THE ISOLATION, FROM *MYROTHECIUM* SPECIES, AND LONG-RANGE SELECTIVE PROTON DECOUPLING ^{13}C-NMR OF RHIZONIC ACID[1]

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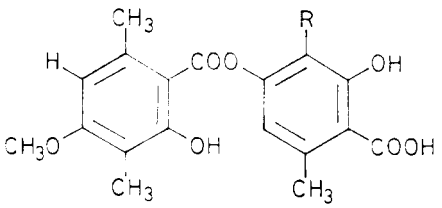
ABSTRACT.—Rhizonic acid, which has a partial structure of lichen depsides and depsidones, was isolated for the first time from a natural origin, a possibly new fungal species of genus *Myrothecium*. Low power ^{1}H irradiation methods including long-range selective proton decoupling (lspd), selective ^{13}C{^{1}H} noe, and ^{13}C{^{1}H,^{1}H} triple resonance (triple lspd) were applied for the structural comfirmation.

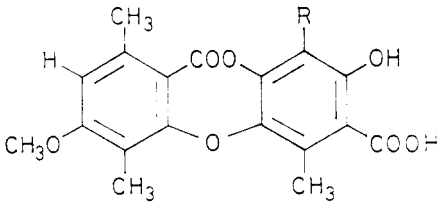
Rhizonic acid (2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid) is known as a structural component of lichen depsides, such as obtusatic acid (**1**) (2) and barbatic acid (**2**) (3), as well as lichen depsidones, such as notatic acid (**3**) (4, 5) and 4-*O*-methylhypoprotocetraric acid (**4**) (4, 5). However, as far as we are aware, no report on the isolation from natural sources of rhizonic acid has been made until the present. Now we report the isolation of this compound from the liquid culture of a possibly new species of the genus *Myrothecium* (Fungi, Deuteromycotina, Hyphomycetes).

In the course of the structural identification of rhizonic acid, the authors applied not only the ordinary ^{1}H{^{1}H} nuclear Overhauser effect (noe) but also several new techniques in the field of ^{13}C-nmr, *i.e.*, long-range selective proton decoupling (lspd), selective ^{13}C{^{1}H} noe, and ^{13}C{^{1}H, ^{1}H} triple resonance (triple lspd). These new techniques, which have been developed recently by one of the authors (6), were found to have good applicability with this compound as is reported herein.



1 R = H

2 R = CH_3



3 R = H

4 R = CH_3

[1]Part 7 in the series "Studies on Fungal Products." For part 6 see LITERATURE CITED 1.

EXPERIMENTAL[2]

ORGANISM.—*Myrothecium* sp. NHL 2822 used in this experiment was originally isolated by Mr. T. Awao[3] from the soil of Hiekawa, Naka-izu-cho, Tagata-gun, Shizuoka-ken, Japan, in February 1973, and the living culture has been deposited in the National Institute of Hygienic Sciences (NHL), Tokyo. Since the morphology is different from any previously known species of the genus, it seems to be a new species. Its taxonomical aspects are currently under study in the Mycology Laboratory, NHL.

The organism was grown on potato-dextrose agar, potato-carrot agar, and other common mycological media. It was maintained on potato extract slants containing 0.03 g per ml of sucrose; 0.03 g per ml of sodium chloride; 0.027 g per ml of agar; and NaOH to bring the pH to 6.0.

CULTURE.—The organism was grown in a medium containing 3% sucrose; 0.6% $NaNO_3$; 0.1% K_2HPO_4; 0.05% KCl; 0.05% $MgSO_4 \cdot 7H_2O$; 0.01% corn steep liquor; 0.01% trace element solution[4]; and 0.0001% biotin in water. The pH of the medium was adjusted to 5.0 by the addition of dilute hydrochloric acid. The medium was distributed into Roux bottles (200 ml per each bottle); the bottles were stoppered with cotton plugs and autoclaved at 121°C, 15 psi for 30 minutes. Each bottle was inoculated with a heavy spore suspension which was prepared by adding sterile water to the seed culture on the potato extract agar. Five hundred Roux bottles were used for the stationary culture at 27°C for 26 days.

