รายงานวิจัยฉบับสมบูรณ์

โครงการ สารเมทาบอไลท์ทุติยภูมิที่แสดงฤทธิ์ทางชีวภาพจากราดิน *Botryosphaeria rhodina* NSTRU-PN1.4 และ *Trichoderma virens* NSTRU-PP3.8

(Bioactive Secondary Metabolites from the Soil Fungi *Botryosphaeria rhodina* NSTRU-PN1.4 and *Trichoderma virens* NSTRU-PP3.8)

โดย

ดร.จิราพร อรุณพานิชเลิศ

กันยายน 2563

สัญญาเลขที่ MRG5980088

รายงานวิจัยฉบับสมบูรณ์

โครงการ สารเมทาบอไลท์ทุติยภูมิที่แสดงฤทธิ์ทางชีวภาพจากราดิน *Botryosphaeria rhodina* NSTRU-PN1.4 และ *Trichoderma virens* NSTRU-PP3.8

(Bioactive Secondary Metabolites from the Soil Fungi *Botryosphaeria rhodina* NSTRU-PN1.4 and *Trichoderma virens* NSTRU-PP3.8)

ดร.จิราพร อรุณพานิชเลิศ มหาวิทยาลัยธรรมศาสตร์

สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษาและสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกอ. และ สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

รหัสโครงการ: MRG5980088

ชื่อโครงการ: สารเมทาบอไลท์ทุติยภูมิที่แสดงฤทธิ์ทางชีวภาพจากราดิน *Botryosphaeria rhodina* NSTRU-PN1.4 และ *Trichoderma virens* NSTRU-PP3.8

ชื่อนักวิจัย: ดร.จิราพร อรุณพานิชเลิศ

สถาบัน: มหาวิทยาลัยธรรมศาสตร์

อีเมล์: jira550a@gmail.com

ระยะเวลาโครงการ: 2 ปี (2 พฤษภาคม พ.ศ. 2559 ถึง 1 พฤษภาคม พ.ศ. 2561)

Project Code: MRG5980088

Project Title: Bioactive Secondary Metabolites from the Soil Fungi *Botryosphaeria rhodina* NSTRU-PN1.4 and *Trichoderma virens* NSTRU-PP3.8

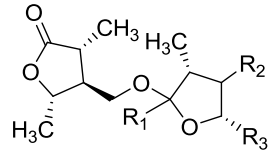
Investigator: Dr. Jiraporn Arunpanichlert, Thammasat University

E-mail Address: jira550a@gmail.com

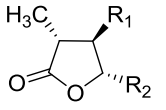
Project Period: 2 years (May 2, 2016 – May 1, 2018)

บทคัดย่อ

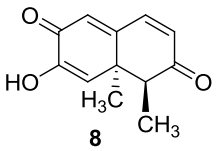
งานวิจัยนี้ศึกษาสารเมทาบอไลท์ทุติยภูมิที่แสดงฤทธิ์ทางชีวภาพจากราดิน *Botryosphaeria rhodina* NSTRU-PN1.4 และ *Trichoderma virens* NSTRU-PP3.8 โดยทำการแยกส่วนสกัดหยาบจากราดินทั้งสองชนิดด้วยเทคนิคทางโครมาโทกราฟีแบบต่างๆ ได้สารบริสุทธิ์ทั้งหมด 19 สาร โดยเป็นสารใหม่จำนวน 3 สาร (**4-5** และ **13**) และสารที่มีการรายงานโครงสร้างแล้วจำนวน 16 สาร วิเคราะห์โครงสร้างของสารบริสุทธิ์ด้วยข้อมูลทางสเปกโทรสโกปี นำสารที่แยกได้ซึ่งมีปริมาณเพียงพอมาทำการทดสอบฤทธิ์ทางชีวภาพ โดยนำ (3*R*,4*R*)-4-acetyl-3-methyl-2(3*H*)-dihydrofuranone (**7**) ที่แยกได้จากเชื้อรา *B. rhodina* NSTRU-PN1.4 มาทดสอบฤทธิ์ต้านเชื้อแบคทีเรียและต้านเชื้อรา พบว่า สาร **7** แสดงฤทธิ์ต้านเชื้อรา *Cryptococcus neoformans* ATCC90113 ในระดับที่ต่ำด้วยค่า MIC 200 μg/mL นอกจากนี้พบว่าสารดังกล่าวไม่แสดงฤทธิ์ต้านเชื้อมาลาเรีย (*Plasmodium falciparum*) และต้านเซลล์มะเร็ง (KB cell lines) สำหรับสาร **14-15** และ **17** ซึ่งแยกได้จากเชื้อรา *T. virens* NSTRU-PP3.8 ถูกนำมาทดสอบฤทธิ์ต้านเชื้อแบคทีเรีย ต้านเชื้อรา ต้านเซลล์มะเร็งเต้านม ต้านเซลล์มะเร็งลำไส้ใหญ่ และต้านการอักเสบ พบว่าสารทั้งสามชนิดไม่แสดงฤทธิ์ต่อการทดสอบทั้งหมดข้างต้น



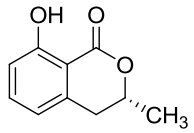
1: R_1 = α-H, R_2 = β-CH_2OH, R_3 = CH_3
2: R_1 = β-H, R_2 = β-CH_2OH, R_3 = CH_3
3: R_1 = α-H, R_2 = β-$COCH_3$, R_3 = H
4: R_1 = α-H, R_2 = α-$COCH_3$, R_3 = H
5: R_1 = β-H, R_2 = α-$COCH_3$, R_3 = H



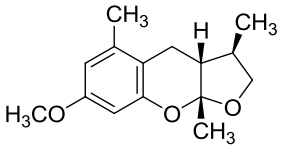
6: R_1 = CH_2OH, R_2 = CH_3
7: R_1 = $COCH_3$, R_2 = H



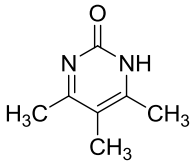
8



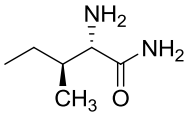
9



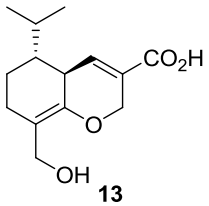
10



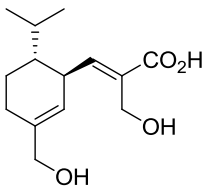
11



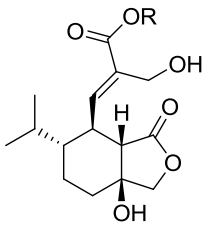
12



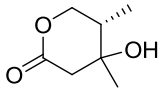
13



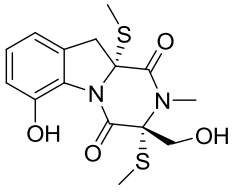
14



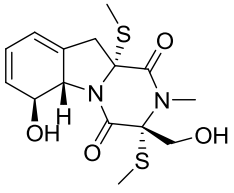
15: R = H
16: R = CH_3



17



18

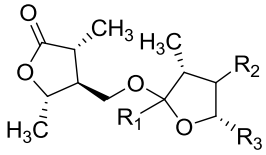


19

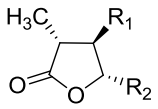
คำหลัก : ราดิน *Botryosphaeria rhodina Trichoderma virens* ฤทธิ์ต้านเชื้อรา

Abstract

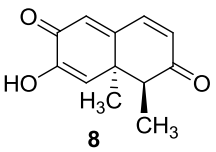
This research project involved investigation of bioactive secondary metabolites from the soil fungi *Botryosphaeria rhodina* NSTRU-PN1.4 and *Trichoderma virens* NSTRU-PP3.8. Purification of the crude extracts by various chromatographic techniques resulted in the isolation of nineteen compounds including three new metabolites (**4**-**5** and **13**) as well as sixteen known ones. Their structures were elucidated by extensive spectroscopic analysis. The isolated compounds with sufficient amount were tested for biological activities. (3*R*,4*R*)-4-acetyl-3-methyl-2(3*H*)-dihydrofuranone (**7**) isolated from *B. rhodina* NSTRU-PN1.4 was tested for antibacterial and antifungal activities. It exhibited weak antifungal activity against *Cryptococcus neoformans* ATCC90113 with the MIC value of 200 μg/mL. In addition, **7** was evaluated for antimalarial (*Plasmodium falciparum*) and anticancer (KB cell lines) activities. However, it was inactive against both assays. Compounds **14**-**15** and **17** obtained from *T. virens* NSTRU-PP3.8 were evaluated for antibacterial, antifungal, anti-breast cancer (MDA-MB-231 cells), anti-colorectal cancer and anti β-cell inflammation activities. Unfortunately, they were inactive against all assays.



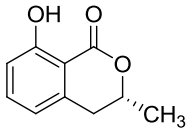
1: R_1 = α-H, R_2 = β-CH_2OH, R_3 = CH_3
2: R_1 = β-H, R_2 = β-CH_2OH, R_3 = CH_3
3: R_1 = α-H, R_2 = β-$COCH_3$, R_3 = H
4: R_1 = α-H, R_2 = α-$COCH_3$, R_3 = H
5: R_1 = β-H, R_2 = α-$COCH_3$, R_3 = H



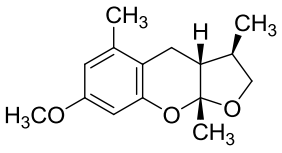
6: R_1 = CH_2OH, R_2 = CH_3
7: R_1 = $COCH_3$, R_2 = H



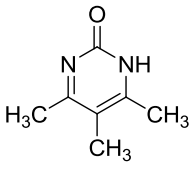
8



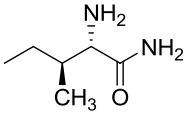
9



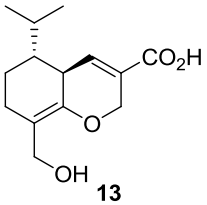
10



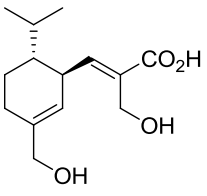
11



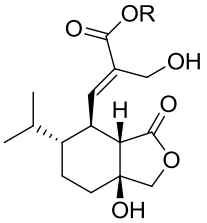
12



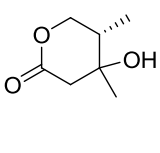
13



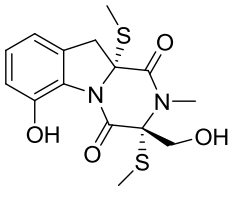
14



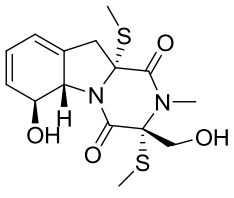
15: R = H
16: R = CH_3



17



18



19

Keywords: Soil fungi, *Botryosphaeria rhodina*, *Trichoderma virens*, Antifungal activity

Executive summary

This research project involved investigation of bioactive secondary metabolites from the soil fungi *Botryosphaeria rhodina* NSTRU-PN1.4 and *Trichoderma virens* NSTRU-PP3.8 isolated from a soil sample collected from Paknakhon mangrove in Nakhon Si Thammarat Province, Thailand. Preliminary investigation revealed that the extracts from the fungi NSTRU-PN1.4 and NSTRU-PP3.8 selectively displayed antimalarial and antimycobacterial activities, respectively. Both extracts were weakly active against noncancerous (Vero) cell lines. Accordingly, the crude extracts were purified using various chromatographic techniques. The structures of pure compounds were elucidated by the extensive spectroscopic methods including UV, IR, NMR and MS. The relative configuration was assigned according to NOEDIFF data and/or coupling constants while the absolute configuration was determined by comparison of the specific rotation or ECD data with those of the known or structurally related compounds. The isolated compounds with sufficient amount were tested for antibacterial, antifungal, anticancer, antimalarial and anti β-cell inflammation activities.

Twelve metabolites including five dimeric γ-lactones, two new botryosphaerilactones D and E (**4** and **5**) and three known botryosphaerilactones A (**1**), B (**3**) and C (**2**), as well as seven known compounds (**6**-**12**) were isolated from *B. rhodina* NSTRU-PN1.4. Known (3*R*,4*R*)-4-acetyl-3-methyl-2(3*H*)-dihydrofuranone (**7**) with sufficient amount was tested for antimicrobial activity against *Staphylococcus aureus* ATCC25923, methicillin-resistant *S. aureus* SK1 (a clinical isolate), *Pseudomonas aeruginosa* ATCC27853, *Escherichia coli* ATCC25922, *Candida albicans* NCPF3153, *Cryptococcus neoformans* ATCC90113, *Microsporum gypseum* and *Penicillium marnefeii* clinical isolate. It exhibited weak antifungal activity against *C. neoformans* ATCC90113 with the MIC value of 200 μg/mL. In addition, **7** was evaluated for antimalarial (*Plasmodium falciparum*) and anticancer (KB cell lines) activities. It was inactive against both assays.

One new sesquiterpene derivative (**13**) and six known compounds (**14**-**19**) were isolated from *T. virens* NSTRU-PP3.8. Compounds **14**-**15** and **17** were tested for antibacterial (against *S. aureus*, methicillin-resistant *S. aureus*, *P. aeruginosa*, *E. coli*, *Acinetobacter baumannii* NPRC005 and *A. baumannii* NPRC007), antifungal (against *C. albicans* ATCC90028, *C. albicans* NCPF3153, *C. neoformans* ATCC90112, *C. neoformans* ATCC90113, *M. gypeum* and *Talaromyces marneffei* PSU-SKH1), anti-breast cancer (MDA-MB-231 cells), anti-colorectal cancer and anti β-cell inflammation activities. Unfortunately, they were inactive against all assays.

Research Project

Project title: Bioactive secondary metabolites from the soil fungi *Botryosphaeria rhodina* NSTRU-PN1.4 and *Trichoderma virens* NSTRU-PP3.8

Objectives

- To isolate secondary metabolites from two soil fungi, *B. rhodina* NSTRU-PN1.4 and *T. virens* NSTRU-PP3.8.

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

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

Scope of research

Isolation of secondary metabolites from *B. rhodina* NSTRU-PN1.4 and *T. virens* NSTRU-PP3.8, structural determination of isolated compounds and evaluation for their antibacterial, antifungal, anticancer, antimalarial and anti β-cell inflammation activities.

Methodology

1. Fermentation

The fungi NSTRU-PN1.4 and NSTRU-PP3.8 were grown on potato dextrose agar (PDA) at 25^{o}C for 5 days. Five pieces (0.5 x 0.5 cm^{2}) of mycelial agar plugs were inoculated into 50 flasks of 500 mL Erlenmeyer flasks containing 300 mL of potato dextrose broth (PDB) 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 Na_2SO_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 a 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 a mycelial ethyl acetate extract.

- Each crude extract was fractionated by column chromatography over Sephadex-LH20. 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 data, including UV, IR, NMR and MS and/or X-ray diffraction. The absolute configuration of isolated compounds was determined by comparison of the specific rotation and/or ECD data with those of the known or structurally related compounds.

4. Bioassays

- The crude extracts were tested for antibacterial (against *S. aureus* ATCC25923, methicillin-resistance *S. aureus*, *P. aeruginosa* ATCC27853 and *E. coli* ATCC25922), antifungal (against *C. albicans* NCPF3153, *C. neoformans* ATCC90113, *M. gypseum* clinical isolate and *P. marnefeii* clinical isolate), anticancer (oral human carcinoma cells (KB) and human breast cancer cells (MCF-7)), cytotoxic (Vero cells), antimalarial (against *P. falciparum*) and antimycobacterial (against *M. tuberculosis* strain H_{37}Ra strain) activities.

- The isolated compounds with sufficient amount were tested for antibacterial (against *S. aureus* ATCC25923, methicillin-resistance *S. aureus*, *P. aeruginosa* ATCC27853, *E. coli* ATCC25922, *A. baumannii* NPRC005 and *A. baumannii* NPRC007), antifungal (against *C. albicans* NCPF3153, *C. albicans* ATCC90028, *C. neoformans* ATCC90112, *C. neoformans* ATCC90113, *M. gypseum* clinical isolate, *P. marnefeii* clinical isolate and *T. marneffei* PSU-SKH1), anticancer, antimalarial and anti β-cell inflammation activities.

