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The Isolation and Structure Determination of Violaceic Acid, A New Biphenyl Ether Type Metabolite from *Emericella violacea*

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A new phenolic metabolite (**1**) named violaceic acid was isolated from a strain of *Emericella violacea* and shown to be a novel biphenyl ether compound having a carboxyl group and an aldehyde group by using ^{13}C-^{1}H indirect spin coupling data obtained from ^{13}C-nuclear magnetic resonance spectroscopic analysis.

Keywords——violaceic acid; fungal metabolite; *Emericella violacea*; phenolics; structure determination; ^{13}C-NMR spectrum; ^{13}C-^{1}H indirect spin coupling

During a survey of sterigmatocystin-producing fungi,[1] a strain of *Emericella violacea* (IFO 8106) has been found to be toxigenic to experimental animals and to produce some new phenolic metabolites. The ethyl acetate extract of the mycelia grown on sterilized rice exhibited a lethal effect on mice upon intraperitoneal injection at a dose of 500 mg/kg. As a result, a toxic phenolic metabolite, named violaceol was isolated as indicated in Chart 1 by repeated silica gel column chromatography of the extract, together with another phenolic compound, non-toxic violaceic acid.

In this paper, the structure determination of violaceic acid is described. The structure determination of violaceol will be reported in the next paper.

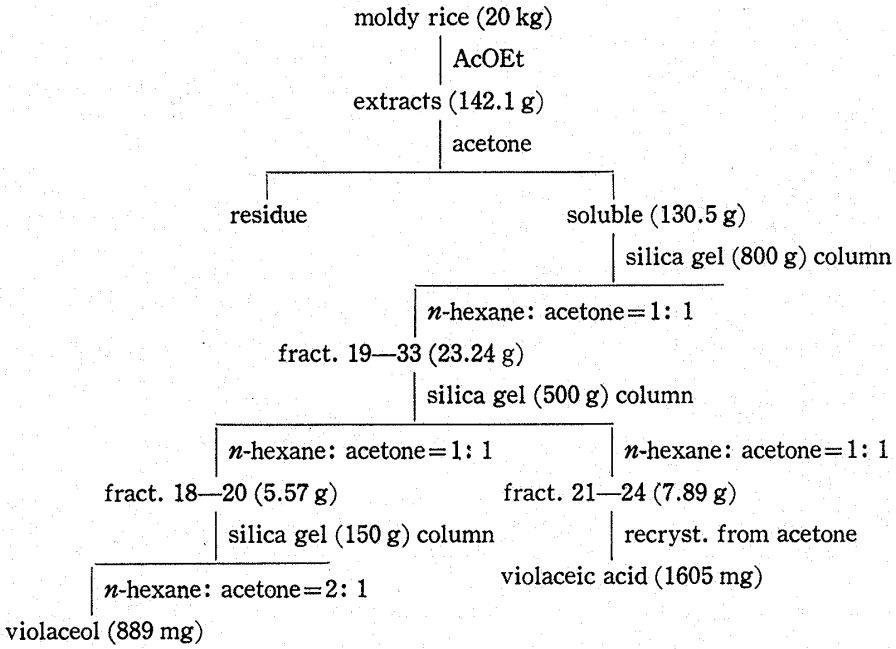

Chart 1. Isolation of Metabolites from Moldy Rice infected with *Emericella violacea*

The non-toxic metabolite of *E. violacea*, violaceic acid (**1**), was isolated as pale yellow fine needles, mp 223—225°C (acetone), $C_{15}H_{12}O_6$. The absorption maxima in the ultraviolet (UV) spectrum, UV λ_{max}^{MeOH} 232, 253, 276, 283 and 310 nm, indicated that **1** contained a substituted aromatic ring system in the molecule. The presence of a phenolic hydroxyl group was

