NEW AZASTEROIDAL ANTIFUNGAL ANTIBIOTICS FROM *GEOTRICHUM FLAVO-BRUNNEUM*

I. DISCOVERY AND FERMENTATION STUDIES

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Although *Geotrichum* species occur ubiquitously, antibiotic production by members of this genus has not previously been reported. The antibiotic complex designated A25822, consisting of one major and six minor structurally-related components active primarily against *Candida* and *Trichophyton*, represents a new family of naturally-occurring compounds. Approximately 90% of the antibiotic activity synthesized remained associated with the fungal cell mass, from which it was recovered by multiple methanolic extractions for quantitation. Antibiotic production was enhanced by tryptophan, iron, zinc, and high levels of dextrin.

The continuing search for more effective antibiotics led to the discovery of A25822, a complex of antifungal compounds active *in vivo* against *Candida* and *Trichophyton*[1]. The producing organism, classified taxonomically as *Geotrichum flavo-brunneum*, NRRL-3862, was isolated by a standard serial dilution technique from a soil sample obtained in the Grand Teton National Park region of Wyoming. The genus *Geotrichum* represents ubiquitous fungi of known importance to man because of their association with slime accumulations in streams and paper mills, sludge bulking in waste disposal systems, plant diseases, mycoses of animals, and human intestinal disorders[2]. Discovery of the A25822 antibiotic complex extends the importance of *Geotrichum* to include antibiotic biosynthesis, which has not previously been reported for this group of fungi.

A25822 is a complex of nitrogenous, polycarbocyclic, structurally related substances consisting of one major component, A25822B, and six minor components. A25822B is a crystalline compound with an empirical formula of $C_{28}H_{45}NO$ and a molecular weight of 411[3]. The antibiotic complex represents a novel family of metabolites, being the first example of naturally-occurring azasteroids[4].

This communication reports the discovery of A25822 and initial fermentation studies.

Culture

Fig. 1 shows a typical giant colony of the novel *G. flavo-brunneum* culture that produces the A25822 antibiotic complex. This colony was grown at 25°C on a casein-corn steep agar medium. Microscopic examination revealed a low-growing, loosely-matted network of translucent, funiculose hyphae with prominent septa. The culture exhibits the characteristic morphology of the genus *Geotrichum*, which is classified taxonomically as a member of the Fungi Imperfecti. Conidia are formed by segmentation of older mycelia into thick-walled reproduc-

tive cells of two types, arthrospores and intercalary chlamydospores.

The culture forms thin, soft, spreading colonies on a variety of solid media. Growth, never abundant, occurs over a temperature range of 20~34°C with a broad optimum at 25~30°C. While vegetative hyphae are uncolored or very lightly pigmented, a soluble brown pigment is frequently discernible in the agar medium beneath colonies.

Antimicrobial Spectrum

Growth inhibition of fungal organisms, including *Trichophyton mentagrophytes* and *Candida albicans*, was first discovered in filtrates of the fermentation broth and in methanolic extracts of the mycelia (Table 1).

Microbiological Assay

Microbiological assay of A25822 was accomplished by a conventional disc-plate agar diffusion procedure employing *Trichophyton mentagrophytes*, NIH-9129, as the test organism. The assay medium contained 0.15% beef extract, 0.6% peptone, 0.6% yeast extract, 0.4% N-Z-Case, 2.0% agar, and 4 mcg/ml tetracycline. Ten ml of inoculated medium was dispensed into 85 mm circular plates. Test samples were diluted in methanol-water (1 : 9) and measured against a freeze-dried broth standard containing predominantly factor B. This standard was later replaced by a crystalline factor B standard. Assay plates were incubated at 30°C for 64~72 hours.

Fig. 1. Giant colony of A25822-producing *Geotrichum flavo-brunneum* culture

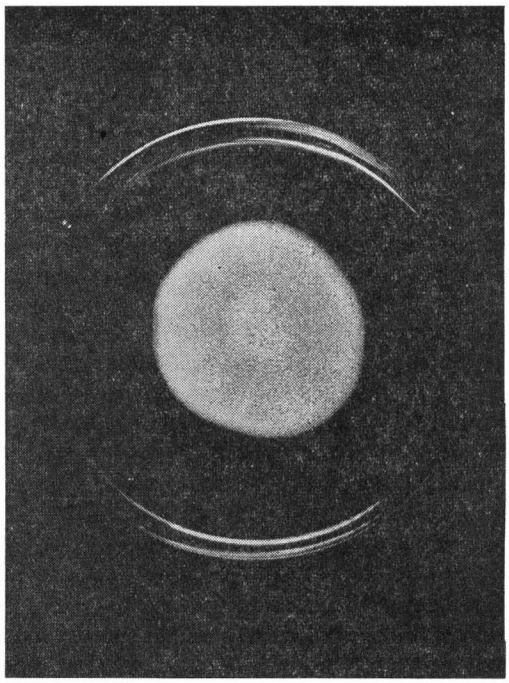


Table 1. *In vitro* antifungal agar diffusion spectrum of A25822 fermentation samples

