180 r/min. Then, 10 ml of the preculture was inoculated into a 500 ml *Erlenmeyer* flask containing 150 ml of the above culture medium and incubated (375 flasks) for 8 days under the same conditions. Genomic DNA isolation, PCR amplification of the ITS sequence, and the sequencing were performed with conventional methods. Sequence analysis of ITS sequences was performed using BLASTN. Phylogenetic tree was constructed using neighbor-joining method of MEGA (version 5.0). Tree topologies were evaluated by bootstrap analysis with 1000 replicates. *Myrothecium verrucaria* Hmp-F73 was a gift from Prof. Dr. *Jiangchun Hu* from the Institute of Applied Ecology, Chinese Academy of Sciences.

Extraction and Isolation

The fermented broth (56 l) was filtered through cheese cloth to yield the supernate and mycelia. The supernate was subjected to *HPD100* macroporous absorption resin, gradiently eluted with EtOH/H_2O 30%, 70%, 100% to yield three fractions. Fraction 2 was fractioned on a SiO_2 column eluting with CH_2Cl_2/MeOH (100: 0-0: 100) and was further separated by a flash SiO_2 column (eluted with petroleum ether/acetone, 10:1) to give three subfractions (A, B, and C). Subfr. A was further purified by RP-HPLC (MeCN/H_2O (65%), 2.0 ml/min) to yield myrothecoside (13.3 mg).

Myrothecoside (= (3*R*,5*R*,6*E*)-3-Hydroxy-5-[(4-*O*-methyl-β-D-mannopyranosyl)oxy]-7-[(1*R*,2*R*,4a*S*,6*S*,8*S*,8a*S*)-2,6,8-trimethyl-1,2,4a,5,6,7,8,8a-octahydronaphthalen-1-yl]hept-6-enamide). Colorless solid. M.P. 150°. $[\alpha]_D^{20} = -8.77$ (c=0.11, MeOH). UV (MeOH): 255, 224. IR (KBr): 3380, 2948, 2905, 1665. ^{1}H- and ^{13}C-NMR: see the *Table*. ESI-MS: 512.31 ($[M + H]^+$). HR-ES-MS: 512.3221 ($[M + H]^+$, $C_{27}H_{46}NO_8^+$; calc. 512.3218).

Acid Hydrolysis and Sugar Determination of Myrothecoside

Myrothecoside (5 mg) was hydrolyzed with 2N HCl (2 ml) for 16 h at room temperature. The reaction mixture was diluted with H_2O (5 ml), and then extracted with $CHCl_3$ (5 ml) three times. The combined $CHCl_3$ extraction was washed with H_2O and evaporated to afford a final mixture. The aqueous layer was repeatedly evaporated to dryness with MeOH until the solvent showed a neutral reaction. The residue was purified through *Sephadex LH-20* column (1:1 $CHCl_3$/MeOH) to afford 2 mg of sugar. By comparing the optical rotation with D-mannose, the sugar was determined as D-form for its optical rotation $[\alpha]_D^{23} = +6.79$ (c = 0.093, H_2O).

In vitro Biological Activity Assay

The *in vitro* cell growth inhibitory activities of crude extracts were evaluated by the Trypan blue method using human promyelocytic leukemia HL-60 cell lines. The cell growth inhibitory activities of pure compound was assayed by the Trypan blue method using human breast adenocarcinoma MCF-7 cell lines and human promyelocytic leukemia HL-60 cell lines, and the MTT method using human prostate cancer PC-3 cell lines as previously described [18]. The cell lines obtained from *American Type Culture Collection*, Rockville, MD, USA were cultured in RPMI-1640 medium (*Gibco*, New York, NY, USA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mmol glutamine, and 10% heat-inactivated fetal bovine serum. Standard agar diffusion tests are used to assess the bioactivity in a series of antimicrobial. All microorganisms were handled under standard method. Plant pathogenic fungi *Rhizoctonia solani* and *Fusarium oxysporum* were grown in PDA medium (20% potato sliced washed unpeeled, 2% dextrose).

REFERENCES

[1] Z. E. Wilson, M. A. Brimble, *Nat. Prod. Rep.* **2009**, *26*, 44.
[2] Y. Dong, C.-B. Cui, C.-W. Li, W. Hua, C.-J. Wu, T.-J. Zhu, Q.-Q. Gu, *Mar. Drugs* **2014**, *12*, 4326.
[3] H. K. Tam, J. Härle, S. Gerhardt, J. Rohr, G. Wang, J. S. Thorson, A. Bigot, M. Lutterbeck, W. Seiche, B. Breit, A. Bechthold, O. Einsle, *Angew. Chem. Int. Ed.* **2015**, *54*, 2811.
[4] S. I. Elshahawi, K. A. Shaaban, M. K. Kharel, J. S. Thorson, *Chem. Soc. Rev.* **2015**, *44*, 7591.
[5] A. Schueffler, T. Anke, *Nat. Prod. Rep.* **2014**, *31*, 1425.
[6] M. Saleem, M. S. Ali, S. Hussain, A. Jabbar, M. Ashraf, Y. S. Lee, *Nat. Prod. Rep.* **2007**, *24*, 1142.
[7] T. Liu, J. Zhu, S.-Y. Zhang, Z.-L. Li, L.-P. Guan, H.-Q. Pan, X. Wu, J. Bai, H.-M. Hua, *Molecules* **2013**, *18*, 15126.
[8] S.-Y. Zhang, Z.-L. Li, L.-P. Guan, X. Wu, H.-Q. Pan, J. Bai, H.-M. Hua, *Magn. Reson. Chem.* **2012**, *50*, 632.
[9] T. Liu, S. Zhang, J. Zhu, H. Pan, J. Bai, Z. Li, L. Guan, G. Liu, C. Yuan, X. Wu, H. Hua, *J. Antibiot.* **2015**, *68*, 267.
[10] K. P. Vyas, P. H. Kari, S. M. Pitzenberger, R. A. Halpin, H. G. Ramjit, B. Arison, J. S. Murphy, W. F. Hoffman, M. S. Schwartz, E. H. Ulm, *Drug Metab. Dispos.* **1990**, *18*, 203.
[11] A. Endo, K. Hasumi, T. Nakamura, M. Kunishima, M. Masuda, *J. Antibiot.* **1985**, *38*, 321.
[12] M. Kuramoto, K. Yamada, M. Shikano, K. Yazawa, H. Arimoto, T. Okamura, D. Uemura, *Chem. Lett.* **1997**, *26*, 885.
[13] N. Saitou, M. Nei, *Mol. Biol. Evol.* **1987**, *4*, 406.
[14] Y. Shan, L. Xu, Y. Lu, X. Wang, Q. Zheng, L. Kong, M. Niwa, *Chem. Pharm. Bull.* **2008**, *56*, 52.
[15] T. Amagata, C. Rath, J. F. Rigot, N. Tarlov, K. Tenney, F. A. Valeriote, P. Crews, *J. Med. Chem.* **2003**, *46*, 4342.
[16] K.-H. Rhee, *Int. J. Antimicrob. Agents* **2004**, *24*, 423.
[17] T. J. White, T. Bruns, S. Lee, J. W. Taylor, in 'PCR Protocols: A Guide to Methods and Applications', Eds. M. A. Innis, D. H. Gelfand, J. J. Sninsky, T. J. White, Academic Press, Inc., New York, 1990, p. 315.
[18] F. Wang, H. Hua, Y. Pei, D. Chen, Y. Jing, *J. Nat. Prod.* **2006**, *69*, 807.

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