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Acyclic Congeners from *Actinoalloteichus cyanogriseus* Provide Insights into Cyclic Bipyridine Glycoside Formation

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Supporting Information

ABSTRACT: Inactivation of the *O*-methyltransferase gene *crmM* of *Actinoalloteichus cyanogriseus* WH1-2216-6 led to a mutant that produced three new acyclic bipyridine glycosides, cyanogrisides E−G (**1**−**3**). Further chemical analysis of the wild strain yielded **1** and another new analogue, cyanogriside H (**4**). Compounds **1**−**4** possess a skeleton consisting of a 2,2′-bipyridine and a D-quinovose or L-rhamnose sugar moiety. Cyanogriside G (**3**) was considered to be a key biosynthetic intermediate of the cyclic bipyridine glycosides cyanogrisides A−D. Compounds **2** and **3** showed cytotoxicities against HCT116 and HL-60 cells, and compounds **1** and **4** were cytotoxic on K562 cells.

Caerulomycins, a family of alkaloids featuring a 2,2′-bipyridine skeleton, were first discovered from *Streptomyces caeruleus*.[1] Their interesting activities, such as antibiotic,[1a,2] phytotoxic[3] and immunosuppressant activities,[4] and their unique structure have attracted great attention of chemists and biologists.[5] Recently, we reported a number of new caerulomycin analogues, such as caerulomycins F−K[6] and cyanogrisides A−D,[7] from the marine-derived actinomycete strain *Actinoalloteichus cyanogriseus* WH1-2216-6. Several new caerulomycin analogues showed significant antitumor activities.[6,7] We and others have also unveiled the molecular and genetic basis for constructing the bipyridine core of caerulomycins and their closely related analogues collismycins, revealing a common origin of a hybrid polyketide synthase/nonribosomal peptide synthetase pathway.[8] The biosynthesis of caerulomycins was also demonstrated to involve an unusual amidohydrolase CrmL capable of removing an L-leucine residue[8a] and a unique two-component monooxygenase CrmH for the oxime formation.[9] Although the biosynthetic pathway for caerulomycin A has been well-established, the formation of the cyclic bipyridine glycosides, cyanogrisides A−D, remains enigmatic.[7] In this study, we report the isolation and structural characterization of four new acyclic bipyridine glycosides, cyanogrisides E−H (**1**−**4**, Figure 1), from the wild *A. cyanogriseus* WH1-2216-6 and a *crmM* gene (encoding an *O*-methyltransferase[8a]) disrupted mutant CRM05. Cyanogriside G (**3**) was found as a key biosynthetic intermediate to support our previously proposed biogenetic pathway of the cyclic bipyridine glycosides cyanogrisides A−D.[7]

Glycosylation of the 4-OH was proposed as a key step leading to cyanogrisides A−D.[7] However, caerulomycin A was isolated as a dominant product in *A. cyanogriseus* WH1-2216-6,[6,7] indicating that methylation of the 4-OH in caerulomycin A

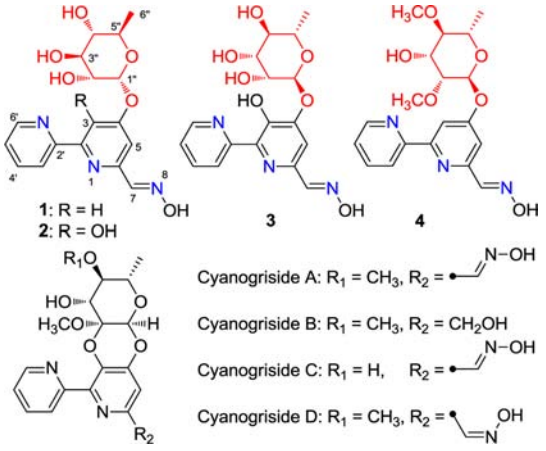

Figure 1. Structures of cyanogrisides E−H (**1**−**4**) and cyanogrisides A−D.[7]

