8-Methyl-Pyridoxatin: A Novel *N*-Hydroxy Pyridone from Fungus OS-F61800 That Induces Erythropoietin in Human Cells

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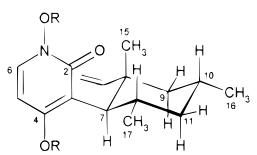
In the course of screening for small-molecule modulators of erythropoietin gene expression, a novel *N*-hydroxy pyridone was isolated from a culture of OS-F61800. Its structure was elucidated by extensive ^{1}H and ^{13}C NMR spectroscopic and chemical studies. This compound induced erythropoietin gene expression fivefold at a concentration of 0.3 μM, which is about threefold greater potency than our previously identified erythropoietin inducers.

As described in previous communications,[1,2] we have screened for inducers of erythropoietin (EPO) gene expression using luciferase-reporter technology.[3,4] Continued screening resulted in the isolation of a novel pyridone (**1**) from the fermentation extract of fungus OS-F61800, a sterile-hyaline culture, collected from Six Mile Cypress Slough in Florida. In this note, we report the isolation and structure elucidation of this cyclic hydroxamic acid. In addition, we provide data on the ability of **1** and related compounds to induce human EPO in cell-based assays.

The dried methanol extract from a 3-L fermentation culture of OS-F61800 was partitioned into hexane, chloroform, and methanol−water. The $CHCl_3$ fraction was subjected to isolation by LH-20 flash chromatography, followed by reversed-phase HPLC to yield **1** as a white powder. Elemental analysis established a molecular formula of $C_{16}H_{23}NO_3$, which was confirmed by HRFABMS. The IR spectrum of **1** had a carbonyl absorption at 1625 cm^{-1}. The presence of a pyridone ring was supported by both ^{1}H and ^{13}C NMR data. In the ^{1}H NMR spectrum, **1** displayed a pair of coupled doublets at δ 7.53 (d, J = 8.5, H-6) and 5.91 (d, J = 8.5, H-5), which correlated to the carbons at δ 132.7 and 98.8, respectively, in the HMQC spectrum. The proton at δ 5.91 displayed two- and three-bond correlations to the quaternary carbons at δ 164.6 (C-4) and 113.4 (C-3), respectively, in the HMBC spectrum; the proton resonance at δ 7.53 had three-bond correlations to carbons corresponding to δ 164.6 (C-4) and 161.1 (C-2), suggesting a 3-alkyl-4-hydroxy-2-pyridone substructure. In addition to the pyridone protons, the DQCOSY spectrum of **1** also demonstrated the presence of two isolated proton-spin systems. One of these was a monosubstituted double bond, in which an olefinic doublet of doublets (δ 4.87, dd, J = 17, 10 Hz, H-13) coupled to two olefinic protons at δ 4.69 (dd, J = 17, 1.5 Hz, H-14$_a$) and 4.67 (dd, J = 10, 1.5 Hz, H-14$_b$), respectively. The second proton-spin system consisted of four methylene protons, three methine protons, and two groups of methyl protons; the most downfield methine-proton doublet at δ 2.61 (H-7) coupled to the methine-proton multiplet at δ 2.89 (H-12), which in turn coupled to a methyl-proton doublet at δ 0.63 (H-17) and one methylene proton at δ 0.66 (H-11′). This methylene proton was also coupled to its geminal partner at δ 1.75 (H-11) and to the most upfield methine proton at δ 1.78 (H-10), which in turn coupled to the methyl doublets at δ 0.88 (H-16) and a pair of methylene protons at δ 1.12 (H-9′) and 1.32 (H-9), respectively. A cyclohexane substructure was suggested by those correlations and by the observed HMBC correlations of a quaternary carbon at δ 45.3 (C-8) with both H-7 and H-9. The monosubstituted double bond and a methyl group were attached to a common quaternary carbon, as indicated by HMBC correlations of C-8 (δ 45.3) to the double-bond proton at δ 4.68 (H-14) and the methyl-proton singlet at δ 1.07 (H-15). Clear HMBC correlations of the methine proton at δ 2.61 (H-7) with the carbons at δ 113.4 (C-3), 164.6 (C-4), and 161.1 (C=O, C-2) revealed the connection of the cyclohexane ring to the pyridone C-3 position.

