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Metabolites from the endophytic fungi *Botryosphaeria rhodina* PSU-M35 and PSU-M114

Vatcharin Rukachaisirikul [a,*], Jiraporn Arunpanichlert [a], Yaowapa Sukpondma [a], Souwalak Phongpaichit [b], Jariya Sakayaroj [c]

[a] Department of Chemistry and Center for Innovation in Chemistry, Faculty of Science, Prince of Songkla University, Songkhla 90112, Thailand
[b] Department of Microbiology, Faculty of Science, Prince of Songkla University, Songkhla 90112, Thailand
[c] National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand Science Park, Klong Luang, Pathumthani 12120, Thailand

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ABSTRACT

Three dimeric γ-lactones (**1**–**3**), one dihydronaphthalene-2,6-dione (**4**), one hexahydroindenofuran (**5**), one cyclopentanone (**6**), and one lasiodiplodin (**7**) were isolated from the endophytic fungi *Botryosphaeria rhodina* PSU-M35 and PSU-M114 along with twelve known metabolites. The structures and the proposed stereochemistry of the new metabolites were established by spectral data analysis. The isolated compounds were submitted for evaluation of the antibacterial activity against *Staphylococcus aureus*, both standard and methicillin-resistant strains.

1. Introduction

Various types of compounds were isolated from the genus *Botryosphaeria*. Some of them showed interesting biological activities such as antibacterial primin,[1] antiseptic mellein,[2] phytotoxic 4-hydroxymellein,[3] and antimicrobial lasiodiplodin.[4] In our ongoing search on bioactive metabolites from endophytic fungi, the broth extracts of two endophytic fungi, *B. rhodina* PSU-M35 and PSU-M114, displayed antibacterial activity against *Staphylococcus aureus*, both standard ATCC 25922 (SA) and methicillin-resistant (MRSA) strains. We describe herein the isolation of three new dimeric γ-lactones (**1**–**3**), one new dihydronaphthalene-2,6-dione (**4**), one new hexahydroindenofuran (**5**) together with two known compounds, dihydro-4-(hydroxymethyl)-3,5-dimethyl-2(3*H*)-furanone (**8**)[5] and (3*S*,4*S*)-(-)-4-acetyl-3-methyl-2(3*H*)-dihydrofuranone (**9**)[6] from *B. rhodina* PSU-M35. In addition, one new carboxylic acid derivative (**6**) and one new lasiodiplodin derivative (**7**) as well as ten known ones, (3*S*)-lasiodiplodin (**10**),[7] (5*R*)-hydroxylasiodiplodin (**11**),[8] (5*S*)-hydroxylasiodiplodin (**12**),[8] (*R*)-(-)-mellein (**13**),[9] *cis*-(3*R*,4*R*)-(-)-4-hydroxymellein (**14**),[10] *trans*-(3*R*,4*S*)-(-)-4-hydroxymellein (**15**),[10] (*R*)-(-)-5-hydroxymellein (**16**),[11] (*R*)-(-)-2-octeno-δ-lactone (**17**),[12] tetrahydro-4-hydroxy-6-propylpyran-2-one (**18**),[13] and 6-methylsalicylic acid (**19**)[14] were obtained from *B. rhodina* PSU-M114. Their antibacterial activity against SA and MRSA strains was examined.

2. Results and discussion

The endophytic fungi PSU-M35 and PSU-M114 were isolated from the leaves of *Garcinia mangostana*. As neither conidia nor spores were observed, these fungi were identified based on the analyses of the nuclear ribosomal internal transcribed spacer (ITS) regions. The ITS sequences of PSU-M35 (EF564146) and PSU-M114 (EF564147) are well placed in the *B. rhodina* subclade comprising nine sequences of *B. rhodina*. (DQ008312, DQ008311, DQ008310, DQ008309, DQ008308, DQ307677, AY568635, AY9412180 and AY745998) with 89% statistical support and sequence similarity between 99.2 and 100.0%. Moreover, sequence similarity between PSU-M35 and PSU-M114 was 98.7%. These fungi were then identified as *B. rhodina*.

The crude EtOAc extract from the culture broth of the fungus PSU-M35 was subjected to various chromatographic techniques, leading to the isolation of five new (**1**–**5**) and two known (**8**–**9**) compounds. All metabolites except for compound **4** were γ-lactone derivatives. One new compound (**6**) along with ten known ones (**10**–**19**) were obtained from the broth extract of the fungus PSU-M114. In addition, investigation of the mycelial extract afforded one new metabolite (**7**). Compounds **11** and **12** were isolated as their

* Corresponding author. Tel.: +66 74 288 435; fax: +66 74 212 918.
E-mail address: vatcharin.r@psu.ac.th (V. Rukachaisirikul).

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acetate derivative. Their structures were elucidated by analysis of spectroscopic data, including IR, UV, NMR and MS. The relative configuration was assigned according to NOEDIFF results. In addition, the absolute configuration of compounds **1**–**3** and **8** was proposed on the basis of the known absolute configuration of **9**. It is worth to note that compounds **1**–**3** were not artifacts formed from the monomers **8** and **9** as their anomeric protons (H-10) were present in the ^{1}H NMR spectrum of the fraction obtained from the first chromatographic separation of the crude extract.

