L-*THREO*-β-HYDROXYASPARTIC ACID AS AN ANTIBIOTIC AMINO ACID

TADAYUKI ISHIYAMA, TAKAKI FURUTA, MAKOTO TAKAI and YOICHIRO OKIMOTO

Department of Agriculture, Tamagawa University, Tamagawa-gakuen, Machida, 194 Japan

SHOJIRO AIZAWA,* AKIRA SHIMAZU and HIROSHI YONEHARA

Institute of Applied Microbiology, The University of Tokyo, Bunkyo-ku, Tokyo, 113 Japan

(Received for publication June 16, 1975)

In the course of our screening for new antibiotics, *Arthrinium phaeospermum* T-53 and *Streptomyces* sp. 7540-MC_1 were found to produce an antibiotic substance. This paper describes the production, isolation, chemical and biological properties of the antibiotic.

Organisms and Fermentation

A strain of *A. phaeospermum*[1,2] T-53 was isolated from a fruiting body of *Psathyrella obtusata* (mushroom) and maintained on agar slants containing glucose 2 %, peptone 0.2 %, $MgSO_4$ 0.05 %, KH_2PO_4 0.06 %, K_2HPO_4 0.1 % and agar 1.5 %. The fungus was cultured on a reciprocal shaker at 28°C in a vegetative medium containing the following ingredients; soybean meal 1.5 %, dry yeast 0.2 %, soluble starch 2.5 %, $CaCO_3$ 0.4 % and NaCl 0.5 %. Two ml of 2 day old culture was transferred into 100 ml of the same medium in a 500-ml Erlenmeyer flask and was cultivated for 3 days on a rotary shaker at 28°C.

Bacillus subtilis was used as a test organism in the medium of peptone 0.5 % and agar 1.2 %, pH 7.0. *Streptomyces* sp. 7540-MC_1 was isolated from a soil sample collected at Nagano Prefecture, Japan and was cultivated for 3 days in the same manner as described above for *A. phaeospermum*.

Isolation Procedure

The broth filtrate was passed through columns of activated carbon and Amberlite IRC-50 (H^+ type), successively. The effluent was charged onto an anion-exchange column (Dowex 1×8 or IRA 410 (OH^- type)) and the column was washed with water and developed with 0.04 N HCl. The active eluate was concentrated *in vacuo* to a small volume and ethanol was added to yield crude precipitates. These were further purified through a column of Amberlite XAD-2 using water as a solvent to give active crystals. As an alternate of this step, the IRC-50 effluent was adsorbed on Amberlite IR-120 (H^+ type) and eluted with 0.3 N NH_4OH. The crystals thus obtained were recrystallized from water-ethanol.

Physicochemical Properties

The active component was colorless crystals of mp. ~210°C (dec.). It was soluble in hot water and conc. HCl, slightly soluble in water and almost insoluble in most organic solvents. It gave a positive color reaction with ninhydrin reagent but negative with $FeCl_3$, 2, 4-dinitrophenyl hydrazine and phenol red reagents, and negatine MOLISCH, FEHLING, BIURET and SAKAGUCHI reactions. The Rf values on paper-chromatograms were as follows; BuOH - AcOH - H_2O (2 : 2 : 1) 0.41, BuOH - AcOH - H_2O (2 : 1 : 1) 0.24 and BuOH - EtOH - H_2O (5 : 2 : 3) 0.24.

In the UV region it showed no characteristic absorption and in the NMR spectrum (100 MC, DCl) it exhibited two methine protons at δ 4.71 and 5.08 with small coupling constant (~2 Hz). The IR spectrum is shown in Fig. 1. Elemental analysis suggests $C_4H_7O_5N$ for its empirical formula.

Calcd. for $C_4H_7O_5N$:	C 32.22, H 4.73, O 53.66, N 9.40 (MW 154)
Found:	C 32.52, H 4.65, O 53.68, N 9.01 (MW 149.1, Titration)

Methylation

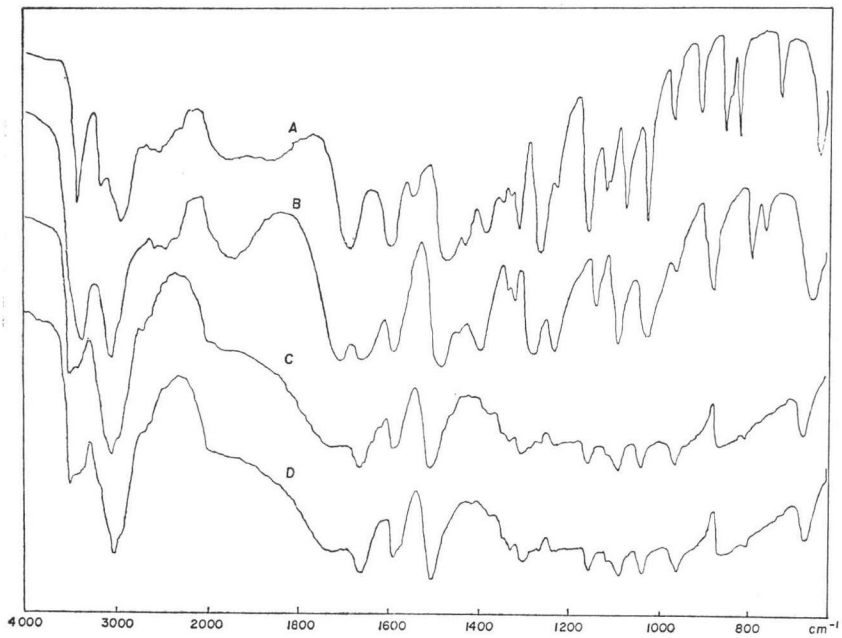
Methylation of this material in boiling 3 N HCl-MeOH for 3 hours gave several products on a paper chromatogram (BuOH - AcOH - H_2O, 2 : 1 : 1). Preparative precipitate and repeated crystallizations from hot water gave a monomethyl ester as a main product of $C_5H_9O_5N$. mp. 143~145°C, IR 1725, 1765 cm^{-1}, NMR (DCl); 3.90 (3H, s), 4.72 (1H, d) and 5.05 (1H, d) ppm.