The complete ^{1}H and ^{13}C assignments agreed with a molecular formula of $C_{16}H_{22}NO_2$, one oxygen less than that obtained from elemental analysis and HRMS. Because ^{1}H and ^{13}C chemical shift analyses eliminated the possibility of another carbon-bonded oxygen, we expected that the oxygen must be attached to the pyridone nitrogen, and this hypothesis was supported by the results of diazomethane methylation. Treatment of **1** with freshly prepared CH_2N_2 produced two methylated derivatives (**2**, **3**). Both compounds had a molecular ion of m/z 305 in the positive ESMS spectrum, suggesting that methylation occurred at both the C-hydroxyl and the N-hydroxyl groups. The UV absorption spectra of the two products were characteristic of the structures; the major product (**2**) had an absorption spectrum similar to **1** (λ_{max} 290 nm), whereas the maximal UV absorption of the minor product shifted to 272 nm, indicative of the tautomer, a 4-pyridone (**3**).



1 R = H

2 R = CH_3

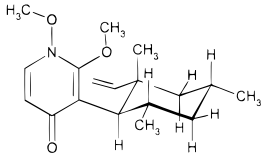


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Table 1. ^{13}C and ^{1}H NMR Data (δ) of Compound **1** in CD_3OD

assignment	δ, ^{13}C (mult.)	δ, ^{1}H (mult., J_{H-H} Hz)	HMBC δ, $^{1}H/^{13}C$
2	161.1 (s)		
3	113.4 (s)		
4	164.6 (s)		
5	98.8 (d)	5.91 (d, 9.0)	5.91/164.6, 132.8, 113.4
6	132.7 (d)	7.53 (d, 9.0)	7.53/164.6, 161.1, 98.8
7	50.6 (d)	2.61 (d,11.5)	2.61/164, 161, 151, 113, 45, 29, 20
8	45.3 (s)		
9	50.9 (t)	1.32 (dd, 12.0, 5.0)	1.32/50.6, 46.7
		1.12 (dd, 12.0, 11.0)	
10	29.1 (d)	1.78 (m)	1.78/50.6,29.1
11	46.7 (t)	1.75 (ddd, 13, 4.0, 2.0)	1.75/50.6, 29.1
		0.66 (ddd, 13, 12, 12)	
12	29.4 (d)	2.89 (m)	2.89/46.7, 21.8
13	151.5 (d)	5.87 (d, 10.0)	
14	109.3 (t)	4.67 (dd, 17.5, 1.5)	4.67/151.5, 45.2
		4.69 (dd, 10.5, 1.5)	
15	20.1 (q)	1.07 (s)	1.07/151.5, 50.8, 45.2
16	23.4 (q)	0.88 (d, 6.5)	0.88/50.9, 46.7, 29.1
17	21.8 (q)	0.63 (d, 6.5)	0.63/50.6, 46.7, 29.4

Table 2. Induction of Human EPO in Hep3B (epo-3) Cells by Compound **1** and Its Analoges

	epo-luci reporter[a,b]		ELISA epo assay[c]	cytotoxicity (48 h)[d]
compound	TIR5x (μM)	TIRmax (μM)	mU/mL protein (μM)	MTT IC_{90}, μM
1	0.3	46 (0.7)	120 (10)	> 20
2	IA	IA		
Ciclopiroxolamine	3.0	31 (15)	99 (10)	> 20

[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). IA = inactive. All compounds were tested in triplicate in at least three experiments at 0.1 to 30 μM. [b] TIR_{5x} is the concentration of compound required to produce a fivefold induction in epo-3 cells over control (0.5% DMSO). Potency values are ±15%. Minimum concentration is shown that generated maximum-fold induction (TIR_{max}) for each compound. [c] ELISA assay was conducted at a single concentration of 10 μM. 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.