largely inhibited the production of cyanogrisides A−D. We expected that the inactivation of the *O*-methyltransferase-encoding gene *crmM* would free the 4-OH for glycosylation to produce a higher titer of cyanogrisides A−D and their putative biosynthetic precursors. To this end, we inactivated the *crmM* gene by a PCR-targeting strategy as previously described[8a] to afford the Δ*crmM* mutant CRM05 (Figure S2). HPLC analysis revealed that the Δ*crmM* mutant strain CRM05 produced caerulomycin H (**5**)[6] as the dominant product (Figure 2). Next, a 15 L scale fermentation of CRM05 afforded three new acyclic bipyridine glycosides, cyanogrisides E−G (**1**−**3**), and three

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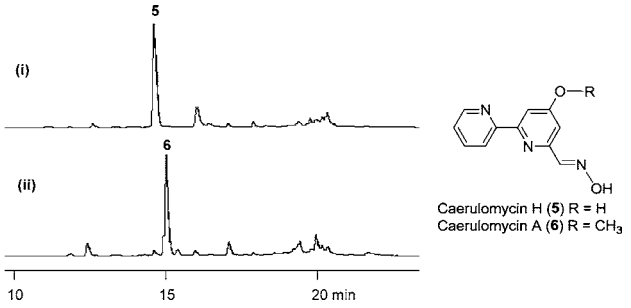

Figure 2. HPLC analysis of metabolite profiles for (i) Δ*crmM* mutant CRM05 and (ii) wild-type *A. cyanogriseus* WH1-2216-6.

known compounds, cyanogrisides A and C (Figure 1)[7] and caerulomycin H.[6] To identify whether the wild strain produced these acyclic bipyridine glycosides, the secondary metabolites[6,7] of the wild strain were rechecked, and this led to the identification of two minor analogues, cyanogriside E (**1**) and the new cyanogriside H (**4**) (Figure 1).

Cyanogriside E (**1**) was obtained as a yellow oil.[10] Its molecular formula was determined as $C_{17}H_{19}N_3O_6$ according to its HRESIMS peak at *m/z* 384.1166 $[M + Na]^+$. The IR spectrum showed the presence of a hydroxy group (3387 cm^{-1}) and an aromatic system (1586 cm^{-1}). The ^1H NMR spectrum (Table 1) showed nine signals at δ_H 5.67−1.10, and the ^{13}C NMR spectrum (Table 1) showed six signals at δ_C 97.8−18.5, which were classified by DEPT and HMQC spectra as one methyl signal ($\delta_{C/H}$ 18.5/1.10), five oxygenated methine signals ($\delta_{C/H}$ 69.6/3.52, 72.0/3.46, 73.3/3.60, 75.9/2.93, 97.8/5.67), and three hydroxyl groups (δ_H 5.22, 5.13, 5.07). These signals suggested the presence of a 6-deoxyhexose unit. $^1H-^1H$ COSY correlations of H-1″/H-2″/H-3″/H-4″/H-5″/H-6″, H-2″/2″-OH, H-3″/3″-OH, and H-4″/4″-OH and the key HMBC correlations of H-1″ to C-5″ and H-6″ to C-4″/C-5″ identified the structure of the sugar fragment (Figure 3). Six proton signals at 7.47 (d, *J* = 2.3), 7.48 (dd, *J* = 7.7, 4.5), 7.96 (td, *J* = 7.8, 1.9), 8.03 (d, *J* = 2.3), 8.39 (d, *J* = 7.8), and 8.69 (d, *J* = 4.6) and 10 carbon signals at δ_C 165.0−107.8 (Table 1) suggested the presence of a bipyridine nucleus.[6,7] $^1H-^1H$ COSY correlations of H-3′/H-4′/H-5′/H-6′ and HMBC correlations of H-3′ to C-5′/C-2, H-4′ to C-2′/C-6′, H-5 to C-3/C-4 and H-7 to C-5/C-6 constituted the bipyridine aldoxime moiety (Figure 3). The linkage between the sugar and the bipyridine core was confirmed by the key HMBC correlation of H-1″ to C-4. The coupling constants and chemical shifts of the sugar unit (Figure 3) were consistent with an α- quinovosyl.[11] Its 1D NMR data of the oxime moiety were very similar to those of caerulomycin A,[6] indicating that the oxime moiety of **1** was also *E*-configuration.

