Bioorganic & Medicinal Chemistry Letters 18 (2008) 6050–6053

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Pyrrospirones A and B, apoptosis inducers in HL-60 cells, from an endophytic fungus, *Neonectria ramulariae* Wollenw KS-246

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ARTICLE INFO

Article history:
Received 13 June 2008
Revised 10 September 2008
Accepted 8 October 2008
Available online 11 October 2008

Keywords:
Neonectria ramulariae
Endophyte
Pyrrospirones
Pyrrocidines

ABSTRACT

Pyrrospirones A and B have been isolated from unpolished rice cultures of the endophytic fungus *Neonectria ramulariae* Wollenw KS-246. Their absolute stereostructures (**1** and **2**) were elucidated through spectroscopic methods using 1D and 2D NMR techniques and chemical transformations, including the modified Mosher's method. The compounds exhibited cytotoxicity and induced apoptosis in human promyelocytic leukemia HL-60 cells.

Endophytic fungi are recognized as potentially prolific sources of bioactive secondary metabolites with a high level of structural diversity.[1] In the search for biologically active natural products from endophytic fungi, new metabolites from strains of *Xylaria* sp. YUA-026,[2,3] *Anthracobia* sp. YST-55,[4] and *Fusarium* sp. YG-45,[5] which were isolated from the plant samples (twigs and petiols), have recently been reported. Further studies on bioactive compounds obtained from endophytic fungi have led to the isolation of two new alkaloids, pyrrospirones A (**1**) and B (**2**). These are in addition to the known pyrrocidines A (**3**) and B (**4**) obtained from the extract of an unpolished rice culture of *Neonectria ramulariae* Wollenw KS-246. In this letter, we describe the fermentation of the producing strain and the isolation, structural elucidation, and biological characterization of the newly discovered compounds (**1** and **2**).

The fungal strain *N. ramulariae* Wollenw KS-246 was isolated from a dead branch collected from Mt. Gassan, Yamagata, Japan, and has been deposited at the laboratory of the Faculty of Agriculture, Yamagata University. This producing fungus was stationarily cultured at 25 °C for 3 weeks in unpolished rice (600 g). The MeOH extract of the moldy, unpolished rice was evaporated to an aqueous concentrate and then partitioned between EtOAc and H_2O. The EtOAc extract was concentrated in vacuo to give a crude extract (5.0 g). The crude extract was subjected to silica gel column chromatography and eluted with stepwise elusion of *n*-hexane–EtOAc and EtOAc–MeOH, respectively. The fraction eluted with *n*-hexane–EtOAc (30:70) was crystallized from EtOAc to yield pyrrocidine A (**3**) (114 mg). The fraction eluted with 100% EtOAc was further separated by ODS chromatography using 90% MeCN as the eluting solvent to afford pyrrocidine B (**4**) (5.7 mg). The fraction eluted with 100% MeOH was purified by preparative HPLC using an ODS column with 90% MeOH as the eluting solvent to afford pyrrospirones A (**1**, 10.0 mg) and B (**2**, 15.0 mg).

The structures of pyrrocidines A (**3**) and B (**4**) were determined on the basis of HREIMS and ^{1}H, ^{13}C, and 2D NMR data. These data were identical to those previously reported in the literature.[6]

Pyrrospirone A (**1**) has the molecular formula $C_{31}H_{39}NO_5$ as established by HRFABMS. The UV spectrum shows an absorption maximum at 229 nm, which suggests the presence of a phenyl group. The IR spectrum exhibits absorption bands at 1716 (CO), 1671 (NHCO), 1606, and 1508 cm^{-1} (aromatic ring). The presence of the hydroxyl and amide groups was confirmed by the existence of two exchangeable downfield protons that have chemical shifts at δ_H 8.02 and 9.82 in the ^{1}H NMR spectrum. The ^{13}C NMR spectrum shows 31 carbons (Table 1) that are classified by an analysis of the DEPT spectra into 4 methyls, 4 methylenes, 15 methines, 6 quaternary carbons, and 2 carbonyls; this classification agrees with the molecular formula. The chemical formula of **1** requires 13 rings or unsaturation equivalents. Since 6 out of the 13 unsaturation equivalents are accounted for by the ^{13}C NMR data, it is inferred that a molecule of **1** should have seven rings. The ^{1}H NMR spectrum (Table 1) of **1** displayed a quaternary methyl [δ_H 1.33 (3H, s, Me-31)], two secondary methyls [δ_H 0.89 (3H, d, J = 6.1 Hz,

