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Cryptocandin, a potent antimycotic from the endophytic fungus *Cryptosporiopsis* cf. *quercina*

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A unique lipopeptide antimycotic, termed cryptocandin, is described from *Cryptosporiopsis* cf. *quercina*, an endophytic fungus. Cryptocandin, with a molecular mass of 1079 Da, contains equimolar amounts of 3,4-dihydroxyhomotyrosine, 4-hydroxyproline, threonine, glutamine, 3-hydroxy-4-hydroxymethylproline, 4,5-dihydroxyornithine and palmitic acid. Cryptocandin is chemically related to well-known antimycotics, the echinocandins and pneumocandins, which are produced by such fungi as *Zalerion arboricola*, *Pezicula* spp. and *Aspergillus* spp. Cryptocandin has minimal inhibitory concentration values of 0·03–0·07 μg ml^{-1} against isolates of *Candida albicans*, *Trichophyton mentagrophytes* and *Trichophyton rubrum*. Cryptocandin is also active against a number of plant-pathogenic fungi including *Sclerotinia sclerotiorum* and *Botrytis cinerea*.

Keywords: *Trichophyton*, endophyte, lipopeptide, 4,4-dimethoxytrityl chloride

INTRODUCTION

Cryptosporiopsis quercina is the imperfect stage of *Pezicula cinnamomea*, a fungus commonly associated with hardwood species in Europe (Sutton, 1980). This fungus and related species occur as endophytes and plant pathogens in many parts of the world (Sutton, 1980). Certain *Pezicula* spp. and *Zalerion arboricola* produce one or more members of a family of lipopeptide antimycotics known as the pneumocandins. Related lipopeptides, the echinocandins, are also produced by *Aspergillus* species.

A fungus taxonomically related to *Cryptosporiopsis quercina* was isolated as an endophyte from *Tripterigeum wilfordii*, a medicinal plant belonging to the family Celastraceae that is native to Eurasia. Extracts of the culture medium of this fungus demonstrated excellent antifungal activity, especially against *Candida albicans* and *Trichophyton* spp. Human and plant infections caused by pathogenic fungi are a continuing and serious problem. Thus, the discovery and characterization of novel, effective antimycotics is important. In the case of humans, the increase in fungal infections has resulted, in part, from the frequent use of antibacterial compounds, which enhances opportunities for fungal infections. Furthermore, there is a worldwide increase in the number of immunocompromised patients, who are susceptible to fungal infections. This patient population has resulted from the AIDS epidemic, chemotherapy of cancer patients, and the increasing numbers of organ transplant patients (Miller *et al.*, 1998).

This report provides spectroscopic, chemical, structural and biological evidence for the existence of a potent new antifungal agent, cryptocandin, produced in cultures of *Cryptosporiopsis* cf. *quercina*. The bioactivity of this agent suggests that it would be useful clinically for the treatment of a variety of mycoses.

Abbreviation: DMT, 4,4-dimethoxytrityl chloride.

METHODS

Fungal isolation and inoculation. Fungi were isolated from *Tripterigeum wilfordii* stems about 1·0 cm in diameter by 15 cm in length. The stems were surface treated with 70% ethanol, the outer bark removed with a sterilized sharp blade, and the pieces of inner bark/phloem/cambium tissues plated on water agar in Petri plates. After several days' incubation at 23 °C, individual hyphal tips of the developing fungal colonies

were removed and placed onto potato dextrose agar (PDA), incubated for 8–10 d, and periodically checked for culture purity. Eventually, pure cultures were transferred again, by hyphal tipping, to Petri plates containing water agar with small pieces of sterilized carnation leaves and incubated in an illuminated incubator cycling 12 h light and 12 h dark at 18 °C. The gamma-irradiated leaves commonly encourage the development of fungal fruiting structures which aid in their identification. The fungal isolate of interest was numbered and stored in distilled water at 4 °C as agar plugs in PDA slants covered with mineral oil at 23 °C, and in 15% (v/v) glycerol at −70 °C as spores and mycelium. The fungus was deposited as culture no. 2039 of the Montana State University culture collection.

Fungal identification by microscopy. Fungal spores and fruiting bodies appearing on the carnation leaf fragments were examined by stereo and light microscopy for measurement and identification. Reference strains from the Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands, were used for comparison. Comparisons of the culture characteristics were made by standard methods after growing the organisms on PDA at 23 °C. Fruiting structures were fixed and processed using the methods of Upadhyay *et al.* (1991), except that they were placed in 2% (v/v) glutaraldehyde in 0·1 M sodium cacodylate buffer (pH 7·2). The samples were critical-point dried, gold coated with a sputter coater and observed and photographed with a JEOL 6100 scanning electron microscope. Assistance in fungal identification was also provided by Dr R. A. Samson of the CBS.

