A NOVEL PHENAZINE ANTIFUNGAL ANTIBIOTIC, 1,6-DIHYDROXY-2-CHLOROPHENAZINE

FERMENTATION, ISOLATION, STRUCTURE AND BIOLOGICAL PROPERTIES

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A novel, solvent extractable, antibiotic complex has been purified from the fermentation broth of an unusual member of the genus *Streptosporangium*. Two of the major components were isolated from the complex by alumina column chromatography. One of the components was identified as a previously reported compound, 1,6-dihydroxyphenazine. The other component was a novel chlorine containing phenazine, 1,6-dihydroxy-2-chlorophenazine, which exhibited broad spectrum antifungal activity *in vitro* against dermatophytes and *Candida*.

In our continuing search for novel antibiotics from actinomycetes, a novel solvent extractable antibiotic complex has been obtained from the fermentation broth of an organism identified as a member of the genus *Streptosporangium*. The antibiotic producing organism was isolated from a soil sample collected in the Guatemalan jungle and has been deposited in the Schering Central Culture Collection under the accession number 1656. In addition to its antibiotic production, this organism has the ability to produce large amounts of iodinin[1], both on agar and in broth. Detailed taxonomic analysis will be presented in a forthcoming paper.

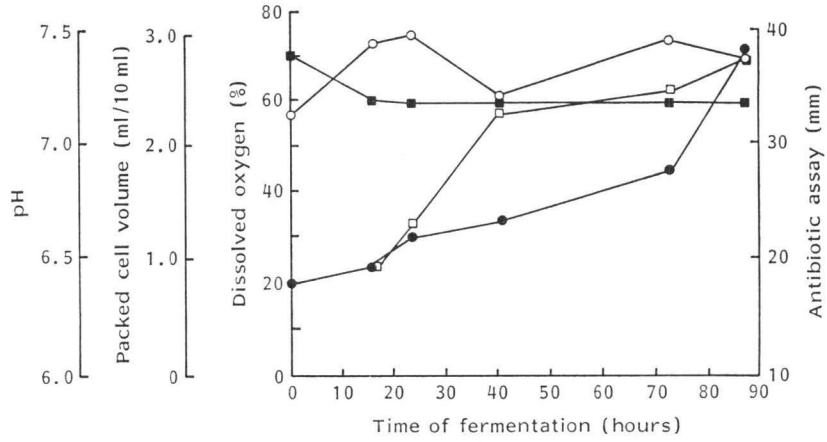
The present paper describes the fermentation, isolation, structure elucidation and biological properties of the two major components of this antibiotic complex.

Fermentation

The inoculum for antibiotic production was prepared in a medium containing yeast extract 0.5%, Cerelose 0.5%, dextrin 3%, corn steep flour 0.5% and cobalt chloride 0.024%. The pH of the medium was adjusted to 7.0 prior to sterilization. A 300-ml Erlenmeyer flask containing 50 ml of this medium was inoculated with 5 ml of a stock suspension of the producing strain which had been maintained at −20°C. The flask was incubated at 30°C, on a rotary shaker at 300 rpm, for 72 hours. The resulting seed culture was transferred to a 2-liter Erlenmeyer flask containing 500 ml of the above medium and incubated as above. The entire contents were used to inoculate a 14-liter New Brunswick Scientific laboratory fermentor containing 10 liters of the same medium.

The fermentation was carried out at 30°C with 10 liters of air per minute and agitation at 350 rpm. Total antibiotic activity was monitored at regular intervals by bioassay against *Candida albicans*. The pH, dissolved oxygen levels, and growth profile of the organism during fermentation were also monitored (Fig. 1).

Fig. 1. Time course study of the fermentation.
○ pH, ■ dissolved oxygen, ● packed cell volume, □ antibiotic assay.



