XYLOCANDIN: A NEW COMPLEX OF ANTIFUNGAL PEPTIDES

I. TAXONOMY, ISOLATION AND BIOLOGICAL ACTIVITY

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Xylocandin is a complex of novel peptides with potent antifungal activity that is produced by *Pseudomonas cepacia* ATCC 39277. The complex was isolated from the fermentation broth by extraction with butanol - methanol, 9 : 1, followed by collection of the precipitate formed upon concentration of the solvent extract. Purification was effected by chromatography on reversed phase and size exclusion gels followed by TLC on silica gel. These techniques afforded eight components: A_1, A_2, B_1, B_2, C_1, C_2, D_1 and D_2. A mixture of the two closely related components, xylocandins A_1 and A_2, displayed potent anticandidal and antidermatophytic activities *in vitro*. The activity was diminished by the presence of serum or vaginal washings. No antibacterial activity was demonstrable.

Two novel anticandidal antibiotic complexes were found in the course of screening bacteria for antifungal agents, these being catacandin and xylocandin. The former is an acyltetramic acid derivative and was reported recently in this journal[1]; the latter, xylocandin, is a complex of peptidic components and forms the subject of this report. Results of structural studies and structure modification studies are presented in the accompanying report[2].

Taxonomy

Xylocandin is produced by an organism isolated from a soil sample taken in Cranbury, New Jersey. The organism is a Gram-negative rod that is motile by means of polar, multitrichous flagella. It is oxidative and cytochrome positive. These characteristics serve to identify the organism as a pseudomonad.

A summary of the key characteristics and the carbohydrate utilization pattern for growth is shown in Table 1. These characteristics agree with the description for *Pseudomonas cepacia* given by BALLARD *et al.*[3]. Consequently, the xylocandin producing organism was classified as *P. cepacia* and deposited in the American Type Culture Collection, Rockville, Maryland, U.S.A., with the accession number ATCC 39277.

Production

Cultures of *P. cepacia* ATCC 39277 were maintained at −85°C as suspensions in deionized water containing 7% glycerol. When needed, a vial was thawed and used to inoculate agar slants composed of yeast extract 0.1%, NZ-amine A 0.2%, glucose 1% and agar 1.5%. The growth of an 18-hour culture incubated at 25°C was washed off the slants and used to inoculate germinator flasks containing medium of the following composition; yeast extract 0.4%, malt extract 1% and Cerelose 0.4%. The germinators were incubated 24 hours on a rotary shaker (300 rpm, 5 cm-stroke) at 25°C,

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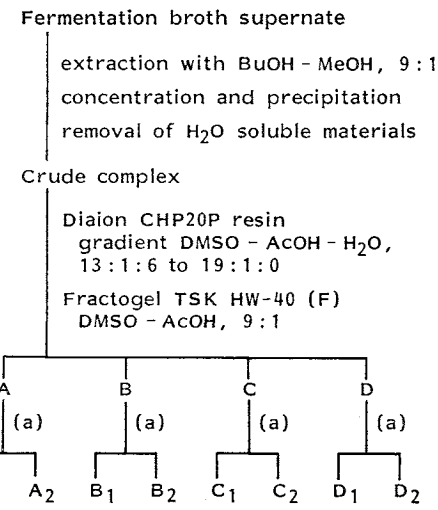
Table 1. Characteristics of *Pseudomonas cepacia* ATCC 39277.

Gram stain	Negative
Morphology	Rod with polar flagella, flagella number >1
Biochemical characteristics:	
Oxidative	Positive
Cytochrome oxidase	Positive
Catalase	Positive
Gelatinase	Positive
Lysine decarboxylase	Positive
Poly β-hydroxybutyrate accumulation	Positive
Arginine dihydrolase	Negative
Nitrate reduction	Negative
Diffusible pigment	Negative
Growth at 41°C	Positive
Growth at 4°C	Negative
Carbon utilization:	
Positive: Arabinose, ribose, xylose, glucose, fructose, sucrose, cellobiose, tryptamine, mannitol, sorbitol, salicin, acetate, citrate, threonine, putrescine	
Negative: D-Fucose, rhamnose, maltose, lactose, erythritol, D-tartrate	

and the growth then used to inoculate the next germinator stage. An inoculum level of 1.6% was used. This stage consisted of a 380-liter stainless steel vessel containing 250 liters of the same medium as just described, except for the addition of 0.05% Ucon LB-625 lubricant to control foaming. Growth was allowed to proceed at 25°C for 19 hours while maintaining an air flow of 280 liters/minute at 0.70 kg/cm² and an agitation rate of 155 rpm. The final fermentation stage was done in a 5,000-liter capacity, stainless steel vessel containing 3,000 liters of the same medium described for the previous step and with an air flow of 3,400 liters/minute at 0.70 kg/cm², and an agitation rate of 120 rpm. An inoculum of 1.6% was used. The fermentation was harvested after 20 hours at 25°C. Progress of the fermentation was monitored by paper-disc agar diffusion assay with *Candida albicans* SC5314 as the assay organism.

