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Three new resorcylic acid derivatives from *Sporotrichum laxum*

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Abstract

Sporotrichum laxum ATCC 15155 is the producing strain of the potent anti-*Helicobacter pylori* natural product spiroloxine (**1**). Investigation of the secondary metabolites in this fungus led to the isolation of five phthalides (**1**, **2**, **3**, **6** and **9**) and five resorcylic acid derivatives (**4**, **5**, **7**, **8** and **10**), among which **5**, **7** and **8** are new compounds. The structures were elucidated by spectroscopic analyses, and the absolute configurations of **7** and **8** were determined by Mosher's method.

Addition of soy flour into the potato dextrose agar has led to the increased production of **4–10**. A biosynthetic pathway consisting of a highly reducing polyketide synthase (PKS), a non-reducing PKS and a series of tailoring enzymes was proposed to produce these fungal natural products. The resorcylic acid derivatives are proposed to result from early hydrolysis of the polyketide chain or incorporation of a longer fatty acyl starter unit.

Keywords

Sporotrichum laxum; Phthalide; Resorcyate; Biosynthetic pathway; Polyketide

Spiroloxine (**1**) and sporotricale (**2**) (Fig. 1) are two secondary metabolites produced by *Sporotrichum laxum* ATCC 15155 (Basidiomycetae). These natural products contain a 5-hydroxy-3-methoxyphthalide moiety linked through an oligomethylene chain to a spiroacetal or a hemiketal.¹ Besides **1** and **2**, two 5,5-spiroacetal phthalides, CJ-12,954 and CJ-13,014, have been isolated from the culture of *Phanerochaete velutina*.² All these compounds exhibited significant anti-*Helicobacter pylori* activity, thus providing promising leads for the treatment of *H. pylori*-related diseases. The structure-activity relationship of phthalide-containing spiroacetal compounds showed that the presence of a spiroketal part in addition to the phthalide nucleus greatly enhanced the activity. Furthermore, these phthalide compounds appeared to be specific for *H. pylori*, since they did not show any antibacterial activities against other bacteria. Additionally, **1** has also been reported to exhibit cholesterol-lowering and anti-tumor activities.^{3, 4}

Because the particular structure and interesting biological activities, structural modification of **1** has been attempted. For instance, incubation of **1** with *Bacillus megaterium* afforded two new monohydroxylated and one new dihydroxylated derivatives.⁵ Chemical synthesis has also been attempted to synthesize analogues of **1** for new anti-*H. pylori* agents.⁶ To further understand the producing strain and obtain some information about the biosynthetic

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pathway of **1**, we investigated the secondary metabolites of *S. laxum* ATCC 15155. In this paper, we report the isolation and structural characterization of 10 natural products including three new resorcylic acid derivatives. A polyketide biosynthetic pathway leading to the production of these compounds is proposed.

S. laxum ATCC 15155 was inoculated onto 30 mL of PDA (potato dextrose agar) in Petri dishes, and incubated at 28°C for 10 days. The culture was extracted twice with ethyl acetate containing 1% methanol for product analysis. The strain was also cultured on PDA supplemented with 2% soy flour to test its effects on the production of secondary metabolites. The extracts were analyzed by reversed-phase HPLC with an Agilent Eclipse XDB-C18 column (5 μ m, 250 mm $\times$ 4.6 mm). The samples were eluted with acetonitrile-water (10% to 95% over 60 min) at a flow rate of 1 mL/min, and the products were detected at 254 nm. As shown in Figure 2, a total of 10 major metabolites were detected in the extracts. When the strain grew on PDA, **1–4** were the major products, while **5–10** were produced only in small amounts. When 2% soy flour was added into PDA, the production of **4–10** was significantly increased. The 10 products were divided into two groups based on their UV absorptions. The first group including **1**, **2**, **3**, **6** and **9** showed the maximum UV absorptions at 218, 256 and 290 nm, similar to those reported for spiroloxine.¹ The second group contains **4**, **5**, **7**, **8** and **10**, which has the characteristic UV absorptions of resorcylic acid at 218, 263 and 303 nm, suggesting that they belong to the family of resorcyates. This fungus was then cultured on 5 L of PDA with or without 2% soy flour to isolate **1–10** for structure elucidation.⁷

