# Russuphelin A, a New Cytotoxic Substance from the Mushroom *Russula subnigricans* HONGO

Akira TAKAHASHI,[a] Tsutomu AGATSUMA,[a] Mamoru MATSUDA,[a] Tomihisa OHTA,[a] Tetsuji NUNOZAWA,[b] Takeshi ENDO[b] and Shigeo NOZOE*,[a]

*Faculty of Pharmaceutical Sciences, Tohoku University,[a] Aoba-ku, Sendai 980, Japan and Fuji Chemical Industry Co., Ltd.,[b] Nakaniikawa-gun, Toyama 930–03, Japan.* *Received May 21, 1992*

**A new cytotoxic substance, designated russuphelin A (1), has been isolated from the mushroom *Russula subnigricans* HONGO (Basidiomycetes). The structure was elucidated as 2,6-bis(2,6-dichloro-4-hydroxyphenyloxy)-1,4-dimethoxybenzene on the basis of spectroscopic data and confirmed by total synthesis.**

**Keywords** cytotoxic substance; russuphelin A; 2,6-bis(2,6-dichloro-4-hydroxyphenyloxy)-1,4-dimethoxybenzene; basidiomycete; mushroon; *Russula subnigricans*; structure elucidation; total synthesis

Previously, we reported the characterization and structure elucidation of (2*S*,3*R*)-(−)-3-hydroxybaikiain, a new amino acid isolated from the mushroom *Russula subnigricans* HONGO (Basidiomycetes) (Japanese name: nisekurohatsu).[1)] In our continuing search for biologically active metabolites, an intriguing chlorinated triphenylether, designated russuphelin A (**1**), has been isolated from the same fungus as a new cytotoxic substance. In this paper, we wish to describe the isolation, structure elucidation and cytotoxic activity of russuphelin A (**1**), as well as its total synthesis.

## Results and Discussion

**Isolation** The fruiting bodies (1.18 kg) of *R. subnigricans* HONGO, collected at Miyagi Prefecture in August 1989, were extracted twice with methanol (5 l) at room temperature for 2 d. The extract was concentrated *in vacuo* to an aqueous suspension, which was subsequently partitioned between ethyl acetate and *n*-butanol. After evaporation, the ethyl acetate-soluble material (5.63 g) was applied to a silica gel column (60 g: 3.5 cm i.d. × 14.5 cm), and the active fractions eluted with chloroform–ethyl acetate (9 : 1 and 8 : 2) were rechromatographed on silica gel with chloroform–ethyl acetate mixture, followed by recrystallization from chloroform–methanol to give russuphelin A (**1**, 292.1 mg) as colorless needles: mp 293—294 °C.

**Structure Elucidation** The physico-chemical properties of russuphelin A (**1**) are summarized in Table I. The molecular formula was deduced to be $C_{20}H_{14}O_6Cl_4$ based on the high resolution (HR) electron impact (EI) mass spectrum (MS) of **1**. The presence of four chlorine atoms was also indicated by the molecular ion cluster at *m/z* 490, 492, 494, 496, 498. The phenolic nature of **1** was suggested by the IR absorptions (3700—2900, 1610, 1580, 1510 cm$^{-1}$) and the existence of two hydroxyl groups was confirmed by the formation of the diacetate (**2**) on treatment of **1** with $Ac_2O$ in pyridine. The $^1$H- and $^{13}$C-NMR spectral data for **1** are shown in Table II. The $^1$H-NMR spectrum displayed only four singlets assignable to two methoxyls (δ 4.01; 3.48) and six aromatic protons [δ 6.92 (4H); 5.61 (2H)]. Irradiation of the H-3 (H-5) signal at δ 5.61 resulted in a 10% nuclear Overhauser effect (NOE) for the methoxyl protons (δ 3.48), indicating that one of the methoxyl groups was located on C-4. The $^{13}$C-NMR spectrum of **1** revealed only ten carbon signals due to two methoxyl and eight aromatic carbons, accounting for half of the carbons. The

