Cytotoxic Bipyridines from the Marine-Derived Actinomycete Actinoalloteichus cyanogriseus WH1-2216-6

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ABSTRACT: Five new bipyridine alkaloids (1–5) and a new phenylpyridine alkaloid (6), which we name caerulomycins F–K, along with five known analogues (7–11), were isolated from the marine-derived actinomycete Actinoalloteichus cyanogriseus WH1-2216-6. The structures of 1–6 were established on the basis of spectroscopic analyses and chemical methods. Compounds 1–10 showed cytotoxicity against the HL-60, K562, KB, and A549 cell lines, with IC_{50} values of 0.26 to 15.7 μM. Compounds 7 and 8 also showed antimicrobial activities against Escherichia coli, Aerobacter aerogenes, Pseudomonas aeruginosa, and Candida albicans, with MIC values of 9.7 to 38.6 μM.

RESULTS AND DISCUSSION

The bioactive EtOAc extract of A. cyanogriseus WH1-2216-6 was chromatographed on a silica gel column, and extensive reversed-phase preparative HPLC was carried out to give compounds 1–11. Among them, 7 was the major compound isolated (4 g from a 50 L culture, compared to all other compounds, which were isolated in quantities less than 50 mg). Its molecular formula was tentatively assigned as 

\[ 	ext{C}_{12}	ext{H}_{11}	ext{N}_{3}	ext{O}_{2} \] 

\[ \text{M} + \text{H} \] 

observed by ESIMS. Analysis of the 1D ^{13}C NMR data for 7 revealed four quaternary carbons, seven methylene carbons, and one methoxyl carbon. The ^{1}H NMR spectrum (Table S1) showed four signals at 8.40
Caerulomycin F (1) was obtained as a white, amorphous powder. Its molecular formula was assigned as C_{12}H_{13}N_{2}O_{2} from the HRESIMS peak at m/z 217.0976 [M + H]^+ which requires eight degrees of unsaturation. The IR spectrum showed the presence of a hydroxy group (3450 cm^{-1}) and an aromatic system (1652 cm^{-1}). With the exception that an oxygenated methylene signal (δ_{C/H} 64.2/4.62) in 1 replaced an oxime methine signal (δ_{C/H} 148.8/8.13) in 7, the 1H and 13C NMR spectra of 1 (Table 1) were very similar to those of 7 (Table S1). This suggested that the same 4-methoxy-2,2'-bipyridine skeleton found in 7 was present in 1. Upfield shifts for C-2 to C-6 indicated that 1 was the hydroxymethyl-substituted derivative of 7. 2D NMR correlations (Figure 1) also support the structure of 1. Thus, the structure of compound 1 was determined to be 4-methoxy-2,2'-bipyridine-6-methanol.

Caerulomycin G (2) was obtained as colorless needles. The molecular formula was determined to be C_{15}H_{14}N_{3}O_{3} from the HRESIMS peak at m/z 247.1079 in the HRESIMS spectrum. The IR spectrum established the presence of hydroxy groups (3622 cm^{-1}) and an aromatic system (1553 cm^{-1}). The 1H and 13C NMR data (Table 1) were almost identical to those of 3 (Table S1). A methoxy signal at δ_{C/H} 56.8/3.78 vs 63.1/3.71 in the 13C NMR spectrum (Table 1). Further comparisons of 1H NMR data for a methylene (δ_{C/H} 4.30/4.30) and an acetyl group (δ_{C/H} 21.0 and 23.2/1.92), while the oxime methine signals (δ_{C/H} 149.6/8.09) in 3 were not observed. These observations suggested that the oxime group of 3 was reduced to the corresponding ammonium in 5. This postulation was confirmed by a key 1H-1H COSY correlation in 5 between the NH (δ_{C/H} 4.46) and CH2 (δ_{C/H} 4.30). Key HMBC correlations between the NH and C-6 (δ_{C/H} 159.9) and between the CH2 and C-5 (δ_{C/H} 103.9) were also observed. HMBC correlations observed between the CH2 and the acetyl carbon indicated the presence of a hydroxy group in 3. Thus, the structure of 5 was elucidated as N-[(4-hydroxy-2,2'-bipyridine-6-yl)methyl]-acetamide.

