Rapid Paper

Isolation and Identification of
(+)-Hexylitaconic Acid as a
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(+)-Hexylitaconic acid was isolated as a root-growth stimulating substance from a culture filtrate of *Aspergillus niger* K-88.

In our screening search for plant growth regulators among the fungal metabolites, we found that *Aspergillus niger* K-88 produced substance(s) which stimulated the root growth of lettuce seedlings. The active principle was isolated from the culture filtrate and identified as (+)-hexylitaconic acid (**1**). In this paper we wish to describe the isolation, identification and biological activity of **1**.

Aspergillus niger K-88 was cultured in a Czapek-Dox medium at 26.5°C for ten days. The active principle (**1**) was isolated from the broth filtrate with solvent extraction and silicic acid column chromatography, followed by preparative silicic acid TLC. The molecular formula of **1** was determined as C₁₁H₁₈O₄ from its high resolution mass spectrum and ¹³C-NMR spectrum. The ¹³C- and ¹H-NMR spectra of **1** indicated the presence of the α-methylene-carboxylic acid moiety (δ_C 171.7 (s), 137.5 (s), 129.6 (t); δ_H 5.80 (1H, s), 6.40 (1H, s) and one more carboxyl function (δ_C 179.6 (s)). The presence of the *n*-hexyl side chain was established from the intense peak of M-C₆H₁₃ in the mass spectrum and the following signals in the ¹³C- and ¹H-NMR spectra: δ_C 14.1 (q), 22.6 (t), 27.4 (t), 29.0 (t), 29.9

(t), 31.7 (t); δ_H 0.83 (3H, t, *J* = 6 Hz), 1.25 (8H, br s), 1.80 (2H, m). The methine carbon at δ_C 47.0, which bears the second carboxyl moiety, was thought to be adjacent to the α-methylene-carboxylic acid group and connected with the *n*-hexyl moiety. This was established by the fact that the methine signal at δ_H 3.35 (1H, t, *J* = 7 Hz) in **1** shifted to δ_H 2.7 in the dihydroderivative **2**, which had been obtained by catalytic hydrogenation of **1**. The assignment of the carbonyl carbon at δ_C (CDCl₃) 171.7 (δ_C (CD₃OD) 169.4) to the C-4 carbon in **1** was confirmed by the downfield shift of the signal in **2** (δ_C (CD₃OD) 177.8 or 178.5). Treatment of **1** with methanolic hydrogen chloride gave mainly its mono methyl ester **3**. The carbonyl signal at δ_C 179.6 (C-1) in **1** shifted to δ_C 173.8 in **3**, indicating that the isolated carboxylic acid (C-1) was esterified. Thus, the structure of **1** was established to be (+)-2-hexyl-3-methylene-succinic acid ((+)-hexylitaconic acid).¹⁾

- 1 R₁ = H, R₂ = CH₂
 2 R₁ = H, R₂ = CH₃, H
 3 R₁ = CH₃, R₂ = CH₂

(+)-Hexylitaconic acid (**1**) stimulated the root growth of lettuce seedlings at concentrations between 2 and 20 ppm, and especially at the concentration of 20 ppm, the roots of lettuce seedlings grew more than three times as long as those in the control experiment. On the other hand, the dihydroderivative **2** and the half methyl ester **3** did not show such a marked root growth-stimulating activity as **1** did. Those are illustrated in Fig. 1. All the compounds **1**, **2** and **3**, however, significantly inhibited the root growth of lettuce seedlings at concentrations of more than 100 ppm. (+)-Hexylitaconic acid (**1**) also stimulated the root growth of rice seedlings at a similar concentration to that for lettuce seedlings (Fig. 2). In the case of a concentration of 20 ppm, the

FIG. 1. Effect of (+)-Hexylitaconic Acid (1) and Its Two Derivatives 2 and 3 on the Root Growth of Lettuce Seedlings.

Root length is indicated as a relative value (%) to the length of the control experiment.

FIG. 2. Effect of (+)-Hexylitaconic Acid (1) on the Growth of Rice Seedlings.

Figures in the photograph indicate the concentration (ppm) of 1 in the assay solution.

growth of leaves of the rice seedlings also increased by 20~30% as compared with the control experiment.

(+)-Hexylitaconic acid (1) has already been synthesized and proved to be a precursor of canadensolide and its related compound,¹⁾ but, to our knowledge, this is first report on the isolation of 1 as a natural product. The root growth-stimulating activity of 1 is very marked, although the mechanism for action is still

uncertain. It is worth while noting that 1 has some structural similarity to radiclonic acid, which has been isolated as a root growth-stimulating substance from an unidentified fungus.²⁾

EXPERIMENTAL

Melting points were measured with a Yanagimoto micro melting point apparatus and are uncorrected. IR spectra were recorded on a JASCO A-202 spectrophotometer and optical rotations were obtained with a JASCO DIP-4 spectrometer. Unit resolution and high resolution mass spectra were measured with a Hitachi RMU-6 and JEOL DX-300 mass spectrometer, respectively. ¹³C- and ¹H-NMR spectra were recorded with a JEOL JNM FX-100 and JEOL JNM MH-100 spectrometer, respectively.

