NEW AZASTEROIDAL ANTIFUNGAL ANTIBIOTICS FROM *GEOTRICHUM FLAVO-BRUNNEUM*

III. BIOLOGICAL ACTIVITY

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The A25822 antibiotic complex consists of seven biologically active factors. A comparative study of these factors determined that factor B possessed the greatest antifungal activity. The minimal inhibitory concentration of A25822B against isolates of *Candida albicans* was $<0.312\sim5.0\,\mu g/ml$, *Trichophyton mentagrophytes* was inhibited at $<0.0312\,\mu g/ml$. Other pathogenic fungi such as *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Sporotrichum schenckii*, and *Microsporium gypseum* were very susceptible to A25822B. Only limited antibacterial activity of A25822B was found. Parenteral or oral administration of 50 mg/kg of A25822B significantly extended the average survival time of mice infected with *C. albicans*. Doses of 20 mg/kg of A25822B caused a greater than ten-fold reduction in the number of *Candida* cells recovered from kidneys of infected mice. A solution of 0.5% or 0.25% A25822B applied topically was effective against an experimental dermatophyte infection on guinea pigs. A peak blood level of $3\,\mu g/ml$ was achieved in mice following a 100 mg/kg dose of A25822B. Combination of A25822B with a polyene antibiotic *in vitro* showed antagonism.

Azasteroids have been reported to produce a diversity of effects in biological systems. Perhaps the greatest emphasis has been in antifertility where azasteroids reduced the synthesis of cholesterol derived androgenic and estrogenic hormones[1]. Alterations in cholesterol synthesis by azasteroids have also been reported in chickens and insects[2,3]. Other investigators have reported the antineoplastic action of a homo-aza-steroidal ester which was less toxic than nitrogen mustard[4]. A reduction in transport of macromolecular precursors with analogues of azasteroids has also been reported[5]. Tomatine is a naturally occurring azasteroid with antifungal activity isolated from tomato plants[6]. Antibacterial activity of nitrogen-containing steroids was reported; this antibacterial action may be directed at the membrane-transport sites[7,8,9]. The chemical structure of A25822B and the isolation and purification of the A25822 antibiotic complex have been reported[10,11]. The antifungal activity of the A25822 complex is reviewed in this report.

Materials and Methods

In Vitro Studies.

Fungal cultures were maintained on SABOURAUD's dextrose agar as described by EMMONS[12]. Susceptibility studies using *Candida albicans* were carried out in filter sterilized Yeast Nitrogen Base (Difco) plus dextrose (10 g/liter) and asparagine (1.5 g/liter). *Histoplasma capsulatum* and *Blastomyces dermatitidis* were tested in SABOURAUD's dextrose agar and incubated at 37°C. All other fungal cultures were incubated at 30°C. Minimal inhibitory concentrations (MIC) of A25822 were determined using disc-plate and agar-dilution techniques described previously[13].

Bacterial cultures were incubated at 30°C, maintained on peptone (30 g), NZ Case (20 g), yeast extract (30 g), beef extract (7.5 g), agar (100 g), and water (1 liter). A25822B activity in mouse blood and urine was determined by disc-diffusion technique using *Trichophyton mentagrophytes* #6 as the assay organism[14].

In Vivo Studies.

A25822 was evaluated in mice infected intravenously with 0.1 ml of *C. albicans* A-26 as previously described[15,16]. Mice were X-irradiated with 400 r 24 hours prior to infection with 1.5×10^6 cells. Antibiotics were suspended in 0.125% methylcellulose (Dow Chemical Company) and administered intraperitoneally, subcutaneously, or orally in 0.25-ml aliquots. At each drug concentration, six infected and two uninfected toxicity control mice were treated at 0 and 2 hours post-infection. Supplemental treatments were administered at 24-hour intervals. After 7 days post-infection, the average survival time of treated mice was compared with untreated controls; surviving mice were arbitrarily given a survival time of 8 days. The significance of treatment administered to infected mice was determined by the *t* test and *p* values. The virulence of each infective dose of *C. albicans* was estimated by calculating the number of LD_{50}'s administered.