- The antibacterial and antifungal activities were performed by Prof. Souwalak Phongpaichit, Division of Biological Science, Faculty of Science, Prince of Songkla University. Anticancer (KB and MCF-7), cytotoxic (Vero cells), antimalarial and antimycobacterial activities were performed at the National Center for Genetic Engineering and Biotechnology (BIOTEC) while anti-breast cancer (MDA-MB-231 cells), anti-colorectal cancer and anti β-cell inflammation activities were carried out at the Excellent Center for Drug Discovery.

4.1 Antimicrobial activity

Antimicrobial activity was determined as described by the Clinical and Laboratory Standards Institute (Drummond and Waigh 2000; Clinical and Laboratory Standards Institute 2002a, 2002b, 2002c). Vancomycin and gentamicin were used as positive controls for bacteria while amphotericin B and clotrimazole were positive control drugs for yeasts and fungus, respectively.

4.2 Cytotoxic and anticancer activities

Cytotoxic assay against Vero cells was performed employing the colorimetric method (Hunt et al., 1999), while the activities against human oral cavity (KB) and human breast (MCF7) 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 whereas those for MCF-7 cell lines were tamoxifen and doxorubicin. Anti-breast cancer (MDA-MB-231 cells) and anti-colorectal cancer

activities were carried out using the MTT (colorimetric assay) (Zhang et al., 1999). Doxorubicin was used as a positive control.

4.3 Antimalarial activity

Antimalarial activity was performed in vitro against the parasite *P. falciparum* (K1, multidrug resistant strain) using the microculture radioisotope technique (Desjardins et al., 1979). Dihydroartemisinine and mefloquine were used as standard compounds.

4.4 Antimycobacterial activity

Antimycobacterial activity was determined against *M. tuberculosis* $H_{37}Ra$ strain using green fluorescent protein (GFP)-based fluorescent detection (Changsen et al., 2003). Rifampicin was used as a positive control.

4.5 Anti β-cell inflammation activity

In anti β-cell inflammation assay, the trafficking of NF-κB from cytoplasm (Normal cells) to nucleus (Inflammatory cells) was used as a major parameter for detection and analysis by using high-content imaging system (Zhang et al., 1999).

Results and Discussions

Soil fungi *B. rhodina* NSTRU-PN1.4 and *T. virens* NSTRU-PP3.8 were isolated from soil in mangroves collected in Nakhon Si Thammarat Province, Thailand. The broth ethyl acetate extracts of the fungi NSTRU-PN1.4 and both broth and mycelia ethyl acetate extracts of NSTRU-PP3.8 were selectively active against *P. falciparum* and *M. tuberculosis*, respectively, with low cytotoxic activity to noncancerous (Vero) cells (**Table 1**).

Table 1 The biological activities of the crude extracts from *B. rhodina* NSTRU-PN1.4 and *T. virens* NSTRU-PP3.8

Fungi/ standard drugs	Crude extracts[1]	Antimicrobial activity[2] (MIC, μg/mL)								Antimalarial (IC_{50}, μg/mL)	Antimycobacterial (MIC, μg/mL)	Cytotoxic (IC_{50}, μg/mL)		
		SA	MRSA	PA	EC	CA	CN	MG	PM	*P. falciparum*[3]	*M. tuberculosis*[4]	KB[5]	MCF-7[6]	Vero[7]
NSTRU-PN1.4	BE	IN	IN	IN	IN	IN	IN	IN	IN	5.21	IN	IN	IN	24.87
	CE	IN	IN	IN	IN	IN	IN	IN	IN	IN	IN	IN	IN	IN
NSTRU-PP3.8	BE	IN	IN	IN	IN	IN	IN	IN	IN	IN	25.00	IN	IN	36.89
	CE	IN	IN	IN	IN	IN	IN	IN	IN	IN	25.00	IN	IN	48.82
Vancomycin		0.5	1.0											
Gentamicin				0.25	0.5									
Amphotericin B						0.25	0.12		2.0					
Clotrimazole								0.5						
Dihydroartemisinine										2.32[8]				
Rifampicin											0.0125			
Ellipticine												1.18[9]		0.73[9]
Tamoxifen													9.53[9]	

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

[2]SA = *Staphylococcus aureus* ATCC25923, MRSA = Methicillin-resistant *Staphylococcus aureus*, PA = *Pseudomonas aeruginosa* ATCC27853, EC = *Escherichia coli* ATCC25922, CA = *Candida albicans* NCPF3153, CN90113 = *Cryptococcus neoformans* ATCC90113, MG = *Microsporum gypseum* clinical isolate, PM = *Penicillium marnefeii* clinical isolate

[3]*P. falciparum* = *Plasmodium falciparum* (K1, multidrug resistant strain)

[4]*M. tuberculosis* = *Mycobacterium tuberculosis* strain $H_{37}Ra$ strain

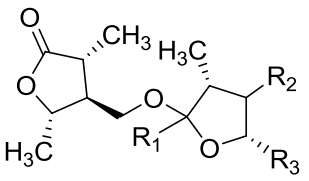
[5]KB = Oral human carcinoma cell lines, [6]MCF-7 = Human breast cancer cell lines

[7]Vero = African green monkey kidney fibroblasts cell lines

[8]nM, [9]μM, IN = Inactive

Botryosphaeria rhodina **NSTRU-PN1.4**

The isolation and characterization of secondary metabolites from the broth extract of the fungus NSTRU-PN1.4 were described in Appendix I. Purification of the broth EtOAc extract led to the isolation of twelve metabolites including five dimeric γ-lactones, two new botryosphaerilactones D and E (**4** and **5**) and three known botryosphaerilactones A (**1**), B (**3**) and C (**2**), as well as seven known compounds, (3*R*,4*S*,5*S*)-dihydro-4-(hydroxymethyl)-3,5-dimethyl-2(3*H*)-furanone (**6**) (Ravi et al. 1979; Andolfi et al. 2014), (3*R*,4*R*)-4-acetyl-3-methyl-2(3*H*)-dihydrofuranone (**7**) (Forzato et al. 2005, 2007), botryosphaeridione (**8**) (Rukachaisirikul et al. 2009), (*R*)-(—)-mellein (**9**) (Rukachaisirikul et al. 2009), *O*-methyl alboatrin (**10**) (Biswas et al. 2008), 4,5,6-trimethyl-2(1*H*)-pyrimidinone (**11**) (Lee et al. 1984) and L-isoleucinamide (**12**) (Smith et al. 1952) (**Figure 1**).



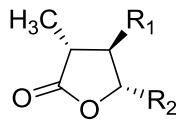
1: R_1 = α-H, R_2 = β-CH_2OH, R_3 = CH_3

2: R_1 = β-H, R_2 = β-CH_2OH, R_3 = CH_3

3: R_1 = α-H, R_2 = β-$COCH_3$, R_3 = H

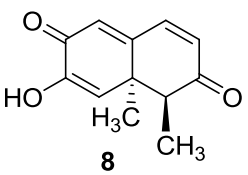
4: R_1 = α-H, R_2 = α-$COCH_3$, R_3 = H

5: R_1 = β-H, R_2 = α-$COCH_3$, R_3 = H

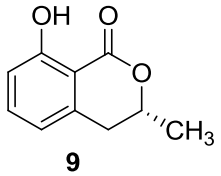


6: R_1 = CH_2OH, R_2 = CH_3

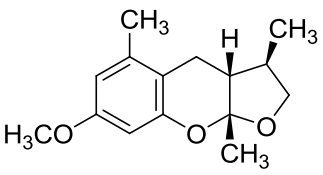
7: R_1 = $COCH_3$, R_2 = H



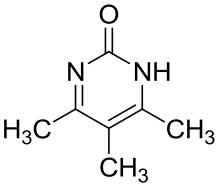
8



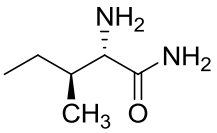
9



10



11



12

Figure 1. Structures of compounds **1-12** isolated from *Botryosphaeria rhodina* NSTRU-PN1.4.

The isolated compound **7** with sufficient amount was tested for antimicrobial activity against *S. aureus* ATCC25923, methicillin-resistant *S. aureus* SK1 (a clinical isolate), *P. aeruginosa* ATCC27853, *E. coli* ATCC25922, *C. albicans* NCPF3153, *C. neoformans* ATCC90113, *M. gypseum* and *P. marnefeii* clinical isolate. It exhibited weak antifungal activity against *C. neoformans* ATCC90113 with the MIC value of 200 μg/mL. In addition, **7** was evaluated for antimalarial (*P. falciparum*) and anticancer (KB cell lines) activities. It was inactive against both assays.

***Trichoderma virens* NSTRU-PP3.8**

The isolation and characterization of secondary metabolites from the broth and mycelial extracts of the fungus NSTRU-PP3.8 were described in Appendix II. Chromatographic separation of the crude extracts from *T. virens* NSTRU-PP3.8 led to the isolation of one new sesquiterpene derivative (**13**) and six known compounds which were identified as gliocladic acid (**14**) (Rukachaisirikul et al., 2019), hydroheptelidic acid (**15**) (Liu et al., 2018), methylhydroheptelidate (**16**) (Calhoun et al., 1992), *β*,*γ*-dimethyl-*β*-hydroxy-*δ*-valerolactone (**17**) (Tamura and Takai, 1957) bisdethiobis(methylthio)dehydrogliotoxin (**18**) (Luo et al., 2017) and bisdethiobis(methylthio)gliotoxin (**19**) (Luo et al., 2017) (**Figure 2**).

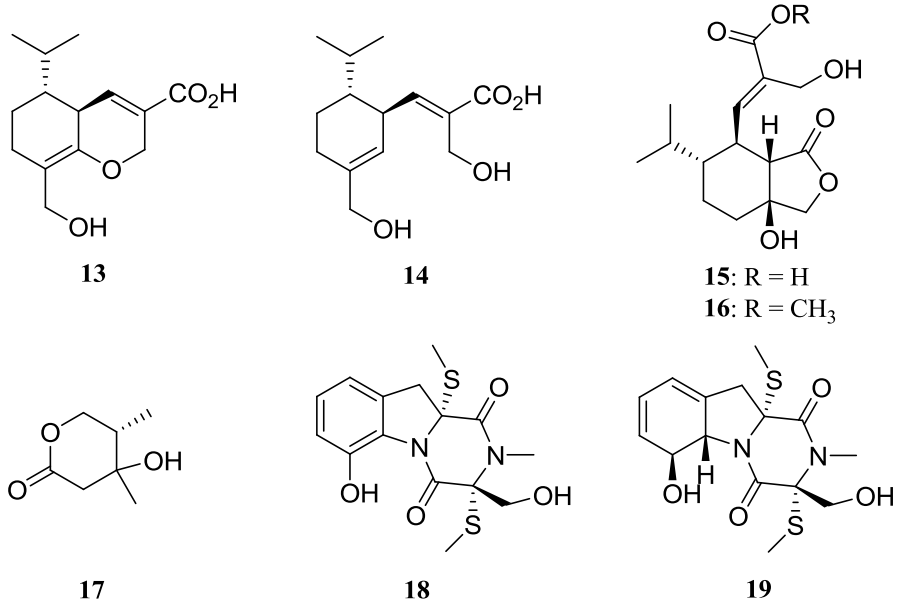


Figure 2. Structures of compounds **13-19** isolated from *Trichoderma virens* NSTRU-PP3.8.

The isolated compounds **14-15** and **17** were tested for antibacterial (against *S. aureus* ATCC25923, methicillin-resistant *S. aureus* SK1, *P. aeruginosa* ATCC27853, *E. coli* ATCC25922, *A. baumannii* NPRC005 and *A. baumannii* NPRC007), antifungal (against *C. albicans* ATCC90028, *C. albicans* NCPF3153, *C. neoformans* ATCC90112, *C. neoformans* ATCC90113, *M. gypeum* SK-MU4

and *T. marneffei* PSU-SKH1), anti-breast cancer (MDA-MB-231 cells), anti-colorectal cancer and anti β-cell inflammation activities. Unfortunately, they were inactive against all assays.

References

Andolfi A, Maddau L, Cimmino A, Linaldeddu BT, Basso S, Deidda A, Serra S, Evidente A. 2014. Lasiojasmonates A-C, three jasmonic acid esters produced by *Lasiodiplodia* sp., a grapevine pathogen. Phytochemistry. 103:145-153.

Biswas B, Sarkar D, Venkateswaran RV. 2008. Total synthesis of alboatrin, a phytotoxic metabolite from *Verticillium alboatrum*. Tetrahedron. 64:3212-3216.

Calhoun LA, Findlay JA, Miller JD, Whitney NJ. 1992. Metabolites toxic to spruce budworm from balsam fir needle endopytes. Mycol Res. 14:281-286.

Changsen C, Franzblau SG, Palittapongarnpim P. 2003. Improved green fluorescent protein reporter gene-based microplate screening for antituberculosis compounds by utilizing an acetamidase promoter. Antimicrob Agents Chemother. 47:3682-3687.

Clinical and Laboratory Standards Institute (CLSI). 2002a. Reference method for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved standard M7-A4. Wayne, PA: Clinical and Laboratory Standards Institute.

Clinical and Laboratory Standards Institute (CLSI). 2002b. Reference method for broth dilution antifungal susceptibility testing of yeasts; Approved Standard M27-A2. 2nd ed. Wayne, PA: Clinical and Laboratory Standards Institute.

Clinical and Laboratory Standards Institute (CLSI). 2002c. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; Approved Standard. CLSI Documents M38-A. Wayne, PA: Clinical and Laboratory Standards Institute.

Desjardins RE, Canfield CJ, Haynes JD, Chulay JD. 1979. Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. Antimicrob Agents Chemother. 16:710-718.

Drummond AJ, Waigh RD. 2000. Recent research developments in phytochemistry. 4:143-152.

Forzato C, Furlan G, Ghelfi F, Nitti P, Pitacco G, Roncaglia F, Valentin E. 2007. Lipase-catalysed deacetylation of botryodiplodin acetate. Tetrahedron Asymmetry. 18:447-450.

Forzato C, Furlan G, Nitti P, Pitacco G, Marchesan D, Coriani S, Valentin E. 2005. A combined experimental and computational strategy in the assignment of absolute configurations of 4-methyl-5-oxo-tetrahydrofuran-3-carboxylic acids and their esters. Tetrahedron Asymmetry. 16:3011-3023.

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

Lee TWS, Rettig SJ, Stewart R, Trotter J. 1984. Six pyrimidonium salts: determination of their crystal structures and studies of their prototropic behavior in solution. Can J Chem. 62:1194-1202.

Liu L, Han JJ, Xu TS, Liu RX, Bao L, Liu HW. 2018. Three new heptelidic acid derivatives from the culture of mashroom *Lentinellus ursinus*. Nat Products Bioprospect. 8:355-360.

Luo X, Zhou X, Lin X, Qin X, Zhang T, Wang J, Tu Z, Yang B, Liao S, Tian Y, Pang X, Kaliyaperumal K, Li JL, Tao H, Liu Y. 2017. Antituberculosis compounds from a deep-sea-derived fungus *Aspergillus* sp. SCSIO Ind09F01. Nat Prod Res. 31:1958-1962.

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. Eur J Biochem. 267:5421-5426.

Ravi BN, Armstrong RW, Faulkner DJ. 1979. Some aromatic compounds from the marine sponge *Plakortis halichlondriodes*. J Org Chem. 44:3109-3113.

Rukachaisirikul V, Arunpanichlert J, Sukpondma Y, Phongpaichit S, Sakayaroj J. 2009. Metabolites from the endophytic fungi *Botryosphaeria rhodina* PSU-M35 and PSU-M114. Tetrahedron. 65:10590-10595.

Rukachaisirikul V, Chinpha S, Phongpaichit S, Saikhwan N, Sakayaroj J, Preedanon S. 2019. Sesquiterpene and monoterpene derivatives from the soil-derived fungus *Trichoderma reesei* PSU-SPSF013. Phytochem Lett. 30:124-129.