Isolation

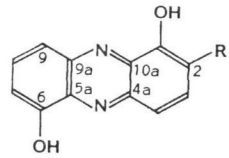
The whole broth from six, 10-liter, fermentations was extracted twice with equal volumes of ethyl acetate. The solvent layer was concentrated to an oil and precipitated with petroleum ether. The resulting reddish purple solid (2 g) was filtered and dried under vacuum. Silica gel thin-layer chromatography of the complex in benzene - methanol (9: 1) followed by bioautography against *C. albicans* resulted in four biologically active components. The components were labelled **1** through **4** based on decreasing Rf values.

Components **3** and **4**, the major components, were purified by column chromatography on neutral alumina utilizing chloroform - methanol - water (2: 2: 1, lower phase) as the eluting solvent. The active fractions were monitored by thin-layer chromatography on silica gel, using benzene - methanol (9: 1) as the developing system, followed by bioautography against *C. albicans*. The fractions containing either component **3** or **4** were combined and concentrated. Crystallization of both components **3** and **4** from methylene chloride yielded 504 mg of pale yellow crystals (component **3**) and 160 mg of reddish purple crystals (component **4**).

Structure Elucidation

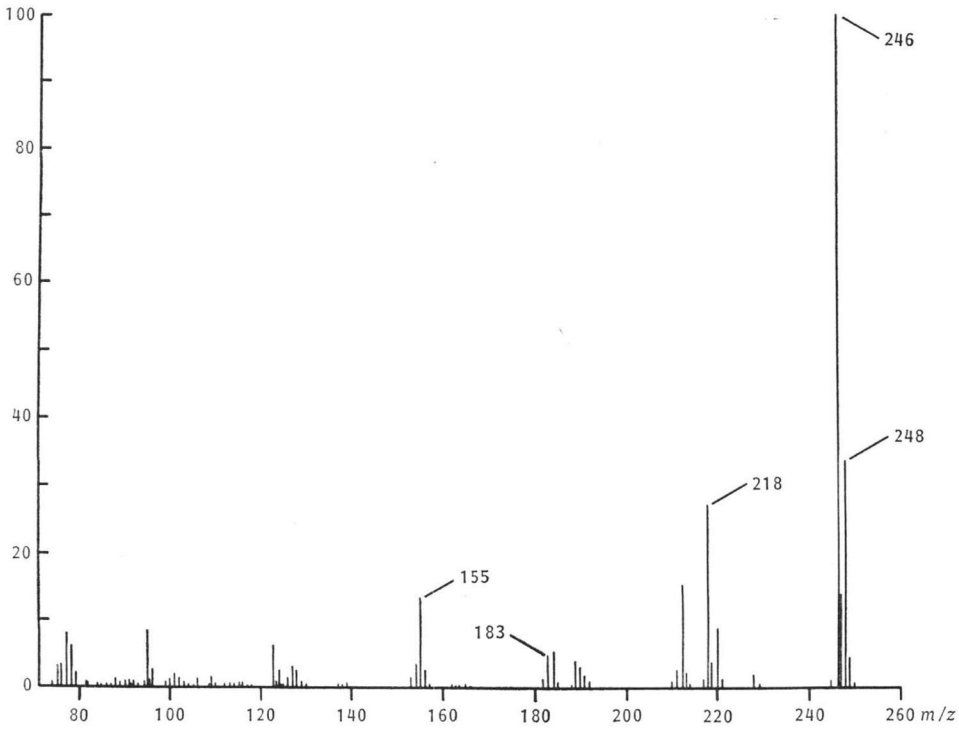
Component **3** (Fig. 2, R=H) was found to be identical to 1,6-dihydroxyphenazine[2] based on the following physico-chemical and spectroscopic properties: mp 245°C (dec); *Anal*, calcd for $C_{12}H_8N_2O_2$: C 67.91, H 3.80, N 13.21, found: C 67.78, H 3.84, N 13.13; EI-MS *m/z* 212.0591 (M, $C_{12}H_8N_2O_2$); UV λ_{max}^{MeOH} nm (ε) 272 (53,600), 373 (3,130), 442 (2,140); UV $\lambda_{max}^{0.1N\ NaOH}$ (ε) 262 (13,100), 291 (30,300); IR ν_{max}^{KBr} cm^{-1} 3400 (br, OH), 1640, 1530, 1522, 1485, 1440 (s, C=C, C=N), 1207 (s, phenolic OH), 804 (s, aromatic H); ^1H NMR (100 MHz, DMSO-d_6) δ 7.20 (2H, m, 2,7-H), 7.72~7.78 (4H, m, 3,4,8,9-H), 10.45 (2H, br s, phenolic OH's); ^{13}C NMR (25.2 MHz, DMSO-d_6) δ 110.5 (C-2,7), 119.2 (C-4,9), 131.2 (C-3,8), 135.7 (C-5a, 10a), 142.1 (C-4a, 9a), 153.3 (C-1,6).

