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BRIEF COMMUNICATION

GKK1032C, a new alkaloid compound from the endophytic fungus *Penicillium* sp. CPCC 400817 with activity against methicillin-resistant *S. aureus*

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Abstract

Five alkaloid compounds (**1–5**), including one new compound, GKK1032C (**1**), and four known compounds, pyrrospirones E (**2**) and F (**3**), and GKK1032B (**4**) and A2 (**5**) were isolated from the culture of endophytic fungus *Penicillium* sp. CPCC 400817. The planar structures of all compounds were elucidated by NMR and MS spectra. The relative configuration of new compound GKK1032C (**1**) was deduced from the vicinal *J*-values and ROESY spectral data. Compound **1** exhibited potent antibacterial activity against methicillin-resistant *Staphylococcus aureus* with an MIC value of 1.6 μg ml^{-1}.

Microorganisms produce a remarkable array of small bioactive molecules that represent most of our new drugs, especially antibiotics [1]. The emergence and spread of antibacterial resistance are jeopardizing the effectiveness of most antibiotics in clinical use and are threatening to the public health worldwide; therefore it is imperative to develop new antibiotics to combat resistant pathogens [2]. In our ongoing screening program to discover new compounds against drug-resistant pathogens from microbes [3–6], it was found that the culture of strain CPCC 400817 exhibited good antibacterial activity. CPCC 400817, an endophytic *Penicillium* sp. strain, was isolated from a mangrove plant collected in Dongzhai harbor of Hainan province, which is deposited at the China Pharmaceutical Culture Collection (No. CPCC 400817). Investigation of the bioactive metabolites of CPCC 400817 led to the discovery of five alkaloid compounds, including one new compound, GKK1032C (**1**), and four known compounds (Fig. 1), pyrrospirones E (**2**) and F (**3**) [7], as well as GKK1032B (**4**) [8] and A2 (**5**) [9] (see the Supporting Information). Here we report their isolation, purification, structural elucidation and bioassay.

The strain was cultured on a PDA slant containing 0.3% potato extract, 2% glucose, and 1.5% agar at 25 °C for 5 days, then inoculated in 500-ml Erlenmeyer flasks containing 100 ml of F1 medium (2.0% glucose, 1.0% glycerol, 0.2% soybean powder, 1.0% sucrose, 1.0% peptone, 0.25% PEG (6000), 0.03%K_2HPO_4, 0.3% $(NH_4)_2SO_4$, 0.3% $NaNO_3$, pH 6). The flasks were placed in dark at 25 °C for 30 days without shaking. The culture (10 l) was filtered to separate the mycelia from the supernatant. The mycelia were extracted with acetone (3 × 10 l), and after recovering the organic solvent, the crude material was further extracted with EtOAc (3 × 3 l). The EtOAc-soluble fraction (8.7 g) was subjected to ODS column chromatography (MeOH/H_2O, v/v, 3:7 → 7:3 → 10:0) to finally yield six combined fractions (Fr. A to F). By repeated purification using semipreparative HPLC on a ReproSil-Pur Basic C18 column (5 μm, 250 × 10 mm) with an isocratic elution (MeOH-H_2O) at a flow rate of 2.5 ml min^{-1}, compounds **1** (4.3 mg), **2** (5.3 mg), **3** (5.6 mg), and **5** (10 mg) were obtained from Fr. D, and compound **4** (6 mg) was obtained from Fr. C.

