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Cyanogramide with a New Spiro[indolinone-pyrroloimidazole] Skeleton from *Actinoalloteichus cyanogriseus*

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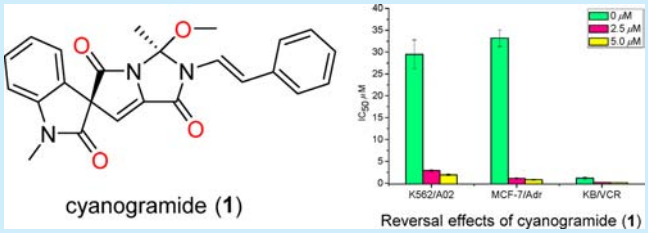
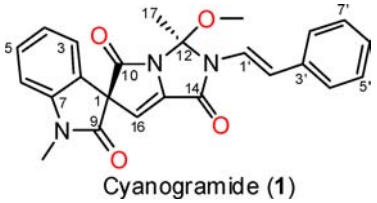
Supporting Information

ABSTRACT: Cyanogramide (**1**), an unprecedented alkaloid bearing a novel spirocyclic pyrrolo[1,2-*c*]imidazole skeleton, was identified from the fermentation broth of the marine-derived *Actinoalloteichus cyanogriseus* WH1-2216-6. The structure was fully determined by spectroscopic analysis, an exciton chirality CD method, and quantum mechanical calculations. Cyanogramide (**1**) could efficiently reverse the adriamycin-induced resistance of K562/A02 and MCF-7/Adr cells, and the vincristine-induced resistance of KB/VCR cells at a concentration of 5 μM, with the reversal fold values of 15.5, 41.5, and 9.7, respectively.



Through the years chemotherapy has been an important approach to the treatment of cancer. The development of the natural and molecular targeted anticancer drugs has improved the effect and reduced side effects of anticancer treatment.[1] However, the use of a large number of anticancer drugs made tumor cells develop the resistance that has become a perplexing problem to successful chemotherapy.[2] Usually, the resistance is stimulated by not only a single cytotoxic drug used but also a variety of different drugs.[3] This phenomenon known as multidrug resistance (MDR) has been a major factor in the failure of many forms of chemotherapy.[4] To overcome the MDR, higher doses were used and thus further increased the toxic side effects and further induced the drug resistance.[5] Up to now, some mechanisms involved in resistance to cancer chemotherapy have been clarified, which include increased drug efflux, reduced uptake of drugs, diminished drug-target interactions, increased DNA repair, altered cell-cycle regulation, etc.[6] The major mechanism of MDR in cultured cancer cells is the expression of an energy-dependent drug efflux pump, known as P-glycoprotein (P-gp).[7] P-gp can unilaterally transport intracellular drugs out of the cells to acquire drug resistance.[8] Thus, it is necessary to develop some agents which can reverse the P-gp-mediated MDR.

In order to identify molecules with the potency to reverse the P-gp-mediated MDR, crude extracts of marine-derived actinomycetes were employed to test this possibility using adriamycin-resistant K562/A02 cells with high P-gp expression. Among them, the EtOAc extract of *Actinoalloteichus cyanogriseus* WH1-2216-6 showed strong potency to increase adriamycin cytotoxicity toward K562/A02 cells with the reversal fold of 5.2 (0.25 μg/mL). This extract also showed a significant cytotoxic effect on the K562 cell line. A bioassay-guided chemical investigation resulted in the isolation of a novel spirocyclic pyrrolo[1,2-*c*]imidazole alkaloid, which we have named cyanogramide (**1**), and 15 bipyridine alkaloids.[9,10] Cyanogramide (**1**) exhibited significant ability to reverse the drug resistance of MCF-7, K562, and KB cell lines; meanwhile, some bipyridine alkaloids showed potent cytotoxicity.[9,10]