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suggested by the positive $FeCl_3$ test. The presence of a carboxyl group was suggested by the appearance of characteristic absorption bands at 2900—2400 cm^{-1} in the infrared (IR) spectrum and the solubility of this compound in 1 N $NaHCO_3$ solution. Methylation of **1** with diazomethane afforded two products, **2** and **3**. In the proton magnetic resonance (^{1}H-NMR) and mass spectra, the presence of two and three *O*-methyl groups in **2** and **3** was observed, respectively. In the $FeCl_3$ test, **2** was positive but **3** was negative. From these results, it was evident that the methylation had occurred on the carboxyl group first and then on the phenolic hydroxyl group. A singlet at δ_H 3.93 (3H) in the ^{1}H-NMR spectrum of **1** was assigned to the methoxyl group. The presence of an aldehyde group was also suggested by the appearance of a singlet at δ_H 9.65 in the ^{1}H-NMR spectrum and a doublet at δ_C 190.61 in the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum in the ^{1}H single-frequency off-resonance decoupling (SFORD) mode. The signals of six aromatic protons were observed in the ^{1}H-NMR spectrum: δ_H 7.16 (1H, d, J=9 Hz), 7.24 (1H, d, J=9 Hz), 7.26 (1H, d, J=2 Hz), 7.28 (1H, d, J=2 Hz), 7.64 (1H, dd, J=9 and 2 Hz), 7.76 (1H, dd, J=9 and 2 Hz). These findings suggested the presence of two aromatic rings, both of which might be substituted at the 1,2,4-positions. The role of the remaining one oxygen atom might be to form a linkage between the two aromatic rings to afford the biphenyl ether structure.

The ^{13}C-^{1}H indirect spin coupling in the ^{13}C-NMR spectrum has recently been used for the structure elucidation of aromatic compounds in several reports.[2-5] It is known that $J_{C,H}$ values are generally observed as $^1J > ^3J > ^2J$. The ^{13}C-NMR spectrum showed fifteen peaks due to all the carbons in the structure of **1**. The coupling patterns of these signals (except for OCH_3) are shown in Fig. 1.

