Destruxin-A4 Chlorohydrin, a Novel Destruxin from Fungus OS-F68576: Isolation, Structure Determination, and Biological Activity as an Inducer of Erythropoietin

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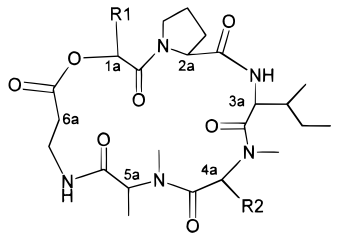
Received October 22, 1997

In the course of screening for small-molecule modulators of erythropoietin gene expression, five destruxins were isolated from the fungal culture of OS-F68576. The structures were elucidated by extensive ^{1}H and ^{13}C NMR spectroscopy and by hydrolytic modification. One compound (destruxin-A4 chlorohydrin, **1**) is a novel destruxin. All these compounds induced erythropoietin gene expression 5-fold at concentration of 0.2−2 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 the EPO receptor on committed progenitor cell 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 endogenous EPO gene and, thereby, increase the levels of circulating of erythropoietin.[2]

To screen for inducers of EPO gene expression, we constructed a luciferase reporter plasmid containing 6-kb 5′-flanking sequence and 300 bp of proximal 3′-sequence of the human EPO gene; this sequence contains the hypoxia-inducible enhancer element.[1] This plasmid was electroporated into Hep3B cells, and the resulting tester-cell clone was designated epo-3. In the epo-3 cells, 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 fungal extracts and discovered the extract obtained from the fermentation of OS-F68576 showed consistent EPO-induction activity. Bioassay-directed fractionation of extract OS-F68576 uncovered five destruxins (**1**−**5**).

Compound **1** is an amorphous solid and has a maximum UV absorption at 208 nm. The high-resolution FAB mass spectrum gave a molecular ion at *m*/*z* of 644.3426 ([M + H$^+$]) and a isotopic peak at *m*/*z* of 646



1. R1=-$CH_2CH(OH)CH_2Cl$ R2=-$CH(CH_3)CH_2CH_3$
2. R1=-$CH_2CH{=}CH_2$ R2=-$CH(CH_3)_2$
3. R1=-$CH_2CH{=}CH_2$ R2=-$CH(CH_3)CH_2CH_3$
4. R1=-$CH_2CH(CH_3)_2$ R2=-$CH(CH_3)_2$
5. R1=-$CH_2CH(CH_3)_2$ R2=-$CH(CH_3)CH_2CH_3$

with a ratio of 3:1, suggesting a molecular formula of $C_{30}H_{50}N_5O_8Cl$. The presence of a chlorine in **1** was confirmed by NMR data. The ^{13}C NMR spectrum of **1** displayed a total of 30 carbon signals, which agreed with the molecular formula obtained from HRMS. These carbon resonances were classified as seven methyl carbons, nine methylene carbons, eight methine carbons, and six carbonyl carbons by DEPT experiments. The presence of six amide (or ester) carbonyl resonances in the range of 169−175 ppm and the characteristic signals of two NCH_3 protons and two NH protons of destruxins in the ^{1}H NMR suggested that compound **1** was a member of the destruxin family.[3]

The structure of **1** was further elucidated on the basis of 2D-NMR data. In the ^{1}H−^{1}H COSY spectrum, a proton triplet at δ 5.09, which correlated to the methine carbon at δ 70.4 in the HMQC spectrum, showed correlations to one pair of methylene protons at δ 2.13−2.07; these two protons in turn coupled with a methine proton at δ 4.06, which continued the coupling network to another pair of methylene protons at δ 3.53 and 3.62. This spin system suggested a −CH(OR)CH_2CH(OR′)-CH_2X substructure for the α-hydroxy acid moiety, in which X was a chlorine, as suggested by the methylene carbon chemical shift (−CH_2X: δ 49.8). The presence of the β-alanine (residue 6) was indicated by the proton spin system starting with the NH proton resonance at δ 8.16. This broad NH proton doublet had a strong correlation to a pair of methylene protons at δ 4.04 and

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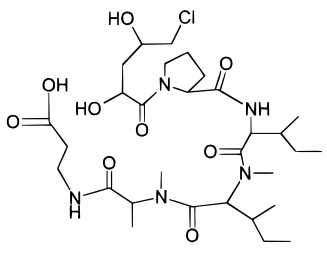
S0163-3864(97)00475-8 CCC: $15.00
Published on Web 02/10/1998