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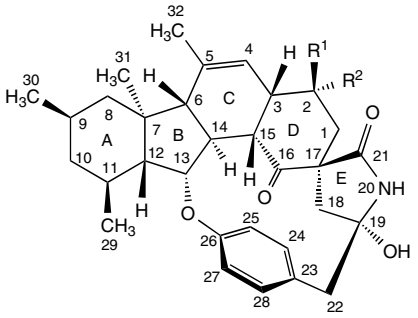
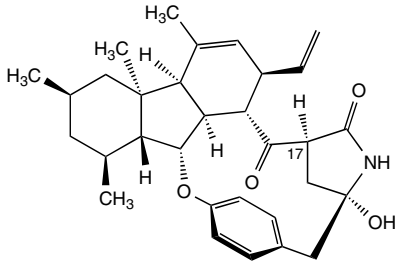
doi:10.1016/j.bmcl.2008.10.032



Pyrrospirone A (**1**): R^1 = H, R^2 = OH
Pyrrospirone B (**2**): R^1 = OH, R^2 = H



3: 17,18-dehydro

4

Me-30), 1.24 (3H, d, *J* = 6.2 Hz, Me-29)], an olefinic methyl [δ_H 1.92 (3H, s, Me-32)], two oxygenated methines [δ_H 4.57 (1H, t, *J* = 7.9 Hz, H-13), 6.04 (1H, m, H-2)], four aromatic protons [δ_H 7.12 (2H, m, H-25, 27), 7.30 (1H, d, *J* = 6.4 Hz, H-24), 7.51 (1H, d, *J* = 6.4 Hz, H-28)], and a trisubstituted olefinic proton [(δ_H 5.95 (1H, br s, H-4)]. Detailed analyses of the ^{1}H–^{1}H COSY spectrum disclosed the presence of a partial structure shown as a bold line in Figure 1. To establish the connectivity of this fragment demarcated by bold lines in the figure, HMBC experiments were carried out (Fig. 1). The olefinic methyl (Me-32) was inferred to be connected to C-5 based on HMBC correlations of the signal of Me-32 with the signals of C-4, C-5, and C-6. The connections among C-6, C-8, C-12, and C-31 through C-7 were inferred from HMBC correlations of the signal of Me-31 with the signals of C-6, C-7, and C-8 and from the correlation of the signal of H-12 with that of C-31. Furthermore, the signals of H-1 and H-3 were correlated with the signal of C-16, and the signals of H-2 and H-15 with the signal of C-17. These data allowed us to construct the cyclohexano[*a*]decahydrofluoren-1-one (6/5/6/6 rings) skeleton. Furthermore, the HMBC correlations of the signal of H-18 with the signals of C-1, C-16, and C-17 and of the signal of 20-NH with the signals of C-17 and C-18 indicate that cyclohexano[*a*]decahydrofluoren-1-one and γ-lactam (2-pyrrolidone) share the same carbon (C-17) and therefore form spiro[cyclohexano[*a*]decahydrofluoren-2,3′-pyrrole]-1,2′-dione (6/5/6/6-5 rings). The HMBC correlations of the signal of H-22 with the signals of C-23, C-24, and C-28 as well as of the signals of H-24 and H-28 with those of C-22 and C-26 allowed the attribution of these signals to the 1,4-disubstituted benzyl moiety. Additionally, the signals of H-18, H-22, and H-13 were correlated with those of C-22, C-18, and C-26, respectively. These HMBC correlations indicate the connectivity between the benzyl moiety and the 6/5/6/6-5 ring substructure through an ether linkage to form a 13-membered macrocyclic ring. Rotation of the benzene ring along the C(23)–C(26) axis seems to be hindered, and this hinderance may persist even in a solution, as the proton signals of the

Table 1
^{1}H and ^{13}C NMR data of Pyrrospirones A (**1**) and B (**2**) (400 MHz, Py-d_5)