Botryosphaerilactone A (**1**) was obtained as a colorless gum with $[\alpha]_D^{27}$ −1.2 (*c* 0.77, MeOH). The UV spectrum showed a maximum absorption band at λ_{max} 286 nm. Its IR spectrum exhibited absorption bands at 3438 and 1770 cm^{-1} for hydroxyl and γ-lactone carbonyl groups, respectively. The HREIMS showed the molecular formula $C_{14}H_{24}O_5$, corresponding to three degrees of unsaturation. The ^{1}H NMR spectral data (Table 1) consisted of signals for the γ-lactone identical to those of **8**, three methine protons [δ_H 4.31 (dq, *J*=9.0 and 6.5 Hz, 1H), 2.54 (dq, *J*=11.5 and 7.2 Hz, 1H) and 1.92 (m, 1H)], two nonequivalent oxymethylene protons [δ_H 3.85 (dd, *J*=10.2 and 4.2 Hz, 1H) and 3.41 (dd, *J*=10.2 and 5.4 Hz, 1H)] and two methyl groups [δ_H 1.43 (d, *J*=6.5 Hz, 3H) and 1.27 (d, *J*=7.2 Hz, 3H)]. The ^{1}H–^{1}H COSY and HMBC data as well as the ^{13}C NMR data supported the presence of this moiety.

The remaining proton signals belonged to a γ-lactol derivative of **8**. The following ^{1}H–^{1}H COSY and HMBC correlations were in agreement with this conclusion. In the ^{1}H–^{1}H COSY spectrum, the anomeric proton, H-10 (δ_H 4.68), was coupled with H-14 (δ_H 2.03), which was further coupled with H-13 (δ_H 1.54) and H$_3$-15 (δ_H 1.14). The same correlations of H-13/H-12 (δ_H 3.98), H-14 and H$_2$-16 (δ_H 3.71 and 3.68) and H-12/H$_3$–17 (δ_H 1.35) were also observed. A ^3J HMBC correlation of H-10 with C-12 (δ_C 77.5) together with the chemical shift of C-10 (δ_C 109.9) established the lactol unit having the methyl groups at C-12 and C-14 and the hydroxymethyl group at C-13. The ether linkage between C-8 (δ_C 65.4) of the lactone unit with C-10 of the lactol ring was established according to a HMBC correlation of H$_2$-8 with C-10 as well as that of H-10 with C-8.

The relative configuration of the lactone ring was identical to that of **8** on the basis of signal enhancement of H$_3$-6 and H$_3$-7 upon irradiation of H-4 in the NOEDIFF experiment. In addition, the lactol unit displayed signal enhancement of H$_3$-15 and H$_3$-17 after irradiation H-13. These NOEDIFF results were identical to those of the lactone ring. Moreover, irradiation of H$_3$-15 affected the signal intensity of H-10, thus indicating their *cis*-orientation. As compounds **1** and **8** were co-metabolites, we proposed that **1** would be derived in nature from two molecules of **8**. The spatial arrangement of both rings would be the same as that of **8**. Consequently, the absolute configuration in **1** was proposed to be 3*S*, 4*R*, 5*R*, 10*S*, 12*R*, 13*R* and 14*S* on the basis of known absolute configuration of its cometabolite **8**. Consequently, botryosphaerilactone A had the structure **1** (Fig. 1).

Botryosphaerilactone B (**2**) was obtained as a colorless gum with $[\alpha]_D^{26}$ +4.5 (*c* 0.57, MeOH). The UV and IR spectra were similar to those of **1**. The HREIMS showed the molecular formula $C_{14}H_{22}O_5$, indicating that **2** contained one degree of unsaturation more than **1**. The ^{1}H NMR spectral data (Table 1) were similar to those of **1** except for proton signals of the lactol ring. The hydroxymethylene protons [δ_H 3.71 (dd, *J*=10.0 and 5.0 Hz, 1H) and 3.68 (dd, *J*=10.0 and 5.0 Hz, 1H)] in **1** were replaced, in **2**, by the methyl protons of an acetyl group (δ_H 2.19, s, 3H). The attachment of the acetyl group at C-13 (δ_C 58.5) of the lactol unit was supported by a HMBC correlation of H$_3$-17 with C-13. In addition, the oxymethine proton (δ_H 3.98) and the coupled methyl protons (δ_H 1.35) in **1** were substituted by two nonequivalent oxymethylene protons [δ_H 4.20 (dd, *J*=9.0 and 7.0 Hz, 1H) and 4.06 (t, *J*=9.0 Hz, 1H)] in **2**. These results established the lactol with the acetyl and methyl groups at C-13 and C-14, respectively. Irradiation of H-14 (δ_H 2.52, m, 1H) in the NOEDIFF experiment enhanced signal intensity of H$_3$-15 (δ_H 1.18) and H$_3$-17 (δ_H 2.19), but not H-10, indicating *trans*-relationship between H-10 and H-14. These results indicated that the configuration of C-10, C-13 and C-14 was identical to that of **1**. Thus, botryosphaerilactone B (**2**) was assigned as a new dimeric γ-lactone, which would be derived from the condensation of **8** and a lactol derivative of **9**.

Table 1
NMR data for botryosphaerilactones A (**1**), B (**2**) and C (**3**)

