GELDANAMYCIN, A NEW ANTIBIOTIC

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A new crystalline antimicrobial compound, geldanamycin, has been discovered in the culture filtrates of *Streptomyces hygroscopicus* var. *geldanus* var. *nova*. Geldanamycin is moderately active *in vitro* against protozoa, bacteria and fungi. It is also active against L-1210 and KB cells growing in culture and against the parasite *Syphacia oblevata*, *in vivo*. The fermentation, assay, chromatography and isolation as well as its biological and chemical properties were investigated. On the basis of its physical and chemical properties, geldanamycin is a complex molecule consisting of an unsaturated moiety attached to a quinone.

In the process of screening for substances which inhibit the growth and multiplication of protozoa, a new compound was isolated which was primarily active against *Tetrahymena pyriformis* and *Crithidia fasciculata*. This substance was produced in submerged culture by a new actinomycete found in a Kalamazoo soil and was identified as *Streptomyces hygroscopicus* var. *geldanus* var. *nova* (UC-5208).*

Production of Geldanamycin

The inoculum for seed flasks was obtained from spore preparations of the culture maintained in sterile soil or in liquid N_2. The culture was incubated at 28°C for 48 hours in a 1% glucose monohydrate, 1% peptone (Difco) and ¼% yeast extract (Difco) medium.

A 5% vegetative seed was used to inoculate the fermentation medium consisting of glucose monohydrate (40 g/liter), peptone (2½ g/liter), tryptone (2½ g/liter), yeast extract (2½ g/liter), Gerber's oatmeal (5 g/liter), and Brer Rabbit molasses (10 ml/liter) (Penick and Ford-Gold Label). Prior to sterilization (121°C for 20 minutes) the pH was adjusted to 7.0. All shaken flask fermentations were run in 500 ml Erlenmeyer flasks with 100 ml of medium and incubated at 28°C on a Gump rotary shaker operating at 250 rev/min. with a 6.35-cm (2.5 in.) stroke.

Table 1. Fermentation titer and pH pattern of *S. hygroscopicus* var. *geldanus* var. *nova*

Hours	pH	Assay (biounits)
24	7.3	2
48	7.0	16
72	6.9	64
96	6.65	96

Table 1 shows a typical fermentation pH and antibiotic titer pattern produced by *S. hygroscopicus* var. *geldanus*. Peak yields of *ca.* 100 biounits/ml were obtained in 4~5 days. Continuation of the fermentation longer than 5 days did not increase the titer.

* This organism has been characterized by A. DIETZ of these laboratories.

Assay Procedure

The antibiotic concentrations were determined using a standard disc-plate agar diffusion assay. Dilutions were made with a pH 6.0 phosphate buffer. Samples (0.08 ml) were applied to 12.7 mm paper discs (Schleicher and Schuel) and assayed against *Tetrahymena pyriformis* growing in 1% proteose-peptone #3 (Difco), 1% glucose monohydrate, 0.1% yeastolate medium (Difco), and 0.4% Ionagar No. 2 (Colab). The diameter of zones of growth inhibition were measured to the nearest mm after a 42 hour incubation at 26°C. The antibiotic activity was expressed in biounits. One biounit equals the amount of antibiotic necessary to produce a 20 mm zone of inhibition under standard conditions. One mg of crystalline geldanamycin is the equivalent of 240~350 biounits.

Chromatography

Paper: Geldanamycin was differentiated from other antibiotics by descending paper-chromatography. Approximately 100 mcg of geldanamycin was spotted on Whatman #1 filter paper and developed without equilibration in solvent vapors. The antibiotic was detected with bioautography on trays of agar seeded with *T. pyriformis* in a medium consisting of 1% glucose monohydrate, 1% proteose-peptone #3, 0.1% yeastolate, 0.4% Ionagar (Oxoid) and distilled water. A 20% inoculum was used with a 35~45% light transmission at 530 mμ on a Model 401 Lumetron Colorimeter. Trays were incubated at 26°C for 42 hours before reading.

