รายงานวิจัยฉบับสมบูรณ์

สารเมทาบอไลท์ทุติยภูมิที่แสดงฤทธิ์ทางชีวภาพจากราดิน *Aspergillus unguis* PSU-RSPG199 และ *Trichoderma brevicompactum* PSU-RSPG27

(Bioactive Secondary Metabolites from the Soil Fungi *Aspergillus unguis* PSU-RSPG199 and *Trichoderma brevicompactum* PSU-RSPG27)

โดย

ดร. ศรัณยู ใคลคลาย

กรกฎาคม 2560

สัญญาเลขที่ TRG5880202

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและ

มหาวิทยาลัยสงขลานครินทร์

รหัสโครงการ: TRG5880202

ชื่อโครงการ: สารเมทาบอไลท์ทุติยภูมิที่แสดงฤทธิ์ทางชีวภาพจากราดิน *Aspergillus unguis* PSU-RSPG199 และ *Trichoderma brevicompactum* PSU-RSPG27

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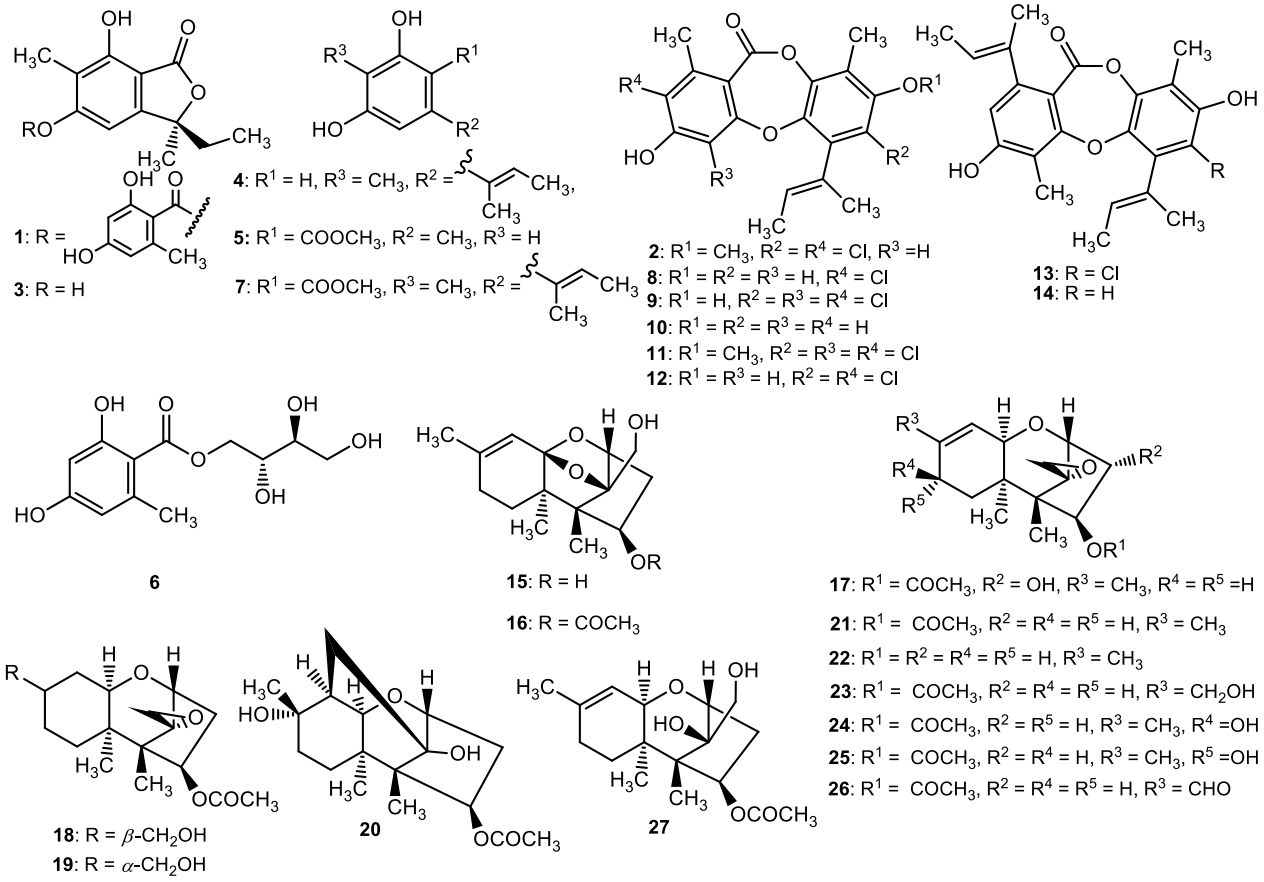
คำสำคัญ: *Aspergillus unguis Trichoderma brevicompactum* ต้านเชื้อจุลินทรีย์ ต้านเชื้อมาลาเรีย ต้านเซลล์มะเร็ง

บทคัดย่อ

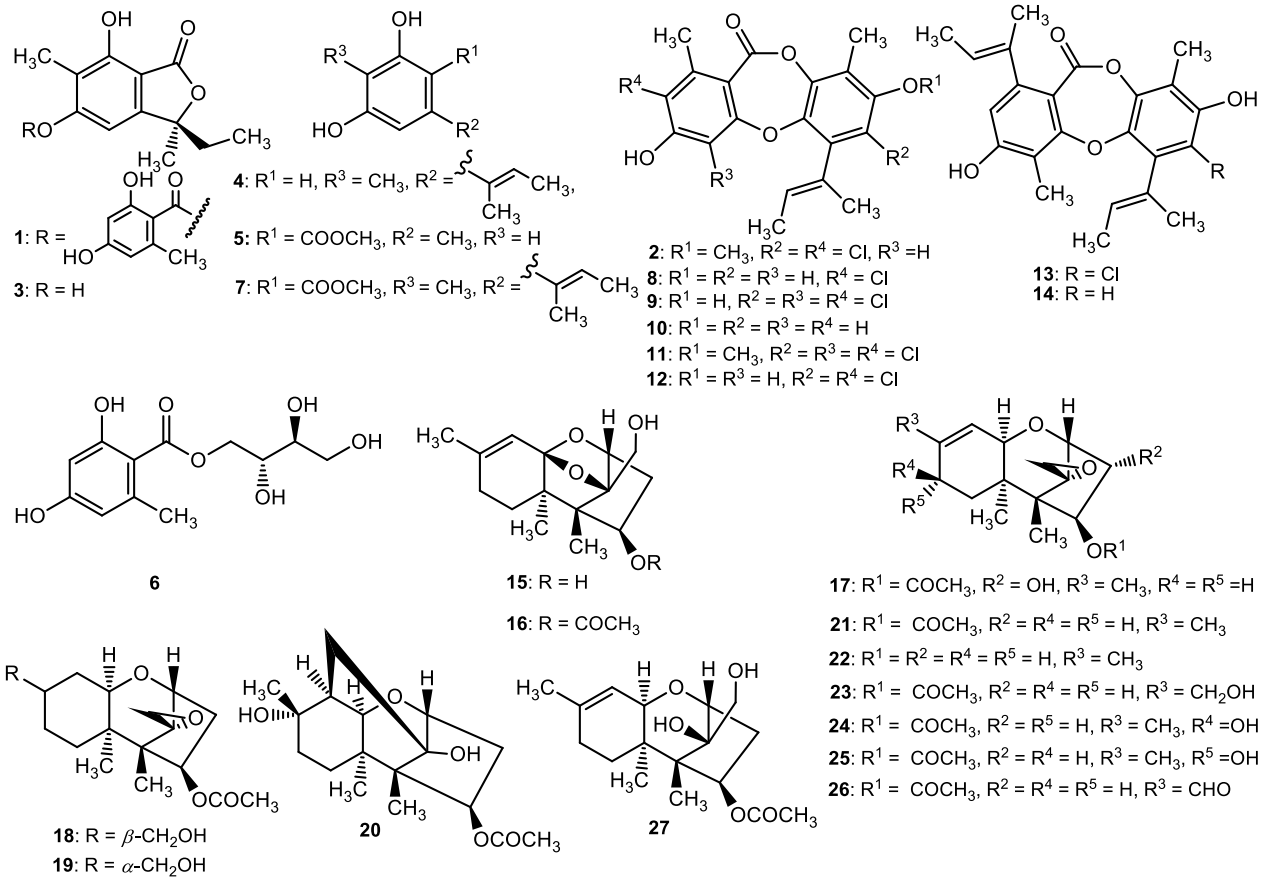
งานวิจัยนี้ศึกษาเมทาบอไลท์ที่แสดงฤทธิ์ทางชีวภาพจากเชื้อราดิน *Aspergillus unguis* PSU-RSPG199 และ *Trichoderma brevicompactum* PSU-RSPG27 แยกส่วนสกัดหยาบด้วยเทคนิคทางโครมาโทกราฟีแบบต่าง ๆ ได้สารบริสุทธิ์ทั้งหมด 27 สาร โดยเป็นสารใหม่จำนวน 8 สาร (**1-2** และ **15-20**) และสารที่มีการรายงานโครงสร้างแล้วจำนวน 19 สาร วิเคราะห์โครงสร้างของสารบริสุทธิ์ด้วยข้อมูลทางสเปกโทรสโกปี emeguisin A (**13**) จากเชื้อรา *A. unguis* PSU-RSPG199 แสดงฤทธิ์ต้านเชื้อ *Staphylococcus aureus* มาตรฐานและที่ดื้อต่อยาเมทิซิลิน และต้านเชื้อรา *Cryptococcus neoformans* ในระดับที่ดีด้วยค่า MIC ที่เท่ากันที่ 0.5 μg/mL pilobolusate (**7**) จากเชื้อราชนิดเดียวกัน แสดงฤทธิ์ต้านเซลล์มะเร็งช่องปาก (KB cell line) ในระดับที่ดีด้วยค่า IC_{50} 4.5 μM โดยที่ไม่แสดงความเป็นพิษต่อเซลล์ไตของลิง (Vero cell line) นอกจากนี้ trichodermin (**21**) จากเชื้อรา *T. brevicompactum* PSU-RSPG27 แสดงฤทธิ์ต้านเชื้อมาลาเรีย (*Plasmodium falciparum*) ในระดับที่ดีด้วยค่า IC_{50} 0.1 μM แต่อย่างไรก็ตามสาร **21** มีความเป็นพิษต่อเซลล์ไตของลิงในระดับที่สูงด้วยค่า IC_{50} 0.4 μM



คำสำคัญ: *Aspergillus unguis Trichoderma brevicompactum* ต้านเชื้อจุลินทรีย์ ต้านเชื้อมาลาเรีย ต้านเซลล์มะเร็ง

Abstract

This research project involved investigation of bioactive secondary metabolites from the soil fungi *Aspergillus unguis* PSU-RSPG199 and *Trichoderma brevicompactum* PSU-RSPG27. Purification of the crude extracts by various chromatographic techniques led to the isolation of twenty-seven compounds including eight new compounds (**1-2** and **15-20**) as well as nineteen known ones. Their structures were elucidated by spectroscopic methods. Emeguisin A (**13**) isolated from the soil fungus *A. unguis* PSU-RSPG199 displayed strong antibacterial activity against *Staphylococcus aureus* and methicillin-resistant strain and antifungal activity against *Cryptococcus neoformans* with equal MIC values of 0.5 μg/mL. Pilobolusate (**7**) derived from the same strain exhibited a potent anticancer activity against KB-oral cell lines with an IC_{50} value of 4.5 μM, but were noncytotoxic against noncancerous (Vero) cell lines. Moreover, trichodermin (**21**) exhibited significant antimalarial activity against *Plasmodium falciparum* with the IC_{50} value of 0.1 μM. However, **21** showed strong cytotoxic activity against Vero cell lines with the IC_{50} value of 0.4 μM.



Keywords: *Aspergillus unguis*, *Trichoderma brevicompactum*, Antimicrobial, Antimalarial, Cytotoxicity

Executive Summary

This research project involved investigation of bioactive secondary metabolites from the soil fungi *Aspergillus unguis* PSU-RSPG199 and *Trichoderma brevicompactum* PSU-RSPG27 isolated from a soil sample collected from the Plant Genetic Conservation Project under the Royal Initiation of Her Royal Highness Princess Maha Chakri Sirindhorn at Ratchaprapa Dam in Surat Thani Province, Thailand. Their culture broth and mycelia from small scale cultivation were extracted with organic solvents. The crude extracts displayed interesting antibacterial, antifungal, antimalarial, anticancer and cytotoxic activities. Accordingly, the extracts from large scale fermentation was prepared and then purified using various chromatographic techniques. The structures of pure compounds were identified using spectroscopic methods including UV, IR, NMR and MS. The isolated compounds were tested for antibacterial, antifungal, antimalarial, anticancer and cytotoxic activities.

Two new compounds including one phthalide (**1**) and one depsidone (**2**) together with twelve known compounds (**3-14**) were isolated from *A. unguis* PSU-RSPG199. Known emeguisin A (**13**) exhibited potent antibacterial activity against *Staphylococcus aureus* and methicillin-resistant *S. aureus* as well as strong antifungal activity against *Cryptococcus neoformans* with equal MIC values of 0.5 μg/mL. In addition, known pilobolusate (**7**) was strongly active against human oral carcinoma (KB) cell lines with an IC_{50} value of 4.5 μM. Interestingly, they were noncytotoxic against noncancerous (Vero) cell lines.

Six new trichothecene analogues (**15-19**) together with seven known trichothecene derivatives (**20-27**) were isolated from *T. brevicompactum* PSU-RSPG27. Trichodermin (**21**) exhibited the most potent antimalarial activity against *Plasmodium falciparum* with an IC_{50} value of 0.1 μM while other trichothecenes (**15**, **18**, **22-23** and **26**) showed the antimalarial activity with the IC_{50} values in the range of 7.1-9.6 μM. However, these compounds (**15**, **21-23** and **26**) displayed strong cytotoxicity against Vero cells the IC_{50} values in the range of 0.1-15.3 μM.

