# Oteromycin: A Novel Antagonist of Endothelin Receptor

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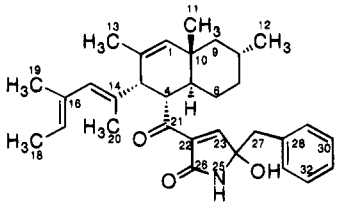
The endothelins (ET-1, ET-2, and ET-3) are a family of 21 amino acid peptides which contain two disulfide bonds between four cysteine residues.[1] Two unique receptor subtypes ($ET_A$ and $ET_B$) of ET have been identified and cloned from mammals.[2] The $ET_A$ receptor subtype mediates vasoconstriction[2] while the $ET_B$ receptor subtype has been implicated in both vasoconstriction and vasodilation.[3] Elevated levels of ET-1 have been found in a number of disease states, including cardiovascular,[4] respiratory,[5] gastric,[6] renal,[4,7] and urological[8] afflictions. Antagonists of the ET receptor have the potential to be safe therapeutic agents against the diseases just mentioned. Therefore, there has been an intense effort to discover novel antagonists to the known receptor subtypes of ET-1, one of the most potent endogenous vasoconstrictors known.[9]

From the screening of microbial extracts, we have isolated oteromycin (**1**), a novel antagonist of the $ET_B$ receptor, from two different strains of an unidentified fungus.[10] Oteromycin is characterized by a bicyclic lipophilic part connected to a modified tetramic acid (hydrophilic) moiety derived from phenylalanine. Oteromycin is an antagonist of the $ET_B$ receptor[11] ($IC_{50}$ 2.5 $\mu$M); it had very little effect in an angiotensin II receptor binding assay ($IC_{50}$ > 25 $\mu$M). In the present study we describe the bioassay-guided isolation, structure elucidation, and biology of oteromycin (**1**).

**Table 1. NMR Assignments 500 MHz of Oteromycin in $CD_2Cl_2$ Solutions**

| position | δC | type | δH | HMBC |
|---|---|---|---|---|
| 1 | 136.98 | CH | 5.40, brs | H-3, 11, 9α, 9β, 13 |
| 2 | 135.81 | $C^0$ | | H-3, 4, 13 |
| 3 | 51.37 | CH | 3.10, brd, $J$ = 8 Hz | H-1, 4, 13, 20 |
| 4 | 50.24 | CH | 3.72, dd, $J$ = 12, 8 Hz | H-3 |
| 5 | 40.29 | CH | 1.76, dt, $J$ = 11, 2.5 Hz | H-1, 3, 4, 7, 11 |
| 6 | 24.70 | $CH_2$ | 1.64, m, 0.96, m | H-7 |
| 7 | 36.10 | $CH_2$ | 1.73, m, 0.98, m | H-12 |
| 8 | 27.79 | CH | 1.70, m | H-6, 7, 12 |
| 9 | 48.77 | $CH_2$ | 1.50, m, 0.98, m | H-7, 11, 12 |
| 10 | 35.35 | $C^0$ | | H-1, 4, 6, 9, 11 |
| 11 | 20.63 | $CH_3$ | 0.90, s | H-9 |
| 12 | 22.92 | $CH_3$ | 0.86, d, $J$ = 7 Hz | H-7, 9 |
| 13 | 22.04 | $CH_3$ | 1.46, m | H-1, 3 |
| 14 | 130.00 | $C^0$ | | H-3, 20 |
| 15 | 133.81 | CH | 5.46, brs | H-3, 17, 19, 20 |
| 16 | 134.13 | $C^0$ | | H-18, 19 |
| 17 | 124.67 | CH | 5.20, q, $J$ = 6.5 Hz | H-15, 18,19 |
| 18 | 13.62 | $CH_3$ | 1.62, brd, $J$ = 8 Hz | H-17 |
| 19 | 16.56 | $CH_3$ | 1.61, s | H-15, 17 |
| 20 | 15.09 | $CH_3$ | 1.49, d, $J$ = 1.0 Hz | H-3, 15 |
| 21 | 196.30 | $C^0$ | | H-4, 23 |
| 22 | 136.82 | $C^0$ | | H-4 |
| 23 | 154.30 | CH | 7.40, d, $J$ = 2.0 Hz | OH-24 |
| 24 | 86.03 | $C^0$ | | H-23, 27 |
| 25 | | NH | 6.18, brs | |
| 26 | 167.76 | $C^0$ | | H-23 |
| 27 | 43.80 | $CH_2$ | 3.17, d, $J$ = 13.5 Hz<br>3.05, d, $J$ = 13.5 Hz | H-29 (33) |
| 28 | 134.76 | $C^0$ | | H-27, 30 |
| 29(33) | 130.80 | CH | 7.25, m | H-31 |
| 30(32) | 128.80 | CH | 7.33, m | H-31 |
| 31 | 127.70 | CH | 7.30, m | H-29(33) |
| | | OH | 7.23, brs | |



