Russuphelins B, C, D, E and F, New Cytotoxic Substances from the Mushroom *Russula subnigricans* HONGO

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Five new chlorinated phenyl ethers, russuphelins B (2), C (3), D (4), E (5) and F (6) have been isolated from the mushroom *Russula subnigricans* HONGO, and their structures were elucidated by spectroscopic and chemical means. Russuphelins B (2), C (3) and D (4) exhibited cytotoxic activity *in vitro* against P388 leukemia cells.

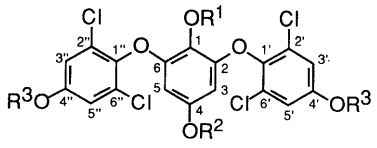
Keywords *Russula subnigricans*; russuphelin; cytotoxic substance; mushroom; basidiomycete; 2,6-dichloro-1,4-hydroquinone

Mushrooms (Basidiomycetes) are good sources of structurally intriguing and biologically active substances.[1] Recently, we have examined the chemical constituents of the toxic mushroom *Russula subnigricans* HONGO and isolated a cytotoxic triphenyl ether named russuphelin A (**1**).[2] During our extensive search for cytotoxic substances from this source, five new chlorinated phenyl ethers, designated russuphelins B (**2**), C (**3**), D (**4**), E (**5**) and F (**6**), have been isolated as congeners of russuphelin A. Of these compounds, russuphelins B, C and D exhibited cytotoxic activity *in vitro* against P388 leukemia cells, with IC_{50} values of 15.4, 0.94 and 12.1 μg/ml, respectively. This paper deals mainly with the structural elucidation of russuphelin B through russuphelin F (**2**—**6**) on the basis of spectral and chemical evidence.

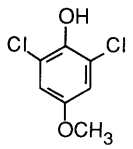
The isolation of russuphelins was guided by the assay of cytotoxic activity *in vitro* against P388 leukemia cells. The EtOAc soluble material (10.2 g) from the MeOH extract of *R. subnigricans* (2.4 kg) was fractionated by column chromatography on silica gel. The fractions eluted with *n*-hexane–EtOAc, $CHCl_3$–EtOAc and $CHCl_3$–MeOH mixtures were re-chromatographed on silica gel to afford russuphelin B through russuphelin F (**2**—**6**), along with russuphelin A (**1**) and 2,6-dichloro-4-methoxyphenol (**7**)[3] (Chart 1). TLC analysis of the acetone extract of the same mushroom revealed several spots detectable under UV (254 nm) irradiation, four of which contained **1**, **2**, **4** and **7**. Therefore, russuphelin A through russuphelin F are considered unlikely to be artifacts of the extraction.

Russuphelin B (**2**) was obtained as colorless needles, mp 295—296 °C. The molecular formula, $C_{19}H_{12}Cl_4O_6$, of **2** was determined from the high-resolution (HR) electron impact (EI) mass spectrum (MS) (M^+, *m/z* 475.9424, Δ +3.7 mμ). The IR spectrum of **2** showed absorptions at 3350, 1610, 1580 and 1510 cm^{-1}, implying the presence of phenolic hydroxyl groups. The ^{1}H-NMR spectrum of **2** (Table I) was similar to that of russuphelin A; however, the decrease in the molecular weight by 14(CH_2) and the loss of a methoxyl signal indicated that **2** was the demethyl compound of **1**. The methoxyl signal at δ 3.99 was assigned to the 1-OCH_3 group by comparing the ^{1}H-NMR spectrum of **2** with that of **1**. Thus, the structure of russuphelin B was concluded to be 2,6-bis(2,6-dichloro-4-hydroxyphenyloxy)-4-hydroxy-1-methoxybenzene (**2**).

Russuphelin C (**3**) was obtained as a pale brown amorphous solid, mp >300 °C (dec.). The HREI-MS exhibited a molecular ion peak at *m/z* 461.9188 (M^+, Δ −4.3 mμ) corresponding to the molecular formula $C_{18}H_{10}Cl_4O_6$. The phenolic nature of **3** was suggested by the IR absorptions (3300, 1620, 1580 and 1515 cm^{-1}). The ^{1}H-NMR spectrum of **3** displayed only five singlet signals assignable to six aromatic protons [δ 5.47 (2H), 6.97 (4H)] and four phenolic hydroxyl protons [δ 8.74 (1H), 8.55 (1H), 10.74 (2H)]. Judging from these results, russuphelin C was deduced to be the 1,4-didemethyl derivative of **1**. The methylation of **3** with methyl iodide/potassium carbonate



russuphelin A (**1**) : $R^1 = R^2 = CH_3$, $R^3 = H$
russuphelin B (**2**) : $R^1 = CH_3$, $R^2 = R^3 = H$
russuphelin C (**3**) : $R^1 = R^2 = R^3 = H$
8 : $R^1 = R^2 = R^3 = CH_3$



