A30641, A NEW EPIDITHIODIKETOPIPERAZINE WITH ANTIFUNGAL ACTIVITY

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A new antibiotic, designated A30641, having *in vitro* activity against Gram-positive bacteria and fungi has been isolated from a strain of *Aspergillus tamarii*. Chemical and physical characterization indicate that it is a member of the class of antibiotics containing the epidithiodiketopiperazine moiety.

The study of metabolites from a new strain of *Aspergillus tamarii* evolved from our interest in fungi as a source of biologically active compounds. An interesting spectrum of antifungal activity exhibited by broth cultures of this organism led to the isolation of a metabolite, A30641, showing *in vitro* activity against fungi and Gram-positive bacteria.

In addition to this new substance, a previously described antifungal compound, canadensolide[1], was isolated from fermentations of the same culture.

Fermentation

The organism, *Aspergillus tamarii* NRRL 8101, was obtained by soil screening procedures from a soil collected in the Netherlands Antilles.

Slant cultures were grown for seven days at 25°C on a medium containing 2% glucose, 0.5% peptone (Difco), 0.05% KH_2PO_4, 0.002% $MgSO_4 \cdot 7\,H_2O$, 0.001% $FeSO_4 \cdot 7\,H_2O$, and 2% agar in deionized water (final pH 5.5). The resulting sporulated cultures were preserved by lyophilization.

Fermentor inoculum was prepared by multiple-stage submerged culture in a medium containing 1% glucose, 1% potato dextrin, 1% Soy Peptone T (Sheffield Chemical), and 0.5% Amber BYF (Amber Laboratories) in tap water (final pH 6.2). The initial stage was grown in 250-ml wide-mouth Erlenmeyer flasks containing 50 ml of media. These flasks were inoculated with lyophilized cultures and incubated for 48 hours at 25°C on a shaker rotating within a 5-cm circle at 250 rpm. Additional stages were incubated in the same manner for 24 hours in 2-liter flasks containing 400 ml of medium. Fermentors were inoculated with 1% (v/v) of the resulting biomass.

Fermented broth for isolation of the active metabolite was produced in stirred fermentors. These vessels were of conventional design, employing two 6-bladed turbine impellers and a 1:1 height-diameter ratio for the 100 liters of medium. Temperature was controlled at 25°C and aeration-agitation was preset to provide a calculated oxygen solution rate of 270 μM per minute. The fermentation medium contained 4% Stadex No. 11 (A. E. Staley), 1% N-Z-Amine A (Sheffield Chemical), 1% blackstrap molasses, 0.05% $MgSO_4 \cdot 7\,H_2O$, and 0.2% $CaCO_3$. After an incubation period of 40~48 hours the pH of the broth was approximately 5.0 and the anti-

biotic titer, measured by a conventional agar-diffusion assay employing *Neurospora crassa* as the test organism, was 30~40 μg/ml.

Fermentation samples were monitored qualitatively by:

1) descending paper chromatography on Whatman No. 1 paper in water - methanol - acetone (12:3:1) that had initially been adjusted to pH 10.5 with NH_4OH and re-adjusted to pH 7.5 with H_3PO_4, and

2) tlc on silica gel plates in $CHCl_3$ - acetone (6:4) (Fig. 1a). Bioactivity was detected on developed chromatograms by a bioautographic technique employing *N. crassa*.

Isolation and Characterization

Both antibiotics could be isolated from filtered broth by extraction with an organic solvent or by adsorption onto a column of Amberlite XAD-2. Active solid preparations were also prepared from methanol extracts of the mycelial solids.

Filtered broth (80 liters) was adjusted to pH 3.0 with dilute acid and extracted twice with 40 liters of chloroform. The extracts were combined and concentrated *in vacuo* to an oily residue which was further purified by column chromatography.