Table 1. ^{13}C and ^{1}H NMR Spectral Data for Compound **1**

position	^{13}C NMR δ (ppm) (multiplicity)	^{1}H NMR δ (ppm) (multiplicity)	important $^{1}H/^{13}C$ long-range correlation from HMBC
1α	70.4 (d)	5.09 (dd, 5, 6)	5.09/173.3, 169.5, 66.9, 34.9
1β	34.9 (t)	2.13 (ddd, 11, 7, 4), 2.07 (m)	2.13/169.5, 70.4, 66.9, 49.8
1γ	66.9 (d)	4.06 (m)	
1δ	49.8 (t)	3.62 (dd, 11, 4), 3.53 (dd, 11, 7)	
1 C=O	169.5 (s)		
2α	61.0 (d)	4.65 (br d, 7)	4.65/170.8, 46.7, 29.2, 24.0
2β	29.2 (t)	2.45 (bdd, 11, 6), 1.95 (m)	2.45/170.8, 46.7, 24.0
2γ	24.0 (t)	2.04 (m), 1.93 (m)	
2δ	46.7 (t)	3.98 (br t 8), 3.65 (m)	
2 C=O	170.8 (s)		
3α	53.7 (d)	4.85 (dd, 9, 6)	4.85/173.6, 170.8, 37.4, 24.4, 15
3β	37.4 (d)	1.92 (m)	
3γ	24.4 (t)	1.45 (m), 1.20 (m)	
3γ′	15.4 (q)	0.84 (d, 7)	
3δ	11.4 (q)	0.82 (t, 7)	
3 C=O	173.6 (s)		
NH		7.09 (d, 8.5)	7.09/173.6, 170.8
4α	56.8 (d)	5.00 (d, 11)	
4β	33.6 (d)	2.02 (m)	
4γ	25.8 (t)	1.40 (m), 0.99 (m)	1.40/56.8, 33.6, 16.2, 11.1
4γ′	16.2 (q)	0.89 (d, 7)	0.89/33.6, 25.8
4δ	11.1 (q)	0.85 (t, 7)	0.86/25.8
4 C=O	171.0 (s)		
N−CH_3	31.0 (q)	3.18 (s)	3.18/173.6
5α	55.5 (d)	5.14 (q, 7)	5.14/169.7, 171.0, 28.1, 15.1
5β	15.2 (q)	1.28 (d, 7)	1.28/169.7, 55.5
5 C=O	169.7 (s)		
N−CH_3	28.1 (q)	2.76 (s)	2.76/171.0
6α	33.2 (t)	2.64 (ddd, 18, 10, 2), 2.54 (dd, 18, 4)	2.64/173.3, 33.2
6β	34.5 (t)	4.04 (m), 3.05 (br d 13)	
6 C=O	173.4 (s)		
NH		8.16 (d, 9)	8.16/169.7, 33.2

3.05 (β-protons); those in turn coupled to another pair of methylene protons at δ 2.64 and 2.54 (α-protons). The remaining amino residues were identified to be proline (residue 2), isoleucine (residue 3), *N*-methylisoleucine (residue 4), and *N*-methylalanine (residue 5), respectively, on the basis of the $^{1}H-^{1}H$ correlations between the α-proton and the β-protons in each amino acid residue (proline, δ 4.65/2.45, 1.93; isoleucine, δ 4.85/1.92; *N*-methylisoleucine, δ 5.00/2.02; *N*-methylalanine, δ 5.14/1.28). These assignments were confirmed by the long-range $^{1}H-^{13}C$ correlations of the α-proton with both β and γ-carbons and the correlations of the NCH_3 protons with the α-carbon in each residue (Table 1).

The linkage sequence of the amino acid residues was deduced from the long-range correlations (HMBC) of the α-proton, NH proton, and/or NCH_3 protons of a given amino residue with the peptidic carbonyl carbon of the adjacent amino acid residue. The HMBC data were supported by mass spectroscopy of the base hydrolysis product. Treating compound **1** with 2 N NaOH produced an acyclicpeptide (**6**), which gave a molecular ion of *m*/*z* 662 and fragment ions of *m*/*z* 573, 488, and 361 by sequential loss of the β-alanine, *N*-methylalanine, and *N*-methylisoleucine residues from the carboxylic acid terminal in positive atmospheric pressure chemical ionization mass spectrum (APCI). On the basis of these spectroscopic data, the structure **1** is proposed, which is a new depsipeptide and named as destruxin-A4 chlorohydrin according to the suggestion of Pais et al.[4] In compound **1**, the halogen is very possibly a product of nucleophilic opening of the corresponding expoxide.[5] However, it is not an artifact of the isolation procedure, because extracts yield equivalent quantities of **1** with or without the use of chlorinated solvents in the isolation scheme. It is likely that the production medium contains some chloride ion, although chloride salts are not deliberately added to the media.



