LACCARIN, A NEW ALKALOID FROM THE MUSHROOM, *LACCARIA VINACEOAVELLANEA*

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Abstract --- A new alkaloid laccarin was isolated from the mushroom, *Laccaria vinaceoavellanea,* and the structure was determined on the basis of a spectroscopic analysis. Laccarin showed phosphodiesterase inhibitory activity.

In the course of our program to find a new biologically active compound from Japanese mushroom (Basidiomycotina),[1] we found a new alkaloid, designated laccarin, from *Laccaria vinaceoavellanea* Hongo, Tricholomataceae, Japanese name: karebakitsunetake. This pale brownish mushroom has a convex cap with the hollow in the center and striate margin.[2] The edibility is not known. In this paper, we wish to report the isolation and the structural elucidation of laccarin from *L. vinaceoavellanea,* and its inhibitory activity against cyclic AMP phosphodiesterase.

The methanol extract of the fruit bodies of *L. vinaceoavellanea* was partitioned between ethyl acetate and water. The ethyl acetate extract was repeatedly subjected to silica gel chromatography using chloroform-ethyl acetate and chloroform-methanol solvent systems to afford laccarin.

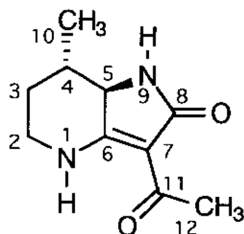

Figure 1. Relative Stereostructure of Laccarin

Laccarin, $[\alpha]_D^{31}$ +188° (c 0.47, $CHCl_3$), was isolated as colorless powder that showed yellow coloration with ninhydrin. The molecular formula $C_{10}H_{14}N_2O_2$ was determined by the combination of HREI ms (*m/z* 194.1049, M^+), FAB ms [*m/z* 195, $(M+H)^+$] and ^{13}C nmr spectra. ^1H Nmr spectrum of laccarin exhibited the signals for a methyl group (δ_H 2.46), a secondary methyl group (δ_H 1.16), two methylene groups (δ_H 1.60, 2.02; 3.49, 3.52), two methine groups (δ_H 1.69; 3.63) and two exchangeable proton signals with D_2O (δ_H 5.40; 8.96). Those exchangeable proton signals were assigned to NH groups since two carbonyls used up two oxygen atoms in the molecule as indicated in the ^{13}C nmr spectrum. ^{13}C Nmr spectrum of laccarin showed 10 signals due to two carbonyls, two sp^2 and six sp^3 carbons as shown in Table 1.

Table 1. ^1H and ^{13}C Nmr Spectral Data for Laccarin.

Position	^{13}C	$^1H^a$	HMBC correlation^b
1		8.96 (1H, br s)	
2	41.2 (t)	3.49 (1H, ddd, *J*=3.5, 11, 13)	C-3, 4, 6
		3.52 (1H, ddd, *J*=1.8, 5.5, 13)	
3	28.1 (t)	1.60 (1H, dddd, *J*=5.5, 11, 11, 14)	C-2, 4, 5, 10
		2.02 (1H, dddd, *J*=1.8, 3.5, 5, 14)	C-4, 5
4	31.8 (d)	1.69 (1H, dddq, *J*=5, 11, 11, 6.5)	
5	58.3 (d)	3.63 (1H, d, *J*=11)	C-3, 4, 6, 8, 10
6	172.7 (s)		
7	100.2 (s)		
8	173.5 (s)		
9		5.40 (1H, br s)	C-5, 7, 8
10	19.1 (q)	1.16 (3H, d, *J*=6.5)	C-3, 4, 5
11	196.2 (s)		
12	27.5 (q)	2.46 (3H, s)	C-11

a) Coupling constants (*J*) were given in Hz.

b) HMBC spectra were taken twice with maximum values of 7 or 9 Hz for $^nJ_{CH}$ in each case.