No	**1**		**2**	
	δ_C	δ_H	δ_C	δ_H
1	50.0 t	1.84–1.93*	49.2 t	1.77–1.83*
		2.49 (1H, dd, 12.3, 5.1)		2.33 (1H, d, 14.2)
2	63.6 d	6.04 (1H, m)	68.9 d	3.83 (1H, dd, 11.1, 3.2)
3	48.6 d	3.26 (1H, m)	55.4 d	3.19 (1H, m)
4	121.4 d	5.95 (1H, br. s)	123.9 d	4.97 (1H, br. s)
5	139.6 s		140.2 s	
6	54.4 d	1.51 (1H, br. d, 12.7)	54.3 d	1.44 (1H, br. d, 12.7)
7	41.8 s		41.7 s	
8	48.5 t	0.71 (1H, t, 12.0)	48.5 t	0.73 (1H, t, 11.9)
		1.84–1.93*		1.77–1.83*
9	28.0 d	1.69–1.79*	28.0 d	1.69–1.73*
10	45.6 t	0.57 (1H, q, 11.8)	45.6 t	0.59 (1H, q, 12.3)
		1.69–1.79*		1.69–1.73*
11	27.4 d	1.98–2.08 (m)	27.4 d	2.00 (1H, m)
12	60.7 d	1.00 (1H, dd, 11.2, 7.9)	60.5 d	0.98 (1H, dd, 11.0, 7.6)
13	87.3 d	4.57 (1H, t, 7.9)	87.8 d	4.51 (1H, t, 7.6)
14	46.0 d	3.13 (1H, m)	45.6 d	2.98 (1H, dt, 12.7, 7.6)
15	45.3 d	3.98 (1H, br t, 6.2)	44.7 d	3.93 (1H, t, 7.6)
16	201.9 s		201.5 s	
17	59.8 s		61.8 s	
18	43.1 d	2.12 (1H, d, 13.9)	43.6 d	2.09 (1H, d, 13.9)
		3.61 (1H, d, 13.9)		3.60 (1H, d, 13.9)
19	88.1 s		88.7 s	
21	175.7 s		177.3 s	
22	47.2 t	3.25 (1H, d, 13.3)	46.8 t	3.26 (1H, d, 13.4)
		3.53 (1H, d, 13.3)		3.53 (1H, d, 13.4)
23	129.5 s		129.2 s	
24	133.3 d	7.30 (1H, d, 6.4)	133.8 d	7.42 (1H, d, 7.0)
25	120.8 d	7.12 (2H, m)*	120.7 d	7.09 (2H, m)*
26	158.3 s		158.3 s	
27	120.8 d	7.12 (2H, m)*	124.7 d	7.09 (2H, m)*
28	133.7 d	7.51 (1H, d, 6.4)	133.3 d	7.30 (1H, d, 7.0)
20-NH		9.82 (1H, br. s)		10.6 (1H, br. s)
2-OH		8.02 (1H, br. s)		7.86 (1H, d, 11.1)
29	19.8 q	1.24 (3H, d, 6.2)	19.8 q	1.24 (1H, d, 6.2)
30	22.8 q	0.89 (3H, d, 6.1)	22.8 q	0.90 (3H, d, 6.1)
31	16.9 q	1.33 (3H, s)	16.7 q	1.28 (3H, s)
32	20.3 q	1.92 (3H, s)	19.9 q	1.85 (3H, s)

* Overlapped signals.

benzene ring in the ^{1}H NMR spectrum of **1** do not exhibit a simple A_2B_2-type coupling pattern but rather complex splitting patterns due to the inequality between (1) H-24 and H-28 and (2) H-25 and H-27.

The relative stereochemistry of **1** was established by a combination of observed coupling constants, NOESY correlations (Fig. 2) and selected NOE differential experiments. The NOESY correlations with Me-31 to Me-32 and H-14, and with Me-29 to H-12 and H-13 were observed. These NOESY data and the large coupling constant ($J_{11,12}$ = 11.2 Hz) suggested a *trans*-juncture between the A and B

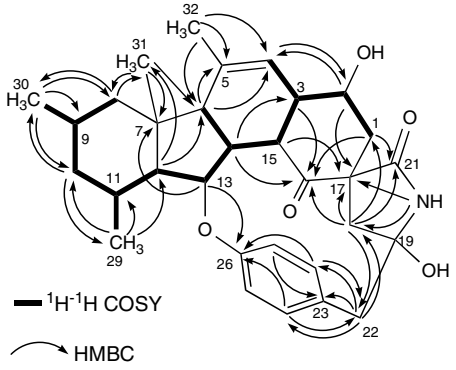


Figure 1. ^{1}H–^{1}H COSY (bold lines) and HMBC (arrows) correlations observed for **1**.

