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Structures and Biomimetic Synthesis of Novel α-Pyrone Polyketides of an Endophytic *Penicillium* sp. in *Catharanthus roseus*

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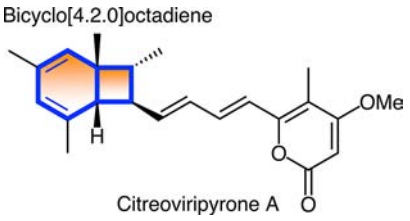
ABSTRACT



Novel polyketides, citreoviripyrone A (1) and B (2), known citreomontanin (3), and (−)-citreoviridin (4) were isolated from the mycelium of the endophytic fungus. The endophytic fungus, which belongs to the genus *Penicillium*, was separated from surface-sterilized healthy leaves of *Catharanthus roseus*. The structures of 1 and 2 were determined on the basis of NMR data, and 1 was characterized as an α-pyrone polyketide featuring bicyclo[4.2.0]octadiene. The biomimetic synthesis of 1 and 2 from 3 elucidated a plausible biosynthetic pathway. Both Zn(II)-type and NAD^+-dependent histone deacetylase inhibitors significantly enhanced the production of 1 and 3.

Bicyclo[4.2.0]octadiene is crucial for structurally and biologically interesting natural products. Several natural products featuring the bicyclo skeleton have been reported from various sources, including the Lauraceaeous plant (endiandric acid D and E),[1] saccoglossan mollusc (ocellapyrone A),[2] *Streptomyces* (SNF4435 C and D),[3] and marine-derived fungus (shimalactone A and B) (Figure 1).[4]

These natural products may arise from the $8\pi-6\pi$ electrocyclization cascade route from their corresponding polyene precursors, most of which are highly methylated.[5]

Several *Penicillium* fungi, such as *P. citreoviride* isolated from "yellowed rice",[6] produce (−)-citreoviridin (**4**)[7] and citreomontanin (**3**).[8] **4** is a well-known mycotoxin composed of three parts: α-pyrone, polyene, and a highly oxidized tetrahydrofuran ring (Figure 2). Due to the structural similarities, **3** is thought to be biosynthetically related to **4**. Although **3** should biosynthetically form compounds with a bicyclo[4.2.0]octadiene in the structure,

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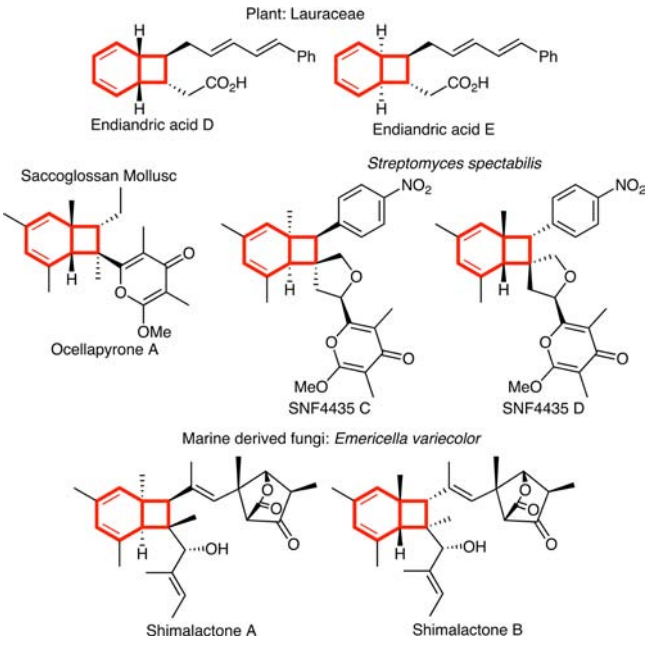

Figure 1. Previously reported compounds possessing a bicyclo-[4.2.0]octadiene core (red bold line).

these types of compounds have yet to be isolated from *Penicillium* fungi.

In our search for novel natural products of endophytic fungi, which are a rich source of structurally and biologically interesting compounds,[9] we have separated an endophytic *Penicillium* sp. CR07 from surface-sterilized healthy leaves of *Catharanthus roseus*, which is a well-known producer of a vinca alkaloid. A chemical study of MeOH extracts of the mycelium of the *Penicillim* sp. CR07 afforded novel α-pyrone polyketides, citreoviripyrone A (**1**) and citreoviripyrone B (**2**), along with **3** and **4**. Herein we report the structures of **1** and **2**, their biomimetic conversion from **3**, and their cell growth inhibitory activity. In addition, we discuss the effects of both Zn(II)-type and NAD^+-dependent HDAC inhibitors on the polyketide production in the *Penicillium* fungus.

