[Chem. Pharm. Bull. 30(2) 514—518 (1982)]

Structure Determination of Violaceol-I and -II, New Fungal Metabolites from a Strain of *Emericella violacea*

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(Received June 17, 1981)

A toxic metabolite, violaceol, which was isolated from the fungus, *E. violacea*, was found to be a mixture of two isomers. Violaceol-I (3) and -II (4) were isolated as tetra-acetates through acetylation and preparative thin layer chromatography. The structures of violaceol-I and -II were determined as shown in Fig. 1 and 2, mainly by the analysis of the ^{1}H- and ^{13}C-nuclear magnetic resonance spectral data, especially the ^{13}C-^{1}H indirect spin coupling constants. Violaceols were lethal to mice and exhibited weak antimicrobial activity.

Keywords——violaceol-I; violaceol-II; fungal metabolite; *Emericella violacea*; biphenyl ether; structure determination; ^{13}C-NMR; ^{13}C-^{1}H indirect spin coupling

The isolation of some new phenolic metabolites from a fungus, *Emericella violacea*, and the structure determination of violaceic acid, one of those metabolites, were previously reported.[1] This paper describes the structure determination of another toxic phenolic metabolite, violaceol, which was isolated from this fungus together with violaceic acid.

Violaceol, a viscous oil, $C_{14}H_{14}O_5$ (M^+ *m/e* 262.0914, calcd. 262.0841), was shown to be phenolic by positive coloring in the $FeCl_3$ test on thin layer chromatography (TLC) plates. The TLC spot of violaceol was however gourd-shaped under some TLC conditions, suggesting that violaceol might be a mixture of two closely related compounds. Further separation of violaceol was attempted through repetition of column chromatography and preparative TLC but the result was not satisfactory. Subsequently, the acetylation of violaceol was carried out, and two acetylated compounds, violaceol-I acetate (**1**) and -II acetate (**2**) were finally isolated by preparative TLC of the product. These two compounds both exhibited a molecular ion peak at *m/e* 430 and similar fragmentation patterns in mass spectrometry. From the molecular ion at *m/e* 430 ($262+42\times4$), it was assumed that violaceol-I (**3**) and -II (**4**) were both converted into tetraacetyl derivatives. Similarly, tetrabenzoates of **3** and **4** (M^+ *m/e* 678) were obtained from the violaceol mixture by treatment with benzoyl chloride.

In the proton magnetic resonance (^{1}H-NMR) spectrum of violaceol-I acetate (**1**), signals corresponding to one aromatic methyl (δ_H 2.20, 3H, s), two acetyl groups (δ_H 2.26, 6H, s) and two aromatic protons at δ_H 6.68 (1H, d), 6.76 (1H, d) were observed. In the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of **1**, signals of one aromatic methyl, two acetyl methyl, two acetyl carbonyl and six aromatic carbons were observed. Compounds **3** and **4** ($C_{14}H_{14}O_5$) were shown to contain four phenolic hydroxyl groups from the mass spectra of the acetylated and benzoylated derivatives. The observations in the ^{1}H-NMR and ^{13}C-NMR spectra suggested that **1** may consist of a symmetric dimer structure containing two aromatic rings.

The role of the remaining one oxygen atom out of five might be to form a linkage between the two aromatic rings to afford a symmetric biphenyl ether structure. The fact that violaceic acid isolated from the same fungus has been demonstrated to have a biphenyl ether linkage supports this possibility.

Among six ^{13}C signals of aromatic carbons observed for **1**, two doublets at δ_C 117.44 ($^{1}J_{C,H}$ =161.8 Hz) and 118.90 ($^{1}J_{C,H}$=165.4 Hz) could be assigned to carbons bearing a hydrogen