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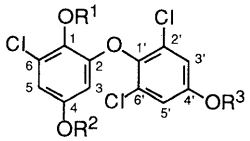

russuphelin D (**4**) : $R^1 = R^2 = CH_3$, $R^3 = H$
russuphelin E (**5**) : $R^1 = CH_3$, $R^2 = H$, $R^3 = CH_3$
russuphelin F (**6**) : $R^1 = H$, $R^2 = R^3 = CH_3$
9 : $R^1 = R^2 = CH_3$, $R^3 = Ac$

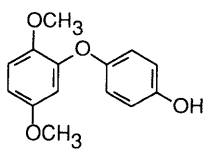

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Chart 1

TABLE I. ^{1}H-NMR Spectral Data for Russuphelin B through Russuphelin F (**2**—**6**) (δ, 500 MHz)[a]

Assignment	2	3	4	5	6
H-3	5.50 (1H, s)	5.47 (1H, s)	5.88 (1H, d, J=3.0)	5.82 (1H, d, J=2.8)	5.96 (1H, d, J=3.0)
H-5	5.50 (1H, s)	5.47 (1H, s)	6.56 (1H, d, J=3.0)	6.55 (1H, d, J=2.8)	6.58 (1H, d, J=3.0)
H-3′,5′	6.88 (4H, s)	6.97 (4H, s)	6.89 (2H, s)	6.95 (2H, s)	6.94 (2H, s)
H-3″,5″	6.88 (4H, s)	6.97 (4H, s)	—	—	—
1-OCH$_3$	3.99 (3H, s)		4.05 (3H, s)	3.99 (3H, s)	
4-OCH$_3$			3.68 (3H, s)		3.66 (3H, s)
4′-OCH$_3$				3.83 (3H, s)	3.82 (3H, s)
1-OH		8.74 (1H, s)			5.62 (1H, br s)
4-OH		8.55 (1H, s)		4.70 (1H, br s)	
4′-OH		10.35 (2H, s)	6.80 (1H, br s)		

a) The spectra of **2**, **3** and **4**—**6** were recorded in CD_3OD, DMSO-d_6 and $CDCl_3$, respectively. Values in parentheses are coupling constants in hertz (Hz).

TABLE II. ^{13}C-NMR Spectral Data for Russuphelin D (**4**) and Its Acetate (**9**) ($CDCl_3$, 125 MHz)[a]

Assignment	4	9
C-1	138.4 (1C, s)	139.2 (1C, s)
C-2	151.4 (1C, s)	151.0 (1C, s)
C-3	100.3 (1C, d)	100.5 (1C, d)
C-4	155.9 (1C, s)	155.7 (1C, s)
C-5	107.3 (1C, d)	107.6 (1C, d)
C-6	129.1 (1C, s)	129.3 (1C, s)
C-1′	139.2 (1C, s)	144.2 (1C, s)
C-2′,6′	129.6 (2C, s)	129.8 (2C, s)
C-3′,5′	116.4 (2C, d)	122.8 (2C, d)
C-4′	154.0 (1C, s)	147.6 (1C, s)
1-OCH$_3$	61.4 (1C, q)	61.2 (1C, q)
4-OCH$_3$	55.8 (1C, q)	55.7 (1C, q)
4′-OCOCH$_3$		168.5 (1C, s)
4′-OCOCH$_3$		21.0 (1C, q)

a) Multiplicities were determined by means of a DEPT experiment.

(CH_3I/K_2CO_3) afforded the tetramethyl ether (**8**), which was identical with that derived from **1**. The structure of russuphelin C was thus elucidated to be 2,6-bis(2,6-dichloro-4-hydroxyphenyloxy)-1,4-dihydroxybenzene (**3**).