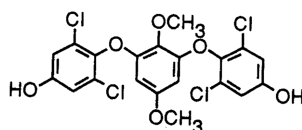


Fig. 1. Structure of Russuphelin A (**1**)

TABLE I. Physico-Chemical Properties of Russuphelin A (**1**)

| | |
|---|---|
| Appearance | Colorless needles |
| mp (°C) | 293—294 |
| EI-MS *m/z* | 490 (M$^+$), 492 (M$^+$+2), 494 (M$^+$+4), 496 (M$^+$+6), 498 (M$^+$+8) |
| Formula | $C_{20}H_{14}Cl_4O_6$ |
| HR EI-MS | Calcd 490.0223<br>Found 490.0225 |
| IR $\nu_{max}^{KBr}$ cm$^{-1}$ | 3700—2900 (OH), 1610, 1580, 1510 |
| UV $\lambda_{max}^{MeOH}$ nm (log ε) | 228 (4.75), 237 (4.55), 283 (4.10), 293 (4.05) |

TABLE II. $^1$H- and $^{13}$C-NMR Spectral Data for Russuphelin A (**1**) in $CD_3OD$ and Its Acetate (**2**) in $CDCl_3$[a)]

| Position | 1 | | 2 | |
|---|---|---|---|---|
| | $^1$H | $^{13}$C[b)] | $^1$H | $^{13}$C[b)] |
| 1 | | 132.3 (1C, s) | | 131.7 (1C, s) |
| 2, 6 | | 153.0 (each 1C, s) | | 150.9 (each 1C, s) |
| 3, 5 | 5.61 (each 1H, s) | 94.8 (each 1C, d) | 5.64 (each 1H, s) | 94.6 (each 1C, d) |
| 4 | | 157.2 (1C, s) | | 155.4 (1C, s) |
| 1′ | | 140.2 (2C, s) | | 144.4 (2C, s) |
| 2′, 6′ | | 130.6 (each 2C, s) | | 129.4 (each 2C, s) |
| 3′, 5′ | 6.92 (each 2H, s) | 117.1 (each 2C, d) | 7.10 (each 2H, s) | 122.5 (each 2C, d) |
| 4′ | | 156.9 (2C, s) | | 147.3 (2C, s) |
| 1-$OCH_3$ | 4.01 (3H, s) | 61.8 (1C, q) | 3.95 (3H, s) | 61.2 (1C, q) |
| 4-$OCH_3$ | 3.48 (3H, s) | 55.8 (1C, q) | 3.42 (3H, s) | 55.3 (1C, q) |
| 4′-$OCOCH_3$ | | | 2.18 (6H, s) | 20.7 (2C, q)<br>168.3 (2C, s) |

*a)* $^1$H- and $^{13}$C-NMR spectra were recorded at 500 MHz and 125 MHz, respectively. *b)* Multiplicities were determined from distortionless enhancement by polarization transfer (DEPT) data.

