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 Streptomyces collinus, 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).

\[
\text{H} \\
\text{H}_2\text{N}-\text{C-C-C-NH-CH}_2\text{-CH-CO-NH-CH-COOH} \\
\text{O H O} \quad \text{NH}_2 \quad \text{CH}_3
\]

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.

\[
\text{HOOC-CH=C-CONH-CH} \\
\text{H CH}_3 \quad \text{COOH}
\]

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 A19009

The characterization of the culture was made by methods suggested by Shirling
and Gottlieb. Comparison with Streptomyces type cultures led to classification of
A19009 as a strain of Streptomyces collinus Lindenbein. A culture similar to A19009
is Streptomyces resistomycificus but differs from A19009 in pigment production in
peptone–yeast extract iron, tyrosine agar, and tryptone–yeast extract broth.

Fermentation

Culture A19009 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% (NH₄)₂SO₄, and 0.2% CaCO₃. 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 A19009, 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 de-
tected on developed chromatograms through a bioautographic technique using
Salmonella gallinarum.

Biological Activity

Although the culture (A19009) 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 com-
plete 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\textsuperscript{4,5).} The significance of these observations is still under study\textsuperscript{*}.

Isolation and Identification of the Antibiotic

The broth was filtered, treated with Norite, and the activity eluted with 30\% acetone. The acetone was removed \textit{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 \textit{in vacuo} for 2 hours at 100\(^\circ\)C, m.p. 275~280\(^\circ\)C (dec).

Calculated for \(\text{C}_{12}\text{H}_{18}\text{O}_{5}\text{N}_{4}\); M.W. : 272.26

\begin{align*}
\text{C} &\quad 44.11, \text{ H} 5.92, \text{ O} 29.38, \text{ N} 20.58. \\
\text{Found:} &\quad \text{C} 44.25, \text{ H} 6.17, \text{ O} 29.36, \text{ N} 20.23. \\
\text{C} &\quad 43.73, \text{ H} 6.08, \text{ O} 29.09, \text{ N} 20.22.
\end{align*}

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\(^\circ\)C (dec).

Calculated for \(\text{C}_{12}\text{H}_{18}\text{O}_{5}\text{N}_{4}\cdot\text{HCl} ; \quad \text{M.W. : 308.73}

\begin{align*}
\text{C} &\quad 38.80, \text{ H} 5.55, \text{ O} 25.91, \text{ N} 18.14, \text{ Cl} 11.49. \\
\text{Found:} &\quad \text{C} 38.60, \text{ H} 5.70, \text{ O} 25.42, \text{ N} 17.88, \text{ Cl} 11.40.
\end{align*}

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 \textmu moles/mg of diaminopropionic acid and 3.47 \textmu moles/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 \mu g/ml, Histoplasma capsulatum 25 \mu g/ml, Blastomyces dermatitidis 25 \mu g/ml, Trichomonas vaginalis 3.9 \mu g/ml, Xanthomonas phaseoli 100 \mu g/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, \( \alpha_M = \lambda_{\text{max}}^{228} = 16,300 \) and \( \lambda_{\text{max}}^{228} = 6,462 \); ORD, \( [\alpha]_{\text{160}} = +107^\circ \); fumaric acid, \( \alpha_M = \lambda_{\text{max}}^{228} = 15,900 \) and \( \lambda_{\text{max}}^{228} = 6,310 \).

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 (1.2x80 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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References