Smith EL, Spackman DH, Polglase WJ. 1952. The specificity of leucine aminopeptidase. III. Action on diastereoisomers. J Biol Chem. 199:801-817.

Tamura S, Takai M. 1957. Synthesis of some homologs of D,L-β-hydroxy-β-methyl-δ-valerolactone. J Agric Chem Soc Jpn. 21:394-395.

Zhang JH, Chung TDY, Oldenberg KR. 1999. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J Biomol Screen. 67-73.

Out put ที่ได้จากโครงการ

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

ผลงานอยู่ระหว่างการพิจารณาตีพิมพ์ในวารสารนานาชาติ ในฐานข้อมูล ISI จำนวน 1 เรื่อง (รายละเอียดตามภาคผนวก ก)

ผู้แต่ง: Jiraporn Arunpanichlert, Vatcharin Rukachaisirikul, Titima Chaiwarin, Yuthana Tantirungrotechai, Nanthaphong Khamthong, Souwalak Phongpaichit, Sumalee Liamthong, Jariya Sakayaroj

ชื่อเรื่อง: Dimeric γ-lactone derivatives from the soil-derived fungus *Lasiodiplodia theobromae* NSTRU-PN1.4

แหล่งพิมพ์: Natural Product Research (IF 2.060, Quartile in Category: Q2)

ปีที่พิมพ์ เล่มที่ หน้า

2. การนำผลงานวิจัยไปใช้ประโยชน์

ด้านวิชาการ

จากการศึกษาสารเมทาบอไลท์ทุติยภูมิจากราดินทั้งสองสายพันธุ์ ทำให้ทราบว่าเชื้อราเหล่านี้สร้างสารหลากหลายชนิดที่น่าสนใจ อีกทั้งยังพบสารใหม่ในกลุ่ม dimeric γ-lactone เป็นครั้งแรกในราดิน โดยองค์ความรู้จากงานวิจัยนี้ สามารถนำสารในกลุ่มที่แยกได้ไปพัฒนาปรับปรุงโครงสร้างเพื่อปรับเปลี่ยนฤทธิ์ทางชีวภาพและใช้ประโยชน์ในเชิงเภสัชวิทยาต่อไป

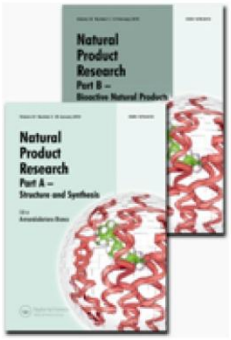
ภาคผนวก ก

(Appendix I)

ผลงานที่อยู่ระหว่างการพิจารณาตีพิมพ์ในวารสารนานาชาติ

ผลงานที่อยู่ระหว่างการพิจารณาตีพิมพ์ในวารสารนานาชาติ

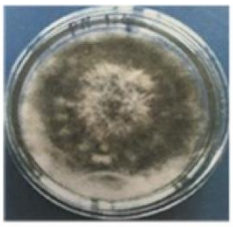
Natural Product Research

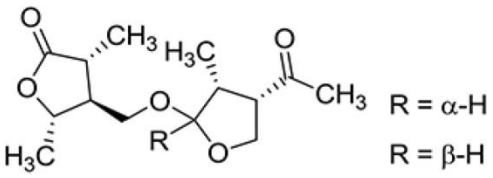


Dimeric γ-lactone derivatives from the soil-derived fungus *Lasiodiplodia theobromae* NSTRU-PN1.4

Journal:	*Natural Product Research*
Manuscript ID	GNPL-2020-2278.R1
Manuscript Type:	Research Article
Date Submitted by the Author:	23-Sep-2020
Complete List of Authors:	Arunpanichlert, Jiraporn; Thammasat University, Chemistry Rukachaisirikul, Vatcharin; Prince of Songkla University, Chemistry Chaiwarin, Titima; Prince of Songkla University Tantirungrotechai, Yuthana; Thammasat University, Chemistry Khamthong, Nanthaphong; Rangsit University Phongpaichit, Souwalak; Prince of Songkla University, Microbiology Liamthong, Sumalee; Nakhon Si Thammarat Rajabhat University Sakayaroj, Jariya; Walailak University
Keywords:	Soil-derived fungus, Lasiodiplodia theobromae, Dimeric γ-lactone, Antifungal activity

SCHOLARONE™
Manuscripts





44x14mm (300 x 300 DPI)

Dimeric γ-lactone derivatives from the soil-derived fungus *Lasiodiplodia theobromae* NSTRU-PN1.4

Jiraporn Arunpanichlert[a,*], Vatcharin Rukachaisirikul[b], Titima Chaiwarin[b], Yuthana Tantirungrotechai[a], Nanthaphong Khamthong[c], Souwalak Phongpaichit[d], Sumalee Liamthong[e] and Jariya Sakayaroj[f]

[a]*Department of Chemistry, Faculty of Science and Technology, Thammasat University, Klong Luang, Pathumthani 12120, Thailand*

[b]*Division of Physical Science and Center of Excellence for Innovation in Chemistry, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand*

[c]*College of Oriental Medicine, Rangsit University, Pathumthani 12000, Thailand*

[d]*Division of Biological Science, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand*

[e]*Department of Biology, Faculty of Science and Technology, Nakhon Si Thammarat Rajabhat University, Nakhon Si Thammarat 80280, Thailand*

[f]*School of Science, Walailak University, Nakhon Si Thammarat 80161, Thailand*

*Corresponding author.

E-mail address: jira_550@tu.ac.th (J. Arunpanichlert).

For Peer Review Only

ABSTRACT

Investigation of the soil-derived fungus *Lasiodiplodia theobromae* NSTRU-PN1.4 resulted in the isolation of five dimeric γ-lactones including two new botryosphaerilactones D and E (**4** and **5**) and three known structurally related analogoues (**1**-**3**) along with seven known compounds. Their structures were elucidated by extensive spectroscopic analysis. The absolute configuration of **1-5** was determined by comparison of the ECD data with those of the structurally related monomeric γ-lactones. For biological evaluation, this is the first report on antifungal activity of the known (3*R*,4*R*)-4-acetyl-3-methyl-2(3*H*)-dihydrofuranone which displayed weak antifungal activity against *Cryptococcus neoformans* with an MIC value of 200 μg/mL.

Keywords: Soil-derived fungus, *Lasiodiplodia theobromae*, Dimeric γ-lactone, Antifungal activity

For Peer Review Only

1. Introduction

Soil-derived fungi have served as an important source of novel and bioactive secondary metabolites, for example, antimalarial radicicol (Tanaka et al. 1998), cytotoxic hamavellones A and B (Isaka et al. 2008), antimycobacterial penicilleremophilane A (Daengrot et al. 2015), antifungal bacilysin (Sansinenea and Ortiz 2011) and antibiotic koninginins A and B (Reino et al. 2008). For the fungus *Lasiodiplodia theobromae*, it has been proven to be a fabulous source of bioactive secondary metabolites, including antibacterial cladospirone B (Kamal et al. 2017), (+)-(*R*)-de-*O*-methyllasiodiplodin (Umeokoli et al. 2019), chloropreussomerins A and B and preussomerins A, F, G and H (Chen et al. 2016). Lasiodiplodin, scytalone and (−)-jasmonic acid showed phytotoxicity (Félix et al. 2018) while cladospirone B and desmethyllasiodiplodin exhibited anti-trypanosomal activity (Kamal et al. 2017). In addition, chloropreussomerins A and B displayed potent cytotoxicity against A549 and MCF-7 human cancer cell lines (Chen et al. 2016). As part of our continuous investigation on chemical and biological diversity of soil-derived fungi, we described herein the isolation of secondary metabolites from *L. theobromae* NSTRU-PN1.4 isolated from soil in mangrove area in Nakhon Si Thammarat Province, Thailand. Purification of the broth EtOAc extract led to the isolation of twelve metabolites, including five dimeric γ-lactones, two new botryosphaerilactones D and E (**4** and **5**) and three known botryosphaerilactones A (**1**), B (**3**) and C (**2**), as well as seven known compounds, (3*R*,4*S*,5*S*)-dihydro-4-(hydroxymethyl)-3,5-dimethyl-2(3*H*)-furanone (**6**) (Ravi et al. 1979; Andolfi et al. 2014), (3*R*,4*R*)-4-acetyl-3-methyl-2(3*H*)-dihydrofuranone (**7**) (Forzato et al. 2005, 2007), botryosphaeridione (**8**) (Rukachaisirikul et al. 2009), (*R*)-(−)-mellein (**9**) (Rukachaisirikul et al. 2009), *O*-methyl alboatrin (**10**) (Biswas et al. 2008), 4,5,6-trimethyl-2(1*H*)-pyrimidinone (**11**) (Lee et al. 1984) and L-isoleucinamide (**12**) (Smith et al. 1952). Additionally, biological activities of **7** which was obtained in sufficient quantity are presented herein.

2. Results and discussion

All metabolites (**1**-**12**) (Figure 1) were purified using chromatographic techniques and their structures were elucidated by analysis of spectroscopic data, including IR, UV, NMR and MS. The relative configuration was assigned according to NOEDIFF data and/or coupling constants while the absolute configuration was determined by comparison of the specific rotation or ECD data with those of the known or structurally related compounds. The absolute configuration of the known dimeric γ-lactones (**1**-**3**) remained unresolved in the

For Peer Review Only

previous studies. We here established the absolute configuration of the dimeric γ-lactones (**1**-**5**) on the basis of the absolute configuration of the monomeric γ-lactones **6** and **7**. The absolute configuration at C-3 of **6** was assigned as *R* configuration according to the ECD spectral data which showed a negative Cotton effect of the n→π* transition of a lactone carbonyl group (Matsumoto et al. 1973; Forzato et al. 2005) at 216 nm ($\Delta\varepsilon$ = -133.37, MeOH), the same sign as that of (−)-(3*R*,3a*S*,6a*S*)-2*H*-hexahydro-3-methylcyclopenta[b]furan -2-one at 215 nm ($\Delta\varepsilon$ = -2173, MeOH) (Forzato et al. 1997). According to the NOEDIFF results together with the established absolute configuration at C-3, the absolute configuration in **6** was thus assigned to be 3*R*, 4*S* and 5*S*. These assigned absolute configurations were confirmed based on the opposite sign of the specific rotation of **6**, $[\alpha]_D^{25}$ = +51.5 (*c* 0.17, MeOH), to that of its enantiomer (3*S*,4*R*,5*R*)-4-hydroxymethyl-3,5-dimethyldihydro-2-furanone, $[\alpha]_D^{25}$ = -18 (*c* 0.3, $CHCl_3$) (Andolfi et al. 2014). For the related monomeric γ-lactone **7**, the absolute configuration at C-3 and C-4 was established to be both *R* based on a mirror-image ECD spectrum of **7**, [216 nm ($\Delta\varepsilon$ = -156.77) and 284 nm ($\Delta\varepsilon$ = +17.88), MeOH], and the opposite sign of the specific rotation, $[\alpha]_D^{24}$ = +81.9 (*c* 0.57, MeOH), to those of its enantiomer, (3*S*,4*S*)-(−)-4-acetyl-3-methyl-2(3*H*)-dihydrofuranone [214 nm ($\Delta\varepsilon$ = +6073) and 281 nm ($\Delta\varepsilon$ = -479), MeOH; $[\alpha]_D^{25}$ = -72.1 (*c* 0.58, MeOH)] (Forzato et al. 2005). The first and second Cotton effects of **7** at λ_{max} 216 and 284 nm were related with the n→π* transition bands of lactone (Matsumoto et al. 1973; Forzato et al. 1997, 2005) and acetyl groups (Forzato et al. 2005, 2007), respectively. It is worth to notify that the substituents at C-3 and C-4 of both **6** and **7** had identical spatial arrangement.

Compound **1** was obtained as a colorless gum with $[\alpha]_D^{24}$ -14.6 (*c* 0.77, MeOH) and the molecular formula $C_{14}H_{24}O_5$ was determined by the HRESIMS that showed the molecular ion at *m/z* 295.1521 $[M+Na]^+$. It exhibited an UV absorption band of a carbonyl chromophore at 286 nm, while the IR absorption bands at 3451 and 1770 cm^{-1} were indicative of the presence of hydroxyl and γ-lactone carbonyl groups (Rukachaisirikul et al. 2009; Andolfi et al. 2014), respectively. The ^1H (Table S1) and ^{13}C NMR (Table S2) spectroscopic data of **1** as well as the ^1H-^1H COSY and HMBC correlations (Figure S1) indicated that **1** had a planar structure which was identical to that of botryosphaerilactones A and C (Rukachaisirikul et al. 2009). Moreover, the following NOEDIFF data of **1** (Figure S2) revealed that **1** and botryosphaerilactone A had an identical relative configuration. Irradiation of H-4 (δ_H 1.91) of the γ-lactone moiety enhanced the signal intensity of H_3-6 (δ_H 1.27) and H_3-8 (δ_H 1.43),

For Peer Review Only

indicating their *cis*-relationship which was identical to that of **6**. In the furan unit, signal enhancement of H-2′ (δ_H 4.68) and H-4′ (δ_H 1.55) upon irradiation of H_3-6′ (δ_H 1.13) was observed whereas irradiation of H-4′ affected signal intensity of H_3-8′ (δ_H 1.35). These results suggested the *cis*-orientation of H-2′, H-4′, H_3-6′ and H_3-8′. The coupling constant of 1.8 Hz between H-2′ and H-3′ (δ_H 2.03) supported their *trans*-relationship (Renauld et al. 1985; Hosoyama et al. 2000 and Cabedo et al. 2007). Thus, the relative configuration of C-5′ (δ_C 77.6), C-4′ (δ_C 55.8) and C-3′ (δ_C 44.0) was identical to that of C-5 (δ_C 77.7), C-4 (δ_C 51.5) and C-3 (δ_C 38.4) of **6**. The absolute configuration at C-3 of **1** was assigned as *R* configuration according to the negative Cotton effect of the lactone n→π* transition at 217 nm ($\Delta\varepsilon$ = -36.98, MeOH) in the ECD spectrum, the same sign as that of **6**. Accordingly, the absolute configurations in the γ-lactone unit of **1** were determined to be 3*R*, 4*S* and 5*S*, respectively, identical to those of **6**. Biosynthetically, we proposed that **1** would be derived from the condensation of two molecules of **6** by nucleophilic addition of 7-OH of one molecule to the lactone carbonyl functionality of the second molecule followed by dehydration and subsequent hydride addition to an oxonium intermediate. Subsequently, the remaining absolute configuration in **1** was proposed to be 2′*R*, 3′*R*, 4′*S* and 5′*S*. It is worth to note that botryosphaerilactone A of which the absolute configuration remained identified displayed the specific rotation closed to zero, $[\alpha]_D^{27}$ -1.2 (*c* 0.77, MeOH) (Rukachaisirikul et al. 2009), indicating that it was obtained as a scalemic mixture. Thus, **1** was (3*R*,4*S*,5*S*,2′*R*,3′*R*,4′*S*,5′*S*)-botryosphaerilactone A.