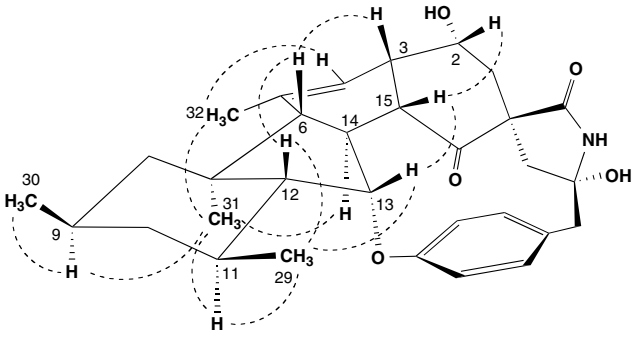
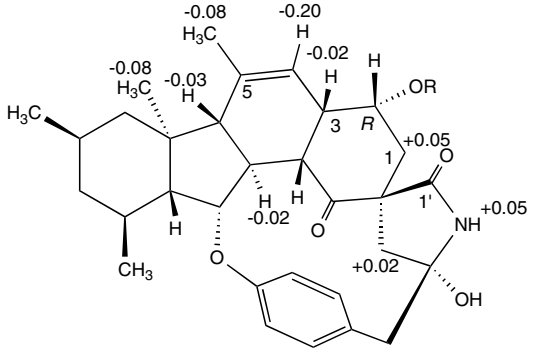


Figure 2. Selected NOESY correlations observed for **1**.

rings with α Me-31 and β H-12. The $J_{6,14}$ value (12.7 Hz) and NOESY correlation with H-6 to H-12 implied that H-6 was arranged pseudoaxially and *trans* to H-14. NOESY correlations with H-3 to H-6 and with H-15 to H-13 and the coupling constant ($J_{3,15}$ = 6.2 Hz) indicated that C/D rings fusion was *cis*. The NOESY data between H-2 and H-15 implied that the hydroxyl group at C-2 was α-oriented. Additionally, NOESY correlations with Me-31 to H-9 and from Me-31 to H-11 suggested that the methyl groups at C-9 and C-11 were in equatorial positions. A chair-like conformation of ring A and ring D that was consistent with the results of the NOESY correlations is shown in Figure 2. Although two aromatic methine signals (H-25 and 27) were unresolved in the ^{1}H NMR spectrum of **1** in pyridine-d_5, the spectrum of (*S*)-(−)-2-methoxy-2-phenyl-2-(trifluoromethyl)acetic acid (MTPA) ester **1a** recorded in $CDCl_3$ enabled these two to be resolved. NOEs from H-22 β to NH-20 and H-28, from H-14 to H-25, and from H-24 to H-18 β and H-22 α, as determined in the NOE difference experiment of **1a**, indicate that the relative configuration of the spiro carbon (C-17) is as shown. Thus, the relative stereochemistry of **1** was established.

The absolute configuration of pyrrospirone A (**1**) was determined by the modified Mosher's method. The ^{1}H chemical shift differences between the (*S*)-(−) and (*R*)-(−)-MTPA esters (**1a** and **1b**) of **1** are shown in Figure 3. The results suggest that the *R* configuration is attributable to the C-2 chirality, thus elucidating the absolute structure of pyrrospirone A (**1**).

The molecular formula of pyrrospirone B (**2**) was found to be $C_{31}H_{39}NO_5$ by HRFABMS, indicating that **2** had the same molecular formula as **1**. The IR, ^{1}H, and ^{13}C NMR spectra (Table 2) of **2** resemble those of **1**. It is possible that **2** is a diastereomer of **1**. A detailed comparison of the chemical shifts and coupling constants between the ^{1}H NMR spectrum of **1** and that of **2** revealed differences only in the signals of the methine proton (H-2 and H-4). These differences suggest that **2** is the C-2 epimer of **1**. This agrees with the COSY, ^{13}C–^{1}H COSY, and HMBC data. This has been confirmed by NOE experiments, in which the correlation of OH-2 with H-15 has been observed; however, no NOE from H-2 to H-15 has been seen, indicating that the structure of **2** is the C-2 epimer of pyrrospirone A (**1**). The unambiguous determination of the origin of the signals in the ^{1}H and ^{13}C NMR spectra was based on HMBC experiments.



1a: R = (*S*)-(-)-MTPA
1b: R = (*R*)-(+)-MTPA

Figure 3. Δδ values [δ(−) − δ(+)] for the MTPA esters (**1a** and **1b**).

Table 2
Cytotoxic activities of compounds **1**–**4**

Compounds	IC_{50} (μM)		
	HL-60	K 562	LNCaP
Pyrrospirone A (**1**)	14.91	9.96	17.97
Pyrrospirone B (**2**)	7.44	9.27	20.24
Pyrrocidine A (**3**)	0.12	0.45	0.48
Pyrrocidine B (**4**)	6.92	8.64	11.74

Although some closely related skeletons—GKK1032A$_1$,[7,8] GKK1032A$_2$,[7,8] GKK1032B,[7,8] and hirsutellones A-F[9,10]—of pyrrocidine A possessing a unique 13-membered macro-ether ring structural feature were isolated as fungal metabolites, compounds containing a spiro ring system like **1** and **2** are unique and belong to a new type of pyrrocidine analogue.