Fig. 2. Structure of 1,6-dihydroxyphenazine (R=H) and 1,6-dihydroxy-2-chlorophenazine (R=Cl).



Component **4** (Fig. 2, R=Cl) was found to

Fig. 3. El-MS of 1,6-dihydroxy-2-chlorophenazine.



be a novel chlorine containing phenazine. The compound decomposed at 252°C and was soluble *in methanol*, ethanol, ethyl acetate and dimethyl sulfoxide, but insoluble in petroleum ether and water. The structure determination of this chlorine containing phenazine was based on the following chemical and spectroscopic data: *Anal*, calcd for $C_{12}H_7N_2O_2Cl$: C 58.53, H 2.87, N 11.38, Cl 14.21, found: C 58.42, H 2.99, N 11.46, Cl 12.58; EI-MS *m/z* 246.0203 (M, $C_{12}H_7N_2O_2Cl$), 218 (M−CO), 183 (M−CO−Cl), 155 (M−2CO−Cl) (Fig. 3); UV λ_{max}^{MeOH} (ε) 275 (71,300), 377 (4,950), 440 (3,400) (Fig. 4); UV $\lambda_{max}^{MeOH+0.1N NaOH}$ nm 265 (22,800), 296 (64,900) (Fig. 4); IR ν_{max}^{KBr} cm^{-1} 3400 (d, br, OH), 1635, 1610, 1555, 1525, 1485 (s, C=C, C=N), 1200 (s, phenolic OH), 805 (s, aromatic C-H), 750 (s, C-Cl) (Fig. 5); ^1H NMR (100 MHz, DMSO-d_6) 7.25 (1H, dd, J=6.0, 3.0, 7-H), 7.80~7.84 (4H, m, 3,4,8,9-H), 10.5 (2H, br s, phenolic OH's) (Fig. 6); ^{13}C NMR (25.2 MHz, DMSO-d_6) δ 110.9 (C-7), 115.6 (C-2), 118.8, 120.6 (C-9, 4), 132.1 (2C-3, 8), 135.1, 135.7 (C-5a, 10a), 140.6, 142.1 (C-4a, 9a), 148.9 (C-1), 153.6 (C-6) (Fig. 7). The UV spectrum clearly indicated that component **4** was a phenazine type compound.[3] The mass spectrum and chemical analysis showed that component **4** contained a chlorine atom. Analysis of the ^1H and ^{13}C NMR spectra could only be interpreted by placing the chlorine atom at position 2.

Fig. 4. UV spectra of 1,6-dihydroxy-2-chlorophenazine.

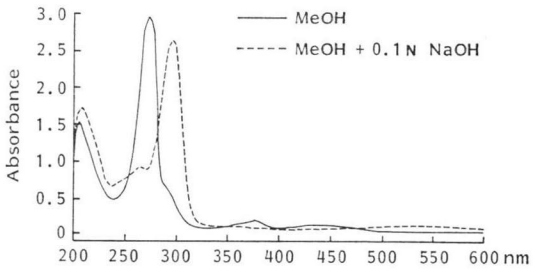


Fig. 5. IR spectrum (KBr) of 1,6-dihydroxy-2-chlorophenazine.

