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Pyrrocidines A and B, new antibiotics produced by a filamentous fungus

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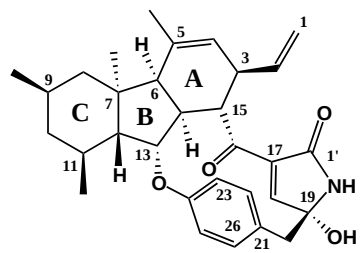
Abstract—Pyrrocidines A (**1**) and B (**2**), two new antibiotics, containing rare 13-membered macrocycles, were isolated from the fermentation broth of a fungus, *LL*-Cyan426. Pyrrocidine A (**1**) exhibited potent activity against Gram-positive bacteria, including drug-resistant strains. The structures of these compounds were established using spectroscopic methods.

In the course of our continuous search for novel antibiotics to combat drug-resistance in antibacterial chemotherapy,[1] we discovered that the production of many biologically active fungal metabolites could be significantly increased by fermentation in heterogeneous phases.[2] Among the several organisms we studied, an unidentified filamentous fungus, *LL*-Cyan426,[3] produced much enhanced antibacterial activity when fermented with this method compared with the regular one-phase liquid or solid medium fermentation methods. Two closely related antibiotics, designated pyrrocidines A (**1**) and B (**2**), were isolated from the fermentation broth and found to be primarily responsible for the activity. Compounds **1** and **2** possess a tricyclic moiety, which is fused to 13-membered macrocycles containing phenyl and pyrrolidinone functions (Fig. 1). Compound **1** showed potent activity against Gram-positive bacteria, including the piperacillin-resistant streptococci and vancomycin-resistant enterococci. In this paper the production, isolation, and structural determination of the new antibiotics pyrrocidines A (**1**) and B (**2**) are reported.

A 14-day culture of *LL*-Cyan426 in heterogeneous phases[4] was extracted by methanol and an assay-guided fractionation[5] of the extract led to the isolation of the hydrophobic pyrrocidines A (**1**) and B (**2**), along with the known ilicicolins A, C, E, and F.[6]

The molecular formula of pyrrocidine A (**1**)[7] was determined to be $C_{31}H_{37}NO_4$ by high resolution Fourier transform ion cyclotron resonance (FTICR) mass spectrometry. The ^{13}C NMR spectrum displayed signals of a ketone carbonyl at δ 202.4 and an amide carbonyl at 168.2, together with 12 sp^2 carbon signals between δ 114.7 and 156.6. The ^{13}C signals at δ 91.0 and 87.6 indicated the presence of two carbons each attached to at least one heteroatom. The ^1H NMR spectrum showed a D_2O exchangeable signal at δ 8.77, assigned to an amide NH proton. There were four double doublet signals belonging to a homonuclear spin system in



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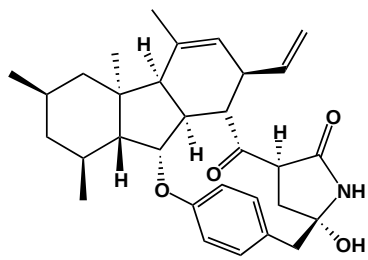

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Figure 1. Structures of cyan426-A (**1**) and B (**2**).

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the range from δ 6.80 to 7.18, indicative of a *para*-substituted benzene ring whose rotation was restricted. In addition, four methyl signals were observed at δ 0.85, 0.94, 1.15, and 1.74. The ^{1}H and ^{13}C NMR spectral data of **1** are listed in Table 1.

Detailed analysis of 2-D ^{1}H–^{1}H COSY and TOCSY, ^{1}H–^{13}C HMBC, and HSQC data for **1** revealed a tricyclic system from C-3 to C-15. The COSY and TOCSY spectra delineated the ^{1}H–^{1}H spin system including H-4, H-3, H-15, H-14, H-6, H-13, H-12, H-11, H_2-10, H-9, and H_2-8. The 2- or 3-bond ^{1}H–^{13}C correlations in an HMBC spectrum between 5-Me (δ 1.68) and C-4 (122.7), C-5 (135.4), and C-6 (51.7), between 7-Me (δ 1.13) and C-6, C-7 (46.9), C-8 (47.8), and C-12 (52.8), between 9-Me (δ 0.85) and C-8, C-9 (27.7) and C-10 (44.3), and between 11-Me (δ 0.94) and C-10, C-11 (26.6), and C-12 established the substituted decahydrofluorene moiety. The attachment of the C-1 and C-2 ethenyl group at C-3 was required by a cross peak between H-2 at δ 5.86 and H-3 at 2.68 in the COSY spectrum, and by related correlations in the HMBC spectrum (Table 1).

