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SHORT REPORTS

2(*S*)-AMINOHEX-5-YNOIC ACID, AN ANTIMETABOLITE FROM *CORTINARIUS CLARICOLOR* VAR. *TENUIPES*

YASUO AOYAGI and TATSUYUKI SUGAHARA

Food Chemistry, Kagawa Nutrition College, Komagome 3-24-3, Toshima-Ku, Tokyo, 170, Japan

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Key Word Index—*Cortinarius claricolor* var. *tenuipes*; Basidiomycetes; mushroom; antimetabolite; non-protein amino acid; acetylenic amino acid; 2(*S*)-aminohex-5-ynoic acid.

Abstract—Screening for antimetabolites in edible mushrooms showed that the hot water extract of fruiting bodies of *Cortinarius claricolor* var. *tenuipes* strongly inhibited the growth of *Bacillus subtilis* B-50 in a chemically defined minimal medium. 2(*S*)-Aminohex-5-ynoic acid was isolated as an active compound.

INTRODUCTION

There have been many reports on antibacterial or antimetabolic substances in the fruiting bodies of Basidiomycetes [1], but there have been almost none in edible mushrooms. We have conducted antimetabolite screening of various Japanese edible mushrooms by examining the antibacterial activity against *Bacillus subtilis* B-50 in a chemically defined minimal medium. In the results of our previous report, we found the existence of antimetabolic amino acids in two species of Basidiomycetes, *Rhodophyllus crassipes* and *Rozites caperata*, and reported that they were 2-amino-3-butenoic acid and *S*-2-aminoethyl-L-cysteine, respectively [2].

In addition, we have now found a substance which strongly inhibits the growth of *B. subtilis* B-50 in the minimal medium, in the hot water extract of the mushroom, *Cortinarius claricolor* [3]. Structural studies led to the identification of its structure as an acetylenic amino acid, 2(*S*)-aminohex-5-ynoic acid. There have been some reports on the isolation of amino acids containing an acetylenic structure in the fruiting bodies of Basidiomycetes [4], bacteria [5] and higher plants [6], but none on this amino acid.

RESULTS AND DISCUSSION

The test for antibacterial activity was carried out using a sensitive disc method using a chemically defined minimal medium [7]. The hot water extract of *C. claricolor* var. *tenuipes* inhibited the growth of *B. subtilis*. The defatted residue of the 70% ethanol extract of the mushrooms also showed the same effect. The active substance was eventually isolated by fractionation using cation and anion exchange resins from this defatted extract. The compound gave a positive reaction with ninhydrin and revealed a single peak just before Val in AA (automated amino acid analysis). The FD mass spectrum of the purified crystals showed the M_r to be 127. From an elementary analysis, the empirical formula was estimated to be $C_6H_9NO_2$. As the ninhydrin reaction was inhibited by Cu^{2+}, the compound was considered to be an α-mono amino acid. The compound absorbed 1.96 mol of H_2 by hydrogenation using Adams PtO_2. TLC and AA of this reaction mixture showed norleucine as the sole ninhydrin positive product. These results showed that the amino acid is an unsaturated norleucine with two double bonds or one triple bond. Oxidation with acidic potassium permanganate to elucidate the unsaturated position, resulted in the production of glutamic acid as the sole ninhydrin positive product. This strongly suggests that the compound is 2-aminohex-5-ynoic acid. The IR showed absorbance at 3300 cm^{-1} (≡C–H) and 2120 cm^{-1} (–C≡C–), which supports the above assumption. Furthermore, the ^1H NMR spectrum was also in good agreement with this structure. The optical rotation values of this amino acid were increased with greater acidity, showing that it belonged to the L-series. Therefore, the active compound was identified as 2(*S*)-aminohex-5-ynoic acid.

As for naturally occurring acetylenic amino acids, the first one reported was 2-amino-4-hydroxyhex-5-ynoic acid from the higher plant, *Euphoria longan* [6]. In mushrooms, the 2-aminohex-4-ynoic acid (*Tricholomopsis rutilans*, *Amanita pseudoporphyria*), 2-amino-6-hydroxyhex-4-ynoic acid (*Amanita onusta*), *threo* and *erythro*-2(*S*)-amino-3-hydroxyhex-4-ynoic acids (*Tricholomopsis rutilans*), 2-aminopent-4-ynoic acid and 2-aminohept-4-ene-6-ynoic acid (*Amanita pseudoporphyria*), have also been reported [4]. However, this is the first time that the isolation of 2(*S*)-aminohex-5-ynoic acid from a natural source has been reported.

The amino acid inhibited the growth of *B. subtilis* B-50 in the minimal medium at 0.1 μg/ml. Further work to

elucidate the mechanism of this antimetabolic effect are in progress.

EXPERIMENTAL

General. TLC was run on a precoated silica gel plates (Kieselgel 60, Merck) using *n*-BuOH–HOAc–H_2O (4:1:2). ^1H NMR was measured in D_2O at 90 MHz with TSP as int. standard.