Russuphelin D (**4**) was obtained as colorless needles, mp 136—138 °C, and russuphelins E (**5**) and F (**6**) were obtained as amorphous solids. In the EI-MS, they exhibited the same molecular ion clusters at m/z 348 (M^+), 350 (M^++2), 352 (M^++4) and 354 (M^++6), the intensity ratio of which indicated the presence of three chlorine atoms in the molecule. Further, the similarity of the structures of **4**—**6** was readily apparent from the ^{1}H-NMR spectral data (Table I), suggesting that the structures of **4**—**6** differ in the positions of two methoxyl groups.

The HREI-MS of **4** showed a molecular ion peak at m/z 347.9720 (M^+, Δ−2.4 mμ), establishing the molecular formula, $C_{14}H_{11}Cl_3O_4$. The existence of a phenolic hydroxyl group was suggested by the IR absorptions (3300, 3025 and 1580 cm^{-1}), and was confirmed by the formation of the monoacetate (**9**) on treatment of **4** with acetic anhydride in pyridine. The ^{13}C-NMR spectrum of **4** (Table II) revealed ten aromatic carbon signals other than two methoxyl carbon signals. The ^{1}H-NMR spectrum exhibited six signals which were assigned to four aromatic protons [δ 5.88 (1H, d, J=3.0 Hz), 6.56 (1H, d, J=3.0 Hz), 6.89 (2H, s)], two methoxyl protons (δ 3.68, 4.05), and a hydroxyl proton (δ 6.80). The signals of these protons and carbons were assigned on the basis of the correlation spectroscopy *via* long-range coupling (COLOC) spectrum with the aid of nuclear Overhauser effect (NOE) and distortionless enhancement by polarization transfer (DEPT) experiments. Irradiation of the methoxyl protons (δ 3.68) resulted in 12.5% and 6.3% NOEs for H-5 (δ 6.56) and H-3 (δ 5.88), respectively, implying that one of the two methoxyl groups is located on C-4. The position of the other methoxyl group was verified to be at C-1 through the COLOC spectrum (Fig. 1), in which the following cross peaks were observed: 1-OCH$_3$/C-1, H-3/C-1, C-2, C-4, C-5, H-5/C-1, C-3, C-6, and 4-OCH$_3$/C-4. On the other hand, in the ^{1}H- and ^{13}C- (Table II) NMR spectra of **9**, acetylation shifts were observed in the signals due to H-3′,5′ (Δ+0.34), C-4′ (Δ−6.4), and C-3′,5′ (Δ+6.4), which suggested that the hydroxyl group is attached to C-4′. Taking into consideration these results and the molecular formula, russuphelin D should contain the two partial structures A and B. The partial structures A and B account for $C_{14}H_{11}Cl_2O_3$ with eight degrees of unsaturation, and hence one oxygen atom and one chlorine atom remained to be assigned. The former should be placed as an ether linkage in the structure of **4**, and thus the latter must be located at C-6. Consequently, the structure of russuphelin D was concluded to be 2-(2,6-dichloro-4-hydroxyphenyloxy)-6-chloro-1,4-dimethoxybenzene (**4**). The structure of **4** was substantiated by the formation of the dechlorinated compound (**10**) upon hydrogenolysis over Pd–C.

The structures of russuphelins E and F were elucidated to be 2-(2,6-dichloro-4-methoxyphenyloxy)-6-chloro-4-hydroxy-1-methoxybenzene (**5**) and 2-(2,6-dichloro-4-methoxyphenyloxy)-6-chloro-1-hydroxy-4-methoxybenzene (**6**), respectively, by comparing their ^{1}H-NMR spectra with that of **4**. Namely, clear differences exist in the chemical shifts of three methoxyl signals (1,4,4′-OCH$_3$), which allowed us to determine the locations of a hydroxyl and two methoxyl groups as depicted in **5** and **6**.

In the ^{1}H- and ^{13}C-NMR spectra of **1**—**6**, the signals due to H-3(5) and C-3(5) were observed at characteristically high field. These unusually high chemical shifts for aromatic protons and carbons can probably be attributed to the shielding effects of the ring currents of adjacent rings, which suggests strongly that the conformations of the phenyl groups are nearly orthogonal, as found in vancomycin[4] and related compounds (Fig. 2). Dechlorination of russuphelins resulted in the disappearance of these anisotropic effects as well as weakening of the cytotoxic activities, and hence the conformation of the russuphelin