This tricyclic system was fused to an unusual 13-membered macrocycle through ether and ketone linkages. In the HMBC spectrum, a weak correlation from H-13 at δ 4.25 to C-24 at 156.6 implied that C-13 and C-24 were connected through an ether linkage. On the other hand, the evidence for the ketone linkage was found in the 2- and 3-bond correlations from H-14 at δ 2.10, H-15 at 2.76, and H-18 at 6.67 to the C-16 ketone signal at δ 202.4. The 5-membered pyrrolidinone ring was identified based on the chemical shift data, and the HMBC correlations from the amide proton at δ 8.77 to carbons C-1′ at δ 168.2, C-17 at 138.5 and C-18 at 151.3, C-19 at 87.6, and from H-18 to C-17, C-19, and C-1′. The correlations from H_2-20 at δ 3.10 and 3.17 to C-19, C-21 at 130.9 and C-26 at 131.6 indicated that the phenyl and pyrrolidinone functions were connected through a methylene group. Thus, the elucidation of the planar structure of pyrrocidine A (**1**) was com-

Table 1. ^{1}H and ^{13}C NMR spectral data for pyrrocidine A (**1**), in DMSO-d_6

Atom	^{1}H (400 MHz, mult., J in Hz)	^{13}C (100 MHz)	HMBC (J=8 Hz)
1	4.95 (*cis*, d, 9.5)	114.7 (CH_2)	C-2, C-3
	4.98 (*trans*, d, 16.5)		C-2, C-3
2	5.86 (m)	141.6 (CH)	C-3, C-4, C-15
3	2.68 (m)	37.2 (CH)	C-2, C-4, C-5, C-14, C-15, C-16
4	5.42 (br d, 6.4)	122.7 (CH)	C-3, 5-Me, C-6
5		135.4 (C)	
5-Me	1.74 (3H, s)	25.0 (CH_3)	C-4, C-5, C-6
6	2.11 (m)	51.7 (CH)	C-4, C-5, C-7, 7-Me, C-12, C-13, C-14
7		46.9 (C)	
7-Me	1.15 (3H, s)	23.5 (CH_3)	C-6, C-7, C-8, C-12
8	0.88 (m)	47.8 (CH_2)	C-7, 7-Me, C-9
	1.69 (m)		C-7, C-9, C-10, C-12
9	1.70 (m)	27.7 (CH)	C-10
9-Me	0.85 (3H, d, 6.2)	22.8 (CH_3)	C-8, C-9, C-10
10	0.43 (ddd, 11.7, 11.7, 11.7)	44.3 (CH_2)	C-8, C-9, 9-Me, C-11, 11-Me, C-12
	1.67 (m)		C-8, C-9, C-12
11	1.78 (m)	26.6 (CH)	11-Me, C-10
11-Me	0.94 (3H, d, 6.2)	19.7 (CH_3)	C-10, C-11, C-12
12	1.10 (dd, 11.4, 6.1)	52.8 (CH)	C-6, C-7, 7-Me, C-8, C-11, 11-Me
13	4.25 (br d, 6.1)	91.0 (CH)	C-7, C-12, C-15, C-24
14	2.10 (m)	41.4 (CH)	C-3, C-5, C-6, C-13, C-15, C-16
15	2.76 (br s)	50.4 (CH)	C-2, C-3, C-4, C-6, C-13, C-14, C-16
16		202.4 (C)	
17		138.5 (C)	
18	6.67 (s)	151.3 (CH)	C-1′, C-16, C-17, C-19
19		87.6 (C)	
20	3.10 (d, 12.5)	43.8 (CH_2)	C-18, C-19, C-21, C-26
	3.17 (d, 13.7)		C-18, C-19, C-21, C-26
21		130.9 (C)	
22	7.15 (br d, 7.3)	130.0 (CH)	C-19, C-20, C-24, C-26
23	7.02 (br d, 7.9)	121.4 (CH)	C-21, C-24, C-25
24		156.6 (C)	
25	6.8 (br d, 7.8)	124.4 (CH)	C-21, C-23, C-24
26	7.17 (br d, 6.9)	131.6 (CH)	C-19, C-20, C-22, C-24
1′		168.2 (C)	
NH	8.77 (s)		C-1′, C-17, C-18, C-19

pleted. Compound **1** was treated with 0.2 M HCl in 1:5 MeOH/Et_2O (rt, 24 h) to give a methyl ether derivative. The ^1H NMR spectrum of the product showed an additional methyl signal at δ 3.06, which was correlated to ^{13}C signal at δ 92.3 in an HMBC spectrum. These data clearly indicated the presence of a methoxy group on C-19 in the methylation product, which confirmed the structural assignment for **1**.

The molecular formula of pyrrocidine B (**2**)[8] was determined by high resolution FTICR mass spectrometry to be $C_{31}H_{39}NO_4$, indicating two additional hydrogen atoms to that of pyrrocidine A (**1**). The analysis of its ^1H and ^{13}C NMR spectral data led to the conclusion that this compound differed **1** only in the pyrrolidinone ring where the double bond was reduced.