© 1992 Pharmaceutical Society of Japan

NII-Electronic Library Service

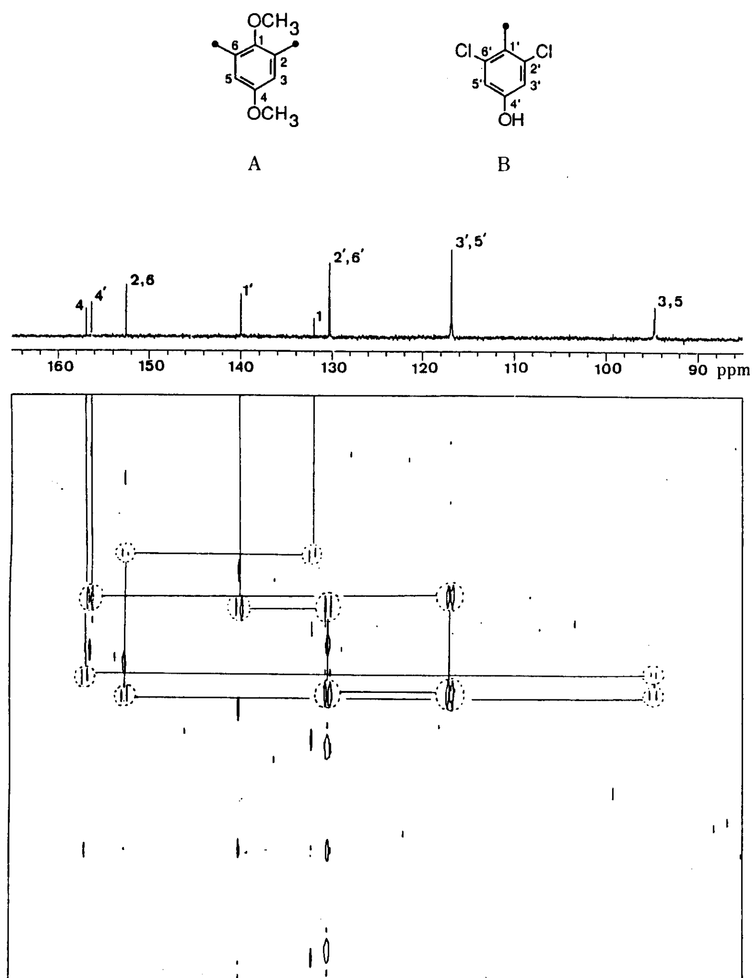


Fig. 2. 2D INADEQUATE Spectrum of Russuphelin A in $CD_3OD$

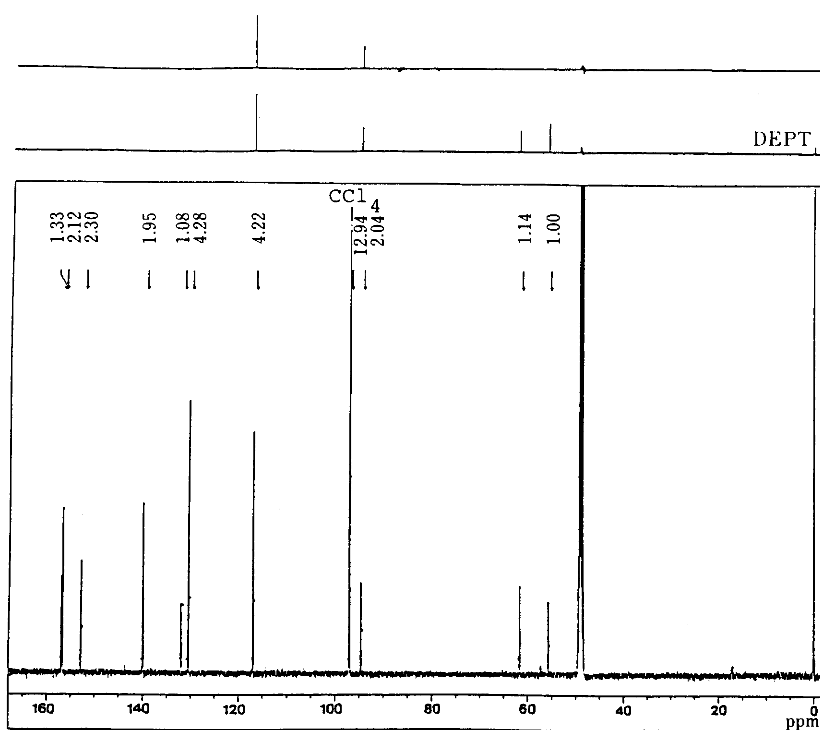


Fig. 3. NOE Suppressed $^{13}$C-NMR Spectrum of Russuphelin A ($CD_3OD$, 125 MHz)

