GARDIMYCIN, A NEW ANTIBIOTIC FROM *ACTINOPLANES*

II. ISOLATION AND PRELIMINARY CHARACTERIZATION

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(Received for publication January 17, 1976)

The strain *Actinoplanes garbadinensis* nov. sp. produces a peptide antibiotic, named gardimycin, which is active *in vitro* and *in vivo* against Gram-positive bacteria. Isolation and purification of the product have been accomplished by extraction from the broth with butanol and dialysis of the crude extract, followed by counter-current distribution.

Gardimycin is an open chain peptide with an approximate minimal formula $C_{84}H_{138}N_{18}S_{3\sim4}O_{34}Na$. The following amino acids have been identified by column chromatography of an acid hydrolysate: serine, glutamic acid, alanine, leucine, isoleucine, glycine, valine and two sulphur-containing amino acids whose structure is presently under study. Tryptophan has been identified in an alkaline hydrolysate.

Gardimycin is a new peptide antibiotic produced by *Actinoplanes garbadinensis* nov. sp.

The product is active *in vitro* against Gram-positive bacteria, inhibiting their growth by interfering with cell-wall synthesis. It is very effective *in vivo* in protecting mice infected with *Streptococcus hemolyticus* and has a very low acute toxicity in mice.

In this report the isolation and preliminary characterization of gardimycin are described. The characteristics of the producing organism, the production of the antibiotic and its biochemical properties are described in companion papers.[1~3]

Isolation

Gardimycin is an amphoteric peptide with acidic net charge and can be extracted from the culture filtrate either as the free acid or as a sodium salt. Extraction is carried out with butanol at pH 8.0 and the antibiotic precipitates from the concentrated solvent as a light-tan powder having 35~40% purity as determined spectrophotometrically. An additional amount of the antibiotic can be obtained from the concentrated solvent by pouring it into a large amount of light petroleum ether, the material obtained being usually 10~20% pure. Purification is accomplished by dialysis of a 10% aqueous solution of the crude material. Gardimycin is recovered from the non-dialyzable portion as a whitish powder with 60~65% purity. Pure gardimycin is obtained as sodium salt by counter-current distribution in the solvent system butanol - 0.45 M phosphate buffer at pH 7.2 - hexane (1:1:0.2).

Physical and Chemical Properties

Gardimycin has not been obtained in crystalline form and the properties reported have been determined for a product which has constant microanalytical values after repeated counter-current distributions.

Gardimycin sodium salt is a white powder that melts with decomposition at 260°C. The

specific rotation is $[\alpha]_D^{25}$—44 (*c* 0.5, DMF). It is soluble in dimethylformamide, dimethylsulfoxide, glacial acetic acid, and in water at pH's higher than 7.0. It is partially soluble in warm methanol and butanol and is insoluble in the other common organic solvents. It gives positive FEHLING and TOLLENS reactions and gives negative $FeCl_3$, MILLON, SCHIFF and maltol reactions; treatment with concentrated H_2SO_4 gives a brown color; neutral aqueous solutions of potassium permanganate are decolorized.

The behavior of gardimycin in paper and thin-layer chromatography is reported in Table 1.

Table 1. Chromatographic behavior of gardimycin

Solvent system	Rf*
Buffer, pH 6.0, saturated with butanol	0.80
Water-saturated *n*-butanol +2% *p*-toluenesulfonic acid	0.80
〃 〃 〃 +2% concentrated ammonia	0.10
n-Butanol saturated with buffer, pH 6.0	0.15
Ammonium chloride (20% w/v in water)	0.80
n-Butanol-methanol-water (40:10:20), containing 0.75 g methyl orange	0.65
n-Butanol-methanol-water (40:10:30)	0.80
Water-acetone (1:1)	0.80
Water-saturated ethyl acetate	0.0
17% Ammonia-ethanol-water (1:8:1)**	0.79

* Paper chromatography on Whatman No. 1 paper, Antibiotic visualized on agar plates seeded with *S. aureus*.

** TLC on Silica gel HF/UV_{254} plates. Spot detected under u.v. light and by spraying with conc. H_2SO_4 containing vanillin and heating at 100°C.