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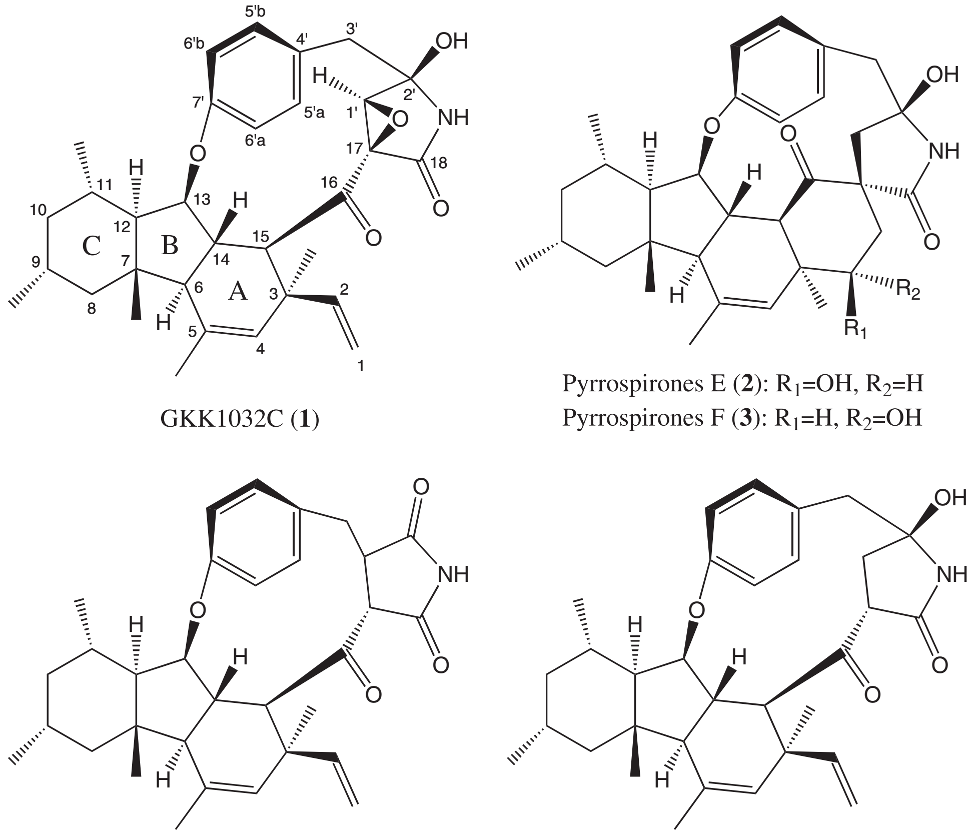

Fig. 1 Structures of compounds **1**-**5** isolated from *Penicillium* sp. CPCC 400817

GKK1032C (**1**) was obtained as white powder and was determined to have a molecular formula of $C_{32}H_{39}O_5N$, based on high resolution electrospray ionization mass spectrometry (HR-ESIMS) $[M - H]^-$ *m/z* 516.2756 and analysis of ^{1}H and ^{13}C NMR data. The planar structure of **1** was deduced by interpretation of ^{1}H and ^{13}C NMR, ^{1}H-^{1}H COSY, HSQC and HMBC spectral data (Table 1) and comparison of these data with those of related alkaloids [9, 10]. Detailed analysis of ^{1}H-^{1}H COSY spectrum disclosed the presence of the spin systems $CH_2 = CH$-, -$CH_2CH(CH_3)CH_2CH(CH_3)CHCHCH(CH)CH$-, and that due to a *para*-substituted benzene ring with restricted free rotation. Further comprehensive analysis of NMR data indicated that **1** contained a γ-lactam moiety (δc-$_{18}$ 168.44) and a decahydrofluorene ring system bearing five methyl groups at C-3, C-5, C-7, C-9, C-11 and an $CH_2 = CH$- group at C-3 (Fig. 2). The γ-lactam system was deduced from the chemical shift data and HMBC correlations from NH to C-2′, C-1′, C-17 and C-18, and from H-1′ to C-2′, C-17 and C-18. The decahydrofluorene ring system was connected at C-15 to the α-carbon (C-17) of the γ-lactam moiety via a ketone (δc-$_{16}$ 203.02), as indicated by the HMBC correlations H-15/C-16, NH/C-18, NH/C-17, NH/C-16. Additionally, the HMBC correlations from H-3′ to C-1′, C-2′, C4′, C-5a′ and C-5b′ suggested that the *para*-substituted benzene ring is attached to γ-lactam system via a methylene group. The presence of the HMBC correlation from H-13 (δ 4.70) to the oxygenated aromatic carbon (C-7′, δ157.72) indicated that the *para*-substituted benzene ring is attached to the decahyrofluorene ring system at C-13 through an oxygen atom. The chemical shifts of the adjacent carbons C-1′ (66.85) and C-17 (58.59) suggested that they were oxygenated and might form an oxirane ring, which is further confirmed by the molecular formula and the unsaturation degrees of the molecule. Thus, the planar structure of **1** was determined.