Caerulomycin H (3) was obtained as a white, amorphous powder, and the molecular formula C_{15}H_{16}N_{3}O_{3} was assigned on the basis of the HRESIMS peak at m/z 216.0781 [M + H]^+. Careful comparison of its 1H and 13C NMR spectra (Table 1) with those of 5 (Table S1) revealed that 3 contained the same 2,2'-bipyridine-6-carboxaldehyde oxime skeleton, but that the CH_{2}O-4 of 7 was replaced by HO-4 in 3. To confirm the structure of compound 3, both HO-8 and HO-4 of 3 and HO-8 of 7 were methylated, which resulted in the production of a common product as expected (3a). 1D NMR data of the oxime moiety in 3 were very similar to those of 7, which indicated that the oxime moiety of 3 was also of the E configuration. Thus, the structure of compound 3 was determined to be (E)-4-hydroxy-2,2'-bipyridine-6-carboxaldehyde oxime.

Caerulomycin I (4) was obtained as colorless needles, and the molecular formula C_{15}H_{13}N_{3}O_{3} was assigned on the basis of the HRESIMS peak at m/z 282.0855 [M + Na]^+. The 1H and 13C NMR spectra (Table 1) of 4 were very similar to those of 9 (Table S1), with the exception that an amide proton signal at δ_{C/H} 9.85 (1H, brs) was replaced by a methoxy signal at δ_{C/H} 63.9/3.78. Furthermore, another amide proton signal was shifted downfield to δ 12.15, suggesting that 4 was the methoxy-substituted derivative of 9 at the amide nitrogen. The nearly identical NMR data of the methoxy to those of O-methylbenzo-hydroxamic acid (δ_{C/H} 63.9/3.78 vs 63.1/3.71) established the placement of the methoxy group on the amido nitrogen, and the assignment was further supported by key cross-peaks from H-3 (δ_{C/H} 8.04) and the amide group proton (δ_{C/H} 12.15) to C-7 (δ_{C/H} 161.3) in the HMBC spectrum (Figure 1). Therefore, the structure of compound 4 was identified as N,4-dimethoxy-2,2'-bipyridine-6-carboxamide.

The molecular formula of caerulomycin J (5, isolated as a yellow oil) was determined as C_{15}H_{14}N_{3}O_{2} on the basis of the molecular ion peak at m/z 266.0905 [M + Na]^+ in the HRESIMS spectrum. With the exception of the oxime moiety, the 1H and 13C NMR spectra of 5 were similar to those of 3 (Table 1), indicating the same molecular skeleton. Compared with 3, the 1H and 13C NMR spectra (Table 1) of 5 showed additional signals for a methylene (δ_{C/H} 44.6/4.30) and an acetyl group (δ_{C/H} 170.1 and 23.2/1.92), while the oxime methine signals (δ_{C/H} 149.6/8.09) in 3 were not observed. These observations suggested that the oxime group of 3 was reduced to the corresponding aminoethyl in 5. This postulation was confirmed by a key 1H-1H COSY correlation between the NH (δ_{C/H} 8.44) and the CH2 (δ_{C/H} 4.30) and key HMBC correlations between the NH and C-6 (δ_{C/H} 159.9) and between the CH2 and C-5 (δ_{C/H} 103.9) (Figure 1). HMBC correlations observed between the CH2 and the acetyl carbon indicated the presence of a hydroxy group in 5. Thus, the structure of 5 was elucidated as N-[(4-hydroxy-2,2'-bipyridine-6-yl)methyl]-acetamide.
Table 1. $^1$H and $^{13}$C NMR Data for 1–6 (600 MHz, 150 MHz, DMSO-$d_6$, TMS, $\delta$ ppm)

