

AMBRUTICIN* (W7783), A NEW ANTIFUNGAL ANTIBIOTIC

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(Received for publication March 7, 1977)

Ambruticin represents a new class of antibiotics isolated from a strain of *Polyangium cellulosum* var. *fulvum*, a bacterium belonging to the class Myxobacteriales. This antibiotic is a cyclopropyl-polyene-pyran acid and is active *in vitro* against fungi.

Myxobacteria are ubiquitous microorganisms found in soil, on the bark of trees, and on animal dung. These organisms are not detected by the routine methods used in culturing bacteria and fungi but require special techniques for their isolation¹. In the course of our program to screen the Myxobacteriales as a source of biologically active compounds, an isolate of *Polyangium cellulosum* var. *fulvum* was found to have interesting antifungal properties. The present communication deals with the production and recovery of the antibiotic substances as well as the isolation and characterization of one of the antibiotics from this organism.

Fermentation

Slant cultures of *Polyangium cellulosum* var. *fulvum* ATCC 25532 were grown for 4 days at 37°C on a medium containing 0.5% soluble starch, 0.25% Casitone (Difco), 0.05% MgSO₄·7H₂O, 0.025% K₂HPO₄, and 1.5% agar in distilled water.

Inoculum for fermentors was prepared by sequential liquid culture in flasks containing 0.5% soluble starch (Staclipse J starch, A. E. Staley), 0.25% Casitone, 0.05% MgSO₄·7H₂O, and 0.025% K₂HPO₄ in distilled water (final pH 7.1). The preliminary stage was grown in a one-liter Erlenmeyer flask containing 400 ml of media, inoculated with cells from a slant culture and incubated for 96 hours at 32°C on a rotary shaker with a two-inch stroke at 150 rpm. The secondary stage was incubated in a similar manner for 72 hours by inoculating 40 ml of primary stage culture into a two-liter baffled Erlenmeyer flask containing one liter of medium.

The fermentation medium consisted of 0.5% soluble starch (Staclipse J starch, A. E. Staley), 0.25% soybean concentrate (Pro-Fam 70/LS, Grain Processing Corp.), 0.05% MgSO₄·7H₂O, 0.025% K₂HPO₄, 0.04 mg/liter CoCl₂·6H₂O, and 0.1% Antifoam C (Dow Corning) in distilled water (final pH 7.1). Fourteen-liter capacity stir vessels (NBS FS-314) containing 10 liters fermentation medium were inoculated with seed culture (5~10% v/v) and incubated at 32°C with 300 rpm agitation and sterile air supplied at 2 liters/minute through a single sparge hole. Peak titers occurred in ~96 hours and harvested broths were pooled and stored at 4°C.

Fermentation samples were monitored by a microbiological disc or cylinder-plate assay procedure using SABOURAUD dextrose agar and *Microsporum fulvum* or *Penicillium* sp. (WLRI 0135) as the assay organism.

* Proposed USAN

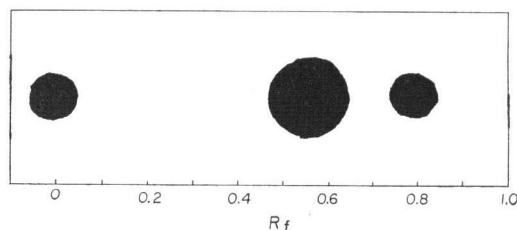
Recovery

Based on 100 gallons, 15 g hydroquinone were added to the whole broth followed by 90 kg granular sodium chloride. The mixture was adjusted to pH 4.0 with acetic acid and stirred with 8 kg Supercel (Johns-Manville) for three hours under a blanket of nitrogen. The mixture was then pumped through a "Sparkler" type filter and the filtrate discarded. The activity from the filter cake was desorbed by recycling with 15 gallons of acetone to which 15 kg anhydrous sodium sulfate were added. The acetone was concentrated under vacuum until the acetone was completely removed, leaving a salt-water residue. This was then extracted with two 80% volumes of ethyl acetate which were combined and dried with 70 g anhydrous sodium sulfate per liter of ethyl acetate. The resulting ethyl acetate extract was concentrated to a volume of 100~200 ml and contained the crude antibiotics.

Fermentation broths were prepared for monitoring of antibiotic components by adsorption onto florasil followed by methanolic desorption. The crude ethyl acetate concentrates as well as the fermentation broths were subjected to thin-layer chromatography on silica gel plates (Quantum Q₁) in ethyl acetate - methanol - acetic acid (95:1:4) and the antibiotics present were detected by bioautography on *Candida parapsilosis* seeded agar trays. Fermentation broths as well as the crude concentrates contained three active components and occasionally an additional active component was detected at origin (Fig. 1). Biological activity for the moving components coincided with spots located by iodine vapor²¹. The major component ambruticin constituted from 70 to 90% of the antibiotic complex and had been referred to as acid "S" by our research group in distinction to the faster moving component.