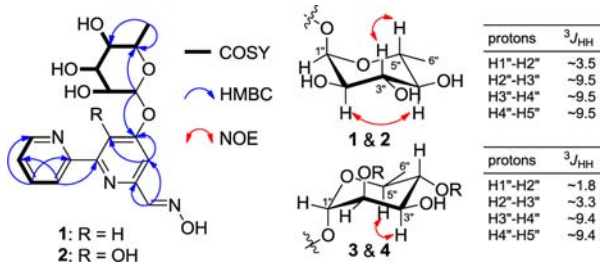

Figure 3. Key HMBC and $^1H-^1H$ COSY correlations for **1** and **2** and the determination of relative configurations.

The molecular formula of cyanogriside F (**2**) was $C_{17}H_{19}N_3O_7$ based on the HRESIMS,[12] which was only one oxygen more than that of **1**. Careful comparison of its 1D NMR spectra (Table 1) with those of **1** revealed that an oxygenated quaternary carbon signal at δ_C 148.5 and a hydroxy signal at δ_H 14.70 in **2** replaced the corresponding CH signals at $\delta_{C/H}$

Table 1. ^1H (600 MHz) and ^{13}C NMR (150 MHz) Data for 1−4 (DMSO-d_6, TMS, δ ppm)

	1		2		3		4	
position	δ_C	δ_H, multi (*J* in Hz)	δ_C	δ_H, multi (*J* in Hz)	δ_C	δ_H, multi (*J* in Hz)	δ_C	δ_H, multi (*J* in Hz)
2	157.5, C		135.6, C		135.7, C		157.5, C	
3	109.1, CH	8.03, d (2.3)	148.5, C		148.3, C		109.0, CH	8.02, d (2.3)
4	165.0, C		153.1, C		152.3, C		164.1, C	
5	107.8, CH	7.47, d (2.3)	107.8, CH	7.59, s	108.0, CH	7.58, s	107.9, CH	7.48, d (2.3)
6	154.1, C		143.7, C		143.7, C		154.1, C	
7	149.3, CH	8.15, s	146.4, CH	8.04, s	146.4, CH	8.04, s	149.3, CH	8.15, s
2′	155.0, C		157.6, C		157.5, C		154.9, C	
3′	121.3, CH	8.39, d (7.8)	121.1, CH	8.51, d (7.6)	121.1, CH	8.51, d (8.3)	121.3, CH	8.38, d (7.8)
4′	137.9, CH	7.96, td (7.8, 1.9)	139.5, CH	8.12, t (7.7)	139.6, CH	8.12, ddd (8.3, 7.4, 1.7)	137.9, CH	7.96, td (7.8, 1.8)
5′	125.1, CH	7.48, dd (7.7, 4.5)	124.4, CH	7.56, dd (7.5, 5.0)	124.5, CH	7.57, ddd (7.5, 5.0, 1.1)	125.2, CH	7.48, ddd (7.5, 4.8, 1.2)
6′	149.9, CH	8.69, d (4.6)	149.3, CH	8.66, d (5.0)	149.2, CH	8.65, d (5.0)	149.9, CH	8.69, d (4.7)
1″	97.8, CH	5.67, d (3.6)	98.2, CH	5.65, d (3.3)	99.3, CH	5.56, d (1.8)	95.4, CH	5.87, d (1.8)
2″	72.0, CH	3.46, dd (9.5, 3.5)	72.2, CH	3.49, dd (9.4, 3.3)	70.6, CH	3.91, dd (3.2, 1.8)	80.6, CH	3.55, dd (3.4, 1.9)
3″	73.3, CH	3.60, t (9.5)	73.0, CH	3.67, t (9.4)	70.7, CH	3.70, dd (9.4, 3.3)	70.4, CH	3.86, ddd (9.5, 6.7, 3.4)
4″	75.9, CH	2.93, td (9.5, 5.6)	75.9, CH	2.93, t (9.4)	72.1, CH	3.31, t (9.4)	82.8, CH	3.01, t (9.4)
5″	69.6, CH	3.52, dq (9.4, 6.4)	69.6, CH	3.58, dq (9.7, 6.2)	70.5, CH	3.30, dq (9.4, 6.0)	69.2, CH	3.42, dq (9.5, 6.3)
6″	18.5, CH_3	1.10, d (6.4)	18.5, CH_3	1.08, d (6.1)	18.4, CH_3	1.10, d (6.0)	18.5, CH_3	1.12, d (6.4)
2″-OH/OCH_3		5.22, d (6.4)		5.13, brs		4.99, brs	60.7, CH_3	3.47, s
3″-OH		5.07, brs		5.13, brs		4.99, brs		5.10, d (6.8)
4″-OH/OCH_3		5.13, d (5.7)		5.13, brs		4.99, brs	59.4, CH_3	3.46, s
8-OH		11.79, s		11.48, brs		11.50, brs		11.77, s
3-OH				14.70, brs		14.76, brs		

