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The Constituents of *Lactarius flavidulus* IMAI

AKIRA TAKAHASHI, GENJIRO KUSANO, TOMIHISA OHTA,
and SHIGEO NOZOE*

Pharmaceutical Institute, Tohoku University,
Aobayama, Sendai 980, Japan

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Three new geranylphenols, flavidulols A, B and C (I, II and III), were isolated from an edible mushroom, *Lactarius flavidulus* IMAI, and their structures were elucidated on the basis of chemical and spectral evidence. Flavidulol A (I) exhibited antimicrobial activity against *Staphylococcus aureus* and other bacteria.

Keywords—Basidiomycetes; *Lactarius flavidulus*; geranylphenol; flavidulol A; flavidulol B; flavidulol C; antimicrobial activity; *Staphylococcus aureus*; *Bacillus subtilis*

Several mushrooms belonging to the *Lactarius* family are known to show antibacterial activity.^{1,2)} This information led us to examine the constituents of *Lactarius flavidulus* IMAI (Japanese name: kihatsudake), which is edible but has a bitter taste.

Chromatography of the acetone and methanol extracts led to the isolation of an antimicrobial compound, which was named flavidulol A (I), along with flavidulol B (II) and flavidulol C (III). In this paper, we described the isolation and structural elucidation of the new geranylphenols, I—III.

Flavidulol A (I), $C_{17}H_{22}O_2$, an oil, was obtained by Florisil and silica gel chromatographies of the fraction soluble in ethyl acetate. The spectroscopic data of I were similar to those of wigandol (I'), which was isolated from *Wigandia kunthii* CHOISY for the first time,³⁾ except for the signal of a methoxyl group in the place of those of the acetoxyl group of I'. Because authentic specimens of wigandol and related compounds were not available for direct comparison, independent confirmation of the presumed structure was performed as follows.

A two-dimensional (2D)- ^{13}C , 1H -correlated (COLOC) technique was extremely valuable in the assignment of all carbons and hydrogens of flavidulol A acetate (IV), especially in confirming the sequences of carbon atoms [3'-2'-1'(OCH₃)-6'(5')-8-7-6(10) and 2'-3'-4'(OCOCH₃)-5'(6')-1-2(9)-3] (Fig. 1). Nuclear Overhauser effect (NOE) difference spectra of the acetate (IV) suggested that both double bonds at 2(3) and 6(7) were *trans*, as shown in formula IV. A 5% signal enhancement of the methyl protons (10-3H) on irradiating the methylene protons (8-2H) was observed, while no enhancement of the methyl protons (9-3H and 10-3H) on irradiating the vinyl protons (3H and 7-H) was recognized.

Flavidulol B (II), $C_{17}H_{22}O_2$, $[\alpha]_D \pm 0^\circ C$, was obtained as a pale brown oil. The infrared (IR) spectrum of II showed absorption bands at 3600, 1640 and 1600 cm^{-1} due to a hydroxy group, double bond(s) and a benzene ring, respectively. The proton nuclear magnetic resonance (1H -NMR) spectrum (Table I) of II showed the presence of the following groups: a methoxy group, two methylene groups, two aromatic hydrogens, a vinyl methyl, an exomethylene and a vinyl group. Compound II was acetylated by treatment with acetic anhydride in pyridine, providing a monoacetate, which was identical with the Cope rearrangement product (V) obtained from I by refluxing in acetic anhydride containing sodium acetate by comparison of thin layer chromatography (TLC) behavior and spectral