Research Project

Project title: Bioactive secondary metabolites from the soil fungi *Aspergillus unguis* PSU-RSPG199 and *Trichoderma brevicompactum* PSU-RSPG27

Objectives

- To isolate secondary metabolites from two soil fungi, *A. unguis* PSU-RSPG199 and *T. brevicompactum* PSU-RSPG27.

- To identify the structures of isolated compounds using spectroscopic data.

- To evaluate for antibacterial, antifungal, anticancer and antimalarial activities of the isolated compounds.

Scope of research

Isolation of secondary metabolites from *A. unguis* PSU-RSPG199 and *T. brevicompactum* PSU-RSPG27, structural determination of isolated compounds and evaluation for their antibacterial, antifungal, anticancer and antimalarial activities.

Methodology

1. Fermentation

- The fungi PSU-RSPG199 and PSU-RSPG27 were grown on potato dextrose agar at 25 ^{o}C for 5 days. Five pieces (0.5 x 0.5 cm^2) of mycelial agar plugs were inoculated into 54 flasks of 500 mL Erlenmeyer flasks containing 300 mL of potato dextrose broth and kept at room temperature for 3 weeks.

2. Extraction and purification

- The cultures were filtered to give filtrate and mycelia.

- The filtrate was extracted with ethyl acetate. The ethyl acetate extract was dried over anhydrous $NaSO_4$, filtered and evaporated to dryness under reduced pressure to afford a broth extract.

- The mycelial cake was extracted with methanol. The methanol extract was concentrated under reduced pressure. Water was added to the extract and the mixture was washed with hexane to give the aqueous and hexane layers. The hexane layer was dried and evaporated to dryness to afford the mycelial hexane extract. The aqueous residue was extracted with ethyl acetate. The ethyl acetate layer was then dried over anhydrous Na_2SO_4 and evaporated to dryness to obtain the mycelial ethyl acetate extract.

- Each crude extract was fractionated by column chromatography over Sephadex LH-20. The selected fractions were further purified by various chromatographic techniques to afford pure compounds.

3. Structure determination

The structures of pure compounds were identified by spectroscopic evidences, including UV, IR, NMR and mass spectroscopy and/or x-ray diffraction. The absolute configurations of isolated compounds were determined by comparison of optical rotations and/or CD data with those of related compounds as well as the modified Mosher method.

4. Bioassays

- The crude extracts was tested for antibacterial (*Staphylococcus aureus* and methicillin-resistance *S. aureus*), antifungal (*Canida albicans* and *Cryptococus neoformans*), anticancer (oral human carcinoma cells (KB) and human breast cancer cells (MCF-7)), cytotoxic (Vero cell) and antimalarial (against *Plasmodium falciparum*) activities.

- The isolated compounds with sufficient amount were tested for antibacterial, antifungal, anticancer, cytotoxic and antimalarial activities.

- The antibacterial and antifungal activities were performed by Assoc. Prof. Souwalak Phongpaichit, Department of Microbiology, Faculty of Science, Prince of Songkla University while other bioassays were carried out at the National Center for Genetic Engineering and Biotechnology (BIOTEC).

4.1 Antimicrobial activity (Phongpaichit et al., 2006)

Antibacterial activity against *S. aureus* ATCC25923 and methicillin-resistant *S. aureus* SK1 (a clinical isolate) was performed using a colorimetric broth microdilution test. Vancomycin is used as a positive control.

Antifungal activity against *C. albicans* NCPF3153 and *C. neoformans* ATCC90113 was carried out using a colorimetric broth microdilution test. MICs were recorded by reading the lowest substance concentration that inhibits visible growth. Amphotericin B was a positive control drug.

4.2 Anticancer and cytotoxic activities

Cytotoxic assays against Vero cells were performed employing the colorimetric method (Hunt et al., 1999), while the activities against human oral cavity (KB) and human breast (MCF-7) cancer cell lines were carried out using the resazurin microplate assay (O'Brien et al., 2000).

The standard compound for Vero and KB cell lines was ellipticine while those for MCF-7 cell lines were tamoxifen and doxorubicin.

4.3 Antimalarial activity

Antimalarial activity was performed in vitro against the parasite *Plasmodium falciparum* (K1, multidrug resistant strain) using the microculture radioisotope technique (Desjardins et al., 1979). The standard compound was dihydroartemisinine.

Results and Discussions

Fungi soil fungi *A. unguis* PSU-RSPG199 and *T. brevicompactum* PSU-RSPG27 were isolated from soil samples collected from the Plant Genetic Conservation Project area under the Royal Initiative of Her Royal Highness Princess Maha Chakri Sirindhorn (RSPG area) at Ratchaprapa Dam in Surat Thani Province in 2010. The crude extracts from these fungi showed interesting biological activities as shown in **Table 1**.

Table 1. The biological activities of the crude extracts from *A. unguis* PSU-RSPG199 and *T. brevicompactum* PSU-RSPG27

Fungi/ standard drugs	Crude extracts[1]	Antimicrobial activity[2] (MIC, μg/mL)				Antimalarial[3] (IC_{50}, μg/mL)	Cytotoxic[4] (IC_{50}, μg/mL)		
		SA	MRSA	CA	CN	*P. falciparum*	KB	MCF-7	Vero cell
PSU-RSPG199	BE	32	32	200	32	8.46	Inactive	Inactive	Inactive
	CE	8	8	64	8	Inactive	36.50	Inactive	19.15
	CH	16	16	>200	128	Inactive	30.78	Inactive	45.99
PSU-RSPG27	BE	32	128	4	16	0.21	1.14	21.57	0.30
	CE	32	64	64	8	2.73	3.06	22.01	7.25
	CH	200	>200	2	8	0.06	1.04	Inactive	0.21
Vancomycin		0.5	0.5						
Amphotericin B				0.25	0.06				
Dihydroartemisinine						2.32[5]			
Ellipticine							1.18[6]		0.73[6]
Tamoxifen								9.53[6]	

[1]BE = Broth extract, CE = Mycelial ethyl acetate extract, CH = Mycelial hexane extract

[2]SA = *Staphylococcus aureus* ATCC25923, MRSA = methicillin-resistant *S. aureus*, CA = *Candida albicans* NCPF3153, CN = *Cryptococcus neoformans* ATCC90113 flucytosine-resistant

[3]Antimalarial activity against parasite *Plasmodium falciparum* (K1, multidrug resistant strain)

[4]Cytotoxicity against African green monkey kidney fibroblasts (Vero) cell lines, KB = Oral human carcinoma cell lines, MCF-7 = Human breast cancer cell lines

[5]nM, [6]μM

***Aspergillus unguis* PSU-RSPG199**

The isolation and characterization of secondary metabolites from the broth and mycelial extracts of the fungus PSU-RSPG199 were described in Appendix I. One new phthalide (asperlide, **1**) together with four known compounds, 3-ethyl-5,7-dihydroxy-3,6-dimethylphthalide (**3**) (Kawahara et al., 1988), aspergillusphenol A (**4**) (Sureram et al., 2012), methyl orsellinate (**5**) (Lopes et al., 2008) and (+)-montagnetol (**6**) (Basset et al., 2010), were isolated from the broth extract. Moreover, one new depsidone (aspersidone, **2**), one known orsellinate, pilobolusate (**7**) (Rajachan et al., 2014), and seven known depsidones, including 3-chlorounguinol (**8**) (Kawahara et al., 1988), nornidulin (**9**) (Kawahara et al., 1988), unguinol (**10**) (Kawahara et al., 1988), nidulin (**11**) (Kawahara et al., 1988), aspergillusidone C (**12**) (Sureram et al., 2013), emeguisin A (**13**) (Kawahara et al., 1988) and folipastatin (**14**) (Hamano et al., 1992), were obtained from the mycelial extract (**Figure 1**).

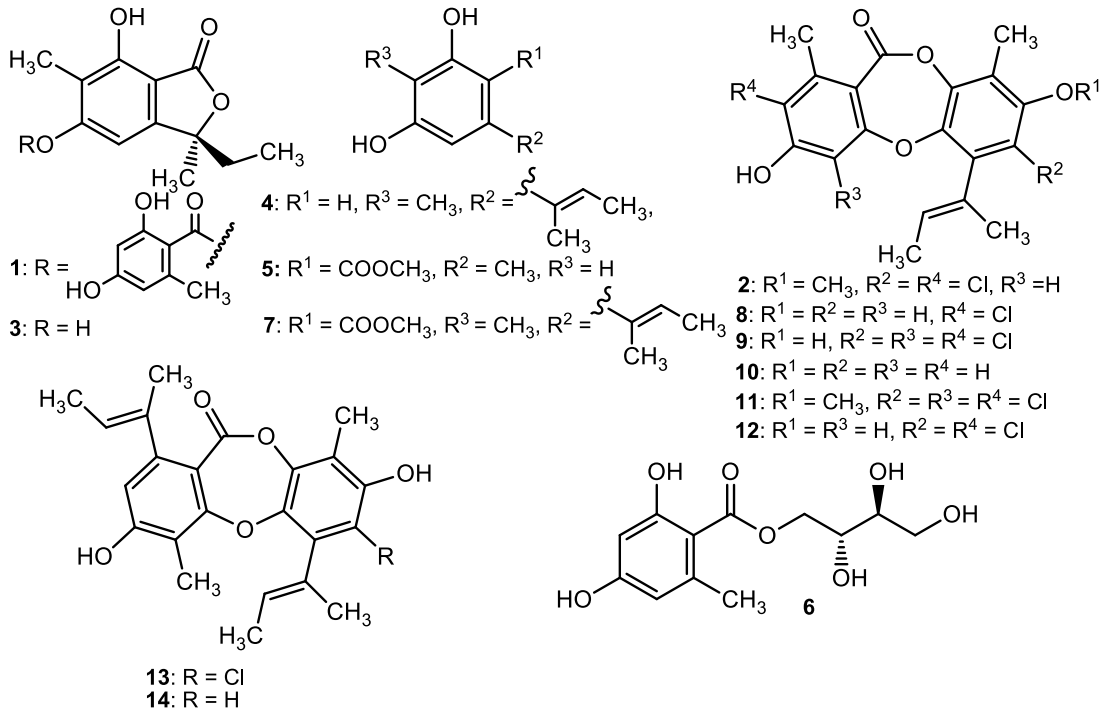


Figure 1. Structures of compounds **1-14** isolated from *Aspergillus unguis* PSU-RSPG199

The isolated compounds (**3, 5, 7-9** and **12-14**) with sufficient amount were evaluated for antimicrobial (against *S. aureus*, methicillin-resistant *S. aureus, C. albicans*, *C. neoformans* and *M. gypseum*), antimalarial (against *P. falciparum*) and cytotoxic (against MCF-7, KB and Vero cells) activities (**Table 2**).

Table 2 Biological activities for compounds **3**, **5**, **7-9** and **12-14**

Compounds/ Standard drugs	Antimicrobial[1] (MIC, μg/mL)					Antimalarial IC_{50} (μM)	Cytotoxicity IC_{50} (μM)		
	SA	**MRSA**	**CA**	**CN**	**MG**	***P. falciparum***	**KB**	**MCF-7**	**Vero**
3	2	4	128	32	8	15.6	51.5	62.6	40.0
5	IN[2]	IN[2]	IN[2]	IN[2]	IN[2]	IN[2]	IN[2]	IN[2]	IN[2]
7	64	64	200	64	64	39.7	4.5	128.5	76.9
8	4	8	8	32	8	IN[2]	IN[2]	IN[2]	85.7
9	2	2	IN[2]	8	IN[2]	IN[2]	IN[2]	IN[2]	110.1
12	2	1	200	128	2	6.3	15.8	27.9	8.1
13	0.5	0.5	IN[2]	0.5	IN[2]	2.2	14.4	60.0	37.7
14	2	1	IN[2]	1	IN[2]	7.7	47.2	33.7	45.4
Vancomycin	0.25	0.25							
Amphotericin B			0.5	0.25					
Miconazole					1				
Dihydroartemisinine						2.2[3]			
Ellipticine							8.2		4.1
Tamoxifen								18.4	

[1]SA = *Staphylococcus aureus* ATCC25923, MRSA = methicillin-resistant *S. aureus*, CA = *Candida albicans* NCPF3153, CN = *Cryptococcus neoformans* ATCC90113 flucytosine-resistant and MG = *Microsporum gypseum* clinical isolate

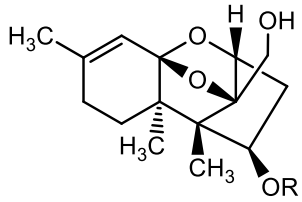
[2]IN = Inactive, [3]nM

Compound **5** was inactive in all tested assays. Interestingly, compound **13** exhibited the most potent antibacterial (against *S. aureus* and methicillin-resistant *S. aureus*) and antifungal (against *C. neoformans*) activities with equal MIC values of 0.5 μg/mL. In addition, compounds **12** and **14** showed two folds more active against methicillin-resistant *S. aureus* than *S. aureus* with equal MIC values of 1 μg/mL. In contrast, compounds **3** and **8** exhibited two folds more active against *S. aureus* than methicillin-resistant strain with MIC values of 2 and 4 μg/mL, respectively. Compounds **7** and **9** displayed activity against both bacterial stains with equal MIC values of 64 and 2 μg/mL, respectively. For antifungal activity, compound **8** was active against *C. albicans* with a MIC value of 8 μg/mL while other tested substances were much less active. Compound **14** showed significant antifungal activity against *C. neoformans* with MIC value of 1 μg/mL while compounds **3**, **7**, **8** and **9** displayed moderate to weak activity with MIC values in the range of 8-128 μg/mL. Compound **12** exhibited antifungal activity toward *M. gypseum* with a MIC value of 2 μg/mL. In addition, compounds **3** and **8** showed eight folds more active against the same fungal pathogen than compound **7** with equal MIC values of 8 μg/mL. Compound **13**

was approximately three folds more active against *P. falciparum* than **12** with an IC_{50} value of 2.2 μM. Moreover, compounds **3**, **7** and **14** displayed the same activity with IC_{50} values in the range of 7.7-39.7 μM. Among the tested compounds, compound **7** exhibited the most potent activity against KB cells with an IC_{50} value of 4.5 μM which was much stronger than the standard drug, ellipticine. Compound **12** showed the strongest activity against Vero cells with an IC_{50} value of 8.1 μM. Other tested compounds except for compounds **8** and **9** which were inactive against KB and MCF-7 cells were much less active toward all cells with the IC_{50} values in the range of 14.4-110.1 μM. These results implied that the chlorine atom in the depsidone was important for antimicrobial activity. Furthermore, this is the first report on the antibacterial activity against *S. aureus* and methicillin-resistant *S. aureus* of compounds **3**, **7-8** and **13-14** as well as antifungal and antimalarial activities for all tested substances.