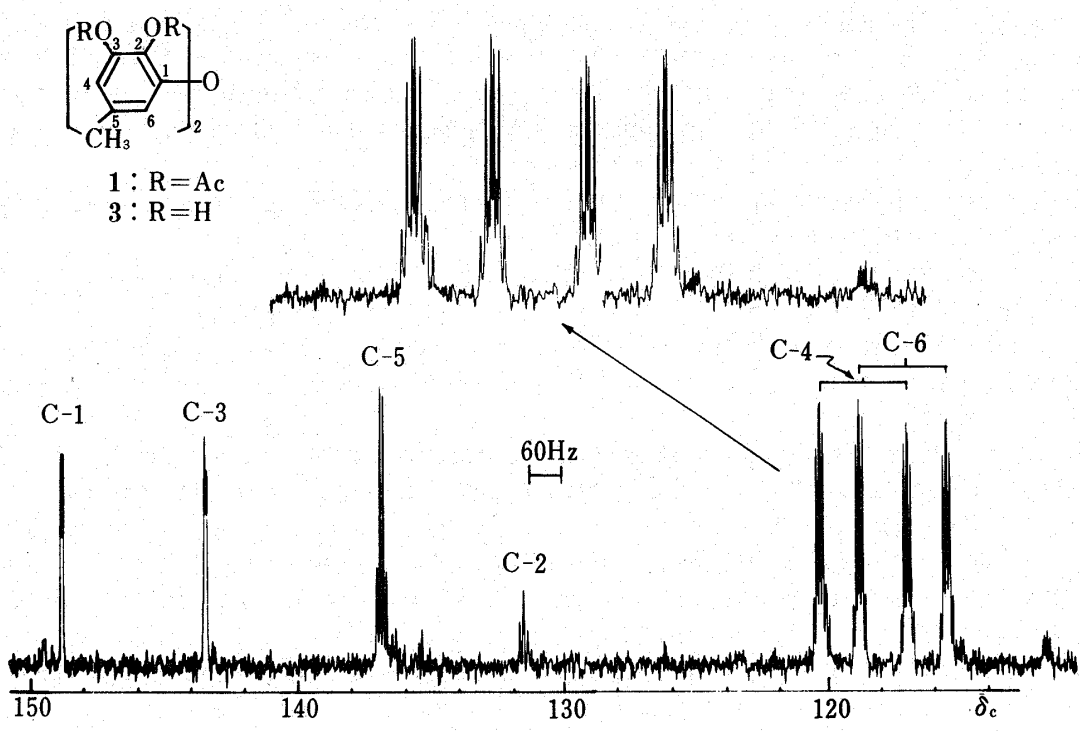

Fig. 1. 50 MHz ^{13}C-NMR Spectrum of Violaceol-I Acetate (Aromatic Carbon Region)

atom (see Fig. 1). These two carbons were expected to be in a *meta* relation, because the aromatic protons (δ_H 6.68 and 6.76) attached to these carbons exhibited *meta*-coupling (doublet, J=2) in the ^{1}H-NMR spectrum. The similar patterns of fine splitting of these two doublets (apparently octets) may be due to the $^3J_{C,H}$ to protons at the *meta*-position and a methyl group. Accordingly, the methyl group should be located on the carbon sandwiched between these two carbons. The two doublets at δ_C 143.47 ($^2J_{C,H}$=3.7 Hz) and 148.86 ($^2J_{C,H}$=2.4 Hz) were changed into singlets by irradiation of the aromatic proton signal in the range of