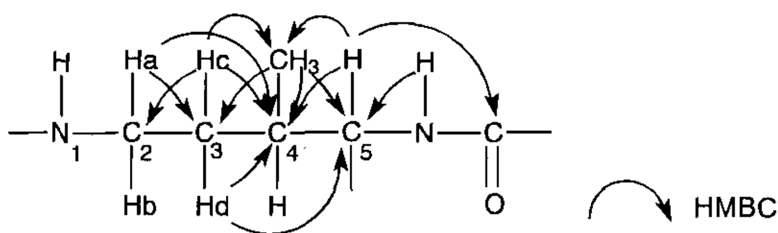

Figure 2. Partial Structure of Laccarin.

The signals at δ_H 2.46 and δ_C 196.2 indicated an acetyl group in the molecule. The carbonyl carbon signal at δ_C 173.5, along with ir absorption at 3270 and 1660 cm^{-1}, implied an amide or a lactam moiety. The methine hydrogen next to the amide nitrogen was inferred to resonate at δ_H 3.63 as a doublet signal (H-5). Signals at similar chemical shift at δ_H 3.49 and 3.52 were assumed to correspond to a methylene next to an amine having last one of the four hetero atoms in the molecule.

2D NMR such as H-H COSY, HMQC and HMBC, and 1D proton double and triple resonance experiments revealed the correlation of the hydrogens as shown in Figure 2. Both of the two amino-hydrogens at δ_H 5.40 and 8.96 were observed as a broad singlet, respectively. The amino-hydrogen signal at δ_H 8.96 observed as a broad singlet with a half-band width of 17.0 Hz sharpened by triple resonance experiment, irradiating aminomethylene signals at δ_H 3.49 and 3.52. The half-band width decreased to 15.5 Hz. The assignment of the signal at δ_H 8.96 to the amino-hydrogen, N-1, was supported by the NOE correlation to H-2 (Figure 3).

The aminomethylene signals at δ_H 3.49 and 3.52 showed a long range correlation to the olefinic carbon signal at δ_C 172.7 in the HMBC spectrum. This quaternary sp^2 carbon signal showed a cross peak with the amidomethine proton signal at δ_H 3.63 in the HMBC spectrum. The above facts and Figure 2 indicated a piperidine ring in the structure of laccarin. Coupling constants around 11 Hz for Hb-2/Hc-3, Hc-3/H-4 and H-4/H-5 indicated trans-diaxal relationships of those hydrogens in six-membered ring having chair conformation. Partial structures thus obtained as illustrated in Figure 3 lacked one unsaturation unit, which lead the conjugated γ-lactam ring. C-7 was the only place left to place the acetyl group. Interestingly, the acetyl-methyl signal at δ_H 2.46 showed C-H long range correlation with only an acetyl-carbonyl carbon signal at δ_C 196.2 in the HMBC spectrum taken with 7 or 9 Hz of maximum long range coupling constant (^nJ). It might be because the acetyl group was twisted in the conjugate system and ^nJ was too small to give any cross peak with olefinic carbon signal. In fact, the acetyl-carbonyl signal showed the coupling with only a methyl signal as a detectable splitting in the INEPT spectrum.

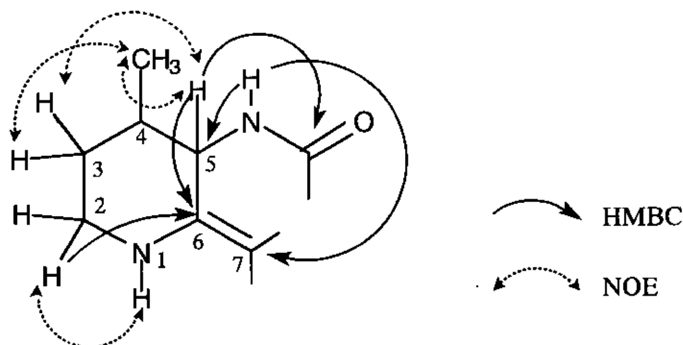

Figure 3. Extended Partial Structure of Laccarin

The conjugate system in laccarin showed strong uv absorptions at λ 235 (log ε 4.05) and 293 (log ε 4.07) nm, similar to those of Fusarium toxin equisetin.[3] Consequently the relative stereo structure of laccarin was established as illustrated in Figure 1.