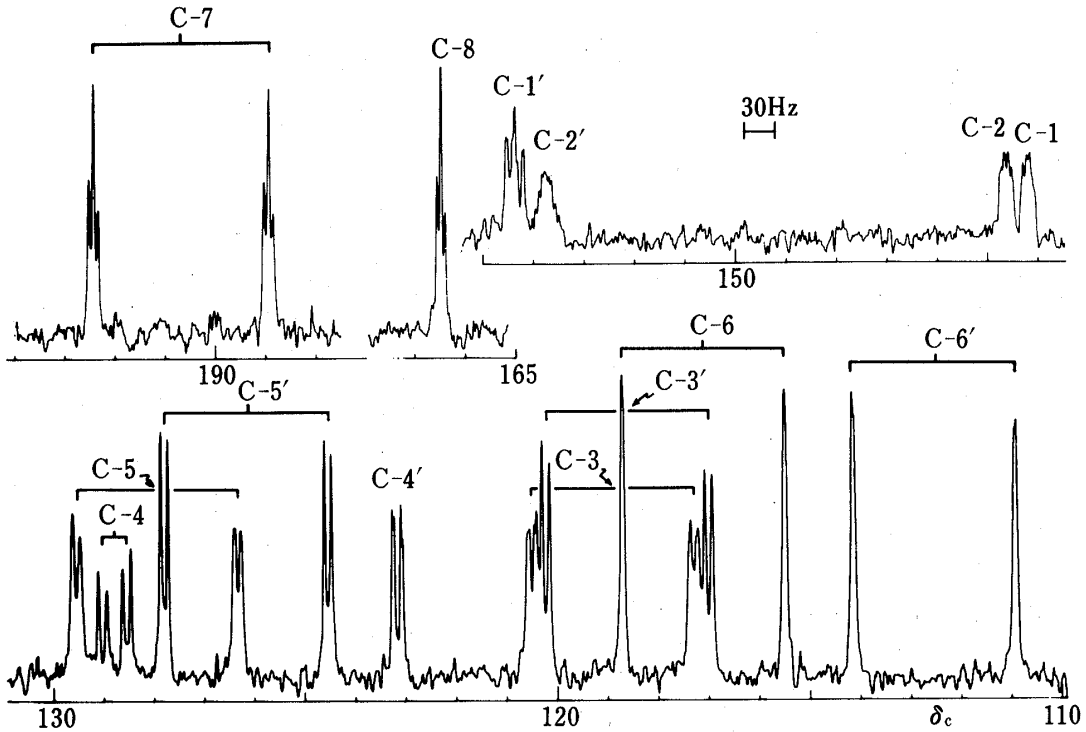

Fig. 1. 50 MHz ^{13}C-NMR Spectrum of Violaceic Acid (Aromatic and Carbonyl Regions)

The ^{13}C-chemical shifts indicated the presence of two carbonyl, twelve aromatic and one methoxyl carbons in the molecule. A doublet of triplets signal at δ_C 190.61 and a triplet at δ_C 166.44 were assigned to –CHO and –COOH, respectively. These two groups were suggested to be located on C-4 and C-4′, because the fine splitting of the two signals could be expected to be due to the indirect spin couplings ($^3J_{C,H}$=4.4 and 5.1 Hz) with two *ortho*-protons (H-3 and H-5 or H-3′ and H-5′). By selective irradiation of one proton at the *ortho*-position, the

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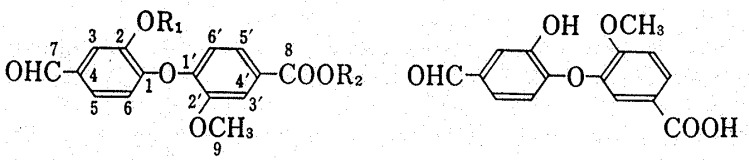
triplet signal of the carboxyl carbon was actually changed into a doublet and the aldehyde carbon signal was changed into a doublet of doublets. Among twelve signals of aromatic carbons, four quaternary carbon signals at δ_C 144.27, 144.65, 153.79 and 154.32 were suggested to be those due to carbons bonding to the oxygenous functions, in view of their chemical shifts. The remaining six doublet signals at δ_c 112.59, 117.09, 118.75, 118.81, 126.17 and 127.83 were anticipated to be those due to carbons bonding to hydrogen atoms. A double doublet at δ_C 128.83 ($^2J_{C,H}$=24; -C<u>H</u>O, $^3J_{C,H}$=7.5 Hz) was observed to be changed into a doublet by selective irradiation of the aldehyde proton. This signal should therefore be assigned to C-4, where the aldehyde group is located. The remaining quaternary carbon signal at δ_C 123.22 was suggested to be due to C-4′, where the carboxyl group is located. On irradiation at δ_H 7.16—7.24, where the signals of H-6 and H-6′ appeared, both of the doublets at δ_C 112.59 ($^1J_{C,H}$=163.4 Hz) and 117.09 ($^1J_{C,H}$=161.2 Hz) were changed into singlets. These signals were therefore assigned to C-6 and C-6′, respectively. No $^3J_{C,H}$ were observed on these two carbons. This result indicates that no OH was present at the positions on both sides of these carbons.

Accordingly, **1** and **4** are proposed as plausible structures of violaceic acid. In the structure **1**, both of the aromatic carbons (C-3′ and C-5′) bond hydrogen atoms. In the ^{13}C-NMR spectrum of violaceic acid, the signal at δ_c 154.32 corresponding to C-1′ was actually observed



1 : R_1=H, R_2=H
2 : R_1=H, R_2=CH_3
3 : R_1=CH_3, R_2=CH_3

4

Fig. 2. Possible Structures of Violaceic Acid and Its Methylates

TABLE I. ^{13}C Chemical Shifts and ^{13}C, ^{1}H Coupling Constants (Hz) of Violaceic Acid (**1**) (in DMSO-d_6)