Cryptocandin isolation procedures. Small agar blocks containing the fruiting structures and mycelia of *Cryptosporiopsis* cf. *quercina* were inoculated into 1000 ml MID culture medium (Pinkerton & Strobel, 1976) and incubated for 3 weeks at 22 °C. The mat that developed was blended along with the broth in an equal volume of 95% ethanol. The mixture was gently shaken overnight and the insoluble materials removed by centrifugation at 10000 ***g*** for 10 min. The supernatant fluid was taken to dryness by flash evaporation. Fifty millilitres of methanol was added to the dry residue. After thorough stirring, the methanol-soluble extract was taken to dryness by flash evaporation and the residue redissolved in 1000 ml H_2O. This solution was passed over an Amberlite XAD-2 column (2·5 × 30 cm) and then rinsed with 500 ml 2-propanol/H_2O (20:80, v/v), followed by 300 ml of a 30:70 (v/v) mixture of the same solvents. Finally, the column was eluted with 800 ml of a mixture of 50:50 (v/v) 2-propanol/H_2O and the solution taken to dryness by flash evaporation. The residue (20 mg) was dissolved in 4 ml 25% (v/v) acetonitrile in water and placed on a preconditioned (pre-washed with methanol and 25% acetonitrile) Alltech Altima mini C-18 column with 300 mg resin. After loading, this column was washed with 3–4 ml 25% acetonitrile in H_2O and finally eluted with 75% acetonitrile in H_2O. The eluate was taken to dryness by flash evaporation. Final separation (0·2–0·3 mg per run) was accomplished by HPLC on an Altima C-18 column (7·8 × 250 mm; Alltech) and eluted with a linear gradient of 25–100% acetonitrile in H_2O over 35 min at 3 ml min^{-1} (method 1). The eluate was monitored at 208 nm and fractions were assayed for their activity against *Candida albicans*. Other elution methods included a 45–100% acetonitrile/H_2O linear gradient (method 2), and a 0–100% gradient of solvent A (methanol/H_2O, 50:50, v/v), and solvent B (acetonitrile/2-propanol, 75:25, v/v) (method 3).

Bioassays. To demonstrate the general antifungal activity of *Cryptosporiopsis* cf. *quercina* extracts, known weights of semi-purified (mini C-18 column) preparations were placed on PDA plates and overlaid with the test organism in 0·4% agar. These tests were conducted with various plant-pathogenic fungi, certain human-pathogenic fungi, and isolates of *Trichophyton* spp. However, a purified preparation (final HPLC methods 1–3) of cryptocandin was used for determination of minimal inhibitory concentrations (MICs). These determinations were done on several selected and commonly occurring fungal pathogens using the microbroth dilution assay as recommended by the Subcommittee on Antifungal Susceptibility Testing of the US National Committee for Clinical Laboratory Standards (NCCLS). Appropriate controls were run concomitantly, with the known antifungal agents echinocandin B and amphotericin B. Simple bioassays were conducted on various extracts and column effluents by spotting 10–20 µl of solvent on PDA plates, drying the agar surface under a hood, and overlaying plates with a suspension (10^4–10^6 spores ml^{-1}) of *Candida albicans*. Bioactivity was recorded as the diameter of the zone of inhibition that resulted (Miller *et al.*, 1998).

Amino acid analysis. Lyophilized HPLC-purified compounds were dissolved in 50% methanol in water, placed in 6 × 50 mm glass tubes, dried *in vacuo* and then placed in a hydrolysis cylinder (Millipore part no. 007603). Approximately 300 µl 6 M HCl was added to the cylinder, which was then alternately purged with nitrogen and evacuated three times before being sealed under vacuum. Vapour-phase hydrolysis was performed by heating at 110 °C for 22 h. After cooling, the cylinders were dried *in vacuo*, opened, and the residue in each tube dissolved in 2% sodium citrate buffer (pH 2·0) and then analysed using a Beckman model 6300 amino acid analyser (Miller *et al.*, 1998). Ninhydrin-positive peaks were detected at both 570 and 440 nm. Comparative analyses were also performed on echinocandin and pneumocandin. With the exceptions of glutamine and threonine, an absolute determination of all residues in cryptocandin was not possible given the unavailability of these amino acid standards.

Fungal strains. Fungi used for testing sensitivity to cryptocandin were obtained from the American Type Culture Collection, the mycological collection at Montana State University, Eli Lilly Co., Indianapolis, Indiana, USA, and Dr Mike Rinaldi's laboratory at the University of Texas Health Science Center, San Antonio, Texas 78284, USA.

Mass spectroscopic analysis. Cryptocandin, derivatized cryptocandin, and methylated amino acids obtained after hydrolysis of cryptocandin (see **Preparation of chemical derivatives** below) were each subjected to electrospray mass spectroscopy analysis by dissolving the sample in methanol/water/acetic acid (50:50:1, by vol.). The samples were injected into Montana State University's custom-built instrument, with a spray flow of 2 µl min^{-1} and a spray voltage of 2·2 kV, by the loop injection method.