Test organism	Zone diameter (mm)[a]	
	Filtered broth	Mycelial extract[b]
Tricophyton mentagrophytes	25	36
Candida albicans	17[c]	34[c]
Neurospora sp.	18	25
Saccharomyces pastorianus	14	30

[a] 7-mm assay discs
[b] Fermentation solids extracted with methanol, volume same as broth volume
[c] Partial inhibition

Quantitation of Antibiotic Activity in Fermentation Samples

Initial observations indicated that, while small quantities of antibiotic activity were elaborated directly into the fermentation broth, larger amounts could be extracted from the mycelial mass with acetone or methanol. Whole broth extracts were inefficient unless large volumes of methanol were employed, suggesting that relatively anhydrous methanol was required for efficient extraction. Comparatively low antibiotic titers initially prevented use of such a procedure. A single methanolic extraction of the mycelial mass consistently removed

substantial activity without requiring large amounts of methanol, but a sizeable percentage of activity still remained in the mycelia. Sonication, grinding, heating, acidifying or basifying the methanol-solids mixture did not increase the percentage of activity extracted. Therefore, antibiotic levels were determined by assaying the fermentation broth and 3 successive methanolic extracts of the mycelia. The titers reported are the sum of these 4 samples, representing at least 95% of the total antibiotic activity produced in the fermentation (Table 2).

Table 2. Quantitation of antibiotic activity in fermentation samples

Sample type	Antibiotic activity	
	μg/ml	Percent of total
Fermentation broth filtrate	3.5	7
Methanolic extraction of mycelia*		
1st extract	31.0	60
2nd extract	10.5	20
3rd extract	4.8	9
4th extract	2.0	4
Total	51.8	100

* Volume of methanol used was equal to original broth volume. Extracted at broth pH for 30 minutes with stirring.

Chromatography

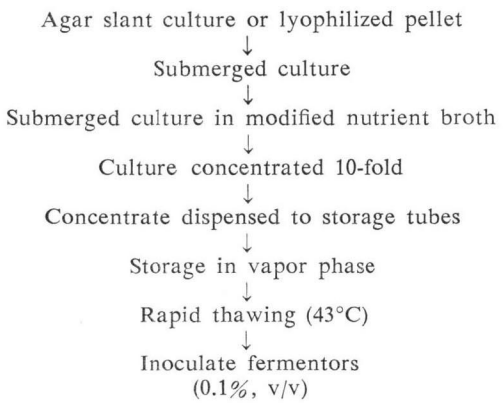
Fermentation samples were monitored qualitatively by both paper and thin-layer chromatography. Chromatograms on Whatmann #1 paper were developed in water-saturated methylisobutylketone - *p*-toluenesulfonic acid (98 : 2). Thin-layer silica gel plates were developed in ether-ethanol (3:1). *T. mentagrophytes* was used as the indicator organism for bioautograms.

Culture and Inoculum Preservation

Stock cultures of *G. flavo-brunneum* were maintained as lyophilized pellets. Cultures for lyophilization were incubated 7 days at 25°C on a medium containing 2.5% sucrose, 3.5% blackstrap molasses, 1% malt extract, 1% N-Z-Case (Sheffield Chemical), 0.6% corn steep liquor, 0.2% K_2HPO_4, and 2.5% agar.

The first stage of fermentor inoculum was grown in a medium containing 4% glucose, 2% dextrin, 1.5% blackstrap molasses, 1.5% cottonseed flour, 1% Peptone 159 (Wilson's Protein Technology), 0.04% NZ Amine A (Sheffield Chemical), 0.05% $MgSO_4 \cdot 7H_2O$, and 0.2% $CaCO_3$. Wide-mouth 250 ml Erlenmeyer flasks, containing 50 ml of inoculated media, were incubated 48 hours at 25°C on a shaker rotating in a 5-cm diameter circle at 250 rpm. The resulting culture was used either (1) to provide a 1% (v/v) inoculum directly to fermentors or (2) was transferred to a nutrient medium containing 0.1% glucose, 0.5% peptone, 0.15% yeast extract, 0.15% beef extract, 0.35% NaCl, 0.37% K_2HPO_4, and 0.13% KH_2PO_4. After 48-hour incubation in the second medium, the mycelial mass was concentrated into a glycerol-lactose solution and stored in the vapor phase of liquid nitrogen[5]

Fig. 2. Liquid nitrogen preservation of *Geotrichum flavo-brunneum* fermentor inoculum



as an alternate source of fermentor inoculum (Fig. 2).

