

NOVEL INHIBITORS OF SUPEROXIDE ANION GENERATION,
OPC-15160 AND OPC-15161

TAXONOMY, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL
PROPERTIES, BIOLOGICAL CHARACTERISTICS AND
STRUCTURE DETERMINATION

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A new inhibitor of superoxide anion generation by guinea pig macrophages, OPC-15160, was isolated from the culture broth of fungus *Thielavia minor* OFR-1561, and a more potent inhibitor, OPC-15161, was obtained as a major degradation product of OPC-15160. OPC-15161 was five times more active than the natural inhibitor and its IC_{50} value was 2.8×10^{-5} M. The structure of OPC-15161 was elucidated by X-ray analysis to be 6-(1*H*-indol-3-ylmethyl)-5-methoxy-3-(2-methylpropyl)-2-(1*H*)-pyrazinone, 4-oxide and had a novel and highly functionalized pyrazine skeleton.

In the course of a screening program for novel inhibitors of superoxide anion generation by guinea pig macrophages, a fermentation broth of fungus *Thielavia minor* OFR-1561 showed inhibitory activity. Bioassay-directed fractionation of the ethyl acetate extract of the culture filtrate led to the isolation of a new inhibitor, OPC-15160. In the purification process a more active inhibitor, OPC-15161, was obtained as a major degradation product of OPC-15160.

In this paper we describe the taxonomy and the fermentation of the producing strain and the isolation, physico-chemical characterization and biological properties of OPC-15160 and OPC-15161, and the structure determination of OPC-15161.

Materials and Methods

General Experimental Procedures

MP's were measured on a Büchi 530 melting point apparatus and are uncorrected. Optical rotations were measured on a Jasco DIP-140 digital polarimeter. A Shimadzu UV-210A spectrophotometer was used for UV spectra and assay of superoxide anion generation. IR spectra were taken on a Jeol JIR-RFX 3002 spectrophotometer. MS were measured on a Shimadzu GCMS-QP 1000 or a Jeol JMS-SX 102 spectrometer. ¹H and ¹³C NMR spectra were recorded on Bruker WH-400, AC-250 and AC-200 spectrometer. Elemental analyses were performed by a Yanaco MT-5 CHN Corder. A Tosoh CCPE system was used for HPLC.

Taxonomic Studies

The methods and media used for these studies are described in the procedures of BOOTH¹⁾.

Strain OFR-1561 was isolated from a soil sample collected at Ishigaki island, Okinawa Prefecture, Japan. The cultures were incubated at 37°C for 10 days before observation.

Fermentation

A loopful of slant culture of strain OFR-1561 was inoculated into 500-ml flasks containing 100 ml of seed medium composed of corn starch 3 g, glucose 0.5 g, soybean meal 0.3 g, yeast extract (Oriental Yeast Co.) 0.1 g, MgSO₄ 0.05 g and CaCO₃ 0.2 g (pH 6.5). The flasks were shaken on a rotary shaker (250 rpm) for 4 days at 28°C. Then 300 ml of broth was transferred into a 30-liter jar fermenter containing 20 liters of the same medium and the fermentation was carried out for 4 days at 28°C.

Assay of Superoxide Anion Generation

Assay of superoxide anion generation was carried out using a modification of the cytochrome C reduction method described by KORCHAK *et al.*²⁾

Macrophages were obtained from peritoneal cavity of a guinea pig treated intraperitoneally with mineral oil. After removal of contaminating red cells by hypotonic hemolysis³⁾, the macrophages, which comprised over 90% of resultant cells, were used for the assay. The test compound dissolved in methanol or a control solution (10 μ l) was added to the cell suspension (1×10^6 macrophages/ml) in HEPES buffer (1 ml) containing cytochrome C (80 μ M) and bovine serum albumin (1 mg/ml). The mixture was preincubated for 1 minute at 37°C and then incubated for 2 minutes after the addition of *N*-formylmethionyl-leucyl-phenylalanine (FMLP, final conc: 10^{-7} M). The optical density of the suspension at 550 nm was recorded continuously. The extent of the superoxide anion generation was expressed in terms of absorbance change after the addition of FMLP, taking the difference of absorbance between the test compound and the control as 100%.