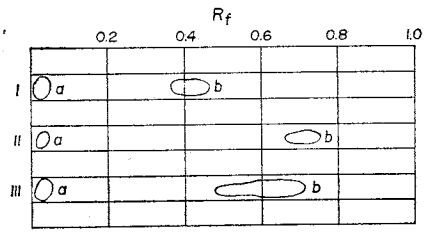


Fig. 1. *Tetrahymena pyriformis* bioautograph pattern of geldanamycin.

Solvent systems: I, 1-butanol-water (84:16) plus 2% *p*-toluene-sulfonic acid, developed 64 hours; II, 0.075 N ammonium hydroxide saturated with methyl isobutyl ketone, developed 5 hours; III, paper strip is equilibrated at 25°C in vapor form mixed solvent composed of benzene-methanol-water (1:1:2) and developed in upper phase 5 hours.

Bioautographs of the culture filtrate revealed two components which moved differently in three solvent systems as shown in Fig. 1. The major component listed as B on the papergram was extracted, crystallized and designated as geldanamycin.

Thin-Layer: On Silica Gel H (E. Merck, A.G., Darmstadt, Germany) tlc plates at neutral pH and a solvent system of 9:1 or 9.5:0.5 chloroform:methanol, the Rf value of geldanamycin was determined to be 0.56. The antibiotic was detected by noting the yellow color or by exposure to U.V. light.

Column: As an alternate method of purification, Silica gel G7734 (E. Merck, A.G. Darmstadt, Germany) was used as the matrix and the same solvent system employed as used in thin-layer chromatography.

Isolation and Purification of Geldanamycin

Two-hundred-forty liters of cultured medium was filtered and the filtrate extracted with 3-133 liter-portions of *n*-butanol. The combined extracts were concentrated by vacuum at 40°C to a volume of 5.5 liters. At this point crude crystals

of approximately 35 % purity formed, and were separated by filtration: weight of dried crystals (I) was 187.5 g; m.p. 257~261°C, assay 120 u/mg. 47.9 g of I was dissolved in 900 ml of boiling chloroform and filtered. The filtrate (II) contained the major part of geldanamycin and was retained for further treatment. The insoluble material was extracted twice using 16 ml of methanol and 50 ml of chloroform each time and filtered (III and IV). The soluble fractions II, III and IV were combined and the solvents evaporated, leaving 32.5 g of solids (V). V was dissolved in 700 ml of boiling chloroform and the solution concentrated to 375 ml under nitrogen at 60°C. Yellow crystals began to form at this point and 28 ml of diethylether was added to complete the crystallization (6.94 g, m.p. 252°~255°C). Recrystallization did not change the melting point. All melting points were determined on a KOFFLER block and corrected.

Physical and Chemical Properties of Geldanamycin

Geldanamycin is soluble in alcohols, aliphatic chlorinated solvents, particularly chloroform, and to a lesser extent, acetone, benzene and ethyl acetate, but only very slightly in water. In solution, it decomposes rather readily with acid, base or heat in the presence of oxygen, but as a dry material it is stable.

The crystalline material forms: yellow needles, m.p. 252°~255°C; $[\alpha]_D^{25°}$ +55° (*c*,

Fig. 2. Mass spectrum of geldanamycin.