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109.1/8.03 in **1**, suggesting that the CH-3 in **1** was oxidized to yield **2**. The HMBC correlation from H-5 to C-3 also supported this change (Figure 3). In addition, the NOE correlations of H-3″/H-5″ and H-2″/4″ and the same coupling constants (Figure 3) indicated the same sugar moiety with **1**.

Cyanogriside G (**3**) was an isomer of **2** according to its HRESIMS peak at m/z 378.1310 $[M + H]^+$.[13] The ^1H and ^{13}C NMR data of **3** (Table 1) were similar to those of **2** except for some differences at the sugar part. The NOE correlations of H-3″/H-5″ and H-4″/6″-CH_3 (Figure 3) and the coupling constants (Figure 3) indicated that the sugar moiety in **3** was rhamnopyranosyl.[14] The coupling constant (1.8 Hz) of H-1″ confirmed the rhamnopyranosyl as an α-glycosidic linkage.[14]

Cyanogriside H (**4**) was obtained as a yellow oil.[15] The molecular formula was $C_{19}H_{23}N_3O_6$ from the molecular ion peak $[M + H]^+$ at m/z 390.1649 (calcd for $C_{19}H_{24}N_3O_6$, 390.1665) in the positive ion HRESIMS. The 1D NMR spectra (Table 1) were similar to those of **1** except that the 2″-OH and 4″-OH of **1** were replaced by the 2″-OCH_3 ($\delta_{C/H}$ 60.7/3.47) and 4″-OCH_3 ($\delta_{C/H}$ 59.4/3.46) of **4**. The NOE correlations of H-2″/H-3″, H-3″/H-5″, H-4″/6″-CH_3, 3″-OH/2″-OCH_3, and 6″-CH_3/2″-OCH_3 (Figure 3) and the coupling constants (Figure 3) confirmed that **4** possessed the same sugar unit as **3**.

Acidic hydrolysis of compounds **2** and **3** liberated D-quinovose and L-rhamnose, respectively. They were determined by HPLC analysis with PMP precolumn derivatization (Figure S3),[16] TLC behaviors, and the specific rotation. The hydrolysates showed the same retention time (t_R 28.2 and 12.1 min), the same R_f values (0.69 and 0.67, 6:4 $CHCl_3$/MeOH), and close $[\alpha]_D$ values ($[\alpha]_D^{22}$ +77 (c 0.3, H_2O), +33 (c 0.1, H_2O)) to those of reported D-quinovose and L-rhamnose,[11,17] respectively. The similar specific rotations for **1** and **2** ($[\alpha]_D^{23}$ −125 (c 0.02, MeOH) vs −69 (c 0.07, MeOH)) and for **3** and **4** ($[\alpha]_D^{23}$ −25 (c 0.07, MeOH) vs −76 (c 0.1, MeOH)) indicated the same absolute configurations of **1** with **2** and **3** with **4**. This result was also consistent with the proposed biogenetic pathway of the cyclic bipyridine glycosides cyanogrisides A–D.[7]