The relative configuration of **1** was elucidated by analysis of vicinal *J*-values and ROESY spectral data (Fig. 3). The large vicinal coupling constants of $J_{8a,\ 9} = 11.9$ Hz, $J_{10a,\ 11} = J_{10a,\ 9} = 12.4$ Hz, $J_{11,\ 12} = 10.5$ Hz and $J_{14,\ 6} = 12.9$ Hz demonstrated that these protons are axial. The ROESY correlations of H-8a/H-10a, H-10a/H-12, H-12/H-6, H-6/H-13, H-13/H-15, and H-6/3-Me indicated that they were cofacial. The same orientation of H-14, 7-Me, H-9, H-11, and $CH_2 = CH$- was suggested by ROE correlations of H-14 with H-2, 7-Me with H-9, H-11, and H-14. On the basis of the above ROEs and coupling constants, the relative stereochemistry of the decahyrofluorene ring system could be established as the *trans*-juncture for A/B and B/C rings. In addition, H-13

Table 1 NMR Data for GKK1032C (**1**) measured at 600 (^{1}H) and 150 (^{13}C) MHz (DMSO-d_6)

Position	δc	δ_H, mult (*J* in Hz)	^{1}H–^{1}H COSY	HMBC
1	114.16	4.79, d (17.6) 4.85, d (10.9)	2	2, 3-Me,
2	144.30	5.65, dd (10.8,17.6)	1	3-Me, 3, 4, 15
3	43.73			
4	128.56	5.16, s		2, 5-Me, 3, 6, 15
5	138.08			
6	52.01	2.05, d (13.2)	14	7-Me, 7, 14, 15, 4, 5
7	41.34			
8	48.15	a 0.86, t (11.9) b 1.90, dd (3.1,12.3)	8b, 9 8a	7-Me, 9-Me, 9, 7, 6 10, 12, 7-Me, 9, 7
9	27.99	1.80, m	8, 10, 9-Me	9-Me, 10
10	45.57	a, 0.62, q (12.4) b, 1.76, m	10b, 11, 9 10a	11-Me, 9-Me, 3-Me, 8, 9, 12
11	27.27	1.76, m	10, 11-Me, 12	
12	59.95	1.27, dd (10.5, 8.4)	11, 13	7, 7-Me, 11, 11-Me, 10, 6, 8
13	86.99	4.70, dd (6.0, 6.9)	12, 14	7, 15, 12, 7′
14	49.83	2.45, dt (6.4,6.4,12.9)	6, 13, 15	6, 15, 13, 5, 16
15	57.41	3.84, d (7.2)	14	3, 3-Me, 4, 13, 14, 16
16	203.02			
17	58.59			
18	168.44			
1′	66.85	3.63, d (1.7)		17, 18, 2′
2′	83.46			
3′	43.99	2.90, d (12.8) 3.01, d (12.8)		1′, 2′, 4′, 5a′, 5b′ 1′, 2′, 4′, 5a′, 5b′
4′	128.73			
5′a	131.89	6.98, d (8.5)	6′a	3′, 6′a, 7′
5′b	132.53	7.19, d (8.2)	6′b	3′, 6′b, 7′
6′a	118.37	7.15, dd (1.9,8.5)	5′a	4′, 5′a, 7′
6′b	121.73	6.51, dd (2.0,8.1)	5′b	4′, 5′b, 7′
7′	157.72			
3-Me	25.97	1.18, s		2, 3, 4, 15, 16
5-Me	20.90	1.84, s		3, 4, 5, 6
7-Me	16.28	1.08, s		6, 7, 8, 12
9-Me	23.16	0.89, d (6.2)	9	8, 9, 10
11-Me	19.91	1.04, d (5.9)	11	11, 12, 10
NH		8.34, s	OH	17, 16, 18, 1’, 2’
OH		6.37, br s	NH	