The relative configurations of these compounds were deduced from the NOE data. For both compounds **1** and **2**, strong cross peaks in ROESY spectra from 7-Me to H-6 and H-14, and signals from H_2-1 to H-12 defined *cis* A/B and *trans* B/C ring junctions. The ROESY cross peaks from H-2 to H-15, from H-13 to H-15, and from H-12 to H-13 required that both H-13 and H-15 have upward orientations. The H-10*ax* at δ 0.43 (ddd, all 11.7 Hz) was diaxial to H-9 and H-11, indicating equatorial positions for 9-Me and 11-Me. Finally, the regiochemistry of the pyrrolidinone moieties was determined by the ROESY correlations from 7-Me to H-25, from H-18 to H-26, and from H-15 to H-22 and H-23. The stereochemistry for these two compounds and the selected ROESY correlations were shown in Fig. 2.

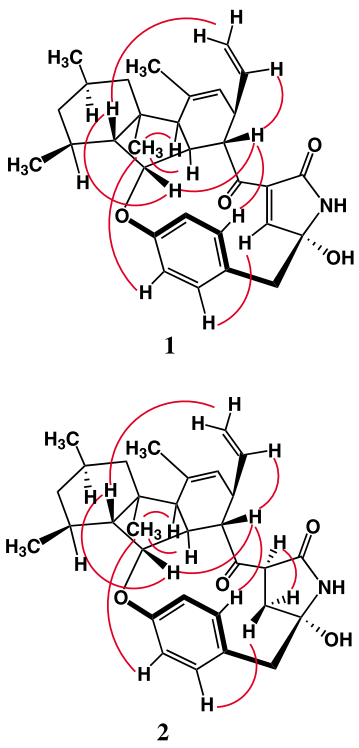

Figure 2. Selected NOEs (indicated by arcs) which define the stereochemistry for pyrrocidines A (**1**) and B (**2**).

Pyrrocidine A (**1**) exhibited potent antibiotic activity against most Gram-positive bacteria, including the drug-resistant strains, but only showed moderate activity against *Streptococcus pneumoniae*. It was also active against the yeast *Candida albicans*. Pyrrocidine B (**2**) showed weaker activity. MIC data obtained by the broth dilution method are listed in Table 2.

The pyrrolidinone function has been reported in other antifungal compounds, the talaroconvolutin A (**3**)[9] and the modulator for synthesis of platelet-activating factor ZG-1494α (**4**),[10] but the 13-membered macrocycle containing ether, phenyl, pyrrolidinone, and ketone functions as in **1** is the first example found in natural products. While preparing for this manuscript, a patent publication covering the antitumor compound, GKK1032 [planar structure, identical to pyrrocidine B (**2**)], came to our attention.[11] Biosynthetically, these compounds are suspected to derive from polyketide and amino acid origins. The ^{13}C labeling experiments designed to study their biosynthesis pathway are currently underway (Fig. 3).

Table 2. Antimicrobial activity of pyrrocidines A and B

Test organism	MIC (μg/ml)[a]	
	A(**1**)	B(**2**)
Staphylococcus aureus (four strains, including two piperacillin-resistant strains)	0.25–2	4–8
S. haemolyticus GC 4546	0.25	8
Enterococcus faecalis (three strains)	0.5	4–8
E. faecium (three strains, including two vancomycin-resistant strains)	0.5–1	4–8
Streptococcus pneumoniae (two strains)	16–64	32–128
Escherichia coli (two strains)	128	>128
Candida albicans	8	128

[a] Broth dilution method in Mueller–Hinton II, incubated at 35°C for 18 h.

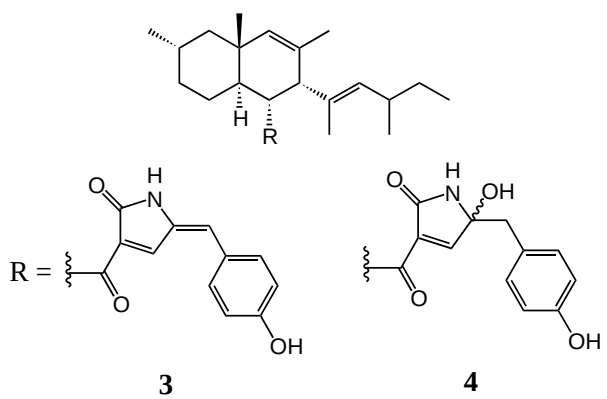

Figure 3. Structures of talaroconvolutin A (**3**) and ZG-1494α (**4**), which contain pyrrolidinone function.