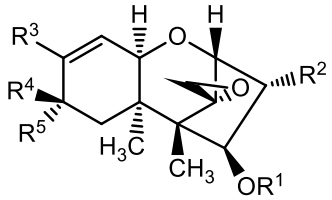
***Trichoderma brevicompactum* PSU-RSPG27**

The isolation and characterization of secondary metabolites from the broth extract of the fungus PSU-RSPG27 were described in Appendix II. Six new trichothecene derivatives, trichodermarins A-F (**16-20**), together with seven known ones, trichodermin (**21**) (Ayer and Miao, 1993), trichodermol (**22**) (Ayer and Miao, 1993), trichoderminol (**23**) (Lee et al., 2016), (2*R*,2'*S*,4*R*,5*S*,5a*R*,7*R*,9a*R*)-2,3,4,5,5a,6,7,9a-octahydro-5,5a,8-trimethyl-4-acetate-spiro[2,5-methano-1-benzo-xepin-10,2'-oxirane]-4,7-diol (**24**), (2*R*,2'*S*,4*R*,5*S*,5a*R*,7*S*,9a*R*)-4-acetate-2,3,4,5,5a,6,7,9a-octahydro-5,5a,8-trimethyl-spiro[2,5-methano-1-benzoxepin-10,2'-oxi-rane]-4,7-diol (**25**) (Xu et al., 2013), (4β)-4-(acetyloxy)-12,13-epoxy-trichothec-9-ene-9-carboxaldehyde (**26**) (Xu et al., 2013) and (2*R*,4*R*,5*S*,5a*R*,9a*R*,10*S*)-4-acetate-2,3,4,5,5a,6,7,9a-octahydro-10-(hydroxylmethyl)-5,5a,8-trime-thyl-2,5-methano-1-benzoxepin-4,10-diol (**27**) (Cheng et al., 2010), were obtained from the broth extract of the fungus (**Figure 2**).



15: R = H

16: R = $COCH_3$



17: $R^1 = COCH_3$, $R^2 = OH$, $R^3 = CH_3$, $R^4 = R^5 = H$

21: $R^1 = COCH_3$, $R^2 = R^4 = R^5 = H$, $R^3 = CH_3$

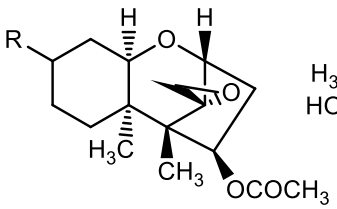
22: $R^1 = R^2 = R^4 = R^5 = H$, $R^3 = CH_3$

23: $R^1 = COCH_3$, $R^2 = R^4 = R^5 = H$, $R^3 = CH_2OH$

24: $R^1 = COCH_3$, $R^2 = R^5 = H$, $R^3 = CH_3$, $R^4 = OH$

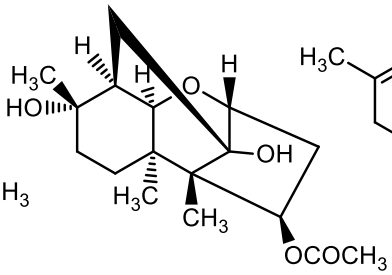
25: $R^1 = COCH_3$, $R^2 = R^4 = H$, $R^3 = CH_3$, $R^5 = OH$

26: $R^1 = COCH_3$, $R^2 = R^4 = R^5 = H$, $R^3 = CHO$



18: R = β-CH_2OH

19: R = α-CH_2OH



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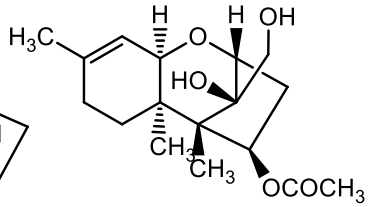

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Figure 2. Structures of compounds **16-27** isolated from *Trichoderma brevicompactum* PSU-RSPG27.

The mycelial extract of this fungus was not investigated because its ^1H NMR spectrum showed trichodermin (**21**) as a major component.

The isolated compounds **15**, **18**, **21-23** and **26**, which were obtained in sufficient amounts, were tested for antifungal (*C. albicans*, *C. neoformans* and *M. gypseum*), antimalarial (*P. falciparum*) and cytotoxic (KB and Vero cell lines) activities (**Table 3**).

Table 3 Biological activities for compounds **15**, **18**, **21-23** and **26**

Compounds/Standard drugs	Antifungal[1] (MIC, μg/mL)			Antimalarial IC_{50} (μM)	Cytotoxicity IC_{50} (μM)	
	CA	**CN**	**MG**	***P. falciparum***	**KB**	**Vero**
15	16	16	IN[2]	7.4	44.7	15.3
18	IN[2]	IN[2]	IN[2]	IN[2]	116.2	45.9
21	1	4	2	0.1	2.4	0.4
22	16	16	IN[2]	7.1	16.1	6.9
23	64	64	IN[2]	8.8	20.6	7.8
26	2	4	IN[2]	9.6	3.7	9.9
Amphotericin B	0.5	0.25				
Miconazole			1			
Dihydroartemisinine				2.2[3]		
Ellipticine					8.2	4.1

[1]CA = *Candida albicans* NCPF3153, CN = *Cryptococcus neoformans* ATCC90113 flucytosine-resistant and MG = *Microsporum gypseum* clinical isolate.[2]IN = Inactive. [3]nM.

Interestingly, **21** exhibited the most potent antifungal activity against *C. albicans*, *C. neoformans* and *M. gypseum* with the MIC values of 1, 4 and 2 μg/mL, respectively. Compound **26** was two fold more active against *C. albicans* than *C. neoformans* with the MIC values of 2 and 4 μg/mL, respectively. Compounds **15** and **22** showed much weaker antifungal activity against *C. albicans* and *C. neoformans* with equal MIC values of 16 μg/mL while compound **23** was four fold less active against these two fungal strains. Among the tested compounds, **21** displayed the most potent antimalarial activity against *P. falciparum* (K1 strain) with an IC_{50} value of 0.1 μM. In addition, the other trichothecenes was less active with the IC_{50} values in the range of 7.1-9.6 μM. Compounds **21** and **26** showed significant cytotoxicity against KB cell lines with the IC_{50} values of 2.4 and 3.7 μM, respectively, which were much stronger than standard drug, ellipticine. Moreover, compounds **22** and **23** were approximately four and five folds less active against the same cell lines than **26**, respectively. However, **21** exhibited much stronger cytotoxic activity (IC_{50} value of 0.4) to Vero cell lines than other tested compounds. These results indicated that the 4-OAc and vinylic methyl functionalities would be important for antifungal, antimalarial and cytotoxic (against KB and Vero cell lines) activities. From these data, we conclude that the biological activities of broth extract might be controlled by **21**, which is the major component in the extract.

References

Ayer, W.A., Miao, S. 1993. Secondary metabolites of the aspen fungus *Stachybotrys cylindrospora*. *Can. J. Chem.* 71, 487–493.

Basset, J.-F., Leslie, C., Hamprecht, D., White, A.J.P., Barrett, A.G.M. 2010. Studies on the resorcylates: biomimetic total syntheses of (+)-montagnetol and (+)-erythrin. *Tetrahedron Lett.* 51, 783–785.

Cheng, J.L., Zhou, Y., Zhao, J.H., Zhang, C., Lin, F.C. 2010. Synthesis and antifungal activity of trichodermin derivatives. *Chin. Chem. Lett.* 21, 1037–1040.

Desjardins, R.E., Canfield, C.J., Haynes, J.D., Chulay, J.D. 1979. Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. *Antimicrob. Agents Chemother.* 16, 710–718.

Hamano, K., Kinoshita-Okami, M., Hemmi, A., Sato, A., Hisamoto, M., Matsuda, K., Yoda, K., Haruyama, H., Hosoya, T., Tanzawa, K. 1992. Folipastatin, a new depsidone compound from Aspergillus unguis as an inhibitor of phospholipase A2. Taxonomy, fermentation, isolation, structure determination and biological properties. *J. Antibiot.* 45, 1195–1201.

Hunt, L., Jordan, M., De Jesus, M., Wurm, F.M. 1999. GFP-expressing mammalian cells for fast, sensitive, noninvasive cell growth assessment in a kinetic mode. *Biotechnol. Bioeng.* 65, 201–205.

Kawahara, N., Nozawa, K., Nakajima, S., Kawai, K., Yamazaki, M. 1988. Isolation and structures of novel fungal depsidones, emeguisins A, B, and C, from *Emericella unguis*. *J. Chem. Soc.* 2611–2614.

Lee, D.H., Kim, J.J., Ryu, K.H., Kim, B.S., Ryu, S.M. 2016. Novel antiviral composition, and method for controlling plant viruses by using same. PCT Patent WO 2016/089166 A1.

Lopes, T.I.B., Coelho, R.G., Yoshida, N.C., Honda, N.K. 2008. Radical scavenging activity of orsellinates. *Chem. Pharm. Bull.* 56, 1551–1554.

O'Brien, J., Wilson, I., Orton, T., Pognan, F. 2000. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity: resazurin as a cytotoxicity assay. *Eur. J. Biochem.* 267, 5421–5426.

Phongpaichit, S., Rungjindamai, N., Rukachaisirikul, V., Sakayaroj, J. 2006. Antimicrobial activity in cultures of endophytic fungi isolated from *Garcinia* species. *FEMS Immunol. Med. Microbiol.* 48, 367–372.

Rajachan, O., Kanokmedhakul, S., Kanokmedhakul, K., Soytong, K. 2014. Bioactive depsidones from the fungus *Pilobolus heterosporus*. *Planta Med.* 80, 1635–1640.

Sureram, S., Kesornpun, C., Mahidol, C., Ruchirawat, S., Kittakoop, P. 2013. Directed biosynthesis through biohalogenation of secondary metabolites of the marine-derived fungus *Aspergillus unguis*. *RSC Adv.* 3, 1781–1788.

Sureram, S., Wiyakrutta, S., Ngamrojanavanich, N., Mahidol, C., Ruchirawat, S., Kittakoop, P. 2012. Depsidones, aromatase inhibitors and radical scavenging agents from the marine-derived fungus *Aspergillus unguis* CRI282-03. *Planta Med.* 78, 582–588.

Xu, X., Cheng, J., Zhou, Y., Zhang, C., Ou, X., Su, W., Zhao, J., Zhu, G. 2013. Synthesis and antifungal activities of trichodermin derivatives as fungicides on rice. *Chem. Biodivers.* 10, 600–611.

Out put ที่ได้จากโครงการ

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

ได้รับการตีพิมพ์ในวารสารนานาชาติ ในฐานข้อมูล ISI จำนวน 1 เรื่อง

ผู้แต่ง Saranyoo Klaiklay, Vatcharin Rukachaisirikul, Weerawat Aungphao, Souwalak Phongpaichit, Jariya Sakayaroj

ชื่อเรื่อง Depsidone and phthalide derivatives from the soil-derived fungus *Aspergillus unguis* PSU-RSPG199

แหล่งพิมพ์ Tetrahedron Letters (IF 2.379, Quartile in Category: Q2)

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2. ผลงานที่อยู่ในระหว่างเตรียม manuscript จำนวน 1 เรื่อง

ชื่อเรื่อง Trichothecene derivatives from the soil-derived fungus *Trichoderma brevicompactum* PSU-RSPG27

การนำผลงานวิจัยไปใช้ประโยชน์

ด้านวิชาการ

จากการศึกษาสารเมทาบอไลท์ที่แสดงฤทธิ์ทางชีวภาพจากเชื้อราดินทั้ง 2 สายพันธุ์ ทำให้ทราบว่าเชื้อราเหล่านี้สร้างสารหลากหลายชนิดที่น่าสนใจ และสารบริสุทธิ์ที่แยกได้นั้นแสดงฤทธิ์ต้านเชื้อแบคทีเรีย เชื้อรา เชื้อมาลาเรีย และต้านเซลล์มะเร็ง ซึ่งจะนำไปสู่การนำสารที่ได้ไปใช้ในเชิงเภสัชวิทยา หรือนำมาเป็นความรู้พื้นฐานในการวิจัยด้านนี้ต่อไป

ภาคผนวก ก

(Appendix I)

ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

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Depsidone and phthalide derivatives from the soil-derived fungus *Aspergillus unguis* PSU-RSPG199

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ABSTRACT

Two new compounds including one phthalide (asperlide, **1**) and one depsidone (aspersidone, **2**) together with twelve known compounds were isolated from the soil-derived fungus *Aspergillus unguis* PSU-RSPG199. Known emeguisin A exhibited potent antibacterial activity against *Staphylococcus aureus* and methicillin-resistant *S. aureus* as well as strong antifungal activity against *Cryptococcus neoformans*, each with MIC values of 0.5 μg/mL. Additionally, known pilobolusate was strongly active against the human oral carcinoma (KB) cell line with an IC_{50} value of 4.5 μM. Interestingly, emeguisin A and pilobolusate were noncytotoxic against noncancerous (Vero) cell lines.

