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Article

Indole Alkaloids from a Soil-Derived *Clonostachys rosea*

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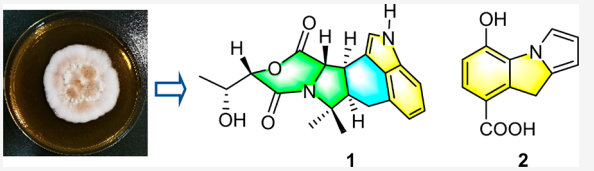
ABSTRACT: Clonorosins A (**1**) and B (**2**), two novel indole alkaloids featuring unprecedented 6/5/6/6/5 and 6/5/5 cores, together with seven known indole-linked 2,5-diketopiperazine alkaloids (**3**–**9**), were isolated from the soil-derived fungus *Clonostachys rosea* YRS-06. The new structures were proposed through HR-MS, NMR, and ECD spectroscopic data. They were established by comparing the calculated NMR, ECD, and specific rotation data with the experimental. To assist in determining the absolute configuration of the chiral carbon in the side chain of 2,5-diketopiperazine derivatives, flexible analogues **3i**–**3iv** were synthesized and analyzed. **1** was active against *Fusarium oxysporum* with an MIC value of 50 μg/mL. **7** and **8** showed excellent activity against human HeLa and HepG2 cells with IC_{50} values of 0.12–0.60 μM.



The indole alkaloids, one of the largest secondary metabolite families widely dispersed in plant and fungi domains, have proven to be an important drug resource.[1] Natural products with 2,5-diketopiperazine (DKP) scaffolds present significant biological activities and complex chemical structures involving fused heterocycles, prenylation, polythio-bridging, dimerization, and oxidation and are produced by a wide range of microorganisms.[2−4] In contrast, the closely related 2,5-diketomorpholine (DKM) motif is rarely reported, and only 12 natural 2,5-diketomorpholine derivatives have been reported, being isolated from *Aspergillus*, *Beauveria*, *Bombyx*, *Bursatella*, *Eupenicillium*, *Gibberella*, and *Isaria*.[5−12]

The soil-derived fungus *Clonostachys rosea* YRS-06, a biological control agent against *Fusarium graminearum* in small grain cereals and maize, is widely distributed. Several rare bisorbicillinoids with a γ-pyrone moiety have been isolated from the cultures of potato sucrose liquid medium with potent antibacterial activity.[13] This report describes our investigation of *C. rosea* YRS-06 grown on rice medium, leading to the isolation of diverse indole alkaloids, including a novel indole-linked 2,5-diketomorpholine alkaloid, clonorosin A (**1**), an unprecedented indole combined with a pyrrole alkaloid, clonorosin B (**2**), and seven known indole-linked 2,5-diketopiperazine alkaloids (**3**–**9**) (Chart 1). The structures were elucidated on the basis of detailed experimental and computational analyses. To determine the absolute configuration of the chiral carbon in the side chain of 2,5-diketopiperazine derivatives, the flexible analogues **3i**–**3iv** were synthesized and analyzed. Compound **1** shows moderate activity against *Fusarium oxysporum* with a MIC value of 50 μg/mL. **3i** and **3iv** inhibited the growth of *Pseudomonas solanacearum* and *Bacillus subtilis*, respectively. **7** and **8** showed excellent activity against human HeLa and HepG2 tumor cells *in vitro* with IC_{50} values of 0.12–0.60 μM, 60–70 times lower than that of cisplatin.

RESULTS AND DISCUSSION

Compound **1**, named clonorosin A, was obtained as a pale yellow powder. The molecular formula was determined to be $C_{20}H_{22}N_2O_4$ (11 degrees of unsaturation) on the basis of HR-ESI-MS data. In the IR spectrum, absorption bands at 1729 and 1657 cm^{-1} indicate the existence of carbonyls, those at 1612 and 1444 cm^{-1} reveal the existence of an aromatic group, and 3333 cm^{-1} implies the presence of exchangeable protons.

An inspection of the ^1H NMR (Table 1) and HSQC spectra shows that each of the signals at δ_H 10.13 (NH) and 4.60 (OH) represented two exchangeable protons. With the help of HSQC correlations, the ^1H NMR data can be considered as representing four aromatic methines [δ_H 7.32 (d, J = 2.0 Hz, H-5′), 7.19 (d, J = 8.4 Hz, H-8′), 7.04 (dd, J = 8.0, 7.2 Hz, H-9′), and 6.81 (d, J = 6.8 Hz, H-10′)], two oxygenated methines [δ_H 4.41 (d, J = 2.8 Hz, H-2) and 4.32 (m, H-3)], one azotizing methine [δ_H 4.56 (d, J = 11.2 Hz, H-2′)], and three methyls, of which one was secondary [δ_H 1.25 (d, J = 6.8 Hz, H_3-4)] and two were singlets [δ_H 1.77 (s, H_3-16′) and 1.61 (s, H_3-17′)]. Analysis of the ^{13}C NMR, DEPT-135 (Table 1), and HSQC NMR data of **1** revealed the presence of 20 carbons, consisting of three methyl groups, a methylene unit connected with an electrophilic group, two carbonyl carbons connected