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<th>position</th>
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<th>5</th>
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<td></td>
<td>$\delta_C$</td>
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<td>5.52, brs</td>
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<td>7.40, dd (7.5, 4.4)</td>
<td>124.9, CH</td>
<td>7.45, t (6.6)</td>
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<td>8.64, d (5.5)</td>
<td>149.8, CH</td>
<td>8.67, d (4.6)</td>
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*The NMR data for the acetyl are $\delta_H$ 1.92 (s, 3H) and $\delta_C$ 23.2 (CH$_3$) and 170.1 (C).*
moiety had the E configuration (Figure 1). Thus, the structure of compound 6 was elucidated as (E)-4-methoxy-2-phenylpyridine-6-carbaldehyde oxime.

It was also established that 11 could be formed from 7 by direct photoisomerization, indicating that E-aldoximes can be transformed into Z-aldoximes using light (Figure S32). Even though 11 was present in the extract before chromatography (Figure S33), it is still possible that 11 could be formed nonenzymatically during the extraction process. The HPLC profile of the EtOAc extract (Figure S33) also showed 4 as a natural product.

Compounds 1–5 and 7–10 are probably biosynthesized from amino acid and polyketide pathways (Scheme 1). Intramolecular reactions of lysine can produce picolinic acid. Picolinic acid (likely as its CoA ester) may condense with acetyl CoA to form picolinoylactetyl CoA, which undergoes condensation with 2-aminoacrylic acid derived from serine to produce 4-hydroxybipyridine-6-carboxylic acid. The bipyridine carboxylic acid can be converted to the corresponding bipyridine amide or acid can undergo reduction to yield the bipyridine product.

Compounds 1–10 were evaluated for their cytotoxicity against the K562, HL-60, and KB cell lines using the MTT method, and the A549 cell line using the SRB method (Table 2). The new compounds 1 and 3–5 were found to be active against K562, HL-60, A549, and KB cells with IC_{50} values ranging from 0.37 to 31.8 μM. Compound 4 had the most potent activity, with an IC_{50} value of 0.37 μM against K562 cells. Caerulomycin A (7) showed potent cytotoxicity against the HL-60 and A549 cell lines, with IC_{50} values of 0.71 and 0.26 μM, respectively. Caerulomycin C (8) showed moderate cytotoxicity against K562 and KB cells, with IC_{50} values of 1.8 and 3.1 μM, respectively. Weak cytotoxicities for caerulomycinonoritile (10) against A549 cells, and 3a against K562 cells, were also observed, with IC_{50} values of 15.0 and 31.8 μM, respectively. The other compounds isolated were found to be inactive (IC_{50} > 50 μM) against the cell lines tested.

Antimicrobial activities against Escherichia coli, Aerobacter aerogenes, Pseudomonas aeruginosa, and Candida albicans were also evaluated for compounds 1, 4, 8, 10, and 3a using an agar dilution method. Compound 7 showed weak antimicrobial activities against E. coli, P. aeruginosa, and C. albicans with MIC values of 10.9, 21.8, and 21.8 μM, respectively. Compound 8 showed weak antimicrobial activities against E. coli, A. aerogenes, P. aeruginosa, and C. albicans with MIC values of 9.7, 19.3, 38.6, and 19.3 μM, respectively. Compound 1 selectively inhibited the growth of E. coli with a MIC value of 41.1 μM. The other compounds did not show antimicrobial activity (MIC ≥ 100 μM). The results showed that the antimicrobial activity of bipyridines is not due to general metal chelation, but more importantly might be related to the oxime moiety.

