Oxysporidinone: A Novel, Antifungal *N*-Methyl-4-hydroxy-2-pyridone from *Fusarium oxysporum*

Jens Breinholt,* Svend Ludvigsen, Birgitte R. Rassing, and Connie N. Rosendahl
Novo Nordisk A/S, DK-2880 Bagsværd, Denmark

Salka E. Nielsen
National Food Agency, Institute of Toxicology, DK-2860 Søborg, Denmark

Carl E. Olsen
Royal Veterinary and Agricultural University, Chemistry Department, DK-1871 Frederiksberg C, Denmark

Received July 23, 1996[⊗]

Oxysporidinone (**1**), a novel 3,5-disubstituted *N*-methyl-4-hydroxy-2-pyridone, was isolated from fermentations of *Fusarium oxysporum* (CBS 330.95) by counter-current chromatography. The structure was determined by spectroscopic methods including NMR, MS, IR, and UV analysis. Oxysporidinone exhibited growth inhibitory activity against several common plant pathogenic fungi.

In screening microbial extracts for secondary metabolites with antifungal activities and potential applications as plant protection agents, *Fusarium oxysporum* (CBS 330.95) was found to produce a novel compound that, in vitro, inhibited the growth of plant pathogenic fungi such as *Botrytis cinerea* and *Venturia inequalis*. Bioassay-guided fractionation led to isolation of the compound, designated oxysporidinone (**1**), responsible for the observed antifungal activity. We wish to report on the fermentation, isolation, structure elucidation, and biological activity of **1**.

F. oxysporum (CBS 330.95) was fermented in submerged cultures in shake flasks on a yeast-extract and sucrose-based growth medium. The antifungal activity observed in EtOAc extracts of the cultures was shown to be associated with a metabolite exhibiting a characteristic UV absorption maximum at 291 nm. After partitioning the EtOAc extract between heptane and aqueous MeOH, the antifungal compound was purified by countercurrent chromatography followed by Si gel column chromatography to yield a homogenous sample of **1**.

The structure of **1** was determined by NMR spectroscopic techniques, including DQF−COSY,[1] TOCSY,[2] NOESY,[3] HMQC,[4] and HMBC[5] experiments, combined with MS, IR, and UV data. Thus, the molecular composition of oxysporidinone was established as $C_{28}H_{43}NO_6$ from HRFABMS measurements in combination with the number of signals detected in its ^{13}C-NMR spectrum. The presence of NMR signals attributable to an *N*-methyl group [δ_H 3.43 (3H, s), δ_C 37.30], a lowfield proton signal [δ_H 7.46(s)], which exhibited a characteristic one-bond coupling constant ($^1J_{CH}$ = 178 Hz) to a carbon atom resonating at δ_C 136.17, combined with long-range ^1H-^{13}C correlations observed in the HMBC spectrum established a 3,5-disubstituted *N*-methyl-4-hydroxy-2-pyridone moiety to constitute the chromophore of oxysporidinone (Figure 1A). The presence of this structural element was supported by the observed UV absorption maximum at 291 nm (log ϵ 3.68) and strong IR absorptions at 1652 and 1568 cm^{-1}, which conform to those reported for funiculosin (**2**) (UV: λmax 290 nm, log ϵ 3.70; IR: 1649 and 1560 cm^{-1}), an antifungal metabolic product of *Penicillium funiculosum*.[6,7]

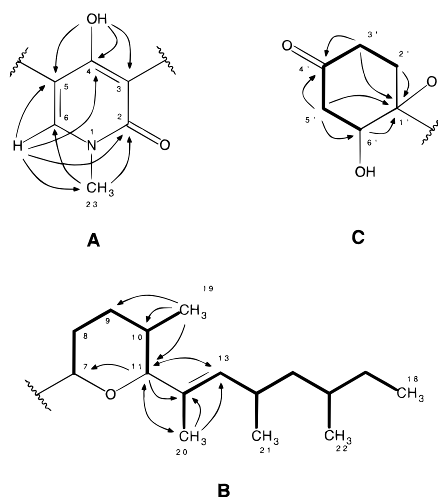
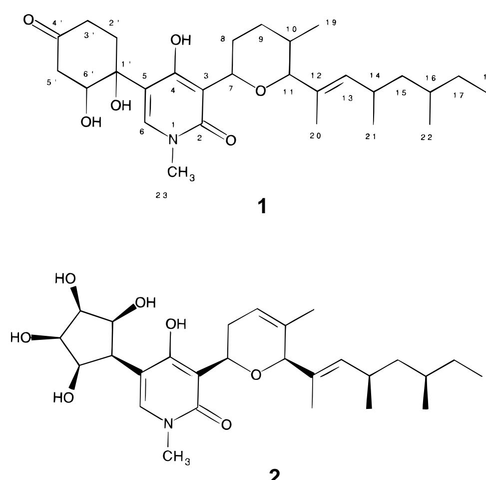