The isolation of caerulomycin H (**5**) as the major product in the Δ*crmM* mutant strain CRM05 suggested that CrmM was an *O*-methyltransferase responsible for the 4-*O*-methylation. For in vitro assays, soluble CrmM proteins were obtained by the fusion with GST tags and were purified using GST·Bind purification kits (Figure S25). Incubation of **5** in the presence of (*S*)-adenosylmethionine (SAM) led to caerulomycin A (**6**, Figure 4A), the yield of which increased with longer incubation time. No change of **5** was observed in control assays lacking CrmM (Figure 4A). These observations unequivocally confirmed CrmM as a 4-*O*-methyltransferase (Figure 4B). However, CrmM displayed stringent substrate specificity toward **5**, with none of the other previously reported caerulomycins (Figure 4C) found to be substrates for CrmM (Figure S26).

Our identification of the first naturally acyclic bipyridine glycosides **1**–**4** confirmed the existence of the putative key biosynthetic intermediates for cyanogrisides A–D (Scheme 1). The glycosyl moiety is probably derived from D-quinovose or L-rhamnose. The glycosylation of 4-OH of **5** with D-quinovose and L-rhamnose produced **1** and the unidentified intermediate (*E*)-4-α-L-rhamnopyranosyl-2,2′-bipyridine-6-aldoxime (α-L-Rha-**5**), respectively. Methylation of α-L-Rha-**5** yielded **4**. Oxidization of **1** and α-L-Rha-**5** at C-3 would lead to compounds **2** and **3**, respectively. Compound **3** could serve as a key biosynthetic intermediate that undergoes oxidation, aldol condensation, and the tailoring modification of sugar-*O*-methylation to yield cyanogrisides A–D.[7]

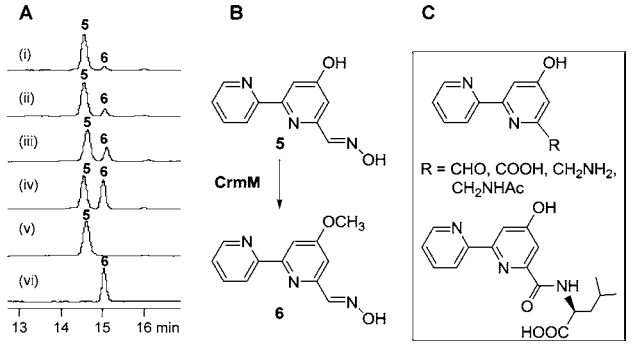
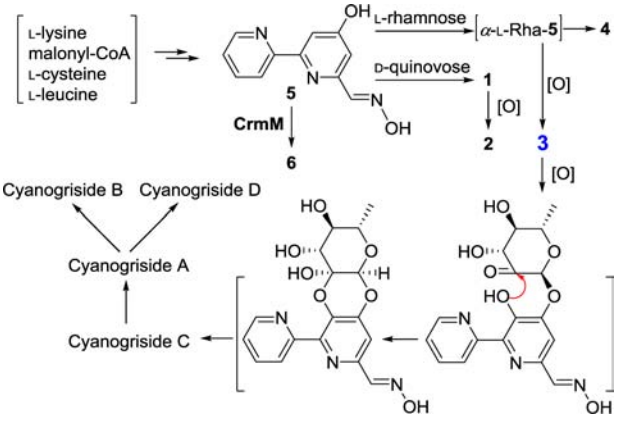


Figure 4. CrmM-catalyzed reaction. (A) HPLC analysis of a CrmM time course assay comprising 100 μM **5**, 10 μM CrmM, and 2 mM SAM in Tris-HCl buffer (50 mM, pH 8.0) at 28 °C for (i) 10 min, 7% conversion; (ii) 30 min, 14% conversion; (iii) 1 h, 26% conversion; (iv) 2 h, 44% conversion; (v) control assay without adding CrmM, and (vi) standard caerulomycin A (**6**). (B) Scheme for CrmM catalysis. (C) Other compounds tested as a CrmM substrate.