**Oteromycin (1)**

Oteromycin (**1**) was isolated, as a white powder, $[\alpha]^{22}_D$ = −60 (*c* 0.5, MeOH), from a methyl ethyl ketone (MEK) extract of the fermentation broth of the fungus[10] by repeated silica gel chromatography followed by HPLC on a Whatman Partisil-10 ODS-3 column.

FAB mass spectral analysis of oteromycin (**1**) gave several secondary molecular ions corresponding to M + H (*m/z* 488), M + Li (*m/z* 494), and M + matrix + Li (base peak, *m/z* 648). HREIMS gave a molecular ion at *m/z* 487.3086 from which a molecular formula of $C_{32}H_{41}NO_3$ (487.3086, 13 DBE) was deduced. The formula was supported by the $^{13}C$ NMR spectrum which showed 32 carbon signals (Table 1). The following carbon types were discerned from DEPT and HMQC experiments: 6 × $CH_3$, 4 × $CH_2$, 13 × CH, one aliphatic quaternary, one oxygenated quaternary, five olefinic/aromatic quaternaries, and two carbonyls. The infrared spectrum showed hydroxyl (3310 $cm^{-1}$) and carbonyl (1723 $cm^{-1}$) absorptions. The UV spectrum in methanol displayed absorption bands at $\lambda_{max}$ ($CH_3OH$): 209 ($\epsilon$ = 16250) and 235 (sh) nm.

The 500 MHz $^1H$ NMR spectrum (Table 1) of oteromycin in $CD_2Cl_2$ displayed a doublet for a secondary methyl,

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(10) Oteromycin was produced by two strains of the same unidentified fungal species, MF 5810 (ATCC 74201) and MF 5811 (74202), isolated from an unidentified dead grass and from leaf litter of *Juniperus deppeana* from Hope, New Mexico and Culberson Co., TX, respectively, in the vicinity of Otero Co., NM; hence the name oteromycin. We believe this fungus is an anamorph of an Ascomycete in the Hypocreales.
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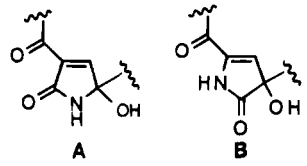
a quaternary methyl singlet, two olefinic methyl singlets, and an olefinic methyl multiplet. The $^1H$ NMR spectrum, beside showing five aromatic protons, also showed a highly deshielded doublet at $\delta$ 7.4, $J = 2.0$ Hz, assignable to a $\beta$-proton of an $\alpha,\beta$-unsaturated carbonyl, two broad singlets corresponding to two olefinic protons and an olefinic proton quartet. The homonuclear $^1H-^1H$ connectivity was determined using $^1H-^1H$ COSY, relayed COSY, and TOCSY experiments, and the results are presented in Figure 1. From these experiments most of the northern half of the molecule and the benzyl unit could be readily assembled. H-23 showed coupling with an NH proton as observed in the COSY spectrum, even though the NH peak still appeared as a broad singlet. The remainder of the structural connectivity of oteromycin was established from an HMBC experiment recorded with $^nJ_{XH} = 7$ Hz in $CD_2Cl_2$. The observed two- and three-bond correlations are listed in Table 1, and some of the key correlations are illustrated in Figure 1. The structure of the lipophilic bicyclic (northern half) unit could be verified from HMBC experiments simply by using the strong correlations from each of the methyl groups. In particular, correlations from H-11 to C-1, C-9, and C-10 established the missing link in the upper part of the structure.