Compounds **1, 2, 3**, and **4** were tested for in vitro cytotoxicity against HL-60 (human promyelocytic leukemia), K562 (human chronic myelogenous leukemia), and LNCaP (human prostate carcinoma) cell lines by using the MTT methods. All the compounds showed cytotoxicity against these cell lines, with IC_{50} values ranging from 0.12 to 20.24 μM; one of the compounds, **3**, was found to be more cytotoxic than the others against HL-60 (IC_{50}: 0.12 μM, Table 2). Although pyrrospirones A (**1**) and B (**2**) appeared less active than **3**, they induced apoptosis against HL-60 cells at a concentration of 30 μM (Fig. 4). In addition, the concentration range in which they induced apoptosis was smaller than that of pyrrocidine A (**3**) (data not shown). The examination of the results allowed us to determine some structural and activity relationships. Higher activity was observed when the structure had a pyrrolidone unit with a double bond (C-17 and C-18). Furthermore, when dissolved in

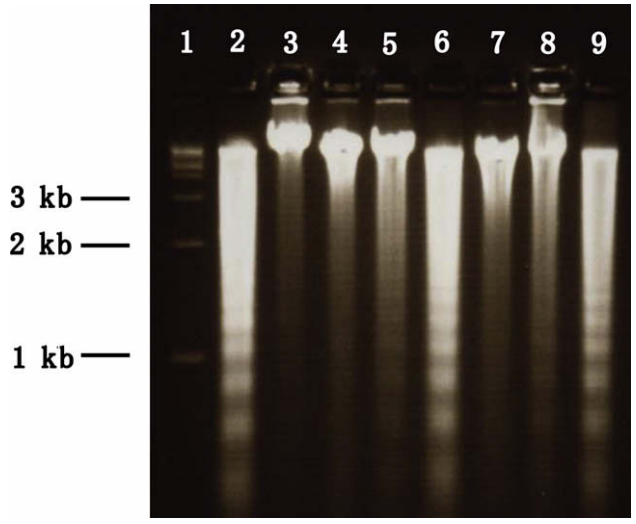


Figure 4. Pyrrospirones A (**1**) and B (**2**) induce DNA fragmentation in HL-60 cells. DNA was prepared from **1** or **2**-treated HL-60 cells at 37 °C for 18 h using an Isoplant (Nippon gene) and was separated by 2% agarose-gel electrophoresis. The panel shows the results for **1** and **2**. Drug concentrations added are: 10, 20, and 30 μM of **1** for lanes 4–6; 10, 20, and 30 μM of 2 for lanes 7–9. Lanes 1, 2, and 3 of the panel comprise 1 kb DNA size markers, control cells treated with 100 nM camptothecin, and samples from vehicle (1% methanol)-treated cells, respectively.

methanol, **3** was easily converted to a methanol adduct by Michael-type addition to the α,β-unsaturated carbonyl group of pyrrolidone. This high reactivity of the double bond of pyrrolidone could be one of the reasons why **3** showed higher activity than, and different behavior from, **1** and **2**. Details on biological activity studies will be described in a future paper.

*Pyrrospirone A (**1**):* Yellow oil, $[\alpha]_D^{20}$ +128° (*c* 0.22, MeOH); IR ν_{max} (KBr) cm^{-1}: 3432, 2948, 1716, 1671, 1652, 1606, 1508; UV λ_{max} nm (log ε) in MeOH: 229 (3.7), 279 sh (3.2); HRFABMS *m/z*: 528.2731 $[M+Na]^+$ ($C_{31}H_{39}NO_5Na$ requires 528.2726); FABMS *m/z*: 528 $[M+Na]^+$; for ^1H and ^{13}C NMR data, see Table 1.

*Pyrrospirone B (**2**):* Yellow oil, $[\alpha]_D^{20}$ +135° (*c* 0.21, MeOH); IR ν_{max} (KBr) cm^{-1}: 3423, 2950, 1718, 1671, 1658, 1604, 1508; UV λ_{max} nm (log ε) in MeOH: 229 (3.7), 278 sh (3.2); HRFABMS *m/z*: 506.2901 $[M+H]^+$ ($C_{31}H_{40}NO_5$ requires 506.2906); FABMS *m/z*: 506 $[M+H]^+$; for ^1H and ^{13}C NMR data, see Table 1.

Acknowledgment

We thank Ms. Teiko Yamada of the Faculty of Agriculture at Tohoku University for NOESY and HRMS measurements.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.10.032.

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