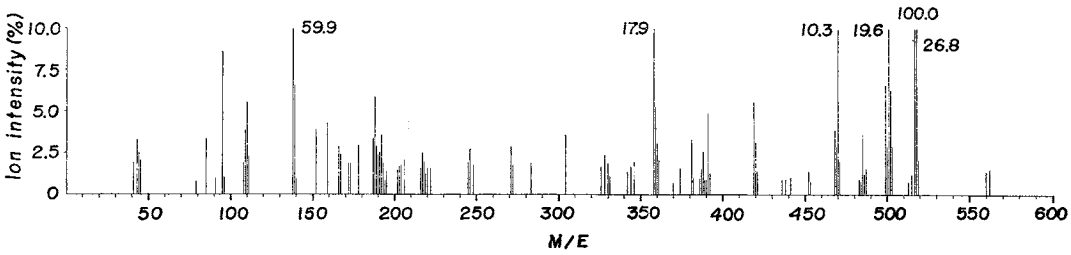
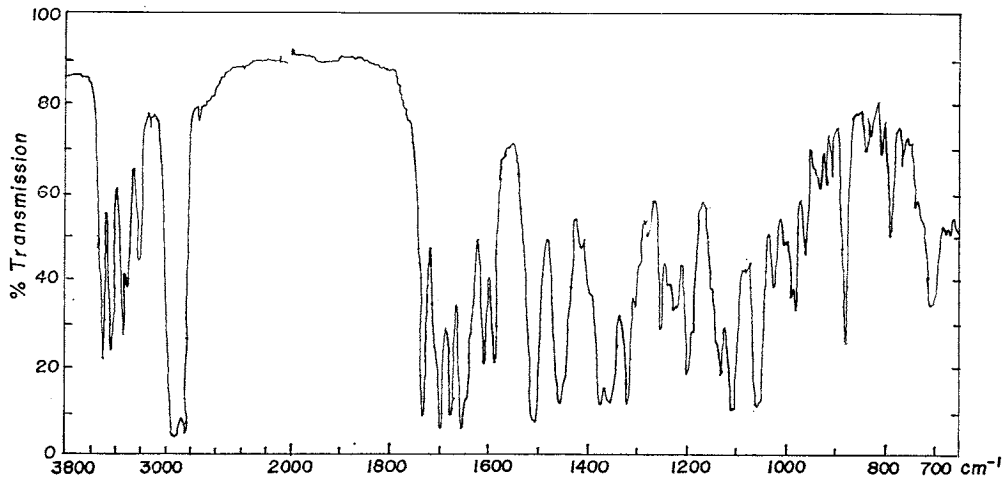


Fig. 3. Infrared absorption spectrum of geldanamycin (Nujol mull).



0.638 $CHCl_3$); mass spectrometric analyses were performed on geldanamycin with a CEC-21-110B mass spectrometer incorporating a direct inlet probe. Most of the spectra were obtained by inserting the sample into the ion source at about 100°C and slowly raising the temperature until ions were obtained (Fig. 2); the mass spectrum of geldanamycin shows a large peak at 517 and weak peaks of about equal intensity at 560 and 562. Accurate mass measurements indicated that the peaks at 562, 560 and 517 corresponded to $C_{29}H_{42}N_2O_9$, $C_{29}H_{40}N_2O_9$ and $C_{28}H_{41}NO_8$ respectively; the 560 and 562 peaks were initially interpreted as being separate real entities differing by two hydrogen atoms, (*i.e.*, a double bond, ketone *vs.* alcohol, *etc.*). Since other spectroscopic and chemical evidence suggest that geldanamycin contains a quinone moiety, and since some quinones yield apparent M^++2 peaks in the ion source of the mass spectrometer,[2,3,4] a logical choice for the empirical formula of geldanamycin is $C_{29}H_{40}N_2O_9$ at mass 560. The peak at mass 517, corresponding to $C_{28}H_{39}NO_8$ represents HOCN loss from the 560 peak; elemental analysis for M.W. 560: Calcd. for $C_{29}H_{40}N_2O_9$: C 61.69, H 7.16, N 5.15, O 25.62. Found: C 62.13, H 7.26, N 5.00, O 25.68; equivalent weight titrates as a monobasic acid having a neutral equivalent of 560; I.R. absorption spectrum was obtained from a Nujol mull (Perkin-Elmer model 421 spectrophotometer). The spectrum has characteristic bands at 3510, 3445, 3350,

Fig. 4. Ultraviolet absorption spectrum of geldanamycin.

