Incarnal. A New Antibacterial Sesquiterpene from Basidiomycetes

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A new sesquiterpene, incarnal, was isolated from culture fluid of *Gloeostereum incarnatum* (Japanese name: Nikawaurokotake). Incarnal inhibited the growth of gram-positive bacteria at 6.25–12.5 µg/ml. The structure of incarnal has been determined to be (1-hydroxy-2,10,10-trimethyl)-3-methylene-tricyclo[6.3.0.0^{2,6}]undec-5,7-diene-4,9-dione by X-ray diffraction and spectroscopic methods.

Keywords sesquiterpene; X-ray diffraction; antibiotic; basidiomycetes; *Gloeostereum incarnatum*; incarnal

An extract of basidioma of *G. incarnatum* inhibits the growth of *Shigella dysenteriae*, *Pseudomonas pyocynea*, *Staphylococcus aureus*, *Escherichia coli* and *Bacillus subtilis*.¹⁾ In the present paper, we describe the isolation, antibacterial activities and three dimensional structure of a new hirsutane-type sesquiterpene, incarnal (1), which was isolated from culture fluid of *G. incarnatum*.

Experimental

The melting point was determined with a Yazawa micro melting point apparatus and is uncorrected. Proton and carbon-13 nuclear magnetic resonance (¹H- and ¹³C-NMR) spectra were obtained on a JEOL JNM-GX270 spectrometer. Chemical shifts (δ) are reported in ppm from internal tetramethylsilane. The mass spectrum (MS) was determined with a JEOL JMS-D300 mass spectrometer. The infrared (IR) spectrum was measured on a JASCO IR-810 spectrometer, and the ultraviolet (UV) spectrum on a Hitachi U-3200.

Fermentation and Isolation Procedure *G. incarnatum* was grown in 10 l of GYP medium composed of (g/l): glucose 50.0, polypeptone 2.5, yeast extract 2.5, KH₂PO₄ 1.0, MgSO₄·7H₂O 0.5, CaCl₂ 0.5, pH 5.0, 121 °C, 15 min. The shaken culture obtained from the spores was kept at 25 °C for 10 d. After fermentation, the mycelia were separated from the culture fluid by filtration. Incarnal was extracted from the culture fluid (9.5 l) with ethyl acetate (4 l). After evaporation of the solvent, the crude product (2.1 g) was applied to a column on silica gel (Fuji Davison 4B) and eluted with *n*-hexane-ether. The fractions containing incarnal were combined to yield 0.70 g of a yellow solid. Pure incarnal (0.57 g) was obtained by crystallization from ether.

A mycelial culture of *G. incarnatum* was also tested for the production of antibacterial activity. For maintenance on agar slants, the strain was grown in potato dextrose agar medium (Difco).

X-Ray Crystallographic Analysis Single crystals were grown from an ether solution by slow evaporation. A yellow prism elongated along *c*, with dimensions of 0.11 × 0.08 × 0.30 mm, was mounted on a Rigaku AFC-5 four-circle diffractometer equipped with a rotating anode (Ni-filtered CuK_α, 40 kV, 200 mA). The lattice parameters were determined with 20 reflections in the range of 36 < 2θ < 41°. Systematic absences were *h*00 for *h* odd, 0*k*0 for *k* odd, 00*l* for *l* odd.

Crystal data: C₁₅H₁₆O₃, *M*_r = 244.29, mp = 405–406 K, orthorhombic, *P*2₁2₁2₁, *a* = 12.019 (1), *b* = 17.690 (1), *c* = 6.282 (1) Å, *V* = 1335.6 (3) Å³, *Z* = 4, *D*_x = 1.215 Mg·m⁻³, CuK_α (λ = 1.54178 Å), μ = 0.64 mm⁻¹, *F*(000) = 520.