Cyanogramide (**1**) was obtained as a yellow oil.[11] Its molecular formula was determined as $C_{24}H_{21}N_3O_4$ according to its HR-ESI-MS peak at *m*/*z* 438.1423 [M + Na]$^+$. The IR spectrum showed the presence of a methyl group (2933, 1375 cm^{-1}), amide group (1713 cm^{-1}), and aromatic system (1607 cm^{-1}). The ^{13}C NMR resolved 24 carbon signals that were classified by DEPT and HMQC spectra as three methyls, 12 olefinic methines, and nine quaternary carbons (Table 1). The proton signals at δ_H 7.44 (1H, ddd, *J* = 7.7, 7.7, 1.2), 7.35 (1H, dd, *J* = 7.4, 1.2), 7.16 (1H, dd, *J* = 7.7, 1.1), and 7.12 (1H, ddd, *J* = 7.5, 7.5, 1.1) revealed the presence of a 1,2-disubstituted phenyl nucleus that was confirmed by ^{1}H−^{1}H COSY correlations of H-3/H-4/H-5/H-6. These data combined with the key HMBC correlations of H-3 to C-1/C-7, H-6 to C-2, and 8-NCH$_3$ to C-7/C-9 constituted the 1-methylindolin-2-one moiety (Unit A, Figure 1). The coupled ^{1}H NMR signals at δ_H 7.18 (1H, d, *J* = 15.2) and 7.04 (1H, d, *J* = 15.2) suggested the

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Table 1. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR Data for Cyanogramide (1) in DMSO-d_6

position	δ_C	δ_H, mult (J in Hz)
1	69.8, C	
2	124.6, C	
3	124.5, CH	7.35, dd (7.4, 1.2)
4	123.1, CH	7.12, ddd (7.5, 7.5, 1.1)
5	130.1, CH	7.44, ddd (7.7, 7.7, 1.2)
6	109.6, CH	7.16, dd (7.7, 1.1)
7	144.8, C	
9	169.5, C	
10	167.4, C	
12	98.7, C	
14	154.6, C	
15	138.6, C	
16	107.2, CH	6.27, s
17	21.8, CH_3	2.08, s
1′	118.9, CH	7.18, d (15.2)
2′	118.0, CH	7.04, d (15.2)
3′	135.5, C	
4′/8′	126.1, CH	7.54, dd (7.8, 1.2)
5′/7′	128.8, CH	7.36, dd (7.7, 7.7)
6′	127.6, CH	7.27, tt (7.4, 1.2)
8-NCH_3	26.9, CH_3	3.22, s
12-OCH_3	49.4, CH_3	3.20, s

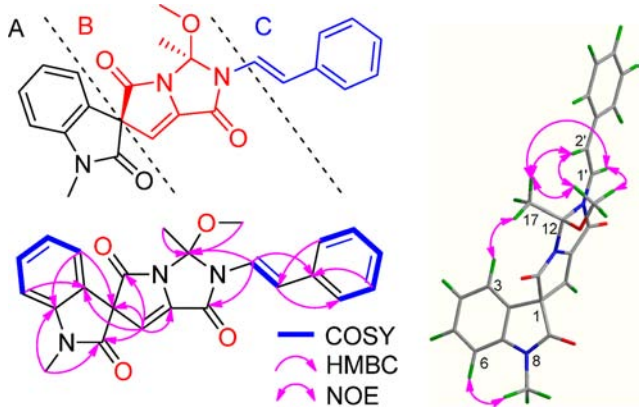

Figure 1. Key HMBC, ^{1}H–^{1}H COSY, and NOE correlations for **1**.

presence of an *E*-1,2-disubstituted ethylene moiety. Moreover, a 1-substituted phenyl moiety could be deduced from the protons at δ_H 7.54 (2H, dd, J = 7.8, 1.2), 7.36 (2H, dd, J = 7.7, 7.7), and 7.27 (1H, tt, J = 7.4, 1.2) and the ^{1}H–^{1}H COSY correlations of H-4′/H-5′/H-6′/H-7′/H-8′. The key HMBC correlations of H-4′ to C-2′ and H-1′ to C-3′ constituted the disubstituted ethylene moiety and the monosubstituted phenyl group as an *E*-styryl unit (Unit C, Figure 1). The other ^{1}H and ^{13}C NMR signals and the HMBC correlations of H-16 (δ_H 6.27, s) to C-1/C-10/C-15/C-14, 12–OCH_3 ($\delta_{C/H}$ 49.4/3.20) to C-12, and H-17 (δ_H 2.08, s) to C-12, combined with the molecular formula, revealed the pyrroloimidazole skeleton (Unit B, Figure 1). These structural fragments were confirmed by the Q-TOF-MS^2 peaks at m/z 279, 295, 364, 379, and 406 (Figures 2 and S1). The linkage among these three units was confirmed by the key HMBC correlations of H-16 to C-2/C-9 and H-1′ to C-12/C-14.