Figure 1. Partial structures A, B, and C of **1**. Solid lines indicate ^1H spin systems identified from COSY and TOCSY data, and arrows indicate selected, diagnostic $^1H-^{13}C$ long-range correlations observed in the HMBC spectrum.



* To whom correspondence should be addressed. Phone: 45-44 42 39 59. FAX: 45-44 44 45 65. E-mail: jbre@novo.dk.

⊗ Abstract published in *Advance ACS Abstracts,* December 15, 1996.

S0163-3864(96)00559-9 CCC: $14.00 © 1997 American Chemical Society and American Society of Pharmacognosy

Analysis of the $^1H-^1H$-COSY and TOCSY spectra allowed identification of the spin systems between H-7 and H-11 and between H_3-20 and H_3-18 (Figure 1B, solid bars). The individual spin systems were connected (C-11 to C-12) based on HMBC correlations from H-11 to C-12, C-13, and C-20, and by correlations to C-11 from both H_3-20 and H-13. The chemical shifts for C-7 and C-11 (δ 78.04 and 92.54, respectively) implied an ether function and a HMBC correlation from H-11 to C-7 confirmed the presence of a 3-methyl-pyran ring (Figure 1B).

From the $^1H-^1H$-COSY spectrum another spin system, H_2-2′ to H-6′, was identified (Figure 1C, solid bars). The small coupling constant observed between H-3′eq and H-5′eq ($J_{3'eq,5'eq}$ = 1.6 Hz) suggested that C-3′ and C-5′ were not directly connected, and the expected link between C-3′ and C-5′ was identified as a carbonyl group (C-4′) by HMBC correlations from H_2-3′ and H_2-5′ to C-4′. Furthermore, correlations from H_2-2′, H_2-3′, H_2-5′, and H-6′ to the only carbon atom not accounted for so far, a quaternary and oxygen-bearing carbon atom (C-1′), established the cyclohexanone fragment (C-1′ to C-6′, Figure 1C). A strong carbonyl band at 1724 cm^{-1} in the IR spectrum of **1** supported the presence of a nonconjugated and nonstrained ketone. After hydroxyl groups were attached to both C-1′ and C-6′, as indicated by the corresponding ^{13}C-chemical shifts (δ 73.69 and 70.69, respectively), fragments A, B, and C account for all elements of the molecular formula. Based on HMBC correlations from H-6 to C-1′ and from H-7 to C-2, C-4, and C-5, substructures A, B, and C were joined (C-5 to C-1′ and C-7 to C-3) to complete the structure **1** for oxysporidinone. This structure was further supported by correlations observed in the HMQC−TOCSY spectrum. Assigned ^1H- and ^{13}C-NMR data for **1** are listed in Table 1.

The relative stereochemistry of **1** was partially unraveled by determination of $^1H-^1H$ coupling constants and through-space proton−proton interactions (NOE) from the 2D−NOESY spectrum. Thus, in the case of the cyclohexanone ring observation of large coupling constants between H-2′ax and H-3′ax and between H-5′ax and H-6′($J_{2',3'ax}$ = 14 Hz and $J_{5'ax,6'}$ = 11 Hz), attributable to trans-diaxial interactions, served to identify protons in pseudo-axial positions (Figure 2). Conclusive evidence for the relative stereochemistry was provided by observed NOE interactions between H-6′ and both H-2′ax and H-5′ pictured in Figure 2 as arrows. The stereochemistry at C-1′ was evident from NOE interactions between H-6 (in the pyridone ring) and both H-6′ and H-2′ax, which can only be explained by placing the pyridinone moiety above the plane of the cyclohexanone ring, that is, in the equatorial position. Furthermore, the chair conformation of the cyclohexanone ring fixes H-3′eq and H-5′eq in a *w* conformation in agreement with the observed long-range coupling between H-3′eq and H-5′eq referred to above.

