PRODUCTION, ISOLATION AND PURIFICATION OF ANTITUMOR ACTIVE MONOGLYCERIDES AND OTHER ANTIBIOTICS FROM *SEPEDONIUM AMPULLOSPORUM*

STUDIES ON ANTIVIRAL AND ANTITUMOR ANTIBIOTICS. IX

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Monoglycerides and fatty acids with antitumor activity against EHRLICH ascites tumor in mice were isolated from the acetone extract of the mycelium of *Sepedonium ampullosporum*. In addition, a new Cl- and N-containing antibacterial antibiotic and rugulosin were isolated from this fungus. Rugulosin is a yellow pigment with strong inhibitory activity against Gram-positive bacteria and *Proteus vulgaris*.

Sepedonium ampullosporum, a fungus classified as Fungi Imperfecti[1], produces antitumor active fatty acids[2] and monoglycerides[3]. In the course of isolating these antitumor active substances, two other antibiotics were found to be produced by this microorganism. Both of these antibiotics inhibited *Sarcina lutea*, and exhibited cytotoxicity in chick embryo fibroblast tissue culture.

In this paper the production, isolation and purification of the fatty acids, the monoglycerides and the two antibiotics are described. Some of the chemical properties and biological activities of the antibacterial antibiotic are also reported.

Production and Isolation of the Antibiotics

For the production of antibiotics a medium consisting of 5 % glucose, 0.5 % peptone, 0.2 % yeast extract, 0.06 % KH_2PO_4, 0.1 % NH_4Cl, 0.04 % $MgSO_4 \cdot 7H_2O$ and 1 % $CaCO_3$ was used. One hundred ml of the medium in a 500-ml flask was inoculated with *Sepedonium ampullosporum* Damon IFO 6622 and shake-cultured at 26.5°C for 4 days. Twenty ml of this culture was then transferred into 800 ml of medium in a 5-liter flask and shake-cultured again at 26.5°C for 4 days. For larger scale production, jar and tank fermenters were employed using the same medium and soybean oil as an antifoam agent. At the end of fermentation, the culture was filtered to collect mycelium. Acetone was added to the mycelial mass to extract intracellular products. After the acetone was removed *in vacuo* the extract was adjusted to pH 3.0 and was shaken two or three times with benzene. The solvent phase was collected, dried over anhydrous sodium sulfate, and concentrated *in vacuo*

to remove the benzene. The extract thus obtained was chromatographed on a silicic acid (Mallinkrodt) column with 20 % (by weight) of Celite 545. The column was first developed with benzene and the eluate was concentrated under reduced pressure to obtain a yellowish oil (Fraction I). The column was then eluted with benzene - methanol (97 : 3, v/v) and fractions having antibacterial activity against *Sarcina lutea* were collected. A brown oil was obtained after removal of the solvent, and was rechromatographed on a silicic acid column with chloroform and methanol as the elution solvents. The antibacterial activity was found in two peaks; one in 99 : 1 chloroform - methanol eluate and the other in 97 : 3 chloroform - methanol eluate. Active fractions in each peak were collected and the solvent was removed *in vacuo*. When the oil obtained from the 99 : 1 chloroform - methanol eluate was dissolved in benzene and allowed to stand overnight at 4°C, white crystals formed (Fraction III) which were collected by filtration. The filtrate was concentrated under reduced pressure to get a brownish orange oil (Fraction II). From the 97 : 3 chloroform - methanol eluate a yellow powder was obtained on evaporation. An outline of the isolation procedure is presented in Fig. 1.