Isolation of (+)-hexylitaconic acid (1). *Aspergillus niger* K-88 was cultivated in field soil at Ninomiya-machi in Kanagawa Prefecture, and cultured in a Czapek-Dox medium at 26.5°C for ten days with a rotary shaker. The broth filtrate (5.2 liters; pH 2.0) was extracted with ethyl acetate and the organic layer was further extracted with aqueous sodium bicarbonate. The aqueous layer was acidified to pH 3 and extracted with ethyl acetate. The organic layer was dried over anhydrous sodium sulfate and evaporated under reduced pressure to give oily acidic substances (740 mg). The oil was applied to a Wakogel C-100 column (φ3.1 × 43 cm) and eluted with hexane-ethyl acetate, increasing stepwise the content of ethyl acetate. The eluate with hexane-ethyl acetate (6:4) was successively divided into four portions of equal volume. The crude active substance from the second portion was further purified with TLC using Kieselgel F₂₅₄ and hexane-ethyl acetate-acetic acid (4:6:0.1) to give the pure active compound 1 (74 mg), mp 46~47°C; [α]_D +15.3 (*c* = 2.0, MeOH). MS *m/z*: 214.1225 (*M*⁺, 5%; calcd. for C₁₁H₁₈O₄, 214.1195), 196.1074 (*M* - H₂O, 5%; calcd. for C₁₁H₁₆O₃, 196.1034), 178.1010 (*M* - 2H₂O, 5%; calcd. for C₁₁H₁₄O₂, 178.0994), 169.1246 (*M* - COOH, 43%; calcd. for C₁₀H₁₇O₂, 169.1229), 129.0117 (*M* - C₆H₁₃, 55%; calcd. for C₅H₅O₄, 129.0188), 112 (*M* - C₆H₁₂ - H₂O, 100%). IR $\nu_{\text{max}}^{\text{film}}$ (cm⁻¹): 3300~2400, 1775, 1705, 1625. ¹³C-NMR δ_{C} (CDCl₃): 179.6 (s, C-1), 171.7 (s, C-4), 137.5 (s, C-3), 129.6 (t, C-11), 47.0 (d, C-2), 31.7 (t, C-8), 29.9 (t, C-5), 29.0 (t, C-7), 27.4 (t, C-6), 22.6 (t, C-9), 14.1 (q, C-10); δ_{C} (CD₃OD): 169.4 (s, C-4), 176.9 (s, C-1). ¹H-NMR δ_{H} (CDCl₃): 0.83 (3H, t, *J* = 6 Hz, H-10), 1.25 (8H, br s, H-6~H-9), 1.80 (2H, m, H-5), 3.35 (1H, t, *J* = 7 Hz, H-2), 5.80 (1H, s, H-11a), 6.40 (1H, s, H-11b), 9.6 (2H, br s, 2 × COOH).

Dihydroderivative 2. Catalytic hydrogenation of 1 (20 mg) with PtO₂ in methanol gave 2 (20 mg), mp

109~112°C (acetone -H₂O). IR $\nu_{\max}^{\text{KBr}}$ (cm⁻¹): 3400~2400, 1690. ¹³C-NMR δ_{C} (CD₃OD): 178.5 (s, C-4 or C-1), 177.8 (s, C-1 or C-4), 50.0 (d, C-2), 43.3 (d, C-3), 32.5 (t, C-8), 31.8 (t, C-5), 29.9 (t, C-7), 28.3 (t, C-6), 23.4 (t, C-9), 15.8 (q, C-11), 14.2 (q, C-10). ¹H-NMR δ_{H} (CDCl₃): 0.83 (3H, t, *J*=6 Hz, H-10), 1.2~1.8 (13H, m, H-5~H-9, H-11), 2.7 (2H, m, H-2, H-3), 8.8 (2H, br s, 2 × COOH).

Half methyl ester 3. Compound **1** (20 mg) was refluxed in methanol containing 5% hydrogen chloride for one hour. The reaction mixture was poured into water and extracted with ethyl acetate. The ethyl acetate layer was then extracted with aqueous sodium bicarbonate, and the aqueous layer was re-extracted with ethyl acetate after being acidified to pH 3. The extract was dried over anhydrous sodium sulfate and concentrated under reduced pressure. The residue was purified with preparative TLC to give **3** (10 mg). MS *m/z*: 228 (M⁺, C₁₂H₂₀O₄), 197 (M - OCH₃), 183 (M - COOH), 143 (M - C₆H₁₃), 112 (M - C₆H₁₂ - CH₃OH). IR $\nu_{\max}^{\text{Film}}$ (cm⁻¹): 3400~2400, 1775, 1740, 1700, 1625. ¹³C-NMR δ_{C} (CDCl₃): 173.8 (s, C-1), 171.5 (s, C-4), 138.1 (s, C-3), 129.1 (t, C-11), 52.1 (q, OCH₃), 46.5 (d, C-2), 31.6 (t, C-8), 31.4 (t, C-5), 29.0 (t, C-7), 27.5 (t, C-6), 22.6 (t, C-9), 14.1 (q, C-10). ¹H-NMR δ_{H} (CDCl₃): 0.82 (3H, t, *J*=6 Hz), 1.2 (8H, br s), 1.8 (2H, m), 3.35 (1H, t, *J*=7 Hz), 3.55 (3H, s, OCH₃), 5.80 (1H, s), 6.40 (1H, s).

Bioassay. For the growth of lettuce seedlings, a filter

paper was placed in a petri dish (φ5.5 cm), to which a defined amount of the sample in acetone was added. After drying the solvent, 2 ml of a Hoagland's solution was added to the petri dish, in which seven lettuce seeds (*Lactuca sativa*, cv. Great Lakes No. 54) were transferred just after germination. The petri dish was kept under a continuous light (ca. 3000 lux) at 25 ± 2°C for three days.

For the growth of rice seedlings, a defined amount of the sample in acetone was added in a test tube (φ2.5 cm) and the solvent was evaporated. To the test tube, 2 ml of an eight fold-diluted Kimura B solution³⁾ and five rice seeds (*Oryza sativa*, cv. Nihonbare) were transferred just after germination. The test tube was covered with a thin plastic film and kept under a continuous light (ca. 2700 lux) at 30 ± 2°C for seven days.

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