TRIENINE, AN ANTITUMOR TRIENE ANTIBIOTIC

ADORJAN ASZALOS, ROBERT S. ROBISON,
PAUL LEMANSKI and BERNARD BERK

The Squibb Institute for Medical Research
New Brunswick, New Jersey, U.S.A.

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Trienine, a new antitumor antibiotic, was isolated from the fermentation broth of *Streptomyces* SC3725. The substance, which contains three conjugated double bonds in a molecule weighing approximately 1,400, is active *in vitro* against gram-positive bacteria, as well as against several yeast and fungi. Trienine is also active as an inhibitor of the growth of the 5 WM tumor.

During a search for antitumor antibiotics among the actinomycetes, a triene antitumor antibiotic was isolated from the culture broth of *Streptomyces* SC3725. Although many antibiotics with conjugated double bonds are described in the literature, only two triene antibiotics are known[1,2].

In the present communication, we wish to report the fermentation, isolation, and determination of the properties of a third triene antibiotic, with a molecular weight of about 1,400.

Fermentation

Streptomyces SC3725 was isolated by conventional plating techniques from a soil sample obtained at Piscataway, New Jersey. It is maintained on an agar slant made with 2 medium containing from 20 g of tomato paste, 20 g of oatmeal, and 500 ml of boiling water. The medium is cooled to a thin gruel, filtered, added to 15 g of agar in 500 ml of water, and sterilized at 121°C for 20 minutes. On this medium, the aerial mycelium is gray colored and a light brown soluble pigment diffuses into the agar.

A portion of the growth from a well-sporulated slant is suspended in 0.01 % Duponal solution and used to inoculate 50 ml of sterilized broth in a 250-ml flask. The broth has the following composition:

Soybean meal	15 g	$(NH_4)_2SO_4$	5 g
Glucose	55 g	K_2HPO_4	1 g
$CaCO_3$	7 g	Corn steep liquor	2.5 g
KH_2PO_4	1 g	Distilled water	908.5 g

After 48 hours incubation at 25°C on a rotary shaker, the contents of the flask are transferred to 1,000 ml of the sterile broth in a 4,000-ml flask and again incubated for 48 hours. The entire contents of the flask are used then to inoculate a 380-liter germinator tank containing 225 liters of broth with 0.01 % defoamer (UCON Lubricant). Growth continues for 48 hours at 25°C, with a superficial air velocity of 30.5 cm/min. (1.0 ft/min.) and agitation equivalent to 0.2 HP/360 liters.

Approximately 55 liters of the germinator contents are used to inoculate 500 liters

of the medium in a 760-liter fermentor. Fermentation is conducted for 120 hours at 25°C, with superficial air velocity of 30.5 cm/min. (1.0 ft/min.) and agitation equivalent to 0.2 HP/360 liters.

Isolation

After 120 hours of fermentation, the whole broth is filtered, after the addition of filter aid. Since most of the antibiotic is in the filter cake, the filtrate is discarded. The wet filter cake is extracted twice with one liter isopropanol (IPA) per kg of cake. The combined IPA extracts are concentrated *in vacuo* to 1/60 of the original volume. To this concentrate, which is an aqueous suspension, four volumes of IPA are added and the suspension is kept at 5°C for 4~6 hours. The resulting precipitate is filtered off and dried *in vacuo* at 30°C. Yield is 250~300 g brown powder from 500 liters of broth.

Further purification of the crude product is achieved by solvent extraction, countercurrent distribution, and reprecipitation. The procedure is as follows:

Ten grams of crude antibiotic are dissolved in 1 liter *n*-butanol, saturated with water, and one liter water, saturated with *n*-butanol, and the pH is adjusted to 2.5 with dilute HCl. The water phase is removed and the *n*-butanol phase is shaken with one liter water, saturated with *n*-butanol. The water phase is removed again and the extraction process is repeated three more times. The pH is kept at 2.5 throughout. After removal of the last water phase, the *n*-butanol solution is neutralized with dilute NaOH and taken to dryness. The yield is about two g. Materials extracted into the water phase have negligible amounts of *in vivo* activity and are discarded.

The extracted material (2 g) is dissolved in a mixture of 125 ml *n*-butanol, saturated with water, and 125 ml water, saturated with *n*-butanol, and the pH is adjusted to 10 with dilute NaOH. A five-step countercurrent distribution process is carried out at pH 10 in separator funnels over a period of 4~6 hours.

After the countercurrent process, the contents of all funnels are neutralized with dilute HCl and taken to dryness. The solids are then tested in the 5 WM tumor system[3].

The fractions (#2 and #3) that have the highest activity *in vivo* are further purified by reprecipitation. The combined yield of these fractions is 0.4 g. Both fractions showed the same activity *in vivo* in the 5 WM system.

Dose (mg/kg/day)	50	25	12	6
T/C	tox.	0.09	0.22	0.41

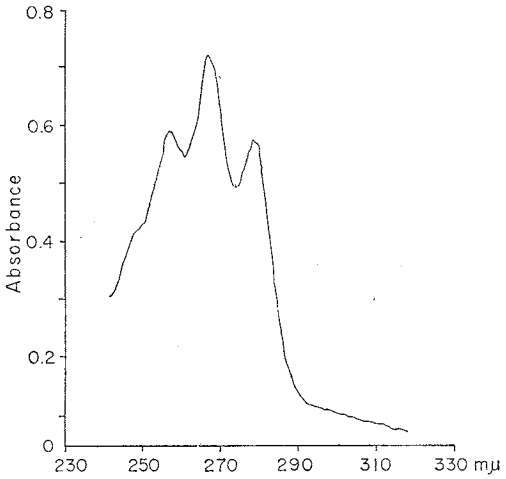
In the reprecipitation process, 2 g of active material of this description are extracted twice with 60 ml pyridine - water (5 : 1). The insoluble residue is discarded and the solution is concentrated to about 10 ml. To this concentrate, four volumes of IPA are added. The precipitate formed is washed with IPA and dried *in vacuo* at 35°C. This reprecipitation process is repeated until there is no change in ultraviolet absorption. Usually two or three reprecipitations are sufficient. The product is an amorphous off-white material (yield is 0.2 g).