NMR spectroscopy. NMR spectroscopy was applied to cryptocandin and other compounds in a Brucker 500 MHz instrument with the sample dissolved in 100% deuterated methanol. Each sample was subjected to 2048 scans with a sweep width of 6024 and 8k real points.

TLC. All comparative TLC analyses were carried out on Merck 0·25 mm silica gel plates developed in the following solvents: A, n-butanol/pyridine/acetic acid/H_2O (15:10:3:12, by vol.); B, methylene dichloride/methanol/acetic acid/H_2O (8:2:0·5:0·5, by vol.); C, n-butanol/picoline/acetic acid/H_2O (15:10:3:12, by vol.); and D, chloroform/acetonitrile/acetic acid/H_2O/methanol (7:3:0·5:1:2, by vol.). Compounds were

detected by a spray reagent consisting of 1% (w/v) vanillin with sulfuric acid after gentle heating (Cardellina, 1991) or with a 0·5% ninhydrin ethanolic solution with gentle heating, or by viewing under short-wave UV light (approx. 254 nm).

Preparation of chemical derivatives. Methylation of lipopeptides and free amino acids was carried out by standard procedures. The methylation reagent was first prepared by the careful addition of acetyl chloride (160 µl), with stirring, to 1 ml methanol over the course of 5 min. Then 50–100 µl of the methylation reagent was incubated with 10–15 µg of peptide or amino acid mixture for 1–2 h. The sample was ultimately blown dry under a stream of N_2 gas and redissolved in 10 µl methanol for mass spectral analysis. This procedure effectively methylates acidic carbon atoms in peptides and in free amino acids.

4,4-Dimethoxytrityl chloride (DMT) was used as a check for the presence of primary alcohol functionalities in cryptocandin with various standard peptides, lipopeptides and amino acid controls (Tuschl *et al.*, 1993). The reaction was carried out by dissolving the anhydrous amino compound (100 µg) in 50 µl pure anhydrous pyridine. An approximately equal molar amount of DMT was then added with stirring. After 2 h incubation at 23 °C, the excess pyridine was removed under a stream of N_2 gas. About 50 µl methanol was added to quench the unreacted DMT, and the methanol and pyridine were removed under a stream of N_2 gas. The products of this reaction were separated by TLC in solvent system D. The separated products were detected on the plates by blowing a steady stream of trifluoroacetic acid vapour onto the plate. The reaction products turned yellowish-orange immediately.

Materials. All solvents used for TLC and HPLC were HPLC grade. Those used for extraction were ACS grade. All other reagents were obtained from Sigma. Standard echinocandin B and pneumocandin L 748-842 were generous gifts of Eli Lilly and Merck, respectively.

RESULTS AND DISCUSSION

Identification of the fungal endophyte

Although stems of *T. wilfordii* yielded a number of endophytic fungi, the fungus of greatest interest best answered to the description of *Cryptosporiopsis quercina*. However, because our isolate did not exactly correspond to the description of *Cryptosporiopsis quercina* by Sutton (1980), we refer to it as *Cryptosporiopsis* cf. *quercina*. The conidiomata were typically 250–300 µm when the fungus was grown on sterile carnation leaves, but ones as large as 500–1000 µm were also noted (Fig. 1a, b). The conidia were comparatively large (19–25 × 8·5–9·7 µm), hyaline, thin-walled, smooth and ellipsoid with an obtuse apex. The base of each spore tapered to a very distinct truncate scar (Fig. 1c, d). The conidiogenous cells appeared to vary in length from 30 to 40 µm, and in diameter from 1·4 to 1·6 µm (Fig. 1c, d). Our isolate differs from the description of *Cryptosporiopsis quercina* by Sutton (1980) primarily in the diameter of the conidiogenous cells (reported as 3–4 µm by Sutton, 1980) and in the diameter of the conidia. Furthermore, although cross-walls in conidia are not shown by Sutton (1980) in *Cryptosporiopsis* spp., they do appear to be present in our isolate (Fig. 1d). They may have been missed, because of the hyaline nature of the conidia. Nevertheless, *Cryptosporiopsis* cf. *quercina* is the name tentatively adopted for this fungus. This appears to be the first mention of any *Cryptosporiopsis* species from a celastraceous plant.

