Epolones: Novel Sesquiterpene-Tropolones from Fungus OS-F69284 That Induce Erythropoietin in Human Cells

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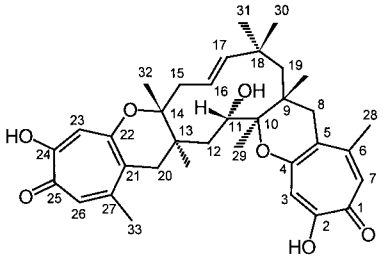
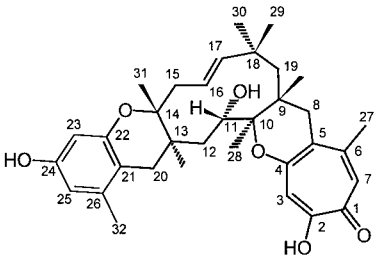
In the course of our screening for small molecule modulators of erythropoietin gene expression, two novel sesquiterpene tropolones and pycnidone were isolated from a culture of OS-F69284 (ATCC 74390). Their structures were elucidated by extensive ^{1}H and ^{13}C NMR spectroscopic studies and chemical reactions. These compounds induced erythropoietin gene expression 5-fold at a concentration of 1−1.6 μM.

Erythropoietin (EPO) is the primary hormone that regulates the proliferation and differentiation of immature erythroid cells. EPO is produced in fetal liver and adult kidney in response to hypoxia and circulates in the bloodstream where it targets EPO receptor on committed progenitor cells in the bone marrow and other hematopoietic tissues.[1] Recombinant human erythropoietin is used currently in the treatment of patients with anemia due to chronic renal failure, cancer chemotherapy, and a variety of other disease states. An alternative to the use of recombinant human erythropoietin, as an intravenous drug, would be an orally active drug that induces endogenous EPO production. Our research has focused on gene transcription as a novel approach to identify small molecules that upregulate the expression of the endogenous EPO gene, thereby increasing circulating EPO levels.[2]

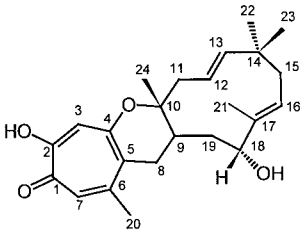
As described in a previous communication,[3] we screened for inducers of EPO gene expression using luciferase-reporter technology. The epo-3 cells (Hep3B) contained the bacterial-luciferase structural gene under the control of the erythropoietin regulatory sequence; luciferase expression directly correlated with the EPO expression of the parent Hep3B cells, such that the luciferase enzyme was inducible by cobalt chloride and hypoxia. Using this luciferase reporter as the primary assay, we screened approximately 60 000 extracts from 10 000 of the fungi in the MYCOsearch collection; each organism was grown in six different media and extracted with methanol after 11 days of fermentation. Extracts from the fermentation of OS-F69284 consistently demonstrated EPO-induction activity. Bioassay-directed fractionation of this extract uncovered three sesquiterpene-tropolones, including pycnidione (**1**)[4] and two novel analogues: epolone A (**2**) and epolone B (**3**). In this paper, we report the isolation and structure elucidation of these three compounds. In addition, we provide data on the ability of these compounds to induce human EPO in cell assays.



Pycnidione (**1**)



Epolone A (**2**)



Epolone B (**3**)

Results and Discussion

The dried methanol extract from a 6 L fermentation culture of OS-F69284 was partitioned into hexane,

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Table 1. ^{1}H NMR Chemical Shifts of Compounds **1**−**3**