Laccarin was found to have a similar chromophore to that of tenuazonic acid having phytotoxic and anti-neoplastic activities.[4] Structural similarity of laccarin to tenuazonic acid implies an analogous pathway in that biogenesis.[5]

Laccarin did not show a cytotoxicity against P388 mouse leukemia nor the inhibitory activity against platelet aggregation. It, however, showed a moderate inhibition on phosphodiesterase activity. This enzyme plays an important role on the hydrolysis of the intracellular cyclic AMP. It is considered that the actions of papaverine, dipyridamole, caffeine and theophylline are due to the inhibition of this enzyme.[6,7] As a standard inhibitor, caffeine was used for a comparison in this study. Caffeine decreased phosphodiesterase activity to 58% at 0.54 mg/ml. Similar concentration of laccarin decreased the activity to 30% at 0.64 mg/ml.

EXPERIMENTAL

The ^{1}H nmr and ^{13}C nmr spectra were recorded on JOEL JNM GX 500 spectrometer (500 MHz for ^{1}H, 125 MHz for ^{13}C) with TMS as an internal standard. High resolution electron impact (HREI) mass spectrum was recorded on a JEOL JMS DX303 spectrometer. Fast atom bombardment (FAB) mass spectrum was recorded on a JEOL JMS DX303 spectrometer using glycerol as a matrix. Optical rotation was recorded on a JASCO DPI-370 spectrometer. Uv spectrum was recorded on a HITACHI U-3200 spectrophotometer.

Isolation The fruiting bodies (1.6 kg) of *L. vinaceoavellanea,* collected in Akita prefecture on September 1993, were extracted twice with methanol (4 l) at room temperature for 1 day. After filtration, the combined solvent

was concentrated *in vacuo* to afford suspension. This suspension was partitioned between ethyl acetate and water. After evaporation, the ethyl acetate soluble fraction (3 g) was subjected to silica gel (50 g) chromatography using the solvent systems chloroform-ethyl acetate and chloroform-methanol to give six fractions. Fraction No. 4 (290 mg), eluted with chloroform-methanol (99:1), was next subjected to silica gel (30 g) chromatography using chloroform-ethyl acetate (20:1) to give six fractions. Fraction No's 4-5 (60 mg) was subjected to silica gel (15 g) chromatography using chloroform-MeOH (99:1) to afford laccarin (12.1 mg), $[\alpha]_D^{31}$ +188^0 (c 0.47, $CHCl_3$). Ir ν_{max} cm^{-1}: 3270, 3150, 1660, 1630, 1590, 1490. Uv λ_{max} nm (log ε): 235 (4.05), 293 (4.07). EI ms: m/z 194 $(M)^+$, 179 $(M-CH_3)^+$. HREI ms: *m/z* 194.1049 $([M]^+$, Calcd for $C_{10}H_{14}N_2O$: 194.1055). ^1H; ^{13}C nmr : see Table 1.

Assay Phosphodiesterase activity was assayed by malachite green method[8] using the following solutions. The enzyme solution : phosphodiesterase (1.48 U/ml, 1 part), 5'-nucleotidase (67 U/ml), $MgCl_2$ (50 mM, 4 parts), 0.5 M Tris-HCl (0.5 M, 8 parts) and water (26 parts). The reaction mixture A : malachite green (1 mM, 2 parts), polyvinyl alchohol (11.6 mg in 500 ml water, 1 part), ammonium molybdate (50 mM in 6N HCl, 1 part) and water (1 part). Sample solution : samples (around 1 mg) were dissolved to water (2.0 ml) and DMSO (2 drops). The reaction was started by the addition of the sample solution (0.5 ml) to the enzyme solution (0.4 ml) at 30 ^{0}C. Cyclic AMP (10 mM, 0.1 ml), the mixture A (1.0 ml) and 25 % sodium citrate (0.2 ml) were added to the above solution successively in every 5 minutes. The absorbance of the color complex was measured at 630 nm against a mixed reagent blank. Laccarin decreased the activity to 30% at 0.64 mg/ml.

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