Intensity data were collected at 25 °C within 2θ_{max} = 120° (0 ≤ *h* ≤ 13, 0 ≤ *k* ≤ 19, 0 ≤ *l* ≤ 7) by the ω-2θ scan method [scan speed 4° min⁻¹ in ω, scan range in ω 1.2° + 0.15° tan θ]. Background was measured for 4 s on either side of the peak; three standard reflections were recorded after every

97 reflections, fluctuations being within 0.8%. Lorentz and polarization corrections were applied, but no absorption correction was made. In total, 1174 unique reflections were measured, 1099 reflections being larger than σ(*F*_o). All unique reflections were used in structure analysis. The structure was solved by MULTAN78 and refined by the block-diagonal least-squares method (non-H atoms anisotropically). The H atoms were determined from a difference Fourier map and refined isotropically; Σw(|*F*_o| - |*F*_c|)² was minimized with *w* = 1.0/[σ(*F*_o)² - 0.0922|*F*_o| + 0.0055|*F*_o|²] for |*F*_o| > 0, *w* = 0.6347 for |*F*_o| = 0. Final *R* = 0.050 for 1174 reflections, *wR* = 0.044, *S* = 1.79. (Δ/σ)_{max} = 0.21 for non-H atoms and 0.74 for H atoms. The residual electron density in the final difference map was within ±0.16 eÅ⁻³. Atomic scattering factors were taken from "International Tables for X-Ray Crystallography".²⁾ Programs: MULTAN78,³⁾ HBLS-V and DAPH,⁴⁾ MOLCON⁵⁾ and ORTEP.⁶⁾ Computations were carried out at the Research Center for Protein Engineering, Institute for Protein Research, Osaka University, and at the Okayama University Computer Center.

Results and Discussion

Physical and Spectroscopic Data Soluble in dimethyl sulfoxide (DMSO), methanol, acetone, ethyl acetate, ether and chloroform; insoluble in water and *n*-hexane; mp 132–133 °C; *R*_f 0.35 (chloroform: ether: acetic acid = 50: 50: 1). *Anal.* Calcd for C₁₅H₁₆O₃: C, 73.77; H, 6.56. Found:

TABLE I. ¹H- and ¹³C-NMR Data (in CDCl₃, 270 and 67.9 MHz)

H-atom	δ multiplicity, J [Hz]	C-atom	δ multiplicity, J [Hz]
5	6.41 s	1	83.67 s
7	7.33 s	2	63.22 s
11	2.04 d 5.1	3	147.60 s
	2.17 d 5.1	4	207.44 s
12	1.29 s	5	128.34 D 78.2
13	5.41 s	6	184.52 s
	5.92 s	7	126.49 D 88.1
14	1.18 s	8	157.40 s
15	1.11 s	9	195.45 s
OH	5.13 s	10	51.10 s
		11	42.52 T 28.8
		13	116.66 T 62.9
		-CH ₃	26.01 Q 22.5
			26.50 Q 22.5
			27.35 Q 23.3

TABLE II. Antibacterial Spectra

Test organism ^{a)}	MIC (µg/ml)
<i>Staphylococcus aureus</i> FDA 209P	6.25
<i>Bacillus subtilis</i> ATCC 6633	12.5
<i>Escherichia coli</i> NIHJ JC-2	> 100
<i>Pseudomonas aeruginosa</i>	> 100

a) Mueller Hinton agar, 36 °C, 18 h. MIC, minimal inhibitory concentration.

Chart 1. Incarnal (1)

TABLE III. Final Atomic Coordinates ($\times 10^4$) and Equivalent Isotropic Thermal Parameters ($\text{\AA}^2$) with e. s. d.'s in Parentheses