proton	**1** ($CDCl_3$) δ ppm (mult, J = Hz)	**2** (CD_3OD) δ ppm (mult, J = Hz)	**3** ($CDCl_3$) δ ppm (mult, J = Hz)
3	6.97 (s)	7.07 (s)	6.97 (s)
7	7.10 (s)	7.09 (s)	7.10 (s)
8	2.63 (m)	2.74 (dd, 18, 5.5)	2.88 (dd, 17, 5.5)
	2.31 (m)	2.48 (dd, 18, 5.5)	2.36 (dd, 17, 12.5)
9	1.65 (m)	1.78 (m)	1.64 (m)
11	3.60 (bd, 10)	3.66 (bd, 10)	2.56 (bd, 14.5)
			2.23 (bd, 14.5, 10)
12	1.60 (bdd, 15, 10)	1.70 (bdd, 15, 10)	5.04 (ddd, 15.5, 10, 3)
	1.14 (m)	1.12 (m)	
13	1.85 (m)	1.89 (m)	5.14 (dd, 15.5, 2)
15	2.69 (dd)	2.62 (bd, 14, 1.2)	2.22 (dd, 12.5, 6)
	2.30 (dd)	2.38 (dd, 14, 11)	1.77 (dd, 12.5, 5)
16	5.38 (m)	5.52 (ddd, 15, 9, 4)	5.20 (dd, 6, 5)
17	5.36 (m)	5.47 (d, 15)	
18			3.94 (bd, 10)
19	1.60 (m)	1.65 (m)	1.72 (dd, 14, 10)
	1.14 (m	1.13 (m)	1.12 (bdd, 14, 8)
20	2.98 (bd, 17, 5)	2.96 (dd, 16.5, 5.5)	2.39 (s, 3H)
	2.26 (bd, 17)	2.24 (bd, 16.5)	
21			1.63 (s, 3H)
22			1.06 (s, 3H)
23	6.93 (s)	6.06 (s)	0.99 (s, 3H)
24			1.10 (s, 3H)
25		6.17 (s)	
26	7.09 (s)		
27		2.43 (s, 3H)	
28	2.36 (s, 3H)	1.24 (s, 3H)	
29	1.24 (s, 3H)	1.12 (s, 3H)	
30	1.05 (s, 3H)	1.05 (s, 3H)	
31	1.08 (s, 3H)	1.17 (s, 3H)	
32	1.20 (s, 3H)	2.15 (s, 3H)	
33	2.39 (s, 3H)		

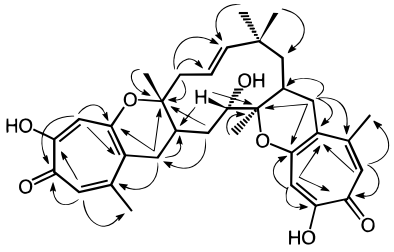

Figure 1. Selected long-range ^{1}H−^{13}C correlation of **1** observed in HMBC experiment.

chloroform, and methanol/water fractions. The chloroform fraction was subjected to isolation by centrifugal partition chromatography (CPC), followed by reversed-phase HPLC to yield pure compounds **1**−**3** as white powders.

The major component, compound **1**, had a molecular formula of $C_{32}H_{40}O_6$ as established by HRFABMS. The UV spectrum of **1** showed maxima at 255, 324 (sh), and 365 nm and was similar in appearance to that reported for 3,7-dihydroxytropolone.[5] Both ^{1}H and ^{13}C NMR spectra of **1** indicated the presence of two identically substituted troplone/pyran rings fused with one 11-membered ring consisting of three isolated proton spin systems (Table 1). Comprehensive analysis of 1D and 2D NMR spectra, including COSY, HMQC, and HMBC (Figure 1), established the structure of **1**. Literature search indicated that **1** has the same 2D-structure as pycnidione, isolated from *Phoma sp*. as a stromelysin inhibitor,[4] and eupenifeldin, isolated from *Eupenicillium brefeldianum* as an antitumor agent.[6] In eupenifeldin, one tropolone/pyran moiety is trans-fused to the central 11-membered sesquiterpene ring (B/C) and the other one is cis fused (C/D); however, in pycnidione, both