Fig. 1. Isolation of antitumor and antibacterial antibiotics

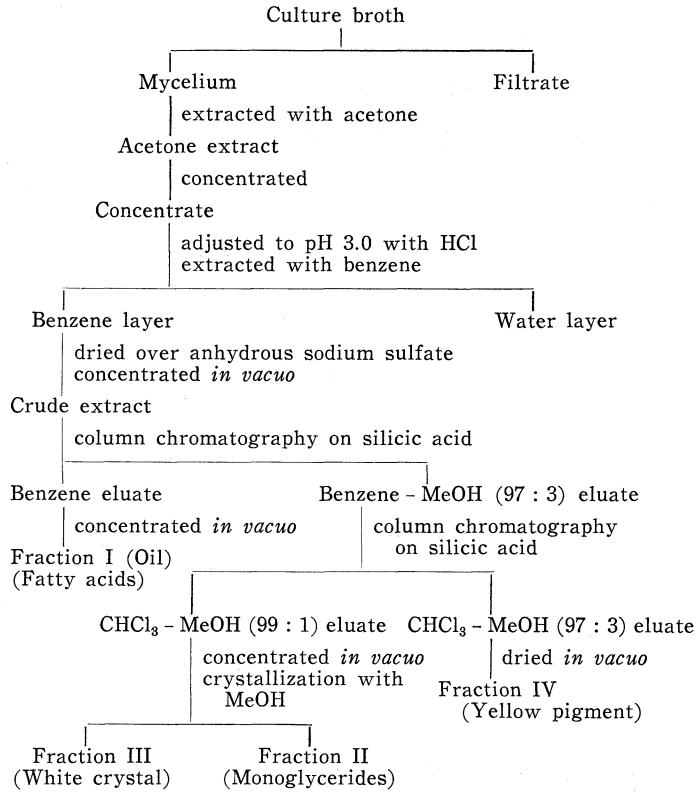


Table 1. Biological activities of each fraction

	Activities against			Cyto-toxicity
	Tumor	Virus	Bacteria	
Fraction I (Fatty acids)	++	−	−	−
Fraction II (Monoglycerides)	+++	−	−	−
Fraction III (White crystal)	−	±	++	+
Fraction IV (Yellow pigment)	−	−	+++	+

Each substance was examined for antitumor activity *in vivo* using EHRLICH ascites tumor in mice, antiviral activity *in vitro* for Newcastle disease virus, antibacterial activity against *Sarcina lutea*, and cytotoxicity against chick fibroblast in tissue culture. The results are summarized in Table 1.

Purification of Antitumor Active Substances

Fraction I was further purified by column chromatography on silicic acid. Active principle was eluted with either benzene or hexane. After the removal of solvents the oil was dried *in vacuo*. The active principle was chemically and spectrometrically identified as a mixture of fatty acids. It showed marked inhibition of tumor growth in mice. The details of the chemical study of these antitumor active fatty acids are reported elsewhere[4,5].

The purification of Fraction II was performed by preparative silica gel thin-layer chromatography. Crude Fraction II was stretched on a silica gel plate and developed with chloroform-methanol (9:1). The substance with the Rf values between 0.55 and 0.90 was recovered and the active principle was extracted with chloroform-methanol (4:1). The active fraction was purified further by silica gel chromatography with hexane-ethylacetate (1:1) as a developing solvent mixture. The material having Rf values between 0.40 and 0.50 was recovered similarly. This material was identified as a mixture of monoglycerides. The details of the identification and chemical and biological properties will be discussed elsewhere[6,7].

Purification and Chemical Properties of the Antibacterial Antibiotics

Thirteen mg of white crystalline powder obtained from 3 liters of culture was purified by recrystallization from benzene. These crystals turned pale yellow when heated to 170°C and sublimed at 200°C. When the crystals were examined for the presence of halogen by BEILSTEIN reaction, bright green color appeared in the flame suggesting the presence of chlorine.

Anal. Found: C 51.28, H 4.69, N 10.27.

Although chlorine could not be determined by the SCHOENIGER flask combustion method[8] for halogen analysis, the presence of halogen in the molecule was shown by mass spectroscopy. The parent peak at m/e 415 was accompanied with a relatively large $p+2$ peak, assigned to the parent peak with chlorine isotope. The infrared

Fig. 2. Infrared spectrum of white crystalline antibiotic.

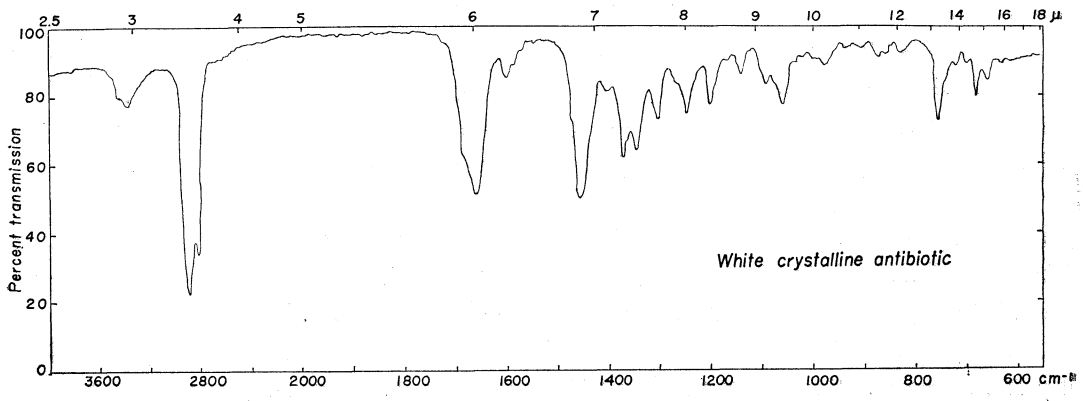


Fig. 3. Infrared spectrum of yellow pigment (Rugulosin).