The antibiotic has amphoteric character, with a p_i=4.2, determined by electrofocusing in ampholine. The molecule contains two acidic functions and one basic function, for a molecular size of about 2,000, as shown by potentiometric titrations of gardimycin free acid. Titration with base in aqueous solution shows two ionizable functions with pKa's of 4.2 and 6.8 and equivalent weight ~2,000; these functions are both acidic, as shown by the pKa values 5.3 and 8.1 obtained, respectively, by titration in methylcellosolve-water (4:1) solution. Titration in non-aqueous solvent with perchloric acid shows a basic function with minimum molecular weight 2,200. These data are suggestive of an open chain peptide.

Microanalytical data obtained with gardimycin sodium salt are as follows: C 48.7, H 6.7, N 12, S 5.7, Na 1.1% (direct determination) 3.3% (as Na_2SO_4 residue). Based on these data, the approximate minimal formula of gardimycin sodium salt is $C_{84}H_{138}N_{18}S_{3\sim4}O_{34}Na$, with molecular weight from 2,062~2,094, in agreement with the results of the potentiometric titrations.

Amino Acid Composition

The infrared spectrum of gardimycin (Fig. 1) is typical for a peptide. Hydrolysis of the antibiotic in 6 N hydrochloric acid in a sealed tube at 110°C gave a mixture of amino acids. Seven have been identified by paper chromatography and quantitative amino acid analysis as serine, glutamic acid, alanine, leucine (one residue), glycine, valine, isoleucine (two residues). Two unknown amino acids containing sulphur are present between glutamic acid and glycine.

In order to confirm these identifications, an acid hydrolysate of gardimycin was chromato-

Fig. 1. Infrared absorption spectrum of gardimycin (nujol mull)

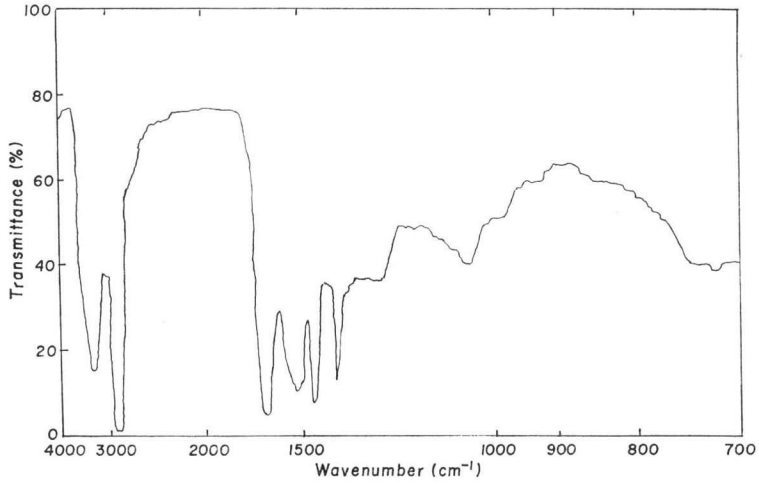
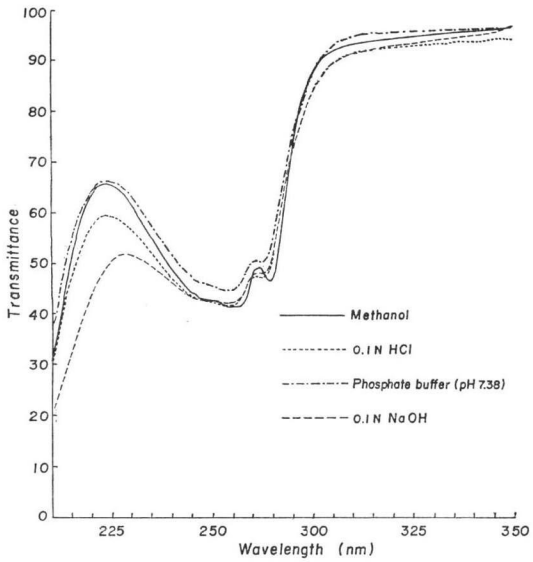


Fig. 2. Ultraviolet absorption spectrum of gardimycin



graphed on ion-exchange resin, the separated amino acids were isolated and their i.r. and p.m.r. spectra were compared with those of standard samples. The identity of the seven amino acids was established. The structure of the two amino acids containing sulphur is presently under study.