Comparison of the spectral data⁸ with the literature allowed the identification of **1**, **2**, **3** and **4** as spiroloxine, sporotricale, sporotricale methylether and phanerosporic acid, respectively.^{1, 9, 10} The structure of **2** is unstable. It was reported that this compound exists as an equimolar mixture of the epimeric cyclic hemiketal (**2a**) and open chain keto (**2**) forms in equilibrium in chloroform-*d*, but in methanol-*d*₄ the open form is absent.¹ We also found the duplicate signals of C-21 and C-22 of **2a** in the ¹H and ¹³C NMR spectra in methanol-*d*₄ due to the presence of the two configurations of 18-OH, and the corresponding resonances were in the ratio of 2:3 (¹H 4.14/3.55 and 1.19/1.10; ¹³C 76.0/78.1 and 21.9/22.6). We assigned the NMR signals of **2a** in methanol-*d*₄ that have not been reported previously. **6** and **9** were confirmed to be CJ-13,104 and CJ-13,108, previously isolated from *P. velutina*,² respectively, and **10** was identified as MC-306,¹¹ based on the ESIMS and NMR data.¹²

5 was obtained as a white powder. The molecular formula was determined as C₂₂H₃₄O₅ on the basis of the [M–H][–] ion peak at *m/z* 377.2340 (calcd. 377.2333) in the HRESIMS spectrum. The IR spectrum suggested the presence of hydroxyl group (3421 cm^{–1}), methyl and methylene groups (2912 and 2848 cm^{–1}), ketone group (1703 cm^{–1}), carboxyl group (1695 cm^{–1}) and phenyl group (1576 and 1512 cm^{–1}). The ¹H NMR spectrum (Table 1) showed two aromatic protons (δ 6.14 and 6.19), four methylene groups (δ 1.54, 1.54, 2.46 and 2.87), a methyl group (δ 2.12), and an overlapped signal corresponding to 18 protons (δ 1.24–1.36). The ¹³C NMR spectrum of **5** exhibited signals attributable to an aromatic ring, a carboxyl group (δ 175.0), a ketone group (δ 212.5), a methyl group (δ 29.9) and a series of methylene carbons. The resorcylic acid core of **5** was established by a comparison of its NMR data with those known resorcyates we isolated from the same strain, which was further supported by the HMBC correlations shown in Figure 3. Further HMBC analysis (Fig. 3) showed the correlations of H-8 at δ 2.87 to C-2, C-6, C-7 and C-9, indicating that the long fatty acyl chain is connected to the resorcylic acid core structure at C-7. The HMBC correlations of H-20 at δ 2.46 and H-22 at δ 2.12 to C-21 confirmed that the ketone group is at C-21. Finally, **5** was identified as 3,5-dihydroxy-7-(14-oxopentadecyl)benzoic acid.

7 and **8** were also obtained as a white powder. The $[M-H]^-$ ion peak at m/z 407.2808 (calcd. 407.2803) in the HRESIMS spectrum of **7** revealed a molecular formula of $C_{24}H_{40}O_5$. The IR spectrum showed the absorption bands of hydroxyl group (3394 cm^{-1}), methyl, methylene and methine groups (2922 and 2827 cm^{-1}), carboxyl group (1693 cm^{-1}) and phenyl group (1599 and 1504 cm^{-1}). The ^1H NMR of **7** (Table 1) revealed two aromatic protons (δ 6.15 and 6.19), a methine proton (δ 3.69), three methylene groups (δ 2.88, 1.56 and 1.41), a methyl group (δ 1.14), and an overlapped signal at δ 1.28–1.36 corresponding to 24 protons. The ^{13}C NMR spectrum of **7** exhibited signals attributable to an aromatic ring, a carboxyl group (δ 174.9), an oxygenated methine group (δ 68.7), a methyl group (δ 23.6), and a series of methylene carbons. HMBC analysis (Fig. 3) revealed that like **5**, the fatty acyl chain is also attached to the aromatic ring at C-7. The hydroxyl group was located at C-23 based on the HMBC correlations of H-22 at δ 1.41 and H-24 at δ 1.14 to C-23, which was further confirmed by the correlations of H-23 to H-22 and H-24 in the ^1H - ^1H COSY spectrum (Fig. 3). According to the molecular formula and the integral area of methylene protons, **7** is two CH_2 units longer than **5**.⁹ To determine the configuration of the 23-OH, **7** was reacted with (*S*)- and (*R*)-MTPA chloride to afford the corresponding (*R*)- and (*S*)-MTPA esters **7R** and **7S**. The ^1H and ^1H - ^1H COSY NMR spectra of these esters were recorded in pyridine- d_5 . The chemical shifts for H-24 and H-22 of **7S** were δ 1.32 and 1.35, respectively, compared to δ 1.25 and 1.65 in **7R**. The ($[\alpha]_D^{25.3}$) values (Fig. 4) confirmed that the absolute configuration of C-23 is *R*. Thus, **7** was identified as (*R*)-3,5-dihydroxy-7-(16-hydroxyheptadecyl)benzoic acid. Its $[\alpha]_D^{25.3}$ value was measured to be -1.7° (methanol, c 0.35).