Similarly, large splittings of the signals corresponding to H-7 and H-11 ($J_{7,8ax}$ = 11 Hz and $J_{10,11}$ = 10 Hz) in combination with NOE interactions revealed the relative configuration of the pyran ring as depicted (Figure 2). The chemical shifts for H-7 and H-11 (δ 4.96 and 3.51) agree with values reported[7] for corresponding protons in dihydrofuniculosin (δ 4.95 and 3.52), supporting the assigned relative stereochemistry for **1**. Due to lack of NOE interactions between protons in the pyran and cyclohexanone rings their stereochemical relation remains undetermined. The *E*-configuration of the 12,13-double bond was indicated by the highfield resonance frequency for CH_3-20 (δ_C 11.75 ppm)[8] and confirmed by an observed NOE interaction between CH_3-20 and H-14 and the absence of NOE between CH_3-20 and H-13.

Including the 4-hydroxy-2-pyridone chromophore, oxysporidinone constitutes a new member of a group of secondary metabolites commonly occurring in fungi. In addition to funiculosin (**2**)[6,7] the group encompasses the fungal metabolites tenellin and bassianin from *Beauveria tenella* and *B. bassiana*,[9−11] ilicicolin H from *Cylindrocladium ilicicola*,[12] harzianopyridone from *Trichoderma harzianum*,[13] leporin A from *Aspergillus leporis*,[14] fischerin from *Neosartorya fischeri*,[15] and apiosporamide from *Apiospora montagnei*.[16]

Table 1. ^1H and ^{13}C NMR Data ($CDCl_3$) for Oxysporidinone (**1**)

no	δ ^{13}C	δ ^1H(multiplicity)
2	161.43[a]	
3	113.90	
4	161.34[a]	10.34 (s,OH)
5	110.78	
6	136.17	7.46(s)
7	78.04	4.96(dd,ax), $J_{7,8ax}$ = 11 Hz, $J_{7,8eq}$ = 1.8 Hz
8	30.66	1.55(m,ax), 2.08(dd,eq), $J_{8ax,8eq}$ = 13 Hz
9	32.12	1.42(dt,ax), 1.95(dd[b],eq), $J_{9ax,9eq}$ = 13 Hz
10	32.41	1.70(m,ax), $J_{10,11}$ = 10 Hz
11	92.54	3.51(d,ax)
12	129.97	
13	138.19	5.22(d), $J_{13,14}$ = 9.4 Hz
14	29.62	2.51(m)
15	44.69	1.05(m), 1.23(m)
16	31.97	1.34(m)
17	28.88	1.06(m)
18	11.20	0.85(t), $J_{17,18}$ = 7.2 Hz
19	17.60	0.77(d), $J_{10,19}$ = 6.6 Hz
20	11.75	1.66(s)
21	20.66	0.94(d), $J_{14,21}$ = 6.6 Hz
22	19.58	0.85(d), $J_{16,22}$ = 6.4 Hz
23	37.30	3.43(s)
1′	73.69	
2′	32.12	2.32(dt,ax), $J_{2'ax,2'eq}$ = 14 Hz, $J_{2'ax,3'ax}$ = 14 Hz, $J_{2'ax,3'eq}$ = 4.8 Hz 2.13(dd,eq), $J_{2'eq,3'ax}$ = 6.5 Hz, $J_{2'eq,3'eq}$ = 1.5
3′	36.36	2.79(dd,ax), 2.24(d[b],eq), $J_{3'ax,3'eq}$ = 14 Hz $J_{3'eq,5'eq}$ = 1.6 Hz
4′	208.82	
5′	45.50	2.76(dd,ax), $J_{5'ax,5'eq}$ = 14 Hz, $J_{5'ax,6'}$ = 11 Hz, 2.67(dd[b],eq), $J_{5'eq,6'}$ = 5.6 Hz
6′	70.69	4.65(m,ax)

[a] Interchangeable. [b] Signal exhibits further fine structure.

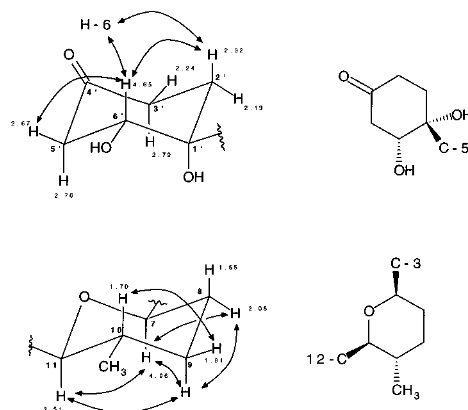


Figure 2. Relative stereochemistry of the cyclohexanone and pyran rings with arrows indicating selected NOE interactions.