The effectiveness of A25822B in reducing the number of *Candida* cells in mice was determined in unirradiated mice infected intravenously with 10^5 cells[17]. Following five daily treatments, plate counts of kidney homogenates from A25822B or amphotericin B treated mice were compared

The effectiveness of topically applied A25822B was evaluated on guinea pigs superficially infected with 10^7 spores of *T. mentagrophytes* 1287. Commencing three days post-infection, lesions were treated twice daily for seven days with A25822B, tolnaftate, or a placebo in polyethylene glycol 300. At designated time intervals, the severity of lesions was scored subjectively on a basis of plus 1 to plus 4[18]. In addition, lesion tissue was cultured on Sabouraud's agar for seven days, and the number of cultures showing visible growth was determined microscopically.

Results and Discussion

The A25822 complex is composed of at least seven biologically-active factors (Table 1). Factors H and L demonstrated the greatest *in vitro* anti-*Candida* activity at 0.625 μg/disc but were inactive *in vivo* in the described tests. Factors A and B were most active against *T.*

Table 1. Antifungal activity of A25822

A25822 Factor	Lowest concentration for a zone (μg/disc) *Candida*/*Trichophyton*	Subcutaneous dose (mg/kg) × 2	Percent extension of survivors beyond controls infected with *Candida albicans*
A	5.0 / 0.0312	25 12.5 6.25	75 (P 0.0005) 58 (P 0.01) 0
B	2.5 /<0.078	12.5 6.25 3.12	97 (P 0.005) 40
D	10.0 / 0.05	50 25	64 (P 0.005) 9
H	0.625/ 0.156	100 50	0 3
L	0.625/ 0.156	100 50	0 0
M	1.0 / 0.25	25 12.5	41 (P 0.025) 24
N	2.5 / 0.312	50 25	43 17

mentagrophytes at 0.0312 and <0.078 μg/disc, respectively. All factors were in the base form except Factor B which was evaluated as the soluble hydrochloride salt. Survival time of infected animals treated with 6.25 mg/kg of Factor B was extended 40% beyond untreated control animals. The lowest level of Factor A demonstrating significant *in vivo* anti-*Candida* activity was 12.5 mg/kg with 58% extension of survival. The LD_{50} of A25822B for mice following a single subcutaneous dose was 74 mg/kg. The *in vitro* and *in vivo* activity of Factors D, M and N were not comparable to that of Factors A or B. Isolates of *T. mentagrophytes*

Table 2. *In vitro* antifungal activity of A25822B

Organism	Broth dilution MIC values (μg/ml) for five isolates each
*Candida albicans**	<0.312~5.0
*Cryptococcus neoformans**	<0.625~2.5
*Trichophyton mentagrophytes**	<0.0312
Histoplasma capsulatum	<0.312~0.625
Blastomyces dermatitidis	0.312~1.25
*Microsporium gypseum**	0.625~1.25
Sporotrichum schenckii	<0.312~1.25

* The *Candida*, *Cryptococcus*, *Trichophyton*, and *Microsporium* isolates were grown at 30°C; all others were incubated at 37°C.

Table 3. *In vitro* antibacterial activity of A25822B

Organism	Agar-dilution MIC (μg/ml)
Staphylococcus aureus	25.0
Streptococcus faecalis	50.0
Klebsiella-Aerobacter sp.	50.0
Pasteurella multocida	<10.0
Erwinia amylovora	6.25
Xanthomonas phaseoli	50.0
Streptococcus faecalis	>100
Salmonella typhosa	>100
Salmonella typhimurium	>100
Proteus sp.	>100
Escherichia coli	>100
Pseudomonas aeruginosa	>100
Pseudomonas solanacaerum	>100

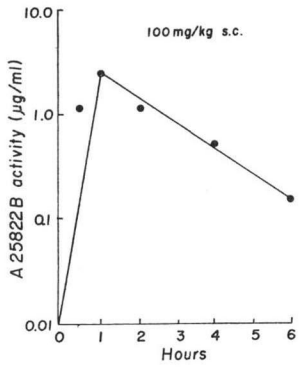
Table 4. Comparison of routes of administration for A25822B

Route	Dose (mg/kg×2)	Percent increase in survivors beyond controls infected with *C. albicans*
Orally	100	64 (P 0.025)
	50	64 (P 0.025)
	25	55 (P 0.1)
	12.5	0
Intraperitoneally	50	68 (P 0.05)
	25	24 (P 0.1)
	12.5	32 (P 0.05)
	6.25	26 (P 0.05)
	3.12	3 (P <0,40)

Table 5. The effect of A25822B on recovery of *Candida* from mice

Antibiotic	Dose SC twice daily (mg/kg)	*Candida* recovered per gram kidney
None	—	8.5×10^5
A25822B	42×3 days	5.2×10^4
	21×3 days	5.8×10^4
Amphotericin B	42×3 days	2.7×10^4

Fig. 1. Antifungal activity of A25822 factor B in mouse blood



were inhibited by $<0.0312\,\mu g/ml$ of Factor B, while *Microsporium gypseum* isolates were susceptible to Factor B at $<0.312\sim5.0\,\mu g/ml$. Only limited gram-positive and gram-negative antibacterial activity was found with A25822B (Table 3).