Purification of cryptocandin

One major peak of antimycotic activity appeared in the effluent of the HPLC-Altima C-18 column between 14 and 17 min (Fig. 2) when 200–300 µg of the material from the mini-C-18 column was injected into it. A sample (10 µl) from the peak eluting at 15–16 min produced a 22 mm zone of inhibition in the *Candida albicans* bioassay test. The eluate between 14 and 17 min was pooled, taken to dryness by flash evaporation, and successively passed through the C-18 column using methods 2 and 3, successively. Method 3 yielded a single peak that was identical to the major peak in method 1 (14–17 min) (Fig. 2). The final product was checked by TLC in solvent systems A, C and D and shown to yield a single UV-absorbing spot under short-wave UV light, a reddish-brown reaction product with the vanillin/sulfuric acid reagent, and a weak yellowish-pink spot with ninhydrin. The compound, which we named cryptocandin, was considered homogeneous and the fungus yielded up to 3–4 mg per 1000 ml of culture medium.

Spectroscopy of cryptocandin

Several spectroscopic evaluations helped confirm the relative purity of the cryptocandin-antimycotic preparation. Electrospray mass spectroscopy revealed an $[M+H]^+$ peak at 1080·6 and $[M+Na]^+$ at 1102·7 with no other contaminating peaks. Thus, the apparent mass of cryptocandin is 1079·6 Da. Cryptocandin possessed UV absorption maxima at 233 and 273 with millimolar absorption coefficients of 2·7 and 1·9, respectively. The ^{1}H-NMR spectrum of cryptocandin was substantially free of signals of contamination (Fig. 3) and this, along with other spectroscopic data, suggested that cryptocandin was an aromatic lipopeptide. Previously described aromatic lipopeptides possessing antimycotic activity include the echinocandins and pneumocandins (*Dictionary of Natural Products on CD ROM*, 1996). Therefore, in order to distinguish cryptocandin from the other aromatic lipopeptides, both analytical and spectroscopic chemical studies were conducted.

Comparative TLC studies

The R_F values of echinocandin B, pneumocandin L 748-842 and cryptocandin illustrated that cryptocandin differs from these earlier established structures (Table 1). The occurrence of two spots, instead of one, in solvent system B with both echinocandin B and cryptocandin may be a result of the racemization of the dihydroxyornithine residue. Each compound absorbed in the short-wave UV range, each gave a yellowish-pink