Results

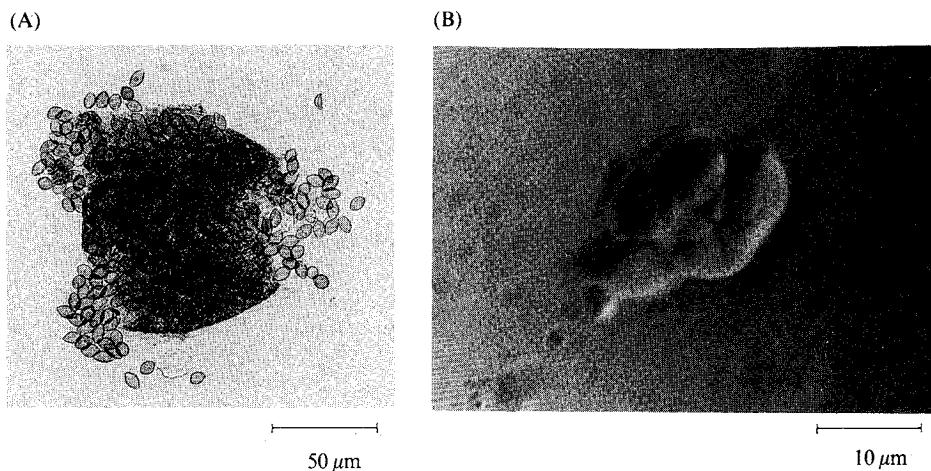
Taxonomy of the Producing Strain

On malt-extract agar, this strain grew abundantly to form cotton-like white to grey colonies with many ascocarps at 37°C. Back side of the colonies were white or dark green.

According to the microscopic observations, the ascocarps were superficial cleistothelia and globose or subglobose, having a size of 40 to 150 μ m i.d., dark brown, nakedness with smooth surface. The peridia were semitransparent. The evanescent asci were 8-spored and wide pyriform or ovoid, having a size of

Fig. 1. Micrograph of *Thielavia minor* OFR-1561.

(A) Ascospores in ascus, (B) ascocarp.



20~25 × 11~15 μm. The 1-celled ascospores with a single germ pore were smooth, ellipsoidal, having a size of 8~11 × 6~8 μm, at first hyaline, then turned to dark olive or dark brown, not dextrinoid. Conidia were not observed (Fig. 1).

Based on these morphological characteristics, the fungus was considered to belong to the genus *Thielavia* Zopf. The fungus was compared with *Thielavia* species described in the literature^{4,5}. As a result, it was found that this strain was closely related to *T. minor* (Rayss & Borut) Malloch & Cain. The strain was named as *T. minor* OFR-1561 and deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the accession No. FERM P-9514.

Isolation of OPC-15160 and OPC-15161

The culture broth was filtered with aid of diatomaceous earth. The filtrate (15 liters) was extracted with ethyl acetate (15 liters), and the extract was evaporated *in vacuo* to yield a brownish residue. The residue dissolved in a small amount of ethyl acetate was charged on a silica gel column (4 × 50 cm) that was developed successively with *n*-hexane-ethyl acetate (3:2) and *n*-hexane-ethyl acetate-methanol (30:20:1). Active fractions were evaporated *in vacuo* and chromatographed on a Sephadex LH-20 column (3 × 50 cm) using chloroform-methanol (3:2) as the eluent, to give a semi-pure solid. This was rechromatographed on an HPLC column (TSK-gel, ODS-80Tm, 2.15 × 30 cm) using methanol-H₂O (7:3) as the eluent. Pure OPC-15160 was obtained as a pale yellow powder (130 mg).

In the process of purifying OPC-15160, a new inhibitor, OPC-15161, was isolated in spite of there being none in the culture broth. This compound was therefore assumed to be a degradation product of the natural compound. After various degradation experiments OPC-15161 was determined to be a major degradation product formed under basic conditions.

The preparation of OPC-15161 was as follow; OPC-15160 (100 mg) was dissolved in 2N NaOH-methanol (1:2). The solution was kept at room temperature for 3 days, neutralized with 2N HCl and extracted with ethyl acetate. The extract was concentrated *in vacuo* to give an oily residue. The residue was chromatographed on a Sephadex LH-20 column (3 × 50 cm) with chloroform-methanol (3:2) to give a yellow powder. Recrystallization from methanol gave OPC-15161 as pale yellow prisms (35 mg).

Table 1. Physico-chemical properties of OPC-15160 and OPC-15161.