ISOLATION OF RHIZONIC ACID.—Cultures of *Myrothecium* sp. NHL 2822 were filtered through cheesecloth, and the mycelium obtained was allowed to air dry for two weeks. The dried mycelium was pulverized in a mortar and weighed 680 g. This was then subjected to continuous extraction for a week with methylene chloride by use of an Asahina-type extractor (7). The dark reddish brown sticky mass (42.2 g) left after evaporation *in vacuo* was mixed with one liter of hexane and warmed to 35°C, and the soluble fatty fraction was removed by decantation. The insoluble part, 5.03 g of brown amorphous powder, was chromatographed on a silica gel[5] column (150 g, 4.5×19 cm); the developing solvent consisted of a mixture of ethyl acetate and hexane in the successively increasing ratio, 1:7 to 1:1, of the former solvent. The seventh to tenth 50-ml fractions gave an orange-colored amorphous powder after removal of the solvent. This amorphous powder was mixed with 100 ml of hexane then boiled and filtered. After evaporation of the solvent, the filtrate gave colorless crystals, which were recrystallized from ethyl acetate to yield colorless prisms (1.2 g). These melted with decomposition at 196°C when heated slowly, but melted at 204°C when heated quickly. *Anal.* Calcd. for $C_{10}H_{12}O_4$: C, 61.19; H, 6.17. Found: C, 61.05; H, 6.01. The ms exhibited ions at *m/e* 196 (M^+, 75%), 178 (100), 150 (100). It showed ir ν max (KBr) 2970, 2860 and 1380 (CH_3), 2860 and 1264 (OCH_3), 3000–2400, 1650–1620 shoulder and 1420 (COOH), 3000–2400 and 1224 (OH), 1605, 1570, 1500 and 880 cm^{-1} (phenyl); ^{1}H-nmr (d_5-pyridine) δ_H 2.37 (3H–7, s), 2.83 (3H–9, s), 3.77 (3H–8, s), 6.46 (1H–5, s), 12.38 (OH and COOH, exchanged with D_2O); ^{13}C-nmr (d_5-pyridine) δ_C 8.4 (C–7, q), 24.6 (C–9, q), 55.4 (C–8, q), 105.8 (C–5, d), 107.2 (C–1, s), 110.4 (C–3, s), 141.1 (C–6, s), 161.3 (C–4, s), 163.3 (C–2, s), 175.9 (C–10, s). The methyl ester was prepared by reaction with diazomethane in ether. Recrystallization from ethanol gave a pure ester as colorless needles, mp 95°C. This ester was identified as methyl rhizonate by mixed fusion with an authentic sample.

RESULTS AND DISCUSSION

Colorless prismatic crystals obtained from *Myrothecium* sp. NHL 2822 showed a melting point that fluctuated with the speed of raising the temperature. The substance is phenolic as it showed a purple color in ethanolic solution upon addition of ferric chloride. This compound, molecular formula $C_{10}H_{12}O_4$ by analytical

[2]Melting points were not corrected. The ir spectrum was obtained on a Hitachi model 215 recording spectrophotometer in KBr pellets. ^{1}H-nmr spectra and ^{13}C-nmr spectra were measured with Varian HA-100 D and JEOL FX-100 spectrometers, respectively. In the experiments of low power ^{1}H irradiation, *i.e.*, lspd, selective ^{13}C{^{1}H} noe and triple lspd, final ^{1}H irradiation amplifier was replaced by a JEOL low power irradiation unit, and the power level was 5~10 mgauss. The ^{1}H irradiation mode was a continuous wave condition. The FT measurements conditions were as follows; spectral width, 5 KHz; data points, 16 K; repetition time, 1.8 sec; flip angle, 45°; number of pulses, 10,000. Samples were measured for 15 w/v% solution in d_5-pyridine in a 10 mm microtube (Shigemi standard joints Ind. Co., Ltd.). Electron impact mass spectrum was obtained with a JEOL JMS-D 300 instrument.

[3]Central Research Laboratories, Ajinomoto Co., Inc.