8 has the same molecular formula as **7**, as revealed by the $[M-H]^-$ peak at m/z 407.2812 (calcd. 407.2803) in the HRESIMS spectrum. The IR spectrum is highly similar to that of **7**, showing the absorption bands at 3390 , 2922 , 2825 , 1691 , 1589 and 1504 cm^{-1} . Its ^1H NMR spectrum of **8** (Table 1) indicated two aromatic protons (δ 6.15 and 6.19), one methine proton (δ 3.43), three methylene protons (δ 2.88, 1.56 and 1.43), a methyl proton (δ 0.93) and an overlapped signal at δ 1.24–1.40 that accounts for 24 protons. The ^{13}C NMR spectrum of **8** demonstrated signals attributable to an aromatic ring, a carboxyl group (δ 174.9), an oxygenated methane group (δ 74.1), a methyl carbon (δ 10.5) and a series of overlapped carbon signals. Apparently, this molecule also has a hydroxylated CH group in the long side chain. HMBC analysis (Fig. 3) confirmed that the side chain is attached to the aromatic ring at C-7. The HMBC correlations of H-24 at δ 0.93 and H-23 at δ 1.43 to C-22 suggested that the hydroxyl group is at C-22. This was further confirmed by the correlations of H-23 to H-22 at δ 3.43 and H-24 in the ^1H - ^1H COSY spectrum (Fig. 3). NMR analysis of the MTPA esters **8R** and **8S** (Fig. 4) revealed that the chemical shift for H-24 of **8S** was δ 0.91 and the H-23 and H-21 signals were overlapped at δ 1.64, while those for H-24, H-23 and H-21 in **8R** were δ 0.80, 1.61 and 1.72, respectively. Based on the values (Fig. 4), the configuration of C-22 in **8** was determined as *R*. Therefore, **8** was identified as (*R*)-3,5-dihydroxy-7-(15-hydroxyheptadecyl)benzoic acid. Its $[\alpha]_D^{25.3}$ value was measured to be -3.3° (methanol, c 0.30).

Iterative polyketide synthases (PKSs) are involved in the biosynthesis of many fungal natural products. With these 10 metabolites isolated from the host, a polyketide biosynthetic pathway was proposed to synthesize these molecules (Fig. 1). A highly reducing PKS (hrPKS) takes 8 units of malonyl-CoA to synthesize an octaketide. This enzyme-bound octaketide **11** is then transferred to a nonreducing PKS (nrPKS), which elongates the chain using three molecules of malonyl-CoA to yield an undecaketide **12**. This nascent polyketide chain is cyclized via an intramolecular C-2/C-7 aldol condensation to form the intermediate **13**. The regioselective C-2/C-7 cyclization by the dedicating nrPKSs is observed in the biosynthesis of various natural products such as zearalenone¹³ and radicicol,¹⁴ while C-3/

C-8 cyclization was reported in the biosynthesis of 10,11-dehydrocurvularin.¹⁵ Recently, we have found that the cyclization pattern is controlled by the product template (PT) domain of fungal nrPKSs and can be reprogrammed by rationally changing the key residues in PTs.¹⁶ The polyketide intermediate **13** can be hydrolyzed and released from the enzyme to yield **4**. Its 21-OH may be oxidized by an endogenous enzyme in *S. laxum* ATCC 15155 to give rise to **5**. When the 3-OH of **4** is methylated, **10** is produced. To synthesize **1**, we propose that **13** is hydroxylated at C-8 and a thioesterase domain in the nrPKS catalyzes the formation of the 5-membered lactone ring to yield **14**. This intermediate is then methylated to yield **15** or **6**. When the 21-OH of **6** is oxidized into a ketone group, **9** is produced. **15** is subsequently hydroxylated at C-18 to afford **16**, which is oxidized to get **2**. **2** is then converted into **2a** to form the methyltetrahydrofuran ring. Then, a hydroxylase introduces a hydroxyl group at C-14 (to afford **17**), which is then used to form the tetrahydropyran ring of **1**. **3** can be generated from **2a** through the methylation of the 5-OH. When the medium is supplemented with soy flour which contains abundant fatty acids, the intermediates from fatty acid biosynthesis/metabolism may be taken by the nrPKS as the starter units to yield new molecules. This has been revealed in a previous work on the zearalenone nrPKS PKS13 from *Gibberella zeae*.¹³ As shown in Figure 1, stearoyl-acyl carrier protein (ACP) (**18**) may be accepted by the nrPKS as a starter unit to synthesize a polyketide intermediate **19**, which undergoes a C-2/C-7 cyclization and is released from the enzyme. Endogenous hydroxylases may hydroxylate the intermediate at C-23 and C-22 to yield **7** and **8**, respectively. It is also possible that **7** is synthesized by the nrPKS from a longer starter unit generated by the hrPKS since it has a similar OH as **4**. To fully understand the biosynthetic mechanism of this pathway, more genetic and biochemical studies are needed.