	OPC-15160	OPC-15161
MP (°C)	191~194	223.5~225.5
$[\alpha]_D^{25}$	775 (c 1, CHCl ₃)	0
UV λ_{max}^{EtOH} nm (ε)	232 (51,550), 287 (16,680), 352 (15,160)	220 (49,390), 279 (10,320), 354 (7,370)
IR (KBr) cm ⁻¹	3352, 2950, 1680, 1610, 1371, 1255, 1240, 750	3250, 2950, 1620, 1370, 1241, 735
EI-MS (m/z)	652 (M ⁺ , 0.4%), 328 (29), 309 (100), 268 (22), 266 (32), 231 (21), 155 (41), 130 (65)	327 (M ⁺ , 20%), 311 (21), 310 (42), 268 (36), 193 (67), 155 (33), 130 (100)
HR-MS (m/z) (M ⁺)	Calcd for C ₃₆ H ₄₀ N ₆ O ₆ : 652.3009	Calcd for C ₁₈ H ₂₁ N ₃ O ₃ : 327.1583
Found:	652.3014	327.1573
Elemental analysis	Calcd for C ₃₆ H ₄₀ N ₆ O ₆ ·H ₂ O: C 64.46, H 6.32, N 12.53	Calcd for C ₁₈ H ₂₁ N ₃ O ₃ : C 66.04, H 6.47, N 12.84
Found:	C 64.26, H 6.15, N 12.42	C 65.81, H 6.30, N 12.83
Rf value ^a	0.40	0.35

^a Silica gel TLC (Merck) solvent: Benzene-EtOAc-MeOH (15:5:1).

Table 2. ^1H NMR spectra of OPC-15160 and OPC-15161.

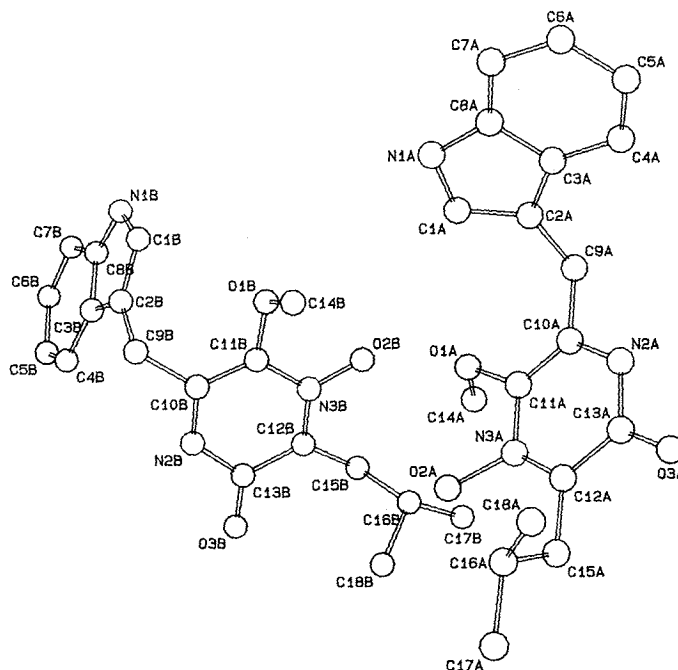
OPC-15160 (δ , DMSO- d_6)	OPC-15161 (δ , DMSO- d_6)
7.36 (1H, d, $J=7.6$), 7.12 (1H, s), 7.05 (1H, t, $J=7.6$), 6.67 (1H, t, $J=7.6$), 6.65 (1H, d, $J=7.6$), 6.03 (1H, br s), 3.86 (1H, d, $J=17.6$), 3.72 (3H, s), 3.47 (1H, d, $J=17.6$), 2.61 (1H, dd, $J=12.7, 7.1$), 2.53 (1H, dd, $J=12.7, 7.1$), 2.05 (1H, m), 0.84 (6H, d, $J=7.1$)	11.96 (1H, br s), 10.94 (1H, s), 7.57 (1H, d, $J=7.9$), 7.34 (1H, d, $J=7.9$), 7.24 (1H, br s), 7.07 (1H, t, $J=7.9$), 6.99 (1H, t, $J=7.9$), 3.92 (2H, s), 3.77 (3H, s), 2.60 (2H, d, $J=7.2$), 2.09 (1H, m), 0.85 (6H, d, $J=6.6$)

$J=\text{Hz}$.