Fungi are a significant source of structurally diverse metabolites, many of which are excellent sources of pharmaceuticals such as cancer drugs.[1] The soil fungi in the genus *Aspergillus* has afforded interesting bioactive compounds such as antiviral merosesquiterpenoids,[2] cholesterol lowering lovastatins,[3] and cytotoxic phenalenone.[4] In our ongoing search for bioactive secondary metabolites from soil fungi, *Aspergillus unguis* PSU-RSPG199 was isolated from a soil sample collected from the Plant Genetic Conservation Project under the Royal Initiation of Her Royal Highness Princess Maha Chakri Sirindhorn at Ratchaprapa Dam in Surat Thani Province, Thailand. The broth extract exhibited antibacterial activity against *Staphylococcus aureus* and methicillin-resistant *S. aureus*, each with minimum inhibitory concentration (MIC) values of 32 μg/mL, while the mycelial extract displayed antibacterial activity against both bacterial stains with MIC values of 8 μg/mL. Both extracts showed weak antifungal activity against *Candida albicans*, *Cryptococcus neoformans*, and *Microsporum gypseum* with MIC values in the range of 64–200 μg/mL. Additionally, the broth extract showed antimalarial activity against *Plasmodium falciparum* (K1 strain) with an IC_{50} value of 8.46 μg/mL, while the mycelial extract was inactive. Regarding the cytotoxic activity against MCF-7 breast cancer, KB oral cavity cancer and noncancerous Vero (African green monkey kidney fibroblasts) cell lines, only the mycelial extract exhibited cytotoxicity toward KB and Vero cell lines with IC_{50} values of 36.6 and 19.15 μg/mL, respectively. Herein, we describe the isolation and characterization of secondary metabolites from the broth and mycelial extracts of the fungus PSU-RSPG199. One new phthalide, asperlide (**1**) together with four known compounds, 3-ethyl-5,7-dihydroxy-3,6-dimethylphthalide (**3**),[5] aspergillusphenol A (**4**),[6] methyl orsellinate (**5**)[7] and (+)-montagnetol (**6**),[8] were isolated from the broth extract. Moreover, one new depsidone, aspersidone (**2**), one known orsellinate, pilobolusate (**7**),[9] and seven known depsidones, 3-chlorounguinol (**8**),[5] nornidulin (**9**),[5] unguinol (**10**),[5] nidulin (**11**),[5] aspergillusidone C (**12**),[10] emeguisin A (**13**),[11] and folipastatin (**14**)[12] were obtained from the mycelial extract. Some of the isolated compounds were evaluated for antimicrobial (against *S. aureus* ATCC25923, methicillin-resistant *S. aureus*, *C. albicans* NCPF3153, *C. neoformans* ATCC90113 flucytosine-resistant, and *M. gypseum* clinical isolate), antimalarial (against *P. falciparum*), anticancer (against MCF-7 and KB cell lines), and cytotoxic (against Vero cell line) activities.

All compounds (**1**–**14**, Fig. 1) were obtained using chromatographic purification, and their structures elucidated using various spectroscopic techniques (ESI). The absolute configuration of compound **3** was assigned for the first time as 3*S* by the opposite sign of

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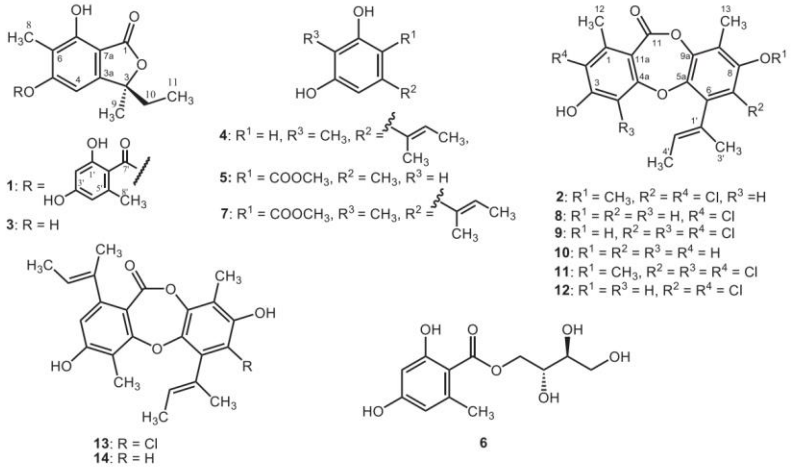

Figure 1. Structures of compounds **1**–**14** isolated from *Aspergillus unguis* PSU-RSPG199.

Table 1
NMR data of asperlide **1**

Position	δ_H,[a] *J* in Hz	δ_C,[b] type	HMBC	NOEDIFF
1		171.3, C		
3		90.2, C		
3a		151.6, C		
4	6.62, s	106.7, CH	C-3, C-5, C-6, C-7a	H_3-9, H_{ab}-10, H_3-11, H_3-8′
5		155.2, C		
6		118.4, C		
7		155.7, C		
7-OH	8.14, s		C-6, C-7, C-7a	
7a		108.9, C		
8	2.14, s	8.9, CH_3	C-5, C-6, C-7	H_3-8′
9	1.65, s	25.5, CH_3	C-3, C-3a, C-10	H-4, H_{ab}-10, H_3-11
10	a: 2.04, dq (14.4, 7.2) b: 1.91, dq (14.4, 7.2)	32.9, CH_2	C-3, C-3a, C-11	
11	0.84, t (7.2)	7.9, CH_3	C-3, C-10	H-4, H_3-9, H_{ab}-10,
1′		166.6, C		
1′-OH	11.30, s		C-1′, C-2′, C-6′	
2′	6.35, s	101.7, CH	C-1′, C-3′, C-4′, C-6′, C-7′	1′-OH
3′		161.6, C		
4′	6.35, s	112.1, CH	C-2′, C-3′, C-6′, C-7′, C-8′	H_3-8′
5′		144.4, C		
6′		104.3, C		
7′		169.5, C		
8′	2.66, s	24.7, CH_3	C-4′, C-5′, C-6′	H-4, H_3-8, H-4′

[a] Recorded at 300 MHz in $CDCl_3$.
[b] Recorded at 75 MHz in $CDCl_3$.

both the CD data ($\Delta\varepsilon$ +12.51 at 211 nm) and specific rotation ($[\alpha]_D^{25}$ −48.9, *c* 0.078, MeOH) to those of (3*R*)-pseudaboydin B ($\Delta\varepsilon$ −7.22 at 211.2 and $[\alpha]_D^{25}$ +53.6, *c* 0.078, MeOH).[13] The absolute configuration of compound **6** was established by comparison of its specific rotation with that reported in the literature.[8]

Asperlide (**1**)[14] was isolated as a colorless gum with the molecular formula $C_{20}H_{20}O_7$ as identified from the HRESIMS peak at *m*/*z* 395.1108 $[M+Na]^+$. It showed UV absorption bands of a conjugated carbonyl chromophore at 270 and 300 nm.[5] The IR spectrum showed characteristic absorption bands of hydroxy, γ-lactone carbonyl, and ester carbonyl groups at 3424, 1757, and 1726 cm^{-1}, respectively.[5,9] The ^1H NMR spectroscopic data (Table 1) were similar to those of compound **3**.[5] The differences in their ^1H NMR spectroscopic data were the presence of signals for a hydrogen bonded hydroxy proton (δ_H 11.30), two aromatic protons of a 1,2,3,5-tetrasubstituted benzene ring (δ_H 6.35, s), and one methyl

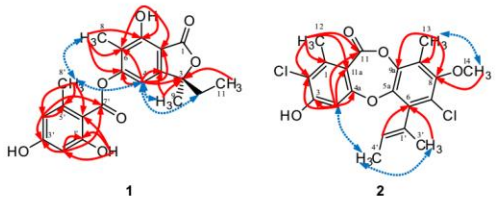

Figure 2. Selected HMBC (↷) and NOEDIFF data (↷) for compounds **1** and **2**.

group (δ_H 2.66, s). The ^{13}C NMR data contained additional signals for an ester carbonyl (δ_C 169.5) as well as four quaternary (δ_C 166.6, 161.6, 144.4 and 104.3), two methine (δ_C 112.1 and 101.7), and one methyl (δ_C 24.7) carbons. The hydrogen bonded

Table 2
NMR data of aspersidone **2**

Position	δ_H,[a] *J* in Hz	δ_C,[b] type	HMBC	NOEDIFF
1		141.6, C		
2		115.6, C		
3		155.1, C		
3-OH	6.12, br s			
4	6.61, s	105.8, CH	C-2, C-3, C-4a, C-11, C-11a	H_3-3′, H_3-4′
4a		161.5, C		
5a		144.6, C		
6		135.7, C		
7		123.8, C		
8		152.1, C		
9		123.3, C		
9a		142.0, C		
11		162.0, C		
11a		119.2, C		
12	2.54, s	18.6, CH_3	C-1, C-2, C-4a, C-11, C-11a	–
13	2.31, s	10.3, CH_3	C-8, C-9, C-9a	H_3-14
14	3.78, s	60.5, CH_3	C-8	H_3-13
1′		129.3, C		
2′	5.38, qq (6.5, 1.0)	127.1, CH	C-6, C-3′, C-4′	H-4, H_3-4′
3′	1.96, brs	17.2, CH_3	C-6, C-1′, C-2′	H_3-4′
4′	1.89, dq (6.5, 0.5)	13.7, CH_3	C-1′, C-2′	H-4, H-2′

[a] Recorded at 500 MHz in $CDCl_3$.
[b] Recorded at 125 MHz in $CDCl_3$.

Table 3
Biological activities for compounds **3**, **5**, **7**–**9**, and **12**–**14**

Compounds	Antimicrobial[a] (MIC, μg/mL)					Antimalarial IC_{50} (μM)	Cytotoxicity IC_{50} (μM)		
	SA	MRSA	CA	CN	MG	*P. falciparum*	KB	MCF-7	Vero
3	2	4	128	32	8	15.6	51.5	62.6	40.0
5	IN[b]	IN[b]	IN[b]	IN[b]	IN[b]	IN[b]	IN[b]	IN[b]	IN[b]
7	64	64	200	64	64	39.7	4.5	128.5	76.9
8	4	8	8	32	8	IN[b]	IN[b]	IN[b]	85.7
9	2	2	IN[b]	8	IN[b]	IN[b]	IN[b]	IN[b]	110.1
12	2	1	200	128	2	6.3	15.8	27.9	8.1
13	0.5	0.5	IN[b]	0.5	IN[b]	2.2	14.4	60.0	37.7
14	2	1	IN[b]	1	IN[b]	7.7	47.2	33.7	45.4
Vancomycin	0.25	0.25							
Amphotericin B			0.5	0.25					
Miconazole					1				
Dihydroartemisinin						2.2[c]			
Ellipticine							8.2		4.1
Tamoxifen								18.4	

[a] SA = *Staphylococcus aureus* ATCC25923, MRSA = methicillin-resistant *S. aureus*, CA = *Candida albicans* NCPF3153, CN = *Cryptococcus neoformans* ATCC90113 flucytosine-resistant and MG = *Microsporum gypseum* clinical isolate.
[b] IN = Inactive.
[c] nM.

hydroxy proton (δ_H 11.30) was located at C-1′ (δ_C 166.6), in a *peri*-position to the ester carbonyl group, and showed HMBC correlations (Fig. 2) with C-1′, C-2′ (δ_C 101.7), and C-6′ (δ_C 104.3). The aromatic proton resonance at δ_H 6.35 was assigned as H-2′ on the basis of an HMQC correlation with C-2′ and showed HMBC correlations with C-1′, C-3′ (δ_C 161.6), C-4′ (δ_C 112.1), C-6′ and C-7′ (δ_C 169.5). Thus, the other aromatic proton (δ_H 6.35) was attributed to H-4′. The substituent at C-3′ was a hydroxy group on the basis of its chemical shift. The methyl group resonating at δ_H 2.66 gave HMBC correlations with C-4′, C-5′ (δ_C 144.4), and C-6′, indicating the attachment of the methyl group at C-5′. Thus, the orsellinate unit with two hydroxy groups at C-1′ and C-3′ and the methyl group at C-5′ was established. The C-5 position of the phthalide moiety was connected to the orsellinate unit *via* an ester linkage on the basis of the molecular formula $C_{20}H_{20}O_7$. Signal enhancement of H-4 (δ_H 6.62) and H_3-8 (δ_H 2.14) after irradiation of H_3-8′ (δ_H 2.66) in the NOEDIFF experiment (Fig. 2) supported the position of the ester moiety. Compound **1** showed similar CD data ($\Delta\varepsilon$ +28.21 at 211 nm) and specific rotation ($[\alpha]_D^{25}$ –48.7, *c* 0.078, MeOH) to those of co-metabolite **3**. Thus, the absolute configuration at C-3 was proposed as an *S*-configuration, identical to that in **3**.

Aspersidone (**2**)[15] was obtained as a pale yellow gum with the molecular formula $C_{20}H_{18}O_5Cl_2$ as determined from the HRESIMS peak at *m*/*z* 431.0429 $[M+Na]^+$. The UV and IR spectra were almost identical to those of compound **12**.[10] Their ^1H and ^{13}C NMR spectroscopic data (Table 2) as well as HMBC correlations were also similar.[10] However, **2** had additional signals of an methoxy group (δ_H 3.78, s; δ_C 60.5). The attachment of a methoxy group at C-8 was assigned on the basis of HMBC correlations (Fig. 2) from the methoxy protons (δ_H 3.78) to C-8 (δ_C 152.1) and the methyl protons (δ_H 2.31, H_3-13) to C-8, C-9 (δ_C 123.3) and C-9a (δ_C 142.0). Signal enhancement of the methoxy protons after irradiation of H_3-13 in the NOEDIFF experiment supported this assignment. The HRESIMS (*m*/*z* 431.0429, 433.0414 and 435.0386 in a 10:6:1 ratio) revealed the presence of two chlorine atoms in **2**. Consequently, compound **2** was identified as a methoxy derivative of compound **12**.