### Scheme 1. Plausible Biogenetic Pathways of 1–5 and 7–10

![Scheme 1](image)

### Table 2. Cytotoxicities against Tumor Cells for 1–10 and 3a (IC_{50}, μM)

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<th>9</th>
<th>10</th>
<th>3a</th>
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<td>HL-60</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>1.6</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>0.71</td>
<td>&gt;50</td>
<td>&gt;50</td>
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<td>&gt;50</td>
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<tr>
<td>K562</td>
<td>15.7</td>
<td>NT</td>
<td>NT</td>
<td>0.37</td>
<td>15.0</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>1.8</td>
<td>NT</td>
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<td>A549</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>8.4</td>
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*NT = not tested.*
General Experimental Procedures. UV spectra were recorded on a Beckman DU 640 spectrophotometer. IR spectra were taken on a Nicolet Nexus 470 spectrophotometer as KBr discs. $^1$H NMR, $^13$C NMR, and DEPT spectra of compounds 1–6 and 2D NMR spectra of compounds 4 and 5 were recorded on a JEOL JNM-ECP 600 spectrometer using TMS as an internal standard, and chemical shifts were recorded as δ values. 2D NMR spectra of compound 6 were recorded on a Bruker Avance 500 spectrometer. 1D NOE spectra were obtained on a Varian INOVA 400 spectrometer. ESIMS utilized a Q-TOF Ultima Global GAA076 LC mass spectrometer. Semipreparative HPLC was performed using an ODS column (YMC-pak ODS-A, 10×250 mm, 5 μm, 4 mL/min). TLC and column chromatography (CC) were performed on plates precoated with silica gel GF254 (10–40 μm) and over silica gel (200–300 mesh, Qingdao Marine Chemical Factory) and Sephadex LH-20 (Amersham Biosciences), respectively. Vacuum liquid chromatography (VLC) was carried out over silica gel H (Qingdao Marine Chemical Factory). Marum salt used is made from the evaporation of seawater collected in Laizhou Bay (Weifang Haisheng Chemical Factory).

Actinomycete Material. The actinomycete Actinoalloteichus cyanogriseus WHI-2216-6 was isolated from marine sediments collected from the seashore of Weihai, China. The marine sediments (2 g) were air dried for 15 days in a 45 mL sterile centrifuge tube. The dried sediments were separated into four subfractions by VLC on RP-18 silica using stepwise gradient elution, with CH2Cl2/MeOH 100% followed by MeOH/CH2Cl2 (0→10%) and 60% MeOH/H2O, respectively. Subfraction 3-2 (650 mg) was further purified by semipreparative HPLC 45% MeOH/H2O to yield 6 (1.1 mg) and 7 (1.1 mg). Subfraction 3 (13 mg) was separated into nine subfractions by semipreparative HPLC 45% MeOH/H2O to yield 7 (1.1 mg) and 6-3 (240 mg) were further purified by semipreparative HPLC to yield 9 (10.0 mg), 10 (8.0 mg) and 11 (1.7 mg), eluting with 40% and 30% MeOH/H2O, respectively. Fraction 7 (1.7 mg) was subjected to CC using a stepwise gradient elution of EtOAc/petroleum ether (25–100%) to afford four subfractions. Subfraction 7-5 (250 mg) was further purified by semipreparative HPLC 35% MeOH/H2O to yield 5 (3.2 mg, 89.9 min).

Caeurulomycin F (1): white, amorphous powder; UV (MeOH) $\lambda_{max}$ (log ε) 203 (2.41), 253 (1.16) nm; IR (KBr) $\nu_{max}$ 3449, 2942, 1646, 1381, 1099, 1056 cm$^{-1}$; $^1$H and $^{13}$C NMR, see Table 1; HRESIMS m/z 217.0977 [M+H]$^+$ (calcd for C8H11N2O2, 217.0977).

Caeurulomycin G (2): colorless needles (MeOH); mp 127 °C; UV (MeOH) $\lambda_{max}$ (log ε) 214 (3.16), 277 (2.62) nm; IR (KBr) $\nu_{max}$ 3458, 2924, 1779, 1692, 1533, 1454, 1069, 670 cm$^{-1}$; $^1$H and $^{13}$C NMR, see Table 1; HRESIMS m/z 247.1083 [M+H]$^+$ (calcd for C11H16N2O4, 247.1083).