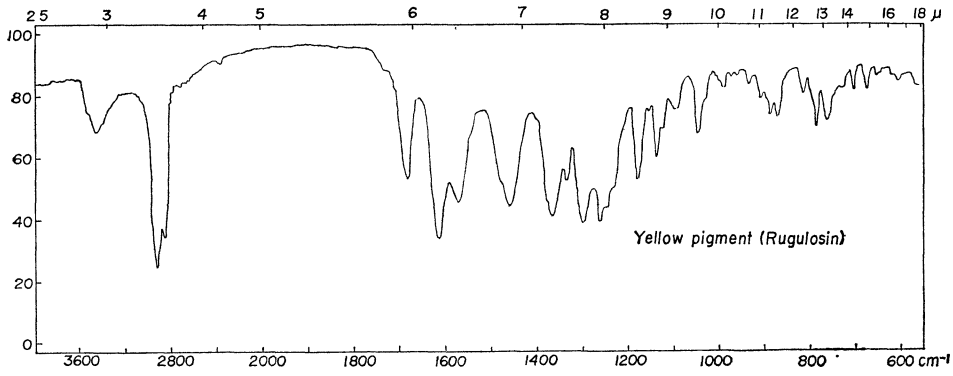


Fig. 4. Ultraviolet spectrum of rugulosin (20 μg/ml methanol).

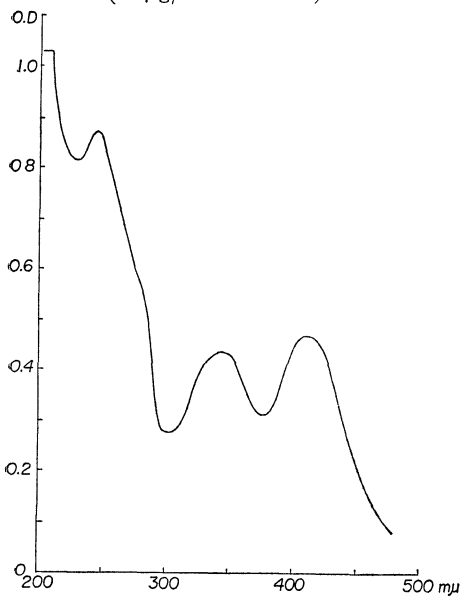
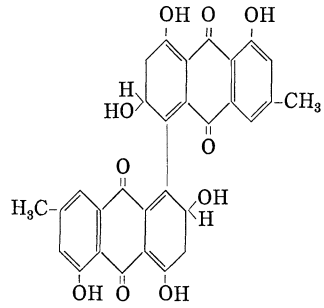


Fig. 5. Structure of rugulosin.



absorption spectrum is shown in Fig. 2, which indicates the presence of a primary amide group and hydroxyl group in the molecule.

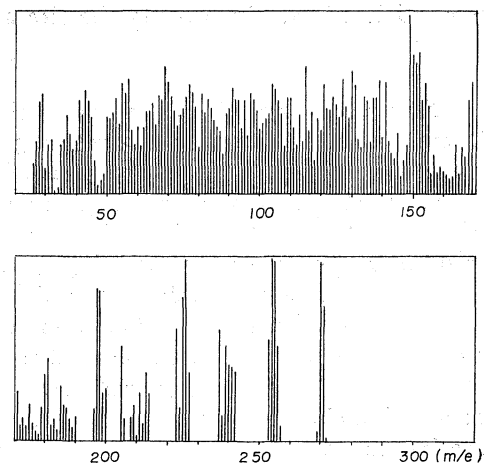
The yellow powder (Fraction IV) was purified by column chromatography on silicic acid with benzene and acetone as elution solvents. Active fractions from the eluate with bezene - acetone (95 : 5) were collected and concentrated *in vacuo*. A bright yellow powder resulted. Pure crystals could also be obtained from the acetone solution. The infrared absorption spectrum of these crystals is shown in Fig. 3. The characteristic bands at 1690 and 1620 cm^{-1} are indicative of a C=O stretching absorption of ketones in quinone compounds[9]. Strong bands at 2960 and 2845 cm^{-1} for -CH stretching vibration and 1574 cm^{-1} for C=C stretching vibration are consistent with quinone structure[9]. The ultraviolet absorption spectrum shown in Fig. 4 is also characteristic for quinone compounds.