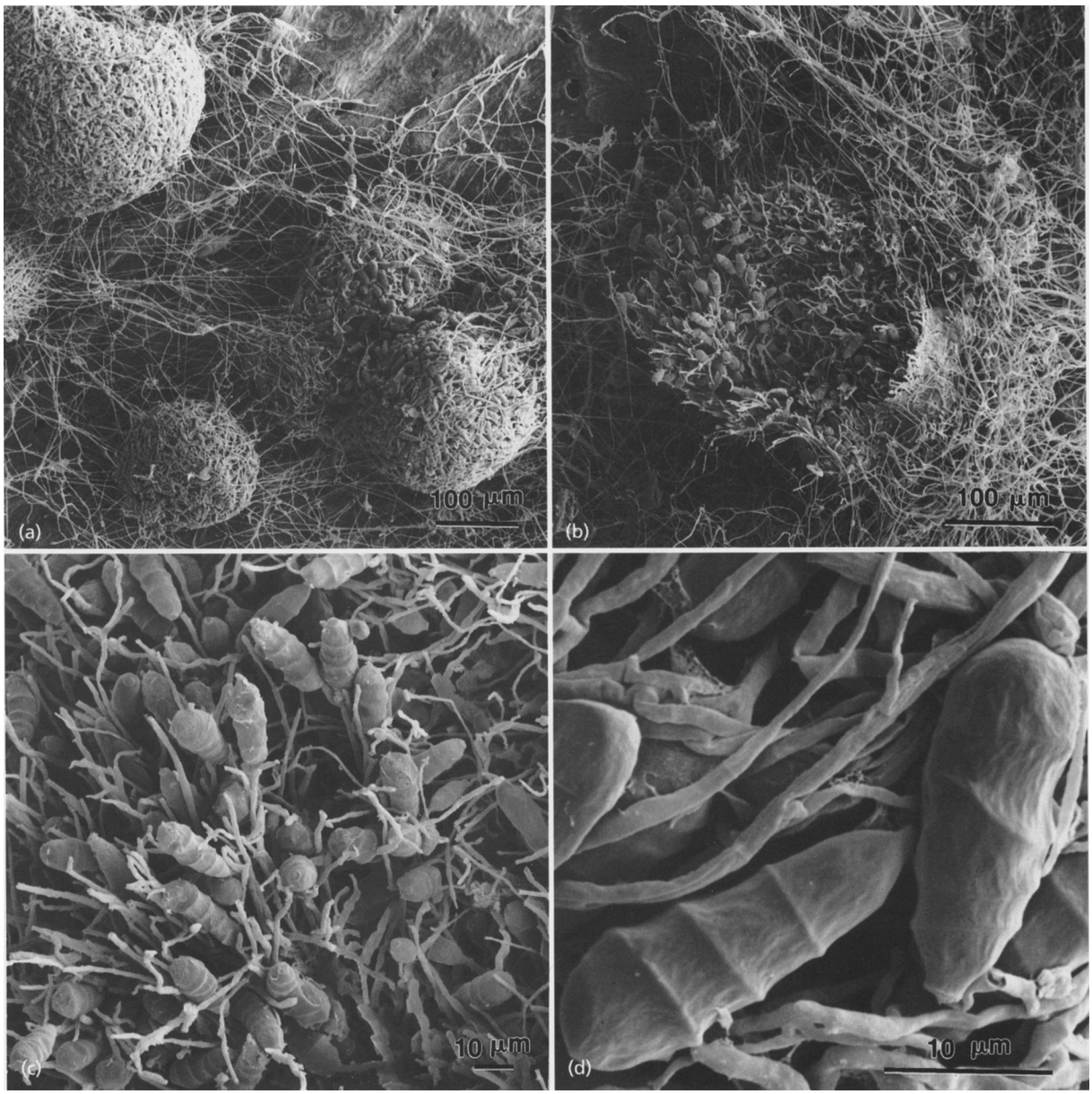

Fig. 1. Conidial structures of *Cryptosporiopsis* cf. *quercina*: (a, b) conidiomata produced on sterile carnation leaves; (c) conidiophores with conidia; (d) conidia.

spot with ninhydrin and each produced a reddish-brown spot with the vanillin/sulfuric acid reagent.

Constituent residues of cryptocandin

The fatty acid content of cryptocandin was determined after methylation of samples followed by gas chromatography/mass spectroscopy (Alltech). Cryptocandin possessed a fatty acid whose retention time and spectral characteristics were identical to methylpalmitate. This is in contrast to pneumocandin A, whose fatty acid moiety is dimethylmyristate, and to echinocandin B, which has linoleic acid as a fatty acid moiety. Several other fatty acids also occur as side chains on the family of echinocandins and pneumocandins (*Dictionary of Natural Products on CD ROM*, 1996).

Amino acid analysis of cryptocandin showed that it contained residues of threonine, 4-hydroxyproline, 3,4-dihydroxyhomotyrosine and 4,5-dihydroxyornithine. These amino acids eluted from the analytical column at 11·19, 9·12, 21·28 and 53·26–55·07 min, respectively (Fig. 4). Threonine and 4-hydroxyproline eluted at times identical to authentic standards. The identities of the other two residues were inferred from comparison of retention times with those amino acids derived by

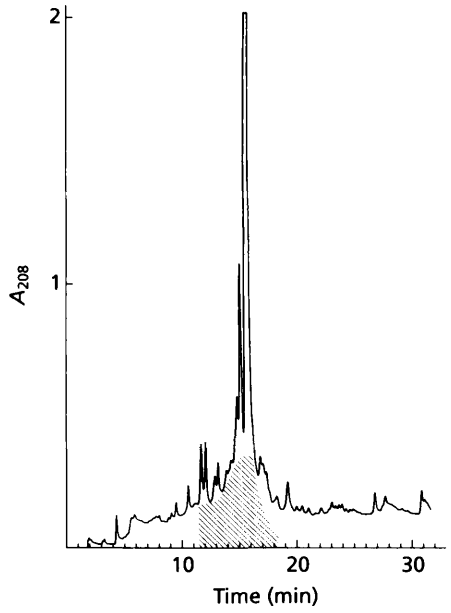

Fig. 2. Elution profile of the antifungal extract of *Cryptosporiopsis* cf. *quercina* from an Altima C-18 HPLC column. The column was monitored at 208 nm and each fraction was checked for antifungal activity against *Candida albicans*. The main peak at 14–17 min possessed the antifungal activity (shaded area). The elution time was 3 ml min^{-1} using solvent system (1) (see Methods).

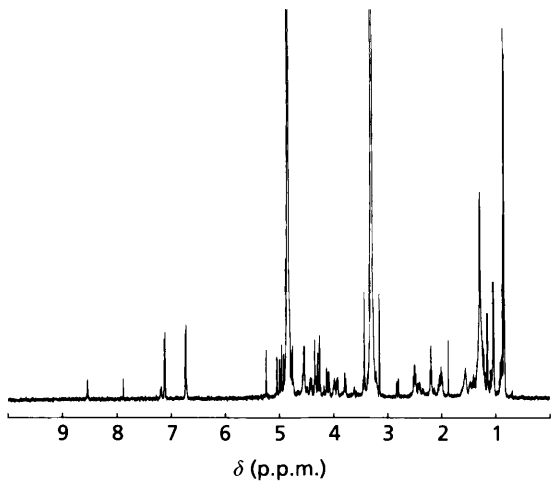

Fig. 3. ^{1}H-NMR spectrum of cryptocandin taken in 100% deuterated methanol.

Table 1. TLC R_F values of cryptocandin, echinocandin and pneumocandin L748-842 in solvents A–D

Details of the solvent systems, separations and detection schemes are given in Methods.