The NOE correlation between H_3-17 and H-3 indicated that the 12-Me (H_3-17) and the benzene nucleus (H-3) of indoline was located on the same side of the pyrrolo[1,2-*c*]imidazole

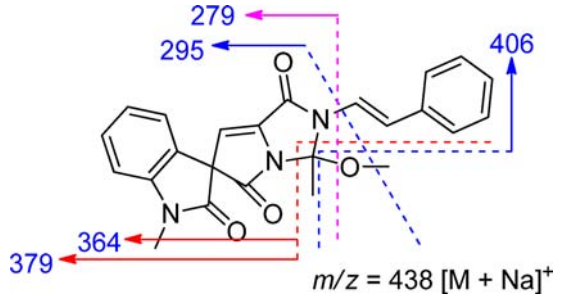

Figure 2. Q-TOF-MS^2 sequence ions (m/z) for protonated molecular $[M + Na]^+$ ion of **1**.

nucleus (Figures 1 and S12), that is (1*R*,12*S*)- or (1*S*,12*R*)-configurations. In order to further confirm the structure and the relative configuration, δ_C values of **1** were calculated at the B3LYP/6-311++G(2d,p)//B3LYP/6-31G(d) level.[12] The magnetic shielding values were converted into chemical shifts after the corrections using the slope and intercept of the linear-square functions,[12] and the relative errors of chemical shifts were computed by subtracting the calculated ^{13}C NMR from the experimental shifts. The relative errors between the measured and the calculated ^{13}C shifts are less than 5.0 ppm in (1*R*,12*S*)-**1**, while the maximum error was 5.9 ppm in (1*R*,12*R*)-**1** (Table S1). Although, there was no significant difference between two possible relative configurations, these results further verified the structure.

The absolute configuration of **1** was determined by CD and ECD spectra. Compound **1** showed a negative CD Cotton effect at λ_{max} ($\Delta\varepsilon$) 222 (−2.6) nm. It was assigned to exciton coupling between the π–π^* transitions of the two chromophores: the α,β-unsaturated ketone and the indolinone unit. Application of the Harada–Nakanishi nonempirical rule for exciton chirality CD predicated the negative and positive bisignate Cotton effects for (1*R*)- and (1*S*)-isomers, respectively (Figure 3).[13] Therefore, the chirality of **1** was

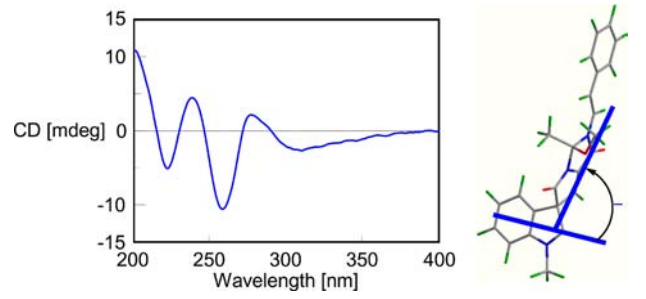

Figure 3. CD spectrum (left) and stereoview (right) of **1**. Bold lines denote the electric dipole of the chromophores.

determined as (1*R*, 12*S*)-. The quantum chemical ECD calculation method[14] was used to further confirm the absolute configuration of **1**. The preliminary conformational distribution search was performed by HyperChem 7.5 software. The corresponding minimum geometries were further fully optimized by using DFT at the B3LYP/6-31G(d) level as implemented in the Gaussian 03 program package. The stable conformers obtained were submitted to ECD calculation by the TDDFT [B3LYP/6-31G(d)] method. The overall predicted ECD spectrum of **1** was subsequently compared with the measured one. The measured CD curve of **1** showed a Cotton effect at λ_{max} ($\Delta\varepsilon$) 310 (−1.4), 277 (+1.1), 258 (−5.3), 238

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Table 2. Reversal Effects of 1 on K562/A02, MCF-7/Adr, and KB/VCR Cells[a]

	K562/A02		MCF-7/Adr		KB/VCR	
treatment	$IC_{50\ Adr}$ (μM)	RF	$IC_{50\ Adr}$ (μM)	RF	$IC_{50\ VCR}$ (μM)	RF
Adr/VCR	29.5 ± 3.3	/	33.2 ± 1.90	/	1.16 ± 0.24	/
Adr/VCR + 1 (2.5 μM)	2.9 ± 0.21	10.2	1.1 ± 0.09	30.2	0.18 ± 0.01	6.4
Adr/VCR + 1 (5 μM)	1.9 ± 0.17	15.5	0.8 ± 0.09	41.5	0.12 ± 0.01	9.7

[a]Data were expressed as means ± SD of three independent experiments.