NII-Electronic Library Service

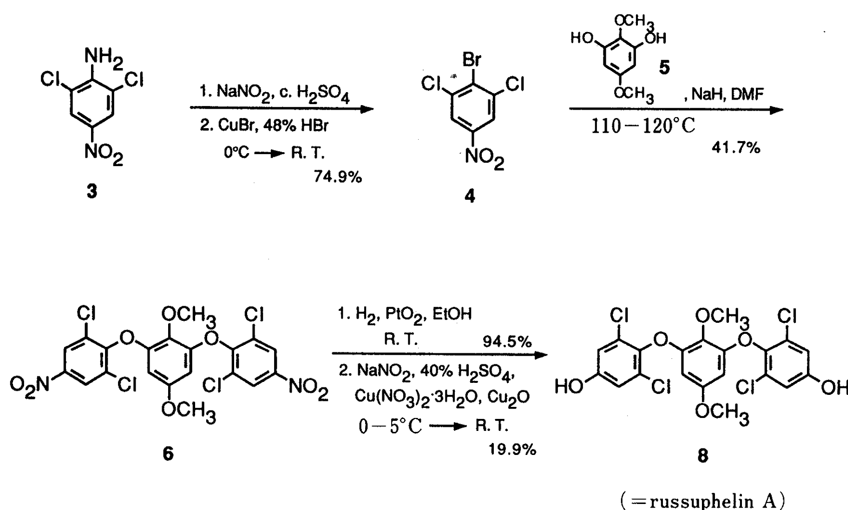


Chart 1

signal at $\delta$ 156.9 was assigned to the carbon (C-4′) bearing a hydroxyl group, since an acetylation shift was observed in the signal at $\delta$ 147.2 due to the corresponding carbon of the acetate (**2**). The connectivities of carbons from C-1 to C-4 and from C-1′ to C-4′ were clarified by the two dimensional (2D) incredible natural abundance double quantum transfer experiment (INADEQUATE) spectrum of **1**, which showed cross peaks for C-1/C-2(C-6), C-2(C-6)/C-3(C-5), C-3(C-5)/C-4, C-1′/C-2′(C-6′), C-2′(C-6′)/C-3′(C-5′), and C-3′(C-5′)/C-4′ (Fig. 2). Further, the signal intensities of carbons were deduced to be as shown in Fig. 3 based on the NOE suppressed $^{13}$C-NMR spectrum of **1**.[2] Taking into consideration these results and the molecular formula, russuphelin A should possess a symmetrical structure containing one partial structure A and two partial structures B. Partial structures A and B (×2) accounted for $C_{20}H_{14}O_4Cl_4$ with twelve degrees of unsaturation and therefore, only two oxygen atoms remained to be assigned. Accordingly, the two partial structures B should be linked to partial structure A with ether linkages. Thus the structure of russuphelin A was concluded to be 2,6-bis(2,6-dichloro-4-hydroxyphenyloxy)-1,4-dimethoxybenzene (**1**), as shown in Fig. 1.

**Total Synthesis** The proposed structure of russuphelin A was confirmed by the total synthesis of **1**. The synthetic route is outlined in Chart 1.

First, commercially available 2,6-dichloro-4-nitroaniline (**3**) was subjected to the Sandmeyer reaction to afford the bromo compound (**4**) in 74.9% yield. The *S*N2 reaction of **4** with the phenoxide obtained from 2,5-dimethoxyresorcinol (**5**) and sodium hydride proceeded at 120 °C for 3 h to give the coupling product (**6**) in 41.7% yield.[3] Compound **5** was prepared from pyrogallol through 5 steps in 19.6% overall yield according to the method of Morita.[4] The triphenylether (**6**) was submitted to the catalytic hydrogenation over $PtO_2$ in ethanol to afford the diamine (**7**) in nearly quantitative yield and then the amino groups of **7** were converted into hydroxyl groups *via* the diazo functions to give **8**,[5] which was identical with natural russuphelin A.

**Cytotoxic Activity of Russuphelin A** The *in vitro* cytotoxic activity of russuphelin A is shown in Table III. Russuphelin A displayed potent cytotoxic activities against various tumor cells, especially human derived carcinoma cells. Therefore, a more detailed evaluation of the antitumor activity of russuphelin A is in progress. The details will be published in the near future.