Identification with L-*threo*-β-Hydroxyaspartic acid

These physicochemical properties suggest

* Present address: Kaken Chemical Co., Kita-ku, Tokyo, 114 Japan.

Fig. 1. IR Spectra of β-hydroxyaspartic acids (KBr)



A: L-*erythro*-β-Hydroxyaspartic acid
B: DL-*threo*-β-Hydroxyaspartic acid
C: L-*threo*-β-Hydroxyaspartic acid
D: The antibiotic substance

Table 1. Antimicrobial spectrum of L-*threo*-β-hydroxyaspartic acid

Test organisms	Concentration (mcg/ml)	Inhibition zone (Diameter, mm)*	Medium**
Bacillus subtilis PCI-219	250	20.0	A
	125	17.0	
	62.5	14.0	
Bacillus cereus IAM-1729	500	0	A
Staphylococcus aureus FDA 209P	500	0	B
Escherichia coli NIHJ	500	0	B
Xanthomonas oryzae NIAS	500	20.0	C
	250	17.0	
Mycobacterium smegmatis ATCC-607	500	0	D
Mycobacterium phlei IID Timothy	250	30.0	D
	125	25.0	
	62.5	20.0	
Candida albicans IAM-4905	500	0	E
Saccharomyces cerevisiae	500	0	E
Botrytis cinerea IAM-5127	125	22.0	F
	62.5	20.0	
	31.25	15.0	
Piricularia oryzae NIAS	500	0	F
Mucor ramanniaus IAM-6128	500	0	F

* Assays were performed with 8 mm filter paper discs.
** A Peptone 0.5%. agar 1.2%, pH 7.0 B Glucose bouillon, pH 7.0
C MUKOW WATANABE pH 7.0 D Glycerin CZAPEK, pH 7.0
E SABOURAUD, pH 7.0 F Yeast ext. 0.2%, starch 1.0%, agar 1.2%, pH 7.0

β-hydroxyaspartic acid for the antibiotic substance. The comparison of this material with *erythro*- and *threo*-β-hydroxyaspartic acid* in IR spectra (Fig. 1) revealed its identity with the *threo* isomer.[3] On high voltage paper electrophoresis (3,500 V, 20 min.) using a buffer of formic acid - acetic acid - water (25 : 75 : 900, v/v), its mobility (2.9 cm), was the same as that of the *threo* isomer (2.9 cm), but different from that of the *erythro* isomer (4.1 cm).

Optical rotations of this antibiotic and L-*threo*-hydroxyaspartic acid were both $[\alpha]_{368nm}^{26}$ +46° (*c* 0.5, 1 N HCl), thus, the antibiotic was identified as L-*threo*-β-hydroxyaspartic acid. It is worth to note that an analogous amino acid, L-*threo*-α-amino-β,γ-dihydroxybutyric acid has been isolated by WESTLEY *et al*,[7] from the fermentation broth of an unidentified *Sreptomyces*.

Biological Properties

This amino acid has been isolated from several organisms,[4,5] but its antimicrobial activity is not described as yet. It shows inhibitory acitvity against *Bacillus subtilis*, *Xanthomonas oryzae*, *Mycobacterium phlei* and *Botrytis cinerea* as summarized in Table 1. It did not show any toxicity at 250 mg/kg (i.v., mice). So far as tested, the biological activity of this antibiotic was not reversed by the addition of known amino acid such serine, threonine, varine and aspartic acid.

Summary

L-*threo*-β-Hydroxyaspartic acid was isolated from cultured broths of *Arthrinium phaeospermum* sp. T-53 and *Streptomyces* sp. 7540-MC_1. The amino acid exhibited broad antimicrobial activity.

Acknowledgement

Authors are indebted to Dr. T. TAKITA, Institute of Microbial Chemistry, for his valuable information and advice. We are also grateful to Prof. T. SHIBA, Faculty of Science, Osaka University, for his generous supply of authentic L-*threo* and *erythro*-β-hydroxyaspartic acids.

References

1) ELLIS, M. B.: Mycological Papers. No. 103, Commonwealth Mycological Institute, Kew, England. 2~46, 1965
2) AINSWORTH, G. C.; F. K. SPARROW & A. S. SUSSMAN: The Fungi. Vol. 4A. Academic Press, New York and London. 1973
3) KORNGUTH, M. L. & H. J. SALLACH: β-Hydroxyaspartic acid: Synthesis and separation of its diastereoisomers. Arch. Biochem. Biophy. 91: 39~41, 1960
4) WIELAND, T. & H. W. SCHNABEL: Über die Giftstoffs des grünen Knollenblätterpilzes. XXI. Die Konstitution des Phallacidins. Ann. Chem. 657: 218~225, 1962
5) WILDING, M. D. & M. A. STAHMANN: Hydroxyaspartic acid from alfalfa and clover roots. Phytochemistry 1: 241, 1962
6) WESTLEY, J. W.; D. L. PRUESS, L. A. VOLPE, T. C. DEMNY & A. STEMPEL: Antimetabolites produced by microorganisms. IV. L-*Threo*-α-amino-β, γ-dihydroxybutyric acid. J. Antibiotics 24: 330~331, 1971

* Authentic L-*erthro* and *threo*-β-hydroxyaspartic acids were kindly presented by Prof. T. SHIBA.