Fusaricide, a New Cytotoxic *N*-Hydroxypyridone from *Fusarium* sp.

Kimberly D. McBrien, Qi Gao, Stella Huang, Steven E. Klohr, Richard R. Wang, Dolores M. Pirnik, Kim M. Neddermann, Isia Bursuker, Kathleen F. Kadow, and John E. Leet*

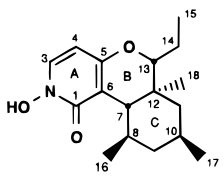
Bristol-Myers Squibb Company, Pharmaceutical Research Institute, 5 Research Parkway, P.O. Box 5100, Wallingford, Connecticut 06492

Received June 18, 1996[⊗]

A new cytotoxic *N*-hydroxypyridone, fusaricide (**1**), was isolated from a *Fusarium* sp. Its structure was solved by X-ray diffraction and spectroscopic analyses.

HIV regulatory proteins such as Rev (regulation of virion expression) are considered to be excellent potential targets for antiviral therapy. A potent and specific inhibitor of the RNA−protein interaction could be an effective antiviral compound.[1−3] An assay that measures inhibition of the specific binding of Rev protein to the Rev-responsive element RNA was used to identify putative inhibitors.[4−8] In the course of screening fermentation extracts for this biological activity, an unknown *Fusarium* sp. (Hyphomycetes) was selected for further evaluation.

The isolation of fusaricide (**1**) was guided by the Rev binding assay.[7,8] The activity was solvent extractable from solid agar fermentations with hexane. Solvent removal and crystallization afforded **1** as colorless diamond-shaped crystals (mp 208 °C). The molecular formula of **1** was established as $C_{17}H_{25}NO_3$ by HR-FABMS ($[M + H]^+$ *m/z* 292.1905, calcd 292.1912), indicating six degrees of unsaturation in the molecule. The UV spectrum exhibited characteristic absorption maxima at 218 and 295 nm (ϵ 25 800, 4200). The IR spectrum displayed amide carbonyl absorption at 1636 cm^{-1}. Apparent in the ^{1}H-NMR spectrum were four methyl signals (δ 0.61 (s), 0.86 (d, $J = 6.3$ Hz), 1.07 (t, $J = 7.3$ Hz), 1.13 (m)), three methine resonances (δ 1.60, 2.00, 2.50), one oxygenated methine (δ 3.75), and three methylenes with nonequivalent protons (δ 1.33 and 1.60, δ 0.64 and 1.60, δ 0.58 and 1.84). A polarized AB system (δ 5.87, 7.53, doublets, $J = 7.4$ Hz) was attributed to two ortho aromatic protons. The ^{13}C-NMR spectrum revealed 17 signals consisting of four methyls, three methylenes, six methines, and four quaternary carbons. In addition to an amide quaternary signal (δ 163.1), there were only four lowfield signals present (δ 98.0, 108.4, 128.2, 156.6), suggesting a heteroaromatic system.



(1)

With suitable crystals at hand, the structure of **1** was readily solved by X-ray crystallography. Single-crystal X-ray analysis revealed a molecular structure containing three fused six-membered rings. The A-ring is planar and consists of a *N*-hydroxy-2-pyridone system. The B-ring adopts a *twist* conformation, and the C-ring is a normal *chair*. The relative stereochemistry was thus determined. In the enantiomer shown (Figure 1), the two methyl groups bonded to C-8 and C-10 are β while the C-12 methyl group and the C-13 ethyl group are in the α orientation. In the crystal, fusaricide exists as a hydrogen-bonded trimer, which is formed between the three molecules related by a 3-fold axis through three-centered hydrogen bonds involving the hydroxyl group and the carbonyl oxygen. The ^{1}H- and ^{13}C-NMR chemical shift assignments for **1** were subsequently made using COSY, HETCOR, and long-range HETCOR data (Table 1).

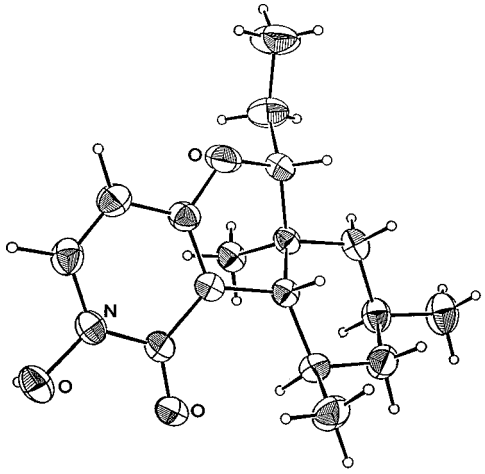


Figure 1. ORTEP drawing of the X-ray structure of fusaricide (**1**). Thermal ellipsoids were drawn at 40% probability level.