Table 2. ^{13}C NMR Chemical Shifts of Compounds **1**−**3**

carbon	**1** δ ppm (multiplicity)	**2** δ ppm (multiplicity)	**3** δ ppm (multiplicity)
1	172.6 (s)	173.4 (s)	172.7 (s)
2	163.3 (s)	165.6 (s)	163.1 (s)
3	112.4 (d)	115.1 (d)	113.0 (d)
4	159.7 (s)	162.6 (s)	160.9 (s)
5	119.9 (s)	125.2 (s)	119.9 (s)
6	149.6 (s)	151.7 (s)	149.8 (s)
7	124.6 (d)	123.4 (d)	124.5 (d)
8	34.0 (t)	34.6 (t)	31.3 (t)
9	31.4 (d)	32.6 (d)	33.4 (d)
10	83.6 (s)	85.9 (s)	81.3 (s)
11	77.0 (d)	77.0 (d)	42.6 (t)
12	33.6 (t)	31.8 (t)	120.3 (d)
13	39.6 (d)	40.9 (d)	142.2 (d)
14	81.5 (s)	80.7 (s)	38.6 (s)
15	48.6 (t)	50.5 (t)	40.6 (t)
16	121.9 (d)	123.9 (d)	123.8 (d)
17	143.7 (d)	144.2 (d)	138.4 (s)
18	36.7 (s)	37.5 (s)	77.9 (d)
19	46.1 (t)	47.1 (t)	38.7 (t)
20	33.9 (t)	35.0 (t)	27.2 (q)
21	121.8 (s)	113.4 (s)	10.6 (q)
22	160.4 (s)	154.8 (s)	30.0 (q)
23	113.5 (d)	101.8 (d)	24.1 (q)
24	163.9 (s)	156.9 (s)	20.1 (q)
25	171.7 (s)	109.9 (d)	
26	124.0 (d)	138.2 (s)	
27	149.4 (s)	27.2 (q)	
28	27.1 (q)	16.5 (q)	
29	15.9 (q)	22.2 (q)	
30	31.2 (q)	31.7 (q)	
31	21.9 (q)	18.2 (q)	
32	18.6 (q)	19.3 (q)	
33	27.3 (q)		

tropolone/pyran moieties are trans fused to the 11-membered ring. For compound **1**, the large coupling constant of H-11 with the downfield H-12 ($J_{H-11/H-12} \approx$ 15 Hz) and the observed NOEs between H-11 with H-9 in a NOESY experiment suggested that H-11 had an axial orientation and occupied the same face as H-9. Since no NOEs were observed between H-9 with the methyl protons of C-29 or between H-13 and the methyl protons of C-32, it suggested that these protons were on the opposite faces and both pyran rings were trans-fused to the 11-membered ring. Further support came from the fact that compound **1** showed the same optical rotation as pycnidione,[4] and therefore, the stereochemistry of **1** should be the same as that of pycnidione.

The molecular weight of one minor compound (**2**) was determined to be 520 by the observation of an ion peak at m/z 521 ($[M + H]^+$) in the positive ESMS and 519 ($[M - H]^-$) in the negative ESMS; this is 28 Da less than compound **1**. The ^{13}C NMR spectrum of **2** showed a striking similarity to that of compound **1**, with the exception of some aromatic signals. Whereas compound **1** displayed a total of 16 unsaturated carbons (14 tropolone-ring carbons and two olefinic carbons) in the ^{13}C NMR spectrum, compound **2** displayed a total of 15 unsaturated carbons, including only one carbonyl carbon signal (δ 173.3, Table 2). In contrast to the proton signals of **1**, the ^{1}H NMR spectrum of **2** displayed only two tropolone protons within the range 6.93−7.10 ppm; additionally, two of the aromatic proton singlets shifted upfield to 6.0−6.2 ppm. This information, together with the 28 Da mass difference between **1** and **2**, suggested that **2** might have only one tropolone ring, with the

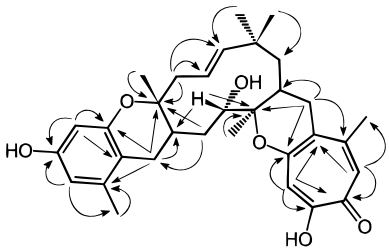

Figure 2. Selected long-range $^1H-^{13}C$ correlation of **2** observed in HMBC experiment.

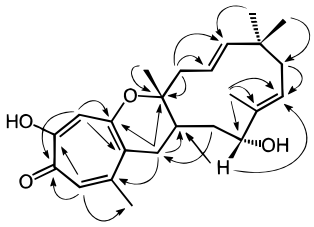

Figure 3. Selected long-range $^1H-^{13}C$ correlation of **3** observed in HMBC experiment.

remaining six aromatic carbons (δ 156.8, s; 154.8, s; 138.2, s; 113.4, s; 109.9, d; and 101.8, d) belonging to a tetrasubstituted benzene ring. Like the tropolone-ring in compound **1**, this benzene ring is connected to the 11-membered ring through methylene C-20 and the ether linkage between C-14 with C-22. This is corroborated by the strong long-range correlations of H-20 (δ 2.96) to the oxygenated carbon of benzene ring at 154.8 ppm (C-22) and one nonprotonated benzene carbon at 138.2 ppm (C-26). A meta arrangement of a methyl group and a hydroxyl group on the benzene ring was suggested by the benzene proton singlets and their long-range correlations with benzene carbons (Figure 2). On the basis of 1D and 2D NMR studies and comparison of the NMR data with that of **1**, structure **2** was proposed for this minor component. This structure is similar to lucidene, isolated from the higher plant *Uvaria Lucida* ssp. *Licida*.[7] The sesquiterpene also possesses the 11-membered ring, but it is fused to two unsubstituted benzopyran systems. The complete ^1H and ^{13}C NMR chemical shift assignments and the important long-range heteronuclear correlations are summarized in Tables 1 and 2, and Figure 2. The stereochemical assignments of **2** at the various asymmetric centers were suggested on the basis of the NOEs, biosynthetic considerations, and comparison with **1**.