For both tenellin[9,17] and ilicicolin H[18] biosynthetic investigations demonstrated that the pyridinone rings are formed by condensation of phenylalanine and polyketide precursors, initially resulting in tetramic acid intermediates, which subsequently undergo rearrangement and ring expansion. It is likely that oxysporidinone (**1**), and the structurally related funiculosin (**2**), are constructed biosynthetically by similar pathways, that is, by condensation of phenylalanine and a tetramethylated heptaketide, extended by modification of the aromatic ring, as assumed for apiosporamide.[16]

Compared to the broad antifungal activity reported for funiculosin (**2**)[6,7] oxysporidinone exhibits a relatively selective target spectrum. Growth inhibitory activity (see Experimental Section) was observed in vitro against the phytopathogenic fungi *Aspergillus niger*, *Botrytis cinerea*, *Alternaria alternata* (asexual ascomycetes), and *Venturia inequalis* (ascomycetes). No activity was observed against bacteria (*Bacillus subtilis* and *Pseudomonas aeruginosa*) and yeasts (*Candida albicans* and *Saccharomyces cervisiae*) when tested at 1 mg/mL.

Experimental Section

General Experimental Procedures. NMR spectra were acquired in $CDCl_3$ at 297 K on Bruker AC300P and AMX600 instruments [DQF−COSY and NOESY (mixing time 400 ms)]. Chemical shifts (δ values) were measured relative to solvent peaks at δ 7.27 (^{1}H) and δ 77.0 (^{13}C). HRFABMS was performed on an AX505W instrument (JEOL). EIMS were recorded on the same instrument at 70eV ionization potential and are presented as *m*/*z* (% rel int). IR spectra were recorded (in KBr) on a Perkin-Elmer 1720 instrument, and UV spectra were obtained on a Phillips PU-8740 instrument. Countercurrent chromatography was performed on a PTR CCC-1000 (Pharma-Tech Research Corp, Baltimore) counter-current chromatograph.

Organism and Fermentation. The producing organism was isolated from plant material collected in Sweden and identified as *F. oxysporum* Schlechtendahl: Fries (Hyphomycetes). A subculture of the organism was deposited at Centraalbureau voor Schimmelcultures (CBS), The Netherlands, under the designation CBS 330.95. The fungus was grown on potato-dextrose agar slants (Difco, 12 mL/slant) at 26 °C for 21 days. Sterile H_2O (10 mL) containing Tween 80 (0.1%) was added and the resulting suspension used for inoculation of 500-mL baffled Erlenmeyer flasks containing 100 mL of yeast extract sucrose medium (2% yeast extract (Difco), 15% sucrose, 18 ppm $ZnSO_4$,7 H_2O, and 8 ppm $CuSO_4$,5 H_2O; pH adjustment to 6.4 and autoclavation at 121 °C for 40 min). The flasks were shaken (200 rpm) for 13 days at 26 °C.

Extraction and Purification of 1. Whole culture broth (3.75 L) from 50 shake flasks was extracted with 7.5 L of EtOAc while being stirred for 2 h. The organic phase (7.35 kg) was concentrated under reduced pressure to a dark, oily residue (5.75 g). The EtOAc extract was further separated by partitioning between heptane (250 mL) and 90% aqueous MeOH (250 mL). Evaporation of the aqueous MeOH phase yielded an oily, red residue (1.36 g).

The extract was in two portions subjected to countercurrent chromatography using a 320-mL column and the solvent system of heptane−EtOAc−MeOH−H_2O (1:1:1:1) with upper phase as stationary phase. The column was rotated at 1000 rpm and eluted at a flow rate of 1 mL/min. Retention of the stationary phase was approximately 50%. The effluent was detected by UV absorption at 290 and 325 nm. Fractions of 20 mL were collected to yield oxysporidinone (**1**, 19.3 mg) in fractions 14, 15, and 16 in almost pure form, though containing minor colored impurities. Final purification was achieved using Si gel column chromatography (Merck Lobar, size B, eluted at a flow rate of 7.5 mL/min with a linear gradient from 100% CH_2Cl_2 to 100% MeOH over 60 min; UV detection at 290 nm). The fractions covering the peak eluting at 24 min containing the active compound were pooled and evaporated to yield homogenous **1** (15 mg) as a colorless glass. ^{1}H and ^{13}C NMR ($CDCl_3$): Table 1. HRFABMS: found 490.3144 $[M + H]^+$, calcd for $C_{28}H_{44}NO_6$ 490.3168. EIMS: 489 (5), 471 (8), 455 (8), 453 (12), 403 (5), 372 (5), 354 (7), 309 (5), 279 (19) 261 (69), 243 (73), 230 (10), 215 (8), 202 (5), 167 (18), 149 (40), 129 (13), 109 (26), 97 (22), 44 (100). UV (MeOH) reported in text above. $[\alpha]_D$ +97° (*c* 0.2, EtOH). IR: 3460, 3260, 2959, 2928, 1724, 1652, 1568, 1447, 1367, 1261, 1153, 1054, and 989 cm^{-1}.