Endophytic *Penicillium* sp. CR07 was cultivated in a potato dextrose broth (PDB) for 21 days at 25 °C. The mycelium (155.6 g) was extracted twice with MeOH, and the MeOH extract (33.9 g) was partitioned between EtOAc and H_2O. The EtOAc extract (10.8 g) was separated by silica gel column chromatography and preparative TLC to afford citreoviripyrone A (**1**, 19.1 mg), B (**2**, 1.2 mg), citreomontanin (**3**, 35.0 mg), and (−)-citreoviridin (**4**, 1.9 g).

The HRFABMS of citreoviripyrone A (**1**) at *m*/*z* 353.2133 $[M + H]^+$ (calcd: *m*/*z* 353.2117) yielded a molecular formula of $C_{23}H_{28}O_3$, which requires ten degrees of unsaturation. The UV absorption at 354 nm ($\log \varepsilon = 4.33$) suggested that the molecule contained an extended

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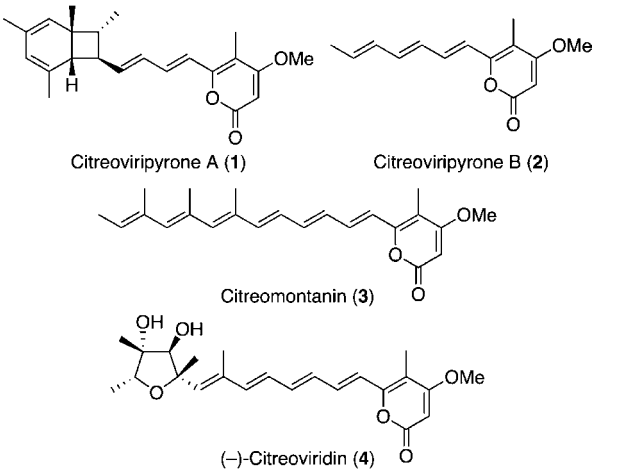

Figure 2. Structures of **1**−**4**.

conjugated system. The ^{13}C NMR and DEPT spectra implied the presence of one ester carbonyl, five quaternary sp^2 carbons, seven tertiary sp^2 carbons, one quaternary carbon, three methines, one methoxy methyl, and five methyls (Table 1). Comparing the ^1H and ^{13}C NMR spectra of **1** to those of **4** indicated the presence of a 3,4,5-trisubstituted 3-methoxy-α-pyrone moiety [δ_C 163.7 (C-1), 88.5 (C-2), 170.7 (C-3), 107.2 (C-4), 154.6 (C-5), and 56.1 (OMe); δ_H 5.48 (s, H-2), 1.94 (s, H_3-22), and 3.82 (s, OMe) (Tables 1 and S1)], which was consistent with the HMBC correlations of H-2/C-1, C-3, C-4; H_3-22/C-3, C-4, C-5; and OMe/C-3 (Figure 3). The $^1H-^1H$ COSY spectrum exhibited sequential correlations of H-6−H-7−H-8−H-9−H-10−H-17−H-18 and H-10−H-11 (Figure 3). The HMBC correlations of H_3-19/C-11, C-15, C-16, C-17; H_3-20/C-13, C-14, C-15; and H_3-21/C-11, C-12, C-13 indicated a pentasubstituted bicyclo[4.2.0]octadiene structure (C-10−C-21) (Figure 3). Additionally, the HMBC correlations of H-6/C-4 and H-6/C-5 determined the C-5/C-6 linkage. The large coupling constants confirmed that the geometries of the C-6/C-7 and C-8/C-9 double bonds were both *E* ($J_{H\text{-}6/H\text{-}7}$ = 15.0 Hz and $J_{H\text{-}8/H\text{-}9}$ = 15.1 Hz).

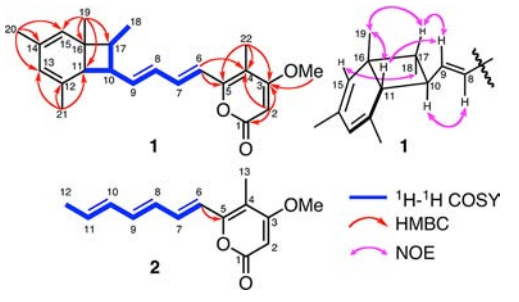

Figure 3. Selected HMBC, $^1H-^1H$ COSY, and NOE correlations of **1** and **2**.