Compound **2** was obtained as a colorless gum with $[\alpha]_D^{24}$ +22.9 (*c* 0.77, MeOH). Its ^{1}H (Table S1) and ^{13}C NMR (Table S2) signals were almost identical to those of **1**. The HRESIMS of **2**, $[M+Na]^+$ ion of *m/z* 295.1521, indicated that **2** had an identical molecular formula to that of **1**. The planar structure of **2** was assigned to be identical to that of **1** and botryosphaerilactones A and C by analysis of the HMQC, ^{1}H-^{1}H COSY and HMBC correlations (Figure S1). However, the coupling constant of 4.8 Hz between H-2′ (δ_H 4.79) and H-3′ (δ_H 2.08) indicated a *cis*-relationship between H-2′ and H-3′ in **2** (Renauld et al. 1985; Hosoyama et al. 2000 and Cabedo et al. 2007), instead of a *trans*-relationship in **1**. This *cis* assignment was supported by signal enhancement of H-2′ upon irradiation of H-3′, not H_3-6′ (δ_H 1.03) as observed in **1**. The remaining NOEDIFF data (Figure S2) were identical to those of **1**. Accordingly, the relative configuration of **2** was identical to botryosphaerilactone C which was obtained as a scalemic mixture based on the specific rotation, $[\alpha]_D^{26}$ +5.2 (*c*

For Peer Review Only

0.77, MeOH) (Rukachaisirikul et al. 2009). The absolute configuration at C-3 (δ_C 38.6) of **2** was assigned to be *R*, identical to that of **1** on the basis of the negative Cotton effect at 218 nm ($\Delta\varepsilon$ = -12.64, MeOH) in the ECD spectrum. Thus, **2** was identified as a C-2′ epimer of **1** which would be biosynthetically derived from the same oxonium intermediate as **1** which would produce both **1** and **2** upon addition of hydride ion. Finally, **2** was (3*R*,4*S*,5*S*,2′*S*,3′*R*,4′*S*,5′*S*)-botryosphaerilactone C.

Compound **3** was isolated as a colorless gum with the molecular formula $C_{14}H_{22}O_5$ determined by the HRESIMS peak at *m/z* 293.1365 $[M+Na]^+$. The UV spectrum was identical to that of **1**, while the IR absorption bands were found at 1775 and 1705 cm^{-1} for γ-lactone and ketone carbonyl groups, respectively. Direct comparison of the ^1H (Table S1) and ^{13}C NMR (Table S2) data of **3** as well as the ^1H-^1H COSY and HMBC correlations (Figure S1) with those of botryosphaerilactone B (Rukachaisirikul et al. 2009) revealed that they had identical planar structure. In addition, the NOEDIFF data of **3** indicated their identical relative configuration. Based on the NOEDIFF data (Figure S2), the relative configuration of the lactone unit in **3** was identical to that of **6**. These results together with the negative Cotton effect of the n→π* transition band of lactone at 218 nm ($\Delta\varepsilon$ = -26.21, MeOH) indicated the absolute configuration of the lactone moiety was the same as those in **6**. In addition, the furan unit displayed signal enhancement of H_3-8′ (δ_H 2.19), but not H-2′ (δ_H 4.70), after irradiation of H-3′ (δ_H 2.52), indicating *trans*-relationship of H-3′ with H-2′ and H-4′ (δ_H 2.70). The absolute configuration at C-4′ (δ_C 59.1) was determined as *R* configuration identical to that of **7** based on the presence of the second positive Cotton effect of the n→π* transition band of an acetyl group at 283 nm ($\Delta\varepsilon$ = +15.45, MeOH), the same sign as that of **7**. Subsequently, the *R* configuration at both C-2′ (δ_C 110.1) and C-3′ (δ_C 43.6) was established. Although **3** showed a relatively low specific rotation value, $[\alpha]_D^{24}$ -8.1 (*c* 0.57, MeOH), it displayed the Cotton effects in the ECD spectrum at 218 and 283 nm of which the amplitude CEs were not close to zero. In general, the specific rotation value of scalemic mixtures would be small value or approached zero. The ECD spectrum of the corresponding mixtures also displayed weak Cotton effects (low-amplitude CEs) or the apparent lack of the Cotton effects (Deng et al. 2017; Odonbayar et al. 2019 and Zhang et al. 2019). Finally, **3** was assigned as (3*R*,4*S*,5*S*,2′*R*,3′*R*,4′*R*)-botryosphaerilactone B based on the opposite sign of the observed specific rotation of **3** to that of botryosphaerilactone B, $[\alpha]_D^{26}$ +4.5 (*c* 0.57, MeOH) (Rukachaisirikul et al. 2009).

For Peer Review Only

Compound **4** was obtained as a colorless gum with $[\alpha]_D^{24}$ -54.3 (*c* 0.57, MeOH). The UV, IR, ^{1}H (Table S1) and ^{13}C NMR (Table S2) data were similar to those of **3**. Its HRESIMS, $[M+Na]^+$ ion of *m/z* 293.1365, indicated their identical molecular formula. The planar structure of **4** was identical to **3** based on the HMQC, ^{1}H-^{1}H COSY and HMBC correlations (Figure S1). The relative stereochemistry was determined using the NOEDIFF data (Figure S2). Irradiation of H_3-6′ (δ_H 0.88) enhanced the signal intensity of H-2′ (δ_H 4.74) and H_3-8′ (δ_H 2.21), suggesting that they were a *cis*-orientation. The remaining NOEDIFF data were identical to those of **3**. Comparison of the ECD spectrum of **4**, [218 nm ($\Delta\varepsilon$ = -22.21) and 286 nm ($\Delta\varepsilon$ = -25.48), MeOH], with that of **3**, [218 nm ($\Delta\varepsilon$ = -26.21) and 283 nm ($\Delta\varepsilon$ = +15.45), MeOH], indicated that the absolute configuration at C-3 (δ_C 38.5) and C-4′ (δ_C 53.7) of **4** was assigned as *R* and *S*, respectively. Accordingly, **4** was a C-4′ epimer of **3** and named as botryosphaerilactone D.

Compound **5** was obtained as a colorless gum with $[\alpha]_D^{25}$ +30.9 (*c* 0.57, MeOH). Its ^{1}H (Table S1) and ^{13}C NMR (Table S2) data were similar to those of **4**, and also gave the same molecular formula as **4** deduced from HRESIMS peak at *m/z* 293.1335 $[M+Na]^+$. The major difference in the ^{1}H NMR spectrum of **5** was the multiplicity of H-2′ (δ_H 4.93) as a doublet with the coupling constant of 4.8 Hz between H-2′ and H-3′ (δ_H 2.63), indicating a *cis*-relationship between these protons. The assigned structure of **5** was supported by ^{1}H-^{1}H COSY and HMBC data as depicted in Figure S1. In the NOEDIFF data (Figure S2), irradiation of H_3-6′ (δ_H 1.02) enhanced only the signal intensity of H_3-8′ (δ_H 2.19), but not that of H-2′, indicating that H_3-6′ was *trans* to H-2′. The absolute configuration at C-3 (δ_C 38.5) and C-4′ (δ_C 53.4) of **5** was identified as *R* and *S*, respectively, identical to those of **4**, on the basis of the two negative Cotton effects at 217 nm ($\Delta\varepsilon$ = -25.24) and 289 nm ($\Delta\varepsilon$ = -8.34), MeOH. These results as well as the NOEDIFF data established the absolute configuration of **5** to be 3*R*, 4*S*, 5*S*, 2′*S*, 3′*R* and 4′*S*. Accordingly, **5** was a C-2′ epimer of **4** and named as botryosphaerilactone E.

Compound **7** with sufficient amount was tested for antimicrobial activity against *Staphylococcus aureus* ATCC25923, methicillin-resistant *S. aureus* SK1 (a clinical isolate), *Pseudomonas aeruginosa* ATCC27853, *Escherichia coli* ATCC25922, *Candida albicans* NCPF3153, *Cryptococcus neoformans* ATCC90113, *Microsporum gypseum* and *Penicillium marnefeii* clinical isolate. It exhibited weak antifungal activity against *C. neoformans* ATCC90113 with the MIC value of 200 μg/mL. In addition, **7** was evaluated for antimalarial

For Peer Review Only

(*Plasmodium falciparum*) and anticancer (KB cell lines) activities. It was inactive against both assays.

3. Experimental

3.1. General experimental procedures

Specific rotations were measured on a JASCO P-1020 polarimeter. Ultraviolet (UV) absorption spectra were measured in MeOH on a Perkin-Elmer Lambda 45 spectrophotometer. Electronic Circular Dichroism (ECD) spectra were recorded on a JASCO model J-810 polarimeter. Infrared (IR) spectra were obtained on a Perkin-Elmer 783 FTS165 FT-IR spectrometer. Mass spectra were recorded on a MAT 95 XL mass spectrometer (Thermo Finnigan) or Shimadzu LCMS-IT-TOF mass spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a 300 or a 500 MHz Bruker FTNMR Ultra Shield spectrometer using tetramethylsilane (TMS) as an internal standard. Thin-layer chromatography (TLC) and precoated TLC (PTLC) 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 reverse phase C_{18} silica gel.

3.2. Fungal material

The fungus NSTRU-PN1.4 was isolated from a soil sample collected from Paknakhon mangrove in Nakhon Si Thammarat province, Thailand. This fungus was deposited at the Nakhon Si Thammarat Rajabhat University Culture Collection, Thailand (NSTRU13118). Its colony on PDA was rapid growth with dark brown coloration. The microscopic morphology showed oval pycnidiospores with striations on the surface of the spore along the long axis. The ITS1-5.8S-ITS2 rDNA sequence (GenBank accession number MG946719) revealed that PN1.4 had affiliation with *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. supported by high bootstrap values and nucleotide identity of 100%. Therefore, PN1.4 should be referred to *Lasiodiplodia theobromae*.

3.3. Fermentation, extraction and purification

The fungus *L. theobromae* NSTRU-PN1.4 was grown on potato dextrose agar (PDA) at 25 °C for 5 days. Five pieces (0.5×0.5 cm^2) of mycelial agar plugs were inoculated into 500 mL Erlenmeyer flasks containing 300 mL of potato dextrose broth (PDB) at room temperature for 3 weeks. The flask culture (19.8 L) was filtered to separate into the filtrate and wet mycelia. The filtrate was divided into 66 portions. Each portion was extracted twice with ethyl acetate

For Peer Review Only

(2 × 300 mL). The organic layer was dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure to afford a dark brown gum (4.51 g). The broth extract was separated by CC over Sephadex LH-20 using MeOH to give four fractions (A-D). Fraction B (2.31 g) was purified by CC over silica gel using a gradient of MeOH/CH_2Cl_2 (1:99 → 100:0) to yield six subfractions (B1-B6). The first subfraction contained **9** (2.4 mg). Subfraction B3 (9.9 mg) was separated by PTLC using EtOAc/hexane (1:19) as a mobile phase (6 runs) to yield **10** (0.9 mg). Separation of subfraction B4 (163.1 mg) by CC over silica gel using a gradient of acetone/hexane (20:80 → 100:0) gave **7** (65.0 mg). Subfraction B5 (1.05 g) was fractionated by CC over silica gel using a gradient of MeOH/CH_2Cl_2 (1:99 → 100:0) to give eight subfractions (B51-B58). Subfraction B52 (27.0 mg) was subjected to CC over silica gel using MeOH/CH_2Cl_2 (1:199) as an eluent, and subsequent PTLC using acetone/CH_2Cl_2 (1:19) as a mobile phase (4 runs) to afford **6** (5.5 mg). Subfraction B54 (77.7 mg) was twice purified by FCC over silica gel using a gradient of EtOAc/hexane (30:70 → 100:0) and CH_2Cl_2/hexane (90:10 → 100:0), respectively, followed by PTLC using $CHCl_3$/hexane (7:3) as a mobile phase (6 runs) to obtain **8** (1.6 mg). Subfraction B55 (81.1 mg) was further purified by CC over reverse phase C_{18} silica gel using a gradient of MeOH/H_2O (40:60 → 100:0) as an eluent, and subsequent PTLC using EtOAc/hexane (3:7) as a mobile phase (7 runs) to afford **11** (1.5 mg). Subfraction B56 (283.9 mg) was chromatographed by CC over Sephadex LH-20 using MeOH/CH_2Cl_2 (1:1) as an eluent, and subsequent CC over silica gel using a gradient of EtOAc/hexane (30:70 → 100:0) to yield six subfractions (B561-B566). The second subfraction (5.0 mg), upon PTLC using MeOH/CH_2Cl_2 (0.5:99.5) as a mobile phase (2 runs), and the fifth subfraction (17.9 mg), upon CC over silica gel using a gradient of acetone/CH_2Cl_2 (2:98 → 100:0) as an eluent, gave **4** (3.3 mg) and **3** (0.7 mg), respectively. Subfraction B57 (340.7 mg) was submitted by CC over Sephadex LH-20 using MeOH/CH_2Cl_2 (1:1) as an eluent, followed by CC over reverse phase C_{18} silica gel using a gradient of MeOH/H_2O (50:50 → 100:0) as an eluent to yield eight subfractions (B571-B578). Subfraction B574 (38.4 mg) was purified by CC over silica gel using acetone/$CHCl_3$/hexane (10:55:35) to afford **1** (3.2 mg) and **2** (1.4 mg). Compound **5** (2.1 mg) was obtained from subfraction B575 (22.0 mg) after purification by CC over silica gel using acetone/$CHCl_3$/hexane (5:60:35). Subfraction B6 (177.5 mg) was fractionated by CC over Sephadex LH-20 using MeOH/CH_2Cl_2 (1:1) as an eluent, and subsequent FCC over silica gel using a gradient of MeOH/CH_2Cl_2 (0.5:99.5 → 100:0) to yield **12** (1.9 mg).

For Peer Review Only

*3.3.1. Compound **1***

Colorless gum; $[\alpha]_D^{24}$ -14.6 (*c* 0.77, MeOH); UV (MeOH) λ_{max} (log ε): 286 (1.30) nm; ECD (MeOH, *c* 0.012 M) λ_{max} ($\Delta\varepsilon$) 217 (-36.98) nm; IR (neat) ν_{max} 3451, 1770 cm^{-1}; ^1H, see Table S1 and ^{13}C NMR data, see Table S2; HRESIMS *m/z*: $[M+Na]^+$ 295.1521 (calcd for $C_{14}H_{24}O_5Na$, 295.1521)

*3.3.2. Compound **2***

Colorless gum; $[\alpha]_D^{24}$ +22.9 (*c* 0.77, MeOH); UV (MeOH) λ_{max} (log ε): 287 (1.31) nm; ECD (MeOH, *c* 0.012 M) λ_{max} ($\Delta\varepsilon$) 218 (-12.64) nm; IR (neat) ν_{max} 3402, 1772 cm^{-1}; ^1H, see Table S1 and ^{13}C NMR data, see Table S2; HRESIMS *m/z*: $[M+Na]^+$ 295.1521 (calcd for $C_{14}H_{24}O_5Na$, 295.1521)

*3.3.3. Compound **3***

Colorless gum; $[\alpha]_D^{24}$ -8.1 (*c* 0.57, MeOH); UV (MeOH) λ_{max} (log ε): 280 (1.61) nm; ECD (MeOH, *c* 0.009 M) λ_{max} ($\Delta\varepsilon$) 218 (-26.21), 283 (+15.45) nm; IR (neat) ν_{max} 1775, 1705 cm^{-1}; ^1H, see Table S1 and ^{13}C NMR data, see Table S2; HRESIMS *m/z*: $[M+Na]^+$ 293.1365 (calcd for $C_{14}H_{22}O_5Na$, 293.1365)

*3.3.4. Botryosphaerilactone D (**4**)*

Colorless gum; $[\alpha]_D^{24}$ -54.3 (*c* 0.57, MeOH); UV (MeOH) λ_{max} (log ε): 287 (2.23) nm; ECD (MeOH, *c* 0.008 M) λ_{max} ($\Delta\varepsilon$) 218 (-22.21), 286 (-25.48) nm; IR (neat) ν_{max} 1772, 1705 cm^{-1}; ^1H, see Table S1 and ^{13}C NMR data, see Table S2; HRESIMS *m/z*: $[M+Na]^+$ 293.1365 (calcd for $C_{14}H_{22}O_5Na$, 293.1365)

*3.3.5. Botryosphaerilactone E (**5**)*

Colorless gum; $[\alpha]_D^{25}$ +30.9 (*c* 0.57, MeOH); UV (MeOH) λ_{max} (log ε): 282 (1.77) nm; ECD (MeOH, *c* 0.008 M) λ_{max} ($\Delta\varepsilon$) 217 (-25.24), 289 (-8.34) nm; IR (neat) ν_{max} 1742, 1698 cm^{-1}; ^1H, see Table S1 and ^{13}C NMR data, see Table S2; HRESIMS *m/z*: $[M+Na]^+$ 293.1335 (calcd for $C_{14}H_{22}O_5Na$, 293.1365)

*3.3.6. (3R,4S,5S)-Dihydro-4-(hydroxymethyl)-3,5-dimethyl-2(3H)-furanone (**6**)*

For Peer Review Only

Pale yellow gum; $[\alpha]_D^{25}$ +51.5 (*c* 0.17, MeOH); ECD (MeOH *c* 0.045 M) λ_{max} ($\Delta\varepsilon$) 216 (-133.37) nm.