The isolated compounds (**3**, **5**, **7**–**9**, and **12**–**14**) with sufficient amount were evaluated for antimicrobial (against *S. aureus*, methicillin-resistant *S. aureus*, *C. albicans*, *C. neoformans*, and

M. gypseum), antimalarial (against *P. falciparum*), and cytotoxic (against MCF-7, KB and Vero cell lines) activities (Table 3). Compound **5** was inactive in all tested assays. Interestingly, compound **13** exhibited the most potent antibacterial (against *S. aureus* and methicillin-resistant *S. aureus*), and antifungal (against *C. neoformans*) activities with MIC values of 0.5 μg/mL. Additionally, compounds **12** and **14** were two fold more active against methicillin-resistant *S. aureus* than *S. aureus* with MIC values of 1 μg/mL. In contrast, compounds **3** and **8** were two fold more active against *S. aureus* than the methicillin-resistant strain with MIC values of 2 and 4 μg/mL, respectively. Compounds **7** and **9** displayed activity against both bacterial stains with MIC values of 64 and 2 μg/mL, respectively. Regarding the antifungal activity, compound **8** was active against *C. albicans* with a MIC value of 8 μg/mL, while other tested substances were much less active. Compound **14** showed significant antifungal activity against *C. neoformans* with a MIC value of 1 μg/mL, while compounds **3**, and **7**–**9** displayed moderate to weak activity with MIC values in the range of 8–128 μg/mL. Compound **12** exhibited antifungal activity toward *M. gypseum* with a MIC value of 2 μg/mL. Furthermore, compounds **3** and **8** were eight fold more active against the same fungal pathogen than compound **7** with MIC values of 8 μg/mL. Compound **13** was approximately three fold more active against *P. falciparum* than **12** with an IC_{50} value of 2.2 μM. Moreover, compounds **3**, **7**, and **14** displayed the same activity with IC_{50} values in the range of 7.7–39.7 μM. Among the tested compounds, compound **7** exhibited the most potent activity against KB cell line with an IC_{50} value of 4.5 μM which was much stronger than the standard drug, ellipticine. Compound **12** showed the strongest activity against Vero cell line with an IC_{50} value of 8.1 μM. Other tested compounds, except for compounds **8** and **9** which were inactive against KB and MCF-7 cell lines, were much less active with IC_{50} values in the range of 14.4–110.1 μM. These results implied that the chlorine atom in the depsidone was important for antimicrobial activity. Furthermore, this is the first report on the antibacterial activity against *S. aureus* and methicillin-resistant *S. aureus* of compounds **3**, **7**–**8**, and **13**–**14** as well as antifungal and antimalarial activities for all tested substances. The antibacterial activity of the depsidones **9** and **12** against methicillin-resistant *S. aureus* has been previously reported.[16]

In conclusion, *A. unguis* PSU-RSPG199 produced two phthalides (**1** and **3**), three orsellinates (**4**, **5** and **7**), one benzoate (**6**), and eight depsidones (**2** and **8**–**14**). The isolated depsidones **8**–**9** and **12**–**14** showed significant antibacterial activity with MIC values in the range of 0.5–8 μg/mL. Furthermore, orsellinate **7** displayed potent cytotoxic activity against the KB cell line with an IC_{50} value of 4.5 μM. All compounds except **12** were considered to be inactive against Vero cell lines.

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Supplementary data

Supplementary data (experimental procedure) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2016.08.040.

References and notes

1. Kornienko, A.; Evidente, A.; Vurro, M.; Mathieu, V.; Cimmino, A.; Evidente, M.; van Otterlo, W. A. L.; Dasari, R.; Lefranc, F.; Kiss, R. *Med. Res. Rev.* **2015**, *35*, 937–967.
2. Wang, J.; Wei, X.; Qin, X.; Tian, X.; Liao, L.; Li, K.; Zhou, X.; Yang, X.; Wang, F.; Zhang, T.; Tu, Z.; Chen, B.; Liu, Y. *J. Nat. Prod.* **2016**, *79*, 59–65.
3. Phainuphong, P.; Rukachaisirikul, V.; Saithong, S.; Phongpaichit, S.; Bowornwiriyapan, K.; Muanprasat, C.; Srimaroeng, C.; Duangjai, A.; Sakayaroj, J. *J. Nat. Prod.* **2016**, *79*, 1500–1507.
4. Rukachaisirikul, V.; Rungsaiwattana, N.; Klaiklay, S.; Phongpaichit, S.; Borwornwiriyapan, K.; Sakayaroj, J. *J. Nat. Prod.* **2014**, *77*, 2375–2382.
5. Kawahara, N.; Nakajima, S.; Satoh, Y.; Yamazaki, M.; Kawai, K.-I. *Chem. Pharm. Bull.* **1988**, *36*, 1970–1975.
6. Sureram, S.; Kesornpun, C.; Mahidol, C.; Ruchirawat, S.; Kittakoop, P. *RSC Adv.* **2013**, *3*, 1781–1788.
7. Lopes, T. I. B.; Coelho, R. G.; Yoshida, N. C.; Honda, N. K. *Chem. Pharm. Bull.* **2008**, *56*, 1551–1554.
8. Basset, J.-F.; Leslie, C.; Hamprecht, D.; White, A. J. P.; Barrett, A. G. M. *Tetrahedron Lett.* **2010**, *51*, 783–785.
9. Rajachan, O.; Kanokmedhakul, S.; Kanokmedhakul, K.; Soytong, K. *Planta Med.* **2014**, *80*, 1635–1640.
10. Sureram, S.; Wiyakrutta, S.; Ngamrojanavanich, N.; Mahidol, C.; Ruchirawat, S.; Kittakoop, P. *Planta Med.* **2012**, *78*, 582–588.
11. Kawahara, N.; Nozawa, K.; Nakajima, S.; Kawai, K.; Yamazaki, M. *J. Chem. Soc.* **1988**, 2611–2614.
12. Hamano, K.; Kinoshita-Okami, M.; Hemmi, A.; Sato, A.; Hisamoto, M.; Matsuda, K.; Yoda, K.; Haruyama, H.; Hosoya, T.; Tanzawa, K. *J. Antibiot.* **1992**, *45*, 1195–1201.
13. Lan, W.-J.; Liu, W.; Liang, W.-L.; Xu, Z.; Le, X.; Xu, J.; Lam, C.-K.; Yang, D.-P.; Li, H.-J.; Wang, L.-Y. *Mar. Drugs* **2014**, *12*, 4188–4199.
14. *Asperlide* (**1**): Colorless gum; $[\alpha]_D^{25}$ −48.7 (*c* 0.078 MeOH); UV (MeOH) λ_{max} (log ε) 300 (3.00), 270 (3.36) nm; CD (*c* 7.30 × 10^{-5} mol/L, MeCN) λ_{max} ($\Delta\varepsilon$) 211 (+28.21) nm; IR (neat) ν_{max} 3424, 1757, 1726 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) and ^{13}C NMR ($CDCl_3$, 75 MHz) data, see Table 1; HRESIMS *m*/*z* 395.1108 [M+Na]$^+$ (calcd for $C_{20}H_{20}O_7Na$, 395.1107).
15. *Aspersidone* (**2**): Pale yellow gum; UV (MeOH) λ_{max} (log ε) 321 (3.43), 287 (3.58), 223 (4.33) nm; IR (neat) ν_{max} 3410, 1736 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) data, see Table 2; HRESIMS *m*/*z* 431.0429 [M+Na]$^+$ (calcd for $C_{20}H_{18}O_5Cl_2Na$, 431.0429).
16. Zhang, Y.; Mu, J.; Feng, Y.; Wen, L.; Han, J. *Nat. Prod. Res.* **2014**, *28*, 503–506.

ภาคผนวก ข

(Appendix II)

ผลงานที่อยู่ในระหว่างเตรียม manuscript

Trichothecene Derivatives from the Soil-Derived Fungus *Trichoderma brevicompactum* PSU-RSPG27

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ABSTRACT. Six new trichothecene analogues (**1-6**) together with seven known trichothecene derivatives (**7-13**) were isolated from the soil-derived fungus *Trichoderma brevicompactum* PSU-RSPG27. Their structures were established using spectroscopic data. The structure of **1** was confirmed by X-ray data. Trichodermin (**7**) exhibited the most potent antimalarial activity against *Plasmodium falciparum* (K1 strain) with an IC_{50} value of 0.1 μM while other trichothecenes (**1**, **4**, **8**-**9** and **12**) were much less active with the IC_{50} values in the range of 7.1-9.6 μM. However, compound **7** displayed strong activity against noncancerous Vero cells with an IC_{50} value of 0.4 μM. The remaining compounds showed moderate to weak activity with the IC_{50} values in the range of 6.9-45.9 μM. Compounds **7** and **8** were strongly active against the human oral carcinoma (KB) cells with the IC_{50} values of 2.4 and 3.7 μM, respectively. Additionally, compounds **7** and **8** also displayed antifungal activity against *Candida albicans* with the respective MIC values of 1 and 2 μg/mL and were active against *Cryptococcus neoformans* with equal MIC values of 4 μg/mL.

KEYWORDS

Trichoderma brevicompactum

Trichothecence

Antifungal

Antimalarial

Cytotoxic

Fungi have gained predominance as a source of interesting bioactive natural products.[1,2] Trichothecenes, a family of sesquiterpenes, were produced by various fungal genera such as *Fusarium*,[3] *Myrothecium*[4] and *Trichoderma*.[5] Trichodermin, one of trichothecence derivatives, displayed antifungal,[6] nematicidal[7] and antiviral[8] activities. Moreover, trichodermin derivatives such as trichodermates, trichothecin and trichodermol, exhibited cytotoxic activity against K562 human myelogenous leukemia,[9] HCT15 colon cancer [10] and SMMC-7721 human carcinoma cell lines.[4] In our ongoing search for biologically active substances from the soil-derived fungi, we chemically investigated a soil-derived fungus *Trichoderma brevicompactum* PSU-RSPG27 isolated from a soil sample collected from the Plant Genetic Conservation Project under the Royal Initiation of Her Royal Highness Princess Maha Chakri Sirindhorn at Ratchaprapa Dam in Surat Thani Province, Thailand. The broth extract showed antifungal activity against *Candida albicans*, *Cryptococcus neoformans* and *Microsporum gypseum* with the MIC values of 4, 16 and 128 μg/mL, respectively. It exhibited interesting antimalarial activity against *Plasmodium falciparum* (K1 strain) with an IC_{50} value of 0.21 μg/mL. In addition, it displayed significant cytotoxicity against KB (oral cavity cancer) and noncancerous Vero (African green monkey kidney fibroblasts) cells with the IC_{50} values of 1.14 and 0.30 μg/mL, respectively. Herein, we describe the isolation and characterization of secondary metabolites from the broth extract of the fungus PSU-RSPG27. Six new trichothecene derivatives, trichodermarins A-F (**1**-**6**) together with seven known ones, trichodermin (**7**),[11] trichodermol (**8**),[11] trichoderminol (**9**),[8] (2*R*,2'*S*,4*R*,5*S*,5a*R*,7*R*,9a*R*)-2,3,4,5,5a,6,7,9a-octahydro-5,5a,8-trimethyl-4-acetatespiro[2,5-methano-1-benzoxepin-10,2'-oxirane]-4,7-diol (**10**),[12,13] (2*R*,2'*S*,4*R*,5*S*,5a*R*,7*S*,9a*R*)-4-acetate-2,3,4,5,5a, 6,7,9a-octahydro-5,5a,8-trimethylspiro[2,5-methano-1-benzoxepin-10,2'-oxirane]-4,7-diol (**11**),[12,13] (4*β*)-4-(acetyloxy)-12,13-epoxytrichothec-9-ene-9-carboxaldehyde (**12**)[12] and (2*R*,4*R*,5*S*,5a*R*,

9a*R*,10*S*)-4-acetate-2,3,4,5,5a,6,7,9a-octahydro-10-(hydroxymethyl)-5,5a,8-trime-thyl-2,5-me-thano-1-benzoxepin-4,10-diol[14] (**13**) were obtained. Antifungal (against *C. albicans*, *C. neoformans* and *M. gypseum*), antimalarial (against *P. falciparum*) and cytotoxic (against KB and Vero cells) activities of isolated compounds were evaluated.

RESULTS AND DISCUSSION

All trichothecene derivatives (**1-13**) (Figure 1) were purified using chromatographic methods, and their structures were elucidated using various spectroscopic techniques. The relative configuration of **1** was determined by NOEDIFF data and confirmed by X-ray data (Figure 2). The absolute configuration of the secondary alcohol at C-4 of **1** was established to be *R* configuration by Mosher's method (Figure 3).[9] For the other isolated compounds, the relative configurations were also assigned according to NOEDIFF data. Consequently, the absolute configurations were confirmed by comparison of their specific rotations with those of known or structurally related compounds.