Table 3. ^{13}C NMR spectra of OPC-15160 and OPC-15161.

OPC-15160 (δ , CD $_3$ OD)	OPC-15161 (δ , DMSO- d_6 at 40°C)
165.0 (s), 150.0 (s), 144.0 (s), 142.6 (s), 131.6 (d), 131.3 (s), 128.8 (s), 126.3 (d), 120.8 (d), 111.4 (d), 85.4 (d), 62.1 (q), 60.0 (s), 37.0 (t), 34.5 (t), 27.1 (d), 23.0 (q)	156.6 (s), 143.4 (s), 138.7 (s), 136.4 (s), 132.1 (s), 126.9 (s), 123.9 (d), 121.2 (d), 118.7 (d), 118.4 (d), 111.6 (d), 109.9 (s), 60.7 (q), 33.0 (t), 25.5 (d), 24.5 (t), 22.7 (q)

Fig. 2. X-Ray molecular structure of OPC-15161.



Physico-chemical Properties of OPC-15160 and OPC-15161

The physico-chemical properties of both compounds are summarized in Tables 1, 2 and 3. Both compounds are soluble in chloroform, ethyl acetate, methanol and dimethyl sulfoxide, but practically insoluble in water. OPC-15160 is

Fig. 3. Structure of OPC-15161.

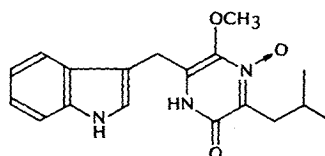
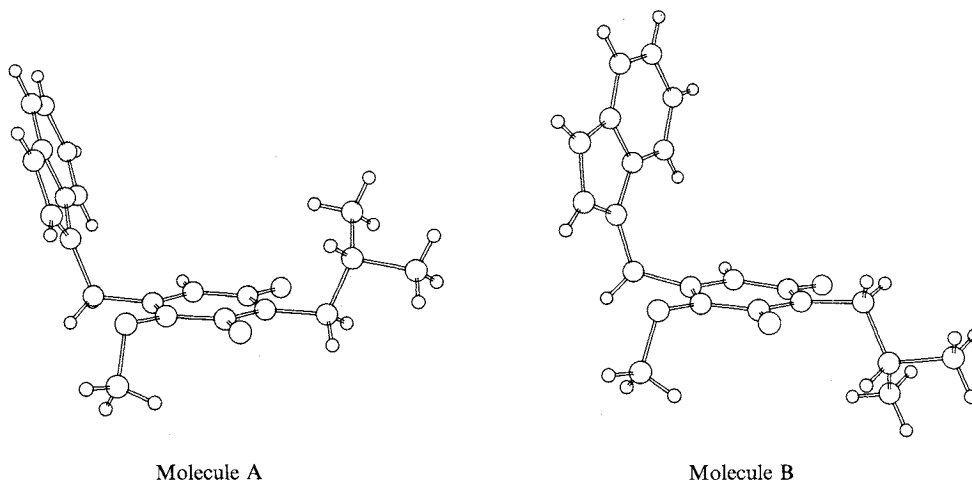


Fig. 4. The shapes of two independent molecules of OPC-15161.



optically active but OPC-15161 is inactive.

OPC-15160 has signals of 20 protons and 18 carbons in ^1H and ^{13}C NMR spectra, and the molecular formula is suggested to be $\text{C}_{36}\text{H}_{40}\text{N}_6\text{O}_6$ by means of MS and elemental analysis. Those results indicate that OPC-15160 should be a dimer of $\text{C}_{18}\text{H}_{20}\text{N}_3\text{O}_3$ unit. But the structure of OPC-15160 is not clear.

Structure Determination of OPC-15161 by X-Ray Analysis

An X-ray crystallographic analysis was carried out to determine the structure of OPC-15161. X-Ray diffraction measurements were performed on a Syntex R3 four circle diffractometer with graphite monochromated $\text{Mo K}\alpha$ radiation. The intensities were measured in the $\omega/2\theta$ scan mode, and corrected for Lorentz and polarization effects but not for absorption.