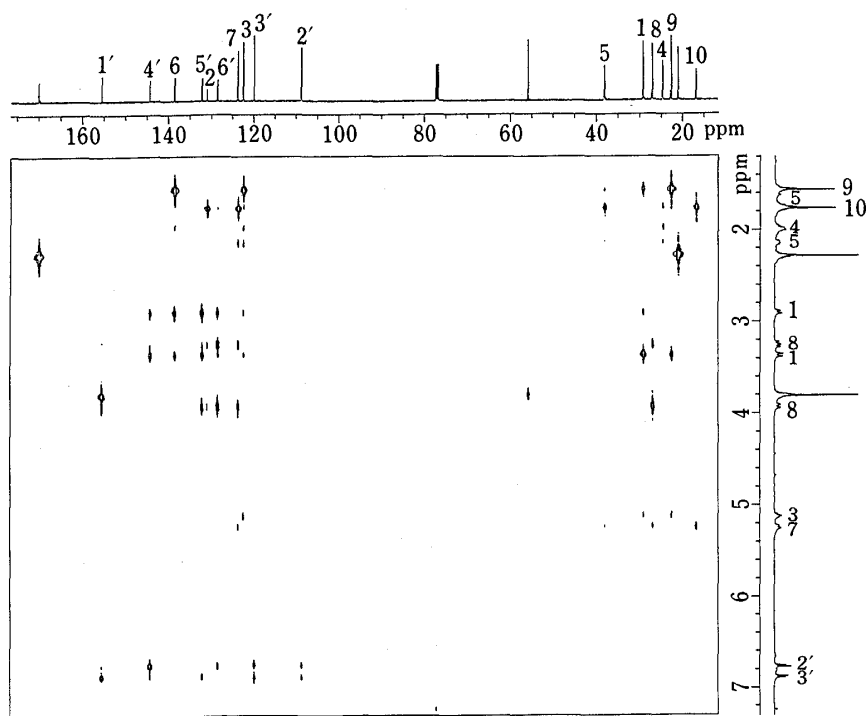


Fig. 1. COLOC Spectrum of Flavidulol A Acetate (IV) in CDCl_3 ($J = 10 \text{ Hz}$)

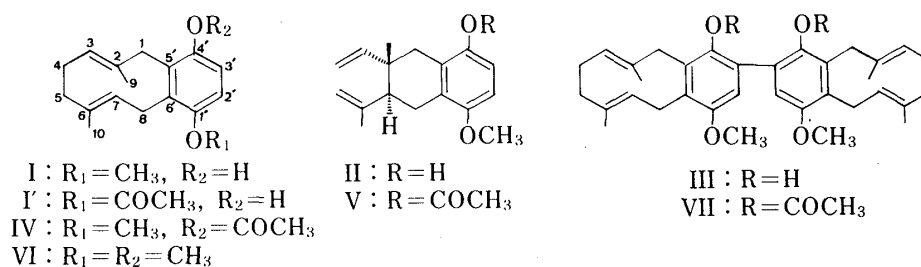


Chart 1

data. The $[\alpha]_D$ value of II was 0° , and therefore, II may be an artifact derived from I. Gómez *et al.* reported that wigandol (I') acetate provided a Cope rearrangement product by pyrolysis and they proposed *cis*-stereochemistry for the product.³⁾ However, it has been reported that compounds containing a *trans-trans* 1,5-diene system afford products of *trans*-stereochemistry by pyrolysis.⁴⁻⁶⁾ We could not obtain any evidence supporting *cis*-stereochemistry for II.

Flavidulol C (III), mp $185\text{--}186^\circ\text{C}$, $\text{C}_{34}\text{H}_{42}\text{O}_4$, was obtained as pale brown needles. The high-resolution mass spectrum (MS) showed the molecular ion peak at m/z 514.306. The ^1H -NMR spectrum was similar to that of I, except for the presence of a singlet at 6.65 ppm due to aromatic protons and a pair of doublets at 3.34 and 3.47 ppm due to the methylene protons in III, instead of a pair of doublets at 6.58 and 6.65 ppm and a singlet at 3.29 ppm in I, respectively. On acetylation with acetic anhydride in pyridine, flavidulol C (III) provided the acetate (VII), mp $175\text{--}177^\circ\text{C}$, $\text{C}_{38}\text{H}_{46}\text{O}_6$, the ^{13}C - and ^1H -NMR spectra of which showed signals due to 19 carbons and 22 hydrogens. These results suggested that III should be a symmetrical dimer composed of two identical units. A 5% signal enhancement of the methoxyl protons on irradiating the aromatic proton was observed in the NOE difference spectra of the acetate (VII). From the results described above, the structure III was proposed for flavidulol C.