3.3.7. (3R,4R)-4-Acetyl-3-methyl-2(3H)-dihydrofuranone (7)

Pale yellow gum; $[\alpha]_D^{24}$ +81.9 (*c* 0.57, MeOH); ECD (MeOH *c* 0.034 M) λ_{max} ($\Delta\varepsilon$) 216 (-156.77), 284 (+17.88) nm.

3.4. Antimicrobial assays

Antimicrobial activity was determined as described by the Clinical and Laboratory Standards Institute (Drummond and Waigh 2000; Clinical and Laboratory Standards Institute 2002a, 2002b, 2002c). Vancomycin, amphotericin B and miconazole were used as positive controls for bacteria, yeasts and fungus with the MIC values of 0.69, 0.27 and 2.40 µM, respectively.

3.5. Antimalarial assay

Antimalarial activity was evaluated against the parasite *P. falciparum* (K1, multi-drug-resistant strain), using the microculture radioisotope technique based on the method described (Desjardins et al. 1979). Dihydroartemisinine and mefloquine were used as standard compounds and exhibited the IC_{50} values of 0.0023 and 0.0269 µM, respectively.

3.6. Cytotoxicity assay

The cytotoxicity assay against KB cell lines was evaluated using the resazurin microplate assay (O'Brien et al. 2000). Ellipticine (IC_{50} 8.24 µM) and doxorubicin (IC_{50} 2.19 µM) were used as standard drugs for KB cell lines.

4. Conclusion

Dimeric γ-lactones, botryosphaerilactones A-C, were firstly reported as secondary metabolites from the endophytic fungi *B. rhodina* PSU-M35 and PSU-M114 which were isolated from leaves of *Garcinia mangostana* (Rukachaisirikul et al. 2009). Interestingly, five dimeric γ-lactones (**1-5**) isolated from the soil-derived fungus *L. theobromae* NSTRU-PN1.4 possessed identical planar structures to those of botryosphaerilactones A-C. Compounds **1-3** were identified to be the enantiomers of botryosphaerilactones A, C and B, respectively, whereas two metabolites **4** and **5** were reported as new diastereomers of **3**. Additionally, this is first time to isolate dimeric γ-lactones from soil-derived fungi.

For Peer Review Only

Acknowledgments

J. Arunpanichlert is grateful to the Thailand Research Fund for a research grant (Grant No. MRG5980088). The Center of Excellence for Innovation in Chemistry (PERCH-CIC), Ministry of Higher Education, Science, Research and Innovation and Thammasat University are gratefully acknowledged for partial support. We are also thankful to Dr. Winai Ieawsuwan, Chulabhorn Research Institute, for 2D NMR data collection. V. Rukachaisirikul thanks the NSTDA Chair Professor grant (the Fourth Grant) of the Crown Property Bureau and National Science and Technology Development Agency. Finally, the National Center for Genetic Engineering and Biotechnology (BIOTEC) is acknowledged for antimalarial and cytotoxic assays.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Thailand Research Fund for a research grant [grant number MRG5980088]; the NSTDA Chair Professor grant (the Fourth Grant) of the Crown Property Bureau and National Science and Technology Development Agency.

References

Andolfi A, Maddau L, Cimmino A, Linaldeddu BT, Basso S, Deidda A, Serra S, Evidente A. 2014. Lasiojasmonates A-C, three jasmonic acid esters produced by *Lasiodiplodia* sp., a grapevine pathogen. Phytochemistry. 103:145-153.

Biswas B, Sarkar D, Venkateswaran RV. 2008. Total synthesis of alboatrin, a phytotoxic metabolite from *Verticillium alboatrum*. Tetrahedron. 64:3212-3216.

Cabedo N, López-Gresa MP, Primo J, Ciavatta ML, González-Mas MC. 2007. Isolation and structural elucidation of eight new related analogues of the mycotoxin (−)-botryodiplodin from *Penicillium coalescens*. J Agric Food Chem. 55:6977-6983.

Chen S, Chen D, Cai R, Cui H, Long Y, Lu Y, Li C, She Z. 2016. Cytotoxic and antibacterial preussomerins from the mangrove endophytic fungus *Lasiodiplodia theobromae* ZJ-HQ1. J Nat Prod. 79:2397-2402.

For Peer Review Only

Clinical and Laboratory Standards Institute (CLSI). 2002a. Reference method for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved standard M7-A4. Wayne, PA: Clinical and Laboratory Standards Institute.

Clinical and Laboratory Standards Institute (CLSI). 2002b. Reference method for broth dilution antifungal susceptibility testing of yeasts; Approved Standard M27-A2. 2nd ed. Wayne, PA: Clinical and Laboratory Standards Institute.

Clinical and Laboratory Standards Institute (CLSI). 2002c. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; Approved Standard. CLSI Documents M38-A. Wayne, PA: Clinical and Laboratory Standards Institute.

Daengrot C, Rukachaisirikul V, Tansakul C, Thongpanchang T, Phongpaichit S, Bowornwiriyapan K, Sakayaroj J. 2015. Eremophilane sesquiterpenes and diphenyl thioethers from the soil fungus *Penicillium copticola* PSU-RSPG138. J Nat Prod. 78: 615-622.

Deng AJ, Zhang HJ, Li Q, Li ZH, Zhang ZH, Wu LQ, Li L, Qin HL. 2017. Six scalemic mixtures of 6-monosubstituted dihydrobenzophenanthridine alkaloids from *Chelidonium majus* and optically active structures of enantiomers. Phytochemistry. 144:159-170.

Desjardins RE, Canfield CJ, Haynes JD, Chulay JD. 1979. Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. Antimicrob Agents Chemother. 16:710-718.

Drummond AJ, Waigh RD. 2000. Recent research developments in phytochemistry. 4:143-152.

Félix C, Salvatore MM, DellaGreca M, Meneses R, Duarte AS, Salvatore F, Naviglio D, Gallo M, Jorrín-Novo JV, Alves A, Andolfi A, Esteves AC. 2018. Production of toxic metabolites by two strains of *Lasiodiplodia theobromae*, isolated from a coconut tree and a human patient. Mycologia. 110:642-653.

Forzato C, Furlan G, Ghelfi F, Nitti P, Pitacco G, Roncaglia F, Valentin E. 2007. Lipase-catalysed deacetylation of botryodiplodin acetate. Tetrahedron Asymmetry. 18:447-450.

Forzato C, Furlan G, Nitti P, Pitacco G, Marchesan D, Coriani S, Valentin E. 2005. A combined experimental and computational strategy in the assignment of absolute configurations of 4-methyl-5-oxo-tetrahydrofuran-3-carboxylic acids and their esters. Tetrahedron Asymmetry. 16:3011-3023.

For Peer Review Only

Forzato C, Nitti P, Pitacco G. 1997. Bicyclic γ-butyrolactones. Relation between conformation of the lactone ring and chiroptical properties. Tetrahedron Asymmetry. 8:4101-4110.

Hosoyama H, Shigemori H, Kobayashi J. 2000. Further unexpected boron trifluoride-catalyzed reactions of taxoids with α- and β-4,20-epoxides. J Chem Soc Perkin Trans 1. 449-451.

Isaka M, Chinthanom P, Veeranondha S, Supothina S, Luangsa-ard JJ. 2008. Novel cyclopropyl diketones and 14-membered macrolides from the soil fungus *Hamigera avellanea* BCC 17816. Tetrahedron. 64:11028-11033.

Kamal N, Viegelmann CV, Clements CJ, Edrada-Ebel R. 2017. Metabolomics-guided isolation of anti-trypanosomal metabolites from the endophytic fungus *Lasiodiplodia theobromae*. Planta Med. 83:565-573.

Lee TWS, Rettig SJ, Stewart R, Trotter J. 1984. Six pyrimidonium salts: determination of their crystal structures and studies of their prototropic behavior in solution. Can J Chem. 62:1194-1202.

Matsumoto T, Okabe T, Fukui K. 1973. Pyrrolizidine alkaloids. The absolute configurations of latifolic acid and its stereoisomers. Chem Lett. 2:773-776.

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. Eur J Biochem. 267:5421-5426.

Odonbayar B, Murata T, Suganuma K, Ishikawa Y, Buyankhishig B, Batkhuu J, Sasaki K. 2019. Acylated lignans isolated from *Brachanthemum gobicum* and their trypanocidal activity. J Nat Prod. 82:774-784.

Ravi BN, Armstrong RW, Faulkner DJ. 1979. Some aromatic compounds from the marine sponge *Plakortis halichlondriodes*. J Org Chem. 44:3109-3113.

Reino JL, Guerrero RF, Hernández-Galán R, Collado IG. 2008. Secondary metabolites from species of the biocontrol agent *Trichoderma*. Phytochem Rev. 7:89-123.

Renauld F, Moreau S, Lablachecombier A, Tiffon B. 1985. Botryodiplodin, a mycotoxin from *Penicillium roqueforti*. Reaction with amino-pyrimidines, amino-purines and 2′-deoxynucleosides. Tetrahedron. 41:955-962.

Rukachaisirikul V, Arunpanichlert J, Sukpondma Y, Phongpaichit S, Sakayaroj J. 2009. Metabolites from the endophytic fungi *Botryosphaeria rhodina* PSU-M35 and PSU-M114. Tetrahedron. 65:10590-10595.

For Peer Review Only

Sansinenea E, Ortiz A. 2011. Secondary metabolites of soil *Bacillus* spp. Biotechnol Lett. 33:1523-1538.

Smith EL, Spackman DH, Polglase WJ. 1952. The specificity of leucine aminopeptidase. III. Action on diastereoisomers. J Biol Chem. 199:801-817.

Tanaka Y, Shiomi K, Kamei K, Sugoh-hagino M, Enomoto Y, Fang F, Yamaguchi Y, Masuma R, Zhang CG, Zhang XW, Ōmura S. 1998. Antimalarial activity of radicicol, heptelidic acid and other fungal metabolites. J Antibiot. 51:153-160.

Umeokoli BO, Ebrahim W, El-Neketi M, Müller WEG, Kalscheuer R, Lin W, Liu Z, Proksch P. 2019. A new depsidone derivative from mangrove sediment derived fungus *Lasiodiplodia theobromae*. Nat Prod Res. 33:2215-2222.

Zhang CY, Gao Y, Zhu RX, Qiao YN, Zhou JC, Zhang JZ, Li Y, Li SW, Fan SH, Lou HX. 2019. Prenylated bibenzyls from the chinese liverwort *Radula constricta* and their mitochondria-derived paraptotic cytotoxic activities. J Nat Prod. 82:1741-1751.

For Peer Review Only

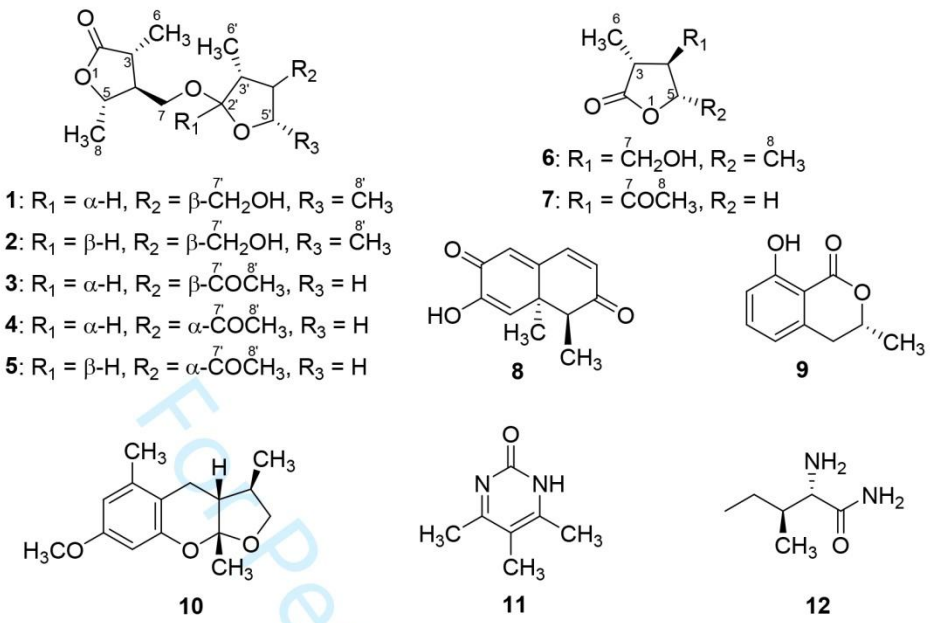


Figure 1. Structures of compounds **1-12** isolated from *Lasiodiplodia theobromae* NSTRU-PN1.4.

For Peer Review Only

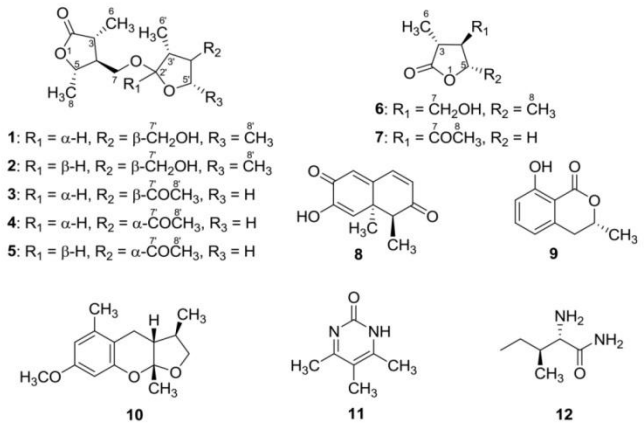


Figure 1. Structures of compounds **1-12** isolated from *Lasiodiplodia theobromae* NSTRU-PN1.4.

173x110mm (300 x 300 DPI)

ภาคผนวก ข

(Appendix II)

The isolation and characterization of secondary metabolites from the fungus *Trichoderma virens* NSTRU-PP3.8

Experimental

1. General experiment procedure

The melting point was measured on a Sanyo Gallenkamp melting point apparatus and reported without correction. Infrared (IR) spectra were recorded on a PerkinElmer spectrum BX FT-IR spectrometer (neat) or a Thermo Scientific NICOLET iS50 FT-IR (neat and KBr). Ultraviolet (UV) spectra were measured on a Shimadzu UV-2600 or a Shimadzu UV-1900 UV-Vis spectrophotometer in MeOH solution. The measurement of specific rotation was performed with a JASCO P-2000 polarimeter in MeOH solution. The ^{1}H and ^{13}C NMR spectra were recorded on a 300 or 400 MHz Bruker FTNMR Ultra Shield^{TM} spectrometer using tetramethylsilane (TMS) as an internal standard. Column chromatography (CC) was conducted on Sephadex LH-20, silica gel (Merck) type 60 (230-400 mesh ASTM) or reverse phase C18. Thin-layer chromatography (TLC) and preparative TLC were carried out on silica gel 60 GF_{254} (Merck). The organic solvents were distilled prior to use.

2. Fermentation, extraction and isolation

The soil fungus *T. virens* NSTRU-PP3.8 was grown on potato dextrose agar (PDA) for five days. Five pieces (0.5×0.5 cm^2) of mycelial agar plugs were inoculated into 50 flasks of 500 mL Erlenmeyer flasks containing 300 mL of potato dextrose broth (PDB) and kept at room temperature for 3 weeks. The cultures were filtered to give filtrate and mycelia. The filtrate was extracted with ethyl acetate. The ethyl acetate layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness under reduced pressure to give a broth ethyl acetate extract (BE). The mycelia were 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 over anhydrous Na_2SO_4, filtered and evaporated to dryness under reduced pressure to afford a mycelia hexane extract (CH). The aqueous layer was further extracted with the same procedure as the filtrate to obtain a mycelia ethyl acetate extract (CE).

