ORGANIC LETTERS

2000
Vol. 2, No. 6
767−770

Cryptocin, a Potent Tetramic Acid Antimycotic from the Endophytic Fungus *Cryptosporiopsis cf. quercina*

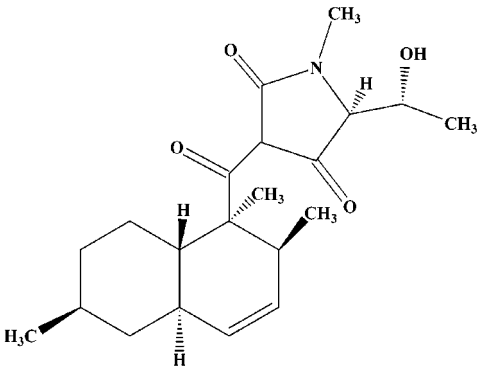
J. Y. Li,[†] Gary Strobel,*[,†] James Harper,[†] Emil Lobkovsky,[‡] and Jon Clardy[‡]

Department of Plant Sciences, Montana State University, Bozeman, Montana 59717, and Department of Chemistry, Baker Laboratory, Cornell University, Ithaca, New York 14853

uplgs@montana.edu

Received January 10, 2000

ABSTRACT



The endophytic fungus *Cryptosporiopsis cf. quercina* produces cryptocin in culture. Among other fungi, this unique tetramic acid displays antimycotic activity against *Pyricularia oryzae*, the causal agent of rice blast disease. Cryptocin also possesses activity against a wide variety of plant pathogenic but not human pathogenic fungi. The fine rhomboid-like crystals of cryptocin allowed structural elucidation by X-ray crystallography. The importance of cryptocin to the symbiotic relationship of *C. quercina* to its hosts is briefly discussed.

Cryptosporiopsis cf. quercina was originally isolated in pure culture from the inner bark of the stems of *Triptergyium wilfordii*.[1] It was obtained from a symptomless 1.0 cm diameter piece of a vine of this plant. The fungus appeared as hyphal growth after a small piece of the inner bark of this vine (surface treated with 70% ethanol) had been placed on a water agar plate. The fungus was obtained in pure culture and then transferred to pieces of γ-irradiated carnation leaves where it formed beautiful asexual fruiting structures called ascervuli. This fungus was primarily identified on the basis of its acervular structures and the hyaline spores contained therein.[1] However, these hyaline spores possessed cross walls making it different than any related fungus previously described. On agar plates, *C. quercina* caused impressive zones of antibiosis against a plethora of other plant-associated fungi including plant pathogenic ones. One of the most interesting biological aspects of this fungus is that it causes no observable external disease-like symptoms on *T. wilfordii* or on *Quercus* sp. (oaks) from which it and related organisms are commonly isolated. As a further check on this important observation, we purposefully inoculated a pure culture of this organism back into the stems of both *Q. alba* and *T. wilfordii*. Even after 1−2 years of incubation of the fungus in these limbs, we did not observe any disease-related symptoms. We therefore suggest that the fungus is existing as an endophyte in the stems of certain plant species and that it may be there in a symbiotic state. While the plant may provide support and nutrition for the fungus, the fungus

[†] Montana State University.
[‡] Cornell University.

(1) Strobel, G. A.; Miller, R. V.; Miller, C.-M.; Condron, M. M.; Teplow, D. B.; *Microbiol.* **1999**, *145*, 1919.

10.1021/ol000008d CCC: $19.00
Published on Web 03/01/2000

may participate in the association by virtue of antifungal agents that it may produce. Thus, one purpose of this report is to show how such endophytic microbes can be isolated, grown in culture, and then be studied for the presence of bioactive compounds. Previously, we had isolated a unique antimycotic in the "candin" series called cryptocandin from this microbe.[1] However, additional antifungal activities were present in extracts of the fungal medium. One of these antifungal agents was isolated and crystallized and its structure determined by X-ray crystallography and shown to be cryptocin, a unique tetramic acid.

Cryptocin was isolated from 4-week-old still cultures of *C. quercina* grown in 500 g of oat seeds plus 400 mL of water in two 2 L flasks at 23 °C. After removal of the fungal mycelium and oat residues by filtration, the culture broth from both flasks was taken to dryness by flash evaporation and successively extracted twice with 500 mL of methanol. The methanol solution was flash evaporated, dissolved in 500 mL of water, and then extracted successively with two 1 L volumes of methylene chloride. The yield of material was 8−10 g. This material was dissolved in a minimal volume of methylene chloride and subjected to two successive silica gel chromatographic steps.[3] Fractions (10 mL) were collected from the eluate of the silica gel columns and were subjected to the bioassay test.[4] The fungus that seemed to be the organism of choice for this test was a rapidly growing isolate of *Sclerotinia sclerotiorum*. The yield of bioactive final product was 63 mg of a whitish powder per flask.