Table 1. ^{13}C (150 MHz) and ^{1}H NMR (600 MHz) Data for **1** and **2**[a]

	1		2	
	^{13}C	^{1}H (multi, J in Hz)	^{13}C	^{1}H (multi, J in Hz)
1	163.7 C	–	163.7 C	–
2	88.5 CH	5.48 (s)	88.5 CH	5.50 (s)
3	170.7 C	–	170.6 C	–
4	107.2 C	–	107.4 C	–
5	154.6 C	–	154.6 C	–
6	117.7 CH	6.26 (d, 15.0)	118.3 CH	6.30 (d, 15.0)
7	136.4 CH	7.13 (dd, 15.0, 10.7)	136.2 CH	7.18 (dd, 15.0, 11.0)
8	128.0 CH	6.15 (dd, 15.1, 10.7)	129.1 CH	6.25 (dd, 15.0, 11.0)
9	144.0 CH	6.05 (dd, 15.1, 8.8)	138.5 CH	6.46 (dd, 15.0, 10.6)
10	51.8 CH	2.46 (q, 8.8)	131.5 CH	6.15 (dd, 15.0, 10.6)
11	49.8 CH	2.19 (d, 8.8)	133.6 CH_3	5.90 (m)
12	135.1 C	–	18.5 CH_3	1.83 (d, 6.6)
13	120.9 CH	5.37 (brs)	8.8 CH_3	1.96 (s)
14	129.9 C	–		
15	122.7 C	4.97 (brs)		
16	40.4 C	–		
17	51.2 CH	2.15 (m)		
18	13.2 CH_3	0.99 (d, 8.8)		
19	28.3 CH_3	1.03 (s)		
20	22.3 CH_3	1.75 (brs)		
21	22.0 CH_3	1.66 (brs)		
22	8.8 CH_3	1.94 (s)		
OMe	56.1 CH_3	3.82 (s)	56.1 CH_3	3.82 (s)

[a] Recorded in $CDCl_3$. Assignments for all compounds were based on DQF-COSY, HMQC, and HMBC experiments.

1D NOE experiments determined the relative stereochemistries around the bicyclo moiety. The NOEs of H-9/H-11, H-17 and H_3-19/H-11, H-17 indicated that the H-11, H-17, H_3-19, and C-9 were situated on the same side. In addition, the NOE of H_3-18/H-15 supported the *anti* relationship between H_3-18 and H_3-19. Citreoviripyrone A (**1**) should be racemic due to the minimal optical rotation of **1** ($[\alpha]_D^{25} = \pm 0.00$ ($c = 0.16$, $CHCl_3$)) and the fact that the CD spectrum did not display a remarkable Cotton effect.

The molecular formula of citreoviripyrone B (**2**) was $C_{14}H_{16}O_3$ [HREIMS: m/z 232.1084 $[M]^+$ (calcd: m/z 232.1099)], which requires seven degrees of unsaturation. Similar to compound **1**, the UV absorption at 356 nm (log $\varepsilon = 4.07$) showed the presence of an extended conjugated system. The ^{13}C NMR and DEPT spectra indicated the presence of one ester carbonyl, three quaternary sp^2 carbons, seven tertiary sp^2 carbons, one methoxy methyl, and two methyls (Table 1). Comparing the ^{13}C NMR spectrum of **2** to that of **1** revealed the presence of a 3,4,5-trisubstituted 3-methoxy-α-pyrone moiety (C-1−C-5, C-13, OMe) (Table 1). Sequential $^{1}H-^{1}H$ COSY correlations (H-6−H_3-12) and HMBC correlations of H-6/C-5 confirmed the triene (C-6−C-12) was linked to C-5 on the α-pyrone (Figure 3). The large coupling constants of H-6/H-7, H-8/H-9, and H-10/H-11 (15.0 Hz for all three J-values) indicated that the three olefins in the triene had *trans* geometries (Table 1). Thus the structure of citreoviripyrone B (**2**) was determined to be as shown Figure 2.

Compounds **1** and **3** have common structural features, including the same molecular formula. Both have the same number of methyl groups at the same substituted positions. Additionally, both have a trisubstituted α-pyrone. Considering these structural similarities, **3** is likely a putative biogenetic precursor of **1** (Scheme 1).