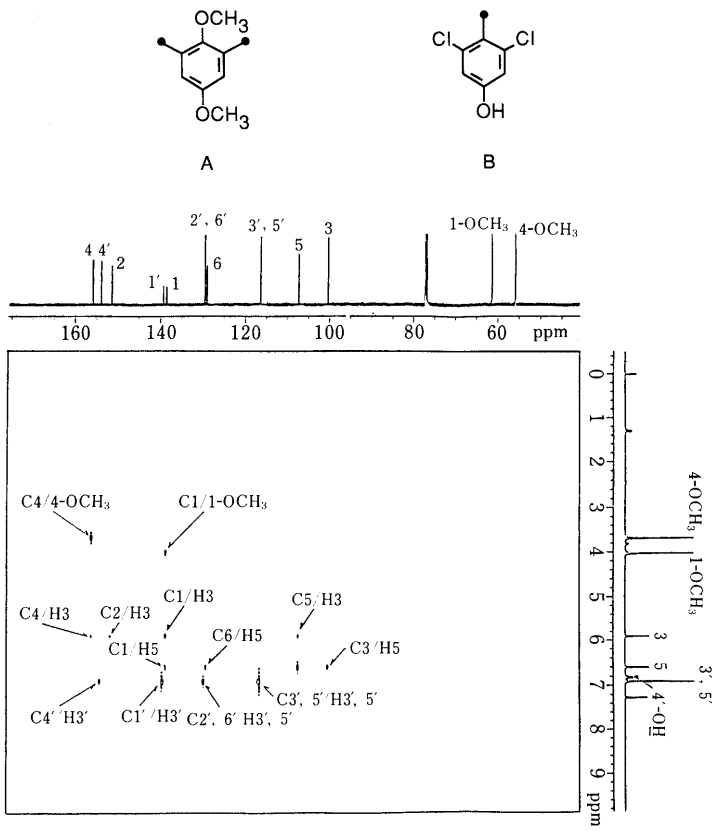

Fig. 1. COLOC Spectrum of Russuphelin D in $CDCl_3$

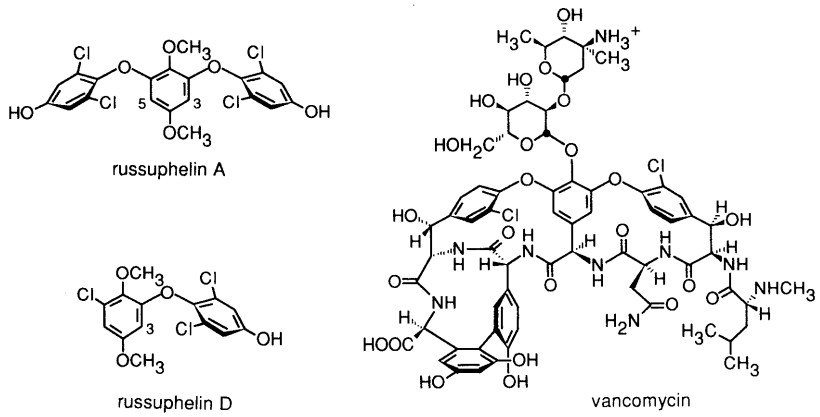

Fig. 2. Probable Conformations for Russuphelins

ring system may be essential for manifestation of the biological activities.

Russuphelin A through russuphelin F are intriguing in that they are presumably biosynthesized from 2,6-dichloro-1,4-hydroquinone and/or its methylether *via* oxidative phenol coupling[5] or *SN*2-type reactions.

Experimental

Melting points were determined on a Yanagimoto micro hot plate and are uncorrected. The spectroscopic data were measured by use of the following instruments: IR spectra, JASCO A-100S infrared spectrometer; UV spectra, Hitachi U-3200 spectrophotometer; mass spectra, JEOL DX-303 spectrometer; ^{1}H- and ^{13}C-NMR spectra, JEOL GX-500 (500 and 125 MHz, respectively). Chemical shifts are shown in δ (ppm) and multiplicities are given as follows: singlet = s, doublet = d, quartet = q and broad = br. Coupling constants (J) are given in hertz (Hz). TLC analyses were performed on Kieselgel 60F_{254} (Merck) and spots were detected under UV irradiation and by heating on a hot plate after spraying 50% sulfuric acid reagent.