[4]This solution was prepared by dissolving 0.99 g $FeSO_4 \cdot 7H_2O$; 0.057 g H_3BO_3; 0.081 g $MnSO_4 \cdot 4H_2O$; 1.065 g $ZnSO_4 \cdot 7H_2O$; 0.049 g $Na_2MoO_4 \cdot 2H_2O$ and 0.259 g $CuSO_4 \cdot 5H_2O$ in one liter of water.

[5]Kanto Kagaku Co., Ltd.

and mass spectroscopic data, possesses a carboxyl function because it is very soluble in sodium bicarbonate solution with effervescence and shows a two-proton singlet at 12.38 ppm exchangeable with D_2O and a carbon singlet at 175.9 ppm in the nmr spectra. The hydroxyl is assigned *ortho* to the carboxyl because the mass spectrum shows a strong dehydration peak at *m/e* 178. The presence of two methyl groups and one methoxyl group was also indicated by proton and ^{13}C-nmr spectra. In the earlier stages of this study, the authors found difficulty in obtaining an authentic sample of the methyl ester of rhizonic acid, and to make the matter more troublesome, the melting point of rhizonic acid varies in literature very widely [from 186°C (8) to 235°C (9)]. Thus, we were obliged to locate the positions of the methyls and the methoxyl on the benzene ring. The intramolecular nuclear Overhauser effects (noe) in d_5-pyridine were measured. As shown in figure 1, irradiation of the proton signals of the methoxy (δ 3.77 ppm) and 9-methyl (δ 2.83 ppm) caused an 18% and 13% increase, respectively, in the integrated intensity of the aromatic proton signal (δ 6.26 ppm), while no effect was observed upon the irradiation of the 7-methyl (δ 2.37 ppm) signal. These results indicated the *meta* positioning of one methyl group to the methoxy group on the benzene ring.

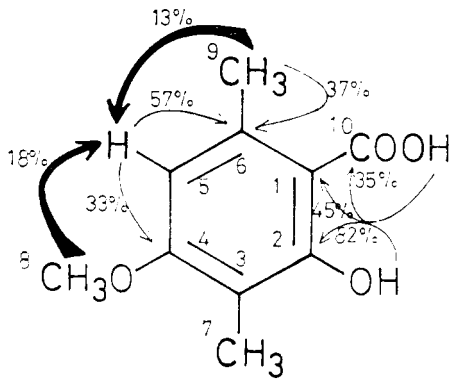


FIG. 1. $^1H\{^1H\}$ and Selective $^{13}C\{^1H\}$ noe of rhizonic acid.

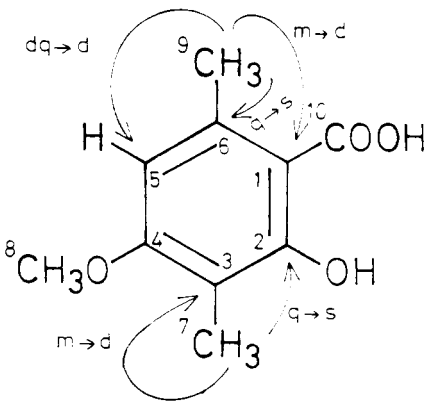


FIG. 2. Lspd in ^{13}C-nmr of rhizonic acid.

For further structural elucidation, several new techniques, including lspd, selective $^{13}C\{^1H\}$ noe and triple lspd, were applied. These techniques have recently become available and involve (6) selective irradiation of protons with low power instead of the high power used commonly for decoupling of protons in ^{13}C-nmr. As shown in figure 1, low power irradiation of the hydroxyl proton caused an enhancement of the integral intensities of the signals at C–1, C–2 and C–10, as much as 45%, 82% and 35%, respectively. These results of selective $^{13}C\{^1H\}$ noe confirmed *ortho* positioning of the carboxyl group to the hydroxyl-bearing one. The positioning of the 9–CH_3 *meta* to the methoxy group was further confirmed by the fact that increases of 57% and 33%, respectively, in the intensities of the signals at C–6 and C–4 were observed upon irradiation of H–5 and that the irradiation of the protons of 9–CH_3 caused the enhancement in the intensities, by as much as 37%, of the signal at C–6.