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Compounds **2**, **3**, **4**, and **5** were identified as destruxin A (**2**),[4] destruxin A4 (**3**),[6] destruxin B (**4**),[4] and homodestruxin B (**5**),[7] respectively, on the basis of the complete agreement of their spectral data with those reported in the literature.

In three independent experiments, all five destruxins isolated from OS-F68576 reproducibly induced erythropoietin gene expression. The transcription induction ratio (TIR) was determined from the luciferase signal obtained from the tested sample versus the luciferase signal obtained from solvent control (Table 2). The TIR_{5X} (concentration resulting in 5-fold induction) of these compounds is in the range of 0.2−2.0 μM; destruxin-A4 chlorohydrin (**1**) was the most potent compound. However, after converting **1** to the corresponding linear opened peptide (**6**) by hydrolysis, the EPO-induction

Table 2. Induction of Human EPO in Epo-3 Cells by Compounds **1**−**6**

compd	TIR_{max}[a] (μM)	TIR_{5X}[b] (μM)	EPO secretion[c] (mU/mL)
1	28 (1.3)	0.2	36
2	28 (2.1)	0.4	37
3	23 (5.0)	0.9	35
4	19 (10)	2.0	35
5	18 (5.0)	0.8	36
6	1.0 (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). [b] TIR_{5X} is the concentration of compound required to produce a 5-fold induction over control (0.5% DMSO). [c] EPO protein secreted in cell control; there was no significant production of epo when cells were incubated with vehicle.

activity was lost completely. To confirm that the apparent induction of erythropoietin by compounds **1**−**5** was not an artifact of our 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 all these destruxins induced secretion by approximately 35−40-fold above DMSO vehicle control in a 24-h assay. When incubated with 10 μM of these depsipeptides, the epo-3 cells produced 30−40 mU of EPO per milliliter of culture; in the absence of the destruxins, the EPO concentration was below the level of detection (Table 2). In the same period of time, $CoCl_2$ could also induce EPO protein; at a concentration of 20 mM, $CoCl_2$ induced the cells to produce 40−60 mU/mL. The cytotoxicity of these destruxins was determined on the same cell line by standard MTT assay; none of these destruxins showed toxicity at the concentrations of ≤20 μM.

Experimental Section

General Experimental Procedures. The ^1H and ^{13}C NMR spectra were recorded using a Varian-500 operating at 500 and 125 MHz, respectively. Chemical shifts are reported in ppm relative to solvent ($CDCl_3$ δ_H 7.24, δ_C 77.0). Mass spectrometric analysis was performed with VG Platform-II (ESP and APCI) and Finnegan MAT-90 (FAB-HRMS) mass spectrometers. IR spectra were determined using a Perkin-Elmer 600. HPLC separations were performed on a Hewlett-Packard 1090 liquid chromatograph with a photodiode-array detector.

EPO-Induction Assays. The luciferase-reporter assay was carried out with the epo-3 line after growth of the cell in DMEM medium for 3 days. The cells were deposited into 96-well plates at 10 000 cells per well in DMEM medium. Experimental compounds were dissolved in DMSO and then incubated with epo-3 cell for 24 h (the final DMSO concentration was 0.5%). For the measurement of secreted EPO the tester cell line was plated in DMEM at 200 000 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.[1]

Culture Description and Storage. The producing fungus OS-F68576 (ATCC 20157), a hyaline *Mycelium sterilium*, was isolated at MYCOsearch from leaf litter collected from a deciduous humid montane forest near Porulha, Guatemala; this culture has been deposited with ATCC. Colonies grown on malt agar were tan and tomentose. The fungus was grown on an agar slant prepared by adding 5.5 mL of a medium containing malt 1.0%, agar 1.8%, chloramphenicol 0.005%, novobiocin 0.005%, and 800 μL of a vitamin solution composed of biotin 0.0005%, *myo*-inositol 0.2%, D-pantothenic acid 0.02%, pyridoxine 0.02%, and thiamine 0.02% into a sterile 16 × 100 mm borosilicate screw capped tube. This medium and all culture media used in this study were steam sterilized. The fungus was stored at room temperature until use.