TABLE I. The 500 MHz ^1H -NMR Assignments of Flaviculols in CDCl_3

Position	I	II ^{a)}	III	IV	V	VII
1	3.29 (s)	2.26—2.92 (m)	3.34 (d, 14.0)	2.90 (d, 13.8)	2.44 (d, 16.3)	2.79 (d, 14.1)
			3.47 (d, 14.0)	3.36 (d, 13.8)	2.61 (d, 16.3)	3.44 (d, 14.1)
3	5.13 (br t, 8.1, 8.3)	5.96 (dd, 10.0, 18.0)	5.13 (br t, 8.4, 8.4)	5.12 (br t, 8.3, 8.3)	5.98 (dd, 11.3, 17.5)	5.15 (br t, 8.4, 8.4)
4	2.00 (m)	4.77 (d, 10.0)	1.77 (m)	1.98 (m)	4.90 (dd, 1.3, 17.5)	1.78 (m)
		4.86 (d, 18.0)	2.17 (m)		4.96 (dd, 1.3, 11.3)	2.16 (br d,
5	1.74 (m)	4.70—4.88 (s)	2.02 (m)	1.72 (m)	4.79 (br s)	2.02 (m)
	2.14 (br d, 11.0)			2.13 (m)	4.88 (br s)	
7	5.26 (br d, 11.5)	2.24 (dd, 6.0, 10.0)	5.28 (br d, 11.2)	5.24 (br d, 11.0)	2.34 (dd, 6.0, 11.9)	5.26 (br d, 11.3)
8	3.23 (dd, 12.0, 17.1)	2.26—2.92 (m)	3.28 (dd, 11.2, 17.0)	3.25 (dd, 11.0, 17.1)	2.64 (dd, 11.9, 18.1)	3.30 (dd, 11.5, 17.1)
	3.90 (br d, 17.1)		3.95 (br d, 17.0)	39.3 (d, 17.1)	2.82 (dd, 6.0, 18.1)	3.94 (br d, 17.1)
9	1.60 (s)	1.09 (s)	1.56 (s)	1.56 (s)	1.17 (s)	1.65 (s)
10	1.78 (s)	1.73 (s)	1.63 (s)	1.76 (s)	1.80 (s)	1.78 (s)
2'	6.65 (d, 8.5)	6.48 (s)	6.64 (s)	6.75 (d, 8.6)	6.67 (d, 9.4)	6.66 (s)
3'	6.58 (d, 8.5)	6.48 (s)		6.87 (d, 8.6)	6.83 (d, 9.4)	
OCH_3	3.75 (s)	3.70 (s)	3.80 (s)	3.79 (s)	3.80 (s)	3.78 (s)
OH^b	4.84 (br s)		4.90 (br s)			
OCOCH_3				2.27 (s)	2.30 (s)	1.91 (s)

a) At 100 MHz. b) D_2O -exchangeable.TABLE II. ^{13}C -NMR^{a)} Data for I, IV and VII