The second minor component from OS-F69284, compound **3**, also showed the characteristic UV absorption of a tropolone. The HRFABMS of **3** exhibited $[M + H]^+$ at *m/z* 385.2336, indicating a molecular formula of $C_{24}H_{32}O_4$ with nine degrees of unsaturation. The ^1H NMR spectrum of **3** revealed two aromatic proton singlets (δ 7.10, 6.97) and three sp^2 proton double-doublets (δ 5.04, 5.14, 5.20), suggesting the presence of one tropolone ring and two double bonds. This was supported by the DEPT and broad-band-decoupled ^{13}C NMR data (Table 2). The ^{13}C NMR spectrum of **3** displayed a total of 24 carbon signals, including those of 11 sp^2 hybridization carbons, which were assigned to seven tropolone-ring carbons (δ 172.7, s, C-1; 163.1, s, C-2; 113.0, d, C-3; 160.9, s, C-4; 119.9, s, C-5; 149.8, s, C-6; 124.5, d, C-7) and four double-bond carbons (δ 120.3, d, C-12; 142.2, d, C-13; 123.8, d, C-16; 138.4, s, C-17). Upon comparison of the ^{13}C NMR data of **3** with that of **1**, we found that **3** lacked one methylene carbon, one methine carbon, and one oxygenated quaternary carbon of a pyran ring; this suggested that **3** had only one tropolone/pyran moiety and agreed with the molecular-weight difference between **3** and **1**. As observed for compounds **1** and **2**, the DQCOSY spectrum of compound **3** displayed three isolated proton spin systems, which connected through three quaternary carbons (C-10, C-14, and C-17) to form the 11-membered C-ring. Together with the corresponding carbon chemical shifts from the HMQC spectrum, these three spin systems were assigned as follows: H11′ (δ 2.23)−H11 (δ 2.56)−H12 (δ 5.04)−H13 (δ 5.14), H15 (δ 2.22)−H15′ (δ 1.77)−H16 (δ 5.20), and H8′ (δ 2.36)−H8 (δ 2.88)−H9 (δ 1.64)−H19/H19′ (δ 1.72/1.12)−H18 (δ 3.94). Their connection positions were deduced from the long-range correlations of H-8, H-9, H-11, and H-19 with C-10 (δ 81.3), H-15 and H-13 with C-14 (δ 38.6), as well as H-15 and H-19 with C-17 (δ 138.4), as observed from the HMBC spectrum (Figure 3). The connection of the 11-membered ring with the tropolone ring through methylene C-8 was evident from the three-bond coupling of H-8 (δ 2.88) with the tropolone ring C-4 (δ 160.9) and C-6 (δ 149.8). Both double bonds in the 11-membered ring were E-configurations; this was established by the large coupling constant between H-12 with H-13 (^3J = 15.5 Hz) and the upfield chemical shift of the methyl group (δ 10.6, C-21) attached to the double bond C-17.[8]

Although there is no direct evidence to support the idea, it is possible that the monotropolone, **3**, is a biosynthetic precursor to the bistropolone **1**. Kinetic studies of the production of **1** and **3** demonstrated that, during the course of fermentation, the ratio of **1** to **3** increases from 1:4 at day 7 to 3:1 at day 11 as determined by quantitative HPLC analysis.