Position	**1**		**2**		**3**		HMBC
	δ_H, mult, *J* in Hz	δ_C, mult.	δ_H, mult, *J* in Hz	δ_C, mult.	δ_H, mult, *J* in Hz	δ_C, mult.	
2		178.7, qC		178.6, qC		178.8, qC	
3	2.54, dq, 11.5, 7.2	38.3, CH	2.52, m	38.1, CH	2.58, dq, 11.5, 7.0	38.2, CH	C-2, C-4, C-7, C-8
4	1.92, m	51.4, CH	1.86, m	51.4, CH	1.93, m	51.6, CH	C-3, C-5, C-6, C-7, C-8
5	4.31, dq, 9.0, 6.5	77.5, CH	4.26, dq, 9.5, 6.0	77.3, CH	4.33, dq, 9.0, 6.0	77.6, CH	C-8
6	1.43, d, 6.5	20.0, CH_3	1.41, d, 6.0	19.9, CH_3	1.45, d, 6.0	20.0, CH_3	C-4, C-5
7	1.27, d, 7.2	14.2, CH_3	1.24, d, 7.0	14.1, CH_3	1.27, d, 7.0	14.0, CH_3	C-2, C-3, C-4
8	a: 3.85, dd, 10.2, 4.2 b: 3.41, dd, 10.2, 5.4	65.4, CH_2	a: 3.78, dd, 10.0, 4.5 b: 3.36, dd, 10.0, 5.5	64.9, CH_2	a: 3.86, dd, 10.0, 5.5 b: 3.40, dd, 10.0, 4.0	64.5, CH_2	C-3, C-4, C-5, C-10
10	4.68, d, 1.5	109.9, CH	4.69, d, 2.0	109.5, CH	4.79, d, 4.5	105.0, CH	C-8, C-12, C-13, C-14
12	3.98, dq, 7.5, 6.0	77.5, CH	a: 4.20, dd, 9.0, 7.0 b: 4.06, t, 9.0	67.0, CH_2	4.06, dq, 8.5, 6.5	79.4, CH	C-10, C-13, C-16
13	1.54, m	55.7, CH	2.70, m	58.5, CH	1.74, m	52.9, CH	C-10, C-15, C-16, C-17
14	2.03, m	43.9, CH	2.52, m	43.3, CH	2.08, m	41.5, CH	C-10, C-15, C-16
15	1.14, d, 7.2	18.5, CH_3	1.18, d, 7.0	18.0, CH_3	1.03, d, 7.0	12.0, CH_3	C-10, C-13, C-14
16	a: 3.71, dd, 10.0, 5.0 b: 3.68, dd, 10.0, 5.0	63.5, CH_2		205.5, qC	a: 3.80, dd, 10.0, 4.5 b: 3.66, dd, 10.0, 6.0	62.6, CH_2	C-12, C-13, C-14
17	1.35, d, 6.0	20.9, CH_3	2.19, s	29.0, CH_3	1.32, d, 6.5	23.2, CH_3	C-12, C-13

Botryosphaerilactone C (**3**) was obtained as a colorless gum with $[\alpha]_D^{26}$ +5.2 (*c* 0.77, MeOH). The UV, IR, ^{1}H (Table 1) and ^{13}C NMR (Table 1) spectra were almost identical to those of **1**. Furthermore, the HREIMS of **1** and **3** displayed the same molecular formula. These results indicated that **3** might be an isomer of **1**. Irradiation of H-14 affected the signal intensity of H-10 (δ_H 4.79) and H$_{ab}$-16 (δ_H 3.80 and 3.66), indicating their *cis*-relationship. Thus, botryosphaerilactone C (**3**) was assigned as a C-10 epimer of **1**.

Botryosphaeridione (**4**) was obtained as a pale yellow gum with $[\alpha]_D^{27}$ −13.2 (*c* 0.68, MeOH). The UV spectrum showed maximum absorption bands at λ_{max} 242 and 289 nm. Its IR spectrum displayed absorption bands at 3354 and 1673 cm^{-1} for hydroxyl and conjugated carbonyl groups, respectively. The HREIMS showed the molecular formula $C_{12}H_{12}O_3$, indicating that compound **4** consisted of seven degrees of unsaturation. The ^{1}H NMR spectral data (Table 2) exhibited signals of *cis*-olefinic protons of an α,β-unsaturated carbonyl unit (δ_H 7.22, d, *J*=10.2 Hz, 1H and 6.20, d, *J*=10.2 Hz, 1H), two olefinic protons of trisubstituted double bonds (δ_H 6.64, s, 1H and

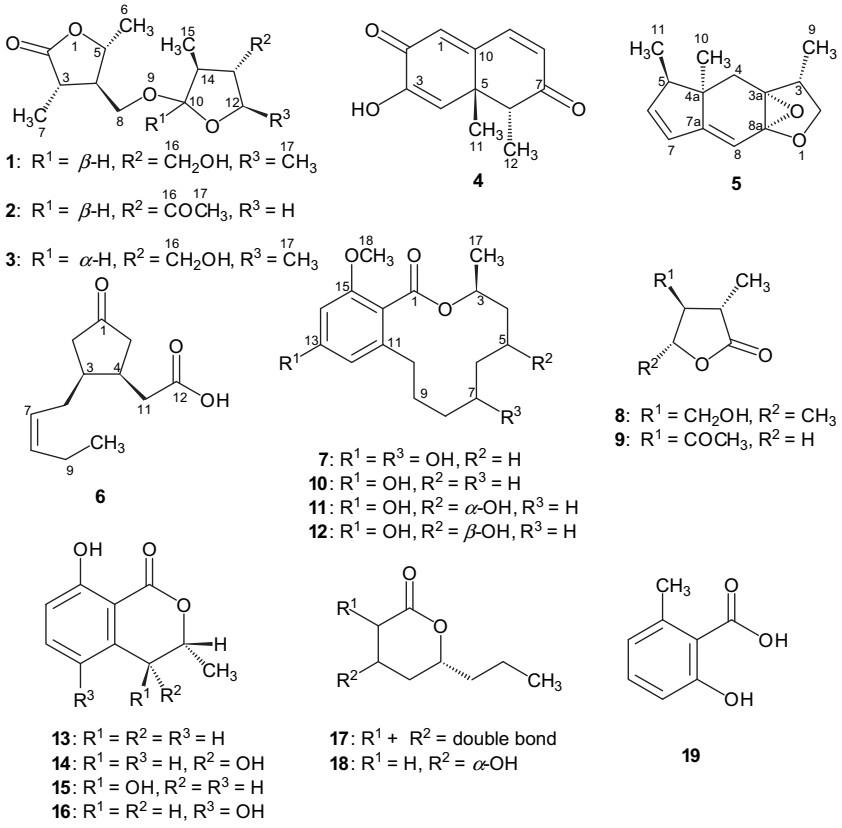

Figure 1. Metabolites isolated from the endophytic fungi *B. rhodina* PSU-M35 and PSU-M114.