Alternatively, 75 liters of filtered broth, adjusted to pH 3.0 with dilute acid, were passed through a 6.5×72.5 cm column of Amberlite XAD-2 resin at a flow rate of 500 ml/min. The column was then washed with water and 50% aqueous methanol, and the active metabolite eluted with 100% methanol. The active fractions were combined and concentrated to an oil, then redissolved in 150 ml methanol and added to 20 volumes of ether to precipitate inactive contaminants. The supernatant was filtered and concentrated to an oil. The oil was dissolved in 150 ml of chloroform and added to 20 volumes of hexane to yield 5.0 g of an active crude precipitate.

The mycelial cake from 100 liters of whole broth was extracted twice with 50 liters of methanol. The extracts were combined and concentrated *in vacuo* to remove the methanol. The aqueous concentrate was diluted to 40 liters with distilled water, adjusted to pH 3.0 with dilute HCl, and extracted twice with 20 liters of chloroform. The extracts were combined and concentrated to an oily residue. The residue was taken up in 500 ml methanol and filtered. The filtrate was concentrated to residue and dissolved in 100 ml chloroform. The chloroform solution was added to 1 liter of hexane or Skellysolve F to precipitate the crude antibiotic mixture (10.3 g).

The active oily residue or crude precipitate was further purified by column chromatography on a silica gel column (Matheson, Coleman and Bell, Grade 62, 60~200 mesh) prepared in benzene. After washing with benzene and 9:1 benzene-ethyl acetate mixture, the dithiodiketopiperazine was eluted with a 4:1 benzene-ethyl acetate mixture. The active fractions were combined and concentrated to an oil which was dissolved in 150 ml of chloroform. The chloroform solution was added to 3 liters of hexane to yield 2.1 g of the pure amorphous dithiodiketopiperazine. Concentration of the 9:1 benzene - ethyl acetate eluant resulted in an oil which could be subsequently chromatographed on silica gel to yield the antibiotic canadensolide[1].

The isolation was monitored by thin-layer chromatography on silica gel plates (E. Merck, 20×20 cm, precoated, F-254, layer thickness 0.25 mm on glass, Brinkmann Instr. Inc.) developed

in 1:1 benzene-ethyl acetate (Fig. 1b). Biological activity coincided with spots located by uv fluorescence (254 nm) quenching, and by spraying with iodine-azide reagent[2]. The untreated spots also turned yellow upon exposure to air. The colorless, chromatographically (tlc) homogenous material could not be crystallized.

Fig. 1. Thin-layer chromatograms of A30641 (A) and canadensolide (C) on silica gel in (*a*) 6:4 chloroform-acetone; and (*b*) 1:1 benzene-ethyl acetate.

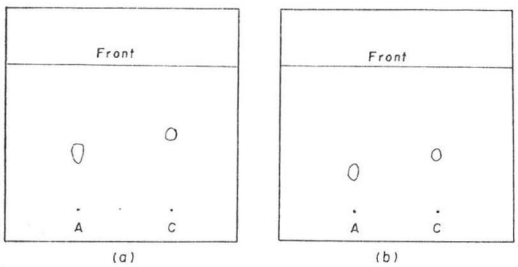
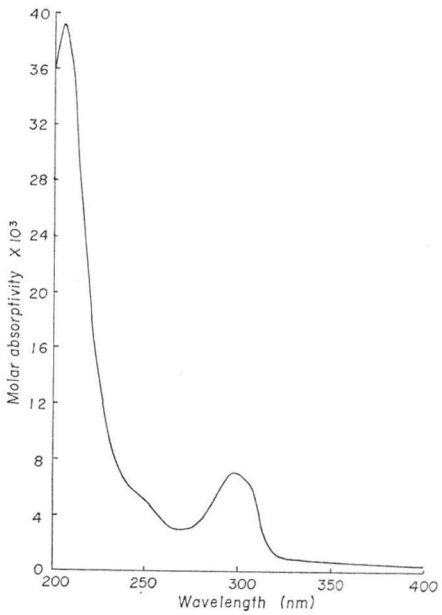


Fig. 2. Ultra-violet absorption spectrum of A30641 in ethanol.