Treating compounds **1**−**3** with freshly prepared diazomethane produced the phenolic methyl ethers; aliphatic hydroxyl methylation products were not observed. Due to tropolone tautomerization, **1** gave four methylated derivatives (1Ma, 1Mb, 1Mc, and 1Md), and all have molecular weights of 576 as determined by LC-ESMS. The major product (1Ma) was isolated and identified as the 1,25-di-*O*-methyl derivative of **1** by its ^1H NMR spectroscopic data (see the Experiment Section). Although not isolated, the three others were expected to be 1,24-di-*O*-methyl, 2,25-di-*O*-methyl, and 2,24-di-*O*-methyl derivatives. Compound **2** produced two methylated derivatives with molecular weights of 548 (LC-ESMS). The major product was isolated and identified by ^1H NMR as 1,24-di-*O*-methyl-**2** (2Ma). Two *O*-methyl derivatives were isolated from the methylation products of **3**, and both had a molecular weight of 398. On the basis of ^1H NMR spectra, they were identified as 1-*O*-methyl-**3** (3Ma) and 2-*O*-methyl-**3** (3Mb), respectively.

In several independent experiments, compounds **1**−**3** reproducibly induced erythropoietin gene expression in epo-3 cells. The TIR_{5X} (concentration resulting in 5-fold induction) of these compounds was in the range of 1.0−1.6 μM, and the maximum induction activity of these compounds reached as high as 30−40-fold (Table 3). However, the methylated derivatives were completely devoid of activity in the same assay. Since **3** contains

Table 3. Induction of Human EPO in Epo-3 Cells (Hep-B3) by Compounds **1**−**3**

compd	epo-luciferase reporter[a,b] TIR_{5X} (μM)	epo-luciferase reporter[a,b] TIR_{max} (μM)	ELISA assay[c] mU/mL of epo protein	cytoxicity[d] (48 h) MTT, IC_{90} (μM)
1	1.6	19 (4.3)	34	>40
2	1.0	26 (4.3)	45	>40
3	1.5	22 (6.5)	40	>40
Me derivatives[e]	0		0	

[a] The TIR is the ratio of light units in the presence of compound to that of a control containing the vehicle (0.5% DMSO). [b] TIR_{5x} is the concentration of compound required to produce a 5-fold induction in epo-3 cells over control (0.5% DMSO). [c] ELISA assay was conducted at the concentration of 10 μM for all compounds. EPO protein secreted in cell control; there was no significant production of epo when cells were incubated with vehicle (0.5% DMSO). [d] Concentration that inhibited 90% of a standard MTT assay in epo-3 cells. [e] All methylated derivatives were tested and all were inactive.

only a single tropolone moiety, we can conclude that only one free tropolone-hydroxyl is requisite for EPO-induction activity. To confirm that the apparent induction of EPO by **1**−**3** was not an artifact of the luciferase reporter, we also assayed EPO protein secretion in the tester cell line. Using a commercially available ELISA kit for human EPO, we were able to demonstrate that these three tropolones induced native-protein secretion by approximately 35−40-fold above the vehicle control in a 24-h assay. When incubated with 10 μM of these tropolones, the epo-3 cells produced 30 −40 mU of EPO per milliter of culture; in the absence of these compounds, the EPO concentration was below the level of detection (Table 3). In both the reporter and secretion assays, the positive control, 10 μM $CoCl_2$, produced a 10−40-fold induction. As indicated in Table 3, these compounds did not demonstrate cytotoxicity at efficacious concentrations.

Experimental Section

General Experimental Procedures. Absorption spectra were measured on a Perkin-Elmer Lambda Bio UV/vis spectrometer. ^1H and ^{13}C NMR spectra were obtained on a Varian VXR-500 spectrometer. LRESMS data were collected on a VG platform-II instrument, and HRFABMS were obtained with a Finnegan MAT-90 spectrometer. CPC separation was carried out using a Sanki high performance centrifugal partition chromatograph model LLB-M instrument, and HPLC was conducted on an Hewlett-Packard 1090 liquid chromatograph equipped with a diode-array detector.

EPO-Induction Assays. To screen for inducers of EPO gene expression, a luciferase reporter plasmid containing 6 kb 5′ flanking sequence and 300 bp of proximal 3′ sequence of the human EPO gene was constructed and was electroporated into Hep3B cells. The resulting tester-cell clone was designated epo-3. The luciferase-reporter assay was carried out with the epo-3 cell line after growth of the cells in DMEM medium for 3 days. The cells were deposited in 96-well plates at 10 000 cell per well in DMEM medium. Experimental compounds were dissolved in DMSO and then incubated with epo-3 cells for 24 h (the final DMSO concentration was 0.05%). For the measurement of secreted EPO, the tester cell line was plated in DMEM at 20 000 cells per well in 24-well dishes. The next day, fresh medium was added, and test compounds were incubated for 24 h as described above. After incubation, culture supernatants were harvested and EPO was measured by ELISA (R&D system) following the manufacturer's protocol.[3]