The southern half of the structure was elucidated as follows. The benzyl unit was firmly established from the HMBC correlations of H-27 to respective aromatic carbons and *vice versa*. H-27 further gave correlations to C-23 and C-24. The latter was also correlated to H-23. H-23 also produced strong correlations to both C-21 and C-26 carbonyls, and the NH proton gave a strong correlation to C-22. On the basis of these two- and three-bond HMBC correlations, two five-membered fragment structures (A and B) could be derived. The biosynthetically more probable[12] structure A,[13] and hence **1** was favored for oteromycin due to a lack of HMBC correlations from H-27 to amide carbonyl (C-26), the relatively high shielding of the amide carbonyl, the deshielding of C-24, and the NOESY correlations of NH to H-27 and H-29.




The connection between the two halves of the molecule was evident from HMBC correlations of H-4 to C-21 and C-22. The proposed structure **1** was further supported by EIMS fragmentation which gave fragment ions at *m/z* 271.2424 ($C_{20}H_{31}$) from cleavage of bond C4–C21 and *m/z* 299 and *m/z* 187 from cleavage of bond C21–C22.

**Stereochemistry.** The relative stereochemistry of oteromycin was determined from NOE measurements by both NOEDS and 2D NOESY methods. The most important NOEs are represented in Figure 2. Irradiation of H-11 of methyl group gave enhancements to H-8 and

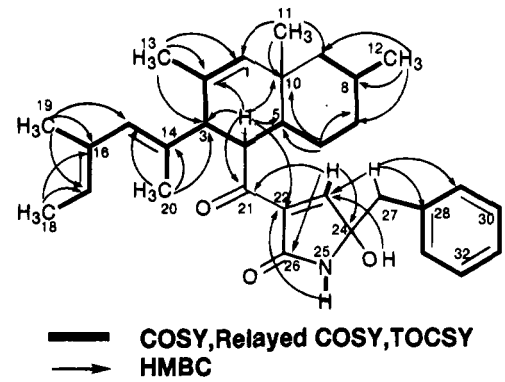



**Figure 1.** $^1H-^1H$ COSY, Relayed COSY, TOCSY, and selected HMBC correlations of oteromycin (**1**).

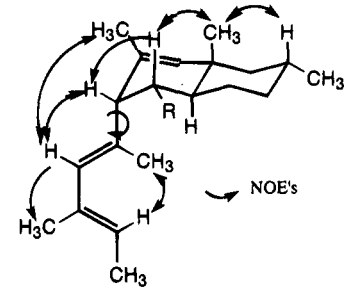



**Figure 2.** Selected NOE enhancements in oteromycin.

H-4 thus establishing their 1,3 diaxial relationship. Irradiation of H-4 gave enhancements of H-3, H-11, and H-23 indicating that H-3 must be equatorially oriented in a *pseudochair* conformation of ring A and placing the side chain in a *axial* orientation. NOEs from H-15 to H-19 and from H-17 to H-20 and the lack of NOEs from H-17 to H-19 and H-15 to H-20 established the *E* olefin geometry for both side chain double bonds. Both olefinic and heterocyclic side chains showed free rotation around bonds C3–C14 and C4–C21 as indicated by NOEs from H-15 to H-13 and H-3, H-4, H-5, and H-20 to H-23, respectively. Thus structure **1** is proposed for oteromycin.