Fig. 1. Isolation of xylocandins.

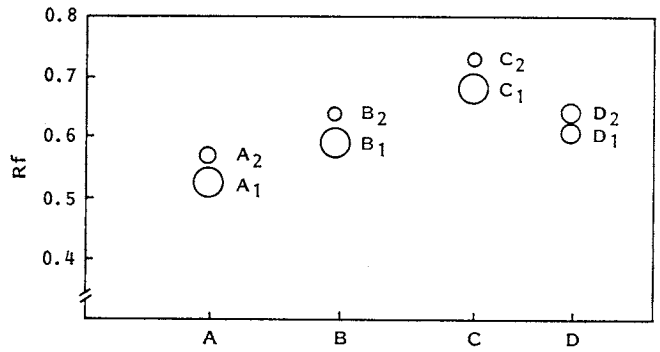


(a) Separation by TLC on silica gel with 2-PrOH - NH_4OH - H_2O (4 : 2 : 1).

Isolation Procedure

Xylocandin was isolated and purified by the procedures outlined in Fig. 1. The cell mass, obtained from the fermentation broth by centrifugation, was discarded because it lacked bioactivity. The supernate, 3,000 liters, was acidified to pH 3 by the addition of hydrochloric acid and then extracted twice with 1,500-liter portions of butanol - methanol, 9 : 1. The two solvent extracts were combined and the pool was then concentrated *in vacuo* to about 60 liters. The precipitate that formed during the concentration process contained the bioactivity, as shown by conventional agar diffusion assay. The concentrate and accompanying precipitate were stored at 5°C for 48 hours to allow the

Fig. 2. TLC[a] of the xylocandins.



[a] Merck Silica gel 60 F_{254}, 2-PrOH - NH_4OH - H_2O (4:2:1).

precipitation to proceed to completion. The precipitate was collected by filtration, washed thoroughly with acetone, and dried to afford 615 g of a light tan powder containing the xylocandin complex. The crude antibiotic complex was washed with water and then dissolved in dimethylformamide. Insoluble material, which proved to be inactive, was removed by filtration and the filtrate was concentrated *in vacuo* to yield the xylocandin complex as 30 g of a dark solid.

The complex was resolved into three major fractions A, B, C and a trace fraction, D, by column chromatography on Diaion CHP20P resin, eluting isocratically with DMSO - acetic acid - water, 13:1:6, followed by a linear gradient starting with this solvent mixture and ending with DMSO - acetic acid, 19:1. The appropriate fractions were combined and concentrated to small volumes. Rapid mixing of these concentrated solutions with acetonitrile or ethyl acetate resulted in the precipitation of the xylocandins as off-white solids. Traces of inactive, pigmented material were removed by chromatography on Fractogel TSK HW-40 (F), eluting with DMSO - acetic acid, 9:1. Combination and concentration of the appropriate fractions *in vacuo* and precipitation with ethyl acetate or acetonitrile afforded each of the xylocandins as white solids.

Xylocandins A, B, C and D were each further resolved into two closely related constituents, *i.e.*, A_1 and A_2, B_1 and B_2, C_1 and C_2, and D_1 and D_2 by TLC on silica gel (Merck Silica gel 60 F_{254}), eluting with 2-propanol - conc ammonia - water, 4:2:1. Visualization of the components was accomplished either by charring after spraying with a solution containing 5% ammonium molybdate and 0.1% ceric ammonium sulfate in 10% sulfuric acid or by spraying with water which results in white, "anti-wetting" zones. TLC of the four fractions is depicted in Fig. 2.

Biological Properties

The antifungal activity of the four antibiotic pairs: A, B, C and D, was determined by conventional agar dilution assay. Xylocandin A was found to be the most potent, having activity against a panel of *Candida* strains equivalent to that of amphotericin B. The others, xylocandins B, C and D were about 20 to 60-fold less active than A, but nonetheless, still exhibited good anticandidal activity (Table 2).

Xylocandin A also proved to be highly active against a panel of dermatophytes, with activity equivalent to that of clotrimazole (Table 3). However, no significant antibacterial activity was detected (MIC >100 μg/ml) when xylocandin A was assayed against a panel composed of Gram-positive and Gram-negative bacteria, *i.e.*: *Staphylococcus aureus*, *Streptococcus faecalis*, *Micrococcus luteus*,

Table 2. Anticandidal activity *in vitro*.