6.06, s, 1H), one hydroxy proton (δ_H 6.41, s, 1H), one methine proton (δ_H 2.64, q, J=7.2 Hz, 1H) and two methyl groups (δ_H 1.39, s, 3H and 0.91, d, J=7.2 Hz, 3H). The ^{13}C NMR spectrum (Table 2) showed two ketone carbonyl (δ_C 201.0 and 181.4), three quaternary (δ_C 158.2, 148.2 and 45.7), five methine (δ_C 141.5, 129.7, 128.1, 123.7 and 53.4) and two methyl (δ_C 29.4 and 15.6) carbons. In the ^{1}H–^{1}H COSY spectrum, the methyl protons, H$_3$-12 (δ_H 0.91), were correlated with H-6 (δ_H 2.64) while one of the *cis*-olefinic protons of the α,β-unsaturated carbonyl unit, H-8 (δ_H 6.20), gave a cross peak with the other one, H-9 (δ_H 7.22). The HMBC correlations of both H-6 and H-9 with C-5 (δ_C 45.7), C-7 (δ_C 201.0) and C-10 (δ_C 158.2) as well as the chemical shift of C-10 established a cyclohexenone unit with the methyl group and an exocyclic double bond at C-6 (δ_C 53.4) and C-10, respectively. The location of the methyl group was confirmed on the basis of the HMBC cross peaks of these methyl protons with C-5, C-6 and C-7. The ^3J HMBC correlations of the olefinic proton, H-1 (δ_H 6.64), with C-5 and C-9 (δ_C 141.5) further implied that the exocyclic double bond was the trisubstituted one.

Table 2
NMR data for botryosphaeridione (**4**)

Position	δ_H, mult, J in Hz	δ_C, mult.	HMBC correlation
1	6.64, s	128.1, CH	C-3, C-5, C-9
2		181.4, qC	
3-OH	6.41, s	148.2, qC	C-2, C-3, C-4
4	6.06, s	123.7, CH	C-2, C-3, C-5, C-6, C-10, C-11
5		45.7, qC	
6	2.64, q, 7.2	53.4, CH	C-5, C-7, C-10, C-11, C-12
7		201.0, qC	
8	6.20, d, 10.2	129.7, CH	C-6, C-9, C-10
9	7.22, d, 10.2	141.5, CH	C-1, C-5, C-7, C-10
10		158.2, qC	
11	1.39, s	29.4, CH$_3$	C-3, C-4, C-5, C-6, C-10
12	0.91, d, 7.2	15.6, CH$_3$	C-5, C-6, C-7

The hydroxy proton, 3-OH (δ_H 6.41), showed HMBC cross peaks with C-2 (δ_C 181.4), C-3 (δ_C 148.2) and C-4 (δ_C 123.7) while the remaining olefinic proton, H-4 (δ_H 6.06), was correlated with C-2. Thus, a 2-hydroxypropenonyl unit was constructed. The HMBC correlations of H-4/C-6 and C-10 and that of H-1/C-3 established a naphthalenedione skeleton by connecting C-1 and C-5 of above cyclohexenone with C-2 and C-4 of the 2-hydroxypropenonyl unit, respectively. The remaining methyl protons, H$_3$-11 (δ_H 1.39), exhibited the HMBC correlations with C-4, C-5 and C-10 (δ_C 158.17), connecting the methyl group at C-5. The relative configuration of compound **4** was assigned by the NOEDIFF results. Irradiation of H-6 enhanced signal intensity of H$_3$-11, indicating their *cis*-relationship. Therefore, botryosphaeridione (**4**) was assigned as a new dihydronaphthalene-2,6-dione derivative.

Botryosphaerihydrofuran (**5**) was obtained as a colorless gum with $[\alpha]_D^{27}$ −23.8 (*c* 0.14, MeOH). The UV spectrum displayed absorption bands at λ_{max} 220 and 280 nm. The IR spectrum showed an absorption band at 1664 cm^{-1} for a double bond functional group. The HREIMS displayed the molecular formula $C_{14}H_{18}O_2$, implying the presence of six degrees of unsaturation. The ^{1}H NMR data (Table 3) consisted of signals of *cis*-olefinic protons [δ_H 6.89 (d, J=10.0 Hz, 1H) and 5.90 (d, J=10.0 Hz, 1H)], one olefinic proton of a trisubstituted double bond (δ_H 6.01, s, 1H), two sets of nonequivalent methylene protons [δ_H 4.15 (t, J=9.0 Hz, 1H) and 3.40 (t, J=9.0 Hz, 1H); 1.76 (d, J=14.5 Hz, 1H) and 1.49 (d, J=14.5 Hz, 1H)], two methine protons [δ_H 2.52 (m, 1H) and 2.31 (m, 1H)] and three methyl groups [δ_H 1.41 (s, 3H), 1.05 (d, J=7.0 Hz, 3H) and 0.99 (d, J=7.0 Hz, 3H)]. The ^{13}C NMR spectrum (Table 3) showed four quaternary (δ_C 139.3, 99.4, 77.0 and 38.8), five methine (δ_C 143.6, 131.7, 126.4, 53.6 and 43.4), two methylene (δ_C 71.3 and 33.1) and three methyl (δ_C 27.8, 14.4 and 8.9) carbons. The ^{1}H–^{1}H COSY spectrum showed cross peaks of H$_3$-11 (δ_H 0.99)/H-5 (δ_H 2.31) and H-6 (δ_H 5.90)/H-5 and H-7 (δ_H 6.89). The HMBC spectrum displayed correlations of H-5/C-4a (δ_C 38.8), C-7a

(δ_C 139.3), C-10 (δ_C 27.8) and C-11 (δ_C 14.4), H-7/C-4a, C-7a and C-8 (δ_C 131.7) and H_3-10 (δ_H 1.41)/C-4 (δ_C 33.1), C-4a, C-5 and C-7a. Furthermore, C-4 and C-8 gave HMQC cross peaks with H_{ab}-4 (δ_H 1.76 and 1.49) and H-8 (δ_H 6.01), respectively. These results together with HMBC correlations of H-8 with C-4a and C-7a and those of H_{ab}-4 with C-4a and C-5 established a cyclopentene having a *cis*-double bond at C-6 and C-7, two methyl groups at C-4a and C-5 as well as an exocyclic trisubstituted double bond and one methylene unit at C-7a and C-4a, respectively.