Microbiological assay. The test for antibacterial activity on *B. subtilis* B-50 in a chemically defined minimal medium, was carried out according to the method of ref. [5] with some modifications. *Bacillus subtilis* was precultured overnight in a meat extract bouillon containing 0.1% Glc at 37°, and washed ×3 with sterilized H_2O. The cells were collected and dispersed in sterilized H_2O (the *A* at 500 nm of the 10-fold dil. suspension was 0.5) to make a bacterial suspension for inoculation. The minimal medium of ref. [7] was prepared by mixing Glc, agar and other reagents which were separately dissolved and sterilized at 120° for 15 min. After cooling to 45°, the above bacterial suspension was inoculated to the medium at 50 ml/l. and 5 ml each was pipetted into a 12 cm^2 laboratory dish. The inoculated plane medium that solidified after standing at room temp, was used for the test. A paper disc (8 mm^2, Toyo Roshi, Ltd.) previously sterilized was soaked in a sample, placed in the centre of the above medium and incubated at 37° for 20 hr. The antimetabolic activity was measured by the size of the inhibition circle around the disc.

Plant material. Fruiting bodies of *C. claricolor* (Fr.) Fr. var. *tenuipes* Hongo collected in October 1983 in one of the forests in Tochigi Prefecture, were washed with dist H_2O and stored after freeze drying. Voucher specimens are kept in the Tottori Mycological Institute (Tottori, Japan).

Isolation. Isolation of the active amino acid was proceeded by testing the antibacterial activity at each extraction and fractionation stage. The powdered dried mushrooms (169 g) were homogenized with 4.5 l. of 70% EtOH and filtered. The residue was further extracted twice with the same soln by refluxing. The combined extract (8 l.) was concd to 500 ml and defatted by washing with Et_2O (500 ml × 3). This active defatted extract was passed through a column of Amberlite IR-120B (H^+ form, 5 cm × 80 cm) and the resin was washed with H_2O. The active compound was eluted with 0.1 N NH_4OH (20 l.). The eluate was dried and the residue dissolved in H_2O (250 ml). The soln was then applied to an active charcoal column (3 cm × 40 cm); the active compound was not adsorbed and was recovered in the aq. effluent (10 l.). The soln was evapd to 100 ml, then subjected to CC on Dowex 1 × 4 column ($^-$OAc form, 5 cm × 60 cm). Elution was carried out successively with 0.1 N HOAc (5 l.), 2 N HOAc (5 l.) and 1 N HCl (5 l.). The antibacterial activity was localized in the 0.1 N HOAc eluate. A third part of the eluate that was concd to a small vol., was fractionated by CC on Dowex 50w × 4 (pyridinium form, 3 cm × 97 cm) by successive elutions with a pH 3.1 pyridine–HOAc buffer (0.2 M pyridine, 1.5 l.) and a linear gradient of a pyridine–HOAc buffer from pH 3.1, 0.2 M pyridine to pH 5.0, 2 M pyridine (3 l., 10 ml/fraction). Tests for antibacterial activity and ninhydrin reaction on each fraction showed that one of the ninhydrin positive peaks, fractions 197–211, was able to just fit the active peak. TLC and AA of the peak, detected an unknown ninhydrin positive compound with some trace substances. Thus, this was assumed to be the active compound. Residual parts were also fractionated by the same chromatographic procedures and the active fractions collected and combined. After purification of the unknown compound by a rechromatography on the same column, crystals were formed by addition of EtOH to the concentrate. Recrystallizations twice from EtOH–H_2O gave 920 mg of colourless crystals, $[\alpha]_D^{20}$ +3.8° (*c* 1.0, H_2O), +27.2° (*c* 0.5, 6 N HCl). FDMS: *m/z* 128 $[M+1]^+$. (Found: C, 54.84; H, 7.23; N, 10.98. Calc. for $C_6H_9NO_2$: C, 56.68; H, 7.13; N, 11.02%). IR ν_{max}^{KBr} 3420, 3300, 3280, 3175, 3000, 2950, 2760, 2630, 2580, 2120, 1616, 1584, 1564, 1533, 1498, 1401, 1352, 1322, 1269, 1190, 1158, 1133, 1080, 970, 873, 804, 750, 703 and 650 cm^{-1}. ^1H NMR (90 MHz, D_2O): δ1.9–2.3 (2H, *m*, H-3), 2.3–2.7 (3H, *m*, H-4, H-6), 3.89 (1H, *dd*, *J* = 5.5, 6.7 Hz, H-2).

Hydrogenation was performed at room temp and pres. Sample (34.3 mg) was dissolved in 8 ml of HOAc and hydrogenated over Adams' PtO_2. Absorption of H_2 was 1.96 mol/mol. TLC and AA of the filtrate of the reaction mixture showed norleucine as the sole ninhydrin positive product.

Degradation. A reaction mixture containing 2.6 mg of sample in 2 ml of 10% H_2SO_4 and 2 ml of 2% $KMnO_4$ was left standing at 4° for 20 hr, then 2 ml of H_2O was added and the reaction mixture filtered. The filtrate was applied to a column of Amberlite IR-120B (H^+ form, 1.5 cm × 6 cm) and, after the resin was washed with 100 ml of H_2O, the product was eluted by 2 N NH_4OH. Glu was detected as the sole ninhydrin positive product by TLC and AA.

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