Assessment of the chemical purity of the bioactive compound was carried out on 10 individual thin layer chromatographic systems.[5] Detection of the compound could be made by its fluorescence at 254 nm and also by spraying the plate with the vanillin sulfuric acid spray reagent followed by gentle heating.[6] The final silica gel step did yield a compound that appeared as a single spot on the TLC plates in each of the solvent systems with R_f values as indicated.[5] In addition, application of the compound (20 μg) to an Altima C-18 reverse phase HPLC column (7.0 × 250 mm) and separation in a linear 30−70% gradient of acetonitrile over 30 min revealed a single UV absorbing (254 nm) peak with a retention time of 14.3 min. By these criteria the compound was considered homogeneous and the name "cryptocin" was coined for it.

Cryptocin had an uncorrected melting point, with decomposition, in the range of 175−180 °C. It has three UV absorption peaks at λ 291, 250, and 204 with millimolar extinction coefficients in methanol of 5.61, 4.64, and 3.23, respectively. Electrospray mass spectroscopy of cryptocin revealed an $(M + H)^+$ peak at 362 and an $(M + Na)^+$ peak at 384. The spectrum also showed a dimer peak at $(2M + Na)^+$, and trimer peaks containing Na^+ and K^+ were also present in the spectrum. All of the data collectively supported the conclusion that the mass of cryptocin is *m*/*z* 361. However, cryptocin yielded an observed molecular ion − H_2O at 343.214012 (calcd MW = 343.214744). Thus, the empirical formula for cryptocin is $C_{21}H_{31}N_1O_4$.

Nearly all of the 21 resonances in the ^{13}C NMR spectrum of cryptocin were doubled, making interpretation difficult. This doubling may arise either from a mixture of two closely related compounds or the presence of an equilibrium between two energetically similar forms of cryptocin. Variation in the ratio of resonances with temperature demonstrates the presence of an equilibrium process rather than contamination from a related compound. A more complete description of the solution NMR of cryptocin will be given elsewhere.

Surprisingly, a slow evaporation of an ethyl acetate−methanol 1:1 (v/v) solution of cryptocin at 23 °C yielded numerous individual slightly yellowish rhomboidal crystals. The crystals belonged to the space group $P2_12_12_1$ with a = 9.4009(3), b = 14.5547(4), and c = 16.1500(4) Å. The molecular structure was determined by single-crystal X-ray diffraction (Figure 1).[7] Cryptocin crystallized as a sodium salt with the sodium coordinating with each of the oxygen atoms in the molecule (Figure 1).

A number of other biologically active tetramic acids have been reported in the chemical literature. Among these are the aurantosides, which are tetramic acid glycosides from a marine sponge which have antifungal activity against *Aspergillus fumigatus* and *Candida albicans*.[8] Goedin A is a novel macrocyclic polyketide lactam tetramic acid from an

(2) Walsh, T. A. Inhibitors of glucan synthesis. In *Emerging Targets in Antibacterial and Antifungal Chemotherapy*; Sutcliffe, J. A., Georgopapadakou, N. H., Eds.; Chapman and Hall: London, 1992; pp 349−373.

(3) The first silica gel chromatography was performed on a 3 × 30 cm column. The organic solvent extract was applied to the column and eluted with 500 mL of chloroform:acetonitrilewater 10:1:0.1 v/v. The first 100 mL eluting from the column was discarded and the next 400 mL to elute from the column (possessing bioactivity) was taken to dryness (350 mg), redissolved in 2.0 mL of methanol, and placed on the second silica gel column (identical in size to the first column). It was eluted with ethyl acetate:methanol 20:10 v/v. Fractions (10 mL) were collected, and the bioactivity appeared at 150−200 mL.

(4) The bioassay test consisted of the placement of an aliquot (20 μL) of each column fraction on a potato dextrose agar (PD) plate and allowing the solution to evaporate to dryness on the surface in a decontaminated (alcohol sprayed) laminar flow hood for 15−20 min. Subsequently, 5 mm plugs of PD agar containing a rapidly growing culture of *Sclerotina sclerotiorum* were placed within 1 cm of the test residue previously deposited on the plate. After 3 days of incubation at 23 °C, fungal colony inhibition could be assessed by virtue of its uneven growth, with little or no growth developing toward the dried residue. Assessment of the chemical purity of the bioactive compound was done on 10 individual thin layer chromatographic systems.[5] Detection of the compound could be made by its fluorescence at 254 nm and also by spraying the plate with the vanillin sulfuric acid spray reagent followed by gentle heating.[6] The final silica gel step did yield a compound that appeared as a single spot on the TLC plates in each of the solvent systems with the R_f values as indicated.[5] In addition, application of the compound (20 μg) to an Altima C-18 reverse phase HPLC column (7.0 × 250 mm) and separation in a linear 30−70% gradient of acetonitrile over 30 min revealed a single UV absorbing (254 nm) peak with a retention time of 14.3 min. By these criteria, the compound was considered homogeneous.