Table 3
NMR data for botryosphaerihydrofuran (**5**)

Position	δ_H, mult, *J* in Hz	δ_C, mult.	HMBC correlation
2	a: 4.15, t, 9.0 b: 3.40, t, 9.0	71.3, CH_2	C-3, C-3a, C-8a, C-9 C-3, C-9
3	2.52, m	43.4, CH	C-3a, C-4
3a		77.0, qC	
4	a: 1.76, d, 14.5 b: 1.49, d, 14.5	33.1, CH_2	C-3a, C-4a, C-5, C-10 C-3a, C-4a, C-5, C-7a, C-8a, C-10
4a		38.8, qC	
5	2.31, m	53.6, CH	C-4a, C-7a, C-10, C-11
6	5.90, d, 10.0	126.4, CH	C-5, C-7a
7	6.89, d, 10.0	143.6, CH	C-4a, C-7a, C-8
7a		139.3, qC	
8	6.01, s	131.7, CH	C-3a, C-4a, C-7
8a		99.4, qC	
9	1.05, d, 7.0	8.9, CH_3	C-2, C-3, C-3a
10	1.41, s	27.8, CH_3	C-4, C-4a, C-5, C-7a
11	0.99, d, 7.0	14.4, CH_3	C-4a, C-5

The methine proton, H-3 (δ_H 2.52), was coupled with H_{ab}-2 (δ_H 4.15 and 3.40) and H_3-9 (δ_H 1.05) in the ^1H–^1H COSY spectrum. The HMBC correlations of H_3-9/C-2 (δ_C 71.3), C-3 (δ_C 43.4) and C-3a (δ_C 77.0) and H_{ab}-2/C-3a, C-8a (δ_C 99.4) and C-9 (δ_C 8.9) together with the chemical shift of C-2 established a tetrahydrofuran moiety with a methyl group at C-3. The chemical shifts at C-3a and C-8a established an epoxide unit at these carbons. The linkage between C-3a and C-4 and that between C-8a and C-8 (δ_C 131.7) were established according to the HMBC correlations of H-4 with C-3a and C-8a and that of H-8 with C-3a to form a tetracyclic skeleton. Irradiation of the methine proton, H-5 (δ_H 2.31), in the NOEDIFF experiment enhanced signal intensity of H_3-10, indicating their *cis*-relationship. In addition, signal intensity of H_a-4 was enhanced after irradiation of either H_3-9 or H_3-11. These results together with the molecular model (Fig. 2) established the relative configuration as shown. Therefore, botryosphaerihydrofuran was assigned to have the structure **5** (Fig. 1).

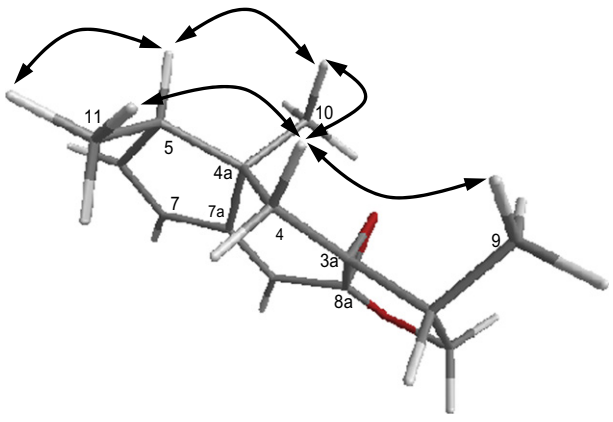

Figure 2. Selected NOEDIFF data of botryosphaerihydrofuran (**5**).