For evaluation of fusaricide in the protein/RNA binding assays, the cyclodextrin-based complexing agent molecusol was necessary to solubilize the crystals. The specificity of the Rev/Rre inhibitory activity was evaluated by the R17 coat protein/R17 RNA binding assay.[6] Fusaricide was active in the primary screen (IC_{50} 40 μg/mL) and was also active in the R17 screen (75 μg/mL). The IC_{50} ratio of R17 to Rev activity was <2, indicating that the compound was not specific for Rev. Fusaricide also showed antifungal activity against *Candida albicans* and *Penicillium chrysogenum* (MIC's 16 and 8 μg/mL, respectively). In addition, fusaricide was tested for antitumor activity against a murine tumor cell line Madison lung carcinoma (M109), as previously described.[9] The compound exhibited *in vitro* antitumor activity at concentrations of 1 μg/mL and above, while no significant activity was observed in the M109 *in vivo* tumor model.

[⊗] Abstract published in *Advance ACS Abstracts,* November 1, 1996.

S0163-3864(96)00521-6 CCC: $12.00

Table 1. ^{1}H and ^{13}C Data ($CDCl_3$) of Fusaricide

carbon	^{13}C (ppm)	^{1}H (ppm) (mult, *J* (Hz))	$^{1}H-^{1}H$ COSY	long range ^{1}H-^{13}C correlations (obsd) (LR-HETCOR)
1	163.1			
3	128.2	7.53 (d, 7.4)	H4	C-1
4	98.0	5.87 (d, 7.4)	H3	
5	156.6			
6	108.4			
7	49.2	2.00 (d, 11.4)	H8	C-5, C-6, C-8, C-12, C-18
8	27.8	2.50 (m)	H7, H9a, H9b, H16	
9	45.0	a. 1.84 (d br, 12.1)	H8, H9b, H10	
		b. 0.58 (d br, 13.0)	H8, H9a, H10	C-8
10	25.9	1.60 (m)	H9a, H9b, H17	C-12
11	45.9	a. 1.60 (m)	H11b	C-7, C-12
		b. 0.64 (d, 12.8)	H11a	C-10, C-12
12	36.9			
13	92.2	3.75 (d, 9.4)	H14b	
14	23.2	a. 1.60 (m)	H14b, H15	C-12
		b. 1.33 (m)	H13, H14a, H15	
15	11.1	1.07 (t, 7.3)	H14a, H14b	C-14
16	24.8	1.13 (m)	H8	
17	22.8	0.86 (d, 6.3)	H10	C-10
18	15.3	0.61 (s)		C-7, C-13

Fusaricide is a new member of a growing list of *N*-hydroxypyridone natural products reported from fungi. While the relative stereochemistry of fusaricide was established in the present study, its absolute configuration remains to be determined. Fusaricide shows structural similarities to the antiinsectan metabolite leporin A, isolated from *Aspergillus leporis*[10], and the free-radical scavenger pyridoxatin, from *Acremonium* sp.[11,12] Most recently, a structural analog PF1140 with unspecified relative stereochemistry, obtained from *Eupenicillium*, was reported in a Japanese patent and claimed to have broad spectrum antifungal activity.[13]

Experimental Section

General Experimental Procedures. Low-resolution MS measurements were performed with a SCIEX API-III tandem quadrupole mass spectrometer, using an IONSPRAY interface. FAB accurate mass measurements were obtained by peak matching using a Kratos MS50 mass spectrometer with a cesium iodide saturated glycerol solution as the reference. The UV spectrum was obtained using a Hewlett-Packard 8452A diode array spectrophotometer. IR measurements were taken on a Perkin-Elmer 2000 Fourier Transform spectrometer. ^{1}H-NMR and ^{13}C-NMR measurements were obtained on a Bruker AM-500 instrument operating at 500.13 and 125.76 MHz, respectively, using a 5-mm broadbanded probe. The long range HETCOR experiment was optimized for 8.3 Hz coupling constant (0.06 s delay time was used). Chemical shifts are reported in ppm relative to solvent ($CDCl_3$, δ_H 7.24, δ_C 77.0). The specific rotation was measured on a Perkin-Elmer 241 polarimeter, and CD data were recorded with a JASCO J-720 spectropolarimeter. X-ray data were acquired at room temperature on an Enraf-Nonius CAD4 diffractometer using graphite-monochromated Cu Kα radiation ($\lambda = 1.5418$ Å).

