

NOTE

Pyrrolizilactone, a new pyrrolizidinone metabolite produced by a fungus

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In the course of our screening program to find structurally unique metabolites from microorganisms on the basis of spectral data collected through LC/MS analysis, a new pyrrolizidinone metabolite, pyrrolizilactone (**1**) (Figure 1), was discovered and isolated from an uncharacterized fungus. The structure of **1** was determined from spectroscopic results. Compound **1** showed moderate cytotoxic activity against HL-60 and HeLa cells.

Microorganisms have a tremendous capacity for producing structurally diverse metabolites, which show various activities.¹ They are important sources of pharmaceutical leads and therapeutic agents,^{2,3} and are also used as bioprobes in chemical biology for the exploration of biological functions.^{4,5} To search for and discover such structurally unique metabolites efficiently and rapidly, we have constructed a microbial metabolite fraction library with a spectral database on the basis of photodiode array detector-attached LC/MS analysis.^{6,7} Through our methodology for the construction of this fraction library, we discovered and identified a 16-membered macrolactam with an unusual β -keto-amide moiety, verticilactam,⁸ 6,6-spiroacetal polyketide, spirotoamides, A and B,⁹ the new fraquinocins, I and J,¹⁰ and 6-dimethylallylindole-3-carbaldehyde.¹¹ These results demonstrate the advantage of the fraction library in isolating novel metabolites from natural sources. We report herein the isolation of a novel compound from a fungi fraction library.

An 18-liter culture broth of an uncharacterized fungus was cultivated to obtain 16.1 g of an ethyl acetate-soluble extract. This was separated into eight fractions through a silica-gel column chromatography, with a stepwise gradient of CHCl₃/MeOH. The second fraction eluted with CHCl₃/MeOH (100:1) was further separated by chromatography using a Sephadex LH-20 column with CHCl₃/MeOH (1:1) to afford three fractions. The second fraction, showing an unidentified peak in LC/MS analysis, was purified by C₁₈-HPLC to afford a colorless amorphous solid (**1**, 5.5 mg). Colorless amorphous; [α]_D²⁶ + 5.9° (c 0.08, MeOH); UV (MeOH) λ_{\max} (log ϵ) 209 (3.34), 236 (3.01) nm; IR (ATR) ν_{\max} (cm⁻¹) 3410, 2920, 2875, 1790, 1715, 1685, 1575, 1450, 1375, 1335, 1280, 1160, 1110, 1020; ¹H NMR and ¹³C NMR data, see Table 1; HRESIMS *m/z*: 416.2433 [M + H]⁺ (calcd for C₂₄H₃₄NO₅: 416.2437).

Compound **1** had the molecular formula C₂₄H₃₃NO₅, as determined by HRESIMS. The IR spectrum implied the presence of hydroxyl (3410 cm⁻¹) and carbonyl (1685, 1715 and 1790 cm⁻¹) groups. The ¹H NMR spectrum showed five methyl signals, which included a singlet, three doublets and a doublet of doublet (1.71 p.p.m., dd, *J* = 0.9, 0.9 Hz) branched at an sp² carbon, and an olefin signal (5.12 p.p.m., br s), suggesting that **1** contained a double bond (Supplementary Figure S1). The ¹³C NMR spectrum showed 24 signals including five methyls, four methylenes, nine methines (including oxygenated and olefin carbons: 81.6 and 131.3 p.p.m.),

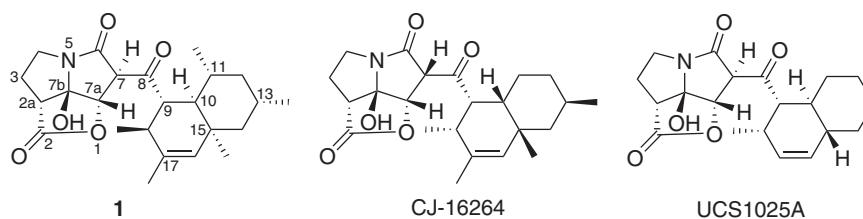


Figure 1 Structures of compound **1** and related compounds.

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Table 1 ^1H and ^{13}C NMR chemical shifts with HMBC and NOESY correlations in CDCl_3