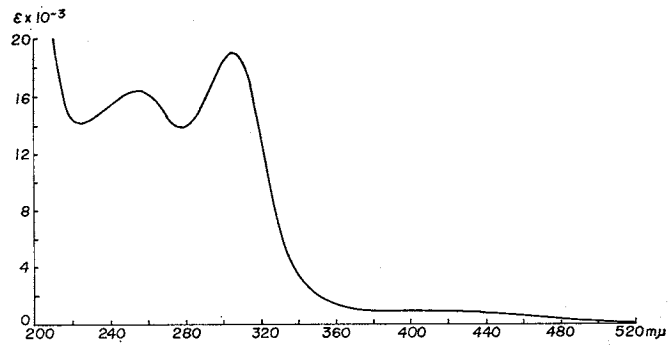
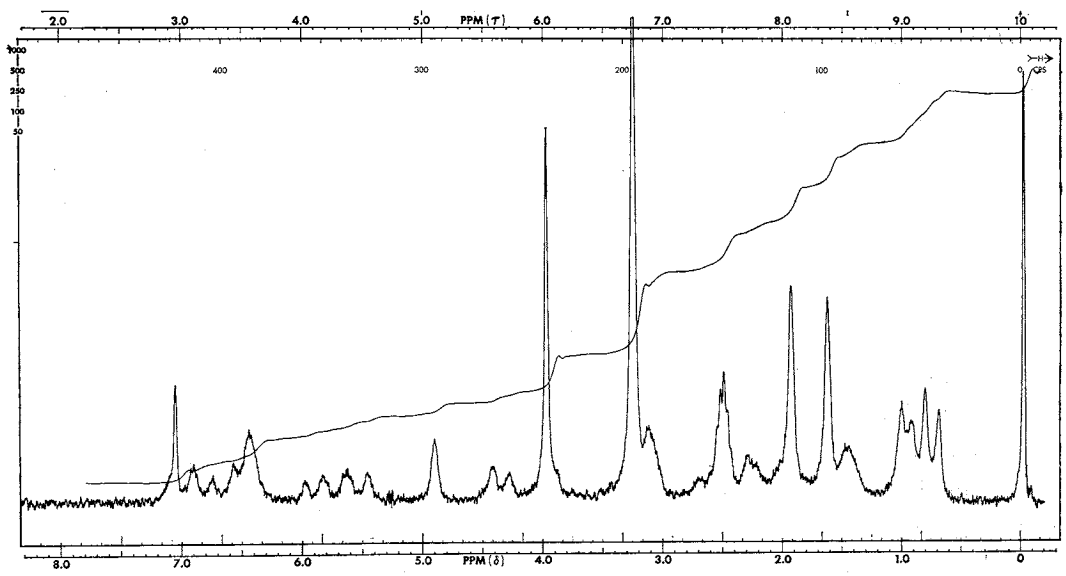


Fig. 5. N.M.R. spectrum of geldanamycin.



3315, 1734, 1700, 1676, 1655, 1635, 1608, 1590, 1510, 1108 and 1056 cm^{-1}. These peaks in conjunction with other physical data are consistent with alcohol, ether, an O-carbamate and a quinone group (see Fig. 3); U. V. spectrum (Fig. 4) of geldanamycin was obtained from a methanol solution using a Cary Model 15 spectrophotometer. Maxima occur at 255 mμ (ε=16,350), 304 mμ (ε=19,300) and a broad, weak shoulder near 400 mμ (ε=980); the N.M.R. spectrum (Fig. 5) was measured on a solution of geldanamycin in deuterated dimethylsulfoxide using a Varian A-60A spectrometer. The multiplet at δ 2.52 is attributed to residual protons in the solvent; the N.M.R. spectrum showed absorptions for 40 hydrogens including three methoxy groups and five additional C-methyl groups:

Acetylation

To 1 g of purified geldanamycin dissolved in 10 ml of pyridine was added 10 ml of acetic anhydride. After stirring for 6 hours the preparation was allowed to stand at room temperature overnight. The solid mass obtained after concentration under vacuum at 40°C was chromatographed over silica gel G #7734 (E. Merck A. G.) using a mixture of 95% chloroform and 5% methanol. The eluates were concentrated to dryness *in vacuo* and the material crystallized and recrystallized from a chloroform solution to which 40% methanol was added. Light yellow crystals (prisms) of the monoacetate m.p. 206°~209°C were obtained and indicated a compound of molecular weight 602.28 by mass spectrometry. The acetate was biologically inactive at 2 mg/ml against *T. pyriformis*.