Rugulosin, a yellow pigment, has been reported by RAISTRICK *et al.*[10] and SHIBATA *et al.*[11] as a metabolite of fungi including *Sepedonium ampullosporum*. It is a bianthraquinone derivative with the molecular weight of 542 whose structure was elucidated by SHIBATA and his co-workers as shown in Fig. 5.

The yellow antibiotic (Fraction IV) was compared with rugulosin kindly furnished by Dr. SHIBATA. Spectroscopic properties and Rf values on thin-layer chromatography

showed identity of the two materials. The mass spectral analysis of the yellow antibiotic is presented in Fig. 6. It shows a strong peak at m/e 270 assigned to a rearranged ion of half a molecule of rugulosin. The base peak at m/e 254 is assigned to an ion formed when the methyl group is lost from the molecule. Characteristic peaks for the quinone compounds formed with the loss of -CO's can clearly be seen at m/e 226 and 198. A relatively large peak at m/e 170 is assigned to an ion formed with the loss of -CO from the hydroxylated carbon.

Fig. 6. Mass spectrum of rugulosin.



Thus the yellow antibiotic was identified as rugulosin.

Biological Activities of Antibacterial Antibiotics

Biological activities of the white crystal and the yellow pigment, rugulosin, were studied. Antibacterial activity against *Sarcina lutea* by paper disk method and antiviral activity against Newcastle disease virus on the chick embryo fibroblast monolayer assayed with agar diffusion method of HERRMANN[12] are shown in Table 2. Both of the antibiotics show inhibitory activity against *Sarcina lutea*. They have cytotoxicity to the chick fibroblast in the monolayer. Antimicrobial spectrum of rugulosin was examined using agar streak method on nutrient agar for bacteria and 1% glucose-nutrient agar for yeasts and fungi with the results demonstrated in Table 3. This antibiotic reveals strong inhibitory activity against the growth of all the Gram-positive bacteria tested. It also showed specific inhibition against *Proteus vulgaris*, but it is not active at all against fungi nor yeasts.

Antitumor activity *in vivo* of rugulosin was studied against EHRLICH ascites tumor in mice. Mice, *ddY* strain, 5 weeks old, were treated with the antibiotic daily by intraperitoneal injection for 5 consecutive days after implantation

Table 2. Inhibition of biological test system by white crystal (Fraction III) and rugulosin

Test system	White crystal (Fraction III)	Rugulosin
S. lutea	0.8 μg/ml	0.8 μg/ml
Newcastle disease virus	3.0	not tested
Cytotoxicity	3.0	100

Table 3. Antimicrobial spectrum of rugulosin. (Agar streak method)

Test organisms	M.I.C. (mcg/ml)
Staphylococcus aureus IAM 1058	0.4
Bacillus megaterium KM	0.2
Bacillus subtilis IAM 1026	12.5
Bacillus cereus IAM 1656	25.0
Micrococcus pyogenes IFO 3340	0.8
Sarcina lutea IAM 1097	6.25
Escherichia coli K-12	>100
Xanthomonas oryzae IAM 1657	>100
Proteus vulgaris IAM 1025	6.25
Pseudomonas aeruginosa IAM 1202	>100
Candida albicans IAM 4888	>100
Saccharomyces cerevisiae IAM 4518	>100
Hansenula anomala IAM 4868	>100
Aspergillus niger IAM 2093	>100
Penicillium chrysogenum Q-176	>100
Trichophyton mentagrophytes strain Ch	>100

of EHRLICH ascites tumor cells. No significant activity against the tumor was noted nor did it effectively prolong the life span of the treated mice.

Discussion

Sepedonium ampullosporum was selected as an antitumor antibiotic-produing fungus in our antitumor screening system using EHRLICH ascites tumor in mice. Examination of the active principle showed two types of materials, fatty acids and monoglycerides. They showed marked inhibition of the tumor growth in mice, and prolongation of the life span of the host was observed. It is interesting to note that active principles of other fungi selected through the same screening system were also fatty acids and monoglycerides[2,3].

In the course of isolating the antitumor antibiotics, two antibacterial antibiotics were obtained, one of which was identified as rugulosin. Antibacterial activity of rugulosin against *Staphylococcus aureus* and other pathogenic bacteria has been reported by RAISTRICK *et al.*[10] In our study, rugulosin shows strong inhibition against Gram-positive bacteria. It also inhibits the growth of *Proteus vulgaris* against which many of known antibiotics are ineffective.

The white crystalline antibiotic (Fraction III) is much interest as it contains as much as 10 % of nitrogen and also chlorine in the molecule. No antibiotic from fungi has been reported to contain both nitrogen and chlorine in its molecule. Antimicrobial spectrum and other biological activities and chemical properties are under investigation.

Acknowledgements

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