No.	I	IV	VII	No.	I	IV	VII
1	28.35 t	29.28 t	29.56 t	1'	151.90 s	155.47 s	155.18 s
2	130.82 s	130.95 s	131.14 s	2'	109.73 d	108.74 d	110.88 d
3	121.93 d	122.42 d	122.54 d	3'	112.60 d	119.88 d	129.61 s
4	24.75 t	24.74 t	24.73 t	4'	149.06 s	144.28 s	141.78 s
5	38.38 t	38.20 t	38.27 t	5'	127.56 s	132.17 s	132.39 s
6	139.41 s	138.54 s	138.39 s	6'	128.86 s	128.53 s	127.97 s
7	123.93 d	123.72 d	123.71 d	OCH_3	56.50 s	55.83 s	55.88 s
8	27.07 t	27.11 t	27.07 t	OCOCH_3		169.94 s	169.56 s
9	22.40 q	22.69 q	22.60 q	OCOCH_3		21.09 q	20.49 q
10	16.89 q	16.88 q	16.86 q				

a) At 125 MHz; chemical shifts in ppm (CDCl_3).

The *in vitro* antimicrobial activities of flaviculol A (I) were assayed by the serial two-fold dilution method and the following values of minimum inhibitory concentration (MIC, $\mu\text{g}/\text{ml}$) were obtained: *Staphylococcus aureus* 209P (6.2), *Bacillus subtilis* ATCC 6633 (6.2), *Escherichia coli* NIHJ (>100), *Candida albicans* (50157) (25), *Trichophyton rubrum* sc. (6.2), *T. mentagrophytes* sc. (6.2).

Experimental

Melting points were determined on a Yanagimoto micro hot plate and are uncorrected. IR spectra were recorded on a JASCO A-100S infrared spectrometer with polystyrene calibration at 1601 cm^{-1} . $[\alpha]_{\text{D}}$ value was measured on a

JASCO DIP-340 polarimeter using methanol as the solvent. Ultraviolet (UV) spectra were recorded on a Hitachi 323 spectrometer. MS were taken on a Hitachi M-52 for low resolution and a JEOL JMS-01SG-2 spectrometer for high resolution. ^1H - and ^{13}C -NMR spectra were recorded in CDCl_3 on a JEOL GX-500 (500 and 125 MHz, respectively) and on a JEOL JNM-FX-100 spectrometer (100 and 25 MHz, respectively) with tetramethylsilane (TMS) as an internal standard. Chemical shifts are recorded in δ (ppm) and the coupling patterns are recorded as follows: singlet = s, doublet = d, triplet = t, multiplet = m, and broad = br. Coupling constants (J) are recorded in hertz (Hz).

TLC was performed on Kieselgel 60F₂₅₄ (Merck), and detected under UV irradiation (254 nm) and by heating on a hot plate after spraying Ehrlich reagent.

Isolation Procedure—The air-dried fruiting bodies (69 g) of *Lactarius flavidulus*, collected at Miyagi prefecture in 1985, were extracted three times with acetone (0.7×3) at room temperature (for 1 d each time). After evaporation of the solvent under reduced pressure, a gummy extract (3.0 g) was obtained. The extract was suspended in water (100 ml) and extracted twice with AcOEt (150 ml \times 2). The combined extracts were washed with water (100 ml), dried over Na_2SO_4 and concentrated to give a syrup (3.2 g). In a similar way, the air-dried fruiting bodies (50 g) were extracted with methanol (250 ml \times 3) and a methanolic gummy extract (8.3 g) was obtained. The extract was suspended in water (100 ml) and extracted twice with AcOEt (150 ml \times 2). The combined extracts were washed with water (100 ml), dried over Na_2SO_4 and concentrated to give a syrup (2.8 g).

After TLC analysis, the AcOEt extracts were combined and applied to a Florisil column (45 g; $3.5 \times \text{h}$ 16.0 cm). The column was eluted successively with *n*-hexane (400 ml: fr. 1), 1% AcOEt in *n*-hexane (300 ml: fr. 2), 5% AcOEt in *n*-hexane (200 ml: fr. 3), AcOEt (150 ml: fr. 4), and MeOH (200 ml: fr. 5).

Fractions 2 and 3 were chromatographed on silica gel using *n*-hexane and *n*-hexane–AcOEt and eluants to afford I (943.3 mg), II (39.2 mg) and III (338.2 mg).