Botryosphaerinone (**6**) was obtained as a colorless gum with $[\alpha]_D^{28}$ −23.2 (*c* 0.14, MeOH). The IR spectrum exhibited absorption bands at 3441 and 1728 cm^{-1} for hydroxyl and carbonyl groups, respectively. The HREIMS showed the molecular formula $C_{12}H_{18}O_3$. The ^1H NMR spectral data (Table 4) exhibited signals of *cis*-olefinic protons [δ_H 5.39 (dtt, *J*=10.5, 7.2 and 1.2 Hz, 1H) and 5.19 (m, 1H)], two methine protons [δ_H 2.29 (m, 1H) and 1.85 (m, 1H)], five sets of methylene protons [δ_H 2.68 (dd, *J*=18.5 and 7.8 Hz, 1H) and 2.28 (m, 1H); 2.31 (m, 2H); 2.25 (m, 1H) and 1.45 (m, 1H); 2.22 (m, 1H) and 2.05 (m, 1H); 1.98 (m, 2H)] and one methyl group [δ_H 0.89 (t, *J*=7.5 Hz, 3H)]. The ^{13}C NMR spectrum (Table 4) showed one ketone carbonyl (δ_C 219.0), one carboxylic carbonyl (δ_C 177.5), four methine (δ_C 134.2, 124.8, 53.9 and 37.8), five methylene (δ_C 38.6, 37.7, 27.2, 25.5 and 20.6) and one methyl (δ_C 14.1) carbons. In the ^1H–^1H COSY spectrum, the methyl protons, H_3-10 (δ_H 0.89), were coupled with H_2-9 (δ_H 1.98), which were further coupled with H-8 (δ_H 5.39). The same correlations of H-7 (δ_H 5.19)/H-8 and H_2-6 (δ_H 2.31) and those of H-3 (δ_H 1.85)/H_{ab}-2 (δ_H 2.22 and 2.05) and H_2-6 were also observed. These results indicated the presence of a $CH_3CH_2CH{=}CHCH_2CHCH_2$ unit. In addition, a CH_2CHCH_2COOH unit was established on the basis of the ^1H–^1H COSY correlations of H-4/H_{ab}-5 (δ_H 2.25 and 1.45) and H_{ab}-11 (δ_H 2.68 and 2.28) as well as the HMBC correlations of both H-4 and H_{ab}-11 with the carboxyl carbon, C-12 (δ_C 177.5). The HMBC correlations of H_{ab}-2 and H-4 with C-1 (δ_C 219.0) and a ^1H–^1H COSY cross peak between H-3 and H-4 established a cyclopentanone ring having a (2*Z*)-pentenyl unit at C-3 (δ_C 53.9) and a carboxymethyl substituent at C-4 (δ_C 37.8). In the NOEDIFF experiment, irradiation of H-3 affected signal intensity of H-4, indicating their *cis*-relationship. Therefore, botryosphaerinone (**6**) was assigned as a new cyclopentanone carboxylic acid.

Table 4
NMR data for botryosphaerinone (**6**)

Position	δ_H, mult, *J* in Hz	δ_C, mult.	HMBC correlation
1		219.0, qC	
2	a: 2.22, m b: 2.05, m	37.7, CH_2	C-1, C-5
3	1.85, m	53.9, CH	C-2, C-4, C-6, C-11
4	2.29, m	37.8, CH	C-1, C-12
5	a: 2.25, m b: 1.45, m	27.2, CH_2	C-2, C-4, C-11
6	2.31, m	25.5, CH_2	C-2, C-3, C-7, C-8
7	5.19, m	124.8, CH	C-6, C-8, C-9
8	5.39, dtt, 10.5, 7.2, 1.2	134.2, CH	C-6, C-7, C-9, C-10
9	1.98, m	20.6, CH_2	C-7, C-8, C-10
10	0.89, t, 7.5	14.1, CH_3	C-8, C-9
11	a: 2.68, dd, 18.5, 7.8 b: 2.28, m	38.6, CH_2	C-3, C-4, C-5, C-12
12		177.5, qC	

Botryosphaeriodiplodin (**7**) was obtained as a colorless gum with $[\alpha]_D^{26}$ −9.8 (*c* 0.70, $CHCl_3$). The UV and IR spectra were similar to those of **10**. The HREIMS showed the molecular formula $C_{17}H_{24}O_5$, 16 mass units higher than that of **10**. The ^1H NMR spectral data (Table 5) were similar to those of **10** except that proton signal of one methylene group in **10** was replaced, in **7**, by an oxymethine proton (δ_H 3.78, m). This was confirmed by the presence of two oxymethine (δ_C 72.2 and 68.0) and six methylene carbons in the ^{13}C NMR spectrum instead of one oxymethine and seven methylene carbons in **10**. This oxymethine proton was attributed to H-7 due to the ^1H–^1H COSY correlations of H_{ab}-10 (δ_H 2.71 and 2.51)/H_{ab}-9 (δ_H 1.92 and 1.52), and H_{ab}-8 (δ_H 1.49 and 1.31)/H-7 and H_{ab}-9. In addition, H-7 displayed a HMBC correlation with C-9 (δ_C 26.6). A hydroxyl group was attached at C-7 (δ_C 68.0) on the basis of the chemical shift of C-7. Thus, botryosphaeriodiplodin (**7**) was assigned as a new lasiodiplodin derivative. The configuration at C-3 was purposed to be identical to that of **10**. However, the NOEDIFF data obtained were inadequate for the assignment of the relative

configuration at C-7. Analysis of the absolute configuration at C-7 was not performed as **7** was obtained in low quantity.

Table 5
NMR data for botryosphaeriodiplodin (**7**)

Position	δ_H, mult, *J* in Hz	δ_C, mult.	HMBC correlation
1		168.5, qC	
3	5.22, m	72.2, CH	C-1, C-4, C-5, C-17
4	a: 1.96, m b: 1.66, m	32.8, CH_2	C-3, C-5, C-6, C-7, C-17
5	a: 1.69, m b: 1.53, m	19.5, CH_2	C-3, C-4, C-6, C-7, C-8
6	a: 1.64, m b: 1.49, m	36.1, CH_2	C-4, C-7
7	3.78, m	68.0, CH	C-5, C-9
8	a: 1.49, m b: 1.31, m	33.5, CH_2	C-6, C-7, C-9, C-10
9	a: 1.92, m b: 1.52, m	26.6, CH_2	C-7, C-8, C-10, C-11
10	a: 2.71, ddd, 14.5, 9.0, 6.0 b: 2.51, dt, 14.5, 6.0	29.8, CH_2	C-8, C-9, C-11, C-12, C-16
11		142.4, qC	
12	6.25, d, 2.0	108.0, CH	C-1, C-10, C-13, C-14, C-16
13		157.3, qC	
14	6.26, d, 2.0	97.1, CH	C-12, C-13, C-16
15		158.0, qC	
16		118.2, qC	
17	1.33, d, 6.5	19.3, CH_3	C-3, C-4
18	3.77, s	55.9, CH_3	C-15

The isolated compounds, except for compounds **2**, **4**, **5**, **7**, **11**–**12** and **19** which were obtained in low quantity, were tested for antibacterial activity against SA and MRSA strains. Among them, compound **10** exhibited the best activity against SA and MRSA with the respective MIC values of 64 and 128 μg/mL. Its antibacterial activity against SA ATCC 27154 with the MIC value of 25 μg/mL[4] was previously reported. Compound **16** was as active as compounds **13**–**15** against both strains,[1] but displayed much weaker activity than compound **10** with the MIC value of >128 μg/mL. The remaining compounds showed no activity at the concentration of 200 μg/mL.

