β-CYANOGLUTAMIC ACID, A NEW ANTIFUNGAL AMINO ACID FROM A STREPTOMYCETE

NOBUAKI NARUSE, SATOSHI YAMAMOTO, HARUAKI YAMAMOTO, SHOICHIRO KONDO, SHINJI MASUYOSHI, KEI-ICHI NUMATA, YASUO FUKAGAWA and TOSHIKAZU OKI

Bristol-Myers Squibb Research Institute, 2-9-3 Shimo-meguro, Meguro-ku, Tokyo 153, Japan

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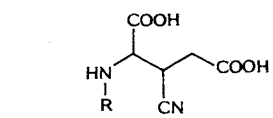
During the course of screening for new antifungal antibiotics, *Streptomyces* sp. K749-42 was found to produce β-cyanoglutamic acid (**1**) as a new amino acid which showed potent activity against *Candida albicans*. This paper briefly describes the fermentation, isolation, structure determination, and antifungal activity of **1**.

Streptomyces sp. strain No. K749-42 was isolated from a soil sample collected in Philippines. A loopful spores of the strain was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of seed medium composed of soluble starch 2%, glucose 0.5%, NZ-case 0.3%, yeast extract 0.2%, fish meat extract D30X 0.5% and $CaCO_3$ 0.3%, pH 7.0 before sterilization, and incubated at 28°C for 4 days on a rotary shaker (200 rpm). For production of the antibiotic, 5 ml of the seed culture was transferred to 100-ml of production medium (corn starch 2%, soy bean meal 3%, $MgSO_4 \cdot 7H_2O$ 0.33% and $CaCO_3$ 1%, pH 7.0) in a 500-ml Erlenmeyer flask and the fermentation was carried out at 28°C for 5 days. The antibiotic production was monitored by the liquid micro-dilution method using *Candida albicans* A9540 as tester. The broth filtrate (8 liters) was stirred with active charcoal (80 g) for one hour and filtered. The filtrate was passed through a column of Dowex 1×2 (350 ml, OH^- form) followed by rinsing with one liter of water. The active fractions collected with a linearly increasing NaCl concentration gradient from 0 to 0.6 M were combined and concentrated *in vacuo*. After desalted by Sephadex LH-20 column chromatography (40 i.d. × 750 mm, 50% methanol), 2.82 g of a crude powder was obtained and dissolved in a small volume of water. The solution was charged on a column of DEAE-Sephadex A-25 (OH^- form, 40 i.d. × 490 mm), and the elution was carried out with a linearly increasing NaCl concentration gradient from 0.05 M to 0.15 M. The active fractions were combined and desalted by Sephadex LH-20 column chromatography with water. The active eluate fractions were charged on Dowex 50 w × 8 (H^+ form, 20 i.d. × 200 mm) and eluted with 3% NH_4OH. Concentration of the eluate to dryness under reduced pressure afforded 272 mg of **1**.

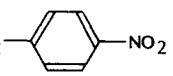
The physico-chemical properties of **1** were as follows: MP 205°C (dec., Na salt); $[\alpha]_D^{27}$ −24.0° (*c* 1, H_2O). The molecular formula of **1** was determined to be $C_6H_8N_2O_4$ by HRFAB-MS ($(M-H)^-$, *m/z* 171.0412, Δ +0.6 mmu); IR ν_{max}^{KBr} 3400 (br), 3200 (br), 2250, 1710 (sh), 1630, 1580, 1400; ^1H NMR (400 MHz, D_2O) δ 2.47 (1H, dd, J=15.3, 5.1 Hz, γ-H), 2.54 (1H, dd, J=15.3, 10.3 Hz, γ-H), 3.36 (1H, dt, J=10.3, 5 Hz, β-H), 3.59 (1H, d, J=4.8 Hz, α-H); ^{13}C NMR (100 MHz, DEPT, D_2O) δ 34.7 (CH), 36.7 (CH_2), 57.2 (CH), 123.0 (C≡N), 178.0 (C=O), 178.5 (C=O); TLC SiO_2 (E. Merck, No. 5715): Rf 0.23 (BuOH-AcOH-H_2O, 3:1:1); detection: ninhydrin.