Fungal Isolation and Culture Conditions. Flowers of sourwood (*Oxydendron arboreum*) were collected from Georgia State Botanical Garden in Athens, GA. The flowers were incubated for several days in a moist chamber containing a sterile filter paper wetted with sterile distilled water. The mycelia were subsequently transferred onto potato dextrose agar (PDA) several times until a pure colony was obtained. Fungal isolate WC-49758 was deposited in the Bristol-Myers Squibb Culture Collection under the accession number SC 15717. Growth on PDA appears white to cream turning yellow to orange with age. The culture reverse is dark wine red with an orange-red diffusible pigment. Aerial mycelium is abundant with chlamydospores present mostly in chains, some apical and measuring an average of 14 μm in diameter. No macroconidia are produced, but it was possible to find a small number of globose microconidia measuring 1.3 × 2.4 μm. Due to the abundance of chlamydospores, the color of the pigment, and the very small numbers of microconidia in the absence of macroconidia, this culture was identified as a degenerated culture of *Fusarium* sp.[14−16]

The culture was grown on a PDA slant for 7 days at 28 °C, and 6 mL of 20% (w/v) glycerol was added. With a sterile swab, a spore suspension was prepared and divided into aliquots, frozen in a dry ice−acetone bath, and stored at −80 °C. From the frozen stock, 0.1 mL was used to inoculate a PDA slant that was incubated at 28 °C for 7 days, and then 6 mL of 0.9% (w/v) NaCl was added to each slant. The culture was agitated with a sterile swab to prepare a spore suspension that was then transferred onto a 20 × 20 cm Nunc plate containing 250 mL of medium. The culture was incubated at 28 °C for 7 days. The medium contained the following per liter of deionized water: 30 g of mashed potato, 10 g of dextrose, 10 g of Esusan Mi-to (defatted soybean meal), 2 g of yeast extract, 2.5 g of NaCl, and 10 g of agar. The pH was adjusted to 7.0 prior to sterilization at 121 °C for 25 min.

Extraction and Isolation. Plates from the solid agar fermentation (20 20 × 20 cm Nunc plates) were pooled. The contents (e.g., fungal biomass and medium) were chopped into small pieces and extracted with hexane (4 L) by soaking at room temperature for 2 h. The hexane extract was decanted and concentrated *in vacuo* to a small volume (10 mL). Further evaporation of the concentrate under a nitrogen stream yielded a crystalline solid. These solids were recovered by filtration and washed with cold hexane, affording **1**, 58 mg, as colorless diamond-shaped crystals suitable for X-ray analysis.

Fusaricide (1): $C_{17}H_{25}NO_3$; colorless crystals (diamond-shaped); mp 208 °C; $[\alpha]_D$ +194° (*c* 0.12, $CHCl_3$);

TLC R_f 0.47 (Si gel 0.25 mm ($CHCl_3$−MeOH−AcOH 95:5:0.5, ceric sulfate detection); HRFABMS m/z 292.1905 ($[M + H]^+$, calcd 292.1912); UV λ_{max} nm (ϵ) (MeOH) 218 (25 800), 295 (4 200); IR ν_{max} (KBr) 2950, 1636, 1594, 1548, 1430, 1374, 1334, 1262, 1230, 1204, 1080, 954, 936, 772 cm^{-1}; CD λ nm ($\Delta\epsilon$) (MeOH) 295 (+0.4), 246sh (+1.6), 218 (+29); ^{1}H-NMR and ^{13}C-NMR data ($CDCl_3$) see Table 1.

Rev Bioassay. The screening methods have been described and reported elsewhere.[7,8] Briefly, the assay utilized an *in vitro* transcribed and ^{33}P-labeled Rev responsive element RNA, and the recombinant Rev protein purified from *E. coli* lysates. When the RNA is uninhibited, it forms a complex with the protein, which can in turn be trapped on nitrocellulose filters and quantified by liquid scintillation counting. The assay was automated and developed into a high-throughput screen. Pure fusaricide was formulated for bioassay by mixing 1 mg/0.3 mL of EtOH solution with 0.5 mL aqueous molecusol (0.5 g/mL solution), followed by lyophilization.