A number of tetramic acid containing natural products are known,[13] but to the best of our knowledge oteromycin is only the second example from natural sources to contain the deoxy tetramic acid heterocyclic system. The only other deoxy compound is $\gamma$-lactam PI-091 produced by *Paecilomyces*.[14] Most of the known compounds incorporating the heterocycle A are the synthons used in the total synthesis of cytochalasins.[15]

## Experimental Section

**General Procedure.** All the reagents and deuterated solvents were obtained from Aldrich Chemical Co. and were used without any purification. The fermentation media components used in this study were obtained from the following sources: yeast extract, Fidco a division of Nestle Co., Inc.; tomato paste, Beatrice/Hunt-Wesson, Inc.; oat flour, Quaker Oat Co. All other materials were reagent grade. E. Merck (Darmstadt) and/or Analtech silica gel plates (0.25 mm) were used for TLC and developed with UV light, iodine vapors, and 3% ceric sulfate in 3 N $H_2SO_4$ spray. Stationary phases used for column chroma-

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**Table 2. Components of the KF Seed Medium**

| seed medium mixture | | trace element mixture | |
|---|---|---|---|
| components | g/L | trace elements | mg/L |
| corn steep liquor | 5 | $FeSO_4 \cdot 7H_2O$ | 1000 |
| tomato paste | 40 | $MnSO_4 \cdot 4H_2O$ | 1000 |
| oat flour | 10 | $CuCl_2 \cdot H_2O$ | 25 |
| glucose | 10 | $CaCl_2$ | 100 |
| | | $H_3BO_3$ | 56 |
| | | $(NH_4)_6Mo_7O_{24} \cdot H_2O$ | 19 |
| | | $ZnSO_4 \cdot 7H_2O$ | 200 |
| trace element mix | 10 mL/L | | |

tography were E. Merck silica gel (70–230 or 40–63 mesh). Melting points are uncorrected.

**Spectral Procedures.** The IR absorption spectra were obtained with a multiple internal reflectance cell (MIR, ZnSe) on neat 10–20 μg samples. Mass spectra were recorded on a JEOL SX-102A (electron impact, EI, 70 eV, and fast atom bombardment, FAB) instrument. Exact mass measurements were performed at high resolution (HR-EI) using perfluorokerosene (PFK) as internal standard. Trimethylsilyl derivatives were prepared with a 1:1 mixture of BSTFA–pyridine at room temperature. The FAB spectrum was run in a matrix of dithiothreitol/dithioerythritol (20/80).

$^1H$ and $^{13}C$ NMR spectra were recorded in $CD_2Cl_2$ solutions, and the chemical shifts are given relative to the solvent peaks at $\delta$ 5.32 and 53.8 ppm, respectively.

$^1H$–$^1H$ COSY were recorded using the standard pulse sequence. The 2K × 2K data set was accumulated in 512 increments with 32 transients respectively for each value of $t_1$ for full phase cycling. HMQC and HMBC experiments were performed using a standard pulse sequence. The 1K × 4K data set was recorded for HMQC experiment on a Unity 400 MHz spectrometer employing Bird nulling of 0.300 s, number of increments = 512, 8 transients per increment, 0.9 s relaxation delay per transient, and $J_{CH}$ = 140 Hz. The HMBC experiment was recorded using a similar experiment with 32 transients per increment, 2.5 s of relaxation delay, and $^nJ_{CH}$ optimized for 7 Hz. The phase sensitive NOESY experiment was recorded on a 400 MHz instrument with 5 s relaxation delay and 500 and 800 ms mixing times. The TOCSY experiment was recorded with a mixing time of 70 ms and relaxation delay of 2.5 s.