Table III. Cytotoxic Activity of Russuphelin A (**1**) *in Vitro*

| Cell line | $IC_{50}$ (μg/ml) |
|---|---|
| L1210 | 2.47 |
| Colon 26 | 10.16 |
| P388 | 4.64 |
| P388/ADM | 2.96 |
| KB | 2.09 |
| B16 | 6.28 |
| A549 | 0.48 |
| DLD-1 | 0.79 |
| K562 | 0.16 |
| CCRF-CEM | 0.49 |

## Experimental

Melting points were determined on a Yanagimoto micro hot plate and are uncorrected. The spectroscopic data were measured by using the following instruments: IR spectra, JASCO A-100S IR spectrometer; UV spectrum, Hitachi U-3200 spectrophotometer; EI and HR MS, JEOL JMS DX-303; $^1$H- and $^{13}$C-NMR spectra, JEOL JNM GX-500 (500 and 125 MHz, respectively). NMR spectra were recorded with tetramethylsilane (TMS) as an internal standard. Chemical shifts are shown in $\delta$ (ppm) and multiplicities are indicated as follows: singlet = s, doublet = d and br = broad. TLC analyses were performed on Kieselgel 60 $F_{254}$ (Merck) and spots were detected under UV irradiation and by heating on a hot plate after spraying 50% sulfuric acid and anisaldehyde–sulfuric acid reagents.

**Acetylation of Russuphelin A (1)** A solution of **1** (30.0 mg) in pyridine (200 μl) was treated with $Ac_2O$ (150 μl) and the reaction mixture was left to stand at room temperature overnight. After usual work-up, the crude crystalline compound obtained was recrystallized from *n*-hexane–$CHCl_3$ to give the diacetate (**2**), 25.2 mg as colorless needles. mp 172—174 °C. EM-MS *m*/*z*: 574 ($M^+$), 576 ($M^+$+2), 578 ($M^+$+4), 580 ($M^+$+6), 582 ($M^+$+8). IR $\nu_{max}^{CHCl_3}$ cm$^{-1}$: 3090, 3040, 3005, 2940, 2840, 1765 (ester C=O), 1600, 1575, 1505. $^1$H-NMR: see Table II.

**Synthesis of Russuphelin A. 2,5-Dimethoxyresorcinol (5)** Compound **5** was prepared from pyrogallol (10.0 g, 79.4 mmol) through 5 steps in 19.6% (2.7 g) overall yield according to the method of Morita.[4] mp 76—78 °C (lit. 60—61 °C). EI-MS *m*/*z*: 170 ($M^+$), 155, 127. IR $\nu_{max}^{CHCl_3}$ cm$^{-1}$: 3400 (br, OH), 3000, 2950, 1630, 1600, 1520, 1510. $^1$H-NMR ($CDCl_3$) $\delta$: 3.72 (3H, s, OC$\underline{H}_3$), 3.81 (3H, s, OC$\underline{H}_3$), 5.40 (2H, br s, O$\underline{H}$), 6.09 (2H, s, H-4, 6).

**1-Bromo-2,6-dichloro-4-nitrobenzene (4)** A stirred solution of $NaNO_2$ (3.4 g, 49.3 mmol) in concentrated $H_2SO_4$ (25 ml) was treated with AcOH

NII-Electronic Library Service

(29 ml) at 10 °C and then 2,6-dichloro-4-nitroaniline (**3**: 10.0 g, 48.5 mmol) was added over 30 min. This reaction mixture was added dropwise to a solution of CuBr (8.3 g, 57.9 mmol) in 48% aqueous HBr (25 ml) with vigorous stirring. Stirring was continued for 1 h, then the reaction mixture was poured into ice-water (500 ml) and the precipitates was collected. The crude crystalline product was dissolved in $CHCl_3$ (200 ml) and the solution was washed with saturated aqueous $NaHCO_3$, water and saturated aqueous NaCl, then dried over anhydrous $Na_2SO_4$. After evaporation of the solvent, the residue was recrystallized from *n*-hexane–$CHCl_3$ to afford the bromo compound (**4**), 9.8 g (74.9%) as yellow needles. mp 89—90 °C. EI-MS *m/z*: 269 ($M^+$). HR-MS *m/z*: 268.8623 ($M^+$); Calcd for $C_6H_2BrCl_2NO_2$: 268.8646. IR $\nu_{max}^{CHCl_3}$ $cm^{-1}$: 3070, 1530 ($NO_2$), 1340 ($NO_2$), 890. $^1$H-NMR ($CDCl_3$) $\delta$: 8.15 (2H, s, H-3, 5).