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References

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2. Details of this fermentation method will be published elsewhere. An example of *LL*-Cyan426 fermentation is described in Ref. 4.
3. Isolated from a mixed Douglas Fir hardwood forest on Crane Island Preserve, Washington, in 1993.
4. The fungal culture *LL*-Cyan426 was plated on Bennett's agar medium from a frozen culture and incubated at 22°C. A small agar slice bearing mycelia was used to inoculate 50 ml of Difco potato-dextrose broth in a 250 ml Erlenmeyer flask. This liquid seed culture was shaken at 200 rpm at 22°C for 1 week, and then used to inoculate the production medium. The sterilized production medium (1 L, 25 g Difco malt extract, 5 g Difco peptone, 0.5 g Difco yeast extract, 20 g Difco agar) was poured into a polypropylene tray. The solidified agar was then overlaid with a sheet of sterilized milk filter paper followed by inoculation with 50 ml of seed culture fluid. After 2 weeks incubation at 22°C, the filter paper bearing prolific mycelial growth was peeled, lyophilized, and extracted with methanol.
5. The methanol extract was separated by reverse phase HPLC on a C18 column (YMC ODS-A, 10 μm particle size, 70×500 mm), using a gradient of 90–100% acetonitrile in water containing 0.02% trifluoroacetic acid (TFA) over 40 min. The early fractions were found to contain the known ilicicolins A, C, E, and F, and the materials from a late fraction at 33 min, active in antibacterial assays, were further separated by a different HPLC system (YMC ODS-A, 5 μm, 10×250 mm column, 70–100% acetonitrile in water with 0.02% TFA over 25 min) to afford pure pyrrocidines B (**2**) (3.1 mg) and A (**1**) (21.8 mg) as yellowish amorphous powders.
6. Minato, H.; Katayama, T.; Hayakawa, S.; Katajiri, K. *J. Antibiot*. **1972**, *25* (5), 315.
7. $[\alpha]_D = +91.2°$ ($c = 0.58$, MeOH), HRFTICRMS (neg.) m/z 486.26486 [$(M-H)^-$, $C_{31}H_{36}NO_4$ requires 486.26498]; ^1H and ^{13}C NMR spectral data, see Table 1.
8. $[\alpha]_D = +88.2°$ ($c = 0.22$, MeOH), HRFTICRMS (pos.) m/z 490.29481 (MH^+, $C_{31}H_{40}NO_4$ requires 490.29573); ^1H NMR (DMSO-d_6, 400 MHz) δ 4.90 (br d, 9.5 Hz, H-1*cis*), 4.94 (br d, 16.1 Hz, H-1*trans*), 5.87 (m, H-2), 3.11 (m, H-3), 5.20 (br d, 6.6 Hz, H-4), 1.68 (3H, s, 5-Me), 2.29 (d, 8.4 Hz, H-6), 1.13 (3H, s, 7-Me), 0.87, 1.66 (m, H_2-8), 1.72 (m, H-9), 0.86 (3H, d, 6.2 Hz, 9-Me), 0.40 (ddd, all 11.8 Hz, H-10*ax*), 1.67 (m, H-10*eq*), 1.77 (m, H-11), 0.96 (3H, d, 6.1 Hz, 11-Me), 0.99 (dd, 11.3, 6.7 Hz, H-12), 4.16 (dd, 6.7, 2.7 Hz, H-13), 2.19 (m, H-14), 1.78 (br s, H-15), 3.13 (dd, 12.7, 6.8 Hz, H-17), 1.73 (m), 2.12 (dd, 14.6, 12.7 Hz, H_2-18), 2.72, 2.96 (d, 13.1, H_2-20), 7.32 (dd, 8.4, 1.8 Hz, H-22), 7.07 (dd, 8.4, 2.1 Hz, H-23), 6.83 (dd, 8.1, 2.1 Hz, H-25), 7.20 (dd, 8.1, 1.8 Hz, H-26), 8.65 (br s, D_2O exchangeable, NH), ^{13}C NMR (DMSO-d_6, 100 MHz) δ 114.1 (C-1), 141.5 (C-2), 36.7 (C-3), 121.7 (C-4), 136.1 (C-5), 24.8 (5-Me), 52.9 (C-6), 46.9 (C-7), 22.9 (7-Me), 47.5 (C-8), 27.7 (C-9), 22.8 (9-Me), 44.5 (C-10), 26.7 (C-11), 19.8 (11-Me), 52.2 (C-12), 90.8 (C-13), 43.5 (C-14), 49.7 (C-15), 209.6 (C-16), 53.3 (C-17), 36.7 (C-18), 87.6 (C-19), 44.6 (C-20), 132.2 (C-21), 133.1 (C-22), 121.8 (C-23), 157.3 (C-24), 124.0 (C-25), 131.7 (C-26), 172.3 (C-1′).
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