**Fermentation Conditions for Production of Oteromycin.** Oteromycin was isolated from two cultures (MF 5810 and MF 5811). The following two-step fermentation protocol was used for both cultures.

**Step 1: Preparation of Seed Culture.** Vegetative mycelia of the culture were prepared by inoculating 54 mL of KF seed medium (Table 2, 10 mL of the freshly prepared trace element mixture was added to 1 L of the seed medium mixture at pH 6.8) in a 250 mL unbaffled Erlenmeyer flask with 2 mL of mycelia in 10% glycerol. The mycelia was stored at −80 °C before inoculation. Seed cultures were incubated for 3 days at 25 °C with 50% relative humidity on a rotary shaker with a 5 cm throw at 220 rpm in a room with constant fluorescent light.

**Step 2: Fermentation Culture.** Two-milliliter portions of the seed culture were used to inoculate 50 mL portions of liquid production medium CYG40 (consisting of, in g/L of water, yellow corn meal, 50; yeast extract, 1; and glucose, 40; no pH adjustment) in 250 mL unbaffled Erlenmeyer flasks. These cultures were incubated at 25 °C at 220 rpm with 50% relative humidity in a room with constant fluorescent light. Maximum production (66 mg/L) of oteromycin was achieved by day 18 using MF 5811. Culture MF 5810 produced only 22 mg/L of oteromycin under optimum conditions.

**Isolation of Oteromycin.** A fermentation batch containing 18 flasks, prepared as described above, were extracted with methyl ethyl ketone (50 mL per flask, stirring for 1 h). After evaporation of the solvent to dryness under reduced pressure, the semisolid residue thus obtained was triturated with 300 mL of methylene chloride and filtered. The biological activity was concentrated in the filtrate. The biologically inactive solid residue was discarded.

The methylene chloride soluble portion from above was first concentrated to 20 mL and then applied onto a 75 mL silica gel column packed in, and equilibrated with, methylene chloride. The adsorbent was washed in sequence with methylene chloride containing increasing amounts of ethyl acetate and then with ethyl acetate containing 0, 5, 10, and 20% v/v methanol. The methylene chloride–ethyl acetate 1:1 washings contained all the biological activity. Evaporation of the solvents under reduced pressure afforded 100 mg of oily residue. Further purification of the residue by preparative TLC on E. Merck silica gel 60 $F_{254}$ plates using a mixture of methylene chloride–methanol–concentrated ammonia 95:5:0.5, v/v/v yielded almost pure oteromycin.

The single active fraction, enriched with oteromycin, thus obtained was dissolved in 0.4 mL of dimethyl sulfoxide–methanol 1:1 and finally purified by preparative high-performance liquid chromatography (HPLC) at room temperature on a Whatman Partisil 10 ODS-3 (9.4 × 250 mm) column. Elution with a 40 min gradient of 60% aqueous acetonitrile to 100% acetonitrile at 4 mL per minute furnished fractions corresponding to 4.25 to 5 column volumes of eluate which contained pure oteromycin. Lyophilization of the pooled fractions gave a colorless amorphous powder of oteromycin (**1**).

The homogeneity of oteromycin was ascertained by $^1H$ and $^{13}C$ NMR spectroscopy, TLC in several normal and reverse phase systems, and on a HPLC (Whatman Partisil 5 ODS-3, 4.6 × 25 cm) column maintained at 40 °C and eluted with 75% aqueous acetonitrile at a flow rate of 1 mL per minute: $k'$ = 4.9. IR: $\nu_{max}$ (ZnSe) 3310, 2949, 2912, 1723, 1606, 1497, 1451, 1377, 1212, 1086, 1038, 955, 858, 702 $cm^{-1}$. Other physical data is reported in the Discussion section. The NMR data is reported in Table 1.

**Supporting Information Available:** $^1H$, $^{13}C$, and NOESY spectra of oteromycin (3 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from ACS; see any current masthead page for ordering information.

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