Compound	R_F in solvent system:			
	A	B	C	D
Cryptocandin	0·72	0·17–0·12*	0·66	0·15
Echinocandin B	0·81	0·27–0·22*	0·76	0·26
Pneumocandin	0·59	0·35	0·56	0·01

* Each of these compounds produced secondary ghost spots in solvent B which may result from the racemization of dihydroxyornithine.

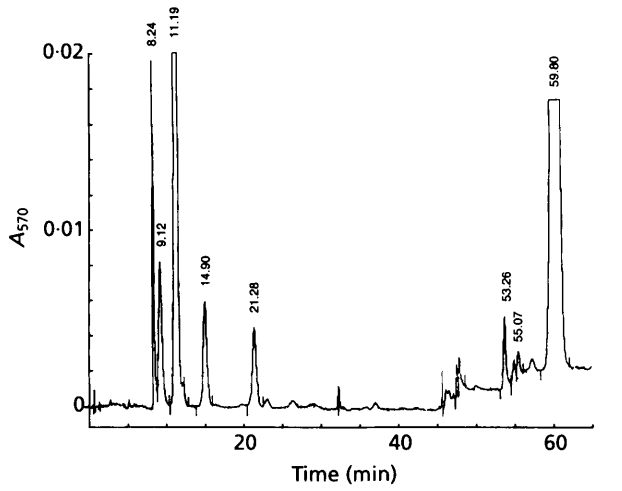

Fig. 4. Amino acid elution profile of the acid hydrolysate of cryptocandin. Detection of column effluent was at 570 nm for the ninhydrin reaction products.

hydrolysis of echinocandin B, which produced peaks with identical retention times as 4,5-dihydroxyornithine and 3,4-dihydroxyhomotyrosine. The former amino acid residue typically appears as multiple peaks as a result of racemization which occurs around the hydroxyaminal functional group and the adjacent carbon on position numbers 4 and 5 (see Fig. 5). Additional evidence for the identity of these amino acids was, in part, provided by electrospray mass spectroscopy of the methylated mixture derived from the hydrolysate of cryptocandin (Table 2).

The remaining peaks at 14·90 and 8·24 min did not occur in the hydrolysates of either echinocandin B or pneumocandin L 748-842, suggesting major structural differences between these molecules and cryptocandin. Since pneumocandin A and cryptocandin have the same molecular mass, and since at least four of the amino acid residues are identical, the other two must, therefore, be different.

Electrospray mass spectroscopy of the methylated cryptocandin revealed an $[M+H]^+$ peak at 1108·2, suggesting that cryptocandin had been di-O-methylated (1079 + 14 + 14 + 1 = 1108). Interestingly, echinocandin B also methylates (M. Rodriguez, Eli Lilly Co., personal communication) on both the acidic phenylhydroxy and

Table 2. Electrospray mass spectroscopy of methylated hydrolysis products of cryptocandin

Methylated amino acid residue	Molecular mass + H^+ (Da)	
	Calculated	Observed
1,2-Dihydroxyhomotyrosine (dimethyl)	242·21	–
4-Hydroxyproline (methyl)	146·15	146·30
Threonine (methyl)	134·14	134·40
Glutamic acid (dimethyl)	176·16	176·10
3-Hydroxy-4-hydroxymethylproline (methyl)	162·18	162·20
4,5-Dihydroxyornithine (dimethyl)	193·19	192·90

hydroxyaminal groups on 3,4-dihydroxyhomotyrosine and 4,5-dihydroxyornithine, respectively. Thus, neither of the two remaining amino acid residues in cryptocandin could have either free carboxyl or acidic OH groups as potential sites of methylation. One peak in the amino acid profile had the identical retention time as Glx (glutamic acid/glutamine) at 14·90 min (as compared to authentic glutamic acid) (Fig. 4). Furthermore, a compound with a mass identical to the di-O-methyl ester of glutamic acid appeared in the methylated hydrolate of cryptocandin (Table 2). Therefore, the peak at 14·9 min must represent glutamine since cryptocandin would have been triply methylated if free glutamic acid were present.

The remaining amino acid residue with a retention of 8·24 min on the amino acid column (Fig. 4) produced a methyl ester with an observed mass of 162 Da. We suggest that this compound may be dihydroxymethylproline. This amino acid residue, along with 4-hydroxyproline, produced a yellowish ninhydrin product when the amino acid analytical column was monitored at 440 nm. Furthermore, a prolyl-like residue is normally conserved in this group of substances (*Dictionary of Natural Products on CD ROM*, 1996).