The BE extract of NSTRU-PP3.8 (4.06 g, a dark brown gum) was fractionated by CC over Sephadex LH-20 using a mixture of MeOH and CH_2Cl_2 in a ratio of 1:1 as an eluent to afford five fractions (PP1-PP5). Fraction PP2 was further purified by CC over silica gel with a gradient of MeOH-CH_2Cl_2 (1:99→1:0) to afford four subfractions (PP21-PP24). Compound **18** (2.2 mg) was obtained from subfraction PP22 after purification by PTLC with a mixture of MeOH-CH_2Cl_2 in a ratio of 1:99. Subfraction PP23 was further purified by PTLC with a mixture of MeOH-CH_2Cl_2 in a ratio of 3:97 to give compound **19** (1.3 mg). Fraction PP3 was further investigated by CC over silica gel with

a gradient of acetone-CH_2Cl_2 (5:95→1:0) to give three subfractions (PP31-PP33). Compound **17** (1.9 mg) was obtained from subfraction PP32 upon purification by PTLC with a mixture of acetone-CH_2Cl_2 in a ratio of 1:9. Fraction PP4 was further purified by CC over reverse phase silica gel (RPCC) with a gradient of MeOH-water (4:6→1:0) to afford five subfractions (PP41-PP45). Subfraction PP42 contained compound **15** (420.8 mg). Subfraction PP43 was further purified by RPCC with a gradient of MeOH-water (1:1→1:0) to yield compound **13** (19.2 mg). Compound **14** (275.9 mg) was obtained from subfraction PP44 after purification by RPCC with a gradient of MeOH-water (1:1→1:0).

The CE extract from NSTRU-PP3.8 (1.01 g, a dark brown gum) was fractionated by CC over Sephadex LH-20 with pure MeOH as an eluent to give five fractions (A-E). Fraction D was further purified by CC over silica gel with a gradient of MeOH-CH_2Cl_2 (1:99→1:0) to afford five subfractions (D1-D5). Compound **17** (22.8 mg) was obtained from subfraction D4 after purification by CC over silica gel with a gradient of acetone-CH_2Cl_2 (2:98→1:0). Subfraction D5 contained compound **14** (24.6 mg). Fraction E was further purified by CC over silica gel with a gradient of acetone-CH_2Cl_2 (1:9→1:0) to give five subfractions (E1-E5). Subfraction E3 was further purified by CC over silica gel with a gradient of acetone-CH_2Cl_2 (1:9→1:0) to yield compound **16** (6.5 mg).

New sesquiterpene derivative (**13**)

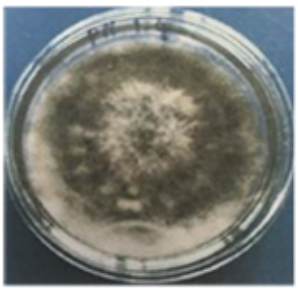
Colorless gum; $[\alpha]_D^{25}$ +158.9 (c 0.17, MeOH); UV (MeOH) λ_{max} nm (log ε): 221 (3.97); FT-IR (neat) ν_{max} cm^{-1}: 3422, 1729; ^{1}H NMR data (300 MHz, $CDCl_3$+CD_3OD): 6.29 (*d*, *J* = 10.8 Hz, 1H), 4.77 (*d*, *J* = 17.4 Hz, 1H), 4.67 (*d*, *J* = 15.6 Hz, 1H), 4.38 (*brs*, 2H), 3.36 (*m*, 1H), 2.37 (*m*, 2H), 1.93 (*m*, 1H), 1.64 (*m*, 1H), 1.53 (*m*, 1H), 0.96 (*d*, *J* = 6.6 Hz, 3H), 0.83 (*d*, *J* = 6.6 Hz, 3H); ^{13}C NMR data (75 MHz, $CDCl_3$+CD_3OD): 174.7 (C), 163.9 (C), 139.2 (CH), 126.5 (C), 72.0 (CH_2), 57.8 (CH_2), 45.4 (CH), 34.3 (CH), 27.7 (CH), 23.1 (CH_2), 21.7 (CH_3), 20.5 (CH_2), 17.7 (CH_3)

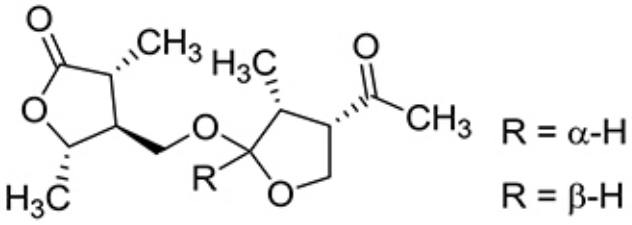
.

Dimeric γ-lactone derivatives from the soil-derived fungus *Lasiodiplodia theobromae* NSTRU-PN1.4

Journal:	*Natural Product Research*
Manuscript ID	GNPL-2020-2278.R1
Manuscript Type:	Research Article
Date Submitted by the Author:	23-Sep-2020
Complete List of Authors:	Arunpanichlert, Jiraporn; Thammasat University, Chemistry Rukachaisirikul, Vatcharin; Prince of Songkla University, Chemistry Chaiwarin, Titima; Prince of Songkla University Tantirungrotechai, Yuthana; Thammasat University, Chemistry Khamthong, Nanthaphong; Rangsit University Phongpaichit, Souwalak; Prince of Songkla University, Microbiology Liamthong, Sumalee; Nakhon Si Thammarat Rajabhat University Sakayaroj, Jariya; Walailak University
Keywords:	Soil-derived fungus, Lasiodiplodia theobromae, Dimeric γ-lactone, Antifungal activity

SCHOLARONE™
Manuscripts





44x14mm (300 x 300 DPI)

Dimeric γ-lactone derivatives from the soil-derived fungus *Lasiodiplodia theobromae* NSTRU-PN1.4

Jiraporn Arunpanichlert[a,*], Vatcharin Rukachaisirikul[b], Titima Chaiwarin[b], Yuthana Tantirungrotechai[a], Nanthaphong Khamthong[c], Souwalak Phongpaichit[d], Sumalee Liamthong[e] and Jariya Sakayaroj[f]

[a]*Department of Chemistry, Faculty of Science and Technology, Thammasat University, Klong Luang, Pathumthani 12120, Thailand*

[b]*Division of Physical Science and Center of Excellence for Innovation in Chemistry, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand*

[c]*College of Oriental Medicine, Rangsit University, Pathumthani 12000, Thailand*

[d]*Division of Biological Science, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand*

[e]*Department of Biology, Faculty of Science and Technology, Nakhon Si Thammarat Rajabhat University, Nakhon Si Thammarat 80280, Thailand*

[f]*School of Science, Walailak University, Nakhon Si Thammarat 80161, Thailand*

*Corresponding author.

E-mail address: jira_550@tu.ac.th (J. Arunpanichlert).

For Peer Review Only

ABSTRACT

Investigation of the soil-derived fungus *Lasiodiplodia theobromae* NSTRU-PN1.4 resulted in the isolation of five dimeric γ-lactones including two new botryosphaerilactones D and E (**4** and **5**) and three known structurally related analogoues (**1**-**3**) along with seven known compounds. Their structures were elucidated by extensive spectroscopic analysis. The absolute configuration of **1-5** was determined by comparison of the ECD data with those of the structurally related monomeric γ-lactones. For biological evaluation, this is the first report on antifungal activity of the known (3*R*,4*R*)-4-acetyl-3-methyl-2(3*H*)-dihydrofuranone which displayed weak antifungal activity against *Cryptococcus neoformans* with an MIC value of 200 μg/mL.

Keywords: Soil-derived fungus, *Lasiodiplodia theobromae*, Dimeric γ-lactone, Antifungal activity

For Peer Review Only

1. Introduction

Soil-derived fungi have served as an important source of novel and bioactive secondary metabolites, for example, antimalarial radicicol (Tanaka et al. 1998), cytotoxic hamavellones A and B (Isaka et al. 2008), antimycobacterial penicilleremophilane A (Daengrot et al. 2015), antifungal bacilysin (Sansinenea and Ortiz 2011) and antibiotic koninginins A and B (Reino et al. 2008). For the fungus *Lasiodiplodia theobromae*, it has been proven to be a fabulous source of bioactive secondary metabolites, including antibacterial cladospirone B (Kamal et al. 2017), (+)-(*R*)-de-*O*-methyllasiodiplodin (Umeokoli et al. 2019), chloropreussomerins A and B and preussomerins A, F, G and H (Chen et al. 2016). Lasiodiplodin, scytalone and (–)-jasmonic acid showed phytotoxicity (Félix et al. 2018) while cladospirone B and desmethyllasiodiplodin exhibited anti-trypanosomal activity (Kamal et al. 2017). In addition, chloropreussomerins A and B displayed potent cytotoxicity against A549 and MCF-7 human cancer cell lines (Chen et al. 2016). As part of our continuous investigation on chemical and biological diversity of soil-derived fungi, we described herein the isolation of secondary metabolites from *L. theobromae* NSTRU-PN1.4 isolated from soil in mangrove area in Nakhon Si Thammarat Province, Thailand. Purification of the broth EtOAc extract led to the isolation of twelve metabolites, including five dimeric γ-lactones, two new botryosphaerilactones D and E (**4** and **5**) and three known botryosphaerilactones A (**1**), B (**3**) and C (**2**), as well as seven known compounds, (3*R*,4*S*,5*S*)-dihydro-4-(hydroxymethyl)-3,5-dimethyl-2(3*H*)-furanone (**6**) (Ravi et al. 1979; Andolfi et al. 2014), (3*R*,4*R*)-4-acetyl-3-methyl-2(3*H*)-dihydrofuranone (**7**) (Forzato et al. 2005, 2007), botryosphaeridione (**8**) (Rukachaisirikul et al. 2009), (*R*)-(–)-mellein (**9**) (Rukachaisirikul et al. 2009), *O*-methyl alboatrin (**10**) (Biswas et al. 2008), 4,5,6-trimethyl-2(1*H*)-pyrimidinone (**11**) (Lee et al. 1984) and L-isoleucinamide (**12**) (Smith et al. 1952). Additionally, biological activities of **7** which was obtained in sufficient quantity are presented herein.

2. Results and discussion

All metabolites (**1-12**) (Figure 1) were purified using chromatographic techniques and their structures were elucidated by analysis of spectroscopic data, including IR, UV, NMR and MS. The relative configuration was assigned according to NOEDIFF data and/or coupling constants while the absolute configuration was determined by comparison of the specific rotation or ECD data with those of the known or structurally related compounds. The absolute configuration of the known dimeric γ-lactones (**1-3**) remained unresolved in the

For Peer Review Only

previous studies. We here established the absolute configuration of the dimeric γ-lactones (**1**-**5**) on the basis of the absolute configuration of the monomeric γ-lactones **6** and **7**. The absolute configuration at C-3 of **6** was assigned as *R* configuration according to the ECD spectral data which showed a negative Cotton effect of the n→π* transition of a lactone carbonyl group (Matsumoto et al. 1973; Forzato et al. 2005) at 216 nm ($\Delta\varepsilon$ = -133.37, MeOH), the same sign as that of (−)-(3*R*,3a*S*,6a*S*)-2*H*-hexahydro-3-methylcyclopenta[b]furan -2-one at 215 nm ($\Delta\varepsilon$ = -2173, MeOH) (Forzato et al. 1997). According to the NOEDIFF results together with the established absolute configuration at C-3, the absolute configuration in **6** was thus assigned to be 3*R*, 4*S* and 5*S*. These assigned absolute configurations were confirmed based on the opposite sign of the specific rotation of **6**, $[\alpha]_D^{25}$ = +51.5 (*c* 0.17, MeOH), to that of its enantiomer (3*S*,4*R*,5*R*)-4-hydroxymethyl-3,5-dimethyldihydro-2-furanone, $[\alpha]_D^{25}$ = -18 (*c* 0.3, $CHCl_3$) (Andolfi et al. 2014). For the related monomeric γ-lactone **7**, the absolute configuration at C-3 and C-4 was established to be both *R* based on a mirror-image ECD spectrum of **7**, [216 nm ($\Delta\varepsilon$ = -156.77) and 284 nm ($\Delta\varepsilon$ = +17.88), MeOH], and the opposite sign of the specific rotation, $[\alpha]_D^{24}$ = +81.9 (*c* 0.57, MeOH), to those of its enantiomer, (3*S*,4*S*)-(−)-4-acetyl-3-methyl-2(3*H*)-dihydrofuranone [214 nm ($\Delta\varepsilon$ = +6073) and 281 nm ($\Delta\varepsilon$ = -479), MeOH; $[\alpha]_D^{25}$ = -72.1 (*c* 0.58, MeOH)] (Forzato et al. 2005). The first and second Cotton effects of **7** at λ_{max} 216 and 284 nm were related with the n→π* transition bands of lactone (Matsumoto et al. 1973; Forzato et al. 1997, 2005) and acetyl groups (Forzato et al. 2005, 2007), respectively. It is worth to notify that the substituents at C-3 and C-4 of both **6** and **7** had identical spatial arrangement.

Compound **1** was obtained as a colorless gum with $[\alpha]_D^{24}$ -14.6 (*c* 0.77, MeOH) and the molecular formula $C_{14}H_{24}O_5$ was determined by the HRESIMS that showed the molecular ion at *m/z* 295.1521 $[M+Na]^+$. It exhibited an UV absorption band of a carbonyl chromophore at 286 nm, while the IR absorption bands at 3451 and 1770 cm^{-1} were indicative of the presence of hydroxyl and γ-lactone carbonyl groups (Rukachaisirikul et al. 2009; Andolfi et al. 2014), respectively. The ^1H (Table S1) and ^{13}C NMR (Table S2) spectroscopic data of **1** as well as the ^1H-^1H COSY and HMBC correlations (Figure S1) indicated that **1** had a planar structure which was identical to that of botryosphaerilactones A and C (Rukachaisirikul et al. 2009). Moreover, the following NOEDIFF data of **1** (Figure S2) revealed that **1** and botryosphaerilactone A had an identical relative configuration. Irradiation of H-4 (δ_H 1.91) of the γ-lactone moiety enhanced the signal intensity of H_3-6 (δ_H 1.27) and H_3-8 (δ_H 1.43),

For Peer Review Only

indicating their *cis*-relationship which was identical to that of **6**. In the furan unit, signal enhancement of H-2′ (δ_H 4.68) and H-4′ (δ_H 1.55) upon irradiation of H_3-6′ (δ_H 1.13) was observed whereas irradiation of H-4′ affected signal intensity of H_3-8′ (δ_H 1.35). These results suggested the *cis*-orientation of H-2′, H-4′, H_3-6′ and H_3-8′. The coupling constant of 1.8 Hz between H-2′ and H-3′ (δ_H 2.03) supported their *trans*-relationship (Renauld et al. 1985; Hosoyama et al. 2000 and Cabedo et al. 2007). Thus, the relative configuration of C-5′ (δ_C 77.6), C-4′ (δ_C 55.8) and C-3′ (δ_C 44.0) was identical to that of C-5 (δ_C 77.7), C-4 (δ_C 51.5) and C-3 (δ_C 38.4) of **6**. The absolute configuration at C-3 of **1** was assigned as *R* configuration according to the negative Cotton effect of the lactone n→π* transition at 217 nm ($\Delta\varepsilon$ = -36.98, MeOH) in the ECD spectrum, the same sign as that of **6**. Accordingly, the absolute configurations in the γ-lactone unit of **1** were determined to be 3*R*, 4*S* and 5*S*, respectively, identical to those of **6**. Biosynthetically, we proposed that **1** would be derived from the condensation of two molecules of **6** by nucleophilic addition of 7-OH of one molecule to the lactone carbonyl functionality of the second molecule followed by dehydration and subsequent hydride addition to an oxonium intermediate. Subsequently, the remaining absolute configuration in **1** was proposed to be 2′*R*, 3′*R*, 4′*S* and 5′*S*. It is worth to note that botryosphaerilactone A of which the absolute configuration remained identified displayed the specific rotation closed to zero, $[\alpha]_D^{27}$ -1.2 (*c* 0.77, MeOH) (Rukachaisirikul et al. 2009), indicating that it was obtained as a scalemic mixture. Thus, **1** was (3*R*,4*S*,5*S*,2′*R*,3′*R*,4′*S*,5′*S*)-botryosphaerilactone A.