X-ray Crystallographic Analysis of 1. A crystal of approximate size 0.20 × 0.25 × 0.26 mm was used for X-ray diffraction experiments carried out at room temperature on an Enraf-Nonius CAD4 diffractometer using graphite-monochromated Cu Kα radiation (λ = 1.5418 Å). The crystals are trigonal, space group $R3$, cell constants $a = b = 21.597(1)$ Å, $c = 8.9085(8)$ Å, $\alpha = \beta = 90$, $\gamma = 120°$, $V = 3598.9(5)$ Å^3 with nine molecules in the unit cell; calculated crystal density $Dx = 1.210$ g/cm^3; absorption coefficient $\mu = 0.62$ mm^{-1}. The structure was solved by direct methods and refined by full-matrix least-squares methods.[17] Final agreement factors $R(F) = 0.033$, $wR(F) = 0.047$, where $w = 1/\sigma^2$ and $S = 1.457$, for 3491 reflections with $I \geq 3\sigma(I)$ and 191 variables. No recognizable residual features ($-0.124 \leq \Delta\rho \geq 0.307$ e Å^{-3}) were observed in the final difference electron density map.[18]

Acknowledgment. The authors thank K. Kodukula for screening support, L.-P. Chang and S. E. Lowe for microbiological and fermentation support, and S. W. Mamber for the antifungal assays. For additional analytical data we thank R. A. Dalterio, K. J. Edinger, M. Cadiz, and J. L. Whitney.

References and Notes

(1) Zapp, M. L.; Stern, S.; Green, M. R. *Cell.* **1993**, *74*, 969−978.
(2) Bartel, D. P.; Zapp, M. L.; Green, M. R.; Szostak, J. W. *Cell.* **1991**, *67*, 529−536.
(3) Cook, K. S.; Fisk, G. J.; Hauber, J.; Usman, N.; Daly, T. J.; Rusche, J. R. *Nucleic Acids Res.* **1991**, *19*, 1577−1583.
(4) Daly, T. J.; Cook, K. S.; Gray, G. S.; Malone, T. E.; Rusche, J. R. *Nature.* **1989**, *342*, 816−819.
(5) Zapp, M. L.; Green, M. R. *Nature.* **1989**, *342*, 714−716.
(6) Peabody, D. S. *J. Biol. Chem.* **1990**, *265*, 5684−5689.
(7) Qian-Cutrone, J.; Huang, S.; Trimble, J.; Li, H.; Lin, P.-F.; Alam, M.; Klohr, S. E.; Kadow, K. F. *J. Nat. Prod.* **1996**, *59*, 196−199.
(8) Qian-Cutrone, J.; Huang, S.; Chang, L.-P.; Pirnik, D.; Klohr, S. E.; Dalterio, R. A.; Hugill, R.; Lowe, S.; Alam, M.; Kadow, K. F. *J. Antibiot.*, **1996**, *49*, accepted for publication.
(9) McBrien, K. D.; Berry, R. L.; Lowe, S. E.; Neddermann, K. M.; Bursuker, I.; Huang, S.; Klohr, S. E.; Leet, J. E. *J. Antibiot.*, **1995**, *48*, 1446−1452.
(10) TePaske, M. R.; Gloer, J. B.; Wicklow, D. T.; Dowd, P. F. *Tetrahedron Lett.*, **1991**, *32*, 5687−5690.
(11) Snider, B. B.; Lu, Q. *Tetrahedron Lett.*, **1994**, *35*, 531−534.
(12) Teshima, Y.; Shin-ya, K.; Shimazu, A.; Furihata, K.; Chul, H. S.; Furihata, K.; Hayakawa, Y.; Nagai, K.; Seto, H. *J. Antibiot.* **1991**, *44*, 685−687.
(13) Hosaya, R.; Yugami, T.; Yoshida, S.; Yaguchi, T.; Komuro, K.; Sasaki, T. Japanese Patent 07,267,962-A; *Chem. Abstr.* **1996**, *124*, 143744k.
(14) Hawksworth, D. L.; Kirk, P. M.; Sutton, B. C.; Pegler, D. N. (1995). *Ainsworth & Bisby's Dictionary of the Fungi, 8th Ed.* CAB International, Wallingford, UK, 1995.
(15) Nirenberg, H. I. *Can. J. Bot.* **1980**, *59*, 1599−1609.
(16) Joffe, A. Z. *Mycologia applicata* **1974**, *53*, 201−228.
(17) Fair, C. K. (1990). *MolEN. An Interactive Intelligent System for Crystal Structure Analysis.* Enraf-Nonius, Delft, The Netherlands.
(18) Atomic coordinates, thermal parameters, bond lengths and angles have been deposited with the Cambridge Crystallographic Data Center and can be obtained, upon request, from Dr. Olga Kennard, University Chemical Laboratory, 12 Union Road, Cambridge Cb2 1EZ, UK.

NP960521T