Mellein derivatives have been isolated from *B. mamane*[1] and *B. obtusa*[11] while *B. rhodina* has produced a lasiodiplodin derivative.[7] This is the first report on the isolation of γ-lactone derivatives from the genus *Botryosphaeria*. Furthermore, compounds **8**, **11**–**12**, and **16**–**19**, were obtained from fungi of this genus for the first time while compound **9** was firstly isolated as a natural product.

3. Experimental

3.1. General experimental procedures

Infrared spectra (IR) were obtained on an FTS165 FTIR spectrometer or a Perkin Elmer Spectrum GX FTIR system and recorded on wavenumber (cm^{-1}). ^1H and ^{13}C Nuclear magnetic resonance spectra were recorded on an FTNMR, Bruker Avance 300 MHz or 500 MHz spectrometers using tetramethylsilane (TMS) as an internal standard. Spectra were recorded as chemical shift parameter (δ) value in ppm down field from TMS. Ultraviolet spectra (UV) were measured with an UV-160A SHIMADSU spectrophotometer. Principle bands (λ_{max}) were recorded as wavelengths (nm) and log ε in MeOH solution. Optical rotations were measured in methanol solution or chloroform solution with sodium D line (590 nm) on an AUTOPOLR® Π automatic polarimeter. Solvents for extraction and chromatography were distilled at their boiling point range prior to use except for ethyl acetate, which was an analytical grade reagent. Thin-layer chromatography (TLC) and precoated TLC plate were performed on silica gel 60 GF_{254} (Merck). Column chromatography was performed on silica gel (Merck) type 100 (70–230 mesh ASTM) with a gradient of MeOH-CH_2Cl_2, Sephadex LH-20 with MeOH, reverse phase C_{18} silica gel with a gradient of MeOH–H_2O or otherwise stated.

3.2. Fungal material

The endophytic fungi *B. rhodina* PSU-M35 (GenBank accession number EF564146) and PSU-M114 (GenBank accession number EEF564147) were isolated from the leaves of *Garcinia mangostana*, collected in Suratthani Province, Thailand in the year 2005. These fungi were deposited as PSU-M35 and PSU-M114 at the Department of Microbiology, Faculty of Science, Prince of Songkla University.

3.3. Fermentation, extraction and isolation

The endophytic fungi *B. rhodina* PSU-M35 and PSU-M114 were separately grown on potato dextrose agar (PDA) at 25 °C for five days. Three pieces (0.5×0.5 cm^2) of mycelial agar plugs were inoculated into 500 mL Erlenmeyer flasks containing 300 mL potato dextrose broth (PDB) at room temperature for four weeks. Each culture (15 L) was filtered to give the filtrate and mycelia. The filtrate and the mycelial cakes were then extracted using the same procedure as described previously.[15] The broth extracts were obtained as a dark brown gum in 2.48 g and 2.13 g from *B. rhodina* PSU-M35 and PSU-M114, respectively, while the mycelia extract of the fungus PSU-M114 was obtained as a brown gum in 796.6 mg.

The broth extract of *B. rhodina* PSU-M35 was separated by CC over Sephadex LH-20 to obtain five fractions (AA-AE). Fraction AB (417.5 mg) was purified by silica gel CC followed by CC over reverse phase silica gel to afford four fractions (AB1–AB4). Fraction AB2 (23.5 mg) was subjected to CC over silica gel, CC over Sephadex LH-20 and then PTLC with 7% acetone–CH_2Cl_2 to yield **5** (1.8 mg). Fraction AB3 (38.3 mg) was separated by CC over silica gel to afford four subfractions. Compound **2** (1.5 mg) was obtained from the first subfraction (12.5 mg) after purification by PTLC with 30% EtOAc–light petroleum. The third subfraction (19.7 mg) afforded **1** (4.3 mg) and **3** (3.8 mg) after separation by CC over silica gel with a gradient of EtOAc–light petroleum. Fraction AC (726.0 mg) was separated by CC over silica gel to give **4** (1.8 mg). Fraction AD was purified using the same procedure as fraction AC to afford five fractions. Compound **9** (13.6 mg) was contained in the second fraction. The fourth fraction (130.6 mg) was further purified by silica gel CC to afford **8** (7.8 mg).

The broth extract of *B. rhodina* PSU-M114 was fractionated by CC over Sephadex LH-20 to afford five fractions (BA-BE). Fraction BB (1.12 g) was then submitted to CC over silica gel to obtain five fractions (BB1-BB5). Fraction BB1 (25.9 mg) was further separated by flash CC over silica gel to afford **13** (4.3 mg). In addition, fraction BB2 contained **17** (138.4 mg). Fraction BB4 (393.3 mg) was purified by CC over silica gel to give six subfractions (BB41–BB46). Compound **10** (12.4 mg) was obtained from subfraction BB42 after purification by CC over silica gel followed by PTLC with 20% EtOAc–light petroleum. Subfraction BB43 (244.1 mg) was further purified by CC over silica gel to yield four subfractions. The second subfraction contained **18** (130.3 mg). The third subfraction (46.6 mg) was further separated using the same procedure as fraction BB43 to yield four subfractions. The third subfraction (9.7 mg) was further subjected to acetylation reaction, followed by purification on PTLC to afford **11** (1.6 mg) and **12** (1.8 mg). Subfraction BB45 contained **6** (4.8 mg). Fraction BC (400.5 mg) was further purified by CC over silica gel followed by PTLC with 20% EtOAc–light petroleum to afford three fractions (BC1–BC3). Fraction BC2 (21.3 mg) was separated by CC over Sephadex LH-20 to yield **14** (4.2 mg) and **15** (5.0 mg). Fraction BD (89.3 mg) was subjected to CC over silica gel with a gradient of EtOAc–CH_2Cl_2 to yield four fractions (BD1-BD4). Fraction BD2 (7.2 mg), on PTLC with 20% EtOAc–