In summary, we have isolated five phthalides (**1**, **2**, **3**, **6** and **9**) and five resorcylic acid derivatives (**4**, **5**, **7**, **8** and **10**) from *S. laxum* ATCC 15155, among which **5**, **7** and **8** are three new compounds. It is noteworthy that **7** and **8** have the longest fatty acyl side chain found in natural resorcylic acid derivatives. Based on the structures of those compounds, we proposed a polyketide biosynthetic pathway that results in the formation of these natural products (Fig. 1).

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7. The cultures were incubated at 28°C for 10 days and then extracted twice with ethyl acetate containing 1% methanol. The crude extract from PDA (1.0 g) was chromatographed on a silica gel column, eluted by chloroform-methanol (from 99:1 to 80:20, v/v), followed by HPLC separation on an Agilent Eclipse XDB-C18 column (5 μ m, 250 mm $\times$ 4.6 mm). The sample was eluted with 37% acetonitrile-water at a flow rate of 1 mL/min for 40 min to yield **1** (17.3 mg), **2** (40.4 mg) and **3** (4.1 mg). Similarly, the extract (1.1 g) from PDA supplemented with 2% soy flour was separated on a silica gel column with the same solvent gradient. The fractions containing **4**, **7** and **8** were further

separated by HPLC with an Agilent Eclipse XDB-C18 column (5 μ m, 250 mm $\times$ 4.6 mm), eluted with 65% acetonitrile-water at a flow rate of 1 mL/min for 40 min to get pure **4** (39.5 mg), **7** (6.0 mg) and **8** (4.9 mg). The fractions containing **6** and **9** were further separated using the same method to yield pure **6** (9.1 mg) and **9** (2.8 mg). The fractions containing **5** and **10** were separated on HPLC, eluted with acetonitrile-water (50% to 100% over 25 min), to afford **5** (4.4 mg) and **10** (2.1 mg).

8. MS and NMR data for **1**: ESIMS (–): $[M-H]^-$ m/z 403.1; 1H NMR (300 MHz, chloroform- d): 6.41 (1H, brs, H-6), 6.39 (1H, brs, H-4), 5.33 (1H, m, H-8), 4.20 (1H, m, H-21), 3.90 (3H, s, OCH₃-3), 3.67 (1H, m, H-14), 2.37 (2H, m, H-9), 2.20–1.08 (20H, overlap, H-10, H-11, H-12, H-13, H-15, H-16, H-17, H-18, H-19, and H-20), 0.87 (3H, t, J = 6.9 Hz, CH₃-22). MS and NMR data for **2** and **2a**: ESIMS (–): $[M-H]^-$ m/z 405.2; 1H NMR (300 MHz, methanol- d_4): 6.44 (1H, d, J = 2.0 Hz, H-4), 6.42 (1H, d, J = 2.0 Hz, H-6), 5.35 (1H, m, H-8), 3.89 (3H, s, OCH₃-3), 4.14/3.55 (1H, m, H-21), 2.35–1.25 (22H, overlap, H-9, H-10, H-11, H-12, H-13, H-14, H-15, H-16, H-17, H-19, and H-20), 1.19/1.10 (3H, d, J = 6.8 Hz, CH₃-22); ^{13}C NMR (75 MHz, methanol- d_4): 171.8 (C-1), 167.8 (C-5), 161.8 (C-3), 157.3 (C-7), 111.2 (C-18), 106.5 (C-2), 101.8 (CH-6), 100.4 (CH-4), 82.2 (CH-8), 76.3/78.1 (2:3, CH-21), 56.3 (OCH₃-3), 35.6–25.5 (CH₂-9, CH₂-10, CH₂-11, CH₂-12, CH₂-13, CH₂-14, CH₂-15, CH₂-16, CH₂-17, CH₂-19, and CH₂-20), 21.9/22.6 (2:3, CH₃-22). MS and NMR data for **3**: ESIMS (–): $[M-H]^-$ m/z 419.1; 1H NMR (300 MHz, methanol- d_4): 6.64 (1H, brs, H-6), 6.58 (1H, brs, H-4), 5.39 (1H, m, H-8), 3.93 and 3.92 (6H, s, OCH₃-3 and OCH₃-5), 3.65 (1H, m, H-21), 2.54 (2H, m, H-9), 2.45–1.25 (20H, overlap, H-10, H-11, H-12, H-13, H-14, H-15, H-16, H-17, H-19 and H-20), 1.19 (3H, brs, CH₃-22). MS and NMR data for **4**: ESIMS (–): $[M-H]^-$ m/z 379.2; 1H NMR (300 MHz, methanol- d_4): 6.18 (1H, d, J = 2.4 Hz, H-6), 6.13 (1H, d, J = 2.4 Hz, H-4), 3.70 (1H, m, H-21), 2.86 (2H, t, J = 7.6 Hz, H-8), 1.60–1.21 (24H, overlap, H-9, H-10, H-11, H-12, H-13, H-14, H-15, H-16, H-17, H-18, H-19 and H-20), 1.12 (3H, d, J = 6.2 Hz, CH₃-22).
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Figure 1.
Proposed polyketide biosynthetic pathway leading to the formation of 1–10.