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Chart 1. Chemical Structures of 1−9

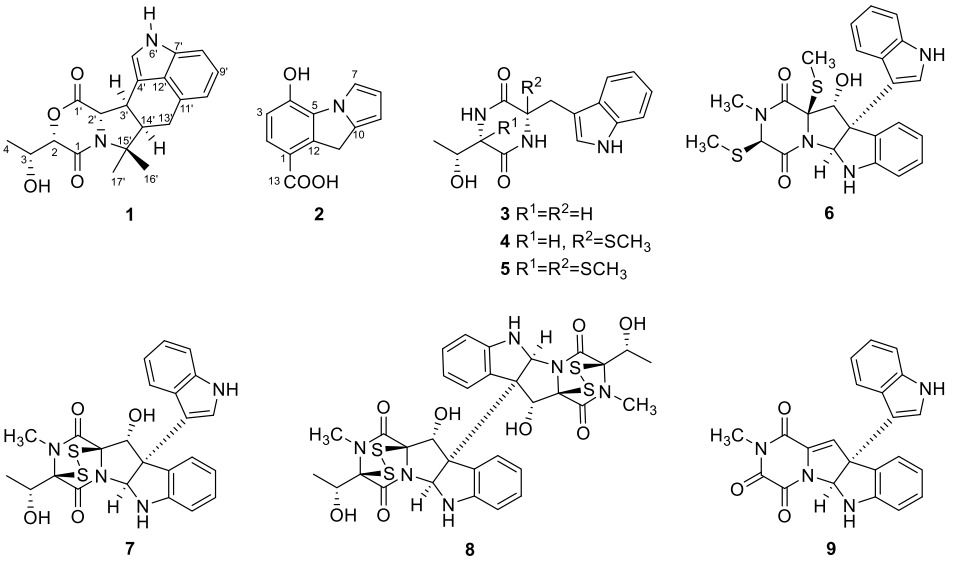

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data for 1

position	δ_C, DEPT[a,b]	δ_H, mult (J in Hz)[c]
1	165.0, C	
2	86.3, CH	4.41, d (2.8)
3	68.9, CH	4.32, m
3-OH		4.60, d (5.2)
4	19.9, CH_3	1.25, d (6.8)
1′	169.5, C	
2′	61.5, CH	4.56, d (11.2)
3′	37.9, CH	3.96, dd (11.2, 6.0)
4′	110.4, C	
5′	123.5, CH	7.32, d (2.0)
6′		10.13, brs
7′	134.3, C	
8′	109.5, CH	7.19, d (8.4)
9′	123.0, CH	7.04, dd (8.0, 7.2)
10′	116.5, CH	6.81, d (6.8)
11′	129.3, C	
12′	127.7, C	
13′a	27.1, CH_2	2.87, dd (16.4, 12.4)
13′b		3.08, dd (16.0, 5.2)
14′	48.8, CH	2.38, dt (12.0, 5.6)
15′	66.2, C	
16′	23.1, CH_3	1.77, s
17′	27.0, CH_3	1.61, s

[a]Recorded in $(CD_3)_2CO$ at 100 MHz. [b]Multiplicities inferred from DEPT-135 and HSQC experiments. [c]Recorded in $(CD_3)_2CO$ at 400 MHz.

with an O or N atom [δ_C 165.0 (C-1) and 169.5 (C-1′)], eight olefinic carbons [δ_C 110.4 (C-4′), 123.5 (CH-5′), 134.3 (C-7′), 109.5 (CH-8′), 123.0 (CH-9′), 116.5 (CH-10′), 129.3 (C-11′), and 127.7 (C-12′)] possessing chemical shifts in the aromatic range of the spectrum, five sp^3-hybridized methine carbons {two joined with O atoms [δ_C 86.3 (CH-2) and 68.9 (CH-3)] and one connected with a N atom [δ_C 61.5 (CH-2′)]}, and one sp^3-hybridized carbon linked with a N atom [δ_C 66.2 (C-15′)].

According to the HMBC correlations (Figure 1) from the methine (H-5′) of the aromatic nucleus to C-4′, C-7′, and C-12′, from H-8′ to C-10′ and C-12′, from H-9′ to C-10′ and C-11′, and from H-10′ to C-8′ and C-12′, together with ^{1}H−^{1}H COSY correlations (Figure 1) of H-8′/H-9′/H-10′ and one NH exchangeable proton, the presence of a disubstituted indole is suggested.[14] Due to HMBC correlations from H-2 to C-1 and C-1′ and from H-2′ to C-1′, a 2,5-diketomorpholine fragment was determined, consistent with the chemical shifts.[15] The ethyl alcohol was joined with C-2 of the 2,5-diketomorpholine by HMBC correlations from H-3-OH and H-4 to C-2. The existence of a hydrogenated benzene in **1** was unambiguously confirmed by HMBC correlations from H-3′ to C-4′, C-13′, and C-14′ and from H-13′b to C-11′ and C-14′ and ^{1}H−^{1}H COSY correlations of H-3′/H-14′/H$_2$-13′. It was connected to the indole as represented by the HMBC correlations from H-13′b to C-10′ and H-3′ to C-4′. ^{1}H−^{1}H COSY correlations of H-2′/H-3′/H-14′ and HMBC correlations from H-14′ to C-2′ and C-13′, from H-3′ to C-2′ and C-14′, and from H-16′ and H-17′ to C-14′ and C-15′ established a dimethyl tetrahydropyrrole system, along with the chemical shift of C-15′ and the degrees of unsaturation. Its position, between the 2,5-diketomorpholine and the hydrogenated benzene was also confirmed by the HMBC correlation from H-3′ to C-1′ and the above-mentioned ^{1}H−^{1}H COSY correlations.