Fermentation Studies

All fermentation studies were conducted at 25°C, either in wide-mouth 250 ml Erlenmeyer flasks or, when so indicated, in stirred and aerated fermentation vessels. Flask fermentors contained 50 ml of media and were incubated on a shaker rotating in a 5-cm diameter circle at 250 rpm. Stirred fermentation vessels were of conventional design, employing two 6-bladed turbine impellers and a 1 : 1 height-diameter ratio for the 25 liters of medium. The initial medium and 3 higher-yielding media are shown in Table 3. Increased titers of antibiotic were

Table 3. Fermentation media employed for production of A25822

Medium component	Medium			
	A	B	C	D
Glucose	2.5*	4.0		
Maltose			6.0	4.0
Corn starch	1.0			
Dextrin 700 (A. E. Staley)		2.0		
Stadex 50 (A.E. Staley)			8.0	12.0
Peptone 159 (Wilson's Protein Technology)	1.0	1.0	1.0	1.0
Cottonseed flour		1.5	1.5	1.5
NZ-Amine A (Sheffield Chemical)	0.4	0.4		
Molasses, blackstrap	0.5	1.5	1.5	1.5
$MgSO_4 \cdot 7H_2O$	0.5	0.5	0.5	0.5
$CaCO_3$	0.2	0.2	0.5	0.5
$FeSO_4 \cdot 7H_2O$				0.2
$ZnSO_4 \cdot 7H_2O$				0.002
L-Tryptophan				0.204
Fermentation time (days)	3	4	7	9
Antibiotic titers (μg/ml)				
Flask fermentors	30	60	300	690
Tank fermentors	45	70	200	490

* Expressed as percent.

obtained through the use of different carbohydrates; increasing the carbohydrate portion of the medium from 3.5% to 16%; inclusion of cottonseed flour and deletion of NZ Amine A; a higher level of $CaCO_3$; and the addition of iron, zinc, and tryptophan. As these modifications were made, the optimum fermentation time was extended from 3 to 9 days. Antibiotic yields from medium A were 50% higher in stirred vessels than in flask fermentors. However, as nutritional studies progressed in shaken flasks this situation was gradually reversed, with medium D being 40% more productive in flasks than in stirred vessels.

Effect of Various Carbohydrates

In order to determine the effect of various carbohydrates on the fermentation, a number of sugars were tested individually in a basal medium from which the standard carbohydrate

was omitted. Growth and antibiotic titers obtained with galactose and the disaccharides sucrose and lactose were equivalent to the control from which carbohydrate had been omitted (Table 4). Glucose increased titers about six-fold. Compounds containing multiple glucose units were superior to glucose, with certain glucose polymers apparently being preferred. Further testing of glucose, maltose, and Stadex 50 substantiated this observation (Table 5). Even low levels of Stadex 50 produced higher antibiotic titers than comparable levels of glucose. This is in contrast to some fermentations where dextrins are helpful only when incorporated into the medium at high levels[6], probably because they exert little or no effect on feedback regulation. Glycerol and oils were utilized for growth as well as, or better than,

Table 4. Effect of various carbohydrates on growth and biosynthesis of A25822 by *Geotrichum flavo-brunneum* in Medium C*

Carbohydrate tested**	Terminal pH	Growth (% Solids)	A25822 Titer (μg/ml)
—	8.5	7	18
Glucose	6.6	15	120
Fructose	6.7	17	105
Galactose	7.7	7	20
Mannose	6.6	15	170
Sorbitol	8.2	8	50
Maltose	7.6	20	195
Sucrose	8.5	7	19
Lactose	8.4	6	17
Corn starch	7.6	30	120
Stadex 11	6.8	17	245
Stadex 50	6.7	23	300
Dextrin 700	7.0	28	180

* See Table 3. Maltose and Stadex 50 were omitted from medium C.