Fig. 1. Thin-layer chromatogram of ambruticin in ethyl acetate - methanol - acetic acid (95:1:4).

Activity detected by bioautography on *C. parapsilosis* seeded agar trays.



Isolation of Ambruticin

The ethyl acetate extract containing the crude antibiotics was decanted onto a column of Quantum Q₁ silica gel, using 500 g silica gel per gram active antibiotic complex as determined by bioassay. The product was eluted with ethyl acetate - isopropanol - water mixtures starting with 97:2:1 and progressing to 85:10:5 until ambruticin was isolated as a single spot on tlc. The ambruticin fractions were concentrated under vacuum and, when triturated with petroleum ether (30~60°C), produced a tan solid.

Characterization of Ambruticin

Spectroscopic methods were as follows: The mass spectrum was run on an AEI-MS902C spectrometer; exact masses were obtained by the peak matching method using perfluorokerosene as the reference. The infrared spectrum was recorded on a Perkin-Elmer 621 spectrometer as a film deposited from chloroform. The proton magnetic resonance spectrum was recorded on a Perkin-Elmer R12B 60 MHz spectrometer with internal lock on TMS; the solvent was CDCl₃ and the concentration was 100 mg/ml.

Elemental analysis showed the absence of nitrogen and the mass spectrum showed M⁺ at 474.

Exact mass gave a composition of $C_{28}H_{42}O_6$ (calcd. 474.2981, found 474.3019). The bar graph of the low resolution spectrum is given in Fig. 2. The infrared spectrum in Fig. 3 showed a hydrogen bonded OH band $3100\sim 3600\text{ cm}^{-1}$, an acid carbonyl at 1715 cm^{-1} , aliphatic C-O bonds at $1030\sim 1130\text{ cm}^{-1}$ and a trans double bond band at 955 cm^{-1} . The UV spectrum showed only end absorption. The pmr spectrum is given in Fig. 4 and it confirmed the presence of olefinic protons by bands in the $5.0\sim 6.0\text{ ppm}$ region. There was broad absorption in the $3.3\sim 4.3$ region, attributable to aliphatic $-\text{CH}-\text{O}$ protons. Several methyl group resonances were detected in the $1.0\sim 1.7$ region. Two components of an ethyl group triplet were found at 0.9 ppm . There were no aromatic protons.

Fig. 2. Mass spectrum of ambruticin.

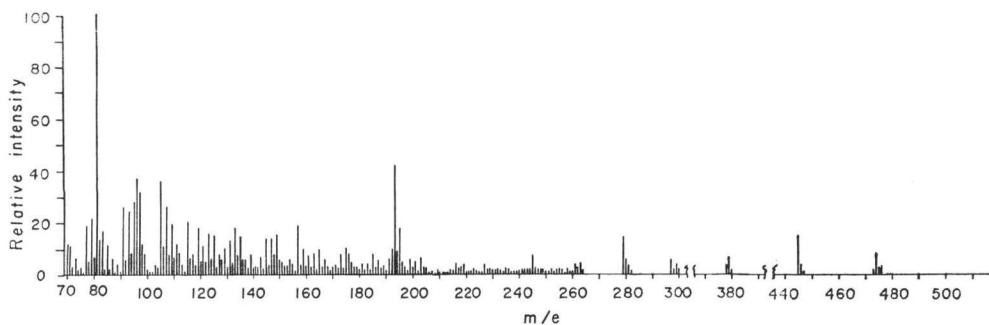


Fig. 3. Infrared absorption spectrum of ambruticin (film).

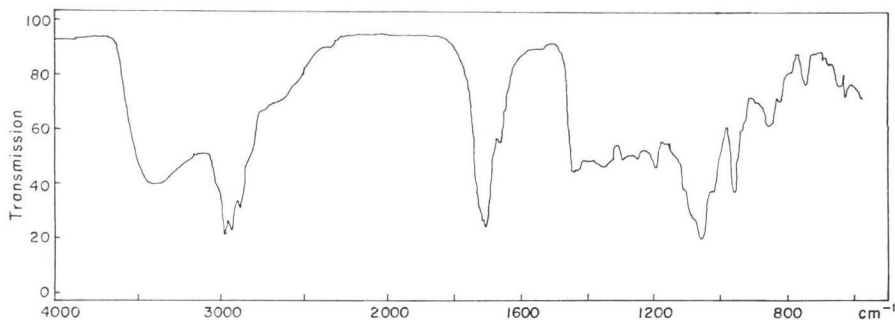


Fig. 4. Proton magnetic resonance spectrum of ambruticin (CDCl_3).