Maintenance and Fermentation of OS-F69284. The fungal strain OS-F69284 was isolated from twigs collected from a seasonal, deciduous alluvial forest at Reserva bíológica Cambuí, Curitiba, Estado Paraná, Brazil, and deposited with the American Type Culture Collection (ATCC 74390). OS-F69284 was grown on several media, and its growth characters are described below. The four media are listed in the *ATCC Catalog of Fungi/Yeasts*, 17th ed., Rockville, MD, 1987, malt extract agar (ATCC No. 323), Czapek's dox agar, $^1/_4$ strength (ATCC No. 312), and yeast extract peptone dextrose agar (ATCC No. 1245). Colony colors were matched with the *Color Standards and Nomenclature*; Robert Ridgway, Washington, DC, 1912. Colonies on $^1/_4$ strength Czapek's agar growing an average of 2 mm diameter in 7 days: floccose to colony margin, colony color, white, no reverse color, hyphae septate, aerial and submerged hyphae 0.5−6.0 m diameter. Colonies on YePD growing an average of 25 mm diameter in 7 days: floccose to colony margin with radial wrinkles, colony color, white, with no reverse color. Mycelium touch at agar surface, hyphae septate, 0.5−6.0 m diameter. Colonies on malt agar growing an average of 57 mm diameter in 7 days: colony minutely tomentose to flat in overlapping waves. Irregular colony margin. Colony color, white, no reverse color: hyphae septate, 0.5−6.0 m diameter.

The fungal strain OS−F69284 (ATCC 74390) was grown on a slant prepared by adding 5.5 mL of a medium containing (per liter of deionized water) 18 g of agar, 10 g of malt, 50 mg of chloramphenicol, 50 mg of novobiocin, and 800 μL of a vitamin solution (biotin 5 mg, myoinositol 2 g, D-pantothenic acid 200 mg, pyridoxine 200 mg, thiamine 200 mg per liter of deionized water), into a sterile 16 × 100 mm borosilicate screw-capped tube. The medium was steam sterilized. After a growth period of 4−12 days, the fungus was stored at room temperature until use. A small piece of culture was transferred from the slant to a seed tube containing 10 mL of medium prepared using 20 g of soypeptone, 20 g of dextose, and 10 g of yeast extract in 1 L of deionized water and steam sterilized. This was incubated on a rotary shaker with a 1 in. throw for 7 days at 22 °C. The contents of the seed tube were transferred to a small homogenizer can and then homogenized at a low setting for 10 s using a Waring blender. The blended culture was added to a flask containing 75 mL of the same medium, and this was incubated on a rotary shaker with a 1 in. throw at 200 rpm and 22 °C. After another 7 days, the culture had grown very well, and 2 mL aliquots were used to inoculate Nunc bioassay dishes containing 500 mL of a medium prepared by adding 9 g of agar, 10 g of soy meal, and 2.5 g of mannitol to 500 mL of deionized water and steam sterilizing. By using a bent glass rod, the culture was spread over the entire surface of the plate. This was incubated at 22 °C in a cabinet incubator for 11 days, at which time the culture was well grown over the entire surface of the plate. This was placed in a freezer at −80 °C and then lyophilized.

Extraction and Isolation. The dried culture was extracted by adding 500 mL of methanol per 500 mL culture and soaking for 4 h. The organic solvent was filtered off and dried using a rotary evaporator. The dried methanol extract from a 6 L fermentation culture of OS-F69284 (~7 g) was partitioned into hexane, chloroform, and methanol/water. The chloroform fraction (1.2 g) was dried and subjected to separation by CPC. CPC was accomplished with a solvent system of hexane/EtOAc /MeOH/H_2O (7:7:6:8) in descending mode with the flow rate of 2 mL per min, at 1400 rpm and 22 °C. The elutes were collected in 10 min/per fraction, and the active fractions (f18−f32) were combined and dried in vacuo. The residues were dissolved in 0.5 mL MeOH, and the supernatant was submitted to further separation by HPLC after removing particulates by centrifugation. Gradient HPLC was conducted with an ODS-hypersil column and a gradient of CH_3CN/H_2O; solvent ratio was increased from 42:58 to 55:45 over 50 min at a flow rate of 1 mL/min. The active fractions (retention times of 22, 24, and 27 min) were collected. After solvents were evaporated, about 18 mg of **1** (t_R = 27 min; yield 0.26%, w/w from the crude extract), 3 mg of **2** (t_R = 24 min; yield 0.04%), and 9 mg of **3** (t_R = 22 min; yield 0.13%) were obtained as white powders. The same HPLC condition was applied for the kinetic study of OS-F69284 fermentation; the absorption coefficients at 254 nm (ϵ) of **1** and **3** were used for the quantitative analysis of **1** and **3** in the crude extracts of days 7−11.