Caeurulomycin H (3): white, amorphous powder; UV (MeOH) $\lambda_{max}$ (log ε) 240 (2.91), 280 (2.75) nm; IR (KBr) $\nu_{max}$ 3711, 2933, 1651, 1502, 1372, 1302, 1016, 987, 797, 738 cm$^{-1}$; $^1$H and $^{13}$C NMR, see Table 1; HRESIMS m/z 216.0781 [M+H]$^+$ (calcd for C11H12N2O3, 216.0773).

Caeurulomycin I (4): colorless needles (MeOH); mp 101 °C; UV (MeOH) $\lambda_{max}$ (log ε) 230 (3.66), 278 (2.32) nm; IR (KBr) $\nu_{max}$ 3508, 3065, 2939, 1676, 1584, 1480, 1426, 1356, 1219, 1034, 875, 780, 671 cm$^{-1}$; $^1$H and $^{13}$C NMR, see Table 1; HRESIMS m/z 282.0855 [M+Na]$^+$ (calcd for C11H12N2O3Na, 282.0855).

Caeurulomycin J (5): yellow oil; UV (MeOH) $\lambda_{max}$ (log ε) 230 (3.83), 273 (3.65) nm; IR (KBr) $\nu_{max}$ 3282, 2986, 1653, 1537, 1442, 1372, 1027, 794, 621 cm$^{-1}$; $^1$H and $^{13}$C NMR, see Table 1; HRESIMS m/z 266.0905 [M+Na]$^+$ (calcd for C11H12N2O2Na, 266.0905).

Chemical Transformations of 3 and 7. A suspension of NaH (1.0 mg) in 1.0 mL of anhydrous DMF was treated with a solution of compound 3 (1.0 mg) in 1.0 mL of DMF at 0 °C. The reaction mixture was stirred at 0 °C for 30 min, and then CH3I (10 μL) was added. After 30 min, a saturated solution of NH4Cl (5.0 mL) was added to quench the reaction. The product was extracted with EtOAc (4×10 mL) and purified by VLC on RP-18 silica to give 3a (1.0 mg, 94%). Using the same procedure, compound 7 also afforded 3a (0.9 mg, 85%). Product 3a was identified as O-methyl-(E)-4-methoxy-2,2′-bipyrindine-6-carboxylic acid by comparison of its 1H NMR spectrum with that reported in the literature.11 Compound 3a: white, amorphous powder; UV (MeOH) $\lambda_{max}$ (log ε) 231 (3.63), 257 (3.41) nm; $^1$H NMR (CDCl3, 600 MHz) $\delta$ 3.98 (s, 3H, OCH3-8), 4.04 (s, 3H, OCH3-4), 7.32 (d, 1H, J=7.7, 1H, J=7.4, 1H, J=7.8), 7.29 (d, 1H, J=7.7, 1H, J=7.4, 1H, J=7.8), 7.25 (s, 1H, J=7.7, 3 H-3), 8.67 (d, 1H, J=4.1 Hz, H-6′); ESIMS m/z 244 [M+H]$^+$.

Photosomerization. The isomerization of 7 (1 mg) was carried out under the following experimental conditions: reaction times, 1, 4, 8, 40 h; solvent (2 mL), Me2CO; temperature, 25 °C; light source, mercury vapor lamp or no light (control). The reaction mixtures obtained from each set of reaction conditions were analyzed by HPLC (50% MeOH) (Figure S32). Compound 7 was found to isomerize to 11 when light was
applied, while it was stable in the absence of light. The proportion of 7 that had isomerized with the application of light after 1, 4, and 40 h was 8.4%, 14%, and 33%, respectively.

■ ASSOCIATED CONTENT

5 Supporting Information. NMR spectra for compounds 1–6, NMR data for compounds 7–11, HPLC analysis of the product of photoisomerization of 7, a description of the bioassay protocols used, and 16S rRNA sequences of A. cyanogriseus WH1-2216-6 are available free of charge via the Internet at http://pubs.acs.org.

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