Trichodermarin A (**1**) was obtained as colorless crystals with the molecular formula $C_{15}H_{22}O_4$ deduced from HRESIMS peak at *m/z* 289.1416 $[M+Na]^+$. The IR spectrum showed an absorption band at 3343 cm^{-1} for a hydroxy group. The ^1H NMR spectroscopic data (Table 1) consist of signals for one olefinic proton of a pentasubstitiuted alkene (δ_H 5.40, s, 1H), two oxymethine protons (δ_H 4.08, brd, J = 6.9 Hz, 1H and 4.20, d, J = 5.4 Hz, 1H), two nonequivalent oxymethylene protons (δ_H 3.99 and 3.93, each d, J = 10.5 Hz, 1H), three sets of nonequivalent methylene protons [δ_H 2.56 (dd, J = 16.5 and 7.5 Hz, 1H)/1.71 (m, 1H), 2.15 (m, 1H)/1.96 (m, 1H) and 1.85 (ddd, J = 12.9, 5.2 and 1.2 Hz)/1.43 (ddd, J = 12.9, 4.2 and 1.2, 1H)] and three methyl groups (δ_H 1.76, 1.05 and 0.89, each s, 3H). The ^{13}C NMR spectrum (Table 2) consisted

of signals for five quaternary (δ_C 145.6, 107.9, 94.7, 54.5 and 47.8), one oxymethylene (δ_C 59.5), three methylene (δ_C 42.0, 30.7 and 28.9), three methine (δ_C, 116.2, 82.7 and 75.6) and three methyl (δ_C 23.0, 14.4 and 10.6) carbons. These data were similar to those of **8**.[12] The obvious differences were the replacement of the oxymethine (C-11) and the oxymethylene (C-13) carbons of the epoxide unit in **8** with one dioxygenated quaternary carbon (δ_C 107.9, C-11) and hydroxymethylene carbon (δ_C 59.5, C-13) in **1**, respectively. This assignment was confirmed by HMBC correlations of H-2 (δ_H 4.20) to C-11, C-12 (δ_C 94.7) and C-13 and those of H_{ab}-13 (δ_H 3.99 and 3.93) with C-2 (δ_C 82.7), C-5 (δ_C 47.8) and C-12. The chemical shifts of C-11 and C-12 together with five degrees of unsaturation established an ether linkage between C-11 and C-12 and the attachment of the hydroxymemthyl group at C-12.[15] In the NOEDIFF experiment (Figure 4), the signal intensity of H_3-15 (δ_H 1.05) was affected by irradiation of H-4, suggesting that they were *cis*-relationship. In addition, irradiation of H_{ab}-13 (δ_H 3.93 and 3.99) enhanced signal intensities of H-2 (δ_H 4.20) and H_3-14 (δ_H 0.89) indicating that theses protons located at the same side of molecule and had *trans* relationship to H-4 and H_3-15. These assignments were confirmed by X-ray data (Figure 2). The absolute configuration of the secondary alcohol at C-4 of **1** was assigned as *R* configuration by Mosher's method (Figure 3),[9] identical to that of **8**. Thus, the remaining absolute configurations were identified as 2*R*, 5*S*, 6*R*, 11*R* and 12*R*. Consequently, **1** was a new trichothecence derivative.

Trichodermarin B (**2**) was obtained as a colorless gum with the molecular formula $C_{17}H_{24}O_5$ deduced from HRESIMS peak at *m/z* 331.1535 $[M+Na]^+$. The IR spectrum showed an absorption band at 1737 cm^{-1} for a carbonyl functionality of an acetoxy group.[11] The ^1H and ^{13}C NMR spectroscopic data were almost identical to **1** except for the present of additional signals of acetyl group [δ_H 2.06, s, 3H, δ_C 170.0 (a carbonyl carbon), and δ_C 21.3 (a methyl carbon)] in **2**. These

results together with the appearance of the oxymethine proton (H-4, δ_H 5.29) in **2** at much lower filed than that in **1**, indicated that the hydroxy group in **1** was replaced by the acetoxy group in **2**. An HMBC correction from H-4 to C-1′ (δ_C 170.0) confirmed this conclusion. Compound **2** displayed similar NOEDIFF data to those of **1**, indicating their identical relative configuration. The absolute configuration was proposed to be identical to that of **1** according to their similar specific rotation (**2**, $[\alpha]_D^{23}$ +10.1, *c* 1.1, CH_2Cl_2 and **1**, $[\alpha]_D^{23}$ +12.7, *c* 1.1, CH_2Cl_2). Thus, **2** was assigned as an acetyl derivative of **1**.

Trichodermarin C (**3**) was obtained as a colorless gum with the molecular formula $C_{17}H_{24}O_5$ deduced from HRESIMS peak at *m/z* 331.1518 $[M+Na]^+$. The IR spectrum showed absorption bands at 3432 and 1733 cm^{-1} for hydroxy and carbonyl functionalities, respectively. The ^1H NMR spectroscopic data were similar to those of **7** except that one set of the methylene protons in **7** was replaced, in **3**, by an oxymethine proton (δ_H 4.19, dd, *J* = 5.0 and 3.0 Hz). The oxymethine proton was attributed to H-3 on the basis of the ^1H-^1H COSY correlations of H-3 with H-2 (δ_H 3.70, d, *J* = 5.0 Hz) and H-4 (δ_H 5.00, d, *J* = 3.0 Hz). The relative configuration was assigned by the NOEDIFF data. Irradiation of H-3 affected signal intensity of H-2, but not that of H-4, indicating β position of H-3. Determination of the absolute configuration at C-3 was not attempted because compound **3** was isolated in low amount. As compound **3** was a co-metabolite of **1**, **2**, **7** and **8**, their absolute configurations were assigned as 2*R*, 4*R*, 5*R*, 11*R* and 12*R*. Thus, the absolute configuration of C-3 was identified as 3*S*. **3** was assigned as a 2-hydroxy derivative of **7**.

Trichodermarin D (**4**) was obtained as a colorless gum with the molecular formula $C_{17}H_{26}O_5$ deduced from HRESIMS peak at *m/z* 333.1668 $[M+Na]^+$. The IR spectrum displayed absorption bands at 3420 and 1730 cm^{-1} for hydroxy and carbonyl functionalities, respectively. Comparison

of the ^{1}H and ^{13}C NMR spectral data of **4** (Table 1 and 2) with those of **7** reveal the similarity of their structures. The significant difference was the replacement of signals for a -CH=C(CH_3)- unit in **7** with signals for a -CH_2CH(CH_2OH)- moiety [δ_H 1.85, m, 1H, δ_C 32.7; δ_H 1.97 and 1.73, each m, 1H, δ_C 28.5; δ_H 3.66 and 3.65, each dd, J = 8.5 and 2.5 Hz, 1H, δ_C 66.8] in **4**. The ^{1}H-^{1}H COSY spectrum displayed the cross peaks of H-9 (δ_H 1.85)/H_{ab}-8 (δ_H 1.68 and 1.62), H_{ab}-10 (δ_H 1.97 and 1.73) and H_{ab}-16 (δ_H 3.66 and 3.65), H_{ab}-8/H_{ab}-7 (δ_H 2.03 and 1.20) and H_{ab}-10/H-11 (δ_H 3.48). These data together with HMBC cross peaks from H_{ab}-16 to C-8 (δ_C 22.8), C-9 (δ_C 32.7), C-10 (δ_C 28.5) and H-9 to C-7 (δ_C 24.2) and C-11 (δ_C 71.8) constructed a fused cyclohexane ring with the hydroxymethylene group at C-9. The relative configuration was assigned by NOEDIFF data (Figure 4). Irradiation of H-11 (δ_H 3.48) enhanced signals of H-9 and H_3-15 (δ_H 1.05), indicating that they were in a *cis*-relationship. In addition, irradiation of H_a-13 (δ_H 3.13) and H_{ab}-16 affected to H_a-10 (δ_H 1.97). Thus, the hydroxylmethyl group was located in a β position. The absolute configurations at C-2, C-4, C-5 and C-6 were proposed to be identical to that of compounds **1**, **2**, **7** and **8**, as they were co-metabolites. Thus, the absolute configuration of C-9 was identified as 9*R*. Consequently, compound **4** was a new trichothecence.

Trichodermarin E (**5**) was obtained as a colorless gum with the same molecular formula as **4** based on HRESIMS peak at *m/z* 333.1678 $[M+Na]^+$. The ^{1}H and ^{13}C NMR spectroscopic data (Table 1 and 2) were similar to those of **4**. These results together with their ^{1}H-^{1}H COSY and HMBC correlations indicated that **5** possessed an identical planar structure to **4**. However, **4** and **5** displayed the different NOEDIFF data (Figure 4). Irradiation of H-11 (δ_H 3.53) affected only signal intensity of H_3-15 (δ_H 1.01), but not H-9 (δ_H 1.80), indicating that hydroxymethyl group in **5** was located in α position. Consequently, **5** was identified as a C-9 epimer of **4**.

Trichodermarin F (**6**) was obtained as a colorless gum with the molecular formula $C_{17}H_{26}O_5$ deduced from HRESIMS peak at *m/z* 333.1678 $[M+Na]^+$. The ^1H and ^{13}C NMR spectroscopic data were similar to those of **7**, indicating that **6** possessed the similar skeleton to that of **7**. The differences in the NMR spectroscopic data were observed in ring A. Signals of two olefinic carbons (C-9 and C-10) in **7** were replaced by a quaternary oxygenated carbon (C-9, δ_C 74.2) and methine carbon (C-10, δ_C 44.7). Moreover, the epoxymethylene proton signals in **7** were replaced by signals for two nonequivalent methylene protons (H_{ab}-13, δ_H 1.91, m and 1.44, dd, *J* = 14.5 and 5.5 Hz). The ^1H-^1H COSY spectrum displayed the cross peaks of H-10 (δ_H 2.11) to H-11 (δ_H 3.48) and H_{ab}-13 (δ_H 1.91 and 1.44). These data together with HMBC cross peaks of H-11 to C-2 (δ_C 80.6), C-5 (δ_C 51.8), C-9 (δ_C 74.1) and C-13 (δ_C 28.0) and H_{ab}-13 to C-2, C-5, C-9, C-10 (δ_C 44.7), C-11 (δ_C 73.7) and C-12 (δ_C 78.6), constructed a methylene bride between C-10 and C-12. The chemical shift of C-12 (δ_C 78.6) attached a hydroxy group at this carbon. In the NOEDIFF experiment (Figure 4), irradiation of H-11 (δ_H 3.48) affected to H_3-15 (δ_H 1.14) whereas signal intensity of H_b-13 was enhanced upon irradiation of H_3-14 and H_3-16 (δ_H 1.23). These results indicated that H-11 and H_3-15 were a *cis*-relationship and *trans* to H_b-13, H_3-14 and H_3-16. In addition, irradiation of H-2 (δ_H 4.01) affected signal intensity of H_a-13 (δ_H 1.91). The results suggested that H-2, H_{ab}-13 and H_3-14 located at the same side of molecule. Consequently, compound **6** was a new trichothecence.

The isolated compounds **1**, **4**, **7-9** and **12**, which were obtained in sufficient amounts, were tested for antifungal (*C. albicans*, *C. neoformans* and *M. gypseum*), antimalarial (*P. falciparum*) and cytotoxic (KB and Vero cell lines) activities (Table 3). Interestingly, **7** exhibited the most potent antifungal activity against *C. albicans*, *C. neoformans* and *M. gypseum* with the MIC values of 1, 4 and 2 μg/mL, respectively. Compound **12** was two fold more active against

C. albicans than *C. neoformans* with the MIC values of 2 and 4 μg/mL, respectively. Compounds **1** and **8** showed much weaker antifungal activity against *C. albicans* and *C. neoformans* with equal MIC values of 16 μg/mL while compound **9** was four fold less active against these two fungal strains. Among the tested compounds, **7** displayed the most potent antimalarial activity against *P. falciparum* (K1 strain) with an IC_{50} value of 0.1 μM. In addition, the other trichothecenes was less active with the IC_{50} values in the range of 7.1-9.6 μM. Compounds **7** and **12** showed significant cytotoxicity against KB cell lines with the IC_{50} values of 2.4 and 3.7 μM, respectively, which were much stronger than standard drug, ellipticine. Moreover, compounds **8** and **9** were approximately four and five folds less active against the same cell lines than **12**, respectively. However, **7** exhibited much stronger cytotoxic activity (IC_{50} value of 0.4) to Vero cell lines than other tested compounds. These results indicated that the 4-OAc and vinylic methyl functionalities would be important for antifungal, antimalarial and cytotoxic (against KB and Vero cell lines) activities. From these data, we conclude that the biological activities of broth extract might be controlled by **7**, which is the major component in the extract. In the previously report, compound **8** showed antimalarial[16] and anticancer (against MCF-7)[17] activities. This is the first report on antifungal (*C. albicans*, *C. neoformans* and *M. gypseum*), antimalarial (against *P. falciparum*) and cytotoxic (KB and Vero cell lines) activities for **7-9** and **12**.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were recorded on a JASCO P-1020 polarimeter. The melting points were determined on an Electrothermal 9100 melting point apparatus and reported without correction. Infrared (IR) spectra were recorded neat using a

Perkin Elmer 783 FTS165 FT-IR spectrometer. Mass spectra were obtained from a liquid chromatograph-mass spectrometer (2090, LCT, Waters, Micromass). ^{1}H and ^{13}C NMR spectra were recorded on a 300 or 500 MHz Bruker FTNMR Ultra Shield spectrometer. Chemical shifts are expressed in δ (parts per million, ppm), referring to the tetramethylsilane peak. Thin layer chromatography (TLC) and preparative TLC were performed on silica gel 60 GF_{254} (Merck). Column chromatography (CC) was carried out on Sephadex LH-20, silica gel (Merck) type 60 (230-400 mesh ASTM) or type 100 (70-230 mesh ASTM), or reversed phase C_{18} silica gel. Petroleum ether has a boiling point range of 40–60 °C.