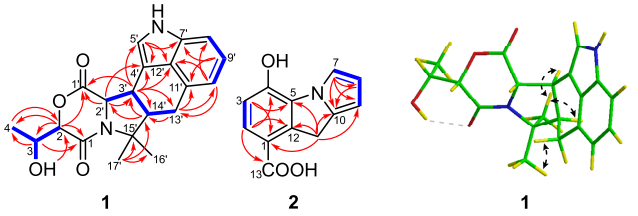

Figure 1. Key NMR correlations of **1** and **2** [blue lines for ^{1}H−^{1}H COSY, red arrows for HMBC (from H to C), dashed two-way arrows for NOE, and gray dashed line for an intramolecular hydrogen bond].

The relative configuration of **1** was partly resolved by analyzing the NOE data (Figure 1) and ^{1}H NMR coupling constants. The strong correlations of H-17′ with H-3′ and H-14′ indicated a *cis* junction between the tetrahydropyrrole and the hydrogenated benzene. Due to the lack of an NOE correlation of H-2′ with H-14′, a *trans* relationship between H-

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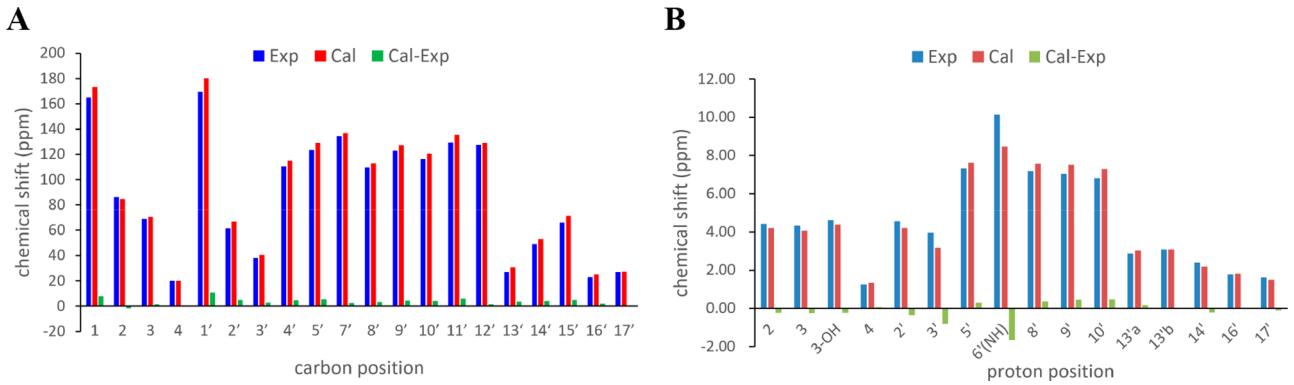

Figure 2. Comparison of calculated ^{13}C (A) and ^{1}H NMR (B) chemical shifts with experimentally observed shifts for **1**.