Pycnidione (1): $[\alpha]^{23}_D$ +270 (c = 0.43, CH_2Cl_2); UV (MeOH) λ_{max} 255 nm (ϵ 67 000), 324 nm (sh), 365 nm (ϵ 24 000); ^1H and ^{13}C NMR ($CDCl_3$) see Tables 1 and 2; ESMS m/z 549 $[M + H]^+$; HRFABMS m/z 549.2812 (calcd for $C_{33}H_{41}O_7$ 549.2852).

Epolone A (2): $[\alpha]^{23}_D$ +230 (c = 0.35, MeOH); UV (MeOH) λ_{max} 255 nm (ϵ 65 000), 324 nm (sh), 365 nm (ϵ 17 000); ^1H and ^{13}C NMR (CD_3OD) see Tables 1 and 2; positive ESMS m/z 521 $[M + H]^+$; negative ESMS m/z 519 $[M-H]^-$.

Epolone B (3): $[\alpha]^{23}_D$ +85 (c = 0.33, CH_2Cl_2); UV (MeOH) λ_{max} 255 nm (ϵ 26 000), 324 nm (sh), 365 nm (ϵ 10 100); ^1H and ^{13}C NMR ($CDCl_3$) see Tables 1 and 2; ESMS m/z 385 $[MH]^+$; HRFABMS m/z 385.2336 (calcd for $C_{24}H_{33}O_4$ 385.2378).

Methylation of Compounds 1−3. To cold CH_2Cl_2 solutions of **1**−**3** (3−5 mg) was added CH_2N_2 freshly generated from Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, 0.2 g) in KOH/EtOH solution at 65 °C. After these solutions were stirred at 0 °C for 2 h, a piece of copper wire was added to quench the action of excess CH_2N_2. The solvents were evaporated under reduced pressure to yield the methylated derivatives. LC-ESMS analysis indicated that compound **1** produced four methylation derivatives with molecular weights of 576 (1M−a, 1M−b, 1M−c, and 1Md) and compunds **2** and **3** produced two methylated derivatives (2Ma, 2Mb, 3Ma, and 3Mb), respectively. The major derivatives (1Ma, 2Ma, 3Ma, and 3Mb) were isolated by silica gel chromatography, and their physicochemical data were determined.

Methylated derivative of compound 1 (1Ma): ^1H NMR ($CDCl_3$) δ 6.87 (1H, s, H-7), 6.84 (1H, s, H-26) 6.48 (2H, s, H-3, H-23), 5.36−5.32 (2H, m, H-17, H-16), 3.89 (3H, s, OCH_3), 3.88 (3H, s, OCH_3) 3.61 (1H, bd, J = 11 Hz, H-11), 3.01 (1H, dd, J = 17, 5 Hz, H-20), 2.67−2.59 (2H, m, H-15, H-8), 2.43 (1H, m, H-8′), 2.36 (3H, s, H-33), 2.29 (2H, m, H-15′, H-20′), 2.27 (3H, s, H-28), 2.12 (1H, m, H-20′), 1.85 (1H, m, H-13), 1.64−1.60 (2H, m, H-9, H-19), 1.26 (3H, s, H-29), 1.25 (3H, s, H-32), 1.09−1.07 (2H, m, H-19′, H-12′), 1.04 (3H, s, H-31), 1.03 (3H, s, H-30); ESMS m/z 577 $[M + H]^+$.