Compound **2** was obtained as a colorless gum with $[\alpha]_D^{24}$ +22.9 (*c* 0.77, MeOH). Its ^{1}H (Table S1) and ^{13}C NMR (Table S2) signals were almost identical to those of **1**. The HRESIMS of **2**, $[M+Na]^+$ ion of *m/z* 295.1521, indicated that **2** had an identical molecular formula to that of **1**. The planar structure of **2** was assigned to be identical to that of **1** and botryosphaerilactones A and C by analysis of the HMQC, ^{1}H-^{1}H COSY and HMBC correlations (Figure S1). However, the coupling constant of 4.8 Hz between H-2′ (δ_H 4.79) and H-3′ (δ_H 2.08) indicated a *cis*-relationship between H-2′ and H-3′ in **2** (Renauld et al. 1985; Hosoyama et al. 2000 and Cabedo et al. 2007), instead of a *trans*-relationship in **1**. This *cis* assignment was supported by signal enhancement of H-2′ upon irradiation of H-3′, not H_3-6′ (δ_H 1.03) as observed in **1**. The remaining NOEDIFF data (Figure S2) were identical to those of **1**. Accordingly, the relative configuration of **2** was identical to botryosphaerilactone C which was obtained as a scalemic mixture based on the specific rotation, $[\alpha]_D^{26}$ +5.2 (*c*

For Peer Review Only

0.77, MeOH) (Rukachaisirikul et al. 2009). The absolute configuration at C-3 (δ_C 38.6) of **2** was assigned to be *R*, identical to that of **1** on the basis of the negative Cotton effect at 218 nm ($\Delta\varepsilon$ = -12.64, MeOH) in the ECD spectrum. Thus, **2** was identified as a C-2′ epimer of **1** which would be biosynthetically derived from the same oxonium intermediate as **1** which would produce both **1** and **2** upon addition of hydride ion. Finally, **2** was (3*R*,4*S*,5*S*,2′*S*,3′*R*,4′*S*,5′*S*)-botryosphaerilactone C.

Compound **3** was isolated as a colorless gum with the molecular formula $C_{14}H_{22}O_5$ determined by the HRESIMS peak at *m/z* 293.1365 $[M+Na]^+$. The UV spectrum was identical to that of **1**, while the IR absorption bands were found at 1775 and 1705 cm^{-1} for γ-lactone and ketone carbonyl groups, respectively. Direct comparison of the ^1H (Table S1) and ^{13}C NMR (Table S2) data of **3** as well as the ^1H-^1H COSY and HMBC correlations (Figure S1) with those of botryosphaerilactone B (Rukachaisirikul et al. 2009) revealed that they had identical planar structure. In addition, the NOEDIFF data of **3** indicated their identical relative configuration. Based on the NOEDIFF data (Figure S2), the relative configuration of the lactone unit in **3** was identical to that of **6**. These results together with the negative Cotton effect of the n→π* transition band of lactone at 218 nm ($\Delta\varepsilon$ = -26.21, MeOH) indicated the absolute configuration of the lactone moiety was the same as those in **6**. In addition, the furan unit displayed signal enhancement of H_3-8′ (δ_H 2.19), but not H-2′ (δ_H 4.70), after irradiation of H-3′ (δ_H 2.52), indicating *trans*-relationship of H-3′ with H-2′ and H-4′ (δ_H 2.70). The absolute configuration at C-4′ (δ_C 59.1) was determined as *R* configuration identical to that of **7** based on the presence of the second positive Cotton effect of the n→π* transition band of an acetyl group at 283 nm ($\Delta\varepsilon$ = +15.45, MeOH), the same sign as that of **7**. Subsequently, the *R* configuration at both C-2′ (δ_C 110.1) and C-3′ (δ_C 43.6) was established. Although **3** showed a relatively low specific rotation value, $[\alpha]_D^{24}$ -8.1 (*c* 0.57, MeOH), it displayed the Cotton effects in the ECD spectrum at 218 and 283 nm of which the amplitude CEs were not close to zero. In general, the specific rotation value of scalemic mixtures would be small value or approached zero. The ECD spectrum of the corresponding mixtures also displayed weak Cotton effects (low-amplitude CEs) or the apparent lack of the Cotton effects (Deng et al. 2017; Odonbayar et al. 2019 and Zhang et al. 2019). Finally, **3** was assigned as (3*R*,4*S*,5*S*,2′*R*,3′*R*,4′*R*)-botryosphaerilactone B based on the opposite sign of the observed specific rotation of **3** to that of botryosphaerilactone B, $[\alpha]_D^{26}$ +4.5 (*c* 0.57, MeOH) (Rukachaisirikul et al. 2009).

For Peer Review Only

Compound **4** was obtained as a colorless gum with $[\alpha]_D^{24}$ -54.3 (*c* 0.57, MeOH). The UV, IR, ^{1}H (Table S1) and ^{13}C NMR (Table S2) data were similar to those of **3**. Its HRESIMS, $[M+Na]^+$ ion of *m/z* 293.1365, indicated their identical molecular formula. The planar structure of **4** was identical to **3** based on the HMQC, ^{1}H-^{1}H COSY and HMBC correlations (Figure S1). The relative stereochemistry was determined using the NOEDIFF data (Figure S2). Irradiation of H_3-6′ (δ_H 0.88) enhanced the signal intensity of H-2′ (δ_H 4.74) and H_3-8′ (δ_H 2.21), suggesting that they were a *cis*-orientation. The remaining NOEDIFF data were identical to those of **3**. Comparison of the ECD spectrum of **4**, [218 nm ($\Delta\varepsilon$ = -22.21) and 286 nm ($\Delta\varepsilon$ = -25.48), MeOH], with that of **3**, [218 nm ($\Delta\varepsilon$ = -26.21) and 283 nm ($\Delta\varepsilon$ = +15.45), MeOH], indicated that the absolute configuration at C-3 (δ_C 38.5) and C-4′ (δ_C 53.7) of **4** was assigned as *R* and *S*, respectively. Accordingly, **4** was a C-4′ epimer of **3** and named as botryosphaerilactone D.

Compound **5** was obtained as a colorless gum with $[\alpha]_D^{25}$ +30.9 (*c* 0.57, MeOH). Its ^{1}H (Table S1) and ^{13}C NMR (Table S2) data were similar to those of **4**, and also gave the same molecular formula as **4** deduced from HRESIMS peak at *m/z* 293.1335 $[M+Na]^+$. The major difference in the ^{1}H NMR spectrum of **5** was the multiplicity of H-2′ (δ_H 4.93) as a doublet with the coupling constant of 4.8 Hz between H-2′ and H-3′ (δ_H 2.63), indicating a *cis*-relationship between these protons. The assigned structure of **5** was supported by ^{1}H-^{1}H COSY and HMBC data as depicted in Figure S1. In the NOEDIFF data (Figure S2), irradiation of H_3-6′ (δ_H 1.02) enhanced only the signal intensity of H_3-8′ (δ_H 2.19), but not that of H-2′, indicating that H_3-6′ was *trans* to H-2′. The absolute configuration at C-3 (δ_C 38.5) and C-4′ (δ_C 53.4) of **5** was identified as *R* and *S*, respectively, identical to those of **4**, on the basis of the two negative Cotton effects at 217 nm ($\Delta\varepsilon$ = -25.24) and 289 nm ($\Delta\varepsilon$ = -8.34), MeOH. These results as well as the NOEDIFF data established the absolute configuration of **5** to be 3*R*, 4*S*, 5*S*, 2′*S*, 3′*R* and 4′*S*. Accordingly, **5** was a C-2′ epimer of **4** and named as botryosphaerilactone E.

Compound **7** with sufficient amount was tested for antimicrobial activity against *Staphylococcus aureus* ATCC25923, methicillin-resistant *S. aureus* SK1 (a clinical isolate), *Pseudomonas aeruginosa* ATCC27853, *Escherichia coli* ATCC25922, *Candida albicans* NCPF3153, *Cryptococcus neoformans* ATCC90113, *Microsporum gypseum* and *Penicillium marnefeii* clinical isolate. It exhibited weak antifungal activity against *C. neoformans* ATCC90113 with the MIC value of 200 μg/mL. In addition, **7** was evaluated for antimalarial

For Peer Review Only

(*Plasmodium falciparum*) and anticancer (KB cell lines) activities. It was inactive against both assays.

3. Experimental

3.1. General experimental procedures

Specific rotations were measured on a JASCO P-1020 polarimeter. Ultraviolet (UV) absorption spectra were measured in MeOH on a Perkin-Elmer Lambda 45 spectrophotometer. Electronic Circular Dichroism (ECD) spectra were recorded on a JASCO model J-810 polarimeter. Infrared (IR) spectra were obtained on a Perkin-Elmer 783 FTS165 FT-IR spectrometer. Mass spectra were recorded on a MAT 95 XL mass spectrometer (Thermo Finnigan) or Shimadzu LCMS-IT-TOF mass spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a 300 or a 500 MHz Bruker FTNMR Ultra Shield spectrometer using tetramethylsilane (TMS) as an internal standard. Thin-layer chromatography (TLC) and precoated TLC (PTLC) 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 reverse phase C_{18} silica gel.

3.2. Fungal material

The fungus NSTRU-PN1.4 was isolated from a soil sample collected from Paknakhon mangrove in Nakhon Si Thammarat province, Thailand. This fungus was deposited at the Nakhon Si Thammarat Rajabhat University Culture Collection, Thailand (NSTRU13118). Its colony on PDA was rapid growth with dark brown coloration. The microscopic morphology showed oval pycnidiospores with striations on the surface of the spore along the long axis. The ITS1-5.8S-ITS2 rDNA sequence (GenBank accession number MG946719) revealed that PN1.4 had affiliation with *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. supported by high bootstrap values and nucleotide identity of 100%. Therefore, PN1.4 should be referred to *Lasiodiplodia theobromae*.

3.3. Fermentation, extraction and purification

The fungus *L. theobromae* NSTRU-PN1.4 was grown on potato dextrose agar (PDA) at 25 °C for 5 days. Five pieces (0.5×0.5 cm^2) of mycelial agar plugs were inoculated into 500 mL Erlenmeyer flasks containing 300 mL of potato dextrose broth (PDB) at room temperature for 3 weeks. The flask culture (19.8 L) was filtered to separate into the filtrate and wet mycelia. The filtrate was divided into 66 portions. Each portion was extracted twice with ethyl acetate

For Peer Review Only

(2 × 300 mL). The organic layer was dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure to afford a dark brown gum (4.51 g). The broth extract was separated by CC over Sephadex LH-20 using MeOH to give four fractions (A-D). Fraction B (2.31 g) was purified by CC over silica gel using a gradient of MeOH/CH_2Cl_2 (1:99 → 100:0) to yield six subfractions (B1-B6). The first subfraction contained **9** (2.4 mg). Subfraction B3 (9.9 mg) was separated by PTLC using EtOAc/hexane (1:19) as a mobile phase (6 runs) to yield **10** (0.9 mg). Separation of subfraction B4 (163.1 mg) by CC over silica gel using a gradient of acetone/hexane (20:80 → 100:0) gave **7** (65.0 mg). Subfraction B5 (1.05 g) was fractionated by CC over silica gel using a gradient of MeOH/CH_2Cl_2 (1:99 → 100:0) to give eight subfractions (B51-B58). Subfraction B52 (27.0 mg) was subjected to CC over silica gel using MeOH/CH_2Cl_2 (1:199) as an eluent, and subsequent PTLC using acetone/CH_2Cl_2 (1:19) as a mobile phase (4 runs) to afford **6** (5.5 mg). Subfraction B54 (77.7 mg) was twice purified by FCC over silica gel using a gradient of EtOAc/hexane (30:70 → 100:0) and CH_2Cl_2/hexane (90:10 → 100:0), respectively, followed by PTLC using $CHCl_3$/hexane (7:3) as a mobile phase (6 runs) to obtain **8** (1.6 mg). Subfraction B55 (81.1 mg) was further purified by CC over reverse phase C_{18} silica gel using a gradient of MeOH/H_2O (40:60 → 100:0) as an eluent, and subsequent PTLC using EtOAc/hexane (3:7) as a mobile phase (7 runs) to afford **11** (1.5 mg). Subfraction B56 (283.9 mg) was chromatographed by CC over Sephadex LH-20 using MeOH/CH_2Cl_2 (1:1) as an eluent, and subsequent CC over silica gel using a gradient of EtOAc/hexane (30:70 → 100:0) to yield six subfractions (B561-B566). The second subfraction (5.0 mg), upon PTLC using MeOH/CH_2Cl_2 (0.5:99.5) as a mobile phase (2 runs), and the fifth subfraction (17.9 mg), upon CC over silica gel using a gradient of acetone/CH_2Cl_2 (2:98 → 100:0) as an eluent, gave **4** (3.3 mg) and **3** (0.7 mg), respectively. Subfraction B57 (340.7 mg) was submitted by CC over Sephadex LH-20 using MeOH/CH_2Cl_2 (1:1) as an eluent, followed by CC over reverse phase C_{18} silica gel using a gradient of MeOH/H_2O (50:50 → 100:0) as an eluent to yield eight subfractions (B571-B578). Subfraction B574 (38.4 mg) was purified by CC over silica gel using acetone/$CHCl_3$/hexane (10:55:35) to afford **1** (3.2 mg) and **2** (1.4 mg). Compound **5** (2.1 mg) was obtained from subfraction B575 (22.0 mg) after purification by CC over silica gel using acetone/$CHCl_3$/hexane (5:60:35). Subfraction B6 (177.5 mg) was fractionated by CC over Sephadex LH-20 using MeOH/CH_2Cl_2 (1:1) as an eluent, and subsequent FCC over silica gel using a gradient of MeOH/CH_2Cl_2 (0.5:99.5 → 100:0) to yield **12** (1.9 mg).

For Peer Review Only

*3.3.1. Compound **1***

Colorless gum; $[\alpha]_D^{24}$ -14.6 (*c* 0.77, MeOH); UV (MeOH) λ_{max} (log ε): 286 (1.30) nm; ECD (MeOH, *c* 0.012 M) λ_{max} ($\Delta\varepsilon$) 217 (-36.98) nm; IR (neat) ν_{max} 3451, 1770 cm^{-1}; ^1H, see Table S1 and ^{13}C NMR data, see Table S2; HRESIMS *m/z*: $[M+Na]^+$ 295.1521 (calcd for $C_{14}H_{24}O_5Na$, 295.1521)

*3.3.2. Compound **2***

Colorless gum; $[\alpha]_D^{24}$ +22.9 (*c* 0.77, MeOH); UV (MeOH) λ_{max} (log ε): 287 (1.31) nm; ECD (MeOH, *c* 0.012 M) λ_{max} ($\Delta\varepsilon$) 218 (-12.64) nm; IR (neat) ν_{max} 3402, 1772 cm^{-1}; ^1H, see Table S1 and ^{13}C NMR data, see Table S2; HRESIMS *m/z*: $[M+Na]^+$ 295.1521 (calcd for $C_{14}H_{24}O_5Na$, 295.1521)

*3.3.3. Compound **3***

Colorless gum; $[\alpha]_D^{24}$ -8.1 (*c* 0.57, MeOH); UV (MeOH) λ_{max} (log ε): 280 (1.61) nm; ECD (MeOH, *c* 0.009 M) λ_{max} ($\Delta\varepsilon$) 218 (-26.21), 283 (+15.45) nm; IR (neat) ν_{max} 1775, 1705 cm^{-1}; ^1H, see Table S1 and ^{13}C NMR data, see Table S2; HRESIMS *m/z*: $[M+Na]^+$ 293.1365 (calcd for $C_{14}H_{22}O_5Na$, 293.1365)

*3.3.4. Botryosphaerilactone D (**4**)*

Colorless gum; $[\alpha]_D^{24}$ -54.3 (*c* 0.57, MeOH); UV (MeOH) λ_{max} (log ε): 287 (2.23) nm; ECD (MeOH, *c* 0.008 M) λ_{max} ($\Delta\varepsilon$) 218 (-22.21), 286 (-25.48) nm; IR (neat) ν_{max} 1772, 1705 cm^{-1}; ^1H, see Table S1 and ^{13}C NMR data, see Table S2; HRESIMS *m/z*: $[M+Na]^+$ 293.1365 (calcd for $C_{14}H_{22}O_5Na$, 293.1365)

*3.3.5. Botryosphaerilactone E (**5**)*

Colorless gum; $[\alpha]_D^{25}$ +30.9 (*c* 0.57, MeOH); UV (MeOH) λ_{max} (log ε): 282 (1.77) nm; ECD (MeOH, *c* 0.008 M) λ_{max} ($\Delta\varepsilon$) 217 (-25.24), 289 (-8.34) nm; IR (neat) ν_{max} 1742, 1698 cm^{-1}; ^1H, see Table S1 and ^{13}C NMR data, see Table S2; HRESIMS *m/z*: $[M+Na]^+$ 293.1335 (calcd for $C_{14}H_{22}O_5Na$, 293.1365)

*3.3.6. (3R,4S,5S)-Dihydro-4-(hydroxymethyl)-3,5-dimethyl-2(3H)-furanone (**6**)*

For Peer Review Only

Pale yellow gum; $[\alpha]_D^{25}$ +51.5 (*c* 0.17, MeOH); ECD (MeOH *c* 0.045 M) λ_{max} ($\Delta\varepsilon$) 216 (-133.37) nm.