Organism	SC No.[a]	MIC (μg/ml)				
		Xylocandin				Amphotericin B
		A[b]	B[b]	C[b]	D[b]	
Candida albicans	5314	0.4	3.1	6.3	3.1	0.4
C. albicans	9177	0.2	3.1	6.3	6.3	0.4
C. albicans	11422	0.4	6.3	6.3	6.3	0.4
C. albicans	10580	0.2	3.1	6.3	6.3	0.4
C. albicans (bacilysin^R)[c]	12734	0.2	3.1	6.3	3.1	0.4
C. tropicalis (amphotericin B^R)[c]	9861	0.2	25.0	12.5	6.3	>100
C. tropicalis	10597	0.05	3.1	3.1	3.1	0.4
C. krusei	2968	0.4	6.3	6.3	6.3	0.8
C. parakrusei	2621	0.4	25.0	6.3	6.3	0.8
C. parakrusei	2966	0.4	6.3	6.3	6.3	0.4
C. pseudotropicalis	11241	0.1	6.3	6.3	3.1	0.4
C. guilliermondii	2210	0.4	6.3	6.3	6.3	0.8
C. stellatoidea	2211	0.4	6.3	6.3	6.3	0.8
C. glabrata	9342	0.1	6.3	6.3	6.3	0.8
C. glabrata	11267	0.2	6.3	6.3	6.3	0.8

[a] Squibb culture.
[b] Fractions A, B, C and D are each two-component mixtures.
[c] Bacilysin^R; bacilysin-resistant, amphotericin B^R; amphotericin B-resistant.

Table 3. Antidermatophyte activity *in vitro*.

Organism	MIC (μg/ml)			
	Xylocandin A[a]	Clotrimazole	Amphotericin B	Tolnaftate
Trichophyton mentagrophytes	0.2	0.4	12.5	0.1
T. rubrum	0.4	0.4	3.1	≤0.05
Epidermophyton floccosum	≤0.05	0.1	≤0.05	≤0.05
Microsporum canis	≤0.05	≤0.05	0.8	≤0.05

[a] Two-component mixture.

Table 4. Effect of serum and vaginal washings on the anticandidal activity of xylocandin A[a].

	MIC (μg/ml)	MFC (μg/ml)[c]
Control	0.2	0.2
+50% human serum	6.3	6.3
+50% rat vaginal lavage fluid[b]	12.5	12.5

[a] Two-component mixture.
[b] 0.85% sterile saline washings. Lavage of 5 mice pooled.
[c] Minimum fungicidal concentration.

Escherichia coli, *Serratia marcescens*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Salmonella typhosa* and *Pseudomonas aeruginosa*.

The effect of human serum upon the anticandidal activity of xylocandin A was determined by a standard microtiter dilution assay prepared in the presence of 50% human serum. Controls consisted of wells without serum. The results are shown in Table 4. It is evident that the activity is diminished about 32-fold by the presence of serum. It was not determined whether the loss of activity was due to serum binding or degradation of the antibiotic. It is also evident from the data in Table 4 that the activity of xylocandin A is fungicidal.

Despite the excellent anticandidal activity *in vitro*, xylocandin A was not efficacious in the rat

Table 5. Effect of vaginal washings on the recovery of xylocandin A[a] activity[b].

Vaginal lavage fluid (%)[c]	Recovery (%)			
	0 hour	1 hour	3 hours	6 hours
0	100	100	100	100
20	78.6	61.9	52.4	67.9
50	≤8	≤8	≤8	≤8
90	≤8	≤8	≤8	≤8

[a] Two-component mixture.
[b] Agar dilution assay against *Candida albicans* SC5314.
[c] Lavage of 5 mice with sterile 0.85% saline. The pooled washings were arbitrarily assigned the value of 100%.

vaginitis model infection described by McRIPLEY *et al.*[4]. We therefore investigated the effect of vaginal washings upon xylocandin A. Saline (0.85%) vaginal washings from five mice pretreated with estradiol were collected and pooled. The pooled washings were arbitrarily assigned the value of 100%. Varying dilutions of the pool were incubated in the presence of 20 μg of xylocandin A for 0, 1, 3 and 6 hours. Reactions were stopped by the addition of one volume of acetonitrile followed by centrifugation. The supernatants were assayed by a conventional agar-well diffusion assay against *C. albicans* SC5314. Recoveries were determined by comparison to saline, acetonitrile-treated standards containing no vaginal lavage fluid. The results are shown in Table 5. An extensive and rapid loss of antibiotic activity due to the presence of the vaginal pool material occurred by 1 hour and did not increase with time. This suggests that inactivation occurs by mechanisms other than enzymatic degradation. This effect was further substantiated by the 60-fold increase in the MIC and MFC values against *C. albicans* SC5314 found when the antibiotic was assayed in the presence of 50% vaginal washings pool (Table 4). Thus, the lack of activity *in vivo* might well be due to the extensive inactivation evidenced by the results of the serum and vaginal lavage fluid studies.

Conclusions

Xylocandins are novel antifungal peptide antibiotics without appreciable antibacterial activity. Marked loss of bioactivity was encountered in the presence of serum or vaginal fluid. The discovery of the xylocandins as well as the catacandins and other antifungal compounds from bacteria does however indicate that bacteria are a good source of novel antifungal antibiotics.

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