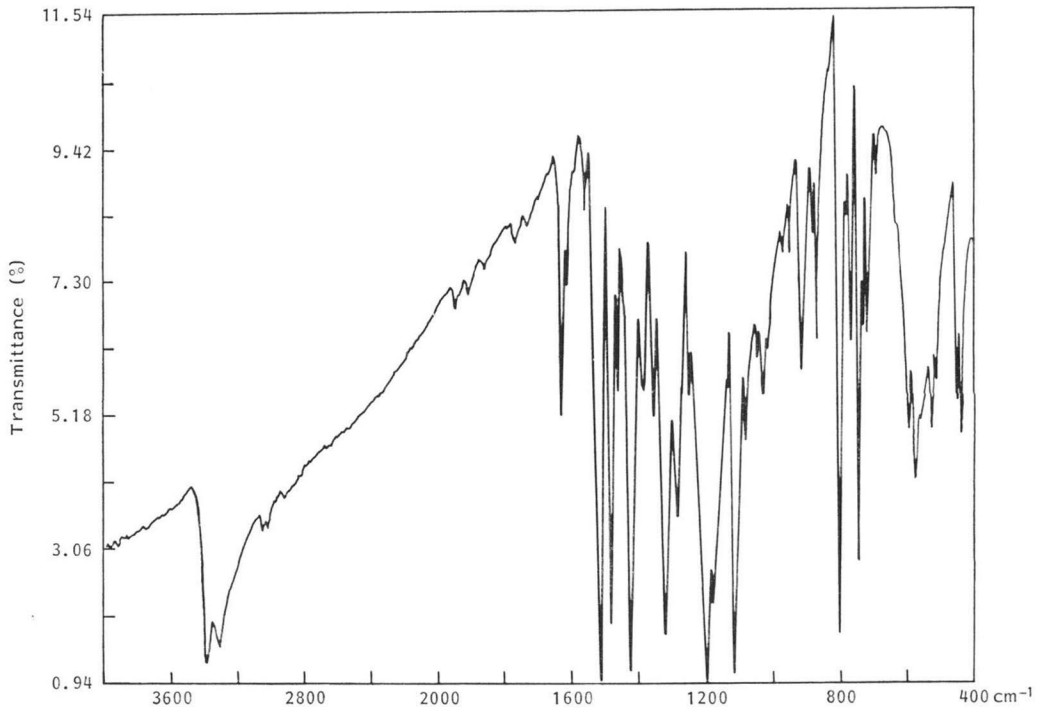
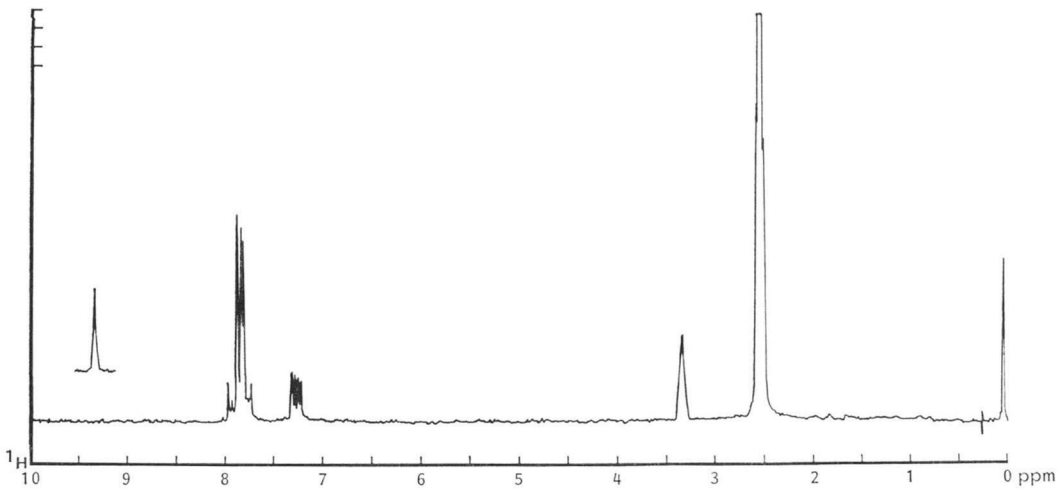


Fig. 6. ^{1}H NMR (100 MHz, DMSO-d_6) of 1,6-dihydroxy-2-chlorophenazine.