Chemical and Physico-chemical Properties

Trienine is an amorphous light-yellow powder that melts at 163~165°C and decomposes at 180°C. It is soluble in methanol, pyridine, and dimethylformamide (DMF) and sparingly soluble in water, but insoluble in most other organic solvents.

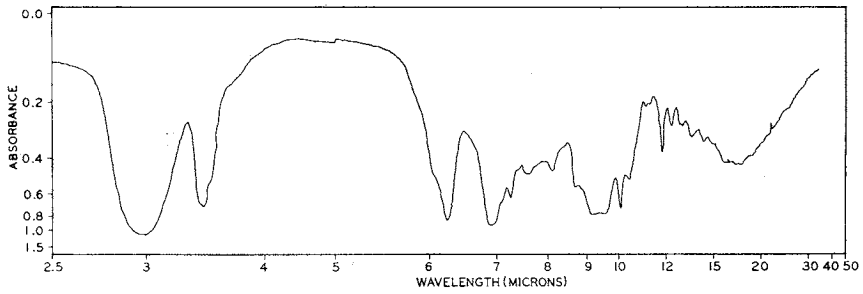
Trienine gives a positive TOLLENS[4] and a negative FEHLING[5] reaction. The ninhydrin reaction is positive only after acid hydrolysis. In the acid hydrolysate, no amino acids could be detected by paper chromatographic methods. Elemental analysis of preparations obtained at two different times gave the following values: C 55.00~54.49, H 7.85~8.13, N 1.34~0.99%. No sulfur or halogen can be detected in trienine preparations. Non-aqueous titrations for acidic function, with potassium methylate, gave an equivalent weight of 1,500 and for basic function, with perchloric acid, gave an equivalent weight of 1,380.

Fig. 1. Ultraviolet spectrum of trienine



Measurements with the analytical ultracentrifuge were carried out according to methods described by KIRSCHBAUM and ASZALOS[6], using the ARCHIBALD approach to sedimentation equilibrium at 42,000 rpm and 75° SCHLIEREN phase bar angle at 20°C. In phosphate buffer, two components were found, with molecular weights of 1,300±130 and 3,200±320. In methanol, only one component was found, with moelcular weight of 1,430±140. The component with molecular weight of 3,200 is believed to be a dimer.

Fig. 2. Infrared spectrum of trienine



The ultraviolet spectrum of trienine in methanol (Fig. 1) shows absorption typical for a triene. The maxima are located at 257, 267, and 278 mμ with $E^{1\%}_{1cm}$ of 332, 390, and 322, respectively.

As calculated from the λ_{max} at 267 mμ of 2,4,6-octratriene[7,8] and from the $E^{1\%}_{1cm}$ at 267 mμ of trienine, the equivalent weight of trienine is 1,360. Trienine loses its ultraviolet characteristics upon standing in methanolic solution exposed to light.

The infrared spectrum of trienine (KBr) shows absorption at 3.0, 3.42, 6.29, 6.90, 7.25, 8.12, 8.70, 10.07, and 11.80 μ, as shown in Fig. 2. (The IR spectra of three separate trienine preparations were superimposable). It is postulated that trienine contains ionized carboxyl and hydroxyl groups. Absorption at 10.07 μ is due to the triene group[1]. The characteristic absorption around 5.8 μ, attributable to the lactone group in other polyenic antibiotics, is not present.

Table 1. Microbiological spectrum of trienine

	M.I.C. mcg/ml
Staphylococcus aureus SC1276	0.37
Streptococcus pyogenes SC3862	0.44
Escherichia coli SC2975	>50
Proteus vulgaris SC3855	>50
Pseudomonas aeruginosa SC3840	>50
Salmonella schottmuelleri SC3850	>50
Geotrichum sp. SC3560	9.4
Saccharomyces cerevisiae SC1600	7.8
Candida tropicalis SC2620	6.3
Candida krusei SC2616	9.4
Candida albicans SC5314	8.6 (14.0)
Trichophyton mentagrophytes SC2637	≥50
F. bulbigenum SC5273	6.3
Aspergillus niger SC2828	6.3

Trienine moves as a single spot in paper chromatographic systems. In the solvent system BuOH - AcOH - H_2O (4 : 1 : 5), trienine has an Rf of 0.55 and, in the solvent system 5 % DMF in MeOH, an Rf of 0.7.

Attempts to determine the purity of the trienine preparations by means of CRAIG countercurrent distribution failed; all *in vivo* biological activity disappeared. Loss of activity may have been due to exposure of trienine to light for the long periods required for disappearance of the emulsions formed by this compound in three different solvent systems tried.

Biological Properties

Trienine is weakly active against microorganisms *in vitro*. The antimicrobiological spectrum is given in Table 1. Trienine is also active against the 5 WM tumor system; activity against this tumor system *in vivo* is as follows (Hazleton Laboratories, Inc., Falls Church, Virginia):

Dose (mg/kg/day)	4	2	1	0.5
T/C	tox.	0.16	0.53	0.72

The loss in ultraviolet absorption in methanolic solution—as mentioned above—is accompanied by a loss in biological activity. In the dry form, at room temperature, trienine is stable for at least four months.

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