position	δ_{C}	δ_{H} (Multiplicity, J in Hz)	HMBC	NOESY
2	174.6	—	—	—
2a	47.6	3.24 (dd, 9.2, 1.8)	C-2, 3, 4, 7b	H-3a, H-3b
3	29.9	2.73 a (dddd, 14.2, 9.6, 5.0, 1.8) 2.56 b (m)	C-2, 2a, 9 C-2, 2a	— —
4	41.8	3.82 a (ddd, 11.9, 9.6, 5.5) 3.35 b (ddd, 11.9, 9.6, 5.0)	— C-3, 7b	H-3b H-3a
6	167.6	—	—	—
7	63.1	4.15 (s)	C-6, 7a, 7b, 8	H-9, H-18
7a	81.6	4.65 (s)	C-2, 6, 7, 7b, 8	H-2a, H-7, H-9
7b	100.7	—	—	—
8	210.3	—	—	—
9	58.8	2.85 (br s)	C-8, 10, 11, 17, 18, Me-18	H-10, Me-11, Me-18
10	46.2	2.10 ax. (br d, 10.5)	C-8, 9, 11, 15, 16, 18, Me-11	H-12ax, H-14ax, Me-11, Me-15
11	32.9	1.57 ax. (m)	—	Me-11, Me-18
12	44.4	0.78 ax. (br ddd, 12.4, 11.5, 11.5) 1.61 eq. (ddd, 12.4, 3.2, 3.2)	— —	— Me-13
13	28.3	1.43 ax. (m)	—	Me-13
14	50.3	0.98 ax. (dd, 12.4, 12.4) 1.40 eq. (dd, 12.4, 2.7)	C-12, 15, 16, Me-15 —	Me-13, Me-15 Me-13, Me-15
15	37.5	—	—	—
16	131.3	5.12 (br s)	C-10, 14, Me-15, Me-17	H-14eq, Me-15, Me-17
17	133.0	—	—	—
18	29.4	2.93 (m)	C-8, 17, Me-18	Me-15, Me-17, Me-18
Me-11	21.0	1.04 (3H, d, 6.4)	C-10, 11, 12	—
Me-13	22.3	0.81 (3H, d, 6.4)	C-12, 13, 14	—
Me-15	29.8	0.62 (3H, s)	C-10, 14, 15, 16	—
Me-17	21.7	1.71 (3H, dd, 0.9, 0.9)	C-17, 18	—
Me-18	21.2	1.11 (3H, d, 7.8)	C-9, 17, 18	—

^1H and ^{13}C NMR spectra were recorded at 500 and 125 MHz, respectively.

and six quaternary carbons including an olefin carbon (133.0 p.p.m.) and three carbonyl ones (167.6, 174.6 and 210.3 p.p.m.), one of which was a ketone (Supplementary Figure S2). These were confirmed by the ^{13}C DEPT experiment and HSQC spectrum (Supplementary Figures S3 and S4). The spectrum also included an unusual quaternary carbon at 100.7 p.p.m., implied to bear both oxygen and nitrogen atoms or two oxygen atoms. These data and the index of hydrogen deficiency of nine suggested that compound **1** consisted of a five-ring system with three carbonyls and one double bond. The planar structure was established through the interpretation of the 2D NMR spectra (Figure 2a). The connections between protons and carbons were confirmed by correlations in the HSQC spectrum. Two proton-spin systems from H-2a to H-4 and from Me-18 to H-14 with methyl branches at C-11 and C-13 were constructed from the correlations in the DQF-COSY and HSQC-TOCSY spectra (Supplementary Figures S5 and S6). The overall structure was established from the long-range correlations in the HMBC spectrum (Supplementary Figure S7). The HMBC correlations from Me-15 to C-10, C-14, C-15 and C-16 showed the attachment of Me-15 at C-15, which was connected to C-10 to construct a cyclohexane substructure, and connectivities from C-14 to C-16. These were confirmed by the HMBC correlations from H-10 to C-15 and C-16, and from H-14 to C-15 and C-16. Me-17, which should be branched at an sp^2 carbon, showed HMBC correlations to C-16, C-17 and C-18, suggesting a methyl branch at C-17 and connectivities from C-16 to C-18. These results in combination with the DQF-COSY result indicated a pentamethyl-octahydronaphthalene structure; this was confirmed by HMBC correlations from H-9 to C-15 and C-17, and from both H-18 and Me-18 to C-17.

A pyrrolizidinone moiety fused with γ -lactone system was confirmed by HMBC correlations and NMR chemical shift values with the consideration of the index of hydrogen deficiency, which suggested the remaining substructure required a three-ring system. The HMBC correlations from H-2a and H-3 to a carbonyl carbon C-2 and from H-2a to C-7b having the unusual ^{13}C NMR chemical shift value of 100.7 p.p.m. were observed. The C-7b was also correlated from H-7 and H-7a in the HMBC spectrum, both of which showed HMBC correlations to another carbonyl carbon C-6. These HMBC correlations and the connectivities from H-2a to H-4 was confirmed by DQF-COSY, as described above, were allowing the connectivities between C-4 and C-6 with a branch of the carbonyl carbon C-2 at C-2a position. The connection between C-7 and C-7a, both of which were observed as a singlet signal in the ^1H NMR spectrum and did not show COSY correlation, was confirmed by an HMBC correlation from H-7 to C-7a and NOE between H-7 and H-7a in the phase-sensitive NOESY spectrum (Supplementary Figure S8). An HMBC correlation from H-7a to C-2 implied the presence of a ring system, and it was confirmed as a γ -lactone by the ^{13}C NMR chemical shift values of C-2 and C-7a (174.6 and 81.6 p.p.m., respectively). Based on the above results and the consideration of molecular formula, the C-7b was supposed to have both oxygen and nitrogen atoms. The ^{13}C NMR chemical shift values of C-4 and C-6 (41.8 and 167.6 p.p.m., respectively) and the substructure being a tricyclo-system allowed the construction of a pyrrolizidinone skeleton, which were also confirmed by an HMBC correlation from H-4 to C-7b. The pyrrolizidinone substructure was connected to the decalin moiety through the ketone C-8, which was confirmed by HMBC correlations from H-7, H-7a,