Hydrogenation

Using PtO_2, 2.4 moles of hydrogen were consumed by geldanamycin and a colorless compound resulted which turned dark rapidly in the presence of air, indicating a hydroquinone had been formed.

Methyl ether derivative

With diazomethane the hydrogenated compound above produced a derivative which did not discolor in the presence of air and showed the loss methanol or water and the introduction of one additional methyl group by mass spectrometry.

In vitro spectrum

The antibacterial spectrum was determined by two-fold dilution endpoints in Bacto Brain Heart Infusion (Difco) broth. The growth inhibition of the protozoa (*C. fasciculata* and *T. pyriformis*) was performed in the same manner in a medium consisting of 1% glucose, 1% Bacto Protease-Peptone #3 (Difco), and 0.1% yeastolate (Difco).

Table 2. Antimicrobial spectrum of geldanamycin

Test organism	MIC* μg/ml
Crithidia fasciculata UC-P23**	4
Tetrahymena pyriformis UC P4	2
Chromobacterium violaceum UC 36	12.5
Flavobacterium suaveolens UC 53***	12.5
Staphylococcus aureus UC 74	12.5
Aeromonas salmonicida UC 897	25
Bacillus cereus UC 8	25
Bacillus subtilis UC 26	25
Pasteurella multocida UC 264	25
Shigella dysenteriae UC 135	25
Salmonella pullorum UC 267	25
Aeromonas liquefacians UC 895	50
Escherichia coli UC 51	50
Klebsiella pneumoniae UC 57	50
Proteus vulgaris UC 93	50
Salmonella schottmuelleri UC 126	50
Salmonella typhosa UC 105	50
Streptococcus faecalis UC 151	50
Aerobacter aerogenes UC 3	100
Erwinia carotovora UC 167	100
Pseudomonas aeruginosa UC 95	>100
Streptococcus viridans UC 155	100

* MIC: Minimal inhibitory concentration
** Upjohn culture collection number
*** Spectrum was run in Difco's Brain Heart Infusion Broth with the exception of *C. fasciculata* and *T. pyriformis* (see text)

A 16-hour growth (37°C) of each bacterial test organism was used as inoculum in a final concentration of 1:40,000. The inoculated broth was incubated for 16 hours at 37°C, at which time the minimum inhibitory concentrations were recorded.

The inhibition of the protozoa was measured in the same way as the bacteria except that they were incubated at 26°C for 48 hours, diluted to a final concentration of 1:1,000 for inoculation, and incubated for 48 hours (26°C). The results are given in Table 2.

It has demonstrated activity against several fungal plant pathogens, *viz*; *Alternaria*, *Pythium*, *Botrytis* and *Penicillium* (<500 ppm).

Geldanamycin is extremely active against KB cells (<0.001 mcg/ml) and L1210 cells (<0.002 mcg/ml). The ID_{50} indicates that geldanamycin is approximately 2~4 times as active as cytosine arabinoside[4].

Geldanamycin is inactive at 0.5 mg/ml against Coxsackie A-21, Parainfluenza type 3, and Herpes simplex viruses, by an agar diffusion test.

In vivo Activity

In mice, geldanamycin was orally active against the parasite *Syphacia oblevata* at 0.5 mg/mouse/day for 4 days, but inactive against *Plasmodium berghei* when given at 20 mg/kg (SQ). In a poultry coccidiostat screen it showed partial control at a 0.0125% concentration in the diet while in dogs it showed only slight anthelminthic activity when given orally.

The acute oral toxicity in rats was determined to be 2,500~5,000 mg/kg while the I.P.$_{LD_{50}}$ in mice was estimated at 1 mg/kg, suggesting a toxic compound which is poorly absorbed.

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