Methylated derivative of compound 2 (2Ma): ^1H NMR ($CDCl_3$) δ 7.01 (1H, s, H-7), 6.61 (1H, s, H-3), 5.86−5.82 (2H, s, H-23, H-25), 5.36−5.32 (2H, m, H-17, H-16), 3.87 (3H, s, OCH_3), 3.85 (3H, s, OCH_3) 3.65 (1H, bd, J = 11 Hz, H-11), 2.93 (1H, dd, J = 17, 5 Hz, H-20), 2.74 (1H, dd, J = 17, 5.5 Hz, H-8), 2.62 (1H, bdd, J = 15, 2 Hz, H-15), 2.51 (1H, m, H-8′), 2.41 (3H, s, H-27), 2.35 (1H, m, H-15′), 2.24(1H, m, H-20′), 2.15 (3H, s, H-32), 1.85 (1H, m, H-8, H-9), 1.78 (1H, m, H9), 1.70 (1H, m, H-12), 1.26 (3H, s, H-28), 1.23 (1H, m, H-19), 1.17 (3H, s, H-31), 1.14−1.12 (2H, m, H-19′, H-12′), 1.11 (3H, s, H-29), 1.05 (3H, s, H-30); ESMS m/z 549 $[M + H]^+$.

Methylated derivative of compound 3: 3Ma ^1H NMR ($CDCl_3$) δ 7.01 (1H, s, H-7), 6.52 (1H, s, H-3), 5.19 (1H, m, H-16), 5.14−5.08 (2H, m, H-12, H-13), 3.94 (1H, bd, J = 11 Hz, H-18), 3.87 (3H, s, OCH_3), 2.86 (1H, dd, J = 17, 5 Hz, H-8), 2.56 (1H, bd, J = 13 Hz, H-11), 2.39 (3H, s, H-20), 2.28−2.20 (3H, m, H-8′, H-11′, H-15), 1.78 (1H, dd, J = 17, 8 Hz, H-15′), 1.72 (1H, m, H-19), 1.64 (3H, s, H-21), 1.63 (1H, m, H-9), 1.12 (1H, m, H-19′), 1.08 (3H, s, H-24), 1.06 (3H, s, H-22), 1.02 (3H, s, H-23); ESMS m/z 399 $[M + H]^+$; **3Mb** ^1H NMR ($CDCl_3$) δ 6.89 (1H, s, H-3), 6.53 (1H, s, H-7), 5.19 (1H, m, H-16), 5.14−5.08 (2H, m, H-12, H-13), 3.94 (1H, bd, J = 11 Hz, H-18), 3.87 (3H, s, OCH_3), 2.86 (1H, dd, J = 17, 5 Hz, H-8), 2.56 (1H, bd, J = 13 Hz, H-11), 2.32 (3H, s, H-20), 2.28−2.20 (3H, m, H8′, H11′, H-15), 1.78 (1H, dd, H=17, 8 Hz, H-15′), 1.72 (1H, m, H-19), 1.64 (3H, s, H-21), 1.63 (1H, m, H-9), 1.12 (1H, m, H-19′), 1.08 (3H, s, H-24), 1.06 (3H, s, H-22), 1.02 (3H, s, H-23); ESMS m/z 399 $[M + H]^+$.

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References and Notes

(1) Krantz, S. B. *Blood* **1991**, *77*, 419−433.
(2) Heguy, A.; Stewart, A. A.; Haley, J. D.; Smith, D. E.; Foulkes, J. G. *Gene Expression* **1995**, *4*, 337−344.
(3) Cai, P.; Smith, D.; Katz, B.; Pearce, C.; Venables, D.; Houck, D. *J. Nat. Prod.* **1998**, *61*, 290−293.
(4) Harris, G. H.; Hoogsteen, K.; Silverman, K. C.; Raghoobar, S. L.; Bills, G. F.; Lingham, B.; Smith, J. L.; Dougherty, H. W.; Cascales, C.; Pelaez, F. *Tetrahedron* **1993**, *49*, 2139−2144.
(5) Sugawara, K.; Ohbayashi, M.; Hatori, M.; Konishi, M.; Oki, T.; Kawaguchi, H. *J. Antibiotic.* **1988**, *41*, 862−868.
(6) Mayerl, F.; Gao, Q.; Huang, S.; Klohr, S. *J. Antibiot.* **1993**, *46*, 1082−1088.
(7) Weegen, H.; Nkunya, M. H. H.; Adul El-Fafl, A.; Harkema, S.; Zwanenburg, B. *J. Org. Chem.* **1990**, *55*, 5107−5109.
(8) Breitmaier, E.; Voelter, W. *Carbon-13 NMR Spectroscopy, High-Resolution Methods and Applications in Organic Chemistry and Biochemistry*; VCH: New York, 1987; pp 115−116.

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