Scheme 1. Plausible Biosynthetic Pathways of Cyanogrisides E–H (1–4) and Cyanogrisides A–D



Compounds **1**–**4** were tested for cytotoxic effects on the A549, K562, MCF-7, HCT116, HeLa, or HL-60 cell lines by the MTT method.[18] Moderate cytotoxicities of **1** and **4** against K562 and **2** and **3** against both HCT116 and HL-60 cells were observed with IC_{50} values of 6.0, 0.8, 0.8/3.6, and 3.1/2.0 μM, respectively (Table 2). Compound **2**, which was the most potent, was also assayed for its inhibitory effect on the A549 cell cycle.[19] The result showed that compound **2** could arrest the A549 cell cycle at S phase at a concentration of 10 μM (Figure S27). The cytotoxicities of acyclic bipyridine glycosides are stronger than the cyclic cyanogrisides A–D.[7] When the glycosyl is replaced by a methyl group (caerulomycin A), the cytotoxicity becomes much stronger.[6] The identification of

Table 2. Cytotoxicities of Compounds 1–4 (IC_{50} μM)

cell lines	A549	MCF-7	K562	HeLa	HCT116	HL-60
1	>50	>50	6.0			
2	33.1	>50	13.6	26.5	0.8	3.1
3	42.0	>50	23.6	44.1	3.6	2.0
4			0.8			

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more analogues could help us to further research the structure−activity relationship.

In summary, we validated CrmM as a 4-*O*-methyltransferase in a caerulomycin biosynthetic pathway and have identified a new acyclic bipyridine glycoside (**3**) with cytotoxicity on HL-60 and HCT116 cells. This has shed light on the biosynthesis of the unique hemiacetal structure in the cyclic bipyridines cyanogrisides A−D.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and the NMR spectra of **1**−**4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(10) Cyanogriside E (**1**): yellow oil, $[\alpha]_D^{23}$ −125 (*c* 0.02, MeOH); UV(MeOH) λ_{max} (log ε) 225 (3.68) nm; IR (KBr) ν_{max} 3387, 2924, 2853, 1586, 1460, 1252, 1047, 843 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRESIMS *m*/*z* 384.1166 $[M + Na]^+$ (calcd for $C_{17}H_{19}N_3O_6$,Na, 384.1172).

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(12) Cyanogriside F (**2**): yellow oil, $[\alpha]_D^{23}$ −69 (*c* 0.07, MeOH); UV(MeOH) λ_{max} (log ε) 222 (3.42), 260 (3.14) nm; IR (KBr) ν_{max} 3424, 1647, 1490, 1382, 1067, 1026, 671 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRESIMS *m*/*z* 378.1303 $[M + H]^+$ (calcd for $C_{17}H_{20}N_3O_7$, 378.1301).

(13) Cyanogriside G (**3**): yellow oil, $[\alpha]_D^{23}$ −25 (*c* 0.07, MeOH); UV(MeOH) λ_{max} (log ε) 222 (3.58), 260 (3.12) nm; IR (KBr) ν_{max} 3427, 1641, 1492, 1382, 1025, 671 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRESIMS *m*/*z* 378.1310 $[M + H]^+$ (calcd for $C_{17}H_{20}N_3O_7$, 378.1301).

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(15) Cyanogriside H (**4**): yellow oil, $[\alpha]_D^{23}$ −76 (*c* 0.1, MeOH); UV(MeOH) λ_{max} (log ε) 226 (3.49) nm; IR (KBr) ν_{max} 3376, 2987, 2936, 2834, 1583, 1428, 1192, 1138, 1097, 1028, 970, 876, 797, 746 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRESIMS *m*/*z* 390.1649 $[M + H]^+$ (calcd for $C_{19}H_{24}N_3O_6$, 390.1665).

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