Carbon No.	δ_C	$^1J_{C,H}$	$^2J_{C,H}$ and $^3J_{C,H}$
7	190.61 (Ddd)[a,b]	175.8	4.4, 5.1 (3-H, 5-H)
8	166.44 (Sdd)		4.4, 5.1 (3′-H, 5′-H)
1′	154.32 (Sdd)		*ca.* 7.5 (3′-H, 5′-H)
2′	153.79 (Sm)		
2	144.65 (Sdd)		
1	144.27 (Sm)		
4	128.83 (Sdd)		24 (7-H), 7.5 (6-H)
5	127.83 (Dbrd)	160.5	6.0 (3-H, 7-H)
5′	126.17 (Dd)	163.4	6.0 (3′-H)
4′	123.22 (Sdd)		*ca.* 2 (3′-H, 5′-H), 7.5 (6′-H)
3	118.81 (Dbrd)	160.5	7.5 (5-H, 7-H)
3′	118.75 (Dd)	160.5	7.5 (5′-H)
6	117.09 (D)	161.2	
6′	112.59 (D)	163.4	
9	55.95 (Q)	145.1	

a) S=singlet, D,d=doublet, Q=quartet, m=multiplet, br=broad.
b) Capital letters refer to the coupling pattern affected by protons directly bonded to the carbon and small letters refer to that affected by ^{13}C-^{1}H indirect spin coupling.

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as a multiplet (triplet-like), not as a simple doublet, indicating that both of the *meta*-positions of this carbon (C-1′) are substituted with hydrogen atoms.

The signal at δ_C 118.75 was observed as a doublet of doublets ($^1J_{C,H}$=160.5, $^3J_{C,H}$=7.5 Hz), indicating that this signal has only one $^3J_{C,H}$ to the hydrogen of the *meta*-position. This signal was therefore assigned to C-3′. The signal of a doublet of broad doublets at δ_C 118.81 ($^1J_{C,H}$=160.5, $^3J_{C,H}$=7.5 Hz) was sharpened by irradiation of the aldehyde proton. This signal should therefore be assigned to C-3. The triplet-like signal at δ_C 154.32 was assigned to C-1′, which couples with H-3′ and H-5′ as mentioned already (this signal was changed into a doublet by the irradiation of H-3′ or H-5′). Furthermore, on irradiation of H-5′ and H-5, the signals at δ_C 126.17 and 127.83 were each changed into a doublet. The latter doublet of broad doublets (δ_C 127.83) was also sharpened by irradiation of the aldehyde proton signal. These two signals should therefore be assigned to C-5′ and C-5. The remaining two quaternary carbons at δ_C 144.65 and 144.27 were assigned to C-2 and C-1. The signal at δ_C 153.79 was observed as a multiplet, and was assigned to C-2′.

Thus, it was expected that the aldehyde group should be located at C-4, the carboxyl at C-4′, the hydroxyl at C-2 and the methoxyl at C-2′ (structure **1** in Fig. 2).

All ^{13}C-chemical shifts and $J_{C,H}$ values are listed in Table I.

Violaceic acid has also been isolated from *Emericella foveolata* in our laboratory.

Experimental

The melting point was measured on a Yanagimoto apparatus and is uncorrected. IR: Hitachi EPI-G3 grating infrared spectrophotometer. UV: Hitachi 323 recording spectrophotometer. ^{1}H-NMR and ^{13}C-NMR spectra: Japan Electron Optics Laboratory JNM-PS-100 and JNM-FX-200 (50.15 MHz) machines. Chemical shifts are given in δ ppm from tetramethylsilane (TMS) added as an internal standard. Mass spectra: a JEOL JMS 01SG-2 mass spectrometer equipped with a JMA 2000 mass data analysis system.