(+2.2), and 222 (−2.6) nm, matching with the calculated ECD curve of (1*R*,12*S*)-**1** (Figure 4). Thus, the absolute configuration of **1** was unambiguously established as (1*R*,12*S*)-.

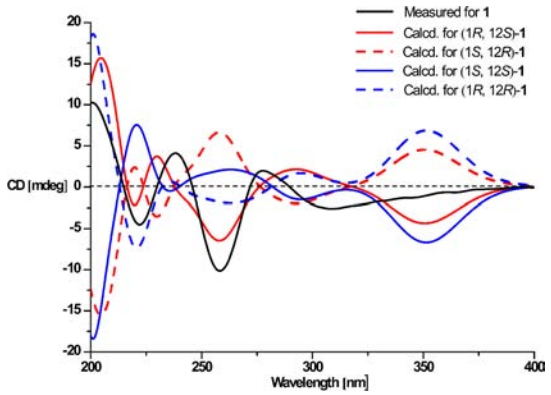
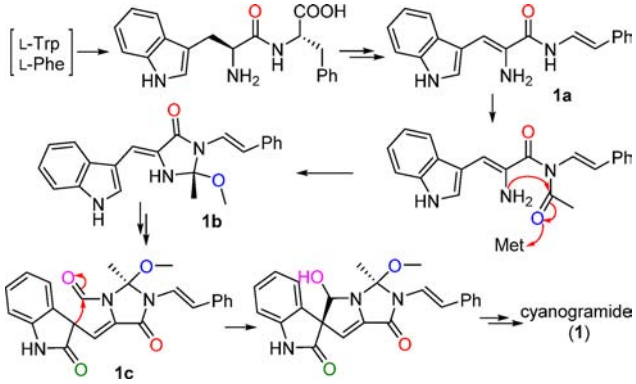


Figure 4. CD and calculated ECD spectra of **1**.

A plausible biosynthetic pathway for cyanogramide (**1**) was postulated (Scheme 1). The dipeptide Trp-Phe derived from L-

Scheme 1. Plausible Biosynthetic Pathway of 1



tryptophan and L-phenylalanine underwent decarboxylation and dehydrogenation to yield an amide intermediate (**1a**). Acetylation of **1a** followed by an intramolecular aldol condensation and an *O*-methylation generated the imidazole derivative (**1b**), which underwent oxidation and *N*-formylation[15] to produce the corresponding indolinone derivative (**1c**). The intermediate **1c** further underwent intramolecular aldol condensation, oxidation, and *N*-methylation to yield cyanogramide (**1**).

The cytotoxic effects of cyanogramide (**1**) on the K562, MCF-7, KB, and their MDR cell lines (K562/A02, MCF-7/Adr, and KB/VCR) were tested by the MTT method.[16] Compound **1** displayed weak cytotoxicity against these six cell lines with IC_{50} values of 12.9, 18.5, 16.8, 10.2, 36.0, and 25.6 μM, respectively. Compound **1** was also assayed for the ability to reverse the adriamycin (Adr)-induced resistance of K562/A02 and MCF-7/Adr cells and the vincristine (VCR)-induced resistance of KB/VCR cells. The reversal effects were evaluated by the values of reversal fold (RF) that was defined as the ratio between the two IC_{50} values of Adr or VCR alone and with compound **1**. The results showed that **1** could efficiently reverse the multiple drug resistance of K562/A02, MCF-7/Adr, and KB/VCR cells at a concentration of 5 μM (Table 2).

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and the NMR spectra of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

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Notes

The authors declare no competing financial interest.

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(11) Cyanogramide (**1**): yellow oil, $[\alpha]_D^{23}$ −96 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 206 (3.96), 253 (3.57), 319 (3.62) nm; CD (MeOH) λ_{max} ($\Delta\varepsilon$) 310 (−1.4), 277 (+1.1), 258 (−5.3), 238 (+2.2), 222 (−2.6) nm; IR (KBr) ν_{max}: 2933, 1713, 1607, 1494, 1375, 1288, 1169, 1056, 944, 745, 685 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRESIMS *m*/*z* 438.1423 $[M + Na]^+$ (calcd for $C_{24}H_{21}N_3O_4Na$, 438.1424).

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