Figure 2.
HPLC analysis of secondary metabolites produced by *S. laxum* ATCC 15155 on PDA (top)
and PDA with 2% soy flour (bottom).

Figure 3.
Key HMBC and ^1H - ^1H COSY correlations for **5**, **7** and **8**.

Figure 4.
Determination of the absolute configurations of **7** and **8** by Mosher's method. (ppm)
values in pyridine-*d*₅ are shown.

Table 1

^1H (300 MHz) and ^{13}C (75 MHz) NMR data for **5**, **7** and **8** (methanol- d_4 in ppm, J in Hz)

5				7				8			
Position	C	H	Position	C	H	Position	C	H	Position	C	H
1	175.0 (C)		1	174.9 (C)		1	174.9 (C)		1	174.9 (C)	
2	105.1 (C)		2	105.1 (C)		2	105.1 (C)		2	105.1 (C)	
3	163.8 (C)		3	163.8 (C)		3	163.8 (C)		3	163.8 (C)	
4	101.9 (CH)	6.14 (1H, brs)	4	101.9 (CH)	6.15 (1H, d, 1.5)	4	101.9 (CH)	6.15 (1H, brs)	4	101.9 (CH)	6.15 (1H, brs)
5	167.0 (C)		5	167.0 (C)		5	167.0 (C)		5	167.0 (C)	
6	111.9 (CH)	6.19 (1H, brs)	6	111.9 (CH)	6.19 (1H, d, 1.5)	6	111.9 (CH)	6.19 (1H, brs)	6	111.9 (CH)	6.19 (1H, brs)
7	150.3 (C)		7	150.3 (C)		7	150.3 (C)		7	150.3 (C)	
8	37.7 (CH ₂)	2.87 (2H, t, 7.6)	8	37.7 (CH ₂)	2.88 (2H, t, 7.5)	8	37.7 (CH ₂)	2.88 (2H, t, 7.5)	8	37.7 (CH ₂)	2.88 (2H, t, 7.5)
9	33.3 (CH ₂)	1.54* (2H, m)	9	33.4 (CH ₂)	1.56 (2H, m)	9	33.4 (CH ₂)	1.56 (2H, m)	9	33.4 (CH ₂)	1.56 (2H, m)
10–18	30.7–31.1*	1.24–1.36*	10–20	30.7–31.1*	1.28–1.36*	10–19	30.7–31.5*	1.24–1.40*	10–19	30.7–31.5*	1.24–1.40*
	(9×CH ₂)	(18H, m)									
19	25.0 (CH ₂)	1.54* (2H, m)	21	27.1 (CH ₂)	1.28–1.36* (2H, m)	20	27.0 (CH ₂)	1.24–1.40* (2H, m)	20	27.0 (CH ₂)	1.24–1.40* (2H, m)
20	44.5 (CH ₂)	2.46 (2H, t, 7.2)									
21	212.5 (C)		22	40.4 (CH ₂)	1.41 (2H, m)	22	74.1 (CH)	3.43 (H, m)	21	38.1 (CH ₂)	1.24–1.40* (2H, m)
22	29.9 (CH ₃)	2.12 (3H, s)	23	68.7 (CH)	3.69 (1H, m)						
			24	23.6 (CH ₃)	1.14 (3H, d, 6.2)	23	31.1 (CH ₂)	1.43 (2H, m)	22	74.1 (CH)	3.43 (H, m)
						24	10.5 (CH ₃)	0.93 (3H, t, 7.2)	23	31.1 (CH ₂)	1.43 (2H, m)

* Overlapped signals.