Isolation of Violaceic Acid (1) from *Emericella violacea*——Strain IFO 8106 was grown in stationary culture on sterilized rice (20 kg) at 25°C for 24 d. The moldy rice was extracted three times with 45 l each of AcOEt. After removal of the solvent under reduced pressure, 142.1 g of residue was obtained. The acetone-soluble fraction (130.5 g) of the extract was chromatographed on a silica gel (800 g) column. The *n*-hexane–acetone (1: 1) eluate fraction (23.24 g) was rechromatographed on a silica gel (500 g) column. Violaceic acid (**1**) was obtained (1605 mg) from the fraction eluted with *n*-hexane–acetone (1: 1) by recrystallization of the product from acetone. Violaceic acid (**1**), pale yellow fine needles, mp 223—225°C. *Anal.* Calcd for $C_{15}H_{12}O_6$ (288): C, 62.50; H, 4.20. Found: C, 62.43; H, 4.21. MS *m/e* (%): 288 (M^+, 100), 270 (14), 257 (11), 239 (56), 152 (24), 119 (13). UV λ_{max}^{MeOH} nm (ε): 232 (24600), 253 (16100), 276 (14300), 283 (13800), 310 (7100). IR ν_{max}^{KBr} cm^{-1}: 3340, 2900—2400, 1693, 1647, 1600, 1510, 1442, 1270, 1210, 1127, 1020, 785, 620. ^{1}H-NMR (in DMSO-d_6) δ_H: 3.93 (3H, s), 7.16 (1H, d, J=9 Hz), 7.24 (1H, d, J=9 Hz), 7.26 (1H, d, J=2 Hz), 7.28 (1H, d, J=2 Hz), 7.64 (1H, dd, J=9 and 2 Hz), 7.76 (1H, dd, J=9 and 2 Hz), 9.65 (1H, s, did not disappear on addition of D_2O). A brown color was observed in the $FeCl_3$ test.

Measurement of the ^{13}C-NMR Spectrum of Violaceic Acid——Solvent=DMSO-d_6. Tube=10 mm. Concentration=130 mg/3 ml. Temp.=room temp. Nucleus; obs.=^{13}C (50.18 MHz), lock=D, irr.=^{1}H (199.56 MHz). Offset; obs.=34.00 kHz, irr.=58.50 kHz. Pulse: width=6 μs. (30°), repetition=1.0 s. Data points=16 K. No. of pulses=4000. Spectral width=12000×1/10.

Methylation of Violaceic Acid (1)——An ether solution of **1** (30 mg) and diazomethane was left with occasional stirring for 1 h at room temperature. The ether was evaporated off, and the residue was separated by preparative TLC (silica gel F_{254}, 5 mm, Merck) using chloroform as the developing solvent to obtain **2** (30 mg) and **3** (16 mg).

Violaceic Acid Dimethyl Ether (**3**): ^{1}H-NMR (in $CDCl_3$) δ_H: 3.76 (3H, s), 3.82 (3H, s), 3.89 (3H, s), 6.94 (1H, d, J=9 Hz), 7.03 (1H, d, J=9 Hz), 7.20 (1H, d, J=2 Hz), 7.51 (1H, d, J=2 Hz), 7.56 (1H, dd, J=9 and 2 Hz), 7.91 (1H, dd, J=9 and 2 Hz), 9.77 (1H, s). MS *m/e* (%): 316 (M^+, 100), 285 (24), 66 (10), 18 (98).

Violaceic Acid Monomethyl Ether (**2**): ^{1}H-NMR (in $CDCl_3$) δ_H: 3.84 (3H, s), 3.87 (3H, s), 7.01 (1H, d, J=9 Hz), 7.12 (1H, d, J=9 Hz), 7.24 (1H, d, J=2 Hz), 7.51 (1H, dd, J=9 and 2 Hz), 7.77 (1H, d, J=2 Hz), 7.91 (1H, dd, J=9 and 2 Hz), 9.75 (1H, s). MS *m/e* (%): 302 (M^+, 100), 271 (29), 270 (41), 239 (69), 135 (25), 84 (26), 66 (31), 46 (10), 18 (42). A brown color was observed in the $FeCl_3$ test.

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