Atoms	x	y	z	B_{eq}
O(1)	1014 (1)	3687 (1)	754 (3)	4.59 (8)
O(2)	4180 (2)	2122 (1)	669 (4)	8.0 (1)
O(3)	743 (2)	5654 (1)	3561 (4)	7.4 (1)
C(1)	1147 (2)	3669 (1)	3019 (4)	3.8 (1)
C(2)	2107 (2)	3127 (1)	3664 (5)	4.4 (1)
C(3)	2425 (2)	2478 (1)	2261 (5)	4.8 (1)
C(4)	3624 (2)	2572 (2)	1695 (5)	5.6 (2)
C(5)	3967 (2)	3309 (2)	2464 (5)	5.7 (2)
C(6)	3100 (2)	3650 (1)	3389 (5)	4.5 (1)
C(7)	2737 (2)	4424 (2)	3787 (5)	4.9 (1)
C(8)	1629 (2)	4429 (1)	3546 (5)	4.3 (1)
C(9)	701 (2)	4965 (1)	3743 (5)	5.0 (1)
C(10)	-354 (2)	4513 (2)	4185 (5)	5.1 (2)
C(11)	27 (2)	3682 (2)	4146 (5)	4.9 (1)
C(12)	2030 (2)	2870 (2)	5996 (5)	6.2 (2)
C(13)	1809 (3)	1906 (2)	1579 (7)	7.8 (2)
C(14)	-1224 (3)	4682 (2)	2492 (7)	8.0 (2)
C(15)	-775 (3)	4746 (2)	6392 (7)	7.5 (2)

$$B_{eq} = (4/3) \sum \beta_{ii} a_i^2$$

TABLE IV. Bond Lengths ($\text{\AA}$) and Angles ($^\circ$) for the Non-Hydrogen Atoms with e. s. d.'s in Parentheses

O(1)-C(1)	1.432 (4)	C(3)-C(15)	1.325 (6)
O(2)-C(4)	1.223 (4)	C(4)-C(5)	1.450 (4)
O(3)-C(9)	1.225 (4)	C(5)-C(6)	1.337 (4)
C(1)-C(2)	1.554 (4)	C(6)-C(7)	1.459 (4)
C(1)-C(8)	1.501 (4)	C(7)-C(8)	1.340 (4)
C(1)-C(11)	1.521 (4)	C(8)-C(9)	1.469 (4)
C(2)-C(3)	1.497 (4)	C(9)-C(10)	1.525 (4)
C(2)-C(6)	1.520 (4)	C(10)-C(11)	1.540 (4)
C(2)-C(12)	1.537 (4)	C(10)-C(14)	1.521 (5)
C(3)-C(4)	1.494 (4)	C(10)-C(15)	1.532 (5)
O(1)-C(1)-C(2)	110.8 (2)	C(4)-C(5)-C(6)	109.2 (3)
O(1)-C(1)-C(8)	104.0 (2)	C(2)-C(6)-C(5)	112.8 (3)
O(1)-C(1)-C(11)	111.3 (2)	C(2)-C(6)-C(7)	108.5 (3)
C(2)-C(1)-C(8)	102.0 (2)	C(5)-C(6)-C(7)	137.0 (3)
C(2)-C(1)-C(11)	123.0 (3)	C(6)-C(7)-C(8)	106.5 (3)
C(8)-C(1)-C(11)	103.0 (2)	C(1)-C(8)-C(7)	113.7 (3)
C(1)-C(2)-C(3)	120.6 (3)	C(1)-C(8)-C(9)	107.7 (3)
C(1)-C(2)-C(6)	100.2 (2)	C(7)-C(8)-C(9)	138.5 (3)
C(1)-C(2)-C(12)	112.7 (2)	O(3)-C(9)-C(8)	127.1 (3)
C(3)-C(2)-C(6)	101.5 (2)	O(3)-C(9)-C(10)	125.0 (3)
C(3)-C(2)-C(12)	110.5 (3)	C(8)-C(9)-C(10)	108.0 (3)
C(6)-C(2)-C(12)	109.6 (2)	C(9)-C(10)-C(11)	104.5 (2)
C(2)-C(3)-C(4)	107.5 (3)	C(9)-C(10)-C(14)	110.0 (3)
C(2)-C(3)-C(13)	129.3 (3)	C(9)-C(10)-C(15)	107.4 (3)
C(4)-C(3)-C(13)	123.2 (3)	C(11)-C(10)-C(14)	112.4 (3)
O(2)-C(4)-C(3)	125.5 (3)	C(11)-C(10)-C(15)	111.7 (3)
O(2)-C(4)-C(5)	127.3 (3)	C(14)-C(10)-C(15)	110.7 (3)
C(3)-C(4)-C(5)	107.2 (3)	C(1)-C(11)-C(10)	106.6 (2)