Fungal Material. The soil-derived fungus PSU-RSPG27 was isolated from a soil sample collected from Surat Thani Province, Thailand, and deposited as BCC56868 at BIOTEC Culture Collection, National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. The fungus PSU-RSPG27 was identified based on morphology and the internal transcribed spacer (ITS1-5.8S-ITS2) rDNA analysis using universal fungal primers.[18] It is a fast growing fungus. Its wooly colony was initially white and became patches green when the conidia were produced. The microscopic morphology showed hyaline septate hyphae, branched conidiophores, flask-shaped phialides and globose to ovoid conidia. Phylogenetic analysis using maximum parsimony method revealed that the fungus PSU-RSPG27 (Genbank accession number KC478544) was closely related and formed a subgroup with various strains of *Trichoderma brevicompactum* (DQ000635, EF417484, HQ596986, FJ610287-610290, EU330934) with 100% nucleotide identity and 99.8-100% bootstrap values. Therefore, the fungus PSU-RSPG27 was identified as *T. brevicompactum*.

Fermentation, Extraction, and Purification. The broth extract (5.3 g) of the fungus PSU-RSPG27 was prepared using the same procedure as described for *Aspergillus sclerotiorum*

PSU-RSPG178.[19] It was subjected to CC over Sepahadex LH-20 using MeOH as a mobile phase to obtain four fractions (A-D). Fraction C (4.7 g) was further separated by CC over silica gel using a gradient of MeOH-CH_2Cl_2 (0:1 to 1:0) to afford nine subfractions (C1-C9). Compound **7** (754.9 mg) was obtained from subfraction C4. Subfraction C5 (1.8 g) was purified using the same procedure as fraction C to provide seven subfractions (C5A-C5G). Subfraction C5E (129.7 mg) was subjected to CC over reversed phase C_{18} silica gel using a gradient of MeOH-H_2O (7:3 to 1:0) to afford five subfractions (C5E1-C5E5). Subfraction C5E1 (36.1 mg) was purified by CC over silica gel using a gradient of acetone-petroleum ether (1:4 to 1:0) to give eight subfrations. Compound **12** (1.1 mg) was obtained after subjecting the first subfraction to preparative TLC using $CHCl_3$-petroleum ether (7:3) as a mobile phase. The fifth subfraction (18.8 mg) was subjected to preparative TLC using acetone-CH_2Cl_2 (1:50) as a mobile phase to provide **4** (8.1 mg). Compound **9** (7.1 mg) was obtained after subjecting the sixth subfraction (16.1 mg) to preparative TLC using acetone-CH_2Cl_2 (1:10) as a mobile phase. Subfraction C5E2 (32.5 mg) was purified using the same procedure as subfraction C5E1 to afford five subfractions. Compounds **2** (3.1 mg), **3** (1.3 mg) and **8** (7.0 mg) were obtained after subjecting the second subfraction (12.8 mg) to preparative TLC using ethyl acetate-petroleum ether (1:5) as a mobile phase. The third subfraction (6.8 mg) was purified using acetone-CH_2Cl_2 (1:20) to afford **13** (3.0 mg). Compound **1** (12.0 mg) was obtained after subjecting subfraction C6 (62.9 mg) to CC over silica gel using a mixture of acetone-CH_2Cl_2-petroleum ether (2:1:7) as a mobile phase. Subfraction C7 (1.2 g) was purified using the same procedure as fraction C to provide seven subfractions (C7A-C7G). Subfraction C7D (482.5 mg) was purified by CC over silica gel using a gradient of ethyl acetate-CH_2Cl_2 (1:20 to 1:0) to afford eight subfractions (C7D1-C7D8). Compound **10** (3.1 mg) was obtained after subjecting subfraction C7D3 (16.1 mg) to preparative

TLC using a mixture of acetone-CH_2Cl_2-hexane (1:1:8) as a mobile phase. Subfraction C7D5 (62.5 mg) was purified by CC over silica gel using the same elution system as subfraction C6 to afford eight subfractions. Compound **11** (3.1 mg) was obtained after subjecting the fifth subfraction to preparative TLC using ethyl acetate-hexane (7:13) as a mobile phase. Subfraction C7E (39.7 mg) was subjected to CC over silica gel using the same eluent system as subfraction C6 to provide eight subfractions. Compound **6** (1.3 mg) was obtained after subjecting the fifth subfraction (5.3 mg) to preparative TLC using a mixture of acetone-CH_2Cl_2-hexane (2:1:7) as a mobile phase. Subfraction C8 (1.3 g) was purified using the same procedure as fraction C to yield eight subfractions (C8A-C8H). Subfraction C8E (129.4 mg) was purified using the same procedure as subfraction C6 to afford six subfractions. Compound **5** (1.7 mg) was obtained after subjecting the sixth subfraction (16.9 mg) to CC over silica gel using ethyl acetate-CH_2Cl_2 (1:4) as a mobile phase followed by purifying using preparative TLC using a mixture of acetone-CH_2Cl_2-hexane (1:1:8) as a mobile phase.

Trichodermarin A (1): Colorless crystals (EtOH); mp 121-126 ºC; $[\alpha]_D^{23}$ +12.7 (*c* 1.1, CH_2Cl_2); IR (neat) ν_{max} 3343, 1441 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m/z* $[M+Na]^+$ 289.1416 (calcd for $C_{15}H_{22}O_4Na$, 289.1416)

Trichodermarin B (2): Colorless gum; $[\alpha]_D^{23}$ +10.1 (*c* 1.1, CH_2Cl_2); IR (neat) ν_{max} 3458, 1737 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m/z* $[M+Na]^+$ 331.1535 (calcd for $C_{17}H_{24}O_5Na$, 331.1521).

Trichodermarin C (3): Colorless gum; $[\alpha]_D^{23}$ $[\alpha]_D^{23}$ +12.3 (*c* 1.1, CH_2Cl_2); IR (neat) ν_{max} 3432, 1733 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m/z* $[M+Na]^+$ 331.1518 (calcd for $C_{17}H_{24}O_5Na$, 331.1521).

Trichodermarin D (4): Colorless gum; $[\alpha]_D^{23}$ -30.1 (*c* 0.1, MeOH); IR (neat) ν_{max} 3420, 1730 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m/z* $[M+Na]^+$ 333.1668 (calcd for $C_{17}H_{26}O_5Na$, 333.1678).

Trichodermarin E (5): Colorless gum; $[\alpha]_D^{23}$ +12.3 (*c* 0.1, MeOH); IR (neat) ν_{max} 3452, 1731 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m/z* $[M+Na]^+$ 333.1678 (calcd for $C_{17}H_{26}O_5Na$, 333.1678).

Trichodermarin F (6): Colorless gum; $[\alpha]_D^{23}$ +3.4 (*c* 0.1, $CHCl_3$); IR (neat) ν_{max} 3409, 1730 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m/z* $[M+Na]^+$ 333.1678 (calcd for $C_{17}H_{26}O_5Na$, 333.1678).

Preparation of the (*R*)- and (*S*)-MTPA Ester Derivatives of 1. Pyridine (100 μL) and (*R*)-(-)-MTPACl (40 μL) were added to a CH_2Cl_2 solution (200 μL) of **1** (2.5 mg). The reaction mixture was stirred at room temperature overnight. After removal of solvent, the mixture was purified by CC over silica gel using a mixture of acetone-CH_2Cl_2-hexane (1:1:8) as a mobile phase to afford (*S*)-MTPA ester (2.7 mg, 60% yield). Compound **1** (2.9 mg) was treated in a same procedure with (*S*)-(+)-MTPACl and, after purification by CC, (*R*)-MTPA ester (1.8 mg, 40% yield) was obtained.

X-ray Crystallographic Analysis of 1. Crystal data for compound **1** was collected on a Bruker Smart Apex CCD diffractometer equipped with a graphite-monochromatic Mo Kα radiation (λ = 0.71073 Å) source at 293(2) K. Cell refinement, data reductions, and absorption correction were performed using SAINT and SADABS. This structure was solved using direct methods with SHELXS[20] and refined with the full-matrix least-squares methods based on F^2 with the SHELXL program.[21] Non-hydrogen atoms were allowed to vibrate anisotropically in cycles of refinement. All hydrogen atoms were placed in calculated, ideal positions and

refined as riding model approximations on their respective parent atoms. The WinGX v2014.1[22] and Mercury[23] programs were used to prepare the materials and molecular graphics for publication. Crystallographic data for the structure for this papar have been deposited with the Cambridge Crystallographic Data Centre (deposit no. CCDC 1554139). Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, U.K. (fax: +44-(0)223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

Antimalarial Assay. Antimalarial assay was evaluated against the parasite *P. falciparum* (K1, multidrug-resistant strain) using the microculture radioisotope technique based on the method described.[24] Dihydroartemisinine was used as a standard compound.

Cytotoxic Assay. The activity against African green monkey kidney fibroblast (Vero) cells was performed in triplicate employing the method described.[25] The activity against KB cell lines was evaluated using the resazurin microplate assay.[26] The positive controls were ellipticine for both Vero and KB cells.

Antifungal Assay. Antifungal activity against *C. albicans* NCPF3153 and *C. neoformans* ATCC90113 was carried out using a colorimetric broth microdilution test.[27] MICs were recorded by reading the lowest substance concentration that inhibited visible growth. Amphotericin B was a positive control drug.

ASSOCIATED CONTENT

Supporting Information. ^{1}H and ^{13}C NMR spectra for trichodermarins A-F (**1**-**6**). This material is available free of charge via the Internet at http://pubs.acs.org.

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REFERRENCES:

(1) Newman, D. J.; Cragg, G. M. *J. Nat. Prod.* **2016**, *79* (3), 629–661.

(2) Dias, D. A.; Urban, S.; Roessner, U. *Metabolites* **2012**, *2* (4), 303–336.

(3) Fruhmann, P.; Mikula, H.; Wiesenberger, G.; Varga, E.; Lumpi, D.; Stöger, B.; Häubl, G.; Lemmens, M.; Berthiller, F.; Krska, R.; Adam, G.; Hametner, C.; Fröhlich, J. *J. Nat. Prod.* **2014**, *77* (1), 188–192.

(4) Shen, L.; Wang, J. S.; Shen, H. J.; Song, Y. C.; Tan, R. X. *Planta Med.* **2010**, *76* (10), 1004–1006.

(5) Nielsen, K. F.; Gräfenhan, T.; Zafari, D.; Thrane, U. *J. Agric. Food Chem.* **2005**, *53* (21), 8190–8196.

(6) Shentu, X.; Zhan, X.; Ma, Z.; Yu, X.; Zhang, C. *Braz. J. Microbiol.* **2014**, *45* (1), 248–254.

(7) Yang, Z.-S.; Li, G.-H.; Zhao, P.-J.; Zheng, X.; Luo, S.-L.; Li, L.; Niu, X.-M.; Zhang, K.-Q. *World J. Microbiol. Biotechnol.* **2010**, *26* (12), 2297–2302.

(8) Lee, D. H.; Kim, J. J.; Ryu, K. H.; Kim, B. S.; Ryu, S. M. PCT Patent WO 2016/089166 A1, 2016.

(9) Li, J.; Ren, J.; Bao, L.; Jin, T.; Wang, W.; Pei, Y.; Liu, X.; Li, E. *Helv. Chim. Acta* **2016**, *99* (1), 63–69.

(10) Choi, S. U.; Choi, E. J.; Kim, K. H.; Kim, N. Y.; Kwon, B.-M.; Kim, S. U.; Bok, S. H.; Lee, S. Y.; Lee, C. O. *Arch. Pharm. Res.* **1996**, *19* (1), 6–11.

(11) Ayer, W. A.; Miao, S. *Can. J. Chem.* **1993**, *71* (4), 487–493.

(12) Xu, X.; Cheng, J.; Zhou, Y.; Zhang, C.; Ou, X.; Su, W.; Zhao, J.; Zhu, G. *Chem. Biodivers.* **2013**, *10* (4), 600–611.

(13) Zheng, M.; Yao, T.-T.; Xu, X.-J.; Cheng, J.-L.; Zhao, J.-H.; Zhu, G.-N. *Bioorg. Med. Chem. Lett.* **2014**, *24* (15), 3565–3568.

(14) Cheng, J. L.; Zhou, Y.; Zhao, J. H.; Zhang, C.; Lin, F. C. *Chin. Chem. Lett.* **2010**, *21* (9), 1037–1040.

(15) Loukaci, A.; Kayser, O.; Bindseil, K.-U.; Siems, K.; Frevert, J.; Abreu, P. M. *J. Nat. Prod.* **2000**, *63* (1), 52–56.

(16) Takashima, J.; Wataya, Y. JP 11228408, 1999.

(17) Wang, Y.; Zhang, L.; Li, G.-T.; Li, Z.-H.; Dong, Z.-J.; Li, Y.; Liu, J.-K. *Nat. Prod. Bioprospecting* **2015**, *5* (1), 47–53.

(18) White, T. J.; Bruns, T.; Lee, S.; Taylor, J. In *PCR Protocols*; Elsevier, 1990; pp 315–322.

(19) Phainuphong, P.; Rukachaisirikul, V.; Saithong, S.; Phongpaichit, S.; Bowornwiriyapan, K.; Muanprasat, C.; Srimaroeng, C.; Duangjai, A.; Sakayaroj, J. *J. Nat. Prod.* **2016**, *79* (6), 1500–1507.

(20) Sheldrick, G. M. *Acta Crystallogr. A* **2008**, *64* (1), 112–122.

(21) Sheldrick, G. M. *Acta Crystallogr. Sect. C Struct. Chem.* **2015**, *71* (1), 3–8.

(22) Farrugia, L. J. *J. Appl. Crystallogr.* **2012**, *45* (4), 849–854.

(23) Macrae, C. F.; Bruno, I. J.; Chisholm, J. A.; Edgington, P. R.; McCabe, P.; Pidcock, E.; Rodriguez-Monge, L.; Taylor, R.; van de Streek, J.; Wood, P. A. *J. Appl. Crystallogr.* **2008**, *41* (2), 466–470.

(24) Desjardins, R. E.; Canfield, C. J.; Haynes, J. D.; Chulay, J. D. *Antimicrob. Agents Chemother.* **1979**, *16* (6), 710–718.