The relative stereochemistry of C-7, C-8, C-10, and C-12 was determined based on proton coupling constants and the NOEs observed between the methine and methyl protons. First, the large coupling constants of H-7 with H-12 (J = 11.5 Hz) and H-10 with H-9′ and H-11′ (J = ca. 11 Hz) required all these protons to be in the axial orientation. Second, the NOEs between the H-7 and the methyl at δ 0.63 (H-17) and between H-17 and the methyl at δ 0.88 (H-16) suggested that these protons occupied the same face of the ring. However, no NOE was detected from H-7 to the methyl proton singlet (H-15). Based on the spectral data, the structure **1** was proposed. Results of a literature search indicated that **1** had a structure similar to the pyridoxatin, previously isolated from *Acremonium* sp.[5,6] Thus, we determined **1** to be 8-methyl-pyridoxatin.

In several independent experiments, compound **1** reproducibly induced EPO gene expression in Hep3B cells (epo-3 cell line). This was demonstrated using both a luciferase-reporter assay and a protein-secretion assay, which were described in two previous communications.[1,2] The TIR_{5X} (concentration resulting in fivefold induction) of **1** was 0.3 μM, and the maximum induction activity of these compounds reached as high as 40−50-fold (Table 2). However, the methylated derivatives (**2** and **3**) were completely devoid of activity in the same assay. We also tested several commercially available pyridones in the EPO-luciferase assays; only ciclopirox olamine[7,8] had weak induction activity. Pyridone compounds without the *N*-hydroxyl group had no detectable activity (Table 2). This result suggested that a free *N*-hydroxyl directly adjacent to the pyridone carbonyl group was requisite for EPO-induction activity. To confirm that the apparent induction of EPO by **1** and ciclopirox olamine 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 compounds **1** and ciclopirox olamine induced native-protein secretion by approximately 100-fold above the vehicle control in a 24-hour assay. When incubated with 10 μM of compound **1** or ciclopirox olamine, the epo-3 cells produced about 100−120 mU of EPO/mL of culture; in the absence of these compounds, the EPO concentration was below the level of detection (Table 2). In both luciferase-reporter and secretion assays, the positive control, 10 μM $CoCl_2$, produced a 10−40-fold induction. In standard MTT assays, compound **1** did not demonstrate cytotoxicity at efficacious concentrations.

Experimental Section

General Experimental Procedures. UV spectra were measured on a Perkin−Elmer Lambda Bio UV/vis spectrometer. ^{1}H 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. HPLC was conducted on an Hewlett−Packard 1090 liquid chromatograph equipped with a diode-array detector. Ciclopirox olamine was purchased from Aldrich Chemical Co.

Biological Assays. A luciferase-reporter assay in Hep3B cells was used for bioassay-guided fractionation. The ability of compounds to induce epo-protein expression was determined using a commercial ELISA kit. The cell lines (epo-3 cells), protocols, and reagents for both assays have been previously described.[1,2]

Organism, OS-F61800. The fungus, a sterile-hyaline culture, was isolated on May 5, 1992, from twigs collected at Six Mile Cypress Slough, Ft. Myers, FL, a hydric-hammock-mixed-swamp forest. Colony growth rate was moderate on malt-extract agar, with mycelium consisting of approximately 2-μm diameter septate hyphae; superficial, thin, black stromata of various sizes were apparent. The fungus is deposited in the corporate culture collection.