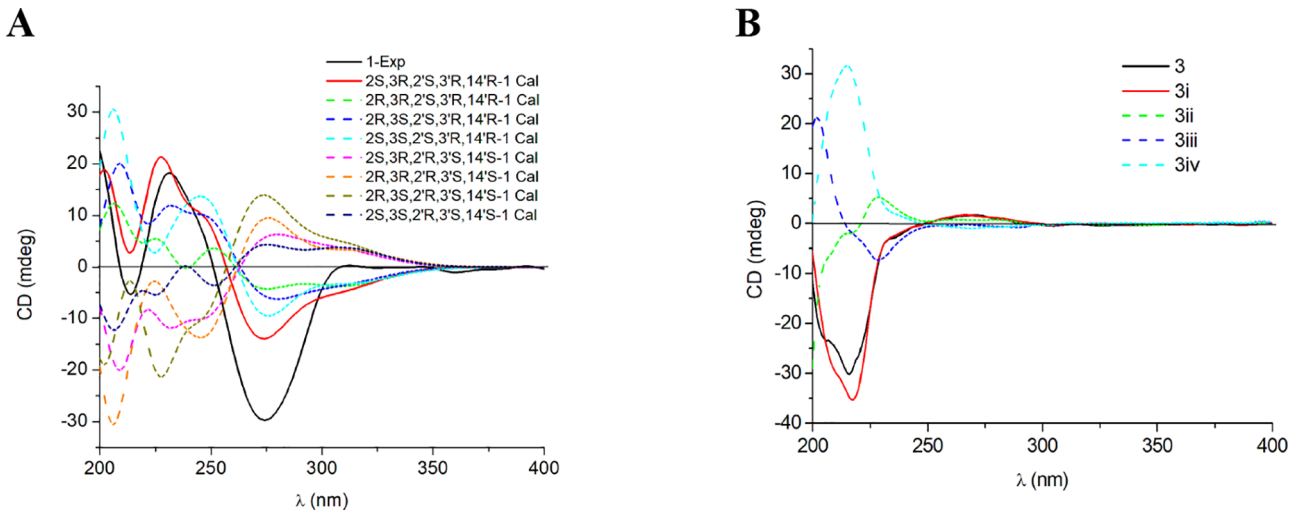

Figure 3. (A) Experimental and calculated ECD spectra of **1**. (B) Experimental ECD spectra of **3** and **3i**−**3iv**.

2′ and H-14′ of the tetrahydropyrrole was proposed. This was supported by the ^{1}H NMR coupling constants ($J_{3',14'}$ = 6.0 Hz and $J_{2',3'}$ = 11.2 Hz), consistent with that of (−)-α-cyclopiazonic acid.[14,16,17] The small ^{1}H NMR coupling constant ($J_{2,3}$ = 2.8 Hz) shows that the dihedral angle of H-2 and H-3 is close to 90 deg, and there could be an intramolecular hydrogen bond between the hydroxy (C-3) and the carbonyl (C-1) (Figure 1). Because of the lack of coupling between H-2 and H-2′, the relative configuration of the 2,5-diketomorpholine ring could not be determined. Based on the above-mentioned analyses, two configurations about C-2′/C-3′/C-14′ are 2′*S*,3′*R*,14′*R* and 2′*R*,3′*S*,14′*S*.

To evaluate the other viable configurational candidates about C-2 and C-3, i.e., 2*S*,3*R*,2′*S*,3′*R*,14′*R*-**1**, 2*R*,3*R*,2′*S*,3′*R*,14′*R*-**1**, 2*R*,3*S*,2′*S*,3′*R*,14′*R*-**1**, and 2*S*,3*S*,2′*S*,3′*R*,14′*R*-**1**, the theoretical ^{13}C and ^{1}H NMR data were predicted with the GIAO methodology (details in the Supporting Information (SI)). The calculated ^{13}C NMR data (Table S4-1 in the SI) for 2*S*,3*R*,2′*S*,3′*R*,14′*R*-**1** are in good agreement with the experimental data (Figure 2A, R^2 = 0.998). Except for the chemical shift of the exchangeable proton for NH, a good match between the experimental and the calculated ^{1}H NMR data (Table S4-2 in the SI) for 2*S*,3*R*,2′*S*,3′*R*,14′*R*-**1** was shown (Figure 2B, R^2 = 0.982). The predicted ^{13}C and ^{1}H NMR data of its enantiomer 2*R*,3*S*,2′*R*,3′*S*,14′*S*-**1** would be the same as that. The same orientation of H-2, H-3, and H-2′ was determined.

The absolute configurations at each of the five chiral carbons was provided by interpreting key Cotton effects at 200 (+22.50), 214 (−5.36), 231 (+18.22), 274 (−29.68), and 309 (+0.10) nm of the ECD spectrum for **1** shown in Figure 3A. The negative Cotton effects at 214 nm show there is a positive dihedral angle for N−C-2′−C-1′−O in the 2,5-diketomorpholine ring, and the absolute configuration of C-2′ was *S*.[18−20] According to the relative configuration, the other chiral carbon stereoconfigurations of **1** were elucidated as 2*S*,3*R*,3′*R*,14′*R*.

Given the above-mentioned configuration analyses by the NOE data and ^{1}H NMR coupling constants, the ECD traces of eight candidates, i.e., 2*S*,3*R*,2′*S*,3′*R*,14′*R*-**1**, 2*R*,3*S*,2′*R*,3′*S*,14′*S*-**1**, 2*R*,3*R*,2′*S*,3′*R*,14′*R*-**1**, 2*S*,3*S*,2′*R*,3′*S*,14′*S*-**1**, 2*R*,3*S*,2′*S*,3′*R*,14′*R*-**1**, 2*S*,3*R*,2′*R*,3′*S*,14′*S*-**1**, 2*S*,3*S*,2′*S*,3′*R*,14′*R*-**1**, and 2*R*,3*R*,2′*R*,3′*S*,14′*S*-**1**, were predicted using time-dependent density functional theory (TDDFT) calculations for the optimized geometries of each structure by the B3LYP/6-31G(d) force field for a methanol solution. The match between the experimental and the calculated key Cotton effects [202 (+18.93), 214 (+2.78), 227 (+21.30), 274 (−13.93), and 309 (−4.94) nm] for 2*S*,3*R*,2′*S*,3′*R*,14′*R*-**1** was excellent, as can be seen in Figure 3A, and the structure of **1** was firmly elucidated as depicted.

