A NEW DIPEPTIDE ANTIBIOTIC FROM *STREPTOMYCES COLLINUS*, LINDENBEIN

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Screening of a new strain of *Streptomyces collinus*, LINDENBEIN, A 19006, against *Salmonella gallinarum* led to the isolation of a new antibiotic. Examination of physical data and identification of hydrolysis products permitted the identification of this new metabolite as fumarylcarboxyamido-L-2,3-diaminopropionyl-L-alanine (I). The Gram-negative activity of the new antibiotic is inhibited by D-glucosamine and to a lesser extent by N-acetylglucosamine.

In the course of the screening program of *Actinomycetes* against a variety of organisms, we became interested in the culture of a new strain of *Streptomycescollinus*, LINDENBEIN, A 19009, which had interesting activity against *Salmonella gallinarum*. The active metabolite was obtained by subjecting the filtered broth to chromatography, followed by crystallization of active fractions. The crystalline compound had activity of 560~600 units/mg, m.p. 275~280°C (dec.). The hydrochloride salt was prepared using methanolic hydrochloric acid and ether. The crystalline solid, m.p. 235~245° (dec.) had the same degree of activity. Examination of physical and analytical data indicated that the substance represents another example of dipeptide antibiotics and is fumarylcarboxyamido-L-2,3-diaminopropionyl-L-alanine (I).

$$H_2N-\underset{\underset{O}{\|}}{C}-\overset{\overset{H}{|}}{C}=\underset{\underset{H}{|}}{C}-\underset{\underset{O}{\|}}{C}-NH-CH_2-\underset{\underset{NH_2}{|}}{CH}-CO-NH-\underset{\underset{CH_3}{|}}{CH}-COOH$$

(I)

Another related compound in this group of antibiotics is fumaryl-D,L-alanine (II), a metabolic product of *Penicillium resticulosum* sp. nov. active against *Staphylococcus aureus*[1].

$$HOOC-\underset{\underset{H}{|}}{CH}=\overset{\overset{H}{|}}{C}-CONH-\overset{\overset{CH_3}{|}}{\underset{\underset{COOH}{|}}{CH}}$$

(II)

The structure of the new antibiotic was confirmed by isolation of fumaric acid, L-2,3-diaminopropionic acid and L-alanine by chromatography of the hydrolysate of the antibiotic. The identity was established by the comparison of X-ray powder data, NMR spectra, and ORD curves of pure hydrolysis products with those of authentic compounds.

Identification of Culture A 19009

The characterization of the culture was made by methods suggested by SHIRLING and GOTTLIEB[2]. Comparison with *Streptomyces* type cultures[3] led to classification of A 19009 as a strain of *Streptomyces collinus* LINDENBEIN. A culture similar to A 19009 is *Streptomyces resistomycificus* but differs from A 19009 in pigment production in peptone-yeast extract iron, tyrosine agar, and tryptone-yeast extract broth.

Fermentation

Culture A 19009 was preserved by lyophilization of spore suspensions prepared in beef serum. Sporulated cultures were obtained by incubation for 7~10 days at 30°C on an agar medium containing 1 % dextrin 700 (A. E. Staley Mfg. Co.), 1 % Proflo (Traders Protein), 0.1 % 2019 yeast (Standard Brands), and 2.5 % agar in distilled water. The pH was adjusted to 7.0 with 5 N NaOH prior to autoclaving.

Fermentor inoculum was prepared by introducing suspensions from sporulated slant cultures into wide-mouth 250-ml Erlenmeyer flasks which contained 50 ml of a germination medium composed of 0.5 % glucose, 1.0 % dextrin 700, 2 % soy peptone powder (Sheffield Chemical), and 0.05 % Nadrisol (National Distillers' Products Co.) in tap water (pH 6.5). After 48-hour incubation at 30°C on a shaker rotating in a 5.08-cm circle at 250 r.p.m., the resulting mycelial suspension was inoculated into the fermentation medium at a 1 % level (v/v).

Preliminary studies in shaken flasks led to development of a production medium containing 2 % glycerol, 2 % dextrin 700, 1 % soy peptone powder, 0.3 % 2019 yeast, 0.2 % $(NH_4)_2SO_4$, and 0.2 % $CaCO_3$. The culture was grown on this medium for 40~48 hours in 40-liter tanks to provide broths for isolation of the biologically active metabolite. While neutral or slightly alkaline media were conducive to maximal growth of A 19009, optimal antibiotic yields were obtained when the harvest pH value of fermentation broths was 4.0~4.5.

Biological activity, present both in culture filtrates and solvent extracts of the mycelial cake, was quantitated by a disc-plate agar diffusion test employing *Salmonella gallinarum*. Fermentation samples were monitored chromatographically on Whatman No. 1 paper in water-saturated 1-butanol plus 2 % *p*-toluenesulfonic acid and in propanol - pyridine - acetic acid - water (15 : 10 : 3 : 12). Antibacterial activity was detected on developed chromatograms through a bioautographic technique using *Salmonella gallinarum*.