light petroleum, and fraction BD3 (18.5 mg), on CC over Sephadex LH-20, gave **16** (3.9 mg) and **19** (2.1 mg), respectively. The mycelial extract was separated by CC over Sephadex LH-20 to afford three fractions. The second fraction (535.7 mg) was further purified by CC over silica gel, followed by PTLC with 40% EtOAc–light petroleum to afford **7** (1.9 mg).

*3.3.1. Botryosphaerilactone A (**1**).* Colorless gum; $[\alpha]_D^{27}$ −1.2 (*c* 0.77, MeOH); UV (MeOH) λ_{max} (log ε) 286 (1.30) nm; IR (neat) ν_{max} 3438, 1770 cm^{-1}; HREIMS *m*/*z* $[M\text{-}H]^+$ 271.1553 (calcd for $C_{14}H_{23}O_5$, 271.1545); ^1H NMR ($CDCl_3$, 500 MHz), see Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz), see Table 1.

*3.3.2. Botryosphaerilactone B (**2**).* Colorless gum; $[\alpha]_D^{26}$ +4.5 (*c* 0.57, MeOH); UV (MeOH) λ_{max} (log ε) 280 (1.61) nm; IR (neat) ν_{max} 3430, 1775 cm^{-1}; HREIMS *m*/*z* $[M]^+$ 270.1479 (calcd for $C_{14}H_{22}O_5$, 270.1467); ^1H NMR ($CDCl_3$, 500 MHz), see Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz), see Table 1.

*3.3.3. Botryosphaerilactone C (**3**).* Colorless gum; $[\alpha]_D^{26}$ +5.2 (*c* 0.77, MeOH); UV (MeOH) λ_{max} (log ε) 273 (2.21) nm; IR (neat) ν_{max} 3438, 1770 cm^{-1}; HREIMS *m*/*z* $[M\text{-}H]^+$ 271.1533 (calcd for $C_{14}H_{23}O_5$, 271.1545); ^1H NMR ($CDCl_3$, 500 MHz), see Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz), see Table 1.

*3.3.4. Botryosphaeridione (**4**).* Pale yellow gum; $[\alpha]_D^{27}$ −13.2 (*c* 0.68, MeOH); UV (MeOH) λ_{max} (log ε) 242 (3.30), 289 (3.18) nm; IR (neat) ν_{max} 3354, 1673 cm^{-1}; HREIMS *m*/*z* $[M]^+$ 204.0793 (calcd for $C_{12}H_{12}O_3$, 204.0786); ^1H NMR ($CDCl_3$, 300 MHz), see Table 2; ^{13}C NMR ($CDCl_3$, 75 MHz), see Table 2.

*3.3.5. Botryosphaerihydrofuran (**5**).* Colorless gum; $[\alpha]_D^{27}$ −23.8 (*c* 0.14, MeOH); UV (MeOH) λ_{max} (log ε) 220 (4.81), 280 (4.91) nm; IR (neat) ν_{max} 1664 cm^{-1}; HREIMS *m*/*z* $[M]^+$ 218.1314 (calcd for $C_{14}H_{18}O_2$, 218.1307); ^1H NMR ($CDCl_3$, 500 MHz), see Table 3; ^{13}C NMR ($CDCl_3$, 125 MHz), see Table 3.

*3.3.6. Botryosphaerinone (**6**).* Colorless gum; $[\alpha]_D^{28}$ −23.2 (*c* 0.14, MeOH); UV (MeOH) λ_{max} (log ε) 212 (2.73) nm; IR (neat) ν_{max} 3441, 1728 cm^{-1}; HREIMS *m*/*z* $[M]^+$ 210.1253 (calcd for $C_{12}H_{18}O_3$, 210.1256); ^1H NMR ($CDCl_3$, 300 MHz), see Table 4; ^{13}C NMR ($CDCl_3$, 75 MHz), see Table 4.

*3.3.7. Botryosphaeriodiplodin (**7**).* Colorless gum; $[\alpha]_D^{26}$ −9.8 (*c* 0.70, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 205 (3.20), 245 (2.44), 280 (2.19) nm; IR (neat) ν_{max} 3440, 1684 cm^{-1}; HREIMS *m*/*z* $[M]^+$ 308.1615 (calcd for $C_{17}H_{24}O_5$, 308.1624); ^1H NMR ($CDCl_3$, 500 MHz), see Table 5; ^{13}C NMR ($CDCl_3$, 125 MHz), see Table 5.

3.4. Antibacterial activity testing

MICs were determined by the agar microdilution method.[16] The test substances were dissolved in DMSO (Merck, Germany). Serial 2-fold dilutions of the test substances were mixed with melted Mueller–Hinton agar (Difco) in the ratio of 1:100 in microtiter plates with flat-bottomed wells (Nunc, Germany). Final concentration of the test substances in agar ranged from 200 to 0.39 μg/mL. MRSA isolated from a clinical specimen, Songklanakarin Hospital, was used as test strain. Inoculum suspensions (10 μL) were spotted on agar-filled wells. The inoculated plates were incubated at 35 °C for 18 h. MICs were recorded by reading the lowest substance concentration that inhibited visible growth. Growth controls were performed on agar containing DMSO.

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