Antibiotic A30641 melted between 160~172°C with decomposition and exhibited $[\alpha]_D^{26}$ +73° (*c* 1, MeOH). High resolution mass spectrometry gave M^+, 359.9670, corresponding to the empirical formula $C_{12}H_9N_2O_5S_2Cl$ (359.9642), and *m/e*=296.0178 ($C_{12}H_9N_2O_5Cl$) indicating the loss of S_2. Elemental analysis gave C, 40.29; H, 2.72; N, 7.67; O, 21.48; S, 17.86; Cl, 9.54. Calcd. C, 39.95; H, 2.51; N, 7.56; O, 22.17; S, 17.77; Cl, 9.83. The ultraviolet spectrum (Fig. 2) shows maximum absorption at 208 nm (ε=36,800) and 300 nm (ε=6,300) under neutral or acid conditions. The latter peak shifts to 314 nm (ε=8,100) under basic conditions. The infrared spectrum (Fig. 3) indicates the presence of OH (3540 cm^{-1}); $-\overset{\overset{O}{\|}}{C}-$ (1730 cm^{-1}); and amide (1630 cm^{-1}). Proton magnetic resonance data are presented in Table 1.

Some structural features may be inferred from these data. The presence of a phenolic hydroxyl is clearly indicated by the ultraviolet absorption shift in base. The presence of

Fig. 3. Infra-red absorption spectrum of A30641 in $CHCl_3$.

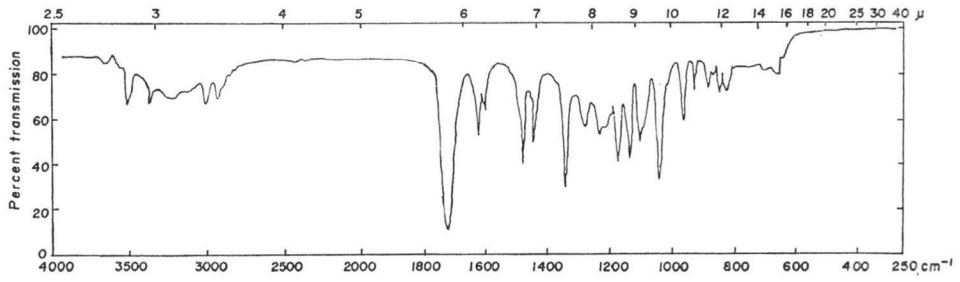


Table 1. PMR spectrum of A30641 in $CDCl_3$

δ(ppm)	No. H	Assignment
3.95 S	3	CH_3-O-
4.88 D (J=1.2)	1	-C-H
5.15 D (J=5)	1	-C-H
6.20 S	1	-OH (Exchangeable)
6.75 S	1	Probably aromatic
7.12 D (J=1.2)	1	Probably aromatic
7.64 D (J=5)	1	-NH (Exchangeable)

carbonyl and amide absorptions in the infrared spectrum together with the presence of two sulfurs infers the possible presence of the epidithiodiketopiperazine ring system. The easy loss of S_2 in the mass spectrometer supports the presence of this moiety.

Table 2. MICs of compound A30641 against different Gram-positive bacteria and fungi

Organism	MIC
Staphylococcus aureus 3055	3.12 μg/ml
Staphylococcus aureus 3074	0.78 〃
Erwinia amylovora	6.25 〃
Bordetella bronchiseptica 16	50 〃
Trichophyton mentagrophytes 27	25 〃
Aspergillus flavus E	25 〃
Ceratocystis ulmi	12.5 〃
Candida albicans	<0.312 μg/Disc
Cryptococcus neoformans	<0.156 μg/ml
Blastomyces dermatitidis	10 〃
Histoplasma capsulatum	10 〃

Assays were carried out by a standard agar dilution procedure except for the *C. albicans* assay, which was by a disc plate procedure.

Biological Activity

The *in vitro* antibacterial and antifungal activities of A30641 are summarized in Table 2. Unlike many dithiodiketopiperazine derivatives, A30641 has only marginal antiviral activity. Its LD_{50} (i.p.) in mice is 106 mg/kg.

Acknowledgements

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References

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