Crystal data: $\text{C}_{18}\text{H}_{21}\text{N}_3\text{O}_3$, triclinic, space group $\text{P}\bar{1}$, $a = 12.786(5)$, $b = 13.564(6)$, $c = 10.804(4)$ Å, $\alpha = 107.00(3)^\circ$, $\beta = 106.93(3)^\circ$, $\gamma = 86.07(3)^\circ$, $Z = 4$, $D_x = 1.27 \text{ g/cm}^3$ and $\mu(\text{Mo K}\alpha) = 1.0 \text{ cm}^{-1}$.

The structure was solved by direct method using MULTAN in Syntex XTL program⁶⁾. All hydrogen atoms except the methyl hydrogen atoms were located near expected positions in a difference Fourier map. The refinements of atomic parameters were carried out by a block-diagonal least-squares method. Thermal parameters were refined anisotropically for all non-hydrogen atoms and isotropic factors were used for the hydrogen atoms. Atomic scattering factors were taken from ref 7. A least-squares refinement on basis of 2,904 observed reflections led to a final $R = 0.078$.

A computer-generated drawing⁸⁾ of the structure is shown in Fig. 2. OPC-15161 is thus 6-(1*H*-indol-3-ylmethyl)-5-methoxy-3-(2-methylpropyl)-2(1*H*)-pyrazinone, 4-oxide (Fig. 3). The final atomic parameters have been deposited with the Crystallographic Data Centre.

There are two independent molecules (A and B) in the asymmetric unit, statistically identical to one another in terms of bond lengths and angles. But there is a clear difference between the conformation of molecule A and that of B. The shapes of the two independent molecules were compared by a least-square fitting technique (calculated by QUANTA program purchased from Polygen Inc., U.S.A.) for the pyrazinone rings of each molecule. The result is shown in Fig. 4. In molecule A the structure is arranged as a boat shape by indolyl methyl group, pyrazinone ring and isobutyl group, while in molecule B it is arranged as

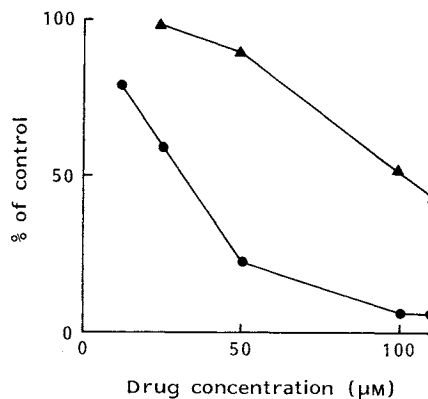
a chair shape with above components. The dihedral angles between indolyl methyl group and pyrazinone ring (C(2)-C(9)-C(10)-C(11)) are similar (-75.37° for molecule A and -94.84° for molecule B), but those for pyrazinone ring and isobutyl group (N(3)-C(12)-C(15)-C(16)) are different (-86.73° and 79.77° , respectively).

Biological Activities of OPC-15160 and OPC-15161

The inhibitory activities of OPC-15160 and OPC-15161 on superoxide anion generation by guinea pig peritoneal macrophages are shown in Fig. 5. Both compounds showed dose dependent inhibitory activity. OPC-15161 was five times as active as OPC-15160. The IC_{50} values of OPC-15160 and OPC-15161 were $1.4 \times 10^{-4} M$ and $2.8 \times 10^{-5} M$, respectively.

Fig. 5. Dose-dependent inhibition of FMLP-induced superoxide anion generation by OPC-15160 and OPC-15161.

Each compound was tested twice. ▲ OPC-15160, ● OPC-15161.



Discussion

In the course of searching for inhibitors of superoxide anion generation by macrophages, we found that fungus *T. minor* OFR-1561 produces a novel inhibitor and that a major degradation product of the natural inhibitor, OPC-15161, has potent inhibitory activity.

This is the first isolation of 4-oxygenated and highly functionalized pyrazine, OPC-15161, though a few natural products possessing the closely related 1-oxygenated-5-methoxy-2-pyrazinone moiety have been isolated from fungi^{9,10}. This novel moiety may contribute to the biological activity of the molecule.

Recent studies suggest that oxygen free radicals, especially superoxide anion released by macrophages or neutrophils, contribute to production of tissue damage in ischemic or inflammatory processes and that inhibitors of superoxide anion generation are effective in protecting against tissue damage in *in vitro* and *in vivo* models of ischemia or inflammation^{11~15}.

These compounds therefore have promise for chemotherapeutic use in the above-mentioned disease.

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