Fermentation. A small piece of the slant culture was transferred to a 50 mL seed tube containing 10 mL of sterile medium comprising soypeptone 2%, dextrose 2%, and yeast extract 1%. This seed culture was incubated on a rotary shaker (1 in. throw and 220 rpm) at 22 °C for 7 days, 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 250 mL Erlenmeyer flask containing 75 mL of the same medium and incubated on a rotary shaker for another 7 days. Two milliliter aliquots of the culture were used to inoculate Nunc plates (20 × 20 cm) holding 500 mL of a medium containing 1.8% agar, 1.0% yeast extracts, 2% dextrose, and 2% casein in deionized water. The culture was spread over the entire surface of the plate using a bent glass rod and then incubated at 22 °C in a cabinet incubator for 11 days, at which time the mycelium was well grown over the entire surface of the plate.

Extraction and Isolation. The cultures were frozen at −80 °C and lyophilized. The dried culture was extracted by adding 500 mL of MeOH and soaking for 4 h. The organic solvent was filtered off and dried using a rotary evaporator. The dried MeOH extracts (58 g) from a 3-L fermentation of OS-F68576 was partitioned into hexane, $CHCl_3$, and aqueous MeOH by Kupchan fractionation. The $CHCl_3$ fraction (3.3 g) was then separated into three parts on a C-18 reversed-phase flash column eluted sequentially with water, 30% aqueous MeOH, and pure MeOH. After the solvents were evaporated, 900 mg of active material was obtained from the MeOH elution. A 200 mg portion of the active mixture was dissolved into 1 mL of MeOH, clarified by centrifugation, and subjected to HPLC separation. HPLC was accomplished with a Zorbax C8 column (9.4 × 250 mm) and a gradient of acetonitrile/H_2O; the solvent ratio was increased linearly from 25/75 to 80/20 over 40 min at a flow rate of 2.5 mL/min. After the solvents were evaporated *in vacuo*, five pure active compounds were obtained as white powders (compound **1**, 3 mg; compound **2**, 2 mg; compound **3**, 3 mg; compound **4**, 6 mg; and compound **5**, 5 mg).

Destruxin-A4 chlorohydrin (1): white solid; HPLC t_R = 28 min; UV (MeOH) λ_{max} 208 nm; IR ($CHCl_3$) ν_{max} 3440, 3330, 2960, 1730, 1672, 1632 cm^{-1}; ^1H NMR and ^{13}C NMR, see Table 1; ESMS *m/z* 644 (100), 646 (33); HRFEBMS *m/z* 644.3450 $[M + H]^+$, calcd for $C_{30}H_{51}N_5O_8$-Cl 644.2086.

Destruxin A (2): white solid; HPLC $t_R = 30$ min; ^{1}H NMR and ^{13}C NMR same as that reported in ref 4; HRFEBMS *m/z* 578.3496 $[M + H]^+$, calcd for $C_{29}H_{48}N_5O_7$ 578.7296.

Destruxin A4 (3): white solid; HPLC $t_R = 32$ min; ^{1}H NMR and ^{13}C NMR as that reported in ref 6; HRFEBMS *m/z* 592.3646 $[M + H]^+$, calcd for $C_{30}H_{50}N_5O_7$ 592.7565.

Destruxin B (4): white solid; HPLC $t_R = 34$ min; ^{1}H NMR and ^{13}C NMR as that reported in ref 4; HRFEBMS *m/z* 594.3791 $[M + H]^+$, calcd for $C_{30}H_{52}N_5O_7$ 594.7724.

Homodestruxin B (5): white solid; HPLC $t_R = 37$ min; ^{1}H NMR and ^{13}C NMR as that reported in ref 7; HRFEBMS *m/z* 608.3984 $[M + H]^+$, calcd for $C_{31}H_{54}N_5O_7$ 608.7993.

Ring Opening of 1. To a methanol solution of compound **1** (2.0 mg in 0.5 mL) was added 2 M NaOH (0.1 mL). The mixture was stirred at room temperature and the reaction was monitored by HPLC. After 14 h, the cyclopeptide was opened completely. The reaction mixture was neutralized with 2 M HCl and extracted with CH_2Cl_2, which afforded 1.5 mg of the pure acyclicpeptide (**6**): APCI-MS *m/z* 662 $[M + H]^+$, 573, 488, 361.

Acknowledgment. We thank Professor Chris Ireland for his helpful and enthusiastic consultations.

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NP970475C