If this residue is a dihydroxymethylproline residue, it represents the only residue in cryptocandin with a primary alcohol functionality. As such, it would be expected to produce a reaction product with the DMT reagent, and cryptocandin did so (Table 3); the electrospray mass spectrum of the reaction products yielded an expected peak with an $[M+H+H]^{2+}$ of 692. A series of peptides, lipopeptides and amino compounds with and without primary alcohol functionalities were also checked for their reactivity with this reagent and the products separated by TLC solvent D (Table 3). Pneumocandin L 748-842, pseudomycin or other peptides not possessing a primary hydroxyl group were not reactive with DMT, but other peptides were reactive (Table 3). Likewise, as expected, serinol, with two primary alcohol groups, produced two reaction products with DMT (Table 3). Although each of the

Table 3. TLC R_F values of DMT derivatives of cryptocandin and other peptides

TLC solvent system D was used. Detection was done by exposing the dried plate to trifluoroacetic acid vapour.

DMT derivative of peptide/compound	R_F value*
Cryptocandin	0·76
Pseudomycin	no product
Echinocandin B	0·46, 0·34†
Pneumocandin	no product
Serinol	0·41, 0·69
Arg-Lys-Asp-Tyr (Sigma A-4777)	no product
Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu (Sigma 1762)	0·07
Ala-Leu-Ile-Leu-Thr-Leu-Val-Ser (Sigma 1061)	0·04
Asp-Ser-Asp-Pro-Arg (Sigma 3526)	0·04

* No DMT products were formed with peptides or lipopeptides lacking a primary alcohol functionality.

† Echinocandin produced at least two DMT derivatives due to its degradation resulting in products with primary alcohols (*Dictionary of Natural Products on CD ROM*, 1996).

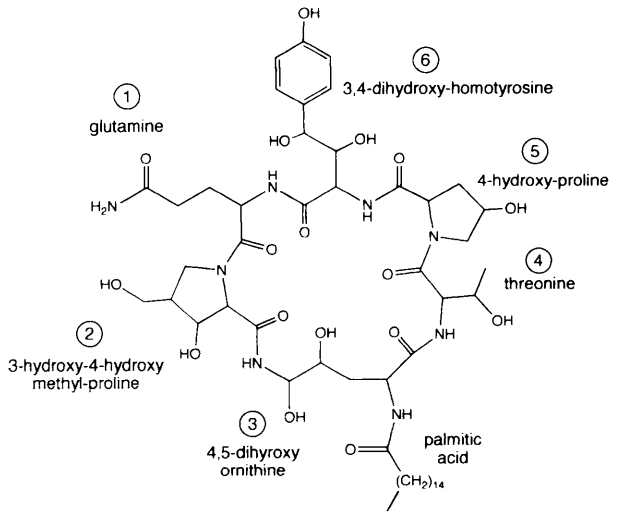

Fig. 5. Proposed structure of cryptocandin (no stereochemistry is implied).

residues is accounted for, bringing the total mass of cryptocandin to 1079 Da, the positions of the hydroxyl groups on dihydroxymethylproline are unknown. We suggest that the structure of this residue is 3-hydroxy-4-hydroxymethylproline based on the 3,4 substitution on this residue that occurs on all other known pneumocandins and echinocandins (Walsh, 1992). Therefore, with no stereochemistry implied, the proposed structure of cryptocandin shows similarity to the other known compounds (Fig. 5). Typically, the order of amino acids in these molecules is conserved with the exception of

Table 4. MICs of cryptocandin (mini C-18 column preparation) against *Trichophyton* isolates

Species	Isolate*	Plate MIC ($\mu g\ ml^{-1}$)†
T. mentagrophytes	ATCC 28185	0·070
	7P-1796	0·035
	7P-1797	0·035
	8P-1027	0·070
T. rubrum	ATCC 28188	0·070
	7P-1794	0·035
	7P-1801	0·035
	7P-1803	0·035
Candida albicans (control)	ATCC 90028	0·035

* ATCC strains are from the American Type Culture Collection; others are clinical isolates from the laboratory of Dr Michael Rinaldi, Fungus Testing Laboratory, University of Texas Health Science Center at San Antonio.

† Plate MIC is the amount required to induce a visible clear zone of inhibition on a PDA plate overlaid with the test organism in 0·4% agar. Results with *Candida albicans* indicate that MICs determined in 96-microwell plates are usually two- to fivefold lower than those on plates (e.g. *C. albicans* with a plate MIC of 0·035 $\mu g\ ml^{-1}$ had a corresponding 96-well MIC of 7 $\mu g\ ml^{-1}$).

position ①; compounds with serinyl, threonyl, or hydroxy glutaminyl substitutes at this position have been described (Walsh, 1992). Also, we have inferred the sequence of these amino acids, based on the striking similarities between the NMR spectra of cryptocandin and echinocandin. Other compounds with varying degrees of hydroxylation at positions ③ and ⑥ are also known and several have been described with demethylation at position ② (Fig. 5). However, a compound with glutamine at position ① and dihydroxymethylproline at position ② has not been previously described (*Dictionary of Natural Products on CD ROM*, 1996). Furthermore, cryptocandin appears to be structurally distinct from the echinocandins and pneumocandins in that one of its amino acids (glutamine) lacks a hydroxyl functionality. As a result of these subtle structural changes, the cryptocandin molecule exhibits unique biological activities.