A25822B was active against *C. albicans* by intraperitoneal or oral administration; greater activity was achieved with lower doses administered intraperitoneally (Table 4).

Compound A25822B was effective in reducing the number of *Candida* cells recovered from kidney homogenates of infected mice (Table 5). Doses of 42 and 21 mg/kg given in two daily doses for 3 days reduced the number of *Candida* cells recovered by one log when compared with untreated control animals. Amphotericin B at the 42 mg/kg dose was two-fold more effective than A25822B in this test.

A single 100 mg/kg subcutaneous dose produced a peak of $3\,\mu g/ml$ of A25822B activity at 1 hour in mouse blood (Fig. 1). This level exceeded the MIC for most fungal pathogens. After 6 hours, $0.3\,\mu g/ml$ of A25822B activity was detected. Ninety minutes after a single 100 mg/kg subcutaneous dose, $8\,\mu g/ml$ of A25822B activity was detected in urine.

Fig. 2. Effect of topical treatments of A25822B on superficial dermatophyte infections in guinea pigs

The height of each bar represents the severity of the dermatophyte infection which was judged on a subjective basis from +1 to +4. The numbers above each column represent culture scores; the denominator is the total number of lesion samples cultured, while the numerator represents the number of lesion samples showing growth.

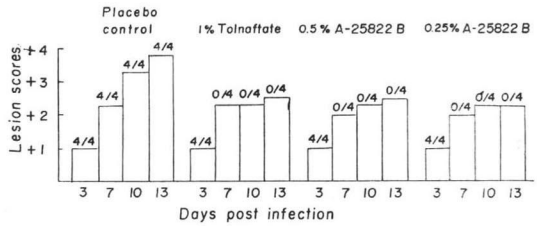
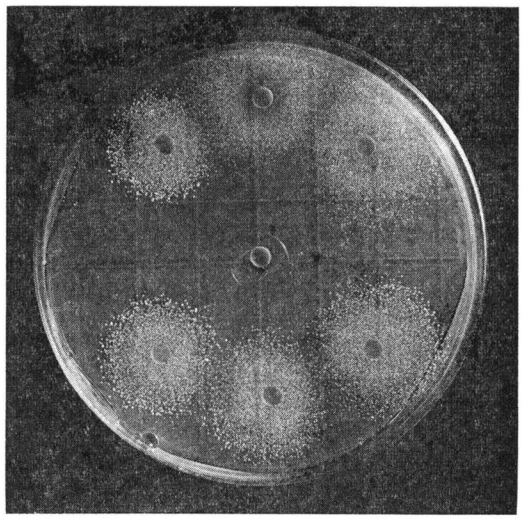


Fig. 3. *In vitro* antagonism of A25822B and pimaricin

SABOURAUD's medium was inoculated with *C. albicans* and contained pimaricin at an inhibitory concentration ($10\,\mu g/ml$). Paper discs saturated with A25822B ($20\sim0.625\,\mu g/disc$) were placed on the agar containing pimaricin with the highest concentration of A25822B in the top center disc with two-fold dilutions placed clockwise around the plate. The presence of both antibiotics in the medium resulted in *zones of growth*



Topical application of A25822B was effective in treating superficial dermatophyte infections in guinea pigs (Fig. 2). The severity of the *T. mentagrophytes* lesions was significantly reduced by A25822B at 0.5 and 0.25% when compared with placebo controls and tolnaftate. After initial isolations from lesion tissue confirmed an established infection, all subsequent post-

treatment culture isolations were negative in antibiotic treated animals. Placebo treated controls had positive culture isolations throughout the experiment.