The calculated specific rotation value for 2*S*,3*R*,2′*S*,3′*R*,14′*R*-**1** is −8.07 at 20 °C in methanol (Table S6 in the SI) and perfectly matches the experimental value

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{[α]$_D^{20}$ −10.00 (*c* 0.30, CH_3OH)}, thus confirming the structure of the new compound.

Compound **2**, named clonorosin B, was obtained as a white powder. The molecular formula was determined to be $C_{12}H_9NO_3$ (9 degrees of unsaturation) on the basis of HR-ESI-MS data. Its IR spectrum exhibits absorption features that corresponded to carbonyl (1720 cm^{-1}), aryl (1595 and 1460 cm^{-1}), and hydroxy (3344 cm^{-1}) groups.

Analysis of the ^1H, ^{13}C, DEPT-135 (Table S2), and HSQC NMR data of **2** revealed the presence of 12 carbons, consisting of a carbonyl carbon, 10 olefinic carbons (including five methines), and a methylene unit. According to the ^1H NMR chemical shifts (δ_H 7.65 and 6.92) and the coupling constant ($J_{2,3}$ = 8.4 Hz), a 1,2,3,4-tetrasubstituted benzene was identified. The remaining aromatic protons accompanied by relatively smaller coupling constants [δ_H 7.31 (dd, *J* = 2.4, 1.8 Hz, H-7), 7.07 (dd, *J* = 1.8, 4.2 Hz, H-9), and 6.42 (dd, *J* = 2.4, 4.2 Hz, H-8)] could be assigned to a 1,2-disubstituted pyrrole.

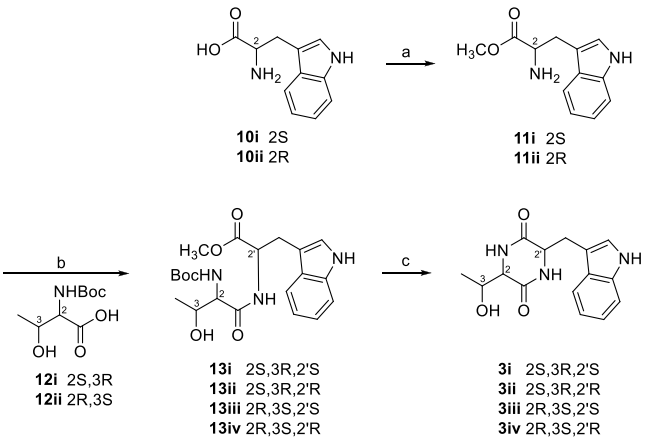
The three substructures, 1,2,3,4-tetrasubstituted benzene, 1,2-disubstituted pyrrole, and 1,2,3,5-tetrasubstituted pyrrole, were assembled by the $^1H-^1H$ COSY and HMBC data (Figure 1). The 1,2,3,4-tetrasubstituted benzene with the carboxyl and a hydroxy unit was affirmed on the basis of the preceding data plus the chemical shift of C-4 (δ_C 150.8), $^1H-^1H$ COSY correlations between H-2 and H-3, and HMBC correlations from H-2 to C-4, C-12, and C-13 and from H-3 to C-1 and C-5. Based on $^1H-^1H$ COSY correlations (H-7/H-8/H-9) and HMBC correlations (H-7 to C-8, C-9, C-10; H-8 to C-10), the 1,2-disubstituted pyrrole was deduced. Finally, according to the HMBC correlations from H-11 to C-5, C-10, and C-12, the 1,2,3,5-tetrasubstituted pyrrole was determined. At this point, it was clear that the C-5−C-12 bond was common to the tetrasubstituted benzene and tetrasubstituted pyrrole substructures. The C-5 (δ_C 142.3) and C-10 (δ_C 130.4) shifts and the key HMBC correlations from H-11 to C-1 and C-9 identified that the junction positions of the three substructures are as shown, representing a novel carbon skeleton.

Given the experimental specific rotation, [α]$_D^{20}$ +80.00 (*c* 0.30, CH_3OH), and the acidic carboxyl group and basic nitrogen atom, it could partly exist as a zwitterion in a methanol solution, with the negative carboxylate ion and the positive pyrrole ion.[21] The computational specific rotation gave a value, [α]$_D^{20}$ +89.29 (CH_3OH), of the zwitterion 6*R*-**2** that is a good match with the experimental data; that of 6*S*-**2** is −11.22 and that of nonionic **2** is −0.07.

Seven other known compounds, glioperazine C (**3**),[22] bionectin D (**4**),[23] glioperazine (**5**),[22] gliocladin A (**6**),[24] bionectin B (**7**),[25] verticillin D (**8**),[25] and gliocladin C (**9**),[24] were also isolated. Their structures were identified by comparing the NMR data with reported values.