Biological activities of cryptocandin

The most impressive antifungal activity of cryptocandin (mini C-18 preparation) was on *Trichophyton* spp. (Table 4). Plate MICs of 0·035–0·07 $\mu g\ ml^{-1}$ were observed for various isolates of *Trichophyton rubrum*, as well as *Trichophyton mentagrophytes* (Table 4).

MIC values for purified cryptocandin were also acquired for some important fungal pathogens of humans (Table 5). The most impressive activity was against *Candida albicans* at 0·03 $\mu g\ ml^{-1}$ and *Histoplasma capsulatum* at 0·01 $\mu g\ ml^{-1}$. These data are virtually identical to those obtained for amphotericin B, the most common clinically used antifungal agent (Table 5). With echinocandin B the MIC for *Candida albicans* was 0·3 $\mu g\ ml^{-1}$ (Table 5). Cryptocandin had little effect on either *Aspergillus fumigatus* or *Cryptococcus neoformans*, in contrast to amphotericin B (Table 5).

Mini C-18 column preparations of cryptocandin were examined for their effects against a range of plant-pathogenic fungi; the most notable susceptible organism in these tests was *Sclerotinia sclerotiorum* (Table 6). However, many of the other organisms tested showed little, no, or weak susceptibility to the cryptocandin preparation.

Cryptocandin

The new antimycotic, cryptocandin, appears to be another member of the growing family of aromatic lipopeptide antifungal agents having such important members as echinocandin and pneumocandin, whose chemical derivatives are already in advanced human trials. Cryptocandin may also have potential as a therapeutic agent, given its impressive MIC values against *Candida albicans* and *Histoplasma capsulatum* (Table 5). However, given the MIC values against *Trichophyton* spp. with only the mini-C-18 column preparation of cryptocandin (Table 4), this compound may have enormous potential for the control of skin and nail diseases in humans caused by *Trichophyton* spp.

Table 5. MICs of purified cryptocandin and other antifungal agents against various human-pathogenic fungi

Test fungal pathogen	MIC ($\mu g\ ml^{-1}$) of antifungal agent:		
	Cryptocandin	Echinocandin B	Amphotericin B
Candida albicans	0·03	0·3	0·04
Cryptococcus neoformans	> 20	> 20	0·04
Aspergillus fumigatus	> 20	> 20	0·03
Candida parapsilosis	2·5	–	0·01
Histoplasma capsulatum	0·01	–	0·01

–, Not tested.

Table 6. Inhibitory effects of cryptocandin (mini C-18 column preparation) against various plant-pathogenic fungi in two replicated experiments

Test fungus	96-well MIC (μg ml $^{-1}$)		Plate test*	
	(test 1)	(test 2)	(test 1)	(test 2)
Geotrichum candidum	3·12	125	none	trace
Rhizoctonia solani	50	31·2	none	none
Sclerotinia sclerotiorum	0·78	15·6	15 mm	15 mm
Fusarium solani	none	none	trace	trace
Botrytis cinerea	6·2	31·2	22 mm	22 mm
Pythium ultimum	50	125	trace	trace
Ustilago hordei	none	none	none	none

* 25 μg semi-purified cryptocandin on PDA plates was overlaid with the test organism and the diameter of the zone of inhibition was measured (see Methods).

(Table 4). Preliminary trials using cryptocandin on nail and skin infections with human volunteers have shown promising results (G. Strobel, unpublished).

Structurally, cryptocandin has the unique aspect of having several amino acids possessing two hydroxy functionalities, and glutamine bearing none (Fig. 5), whereas pneumocandin and echinocandin have each amino acid hydroxylated (Walsh, 1992). Other cryptocandins may occur that differ from each other by virtue of the lipid side chain. This is also a feature of other bioactive bacterially derived lipopeptide antimycotics (Miller *et al.*, 1998; Ballio *et al.*, 1994).

The biological role of cryptocandin and its related antimycotics in nature has not been investigated. However, given the general antimycotic activity of cryptocandin and the endophytic nature of *Cryptosporiopsis* cf. *quercina*, cryptocandin, if it is produced in the plant, may provide protection against invading pathogens. This may happen at an extremely localized cellular level given how sparse endophytes can be relative to the large number of cells in the plant. This work on *Cryptosporiopsis* cf. *quercina* also further exemplifies the value of examining plant endophytes as sources of new potential pharmaceuticals (Strobel & Long, 1998).

NOTE ADDED IN PROOF

Since the initial discovery of cryptocandin, several apparently related compounds have been isolated from *C. quercina*; thus we propose to designate cryptocandin as cryptocandin A.

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