The IR spectrum of **1** suggested the presence of a carboxylate (1580 and 1400 cm^{-1}). The ^1H NMR spectrum revealed a partial structure of –CH–CH–CH_2– and the 4 protons which were not observed in the spectrum were attributed to exchangeable protons of the carboxylic acid and primary amino groups. *p*-Nitrobenzyloxycarbonylation of **1** (Cbz(NO_2)–Cl, $NaHCO_3$, in 50% CH_3CN, 3 hours) gave the mono Cbz(NO_2)-derivative (**2**, FAB-MS *m/z* 350 $(M-H)^-$). In the ^1H NMR spectrum of **2**, one of the methine protons of **1** (δ 3.59) was downfield-shifted to δ 4.42. Based on the ^{13}C chemical shifts of its carbon signals, **1** was deduced to be β-substituted glutamic acid. In

Fig. 1. Structures of β-cyanoglutamic acid (**1**) and its *p*-nitrobenzyloxycarbonyl derivative (**2**).



1 R=H

2 R=–CO–OCH_2–  –NO_2

Correspondence should be addressed to JUN OKUMURA, Bristol-Myers Squibb Research Institute, 2-9-3 Shimo-meguro, Meguro-ku, Tokyo 153, Japan.

Table 1. Comparative antifungal activities of β-cyanoglutamic acid (**1**) and other antifungal agents.

Test organism	MIC (μg/ml)			
	1	Cispentacin	Ketoconazole	Amphotericin B
Saccharomyces cerevisiae ATCC 9763	>100	>20	50	0.4
Candida albicans IAM 4888	25	10	25	0.2
C. albicans A9540	6.3	20	25	0.2
C. albicans ATCC 32354 (B311)	0.8	5	50	0.2
C. albicans 83-2-14	0.8	5	25	0.2
C. tropicalis 85-5	>100	>20	25	0.4
C. tropicalis IFO 10241	>100	>20	50	0.2
Cryptococcus neoformans D 49	>100	>20	25	0.2
C. neoformans IAM 4514	>100	>20	50	0.2
Aspergillus fumigatus IAM 2034	>100	>20	3.1	0.2

Table 2. Effects of nitrogen sources on the antifungal activities of β-cyanoglutamic acid (**1**) and cispentacin.

Nitrogen source (4 mM)	MIC (μg/ml)	
	1	Cispentacin
Ammonium chloride	>100	50
DL-Aminobutyrate	6.3	>100
γ-Aminobutyrate	6.3	50
L-Alanine	12.5	12.5
L-Arginine	>100	12.5
L-Asparagine	>100	25
L-Aspartic acid	>100	6.3
L-Glutamine	>100	25
L-Glutamic acid	>100	25
L-Isoleucine	1.6	12.5
L-Lysine	6.3	3.1
L-Methionine	0.8	3.1
L-Phenylalanine	6.3	3.1
L-Proline	>100	100

addition, the presence of a cyano group in **1** was indicated by ^{13}C NMR (δ 123.0) and IR (2250 cm^{-1}). Thus **1** was concluded to be β-cyanoglutamic acid. The stereochemistry of **1** is under study.

The comparative antifungal activities of **1**, cispentacin, ketoconazole and amphotericin B were studied by the liquid micro-dilution method in Yeast Nitrogen Base (Difco, Cat. No. 0392-15) plus glucose medium (1% glucose, pH 7.0) after 24-hour incubation at 37°C. The inoculum size was 10^5 cells/ml. Table 1 shows that **1** is specifically active against some *C. albicans* strains and has an activity profile similar to cispentacin[1,2].

As an initial approach to the mode-of-action study, the effects of 4 mM nitrogen sources on the antifungal activities of **1** and cispentacin were examined by the liquid micro-dilution method using *C. albicans* A9540 in Yeast Nitrogen Base (Difco, Cat. No. 0335-15) plus glucose medium (without amino acid and ammonium sulfate). After 40 hours incubation at 37°C, MIC's of **1** and cispentacin were read for each nitrogen source (Table 2). As expected from the structural relatedness, the results in Table 2 clearly indicate that the antifungal activity of **1** is significantly antagonized by glutamic acid and related amino acids such as aspartic acid, glutamine, asparagine and proline.

References

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