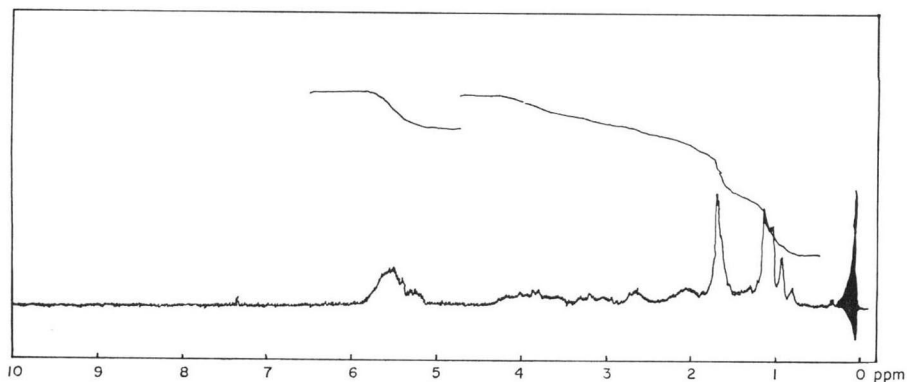
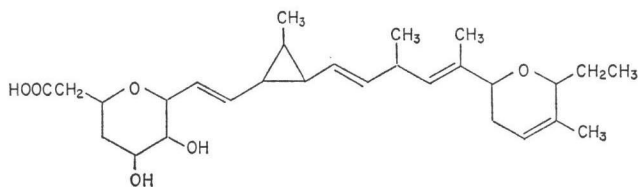


Fig. 5. Structure of ambruticin.



These spectroscopic data on ambruticin did not allow determination of the structure. Single crystal X-ray structure determination of the triformate derivative gave the complete structure as shown in Fig. 5.* This figure does not depict the stereochemical configuration of the asymmetric centers. Ambruticin is a cyclopropyl-polyene-pyran acid and appears to represent a new class of antibiotics.

Ambruticin is not water-soluble but the antibiotic is soluble in polar solvents such as methanol and ethanol as well as in non-polar solvents such as chloroform and petroleum ether. The water-soluble sodium salt of ambruticin is thermostable in aqueous solution at 100°C for at least 30 minutes or in lyophilized form under nitrogen at 45°C for at least 3 months.

Biological Properties of Ambruticin

The *in vitro* antifungal spectrum of ambruticin is summarized in Table 1. This antibiotic is highly active against systemic medical pathogens such as *Coccidioides immitis*, *Histoplasma capsulatum*, and *Blastomyces dermatitidis* as well as the dermatophytic filamentous fungi. Ambruticin is essentially inactive against most gram-positive and gram-negative bacteria. However moderate *in vitro* antibacterial activity was detected against *Streptococcus pyogenes* (MIC 12.5 mcg/ml), *Diplococcus pneumoniae* (MIC 12.5 mcg/ml) and *Bacillus cereus* (MIC 50 mcg/ml). Preliminary tests indicate that the other active components produced by *Polyangium cellulorum* var. *fulvum* appear to have similar antimicrobial spectra *in vitro*.

The acute LD₅₀ values for the sodium salt of ambruticin in mice were: intravenous 315 mg/kg, oral >1,000 mg/kg.

Table 1. Antifungal activity of ambruticin

| Organism | Minimum inhibitory concentration (mcg/ml) |
|--------------------------------------|---|
| <i>Coccidioides immitis</i> | <0.025 |
| <i>Histoplasma capsulatum</i> | 0.195 |
| <i>Blastomyces dermatitidis</i> | 0.04 |
| <i>Cryptococcus neoformans</i> | 100.0 |
| <i>Paracoccidioides brasiliensis</i> | 50.0 |
| <i>Candida albicans</i> | >100.0 |
| <i>Candida parapsilosis</i> | 0.78 |
| <i>Trichophyton mercuriophytes</i> | 0.04 |
| <i>Microsporium audouinii</i> | 0.02 |
| <i>Epidermophyton floccosum</i> | 0.01 |
| <i>Aspergillus flavus</i> | 1.56 |
| <i>Rhizopus nigricans</i> | 0.195 |
| <i>Botrytis cinerea</i> | 0.78 |
| <i>Alternaria tenuis</i> | 3.12 |

Tests carried out by a standard broth dilution procedure.

Acknowledgement

We express our appreciation to Mr. C. LUTOMSKI for antibiotic recovery from fermentation broths, to Ms. U. ZEEK for the elemental analysis, to Dr. E. SCHWARTZ for the animal toxicity data, and to Dr. H. B. LEVINE (Univ. California, Naval Biosciences Laboratory, Oakland, California) for the *C. immitis in vitro* data.

* The X-ray structure determination was done by the Molecular Structure Corporation, College Station, Texas.

References

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