Biological Activity

Although the culture (A 19009) exhibited good activity against *Salmonella gallinarum*, agar-dilution assays using the pure antibiotic indicated very little, if any, activity against several Gram-negative organisms. Examination of the testing procedure indicated a possible influence of media. A thorough study of different media led to the development of an assay using a special agar (No. 1) and the *Salmonella gallinarum* strain with 8 μg/ml of the antibiotic in the agar. This concentration produced complete growth inhibition. Examination of different additives present in various media

led to the conclusion that D-glucosamine and to a lesser extent N-acetylglucosamine inhibited consistently the activity of antibiotic against Gram-negative organisms. It is interesting to note that the activity of bacillin, an antibiotic substance of unknown structure from *Bacillus subtilis*, against a variety of Gram-positive microorganisms is also inhibited by N-acetylglucosamine[4,5]. The significance of these observations is still under study*.

Isolation and Identification of the Antibiotic

The broth was filtered, treated with Norite, and the activity eluted with 30 % acetone. The acetone was removed *in vacuo* and the aqueous solution lyophilized. The dry product had activity of 25~30 units/mg and was chromatographed on acid-washed alumina (Woelm) column (1 : 20) using water as eluent; fractions were collected and examined on thin-layer chromatography plates using ninhydrin. The early slightly colored fractions were inactive and contained fumarodiamide (IR [KBr pellet], NMR [DMSO], and X-ray powder data identical with an authentic specimen [General Aniline Co.]). By lyophilization of active fractions, solids of 350~400 units/mg were obtained. The residues were crystallized by dissolving with slight warming in water and adding an equal amount of methanol. Colorless needles were collected yielding material assaying 560~600 units/mg. For analysis, the antibiotic was dried *in vacuo* for 2 hours at 100°C, m.p. 275~280°C (dec.).

Calculated for $C_{10}H_{16}O_5N_4$; M.W.: 272.26
C 44.11, H 5.92, O 29.38, N 20.58.
Found: C 44.25, H 6.17, O 29.36, N 20.23.
C 43.73, H 6.08, O 29.09, N 20.22.

The hydrochloride was prepared by addition of methanolic hydrochloric acid to a slurry of antibiotic in methanol. Ether was added to the filtered solution and the hydrochloride allowed to crystallize, m.p. 235~245°C (dec.).

Calculated for $C_{10}H_{16}O_5N_4 \cdot HCl$; M.W.: 308.73
C 38.80, H 5.55, O 25.91, N 18.14, Cl 11.49.
Found: C 38.60, H 5.70, O 25.42, N 17.88, Cl 11.40.

Amino acid analysis of the antibiotic showed only one peak; after hydrolysis and extraction with ether (removal of fumaric acid), the residue afforded 3.19 μmoles/mg of diaminopropionic acid and 3.47 μmoles/mg of alanine. Their identity was confirmed by the increase of intensity of corresponding peaks upon the addition of authentic samples.

In the NMR spectrum in D_2O there was one methyl doublet at $\delta=1.6$ p.p.m. coupled to a one-proton quartet at $\delta=4.15$ p.p.m. These two frequencies are in accordance with the presence of alanine moiety. Another characteristic peak was a two-proton singlet at $\delta=6.95$ p.p.m. corresponding to an olefinic proton. This frequency was absent in the spectrum of the amorphous product obtained by catalytic hydrogenation of the antibiotic (PtO_2, H_2; one mole of H_2).

* The i.p. and oral LD_{50} of the antibiotic was >400 mg/kg. The compound has an interesting spectrum of antifungal activities. (MIC : *Cryptococcus neoformans* 100 μg/ml, *Histoplasma capsulatum* 25 μg/ml, *Blastomyces dermatidis* 25 μg/ml, *Trichomonas vaginalis* 3.9 μg/ml, *Xanthomonas phaseoli* 100 μg/ml)

Comparison of the NMR spectrum of the antibiotic in DMSO and D_2O with that of fumaric acid indicated the presence of a singlet at $\delta=6.83$ and 6.75 p.p.m. respectively. The corresponding frequency in the spectrum of maleic acid was found at $\delta=6.42$ p.p.m. The ultraviolet spectrum was also in agreement with the presence of the fumaric acid moiety, $\alpha_M=\lambda_{max}^{213}$ 16,300 and λ_{max}^{241} 6,462; ORD, $[\alpha]_{310}=+107°$; fumaric acid[6], $\alpha_M=\lambda_{max}^{208}=15,900$ and $\lambda_{max}^{258}=6,310$.

Identification of Hydrolysis Products

(1) Isolation of fumaric acid

A solution of 200 mg of A19009 in 50 ml of 6 N HCl was refluxed for 18 hours and evaporated *in vacuo*. The residue (A) was triturated three times with ether, filtered, and chromatographed *"vide infra"*. The ether solution was evaporated and the residue (B) crystallized from 95 % ethanol, subl. 200°C. NMR spectra and X-ray diffraction patterns were identical with those of an authentic sample of fumaric acid.

(2) Isolation of L-alanine and L-2,3-diaminopropionic acid

The residue (A) was chromatographed on 25 g of cellulose powder (Schleicher and Schuell), grade 286 in a column (1.2×80 cm) using propanol - water (7 : 3) as an eluent. Fractions of 12 ml were collected and examined by thin-layer chromatography (cellulose and BuOH - AcOH - H_2O [3 : 1 : 1], ninhydrin spray).

The first eight fractions were discarded. Fraction No. 9 gave 106 mg of crystalline L-alanine, m.p. 175°C. Fraction No. 15 yielded 18 mg of residue which was crystallized from ethanol-water, and gave L-2, 3-diaminopropionic acid, m.p. 220~222°C. The NMR and ORD spectra and X-ray powder data of the two compounds were identical with those of authentic specimens.

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