(5) Thin layer chromatograpy was conducted on Merck 0.25 mm silica gel plates (5 × 10 cm.). The solvents and R_f values recored for each system are as follows: chloroform:acetonitrile, 3:2 v/v, 0.25; chloroform:methanol: acetic acid, 15:1:0.1 v/v, 0.51; ethyl acetate:methanol:ammonium hydroxide, 5:1:0.1 v/v, 0.47; ethyl acetate:methanol, 5:1 v/v, 0.72; chloroform:methanol, 7:1 v/v, 0.68; methylene dichloride:methanol:dimethylforamide, 9:1:0.1 v/v, 0.53; methylene dichloride:tetrahydrofuran, 6:1 v/v, 0.55; ethyl acetate:2-propanol, 95:5 v/v 0.19; ethyl acetate:tetrahydofuran, 3:1 v/v, 0.43; methylene dichloride:acetonitrile, 2:1 v/v, 0.36.

(6) Cardellina, J. H. *J. Chromatogr*. **1991,** *14*, 659.

(7) Supporting Information is available as tables of fractional coordinates, thermal parameters, and interatomic and torsional angles.

(8) Sata, N. U.; Matsunaga, S.; Fusetani, N.; van Soest, R. W. *J. Nat. Prod*. **1999**, *62*, 969.

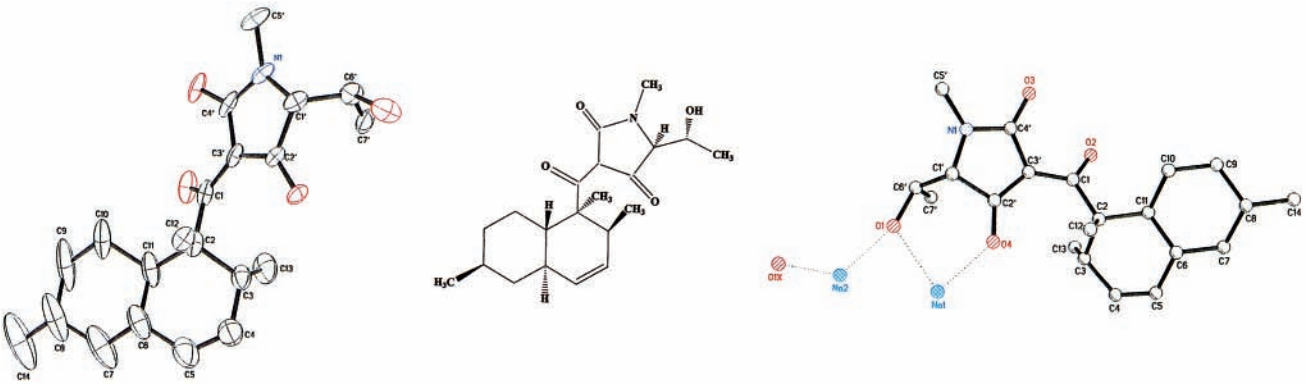


Figure 1. Computer-generated model of cryptocin (left), the structural formula of cryptocin (middle), and the sodiated cryptocin complex (right).

Table 1. Minimum Inhibitory Concentrations of Cryptocin and Pseudomycin A for a Representative Group of Plant Pathogenic Fungi. The Family Name of Each Organism Tested Is Presented Next to the Specific Fungal Name in the Table

fungus (genus and species)	fungal family	cryptocin, μg/mL	pseudomycin A, μg/mL
Pythium ultimum	oomycete	0.78	>12.0
Phytophthora cinnamoni	oomycete	0.78	not active
Phytophthora citrophthora	oomycete	1.56	not active
Sclerotinia sclerotiorum	ascomycete	0.78	1.5
Pyricularia oryzae	ascomycete	0.39	>12.0
Rhizoctonia solani	basidiomycete	6.25	1.5
Geotrichum candidum	fungi imperfecti	1.56	1.5
Fusarium oxysporum	fungi imperfecti	1.56	>12.0

Australian sponge with nematocide activity.[9] Other interesting novel tetramic acids are the aflastatins produced by *Streptomyces* sp. that inhibit aflatoxin production by *Aspergillus parasiticus*.[10] Some other recently described tetramic acids with antibiotic properties are dihydromaltophilin, triandalydigin, and lydicamycin.[11]