**2,6-Bis(2,6-dichloro-4-nitrophenyloxy)-1,4-dimethoxybenzene (6)** A solution of **5** (200 mg, 1.2 mmol) in dry *N,N*-dimethylformamide (DMF) (2 ml) was added to a stirred suspension of NaH (60% in mineral oil: 103.0 mg, 2.6 mmol) in dry DMF (2 ml) and the resulting mixture was stirred at room temperature for 30 min. Then **4** (760 mg, 2.8 mmol) in dry DMF (2 ml) was added and the reaction mixture was heated at 110—120 °C for 3 h. After cooling, the reaction mixture was poured into ice-water and extracted with EtOAc (50 ml × 2). The combined extracts were washed with water and saturated aqueous NaCl, then dried over anhydrous $MgSO_4$. After evaporation of the solvent, the residue was chromatographed on silica gel with *n*-hexane–$CHCl_3$ (1:1, 1:3) as the eluent to give the triphenylether (**6**), 268.8 mg (41.7%) as yellow needles. mp 155—156 °C. EI-MS *m/z*: 548 ($M^+$), 550 ($M^+ + 2$), 552 ($M^+ + 4$), 554 ($M^+ + 6$). HR-MS *m/z*: 547.9364 ($M^+$); Calcd for $C_{20}H_{12}Cl_4N_2O_8$: 547.9349. IR $\nu_{max}^{CHCl_3}$ $cm^{-1}$: 3080, 3000, 1600, 1570, 1530, 1500. $^1$H-NMR ($CDCl_3$) $\delta$: 3.60 (3H, s, $OC\underline{H}_3$), 3.92 (3H, s, $OC\underline{H}_3$), 5.87 (2H, s, H-3, 5), 8.32 (4H, s, H-3′, 5′).

**2,6-Bis(4-amino-2,6-dichlorophenyloxy)-1,4-dimethoxybenzene (7)** The triphenylether (**6**: 95.4 mg, 0.17 mmol) was hydrogenated in EtOH over $PtO_2$ (1.9 mg) for 3 h until a clear solution was obtained. After removal of the catalysts by filtration, the filtrate was concentrated under reduced pressure and the residue was subjected to silica gel chromatography using *n*-hexane–EtOAc as the eluent to give the diamine (**7**), 79.1 mg (94.5%) as colorless needles. mp 109—110 °C. EI-MS *m/z*: 488 ($M^+$), 450 ($M^+ + 2$), 452 ($M^+ + 4$), 454 ($M^+ + 6$). HR-MS *m/z*: 487.9868 ($M^+$); $C_{20}H_{16}Cl_4N_2O_4$: 487.9864. IR $\nu_{max}^{CHCl_3}$ $cm^{-1}$: 3480 (NH), 3400 (NH), 3000, 1620, 1600, 1565, 1500, 1470, 1430. $^1$H-NMR ($CDCl_3$) $\delta$: 3.51 (3H, s, $OC\underline{H}_3$), 3.76 (4H, s, $OC\underline{H}_3$), 4.08 (4H, s, $N\underline{H}_2$), 5.70 (2H, s, H-3, 5), 6.69 (4H, s, H-3′, 5′).