Properties of I—*Rf* 0.28 (*n*-hexane: ethyl acetate = 25:1). MS m/z : 258 (M^+), 243 ($\text{M}^+ - 15$), 189, 150. High-resolution MS m/z : M^+ calcd for $\text{C}_{17}\text{H}_{22}\text{O}_2$: 258.1619. Found: 258.1626. ^1H -NMR: Table I. ^{13}C -NMR: Table II. IR $\nu_{\text{max}}^{\text{CHCl}_3}$ cm^{-1} : 3600, 3350, 3000, 2940, 2850, 1600, 1470, 1435, 1070, 980.

Properties of II—*Rf* 0.21 (*n*-hexane: ethyl acetate = 25:1). MS m/z : 258 (M^+), 243 ($\text{M}^+ - 15$), 189, 150. IR $\nu_{\text{max}}^{\text{CHCl}_3}$ cm^{-1} : 3600, 3350, 3000, 2925, 2825, 1640, 1600, 1480, 1460, 1440, 1080. ^1H -NMR: Table I. ^{13}C -NMR (CDCl_3) ppm: 22.90 (q, C-10), 27.18 (q, C-9), 36.93 (t, C-8), 37.63 (s, C-2), 38.04 (t, C-1), 50.49 (d, C-7), 55.66 (q, OCH_3), 107.56 (d, C-2), 111.90 (d, C-2'), 112.90 (t, C-4), 112.90 (t, C-5), 124.11 (s, C-5'), 126.46 (s, C-6'), 142.43 (d, C-3), 146.72 (s, C-6), 146.89 (s, C-4), 150.89 (s, C-1').

Properties of III—*Rf* 0.46 (*n*-hexane: ethyl acetate = 25:1). mp 185–186 °C. MS m/z : 514 (M^+), 499 ($\text{M}^+ - 15$), 406, 201, 187. High-resolution MS m/z : M^+ calcd for $\text{C}_{34}\text{H}_{42}\text{O}_4$: 514.3081. Found: 514.3059. IR $\nu_{\text{max}}^{\text{CHCl}_3}$ cm^{-1} : 3550, 3000, 2940, 2860, 1605, 1460, 1435, 1105, 1080, 990, 960, 840. ^1H -NMR: Table I.

Acetylation of Flavidulol A (I)—Flavidulol A (I, 40.0 mg) was dissolved in 0.3 ml of pyridine and acetic anhydride (0.2 ml) was added. After standing overnight at room temperature, the reaction solution was poured into ice water (10 ml) and the mixture was extracted with ether (10 ml \times 2). The combined ether layer was washed with 0.5 N HCl (5 ml), saturated NaHCO_3 (10 ml), water (10 ml) and saturated NaCl (10 ml) successively and dried over Na_2SO_4 . After removal of the drying agent and evaporation of the solvent, the residual syrup was chromatographed on silica gel to give the acetate (IV, 41.7 mg) as colorless needles (from *n*-hexane). mp 111–112 °C, $\text{C}_{19}\text{H}_{24}\text{O}_3$. MS m/z : 300 (M^+), 258, 243, 189, 150. IR $\nu_{\text{max}}^{\text{CHCl}_3}$ cm^{-1} : 3015, 2950, 2870, 1750, 1585, 1460, 1435, 1380, 1180, 1070. ^1H -NMR: Table I. ^{13}C -NMR: Table II.