C, 73.86; H, 6.55. UV $\lambda_{\text{max}}^{\text{EtOH}}$ nm (log ϵ): 314.4 (4.21). IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3340 (st), 2950–2870 (m), 1720 (st), 1690 (st), 1650 (st), 1600 (st), 1160 (m), 1000 (m), 940 (m), 860 (m). ^1H - and ^{13}C -NMR spectral data are listed in Table I. MS m/z : 244 (100, M^+), 229 (50.6), 160 (71.5), 132 (99.7), 104 (88.7). The IR, ^1H - and ^{13}C -NMR spectra showed the presence of $\geq \text{C}-\text{OH}$, $2 > \text{C}=\text{CH}-$, $> \text{C}=\text{CH}_2$, $2 > \text{C}=\text{O}$ and $3 -\text{CH}_3$ groups.

Biological Assays The antibacterial spectra obtained by the agar dilution method are shown in Table II. Incarnal inhibited the growth of gram-positive bacteria at 6.25–12.5 $\mu\text{g}/\text{ml}$, but it had no antibacterial activity against

Fig. 1. ORTEP Drawing with Atomic Numbering

Ellipsoids of 50% probability are drawn for the non-H atoms; the H atoms are represented as spheres equivalent to $B=1.0 \text{\AA}^2$.

gram-negative bacteria.

Three Dimensional Structure The final positional and thermal parameters for non-hydrogen atoms are given in Table III. Bond lengths and angles are listed in Table IV. An ORTEP drawing of the molecule with the atomic numbering is shown in Fig. 1.

The molecular skeleton is composed of three five-membered rings. The largest distortions in the skeleton are found around the ring-junction bonds C(2)–C(6) and C(1)–C(8): C(1)–C(2)–C(6)–C(7) $29.4 (3)^\circ$ and C(11)–C(1)–C(8)–C(9) $-31.8 (3)^\circ$. The C(1)–C(2) bond is lengthened by a short 1,4-contact, C(11)···C(12) $3.035 (4) \text{\AA}$. The double bond character of the C(5)–C(6) and C(7)–C(8) bonds is clearly evidenced by their short bond lengths. In the crystal, the molecules related by a 2_1 axis along a are linked by an O–H···O hydrogen bond [O(1)···O(2) $2.776 (4)$, H(1)···O(2) $1.86 (4) \text{\AA}$, O(1)–H(1)···O(2) $172 (4)^\circ$].

Sesquiterpenes comprise the majority of the isoprene metabolites isolated from basidiomycetes.⁷⁾ They are classified into hirsutanes, protoilludanes, illudanes, marasmanes, sterpranes, fomannosanes, illudalanes, secoilludalanes, lactalanes, secolactalanes and isolactalanes. The structure determination revealed that incarnal is a hirsutane-type compound, a category which includes coriolins,⁸⁾ complicitic acid,⁹⁾ hypnophilin¹⁰⁾ and pleuroteic acid.¹⁰⁾ The X-ray structure determination of hexahydrocoriolin has been reported.¹¹⁾ However, the present work is the first to elucidate a sesquiterpene structure composed of highly conjugated five-membered rings. Substances like incarnal have been detected during the synthesis of *dl*-coriolins¹²⁾ and among terpenoid metabolites of mushrooms.⁷⁾ Therefore, it can be assumed that incarnal is also a terpenoid metabolite.

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