(25) Hunt, L.; Jordan, M.; De Jesus, M.; Wurm, F. M. *Biotechnol. Bioeng.* **1999**, *65* (2), 201–205.

(26) O'Brien, J.; Wilson, I.; Orton, T.; Pognan, F. *Eur. J. Biochem.* **2000**, *267* (17), 5421–5426.

(27) Phongpaichit, S.; Rungjindamai, N.; Rukachaisirikul, V.; Sakayaroj, J. *FEMS Immunol. Med. Microbiol.* **2006**, *48* (3), 367–372.

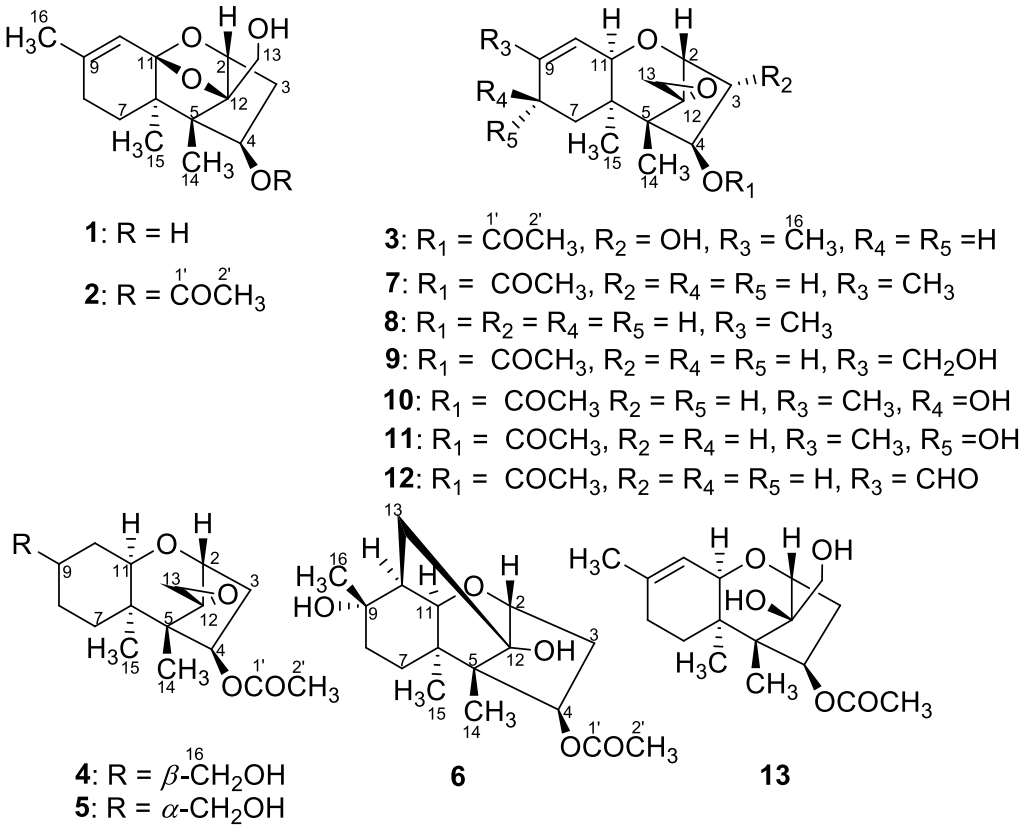

Figure 1. Structures of compounds **1**–**13** isolated from *Trichoderma brevicompactum* PSU-RSPG27.

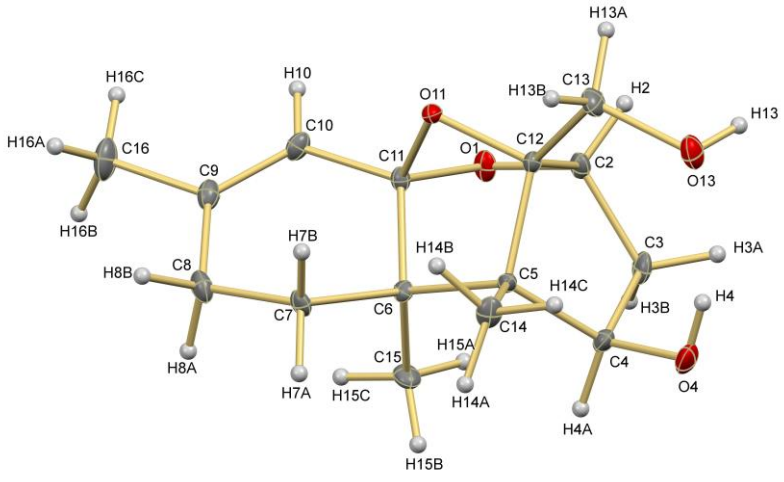

Figure 2. X-ray crystallographic data of compound **1**.

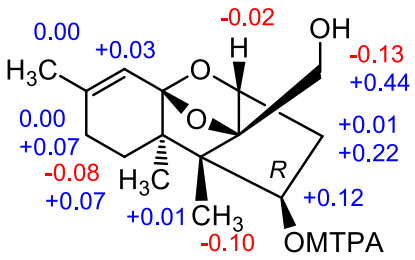

Figure 3. $\Delta\delta$ (δ_S - δ_R) values for (*S*)- and (*R*)-MTPA esters of compound **1**.

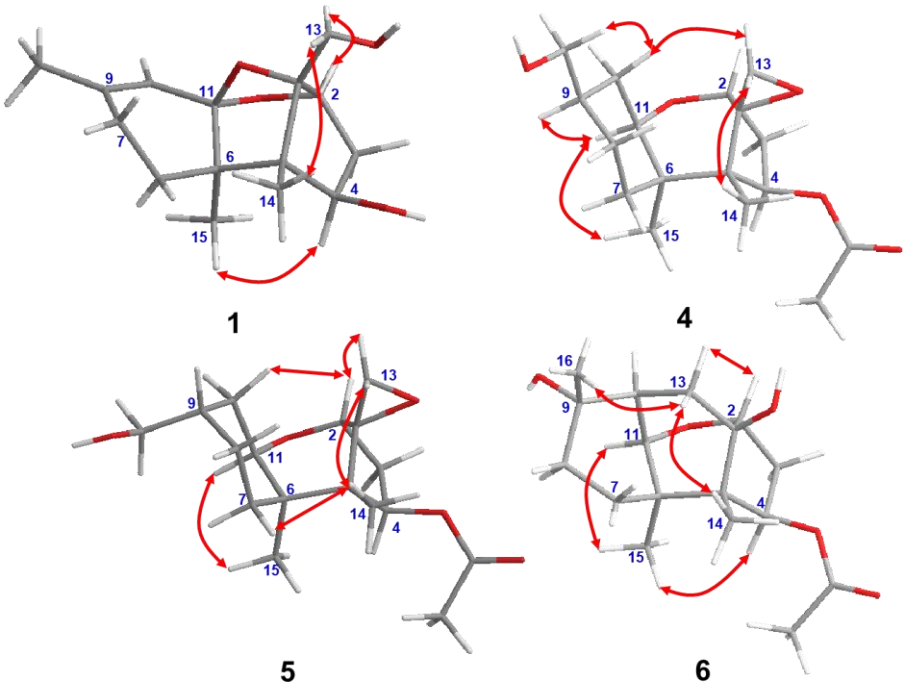

Figure 4. Key NOEDIFF data (⇀↽) of compounds **1, 4, 5** and **6**.

Table 1. The ^{1}H NMR data of trichothecenes **1-6** [$CDCl_3$, δ (ppm), mult (*J* in Hz)]

Position	**1**[a]	**2**[a]	**3**[b]	**4**[a]	**5**[b]	**6**[b]
2	4.20, d (5.4)	4.35, d (5.4)	3.70, d (5.0)	3.83, d (5.0)	3.82, d (5.0)	4.01, d (4.5)
3	a: 2.56, dd (16.5, 7.5)	a: 2.61, dd (16.8, 7.8)	4.19, dd (5.0, 3.0)	a: 2.46, dd (15.5, 8.0)	a: 2.48, dd (15.5, 7.0)	a: 2.32, dd (16.0, 7.5)
	b: 1.71, m	b: 1.85, m		b: 1.96, m	b: 1.97, ddd (15.5, 9.0, 4.0)	b: 1.94, m
4	4.08, brd (6.9)	5.29, dd (7.8, 1.8)	5.00, d (3.0)	5.46, dd (8.0, 3.5)	5.47, dd (8.0, 3.5)	5.52, d (7.0)
7	a: 2.15, m	a: 2.05, m	a: 2.00, m	a: 2.03, m	a: 1.88, td (13.0, 4.0)	a: 1.76, td (14.5, 5.0)
	b: 1.96, m	b: 1.96, m	b: 1.45, m	b: 1.20, m	b: 1.42, brd (14.0)	b: 1.33, dd (9.5, 5.0)
8	a: 1.85, ddd (12.9, 5.2, 1.2)	a: 1.90, m	a: 2.01, m	a: 1.68, m	a: 1.70, m	a: 1.70, m
	b: 1.43, ddd (12.9, 5.2, 1.2)	b: 1.45, dd (12.9, 3.9)	b: 1.45, m	b: 1.62, m	b: 1.43, m	b: 1.51, dd (15.0 5.0)
9				1.85, m	1.80, m	
10	5.40, s	5.44, s	5.52, d (5.0)	a: 1.97, m	a: 1.73, m	2.10, m
				b: 1.73, m	b: 1.17, ddd (15.5, 8.5, 3.5)	
11			3.98, d (5.0)	3.48, m	3.53, m	3.48, d (3.5)

13	a: 3.99, d (10.5)	a: 4.05, d (12.9)	a: 3.06, d (3.5)	a: 3.13, d (4.0)	a: 3.15, d (4.5)	a: 1.91, m
	b: 3.93, d (10.5)	b: 3.84, d (12.9)	b: 2.79, d (3.5)	b: 2.87, d (4.0)	b: 2.86, d (4.5)	b: 1.44, dd (14.5, 5.5)
14	0.89, s	0.91, s	0.78, s	0.68, s	0.69, s	1.02, s
15	1.05, s	0.97, s	0.92, s	1.05, s	1.01, s	1.14, s
16	1.76, s	1.78, s	1.73, s	a: 3.66, dd (8.5, 2.5)	a: 3.49, dd (10.5, 6.0)	1.23, s
				b: 3.65, dd (8.5, 2.5)	b: 3.44, dd (10.5, 6.0)	
2′		2.06, s	2.15, s	2.07, s	2.07, s	2.07, s

[a] Record in 300 MHz

[b] Record in 500 MHz

Table 2. The ^{13}C NMR data of trichothecenes **1-6** [$CDCl_3$, δ (ppm)].

Position	**1**[a]	**2**[a]	**3**[b]	**4**[a]	**5**[b]	**6**[b]
2	82.7, CH	79.5, CH	118.9, CH	79.4, CH	79.5, CH	80.6, CH
3	42.0, CH_2	38.5, CH_2	78.6, CH	36.6, CH_2	36.8, CH_2	40.7, CH_2
4	75.6, CH	78.1, CH	84.6, CH	74.8, CH	74.9, CH	80.5, CH
5	47.8, C	53.1, C	49.0, C	49.5, C	49.4, C	51.8, C
6	54.5, C	48.7, C	41.4, C	41.3, C	41.4, C	40.6, C
7	28.9, CH_2	28.9, CH_2	28.1, CH_2	24.2, CH_2	28.1, CH_2	32.8, CH_2
8	30.7, CH_2	30.5, CH_2	24.6, CH_2	22.8, CH_2	24.7, CH_2	32.1, CH_2
9	145.6, C	145.6, C	139.8, C	32.7, CH	34.6, CH	74.1, C
10	116.2, CH	116.2, CH	118.9, CH	28.5, CH_2	30.2, CH	44.7, CH
11	107.9, C	107.7, C	71.6, CH	71.8, CH	72.3, CH	73.7, CH
12	94.7, C	95.7, C	64.6, C	65.4, C	65.6, C	78.6, C
13	59.5, CH_2	59.2, CH_2	47.2, CH_2	48.3, CH_2	48.5, CH_2	28.0, CH_2
14	10.6, CH_3	10.2, CH_3	6.0, CH_3	5.4, CH_3	5.6, CH_3	8.4, CH_3
15	14.4, CH_3	14.3, CH_3	16.1, CH_3	17.6, CH_3	17.7, CH_3	25.5, CH_3
16	23.0, CH_3	22.9, CH_3	23.3, CH_3	66.8, CH_2	68.1, CH_2	28.1, CH^3
1′		170.0, C	172.6, C	171.0, C	171.0, C	169.6, C
2′		21.1, CH_3	21.1, CH_3	21.1, CH_3	21.1, CH_3	21.2, CH_3

[a] Record in 75 MHz

[b] Record in 125 MHz

Table 3 Biological activities for compounds **1**, **4**, **7-9** and **12**.

Compounds	Antifungal[a] (MIC, μg/mL)			Antimalarial IC_{50} (μM)	Cytotoxicity IC_{50} (μM)	
	CA	**CN**	**MG**	***P. falciparum***	**KB**	**Vero**
1	16	16	IN[b]	7.4	44.7	15.3
4	IN[b]	IN[b]	IN[b]	IN[b]	116.2	45.9
7	1	4	2	0.1	2.4	0.4
8	16	16	IN[b]	7.1	16.1	6.9
9	64	64	IN[b]	8.8	20.6	7.8
12	2	4	IN[b]	9.6	3.7	9.9
Amphotericin B	0.5	0.25				
Miconazole			1			
Dihydroartemisinine				2.2[c]		
Ellipticine					8.2	4.1

[a]CA = *Candida albicans* NCPF3153, CN = *Cryptococcus neoformans* ATCC90113 flucytosine-resistant and MG = *Microsporum gypseum* clinical isolate. [b]IN = Inactive. [c]nM.

ABSTRACT GRAPHIC