Cryptocin possesses impressive biological activity against certain plant pathogenic fungi. Representatives from each of the main taxonomic groups of fungi were selected as test organisms, and a minimum inhibitory concentration (MIC) was obtained for each test organism.[12] Representaive oomycetes (Pythium and Phytophthora) and ascomyctes (Sclerotinia and Pyricularia) each had MIC values of less than 1.0 μg per mL, while other fungi, including a representative basidiomycete (Rhizoctonia) and fungi imperfecti (Fusarium) had MICs greater that 1.0 μg per mL (Table 1). In other tests, all of the major human pathogenic fungi including *Candida albicans*, *Aspergillus fumigatus*, *Candida parapsilosis*, and *Histoplasma capsulatum* had MIC values exceeding 80 μg per mL, while *Cryptococcus neoformans* had a value of 20−40 μg per mL (light growth). These findings make it obvious that cryptocin, while possessing, in part, a tetramic acid structure, does not share biological commonality with the aurantosides that do have activity against *C. albicans*.

The fungus that seemed to be the most sensitive to cryptocin was *P. oryzae* (Table 1). This fungus causes rice blast and continues to be one of the most economically important plant pathogenic fungi in the world.[13] Worldwide annual losses to the rice crop vary from region to region and range from 1.0% to 50%. Many industrial fungicide-screening tests include rice blast disease as one of the top five targeted diseases for fungicide development. We have also observed that cryptocin posseses in vivo activity against the rice blast fungus, making it or a derivative of it a candidate compound for fungicide development. It would appear that compounds derived from natural sources have not been the subject of intensive fungicide development by

(9) Capon, R. J.; Skene, C.; Lacey, E.; Gill, J. H.; Wadsworth, D.; Friedel, T. *J. Nat. Prod.* **1999**, *62*, 1256.

(10) *(10)* Ono, M.; Sakuda, S.; Ikeda, H.; Furihata, K.; Nakayama, J.; Suzuki, A.; Isogai, A. *J. Antibiot.* **1998**, *51*, 1019.

(11) (a) Graupner, P. R.; Thornburgh, S.; Mathieson, J. T.; Chapin, E. L.; Kemmitt, G. M.: Brown, J. M.; Snipes, C. E. *J. Antibiot.* **1997**, *50*, 1014. (b) Hayakawa, Y.; Kanamaru, N.; Morisaki, N.; Furihata, K.; Seto, H. *J. Antibiot.* **1991**, *44*, 288. (c) Karwowski, J. P.; Jackson, M.; Theriault, R. J.; Barlow, G. J.; Coen, L.; Hensey, D. M.; Humphrey, P. E. *J. Antibiot.* **1992**, *45*, 1125.

(12) MIC values for cryptocin were obtained using a standard plate bioassay test method that employs a microtitre plate containing various concentrations of the test compound and the fungus to be tested in PD broth. Each well, after 24 h, was visually observed, and the well showing no growth next to well having some growth was taken as the MIC concentration value. The activity of cryptocin was directly compared with that of a known antimycotic, pseudomycin A. Details of the assay methods are given in ref 1.

(13) Ou, S. H. *Rice Diseases*; C.A.B. Kew: Surrey, England, **1972**.

the agricultural industry throughout the world. With the advent of an increased public concern relative to the hazards of chemically grown produce in the market place, there may be a place for naturally derived compounds to replace the manmade products being used to protect crops and forest species?

While cryptocin expresses antimycotic activity against some fungi (Table 1), the prospect of it having an even broader range of antifungal activity seems likely. Given the fact that *C. quercina* seems to live in an endophytic association with certain higher plants, it may do so symbiotically. Cryptocin is only one of several other antimycotics produced by this fungus.[1] Thus, the contribution of these agents to the host−plant relationship may be to ward off fungi that would normally pose a threat to the plant host. While oomycetes are some of the most pathogenic of all plant fungi, the basidiomycetes, as a group, are also deadly. With further study, it would not be surprising to learn that a wide range of genera in this group of fungi are sensitive to cryptocin given the fact that one representative basidiomycete that was tested (*R. solani)* is sensitive to this compound (Table 1).

This group of fungi (the basidiomycetes) generally includes those fungi causing cankers, heart and stem rots, root rots, and blights of woody and viney plants. Thus, a biological rationale would suggest that if *C. quercina* is acting as a symbiotic endophyte and providing protection to its plant host, the basidiomycetes might, as a group, be the best targets for cryptocin. Combined, a wide range of fungi are targets of cryptocandin and cryptocin, making this fungus one of the most antifungal organisms that we have ever observed.[1]

Acknowledgment. These studies were supported by the Montana Agricultural Experiment Station and NIH Grant CA67786 to J.C. Special help in acquiring NMR data was provided by Dr. Scott Busse of the Department of Chemistry, MSU, and Dr. Joe Sears of that department acquired the mass spectral data on cryptocin shown in this report.

Supporting Information Available: Tables of fractional coordinates, thermal parameters, and interatomic distances and other information on cryptocin. This material is available free of charge via the Internet at http://pubs.acs.org.

OL000008D