Alkaline hydrolysis of the antibiotic with barium hydroxide gave tryptophan, identified by paper chromatography.

The ultraviolet spectrum of gardimycin (Fig. 2) shows the presence of tryptophan in the molecule and the extinction value is consistent for one tryptophan residue in a molecule with molecular weight ~2,000.

Experimental

Extraction of gardimycin from fermentation broth: The culture broth, adjusted to pH 8.0 by addition of 10% NaOH, was filtered with the aid of Hyflo-Supercell, the mycelial cake was discarded and the filtrate extracted once with 50% butanol. The solvent, concentrated *in vacuo* below 45°C to about one fourth the original volume, was washed with a small quantity of water at pH 4.0 and concentrated again to about one-tenth the original volume. The concentrated solution was kept at 4°C for 10~12 hours and a precipitate was obtained that was collected by filtration and washed with a small volume of butanol. The material was dried *in vacuo* at 45°C, giving a light-tan material. The product, assayed spectrophotometrically at the maxima characteristic for tryptophan, contains 35~40% gardimycin. The filtered butanol and the washing were concentrated *in vacuo* to a small volume and poured into a large quantity of light petroleum ether; the precipitate formed, which has a strong tendency to take up water, giving a syrupy material, was thoroughly washed with light petroleum ether and dried *in vacuo*

at 40~45°C. This material contain 10~20 % gardimycin.

Purification of gardimycin: Ten grams of crude material were suspended in water at pH 7.2; NaOH (10 %) was added to the suspension, with stirring, until the pH was constant for at least 30 minutes. The insoluble material was filtered out and the solution was dialyzed against distilled water for 15 hours. The undialyzable fraction was concentrated to a small volume and then lyophylized, yielding 5.6 g of a whitish hygroscopic powder that contained 60~65 % gardimycin. Two grams of the lyophylized material was purified by counter-current distribution with the solvent system; 0.45 M phosphate buffer at pH 7.2 - butanol - hexane (1:1:0.2). The presence of antibiotic was controlled by u.v. analysis at the maxima characteristic for tryptophan. Gardimycin has a K 0.8 with this solvent system, and pure product was obtained after 200 transfers. The product was recovered from the collected fractions by extracting the aqueous layer twice with butanol; the collected butanol extracts were washed with a small quantity of distilled water, concentrated under vacuum to a small volume, and poured into a large amount of light petroleum ether. The solid was filtered and dried at 50°C, under vacuum, giving 750 mg of pure gardimycin ($E^{1\%}_{1cm}$ 26 at λ_{max} 280 nm in CH_3OH solution).

Hydrolysis of gardimycin and isolation of the amino acids: A solution of 1.5 g of gardimycin sodium salt in 150 ml of 6 N HCl was heated at 120°C for 16 hours in a sealed tube under nitrogen.

Excess acid was removed by repeated concentration of the solution under vacuum and the concentrated solution was chromatographed on a 2×45 cm column of IR-120×8 200~400 mesh (H^+ form) resin (160 ml).

Elution with 0.5 N HCl gave serine (effluent volume 200~370 ml), glutamic acid (450~550 ml), glycine (560~685 ml), alanine (695~820 ml), valine (840~1,150 ml), isoleucine (1,200~1,740 ml), and leucine (1,800~2,300 ml), subsequent elution with 2 N HCl gave the two sulphur-containing amino acids (220~480 ml). Concentration of the effluents *in vacuo* gave the amino acid hydrochlorides.

Acknowledgments

The authors wish to thank Dr. L. DORMANN of the Dow Research Laboratories in Midland and Dr. RURALI of Ormonoterapia Richter Laboratories for amino acid analysis with the autoanalyzer; Mr. G. C. ALLIEVI and Mr. G. CAMPANA for potentiometric titrations.

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