Antifungal Activity. Antifungal activity was assessed in agar diffusion assays as described previously.[19] Using this method the minimum inhibitory concentration (MIC) values for oxysporidinone (**1**) against *A. niger*, *B. cinerea*, *A. alternata*, and *V. inequalis* were estimated to 10, 1, 50, and 10 μg/mL, respectively.

Acknowledgment. The authors wish to thank Dr. M. Larsson, Nordreco AB, Sweden, for kindly providing the isolate and Ms. A. Blom, Novo Nordisk A/S, for recording the 600 MHz NMR spectra.

References and Notes

(1) Rance, M.; Sørensen, O. W.; Bodenhausen, G.; Wagner, G.; Ernst, R. R.; Wüthrich, K. *Biochem. Biophys. Res. Commun.* **1983**, *117*, 479.
(2) Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *56*, 355.
(3) Bodenhausen, G.; Kogler, H.; Ernst, R. R. *J. Magn. Reson.* **1984**, *58*, 379.
(4) Bax, A.; Subramanian, S. *J. Magn. Reson.* **1986**, *67*, 565−569.
(5) Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093−2094
(6) Ando, K.; Suzuki, S.; Saeki, T.; Tamura, G.; Arima, K. *J. Antibiotics* **1969**, *22*, 189−194.
(7) Ando, K.; Matsuura, I.; Nawata, Y.; Endo, H.; Sasaki, H.; Okytomi, T.; Saehi, T.; Tamura, G. *J. Antibiotics* **1978**, *31*, 533−538.
(8) Couperus, P. A.; Clague, A. D. H.; van Dongen, J. P. C. M. *Org. Magn. Reson.* **1976**, *8*, 426−431.
(9) McInnes, A. G.; Smith, D. G.; Wat, C.-K.; Vining, L. C.; Wright, J. L. C. *J. Chem. Soc., Chem. Commun.* **1974**, 281−282.
(10) McInnes, A. G.; Smith, D. G.; Walter, J. A.; Vining, L. C.; Wright, J. C. L. *J. Chem. Soc., Chem. Commun.* **1974**, 282−284.
(11) Wat, C.-K.; McInnes, A. G.; Smith, D. G.; Wright, J. L. C.; Vining, L. C. *Can. J. Chem.* **1977**, *55*, 4090−4098.
(12) Matsumoto, M.; Minato, H. *Tetrahedron Lett.* **1976**, 3827−3830.
(13) Dickinson, J. M.; Hanson, J. R.; Hitchcock, P. B. *J. Chem. Soc., Perkin Trans. 1* **1989**, 1885−1887.
(14) Tepaske, M. R.; Gloer, J. B.; Wicklow, D. T.; Dowd, P. F. *Tetrahedron Lett.* **1991**, *32*, 5687−5690.
(15) Fujimoto, H.; Ikeda, M.; Yamamoto, K.; Yamazaki, M. *J. Nat. Prod.* **1993**, *56*, 1268−1275.
(16) Alfatafta, A. A.; Gloer, J. B.; Scott, J. A.; Malloch, D. *J. Nat. Prod.* **1994**, *57*, 1696−1702.
(17) Wright, J. L. C.; Vining, L. C.; McInnes, A. G.; Smith, D. G.; Walter, J. A. *Can. J. Biochem.* **1977**, *55*, 678−685.
(18) Tanabe, M.; Urano, S. *Tetrahedron* **1983**, *39*, 3569−3574.
(19) Berova, N.; Breinholt, J.; Jensen, G. W.; Kjær, A.; Lo, L.-C.; Nakanishi, K.; Nielsen, R. I.; Olsen, C. E.; Pedersen, C.; Stidsen, C. E. *Acta Chem. Scand.* **1994**, *48*, 240−251.

NP9605596