Isolation Procedure The fruiting bodies of *R. subnigricans* HONGO (2.4 kg), collected in Miyagi prefecture in September 1989, were extracted twice with MeOH (6 l). The combined extracts were concentrated under reduced pressure and then the residual aqueous suspension (*ca.* 200 ml) was extracted with EtOAc (250 ml × 2) and *n*-BuOH (150 ml × 2) successively. The EtOAc soluble material (10.2 g) was chromatographed on silica gel (50 g, 2.5 cm i.d. × 30 cm), then the fractions eluted with *n*-hexane–EtOAc (4 : 1), $CHCl_3$–EtOAc (9 : 1, 4 : 1), and $CHCl_3$–MeOH (95 : 5) mixtures were each rechromatographed on silica gel, followed by recrystallization to afford russuphelins D (**4**, 45.4 mg), E (**4**, 1.8 mg), and F (**6**, 30.0 mg) and 2,6-dichloro-4-methoxyphenol (**7**, 296.1 mg) along with russuphelin A (**1**, 323.4 mg). Russuphelins B (**2**, 8.6 mg) and C (**3**, 28.4 mg) were isolated from the fractions eluted with $CHCl_3$–MeOH (9 : 1, 4 : 1), after chromatography over silica gel with $CHCl_3$–MeOH as the eluent.

Russuphelin B (2) Rf = 0.15 ($CHCl_3$: MeOH = 19 : 1), 0.04 ($CHCl_3$: EtOAc = 9 : 1). EI-MS m/z (%): 476 (M^+,76.7), 478 (M^+ + 2, 100.0), 480 (M^+ + 4, 50.7), 482 (M^+ + 6, 12.1). UV λ_{max}^{MeOH} nm (log ε): 228 (4.63), 235

(4.47), 283 (4.06), 294 (3.99). IR ν_{max}^{KBr} cm^{-1}: 3350 (br), 1610, 1580, 1510, 1460, 1440, 1325, 1215. ^{1}H-NMR: Table I.

Russuphelin C (3) Rf=0.10 ($CHCl_3$:MeOH=19:1), 0.03 ($CHCl_3$:EtOAc=9:1). EI-MS m/z (%): 460 (M^+-2, 22.1, quinone form), 462 (M^+, 54.8), 464 (M^++2, 47.8), 466 (M^++4, 20.1), 178 (100.0). FD-MS m/z (%): 462 (M^+, 61.3), 464 (M^++2, 100.0), 466 (M^++4, 53.6), 468 (M^++6, 10.2). UV λ_{max}^{MeOH} nm (log ε): 228 (4.45), 287 (4.10), 294 (4.06). IR ν_{max}^{KBr} cm^{-1}: 3300 (br), 1620, 1580, 1515, 1460, 1435, 1320, 1210. ^{1}H-NMR: Table I.

Russuphelin D (4) Rf=0.46 ($CHCl_3$:MeOH=19:1), 0.58 ($CHCl_3$:EtOAc=9:1). EI-MS m/z (%): 348 (M^+, 100.0), 350 (M^++2, 98.2), 352 (M^++4, 34.3), 354 (M^++6, 4.3). UV λ_{max}^{MeOH} nm (log ε): 286 (3.79), 205 (4.58). IR ν_{max}^{KBr} cm^{-1}: 3300 (br), 3025, 1580, 1495, 1220, 800. ^{1}H-NMR: Table I. ^{13}C-NMR: Table II.

Russuphelin E (5) Rf=0.53 ($CHCl_3$:MeOH=19:1), 0.67 ($CHCl_3$:EtOAc=9:1). EI-MS m/z (%): 348 (M^+, 100.0), 350 (M^++2, 98.8), 352 (M^++4, 34.9), 354 (M^++6, 4.6). HREI-MS m/z: 347.9720 (M^+). Calcd for $C_{14}H_{11}Cl_3O_4$: 347.9723. ^{1}H-NMR: Table I.

Russuphelin F (6) Rf=0.90 ($CHCl_3$:MeOH=19:1), 0.85 ($CHCl_3$:EtOAc=9:1). EI-MS m/z (%): 348 (M^+, 100.0), 350 (M^++2, 98.8), 352 (M^++4, 33.4), 354 (M^++6, 4.2). HREI-MS m/z: 347.9727 (M^+); Calcd for $C_{14}H_{11}Cl_3O_4$: 347.9723. IR ν_{max}^{KBr} cm^{-1}: 3550 (br), 3100, 1600, 1505, 1260, 810. ^{1}H-NMR: Table I.