Maintenance and Fermentation of OS-F61800. The fungal strain OS-F61800 was grown on a slant prepared by adding 5.5 mL of a medium containing (per liter of deionized H_2O) 18 g agar, 10 g malt, 50 mg chloramphenicol, 50 mg novobiocin, and 800 μL of a vitamin solution (biotin 5 mg, myoinositol 2 g, D-pantothenic acid 200 mg, pyridoxone 200 mg, thiamine 200 mg/L of deionized H_2O), into a sterile 16 × 100 mm borosilicate screw-capped tube. The medium was steam sterilized. After a growth period of 4 to 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 soypeptone, 20 g dextose, and 10 g yeast extract in 1 L of deionized H_2O, and steam sterilized. This was incubated on a rotary shaker with a one- inch throw for 7 days at 22 °C. The contents of each seed tube were transferred to a 250-mL Erlenmeyer 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 had clumped into small balls (<5 mm diameter). Approximately 5-mL aliquots of carefully blended culture were used to inoculate 500 mL of medium prepared by adding 10 g soy meal and 2.5 g of mannitol to 500 mL deionized H_2O in a 2.5-L Fernbach flask and steam sterilizing. The flasks were incubated at 22° C in a cabinet incubator without shaking for 11 days, at which time the culture was well grown. The cultures were then frozen at −80 °C and lyophilized.

Extraction and Isolation. The dried culture was extracted by soaking the lyophilized material for 12 to 16 h in 500 mL MeOH (per 500 mL culture). The organic solvent was filtered and dried using a rotary evaporator. The dried MeOH extract from a 3-L fermentation culture of OS-F61800 (ca. 3.9 g) was partitioned into hexane, $CHCl_3$, and MeOH−H_2O. The $CHCl_3$ fraction (360 mg) was dried and subjected to separation by a LH-20 flash column and eluted with CH_2Cl_2−CH_3OH (8:2). All fractions were collected at 8 mL/tube; tubes 6−16 were combined, dried, and then submitted to further separation by HPLC. Gradient HPLC was conducted with a Zorbax C-8 semipreparative column (1 × 30 cm) using a gradient of CH_3CN−H_2O; solvent ratio was increased from 30:70 to 80:20 over 50 min at the flow rate of 4 mL/min. After the active fractions (retention time of 22 min) were collected, and the solvents were evaporated, about 15 mg of pure **1** (t_R = 22 min; yield: 0.43%, w/w from the crude extract) was obtained as a white powder.

Compound 1: $[\alpha]^{23}_D$ −9.4° (*c* 0.43, CH_3OH); UV (MeOH) λ_{max} (ϵ) 289 (6850), 216 (34 000) nm; IR (KBr) ν_{max} 3400−3350, 1625 cm^{-1}; ^1H (CD_3OD, 500 MHz) and ^{13}C (CD_3OD, 125 MHz) NMR, see Table 1; APCI$^+$−MS *m*/*z* 278 $[M + H]^+$; APCI$^-$−MS *m*/*z* 276 $[M - H]^-$; HRFABMS *m*/*z* 278.1731 $([M + H]^+$ (calcd for $C_{16}H_{24}NO_3$ 278.1754); *anal.* C 67.88%, H 7.95%, N 4.81%, calcd for $C_{16}H_{23}NO_3 \cdot 1/2H_2O$, C 67.28%, H 8.30%, N 4.91.

Compounds 2 and 3. To a cold CH_2Cl_2 solution of **1** (4 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 two methylated derivatives (**2** and **3**) indicated by LC−ESMS. The mixture was separated by HPLC on the Zorbax C-8 analytical column (4.6 × 150 mm) and eluted with a gradient of CH_3CN−H_2O (35:65 to 60:40 in 30 min, at 1 mL/min). These compounds were characterized by UV absorption and mass spectroscopic analyses; NMR experiments were not accomplished due to lack of sufficient quantities. Compound **2**: HPLC, t_R = 25.4 min; UV (MeOH) λ_{max} 216, 290 nm; ESMS *m*/*z* 306 $[M + H]^+$. Compound **3**: HPLC, t_R = 21.3 min; UV (MeOH) λ_{max} 210, 272 nm; ESMS *m*/*z* 306 $[M + H]^+$.

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