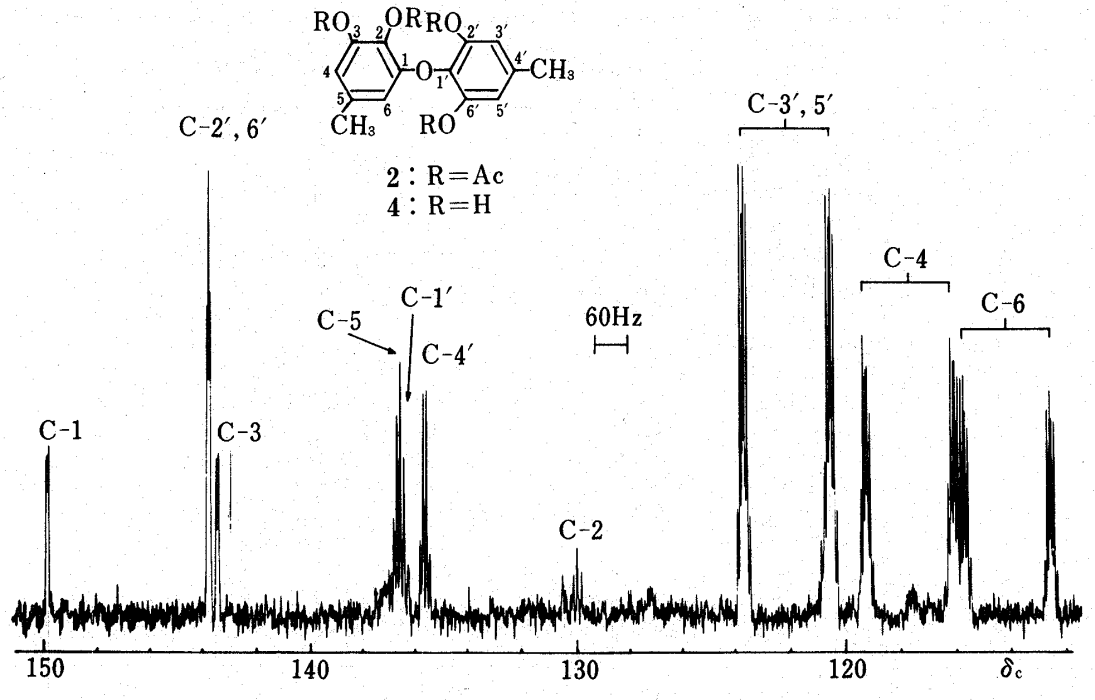

Fig. 2. 50 MHz ^{13}C-NMR Spectrum of Violaceol-II Acetate (Aromatic Carbon Region)

δ_H 6.7—6.8. These carbons were therefore assigned to C-3 and C-1. The quartet at δ_C 136.83 was assigned to C-5. The last aromatic carbon signal was observed at δ_C 131.60 as a triplet. The splitting of this signal may be due to the $^3J_{C,H}$ to the two aromatic protons at the *meta*-positions from this carbon (C-2). In fact, this signal was changed into a singlet by irradiation of the aromatic proton signals at δ_H 6.68 and 6.76.

On the basis of these observations in the ^{1}H and ^{13}C-NMR spectra, the structure **1** (in Fig. 1) is established for violaceol-I acetate.

It was interesting to note that three ^{1}H-signals of aromatic protons were observed at δ_H 6.39 (1H), 6.63 (1H) and 6.87 (2H) for **2**, in contrast to **1**. Two signals at δ_H 6.39 (1H, d, J=2 Hz) and 6.63 (1H, d, J=2 Hz) indicated the presence of two aromatic protons mutually *meta*-coupled. The broad signal at δ_H 6.87 (2H) suggested the presence of two nearly equivalent aromatic protons.

The observation of the $^1J_{C,H}$ of doublets of aromatic carbons at δ_C 113.72 (1C), 117.42 (1C) and 121.90 (2C) in the ^{13}C-NMR of **2** indicated that these carbons are attached to hydrogens (see Fig. 2). The fine splitting (octet-like) of these signals may be due to the $^3J_{C,H}$ to the methyl protons on the neighboring carbon and a proton on the *meta*-position. The signals at δ_C 113.72 and 117.42 were therefore assigned to C-6 and C-4, as in the case of violaceol-I. The signal at δ_C 121.90 (2C) was assigned to two equivalent carbons, C-3′ and C-5′ (see structure **2** in Fig. 2). A triplet-like signal at δ_C 129.78 should be assigned to C-2, because this signal exhibited $^3J_{C,H}$ from the two aromatic protons (H-4 and H-6). Another triplet at δ_C 136.19 was assigned to C-1′. The splitting of this signal may also be due to $^3J_{C,H}$ to two equivalent aromatic protons. Two quartet signals at δ_C 135.37 and 136.34 were assigned to C-4′ and C-5, where the methyl group is attached. Two doublets at δ_C 143.16 and 149.52, which were sharpened into a singlet by irradiation of ^{1}H-signals at δ_H 6.39 and 6.63, were assigned to C-3 and C-1, respectively. Upon selective irradiation of the ^{1}H-signal at δ_H 6.87, both of the doublets close to δ_C 143.48 were decoupled into singlets. Therefore, these signals were assigned to C-2′ and C-6′, where acetoxyl groups should be located. These findings in the ^{13}C-NMR spectrum support the structure **2** for violaceol-II acetate.