3.3.7. (3R,4R)-4-Acetyl-3-methyl-2(3H)-dihydrofuranone (7)

Pale yellow gum; $[\alpha]_D^{24}$ +81.9 (*c* 0.57, MeOH); ECD (MeOH *c* 0.034 M) λ_{max} ($\Delta\varepsilon$) 216 (-156.77), 284 (+17.88) nm.

3.4. Antimicrobial assays

Antimicrobial activity was determined as described by the Clinical and Laboratory Standards Institute (Drummond and Waigh 2000; Clinical and Laboratory Standards Institute 2002a, 2002b, 2002c). Vancomycin, amphotericin B and miconazole were used as positive controls for bacteria, yeasts and fungus with the MIC values of 0.69, 0.27 and 2.40 μM, respectively.

3.5. Antimalarial assay

Antimalarial activity was evaluated against the parasite *P. falciparum* (K1, multi-drug-resistant strain), using the microculture radioisotope technique based on the method described (Desjardins et al. 1979). Dihydroartemisinine and mefloquine were used as standard compounds and exhibited the IC_{50} values of 0.0023 and 0.0269 μM, respectively.

3.6. Cytotoxicity assay

The cytotoxicity assay against KB cell lines was evaluated using the resazurin microplate assay (O'Brien et al. 2000). Ellipticine (IC_{50} 8.24 μM) and doxorubicin (IC_{50} 2.19 μM) were used as standard drugs for KB cell lines.

4. Conclusion

Dimeric γ-lactones, botryosphaerilactones A-C, were firstly reported as secondary metabolites from the endophytic fungi *B. rhodina* PSU-M35 and PSU-M114 which were isolated from leaves of *Garcinia mangostana* (Rukachaisirikul et al. 2009). Interestingly, five dimeric γ-lactones (**1-5**) isolated from the soil-derived fungus *L. theobromae* NSTRU-PN1.4 possessed identical planar structures to those of botryosphaerilactones A-C. Compounds **1-3** were identified to be the enantiomers of botryosphaerilactones A, C and B, respectively, whereas two metabolites **4** and **5** were reported as new diastereomers of **3**. Additionally, this is first time to isolate dimeric γ-lactones from soil-derived fungi.

For Peer Review Only

Acknowledgments

J. Arunpanichlert is grateful to the Thailand Research Fund for a research grant (Grant No. MRG5980088). The Center of Excellence for Innovation in Chemistry (PERCH-CIC), Ministry of Higher Education, Science, Research and Innovation and Thammasat University are gratefully acknowledged for partial support. We are also thankful to Dr. Winai Ieawsuwan, Chulabhorn Research Institute, for 2D NMR data collection. V. Rukachaisirikul thanks the NSTDA Chair Professor grant (the Fourth Grant) of the Crown Property Bureau and National Science and Technology Development Agency. Finally, the National Center for Genetic Engineering and Biotechnology (BIOTEC) is acknowledged for antimalarial and cytotoxic assays.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Thailand Research Fund for a research grant [grant number MRG5980088]; the NSTDA Chair Professor grant (the Fourth Grant) of the Crown Property Bureau and National Science and Technology Development Agency.

References

Andolfi A, Maddau L, Cimmino A, Linaldeddu BT, Basso S, Deidda A, Serra S, Evidente A. 2014. Lasiojasmonates A-C, three jasmonic acid esters produced by *Lasiodiplodia* sp., a grapevine pathogen. Phytochemistry. 103:145-153.

Biswas B, Sarkar D, Venkateswaran RV. 2008. Total synthesis of alboatrin, a phytotoxic metabolite from *Verticillium alboatrum*. Tetrahedron. 64:3212-3216.

Cabedo N, López-Gresa MP, Primo J, Ciavatta ML, González-Mas MC. 2007. Isolation and structural elucidation of eight new related analogues of the mycotoxin (−)-botryodiplodin from *Penicillium coalescens*. J Agric Food Chem. 55:6977-6983.

Chen S, Chen D, Cai R, Cui H, Long Y, Lu Y, Li C, She Z. 2016. Cytotoxic and antibacterial preussomerins from the mangrove endophytic fungus *Lasiodiplodia theobromae* ZJ-HQ1. J Nat Prod. 79:2397-2402.

For Peer Review Only

Clinical and Laboratory Standards Institute (CLSI). 2002a. Reference method for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved standard M7-A4. Wayne, PA: Clinical and Laboratory Standards Institute.

Clinical and Laboratory Standards Institute (CLSI). 2002b. Reference method for broth dilution antifungal susceptibility testing of yeasts; Approved Standard M27-A2. 2nd ed. Wayne, PA: Clinical and Laboratory Standards Institute.

Clinical and Laboratory Standards Institute (CLSI). 2002c. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; Approved Standard. CLSI Documents M38-A. Wayne, PA: Clinical and Laboratory Standards Institute.

Daengrot C, Rukachaisirikul V, Tansakul C, Thongpanchang T, Phongpaichit S, Bowornwiriyapan K, Sakayaroj J. 2015. Eremophilane sesquiterpenes and diphenyl thioethers from the soil fungus *Penicillium copticola* PSU-RSPG138. J Nat Prod. 78: 615-622.

Deng AJ, Zhang HJ, Li Q, Li ZH, Zhang ZH, Wu LQ, Li L, Qin HL. 2017. Six scalemic mixtures of 6-monosubstituted dihydrobenzophenanthridine alkaloids from *Chelidonium majus* and optically active structures of enantiomers. Phytochemistry. 144:159-170.

Desjardins RE, Canfield CJ, Haynes JD, Chulay JD. 1979. Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. Antimicrob Agents Chemother. 16:710-718.

Drummond AJ, Waigh RD. 2000. Recent research developments in phytochemistry. 4:143-152.

Félix C, Salvatore MM, DellaGreca M, Meneses R, Duarte AS, Salvatore F, Naviglio D, Gallo M, Jorrín-Novo JV, Alves A, Andolfi A, Esteves AC. 2018. Production of toxic metabolites by two strains of *Lasiodiplodia theobromae*, isolated from a coconut tree and a human patient. Mycologia. 110:642-653.

Forzato C, Furlan G, Ghelfi F, Nitti P, Pitacco G, Roncaglia F, Valentin E. 2007. Lipase-catalysed deacetylation of botryodiplodin acetate. Tetrahedron Asymmetry. 18:447-450.

Forzato C, Furlan G, Nitti P, Pitacco G, Marchesan D, Coriani S, Valentin E. 2005. A combined experimental and computational strategy in the assignment of absolute configurations of 4-methyl-5-oxo-tetrahydrofuran-3-carboxylic acids and their esters. Tetrahedron Asymmetry. 16:3011-3023.

For Peer Review Only

Forzato C, Nitti P, Pitacco G. 1997. Bicyclic γ-butyrolactones. Relation between conformation of the lactone ring and chiroptical properties. Tetrahedron Asymmetry. 8:4101-4110.

Hosoyama H, Shigemori H, Kobayashi J. 2000. Further unexpected boron trifluoride-catalyzed reactions of taxoids with α- and β-4,20-epoxides. J Chem Soc Perkin Trans 1. 449-451.

Isaka M, Chinthanom P, Veeranondha S, Supothina S, Luangsa-ard JJ. 2008. Novel cyclopropyl diketones and 14-membered macrolides from the soil fungus *Hamigera avellanea* BCC 17816. Tetrahedron. 64:11028-11033.

Kamal N, Viegelmann CV, Clements CJ, Edrada-Ebel R. 2017. Metabolomics-guided isolation of anti-trypanosomal metabolites from the endophytic fungus *Lasiodiplodia theobromae*. Planta Med. 83:565-573.

Lee TWS, Rettig SJ, Stewart R, Trotter J. 1984. Six pyrimidonium salts: determination of their crystal structures and studies of their prototropic behavior in solution. Can J Chem. 62:1194-1202.

Matsumoto T, Okabe T, Fukui K. 1973. Pyrrolizidine alkaloids. The absolute configurations of latifolic acid and its stereoisomers. Chem Lett. 2:773-776.

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. Eur J Biochem. 267:5421-5426.

Odonbayar B, Murata T, Suganuma K, Ishikawa Y, Buyankhishig B, Batkhuu J, Sasaki K. 2019. Acylated lignans isolated from *Brachanthemum gobicum* and their trypanocidal activity. J Nat Prod. 82:774-784.

Ravi BN, Armstrong RW, Faulkner DJ. 1979. Some aromatic compounds from the marine sponge *Plakortis halichlondriodes*. J Org Chem. 44:3109-3113.

Reino JL, Guerrero RF, Hernández-Galán R, Collado IG. 2008. Secondary metabolites from species of the biocontrol agent *Trichoderma*. Phytochem Rev. 7:89-123.

Renauld F, Moreau S, Lablachecombier A, Tiffon B. 1985. Botryodiplodin, a mycotoxin from *Penicillium roqueforti*. Reaction with amino-pyrimidines, amino-purines and 2′-deoxynucleosides. Tetrahedron. 41:955-962.

Rukachaisirikul V, Arunpanichlert J, Sukpondma Y, Phongpaichit S, Sakayaroj J. 2009. Metabolites from the endophytic fungi *Botryosphaeria rhodina* PSU-M35 and PSU-M114. Tetrahedron. 65:10590-10595.

For Peer Review Only

Sansinenea E, Ortiz A. 2011. Secondary metabolites of soil *Bacillus* spp. Biotechnol Lett. 33:1523-1538.

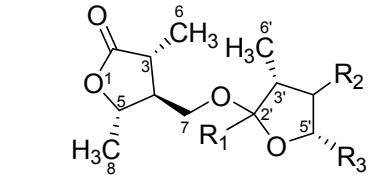
Smith EL, Spackman DH, Polglase WJ. 1952. The specificity of leucine aminopeptidase. III. Action on diastereoisomers. J Biol Chem. 199:801-817.

Tanaka Y, Shiomi K, Kamei K, Sugoh-hagino M, Enomoto Y, Fang F, Yamaguchi Y, Masuma R, Zhang CG, Zhang XW, Ōmura S. 1998. Antimalarial activity of radicicol, heptelidic acid and other fungal metabolites. J Antibiot. 51:153-160.

Umeokoli BO, Ebrahim W, El-Neketi M, Müller WEG, Kalscheuer R, Lin W, Liu Z, Proksch P. 2019. A new depsidone derivative from mangrove sediment derived fungus *Lasiodiplodia theobromae*. Nat Prod Res. 33:2215-2222.

Zhang CY, Gao Y, Zhu RX, Qiao YN, Zhou JC, Zhang JZ, Li Y, Li SW, Fan SH, Lou HX. 2019. Prenylated bibenzyls from the chinese liverwort *Radula constricta* and their mitochondria-derived paraptotic cytotoxic activities. J Nat Prod. 82:1741-1751.

For Peer Review Only



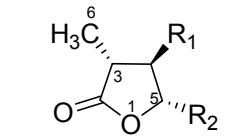
1: R_1 = α-H, R_2 = β-$\overset{7'}{C}H_2OH$, R_3 = $\overset{8'}{C}H_3$

2: R_1 = β-H, R_2 = β-$\overset{7'}{C}H_2OH$, R_3 = $\overset{8'}{C}H_3$

3: R_1 = α-H, R_2 = β-$\overset{7'}{C}O\overset{8'}{C}H_3$, R_3 = H

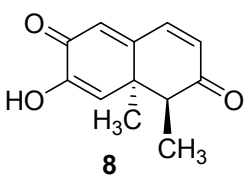
4: R_1 = α-H, R_2 = α-$\overset{7'}{C}O\overset{8'}{C}H_3$, R_3 = H

5: R_1 = β-H, R_2 = α-$\overset{7'}{C}O\overset{8'}{C}H_3$, R_3 = H

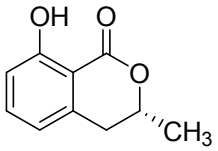


6: R_1 = $\overset{7}{C}H_2OH$, R_2 = $\overset{8}{C}H_3$

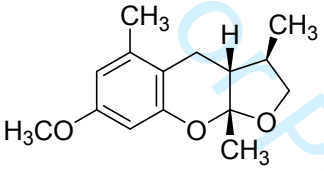
7: R_1 = $\overset{7}{C}O\overset{8}{C}H_3$, R_2 = H



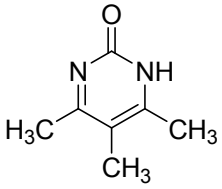
8



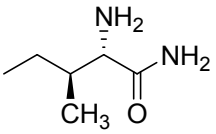
9



10



11



12

Figure 1. Structures of compounds **1-12** isolated from *Lasiodiplodia theobromae* NSTRU-PN1.4.

For Peer Review Only

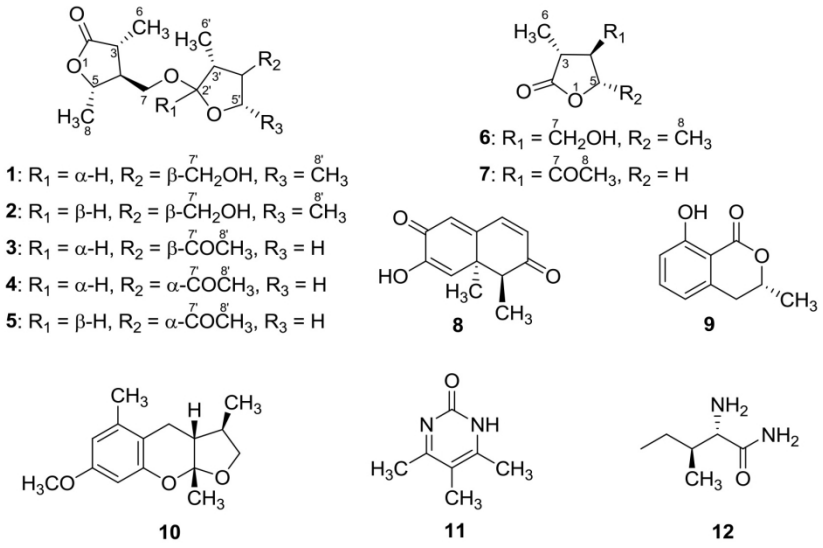


Figure 1. Structures of compounds **1-12** isolated from *Lasiodiplodia theobromae* NSTRU-PN1.4.

173x110mm (300 x 300 DPI)