** Initial concentration of all carbohydrates was 8%.

Table 5. Comparative effects of glucose, maltose, and Stadex 50 on biosynthesis of A25822 in Medium C*

Carbohydrate level (%)	A25822 Titer (μg/ml)		
	Glucose	Maltose	Stadex 50
2	34	47	55
4	42	90	95
6	105	160	185
8	120	195	250
10	125	240	320
12		270	410
14		285	490
16			585
18			660
20			640

* See Table 3. Stadex and maltose were not included in the basal medium.

Table 6. Effect of glycerol and oils on biosynthesis of A25822 by *Geotrichum flavo-brunneum* in medium C[a]

Substrate tested[b]	Medium 1[c]			Medium 2[d]		
	Terminal pH	Growth (% Solids)	A25822 Titer (μg/ml)	Terminal pH	Growth (% Solids)	A25822 Titer (μg/ml)
Glycerol	7.8	10	90			
Corn oil	7.4	15	63			
Cottonseed oil	7.3	18	173	6.4	52	425
Lard oil	7.6	21	210	6.4	60	435
Peanut oil	7.5	19	155			
Refined soybean oil	7.1	21	95	6.3	53	405

[a] See Table 3. Maltose and Stadex 50 were omitted from medium C.

[b] Initial concentration was 4% (v/v).

[c] Contained no carbohydrate.

[d] Contained 10% Stadex 50. Control titers were 350 μg/ml.

glucose. In addition, cottonseed oil, lard oil, and peanut oil produced higher antibiotic titers than glucose (Table 6). However, when examined in combination with Stadex 50, oils did not produce higher titers than an equivalent increase in the level of dextrin.

Incubation Temperature

Antibiotic biosynthesis occurred over the entire temperature range examined, 20~33°C (Table 7). The highest titers were produced in the lower portion of this range, however, with the optimum being 25~27°C.

Mineral Additions

Phosphate and numerous monovalent and divalent cation salts were examined in a modification of medium D. Only iron and zinc were stimulatory, the former increasing antibiotic titers by 10% and the latter by 7%. The same levels of these salts were most effective in combination, their effects apparently being additive (Table 8), producing an 18% stimulation.

Table 7. Effect of incubation temperature on production of A25822 in medium C*

Incubation temperature (°C)	A25822 Titer** (μg/ml)
20	130
25	195
27	200
28	175
29	140
31	115
33	90

* See Table 3.
** Stirred fermentor vessels.

Table 8. Effect of iron and zinc on production of A25822

Mineral addition		Terminal pH	Growth (% Solids)	A25822 Titer (μg/ml)
$FeSO_4$*	$ZnSO_4$*			
—	—	6.6	36	425**
0.2	—	6.7	34	520
—	0.002	6.7	32	505
0.2	0.002	6.5	37	560

* Heptahydrate.
** Medium D minus iron, zinc, and tryptophan. See Table 3.

Table 9. Effect of tryptophan on biosynthesis of A25822

Amino acid addition (Molarity)	A25822 Titer (μg/ml)
	425*
L-Tryptophan, .01	690
DL-Tryptophan, .01	580
DL-Tryptophan, .02	510
D-Tryptophan, .01	485

* Medium D minus iron, zinc, and tryptophan. See Table 3.

Table 10. Effect of cholesterol and cholesterol precursors on biosynthesis of A25822

Medium addition (0.01 M)	A25822 Titer (μg/ml)
	425*
Acetate	365
Iso-valeric acid	505
Squalene	540
Cholesterol	380

* Medium D minus iron, zinc, and tryptophan. See Table 3.

Effect of Amino Acids

The L-isomers of common amino acids were tested, at a level of 1×10^{-2} M, as supplements to the same modification of medium D. A positive effect on antibiotic production was observed with only one, tryptophan (Table 9). The D-isomer and the racemic form of tryptophan were also stimulatory but to a lesser extent than the L-isomer.

Sterol Precursors

Although cholesterol is present in all animal cells and is synthesized by virtually all tissues, sterols have been detected in only one species of true bacteria and in a few species of

PPLO[7] and in yeast. Acetate has long been recognized as the principal precursor of cholesterol in tissues, and squalene is known to undergo cyclization to lanosterol, a precursor of ergosterol in *Saccharomyces cerevisiae*[8]. Acetate, iso-valeric acid, squalene, and cholesterol were, therefore, examined as additions to modified medium D. Acetate and cholesterol depressed antibiotic titers but iso-valeric acid and squalene increased titers, by 19% and 27%, respectively (see Table 10). These results suggest that known precursors of sterol biosynthesis may also be precursors of A25822 in *G. flavo-brunneum*, although the results reported here require verification and expansion, preferrably in a chemically-defined medium.

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