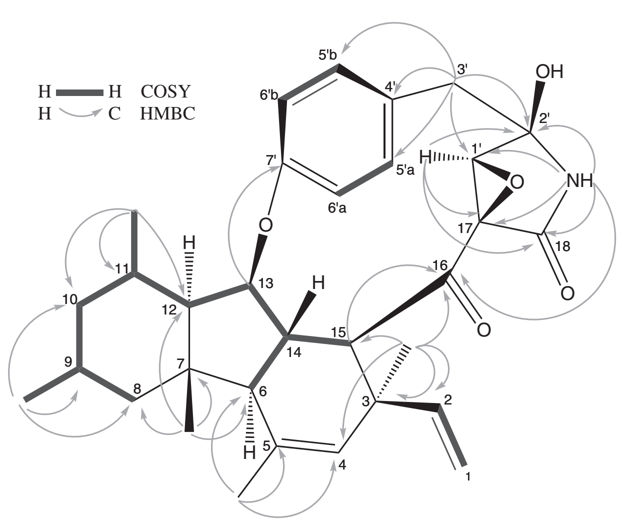

Fig. 2 ^{1}H-^{1}H COSY and key HMBC correlations of GKK1032C (**1**)

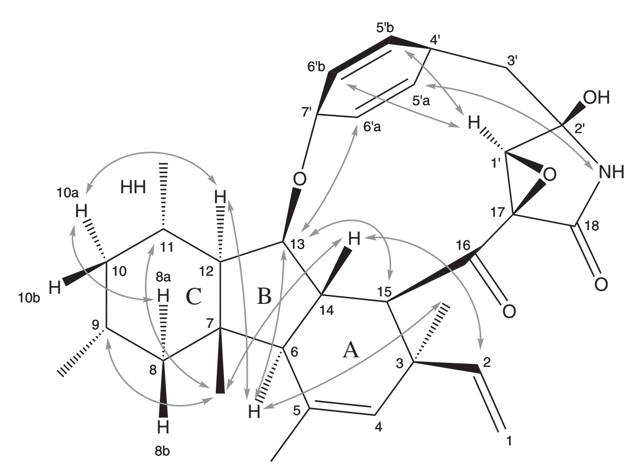

Fig. 3 Key ROSEY correlations of GKK1032C (**1**)

Table 2 Antimicrobial Bioassay Results (MIC, μg ml^{-1}) for compounds **1**–**5**

Microorganisms	Phenotype	1	2	3	4	5	Vancomycin	Meropenem
Staphylococcus aureus ATCC29213		3.2	12.9	25.8	3.225	3.2	1	0.0125
Staphylococcus aureus 67	MRSA[a]	1.6	12.9	25.8	25.8	3.2	2	>32
Escherichia coli ATCC25922		>25.8	>25.8	>25.8	>25.8	>25.8	>32	0.00625
Escherichia coli 46	ESBL[b]	>25.8	>25.8	>25.8	>25.8	>25.8	>32	0.0125
Pseudomonas aeruginosa 38	CR[c]	>25.8	>25.8	>25.8	>25.8	>25.8	>32	32
Klebsiella pneumoniae 19	CR[c]	>25.8	>25.8	>25.8	>25.8	>25.8	>32	0.0125
Acinetobacter baumannii 1	CR[c]	>25.8	>25.8	>25.8	>25.8	>25.8	>32	>32

[a]Methicillin-resistant *S. aureus*

[b]Extended spectrum beta-lactamase-producing strain

[c]Carbapenem-resistant strain

only showed correlations with aromatic H-6′a, but not with H-6′b, confirming that *para*-substituted benzene ring is rotation-restricted and approximately vertical to the rigid decahyrofluorene ring. Furthermore, H-1′ correlated only to H-5′b and H-6′b, whereas NH correlated only to H-5′a, suggesting that the γ-lactam ring is nearly parallel to the benzene ring and affording the relative configuration of the γ-lactam ring. Thus, compound **1** was deduced to share a similar structural framework with GKK1032A2 (**5**), and named as GKK1032C.

GKK1032C (**1**) together with compounds **2**–**4** were assayed for their antibacterial activity by using micro broth dilution method. Vancomycin (against Gram-positive bacteria) and Meropenem (against both Gram-positive and Gram-negative bacteria) were selected for the references. The results (Table 2) disclosed that all tested compounds exhibited potent antibacterial activity against Gram-positive bacteria, including methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* (MIC: 1.6–25.8 μg ml^{-1}), but no activity against Gram-negative bacteria, *Escherichia coli*, *Pseudomonas aeruginosa and Acinetobacter baumannii* (MIC > 25.8 μg ml^{-1}). (Table 2) Among these five compounds, new compound GKK1032C (**1**) afforded the most potent activity against methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* with MIC values of 3.2 and 1.6 μg ml^{-1}, respectively, which were comparable with those of vancomycin.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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