Compound 7 Colorless needles (from $CHCl_3$), mp 75—77 °C. Rf=0.82 ($CHCl_3$:MeOH=19:1), 0.79 ($CHCl_3$:EtOAc=9:1). EI-MS m/z (%): 192 (M^+, 100.0), 194 (M^++2, 66.0), 196 (M^++4, 11.4). HREI-MS m/z: 191.9776 (M^+); Calcd for $C_7H_6Cl_2O_2$: 191.9745. IR ν_{max}^{KBr} cm^{-1}: 3450 (br), 3050, 1570, 1480, 1300, 770. ^{1}H-NMR ($CDCl_3$, 100 MHz) δ: 3.74 (3H, s, OCH_3), 5.46 (1H, br s, OH), 6.84 (2H, s, H-3,5). ^{13}C-NMR ($CDCl_3$, 25 MHz) δ: 56.0 (s, OCH_3), 114.1 (d, C-3,5), 121.1 (s, C-2,6), 142.1 (s, C-1), 152.9 (s, C-4).

Methylation of 1 and 3 A solution of **1** (20.0 mg) in *N,N*-dimethylformamide (DMF, 1.0 ml) and CH_3I (100 μl) were successively added to a suspension of K_2CO_3 (200 mg) in DMF (2 ml) under an N_2 atmosphere, and the reaction mixture was stirred for 3 h at room temperature. To this mixture was added ice water, and the product was extracted with EtOAc (20 ml × 2). After evaporation, the residue was chromatographed on silica gel using *n*-hexane–EtOAc as the eluent followed by recrystallization from *n*-hexane–$CHCl_3$ to afford the methyl ether (**8**, 15.2 mg) as colorless needles. Compound **8** (6.2 mg) was also obtained from **3** (10.0 mg) by the same procedure. The physico-chemical and spectral data of **8** are as follows. mp 215—218 °C. EI-MS m/z: 518 (M^+), 520, 524, 526. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3030, 3000, 2960, 2940, 1600, 1565, 1500. ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 3.51 (3H, s, OCH_3), 3.82 (6H, s, OCH_3), 4.08 (3H, s, OCH_3), 5.67 (2H, s, H-3,5), 6.95 (4H, s, H-3′,5′,3″,5″).

Acetylation of 4 Ac_2O (0.3 ml) was added to a solution of **4** (38.2 mg) in pyridine (0.4 ml) and the mixture was left to stand at room temperature overnight. After usual work-up, the acetate (**9**, 38.7 mg) was obtained as colorless needles after recrystallization from *n*-hexane–$CHCl_3$, mp 125—126 °C. EI-MS m/z (%): 390 (M^+, 100.0), 392 (M^++2, 99.8), 394 (M^++4, 36.0), 396 (M^++6, 4.8). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3050, 2950, 2845, 1770, 1605, 1580, 1495, 1460, 1180. ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 2.32 (3H, s, $OCOCH_3$), 3.68 (3H, s, OCH_3), 3.98 (3H, s, OCH_3), 5.93 (1H, d, J=2.8, H-3), 6.63 (1H, d, J=2.8, H-5), 7.23 (2H, s, H-3′,5′).

Hydrogenolysis of 4 A solution of **4** (10.0 mg) in MeOH (0.5 ml) was hydrogenated over 10% Pd–C (20.0 mg) at room temperature for 24 h. After removal of the catalyst by filtration, the filtrate was concentrated under reduced pressure, and then the residue was purified by chromatography on silica gel with *n*-hexane–EtOAc as the eluent to give the dechlorinated compound (**10**, 5.0 mg) as an amorphous solid. EI-MS m/z (%): 246 (M^+, 100.0), 231 (42.5), 216 (4.5). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600, 3375 (br), 3045, 3005, 2950, 2845, 1605, 1500, 1465, 1445, 835. ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 3.70 (3H, s, OCH_3), 3.82 (3H, s, OCH_3), 6.45 (1H, d, J=2.8, H-3), 6.58 (1H, dd, J=9.0, 2.8, H-5), 6.79 (2H, d, J=9.0, H-3′,5′), 6.90 (2H, d, J=9.0, H-2′,6′), 6.91 (1H, d, J=9.0, H-6).

Cytotoxic Activity Assay for cytotoxic activity against P388 leukemia cells was performed according to the method detailed in the previous papers.[2,6]

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