TABLE I. ^{13}C-Chemical Shifts and ^{13}C, ^{1}H Coupling Constants (Hz) of the Aromatic Carbons of Violaceol-I and -II Acetates

Carbon No.	δ_C	$^1J_{C,H}$	$^2J_{C,H}$ and $^3J_{C,H}$
Violaceol-I acetate (in $CDCl_3$)			
1	148.86 (Sd)[a)]		2.4 (6-H)
2	131.60 (Sdd)		*ca.* 6.5 (4-H, 6-H)
3	143.47 (Sd)		3.7 (4-H)
4	118.90 (Do)	165.4	Octet-like (6-H, 5-C$\underline{H}_3$)
5	136.83 (Sq)		5.5 (5-C$\underline{H}_3$)
6	117.44 (Do)	161.8	Octet-like (4-H, 5-C$\underline{H}_3$)
Violaceol-II acetate (in $CDCl_3$)			
1	149.52 (Sd)		3.0 (6-H)
2	129.78 (Sdd)		*ca.* 7.0 (4-H, 6-H)
3	143.16 (Sd)		2.4 (4H)
4	117.42 (Do)	163.0	Octet-like (6-H, 5-C$\underline{H}_3$)
5	136.34 (Sq)		6.1 (5-C$\underline{H}_3$)
6	113.72 (Do)	161.1	Octet-like (4-H, 5-C$\underline{H}_3$)
1′	136.19 (Sdd)		*ca.* 7.0 (3′-H, 5′-H)
2′, 6′	143.48 (Sd)		1.8 (3′-H or 5′-H)
3′, 5′	121.90 (Do)	162.4	Octet-like (3′ or 5′-H, 4′-C$\underline{H}_3$)
4′	135.37 (Sq)		6.1 (4′-C$\underline{H}_3$)

a) S=singlet, D,d=doublet, q=quartet, o=octet.
Capital letters refer to the coupling pattern affected by protons directly bonded to the carbon and small letters refer to that affected by indirect spin coupling.

Thus, the structures 3 and 4 were proved for violaceol-I and -II, as shown in Fig. 1 and 2, respectively.

Carbon-13 chemical shifts and $J_{C,H}$ values of the acetates of violaceol-I and -II are shown in Table I. The signals were assigned by observing the splitting patterns and using parameters calculated from the substitution effects.[2]

Several fungal biphenyl ethers have so far been isolated: they include geodin hydrate from *Aspergillus terreus*,[3] monomethyl- and dimethylosoic acid and methyl asterrate from *Oospora sulphurea-ochracea*,[4] which are possibly derived from geodin-type compounds biogenetically, and funicin from *Aspergillus funiculosus*.[5] However, the substitution pattern in those metabolites is different from that in violaceic acid and violaceols, indicating that the biogenesis of violaceol may proceed through a different pathway from that of the above compounds (*via* the acetate–malonate pathway).

Violaceols caused peritonitis and were lethal to mice on intraperitoneal administration at the level of 100 mg/kg. Violaceols also exhibited weak antimicrobial activity against *Aspergillus niger*, *Candida stellatoides*, *Bacillus subtilis* and *Escherichia coli*.

Experimental

^{1}H-NMR and ^{13}C-NMR spectra were measured with JNM-PS 100 and JNM-FX 200 machines (Japan Electron Optics Lab.). Chemical shifts are shown in δ ppm from tetramethylsilane (TMS) added as an internal standard. Mass spectra were measured on a JMS-01SG-2 machine equipped with a JMA-2000 mass data analysis system (Japan Electron Optics Lab.).