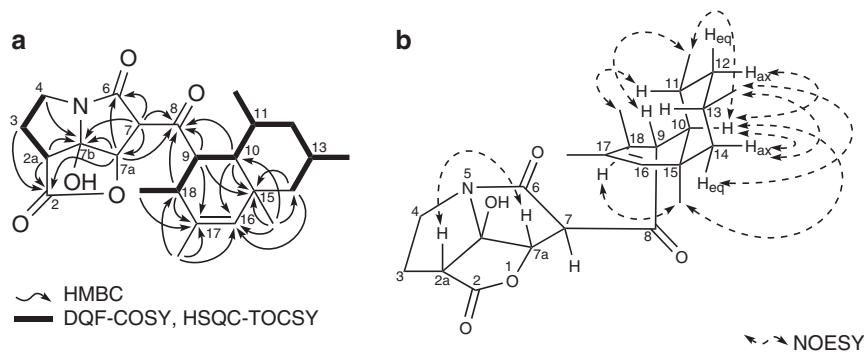


Figure 2 Selected DQF-COSY and HMBC correlations (a) and selected NOESY correlations (b).

H-9, H-10 and H-18 to the remaining carbonyl carbon at 210.3 p.p.m. Therefore, the planar structure of compound **1** was determined.

The relative stereochemistry was examined by the phase-sensitive NOESY spectrum (Figure 2b). H-10 showed NOEs with H-12 at 0.78 p.p.m. and H-14 at 0.98 p.p.m., and these correlations suggested that the cyclohexane had a chair conformation and all three protons were assigned as axials. H-10 also had NOESY correlations with Me-11 and Me-15, allowing the assignments of their equatorial-orientations on the cyclohexane part, and a *cis*-configuration of the decalin moiety, which was also confirmed by NOEs between H-11 and Me-18, between Me-11 and H-9, and between Me-15 and H-18. These NOEs were also confirmed by β -orientation of Me-18 and α -orientation of the pyrrolizidinone moiety at C-9 position. Me-13 was assigned as an equatorial by the large coupling constants of $^3J_{\text{H-12ax/H-13}}$ and $^3J_{\text{H-13/H-14ax}}$, which suggested H-13 was an axial. It was also confirmed by the NOEs with both H-14axial and H-14equatorial. In the pyrrolizidinone moiety, an NOE between H-2a and H-7a was observed suggesting β -orientations of them, which also allowed the assignment of β -orientation of the hydroxyl group at C-7b. H-7 was assigned as α -orientation by the dihedral angle of H-7-C-7—C-7a-H-7a was approximately 90° , which were implied from the weak vicinal coupling between H-7 and H-7a. Based on all the above results, the structure of compound **1** was determined as shown in Figure 1 and designated as pyrrolizilactone.

The configuration between the decalin and pyrrolizidinone moieties could not be determined by NOESY spectrum due to free rotation at C-7 and C-9 positions, but NOEs between H-7 and H-9 and H-7 and H-18 were observed (Table 1). Therefore, there are four possible stereo-structures, which include two enantiomers and two diastereomers by the same relative stereochemistry.

Cytotoxic and antibacterial activities of compound **1** were evaluated *in vitro*. Compound **1** showed moderate cytotoxicities against human promyelocytic leukemia cell line HL-60 and human cervical cancer cell line HeLa with IC_{50} values of 1.1 and $3.1 \mu\text{g ml}^{-1}$, respectively. In contrast, it did not show antibacterial activity against *Escherichia coli* up to $30 \mu\text{g ml}^{-1}$. Two compounds, CJ-16264¹² and UCS1025A,¹³ have been reported in the same class of metabolites (Figure 1), and show broad antibacterial activities against Gram-positive bacteria. It is worth evaluating such activities in future work. We have also observed morphological changes on cancer cell lines, and will report the findings elsewhere.

Compound **1** had a tricyclic skeleton composed of a pyrrolizidinone moiety fused with a γ -lactone, which was connected to a decalin

moiety by a ketone. As mentioned above, only two compounds, UCS1025A and CJ-16264, have been reported in the same class of fungal metabolites, and the differences between them in terms of their planar structure lie in the substitution pattern of methyl groups on the decalin moiety. That of **1** was highly substituted with five methyl groups. UCS1025A, on the other hand, had only one methyl group. In terms of the stereochemistry, the pyrrolizidinone moiety had the same relative configuration as UCS1025A, with a *trans* configuration at H-7 and H-7a. The structure of CJ-16264 was shown to have a *cis*-configuration at H-7 and H-7a.¹² This could be a mistake, because Sugie *et al.*¹² determined the dihedral angle of H-7—C-7—C-7a—H-7a to be 84.9° through molecular mechanics calculations, which clearly suggests a *trans* configuration. The decalin unit of **1** had a *cis*-configuration, which is consistent with CJ-16264 but not with UCS1025A, which has a *trans* configuration.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (<http://www.nature.com/ja>)