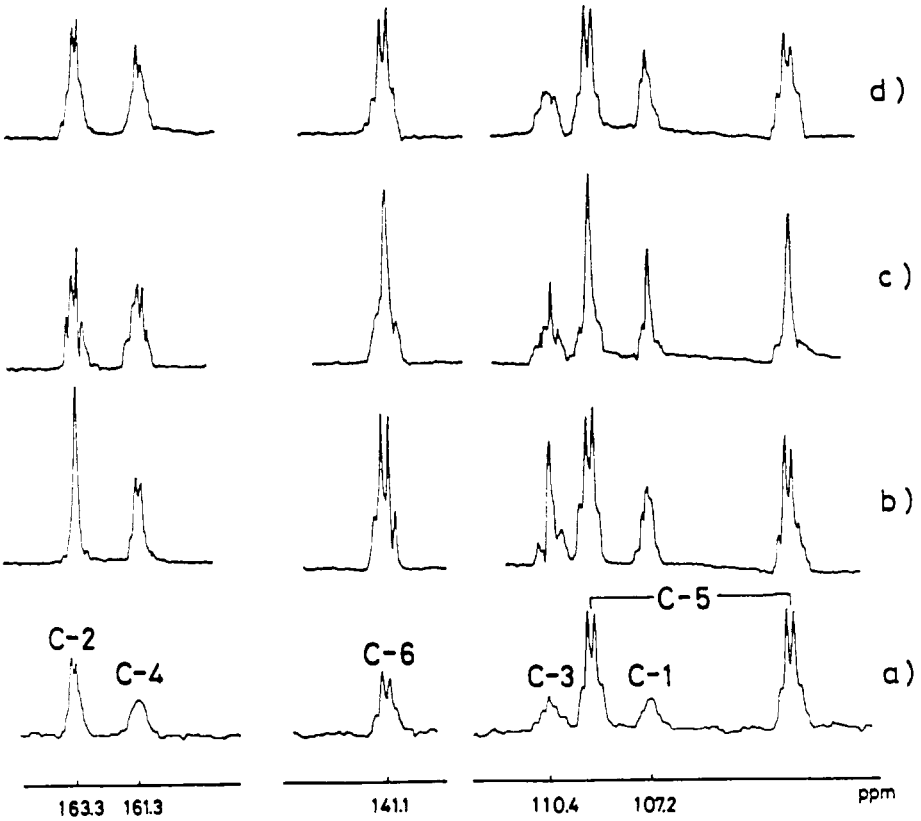


FIG. 3. Triple lspd in ^{13}C-nmr of rhizonic acid; a) non-decoupled b) H-5 and 7-CH_3 irradiated c) H-5 and 9-CH_3 irradiated d) H-5 and OCH_3 irradiated.

Relative positioning, respectively, of 7-CH_3 and 9-CH_3 in comparison with other functional groups was clearly determined by lspd (fig. 2) and triple lspd (fig. 3). Irradiation of the protons of 9-CH_3 with low power caused the change in the shapes of the carbon signals: C-1, multiplet to doublet; C-6, quartet to singlet; C-5, double quartet to doublet (fig. 2). The irradiation at the 7-CH_3 caused collapse of the signals of C-2, quartet into a singlet, and C-3, multiplet into a doublet (fig. 2). Furthermore, double irradiation of 7-CH_3 and H-5 caused the signal of C-3 to change from a multiplet to a singlet; whereas, the double irradiation of 9-CH_3 and H-5 caused the signal of C-1 to change from a multiplet to a singlet (fig. 3). All these results indicated the metabolite to be rhizonic acid. And the final confirmation was made by the preparation of its methyl ester followed by the mixed fusion with an authentic sample.

A survey of the literature revealed that several metabolites such as roridins (10, 11), verrucarins (10) and myrothecin (12) had been isolated from a few strains of *Myrothecium*, but no isolation from *Myrothecium* of a compound, such as rhizonic acid, relating structurally to lichen metabolites had been reported.

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