Flavidulol A (I, 103 mg) was dissolved in acetic anhydride (1.0 ml) and sodium acetate (103 mg) was added. The reaction mixture was heated at 140 °C for 2 h. After cooling, the reaction mixture was poured into water (10 ml) and extracted with ethyl acetate (10 ml) twice. The combined solution was washed with saturated NaHCO_3 , water and saturated NaCl successively, and dried over Na_2SO_4 . After removal of the drying agent and evaporation of the solvent *in vacuo*, the residual syrup was chromatographed on silica gel to afford a Cope rearrangement acetate (V, 116 mg) as colorless needles (from *n*-hexane). mp 62–64 °C, $\text{C}_{19}\text{H}_{24}\text{O}_3$. MS m/z : 300 (M^+), 258, 243, 189, 150. IR $\nu_{\text{max}}^{\text{CHCl}_3}$ cm^{-1} : 3090, 3015, 2965, 2940, 2840, 1750, 1640, 1590, 1470, 1450, 1430, 990, 900, 890. ^1H -NMR: Table I. ^{13}C -NMR (CDCl_3) ppm: 20.78 (q, OCOCH_3), 23.01 (q, C-10), 26.24 (q, C-9), 27.18 (t, C-8), 37.69 (s, C-2), 38.04 (t, C-1), 50.37 (d, C-7), 55.31 (q, OCH_3), 107.03 (d, C-2'), 113.02 (t, C-5), 113.19 (t, C-4), 118.90 (d, C-3'), 126.46 (s, C-6'), 129.05 (s, C-5'), 142.20 (s, C-4'), 146.48 (s, C-6), 154.58 (s, C-1'), 169.61 (s, OCOCH_3).

Acetylation of Flavidulol B (II)—Acetic anhydride (0.1 ml) was added to a solution of flavidulol B (II, 25 mg) in pyridine (0.15 ml) and the reaction mixture was left to stand at room temperature overnight. After usual work-up, the products fraction was chromatographed on silica gel to afford the acetate (V, 27.0 mg) as colorless needles. The MS, IR, ^1H -NMR spectra were identical with those of the Cope rearrangement acetate (V) of flavidulol A.

Acetylation of Flavidulol C (III)—Flavidulol C (III, 55.9 mg) was dissolved in pyridine (0.3 ml) and acetic anhydride (0.2 ml) was added with stirring at room temperature overnight. After usual work-up, an acetate (VII, 48.1 mg) was obtained as colorless needles, mp 175–177 °C, $\text{C}_{38}\text{H}_{46}\text{O}_6$. MS m/z : 598, 556, 514, 187. IR $\nu_{\text{max}}^{\text{CHCl}_3}$ cm^{-1} : 3020, 2945, 2870, 1755, 1600, 1565, 1455, 1435, 1370, 1195, 835. ^1H -NMR: Table I. ^{13}C -NMR: Table II.

Methylation of Flavidulol A (I)—A solution of flavidulol A (26.0 mg) in dry tetrahydrofuran (1 ml) was added dropwise to a suspension of sodium hydride (8.0 mg, 60% dispersion in mineral oil) in tetrahydrofuran (1.0 ml) at 0 °C

under an N₂ atmosphere, and the mixture was stirred at room temperature for half an hour. Methyl iodide (12.5 μ l) was added to the solution with stirring at room temperature. After an hour, ice water (10 ml) was added to the solution and the product was extracted with ethyl acetate (10 ml) twice. The upper layer was washed with water and saturated NaCl, and dried over Na₂SO₄. After removal of the drying agent and evaporation of the solvent, the residual syrup was chromatographed on silica gel and a methyl ether (VI, 23.7 mg) was obtained as a colorless viscous oil. MS m/z : 272 (M⁺), 257, 203, 164. IR $\nu_{\text{max}}^{\text{CHCl}_3}$ cm⁻¹: 3005, 2950, 2870, 1600, 1440, 840, 830. ¹H-NMR (CDCl₃) ppm: 1.54 (3H, s, H-3), 1.80 (3H, s, H=10), 1.80—2.23 (4H, m, H-4, H-5), 3.15—3.50 (2H, m, H-8), 3.63—3.87 (2H, m, H-2), 3.74 (3H, s, OCH₃), 3.78 (3H, s, OCH₃), 4.90—5.43 (2H, m, H-3, H7), 6.66 (2H, s, H-2', H-3').

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