To date, the C-3 absolute configuration of the threonine residue in natural 2,5-diketopiperazine derivatives has not been determined. The similar ^1H NMR coupling constant at C-3 of **1** and **3**, i.e., $J_{2,3}$ = 2.8 Hz in **1** and 3.0 Hz in **3**, suggests that they may have the same configuration. The synthesis of 2,5-diketopiperazine derivatives (**3i**−**3iv**) was carried out (Scheme 1, experimental procedures are elaborated in the SI). The coupling constants of H-2 with H-3 were 3.0 Hz [**3i** (2*S*,3*R*,2′*S*) and **3iv** (2*R*,3*S*,2′*R*)] and 6.0 Hz [**3ii** (2*S*,3*R*,2′*R*) and **3iii** (2*R*,3*S*,2′*S*)] (Table S6, in the SI). The experimental specific OR {[α]$_D^{24}$ −133.33 (*c* 0.30, MeOH)} and ECD spectrum of synthetic **3i** (Figure 3B) are identical

Scheme 1. Synthesis of 2,5-Diketopiperazine Derivatives[a]



[a]Reagents and conditions: (a) MeOH, $SOCl_2$, 0 °C, 20 min; rt, 1 h; 80 °C, 4 h. (b) CH_2Cl_2, EDC, Et_3N, HOBT, **12**, rt, 6 h. (c) 1,4-Dioxane−H_2O, 150 °C, 7 h.

with those of the natural product **3**. Thus, the C-3 configuration is determined as *R* in **3**.

The cytotoxic activities of **1**−**9** against two human cancer cell lines, HeLa and HepG2, were evaluated. Bionectin B (**7**) and verticillin D (**8**) showed excellent activity against those cells (Figure 4). The IC_{50} values of **7** (IC_{50} 0.42 and 0.60 μM)

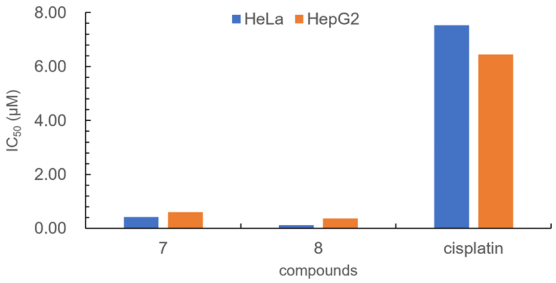


Figure 4. Comparison of the IC_{50} data for **7**, **8**, and cisplatin (positive control) against HeLa and HepG2 cells.

and **8** (IC_{50} 0.12 and 0.37 μM) were 60−70 times lower than that of cisplatin (IC_{50} 7.53 and 6.45 μM), and the IC_{50} values of **1**−**6** and **9** are over 20 μM. The remarkable activity may be attributed to the disulfide bond in **7** and **8** by analyzing the structure−activity relationship.

Compounds **1**−**9** were inactive against *Bacillus cereus*, *Bacillus subtilis*, *Erwinia carotovora* subsp. *carotovora*, *Escherichia coli*, *Pseudomonas solanacearum*, *Pseudomonas syringae* pv. *actinidiae*, *Ralstonia solanacearum*, and *Staphyloccocus aureus* at 100 μg/mL, except that **3i** was active against *P. solanacearum* (MIC 50 μg/mL) and **3iv** against *B. subtilis* (MIC 25 μg/mL).

1, **2**, and **3i**−**3iv** were inactive against *Alternaria alternate*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *Gibperella zeae*, and *Phytophthora capsic*, except that **1** was active against *F. oxysporum* with an MIC value of 50 μg/mL.

EXPERIMENTAL SECTION

General Experimental Procedures. Specific rotations were measured on an A21202-T digital polarimeter (Rudolph Research Analytical, USA) using a 1 cm cell at 20 °C using a sodium lamp (589 nm). IR spectra were recorded on a NEXUS 670 IR spectrometer (Thermo Nicolet, USA). UV spectra were obtained on a UV-

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2800SPC UV/vis spectrometer (Shanghai, China). ECD spectra were obtained using a DSM 1000 spectrometer (OLIS, USA) using a 1 mm cell. NMR spectra were measured on an INOVA-600 (Varian, USA) and an AVANCE III-400 (Bruker, Switzerland) spectrometer. HR-ESI-MS spectra were acquired using an Orbitrap Elite spectrometer equipped with an electrospray ionization (ESI) interface (Thermo, USA). Column chromatography (CC) was performed using silica gel (200−300 mesh) (Qingdao Haiyang Chemical Group Corporation, Qingdao, China) and Sephadex LH-20 (Mitsubishi, Japan). Precoated silica gel plates (GF_{254}, 10−40 μm, Qingdao, China) were used for thin-layer chromatography (TLC). Semipreparative HPLC was performed on a system equipped with a 1525 liquid chromatograph (Waters, USA) and a 2489 UV/visible (254 nm) (Waters, USA) peak detector, using a Synergi C_{18} column (250 × 10 mm, 4 μm, Phenomenex, USA). All solvents were of analytical grade for CC and chromatographic grade for HPLC.

Fungi Material. The isolation and identification of the used fungus were described previously.[13]

Fermentation, Extraction, and Isolation. Erlenmeyer flasks (1000 mL) containing rice (100 g) and distilled water (150 mL) were inoculated with spores after sterilization. The flasks were cultured under static conditions at 26 °C for 26 days.