**2,6-Bis(2,6-dichloro-4-hydroxyphenyloxy)-1,4-dimethoxybenzene (8)** The diamine (**7**: 49.0 mg, 0.1 mmol) was dissolved in hot 40% $H_2SO_4$ (0.5 ml) and then the solution was recooled to 0 °C. To this reaction mixture was added dropwise a solution of $NaNO_2$ (23.0 mg, 0.3 mmol) in cold water (1 ml) at 0—5 °C with stirring. The whole was stirred for 5 min, then excess $NaNO_2$ was decomposed with a few crystals of urea. A solution of $Cu(NO_3)_2 \cdot 3H_2O$ (0.6 g, 2.5 mmol) in water (4 ml) and $Cu_2O$ (15.0 mg, 0.1 mmol) were sequentially added to the above mixture at room temperature. The resulting mixture was stirred at the same temperature for 30 min and then extracted with EtOAc (20 ml × 2). The combined extracts were washed with saturated aqueous $NaHCO_3$, water and saturated aqueous NaCl, and dried over anhydrous $MgSO_4$. After evaporation of the solvent, the residue was subjected to preparative TLC [TLC plate: Kieselgel 60 $F_{254}$, 20 cm × 20 cm × 0.25 mm (Merck), solvent system: $CHCl_3$ : MeOH = 20 : 1] to give **8** (9.8 mg, 19.9%). The spectral data as well as TLC behavior were in good agreement with those of natural russuphelin A.

**Assay for Cytotoxic Activity against Ascites Tumor Cells** RPMI 1640 medium (Nissui) supplemented with 10% fetal bovine serum (Whittaker), 20 μM 2-mercaptoethanol and 100 μg/ml of kanamycin was used for cell culture, and a suspension of tumor cells ($10^4$ cells/ml) in the medium was prepared. The sample was dissolved in dimethylsulfoxide (DMSO) at a concentration of 100, 10, 1.0 or 0.1 μg/ml. The cell suspension (2 ml) in a culture tube (Falcon No. 2054) was incubated in a $CO_2$ (5%) incubator at 37 °C for 5 h, then the sample solution was added. Incubation was continued at 37 °C for a further 72 h, then the cell number in the culture suspension was counted with a Coulter counter (model ZB). The control cell suspension was similarly prepared without the test sample. All assays were performed in triplicate and the 50%-inhibitory concentration ($IC_{50}$) was calculated from the differences of the cell numbers in the culture suspension in the presence and absence of the test material.

**Assay for Cytotoxic Activity against Solid Tumor Cells** MEM medium (Nissui) supplemented with 10% fetal bovine serum (Whittaker) and 60 μg/ml of kanamycin was used for cell culture. A suspension (2 ml) of tumor cells ($5 \times 10^3$ cells/ml) in the medium was prepared in a culture dish (Costar No. 3512) and incubated in a $CO_2$ (5%) incubator at 37 °C for 24 h, then a sample solution in DMSO was added. Incubation was continued at 37 °C for a further 72 h, then the cells were treated with trypsin and the cell number was determined with a Coulter counter. The 50%-inhibitory concentration ($IC_{50}$) was calculated in the same manner as described above.

**Acknowledgements** We thank Mr. K. Kawamura, Miss K. Mushiake and Miss M. Inada of the Central Analysis Room of this Faculty for spectral measurements. We also thank Mr. H. Hayasaka and Mr. K. Ohba of the Medicinal Garden of this Faculty for collecting the mushrooms.

**References**

1) G. Kusano, H. Ogawa, A. Takahashi, S. Nozoe and K. Yokoyama, *Chem. Pharm. Bull.*, **25**, 3482 (1987).
2) E. Breitmaier and W. Voelter, "Carbon-13 NMR Spectroscopy," VCH Publishers, New York, 1987, p. 46—53 and references cited therein.
3) M. Tomita, K. Fujitani and Y. Aoyagi, *Chem. Pharm. Bull.*, **13**, 1341 (1965); D. L. Boger and D. Yohannes, *Tetrahedron Lett.*, **30**, 2053 (1989).
4) N. Morita, *Chem. Pharm. Bull.*, **8**, 66 (1960).
5) T. Cohen, A. G. Dietz, Jr. and J. R. Miser, *J. Org. Chem.*, **42**, 2053 (1977); A. H. Lewin and T. Cohen, *ibid.*, **32**, 3844 (1967).

NII-Electronic Library Service