In vitro combination studies with A25822B and polyene antibiotics revealed an antagonism. Petri plates were prepared with SABOURAUD's medium inoculated with *C. albicans* and containing an inhibitory concentration of 10 μg/ml of the polyene antibiotic pimaricin. Saturated paper discs of A25822B also at inhibitory levels of 20~0.625 μg/disc were placed on the agar surface. Zones of enhanced growth appeared around each disc containing A25822B (Fig. 3). A small zone of inhibition was observed around the disc containing 20 μg with growth occurring at lower antibiotic concentrations away from the disc. *C. albicans* was completely inhibited by pimaricin around the center solvent control disc containing water. Polyenes are believed to disrupt fungal membrane integrity and are used to enhance other antimicrobial compounds which have difficulty in permeating the membrane barrier[18]. An antagonistic relationship between polyenes and A25822B could indicate an interaction between the antibiotic either in solution or at the active site on the cell membrane.

A25822B is a new antifungal antibiotic that is deserving of further evaluation to define its role as a clinical chemotherapeutic agent.

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References

1) GAIND, B. & V. S. MATHUR: Antifertility effects of rats of some compounds related to azasteroids. J. Reprod. Fert. 27: 459~460, 1971
2) SINGH, R. A.: Effect of azasteroids on sterol metabolism in the laying hen. Poult. Sci. 51: 449~456, 1972
3) SVOBODA, J. A. & W. E. ROBBINS: The inhibitive effects of azasteroids on sterol metabolism and growth and development in insects with particular reference to the tobacco hornworm. Lipids 6: 113~119, 1971
4) CATSOULACOS, P. & L. BOUTIS: Antitumor activity of a homo-azasteroidal ester of [*p*-[bis(2-chloroethyl)amino]phenyl]acetic acid (NSC-71964). Cancer Chemother. Rep. 57: 365~367, 1973
5) HIGGINS, M. L.; R. W. CHESNUT, F. R. LEACH, J. G. MORGAN, K. D. BERLIN & N. N. DURHAM: Effect of 15-azasteroid analogues on cell culture growth. Steroids 19: 301~314, 1972
6) FONTAINE, T. D.; G. W. IRVING, Jr., R. MA, J. B. POOLE & S. P. DOOLITTLE: Isolation and partial characterization of crystalline tomatine, an antibiotic agent from the tomato plant. Arch. Biochem. 18: 467~475, 1948
7) CHESNUT, R. W.; D. F. HASLAM, K. D. BERLIN, J. MORGAN & N. N. DURHAM: Enhanced antibacterial activity with a new class of azasteroids and selected antibiotics. Bact. Proc. p. 7, 1971
8) SMITH, R. F.; D. E. SHAY & N. J. DOORENBOS: Antimicrobial action of nitrogen-containing steroids. J. Bact. 85: 1295~1299, 1963
9) SMITH, R. F. & D. E. SHAY: Steroidal lysis of protoplasts and effects of stabilizers and steroid antagonists. Appl. Microbiol 13: 706~712, 1965
10) MICHEL, K. H.; R. L. HAMILL, S. H. LARSEN & R. H. WILLIAMS: New azasteroidal antifungal antibiotics from *Geotrichum flavobrunneum*. II. Isolation and Characterization. J. Antibiotics 28: 102~111, 1975
11) CHAMBERLIN, J. W.; M. O. CHANEY, S. CHEN, P. V. DEMARCO, N. D. JONES & J. L. OCCOLOWITZ: Structure of antibiotic A25822B, a novel nitrogen containing C_{23}-sterol with antifungal properties. J. Antibiotics 27: 992~993, 1974
12) EMMONS, C. W.; C. H. BINFORD & J. P. UTZ: "Medical Mycology". Lea and Febiger, Philadelphia, 1963

13) GORDEE, R. S. & T. R. MATTHEWS: Evaluation of the *in vitro* and *in vivo* antifungal activity of pyrrolnitrin. Antimicr. Agents & Chemoth. -1967: 378~387, 1968
14) KAVANAGH, F.: *In* Analytical microbiology. Vol. 2. pp. 329~338, Academic Press, New York, 1972
15) GORDEE, R. S. & T. R. MATTHEWS: Evaluation of systemic antifungal agents in X-irradiated mice. Appl. Microbiol. 20: 624~629, 1970
16) GORDEE, R. S. & P. J. SIMPSON: Relationships of X-irradiation to the enhancement of *Candida albicans* infection. J. Bact. 94: 6~12, 1967
17) GORDEE, R. S. & T. R. MATTHEWS: Systemic antifungal activity of pyrrolnitrin. Appl. Microbiol. 17: 690~694, 1969
18) HSU CHEN, C. & D. S. FEINGOLD: Selective membrane toxicity of the polyene antibiotics: Studies on natural membranes. Antimicr. Agents & Chemoth. 4: 316~319, 1973