The culture was macerated and extracted three times with Me_2CO−CH_2Cl_2−MeOH (4:3:1), and the extract was first partitioned by liquid−liquid extraction with petroleum ether (15 L), ethyl acetate (15 L), and *n*-butanol (15 L). The EtOAc fraction (53.9 g) was subjected to silica gel open CC (200−300 mesh) eluting with a mixture of CH_2Cl_2−MeOH (50:1, 20:1, 10:1, 5:1, 2:1, 1:1, 0:1, 5.0 L each) to yield 12 fractions (A−G).

Fr.A2 (6.0 g) was further fractionated by silica gel CC eluting with a mixture of petroleum ether−Me_2CO (10:1−0:1) to yield nine fractions (A21−A29). Fr.A25 (556.8 mg) was subjected to silica gel chromatography eluting with a mixture of CH_2Cl_2−Me_2CO (20:1−0:1) and CH_2Cl_2−EtOAc (5:1) to yield **7** (2.1 mg), **8** (13.0 mg), and **9** (4.0 mg). Fr.A28 (411.2 mg) was subjected to a Sephadex LH-20 column eluting with MeOH and silica gel chromatography eluting with a mixture of petroleum ether−Me_2CO (2:1) to yield **6** (1.5 mg).

Fr.B2 (1.7 g) was further fractionated by silica gel chromatography eluting with a mixture of petroleum ether−EtOAc (5:1−1:1) to yield 10 fractions (B21−B210). Fr.B27 (333.4 mg) was subjected to silica gel CC eluting with a mixture of petroleum ether−Me_2CO (4:1−2:1) and a Sephadex LH-20 CC eluting with MeOH to yield **1** (2.4 mg) and **2** (1.0 mg). Fr.B29 (26.5 mg) was repeatedly eluted with methanol to yield insoluble compound **5** (7.1 mg).

Fr.C1 (3.4 g) was further fractionated by Sephadex LH-20 CC eluting with MeOH, semipreparative HPLC (MeOH−H_2O, 40:60, 250 × 10 mm, 4 μm, Synergi 4u Hydro-RP 80A), and Sephadex LH-20 CC eluting with petroleum ether−CH_2Cl_2−MeOH (2:1:1) to yield **4** (3.5 mg). Fr.C2 (2.4 g) was further fractionated by Sephadex LH-20 CC eluting with MeOH, semipreparative HPLC (MeOH−H_2O, 25:75, 250 × 10 mm, 4 μm, Synergi 4u Hydro-RP 80A), and Sephadex LH-20 CC eluting with petroleum ether−CH_2Cl_2−MeOH (2:1:1) to yield **3** (7.0 mg).

*Clonorosin A (**1**):* pale yellow powder; $[\alpha]_D^{20}$ −10.00 (*c* 0.30, CH_3OH); IR (KBr) ν_{max} 3333, 2926, 2855, 1729, 1657, 1612, 1444, 1380, 1265, 1194, 1121, 1094, 1023, 866, 792, 739, 703, 664, 607 cm^{-1}; UV (MeOH) λ_{max} (log ε) 220 (3.93), 274 (3.27) nm; ECD (MeOH) λ_{max} (mdeg, 0.24 mg/mL) 200 (+22.50), 214 (−5.36), 231 (+18.22), 274 (−29.68), and 309 (+0.10) nm; HR-ESI-MS $[M + H]^+$ *m*/*z* 355.1648 (calcd 355.1652); for ^1H NMR and ^{13}C NMR data, see Table 1.

*Clonorosin B (**2**):* white powder; $[\alpha]_D^{20}$ +80.00 (*c* 0.30, CH_3OH); IR (KBr) ν_{max} 3344, 2922, 2852, 1735, 1720, 1638, 1595, 1460, 1385, 1301, 1261, 1095, 854, 794, 738, 660, 615 cm^{-1}; UV (MeOH) λ_{max} (log ε) 206 (3.37), 246 (3.10). 286 (2.91), 341 (3.17) nm; HR-ESI-MS $[M + H]^+$ *m*/*z* 216.0650 (calcd 216.0655); for ^1H NMR and ^{13}C NMR data, see Table S2.