Biological Properties

Minimum inhibitory concentrations (MICs) were determined in Sabouraud dextrose broth pH 5.7, with and without 4% bovine serum albumin (BSA), with various yeasts, dermatophytes and opportunistic fungi. The results are shown in Table 1. Component **4** had broad spectrum antifungal activity with MIC's ranging from 0.25 ~ 8 μg/ml and was much more active than component **3**. In the presence of 4% BSA, component **4** was less active with MICs ranging from 4.0 ~ >16 μg/ml, indicating

Fig. 7. ^{13}C NMR (25.1 MHz, DMSO-d_6) of 1,6-dihydroxy-2-chlorophenazine.

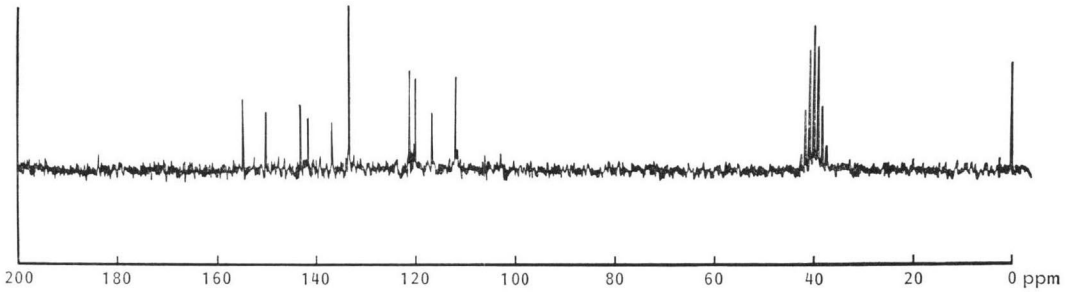


Table 1. *In vitro* microtiter MICs (μg/ml, 48 hours, SDB[a]) of components **3** and **4** against various fungi.

Organism	Component **3**		Component **4**	
	SDB	SDB+4% BSA[b]	SDB	SDB+4% BSA[b]
Candida albicans Burke C40	>16	>16	2	16
〃 〃 Collins C41	>16	>16	2	16
〃 〃 Sparks C42	>16	>16	4	16
〃 〃 Wisconsin C43	>16	>16	2	16
C. tropicalis C44	>16	>16	2	16
C. stellatoidea C45	16	16	0.25	4
C. parapsilosis C53	>16	>16	2	>16
Rhodotorula rubra C47	8	16	0.5	16
Saccharomyces cerevisiae C51	>16	>16	4	>16
Torulopsis glabrata C49	>16	>16	4	>16
Trichophyton mentagrophytes D23	16	>16	0.5	—
T. rubrum D61	16	>16	2	8
Microsporum canis D18	>16	>16	4	16
M. gypseum D16	>16	>16	1	16
Epidermophyton floccosum D58	16	>16	0.25	8
Monosporium apiospermum D17	>16	>16	2	>16
Aspergillus niger ND19	16	>16	8	>16
Geotrichum candidum D15	>16	>16	1	8

[a] Sabouraud dextrose broth.
[b] 4% Bovine serum albumin approximates the concentration of albumin in whole serum.

Table 2. *In vivo* activity of component **4** against a vaginal *C. albicans* infection in hamsters (treatment as insert once daily for 8 days).

Compound	Dose (%)	Culture results (positive/total)			
		Before treatment	Day 2	Day 6	3 days post treatment
Component **4**	2.5	7/7	5/7	5/7	5/7
No treatment	—	7/7	6/7	6/7	6/7
Miconazole	0.25	7/7	6/7	0/7	0/7

high protein binding. There was no significant antibacterial activity found in components **3** and **4**.

The *in vivo* topical activity of component **4** was determined against a vaginal *C. albicans* infection in hamsters[4]. Treatment was as an intravaginal insert once daily for 8 days and the results are shown in Table 2. Component **4** was inactive in this model.

Intravenous LD_{50}s of both components determined in mice were greater than 100 mg/kg.

Acknowledgments

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