Treatment of all-(*E*)-hexaene **3** with dichlorobis(acetonitrile)palladium(II) $[PdCl_2(MeCN)_2]$, which is a well-known reagent to isomerize a conjugated double-bond system,[5c] in DMF at 70 °C for 1 h, afforded **1** in 13% yield with a trace of **2**. In contrast, **2** was isolated in 18% yield as the major product for the same reaction after 14 h (Scheme S1). TLC monitoring (0−14 h) of the reaction showed that **1** was generated initially but was gradually converted into **2**. Presumably, **3** yielded **1** through intermediate **5** by a thermal 8π conrotatory and 6π disrotatory electrocyclization cascade under the Pd(II)-catalyzed isomerization conditions. Then a retro [2 + 2] cycloaddition[5e] of **1** generated **2** (Scheme 1).

Recently, we demonstrated that the addition of either a Zn(II)-type or NAD^+-dependent HDAC inhibitor in the cultivation of fungi, especially 500 μM of suberoyl bishydroxamic acid (SBHA) (a Zn(II) type HDAC inhibitor) and 100 μM of nicotinamide (an NAD^+-dependent HDAC inhibitor), significantly enhanced polyketide production, and we successfully isolated a variety of novel natural products.[10] Thus, we applied these chemicals to the *Penicillium* cultivation. **1** and **3** in the mycelium were notably enriched upon cultivation with SBHA (500 μM) or nicotinamide (100 μM) (Table 2). Upon production of **4**, nicotinamide showed its producing effect, while SBHA did not show a distinct effect.

Scheme 1. Plausible Biogenetic Pathway of **1**, **2**, and **4** from **3**

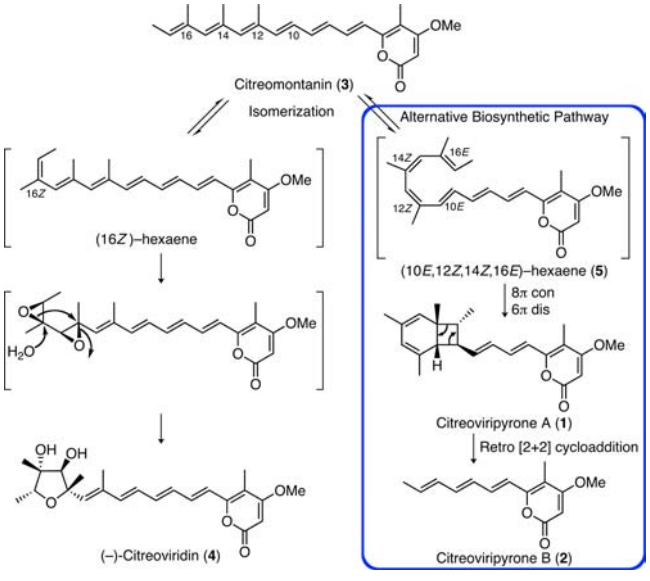

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Table 2. Effects of HDAC Inhibitors on the Concentrations of **1**, **3**, and **4** in the Mycelium (Dry Weight)[a]

condition	**1** (mg/g)	**3** (mg/g)	**4** (mg/g)	mycelium[b] (g)
control	0.13	0.24	7.82	0.39
SBHA (500 μM)	0.72	1.31	8.22	0.36
nicotinamide (100 μM)	1.32	3.07	20.62	0.24

[a] Each compound was quantified against external standards. Quantification was based on peak area in the reversed-HPLC chromatogram. [b] In each condition, the fungus was cultivated in 60 mL of culture medium. Then the mycelia were collected and dried.

The cell growth inhibitory activities of **1**–**4** were evaluated in human HCT 116 cells. Citreoviripyrone A (**1**) exhibited a moderate inhibitory activity on cell growth with a GI_{50} value of 10.4 μM using the luciferin/luciferase assay (Cell Titer Glo, Promega) to quantitate the ATP of live cells. However, **1** did not inhibit growth within 50 μM in an MTT assay.

In conclusion, we isolated a novel α-pyrone polyketide with bicyclo[4.2.0]octadiene, citreoviripyrone A (**1**), from a (−)-citreoviridin (**4**)-producing endophytic *Penicillium* fungus. Using Pd(II) catalyzed polyene isomerization of citreomontanin (**3**) realized the biomimetic formation of **1** and **2** via an uncommon natural product biosynthetic pathway. We also found that both Zn(II)-type and NAD^+-dependent HDAC inhibitors could activate these α-pyrone polyketide productions in the endophytic fungus and verified the availability of HDAC inhibitors in the natural compound production.

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Supporting Information Available. Experimental methods, and NMR spectra of new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.