Synthesis of 2,5-Diketopiperazine Derivatives. To a stirred solution of tryptophan (**10i** or **10ii**) (10 mmol) in 50 mL of dry MeOH, under ice-cooling, was added $SOCl_2$ (2.18 mL, 30 mmol) dropwise over 20 min. After stirring the mixture for 1 h at room temperature, the reaction mixture was kept under reflux (80 °C) for 4 h, and then MeOH was removed under reduced pressure and H_2O (25 mL) added. The pH of the aqueous solution was adjusted to 9−10 with NaOH, the solution was extracted with EtOAc, the organic layer was washed with brine, dried (Na_2SO_4), and filtered, and solvent was evaporated. The residue was purified by column chromatography (CH_2Cl_2−MeOH, 50:1) to produce **11i** or **11ii** (41.1% yield) as a yellow solid. To a solution of **11i** or **11ii** (1.0 mmol) in CH_2Cl_2 were added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (1.2 mmol), triethylamine (Et_3N) (1.2 mmol), 1-hydroxybenzotriazole (HOBT) (1.2 mmol), and *N*-Boc-threonine (**12i** or **12ii**) (1.2 mmol) at room temperature. The solution was stirred at room temperature for 6 h. After completion of the reaction, the solution was washed with brine (20 mL × 3). The organic layer was dried over anhydrous Na_2SO_4 and the solvent evaporated. The products **13i**−**13iv** were obtained by purification with silica gel column chromatography (petroleum ether−acetone, 5:2) in 52.6−59.7% yields. **13i**−**13iv** (1.0 mmol) were dissolved in 6.0 mL of 1,4-dioxane−H_2O (1:3) in a sealed tube. The solution was stirred at 150 °C for 7 h. After completion of the reaction, the crude products were obtained by evaporation of solvent under reduced pressure. The crude product was washed with water to give **3i**−**3iv** in 36.0−49.7% yields. For additional details see the Supporting Information.

ECD, NMR, and Specific Rotation Calculations. The analysis of the absolute configuration of **1** and **2** was supported by the Supercomputing Center of Lanzhou University, China. Conformational searches were performed with molecular dynamics using the xtb-200702 and the ORCA-4.2.1 software. The initial conformers were batch optimized with GFN0-xTB and GFN2-xTB using xtb-200702 in turn and filtered by an energy threshold of 0.5 kcal/mol and geometry threshold of 0.25 Å. The DFT calculations of the resulting conformers of ECD and NMR of **1** were subjected to geometry optimizations and frequency calculations at the B3LYP/6-31G(d) level, while the specific rotations of **1** and **2** were obtained at the PBEPBE/6-31G(d) level using the Gaussian 09 program. The single-point-energy calculations were both performed at the RI-PWPB95/DEF2-QZVPP level using ORCA-4.2.1 with SMD in methanol. All of these programs were used through Molclus-1.9.6 software.[26] The dominant conformations of ECD spectra of **1** were calculated by the TDDFT methodology at the B3LYP/6-311G(d) level with SMD in methanol. ECD spectra were simulated using SpecDis 1.71.[27] The NMR shielding tensors of **1** were computed with the GIAO methodology at the B972/pcSseg-1 level of theory with SMD in acetone, and the Boltzmann-weighted chemical shift data were acquired with Multiwfn-3.8 software.[28] The specific rotation data were calculated by the DFT methodology at the B3LYP/6-31G(d) level for **1** and the B3LYP/ma-def2TZVPP level for **2** with SMD in methanol, and the Boltzmann-weighted data were acquired with SpecDis 1.71. For details see the Supporting Information.

Cell Culture and Cell Viability Assay. HeLa and HepG2 tumor cells were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (September 2019). The cells were maintained in RPMI 1640 medium (containing 100 units/mL of penicillin/streptomycin and 10% FBS). Cell viability was assessed by the Cell Counting Kit-8 assay (CCK-8) (TopScience, China). Cells (8000 cells/well) were seeded into 96-well plates. After culturing for 24 h, cells were incubated with 20 μM compounds **1**−**9** for an additional 48 h. Subsequently, 10% CCK-8 solution was added to each well and incubated for 1.5 h. Absorbance at 450 nm was then measured using a microplate reader (BioTek, USA). Cell viability was calculated as a percentage relative to the DMSO group.

Assay of Antimicrobial Activity. The filter paper diffusion method was used. A 200 μg amount of compounds **1**, **2**, and **3i**−**3iv** was dissolved in 50 μL of DMSO and 950 μL of sterile water, respectively, for the solutions of 200 μg/mL. Five concentration gradients were prepared according to the double-dilution method. Filter papers were impregnated with different concentrations. The filter paper was placed on agar plates inoculated with bacterial and fungal pathogens, including *Bacillus cereus*, *Bacillus subtilis*, *Erwinia*

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carotovora subsp. *carotovora*, *Escherichia coli*, *Pseudomonas solanacearum*, *Pseudomonas syringae* pv. *actinidiae*, *Ralstonia solanacearum*, *Staphyloccocus aureus*, *Alternaria alternate*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *Gibperella zeae*, and *Phytophthora capsici*. Bacteria were cultured at 37 °C for 48 h, and fungi at 30 °C for 72 h, and the zone of growth inhibition was measured. Negative controls were DMSO and sterile water. Relative activity was determined in response to the standard antibiotics used. The antibacterial and antifungal drugs streptomycin sulfate and azoxystrobin (≥95% purity, Sigma, China) were used as the positive controls, respectively.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.1c00457.

Figures of HR-MS, UV, IR, and NMR spectra of **1**, **2**, **3i**−**3iv**, **11i**, **11ii**, and **13i**−**13iv**; computational details for ECD, NMR, and specific rotation of **1** and **2**; and synthesis of **3i**−**3iv** (PDF)

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Notes

The authors declare no competing financial interest.

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