Isolation of Violaceol (Mixture of Violaceol-I (3) and Violaceol-II (4)) from *Emericella violacea*——The fungus (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, the residue (142.1 g) was obtained. The acetone-soluble fraction (130.5 g) of the extract was chromatographed on a silica gel (300 g) column. The *n*-hexane–acetone (1: 1) eluate (23.24 g) was repeatedly chromatographed on a silica gel column (500 g, 150 g). Violaceol (889 mg) was obtained from the fraction eluted with *n*-hexane–acetone (1: 1). Violaceol: viscous oil, $C_{14}H_{15}O_5$ (M^+ *m/e* 262.0914, calcd. 262.0841). A dark blue color was observed in the $FeCl_3$ test.

Acetylation of Violaceol——A solution of violaceol (262 mg) in Ac_2O (1 ml) and pyridine (1 ml) was stirred at room temperature for 24 h. The reaction mixture was poured into ice water and extracted with chloroform. After removal of the solvent, the residue (321 mg) was purified by preparative TLC with *n*-hexane–acetone (2: 1), to give violaceol-I acetate (81 mg) and violaceol-II acetate (85 mg).

Violaceol-I Acetate (1): Viscous oil. ^{1}H-NMR δ_H (in $CDCl_3$): 2.20 (6H, s), 2.26 (12H, s), 6.68 (2H, d, J=2), 6.76 (2H, d, J=2 Hz). MS *m/e* (%): 430 (M^+, 4), 388 (9), 346 (18), 304 (31), 262 (33), 243 (9), 215 (5), 124 (22), 43 (100).

Violaceol-II Acetate (2): Viscous oil. ^{1}H-NMR δ_H (in $CDCl_3$): 2.07 (6H, s), 2.20 (3H, s), 2.26 (6H, s), 2.35 (3H, s), 6.39 (1H, d, J=2), 6.63 (1H, d, J=2 Hz), 6.87 (2H, br s). MS *m/e* (%): 430 (M^+, 6), 388 (22), 346 (32), 304 (59), 262 (76), 243 (34), 215 (27), 124 (100), 43 (100).

Measurement of ^{13}C-NMR Spectrum of Violaceol-I Acetate——Solvent=$CDCl_3$. Tube=egg. Sample=81 mg. Temp.=room temp. Nucleus; obs.=^{13}C (50.18 MHz), lock=D, irr.=^{1}H(199.56 MHz). Offset; obs.=33.0 kHz. irr.=57.2 kHz. Pulse; width=8 μs (45°), repetition=3.0 s. Data points=16K+16K. No. of pulses=2800. Spectral width=10000×1/5 Hz.

Measurement of ^{13}C-NMR Spectrum of Violaceol-II Acetate——Solvent=$CDCl_3$. Tube=egg. Sample=85 mg. Temp.=room temp. Nucleus; obs.=^{13}C (50.18 MHz), lock=D, irr.=^{1}H (199.56 MHz), Offset; obs.=33.0 kHz, irr.=57.2 kHz. Pulse; width=8 μs (45°), repetition=2.0 s. Data points=16K+16K. No. of pulses=2800. Spectral width=10000×1/5 Hz.

Benzoylation of Violaceol——A mixture of violaceol (58 mg), 1 N NaOH (5 ml) and benzoyl chloride (1.5 ml) was stirred at room temperature for 36 h. The reaction mixture was extracted with chloroform. The solvent was washed with 0.5 N NaOH solution and dried over Na_2SO_4. After removal of the solvent by evaporation, the residue (47.3 mg) was separated by preparative TLC, using chloroform as the developing solvent, to give violaceol-I benzoate (10 mg) and violaceol-II benzoate (23 mg).

Violaceol-I Benzoate: Viscous oil. MS *m/e* (%): 678 (M^+, 6.6), 574 (3.7), 452 (3.0), 105 (98.3), 77 (20.6).

Violaceol-II Benzoate: Viscous oil. MS *m/e* (%): 678/(M^+, 1.5), 574 (0.5), 452 (0.5), 105 (100), 77 (40).

Acknowledgement The authors are indebted to Miss K. Takizawa of the Laboratory for Instrumental Analysis of this Institute for measuring the ^{1}H and ^{13}C-NMR spectra, and to Japan Electron Optics Co., Ltd. for help in measuring the 50 MHz ^{13}C-NMR spectra.

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