Ochratoxin A: an antiinsectan metabolite from the sclerotia of *Aspergillus carbonarius* NRRL 369[1]

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Abstract: Ochratoxin A, a known mycotoxin with demonstrated toxicity to insects, has been isolated from the sclerotia of the fungus *Aspergillus carbonarius* NRRL 369. The sclerotia, harvested from a solid substrate fermentation of corn kernels at 28°C, produced quantities of ochratoxin A exceeding 50 ppm/g dry weight of sclerotia. Evidence is presented that ochratoxin A accounts for the activity of the methanol extract against larvae of the detritivorous beetle *Carpophilus hemipterus* (Nitidulidae) (75% reduction in feeding rate) and corn ear worm *Helicoverpa zea* (50% mortality with 99% reduction in weight gain among surviving larvae) when incorporated into a pinto bean diet at levels less than those occurring naturally in the sclerotia.

Key words: ochratoxin A, *Aspergillus carbonarius*, sclerotia, *Helicoverpa zea*, *Carpophilus hemipterus*, antiinsectan, chemical defense.

Résumé : L'ochratoxine A, une mycotoxine dont la toxicité pour les insectes a été démontrée, a été isolée des sclérotes du champignon *Aspergillus carbonarius* NRRL 369. Les sclérotes, récoltés sur un substrat solide de grains de maïs fermentés à 28°C, ont produit des quantités d'ochratoxine A excédant 50 ppm/g de poids sec de sclérotes. L'évidence est présentée que l'ochratoxine A est responsable de l'activité d'extraits de méthanol contre les larves de l'insecte détritivore, le *Carpophilus hemipterus* (Nitidulidés), causant une réduction du taux nutritionnel de 75%, et contre le ver de l'épi du maïs, l'*Helicoverpa zea*, produisant 50% de mortalité et 99% de la réduction du gain en poids chez les survivants, lorsque la mycotoxine est incorporée à une diète de haricots blancs tachetés («pinto bean») à des niveaux inférieurs à ceux qui se trouvent naturellement chez les sclérotes.

Mots clés : ochratoxine A, *Aspergillus carbonarius*, sclérote, *Helicoverpa zea*, *Carpophilus hemipterus*, mycotoxine contre les insectes, défense chimique.
[Traduit par la rédaction]

Introduction

In our investigations of the antiinsectan secondary metabolites from fungal sclerotia, the methanol extract of the sclerotia produced by *Aspergillus carbonarius* (Bainier) Thom NRRL 369 showed antiinsectan activity against the corn earworm *Helicoverpa zea* (Boddie) (Lepidopetera: Noctuidae) and the fungivorous beetle *Carpophilus hemipterus* L. (Nitidulidae: Coleoptera). Carbonarin A (Alfatafta 1994), a novel naphthopyrone, was isolated that caused a 31% feeding reduction by *Carpophilus hemipterus* adults when incorporated into a standard pinto bean test diet at 100 ppm. However, the presence of carbonarin A did not explain the toxicity of the methanol extract to *Carpophilus hemipterus* larvae. Here we report the isolation of ochratoxin A from *Aspergillus carbonarius* sclerotia and offer evidence that this metabolite functions in protecting the fungal sclerotium from insect predation.

Materials and methods

A culture of *Aspergillus carbonarius* NRRL 369 (= Thom No. 4030-1; = WB 369; = CBS 556.65) was obtained from the ARS Culture Collection at the National Center for Agricultural Utilization Research in Peoria, Ill. The sclerotia were produced by solid substrate fermentation of corn kernels (4000 g dry weight) which were distributed among 20 Fernbach flasks, soaked overnight (at 50% moisture), and autoclaved. Flasks were inoculated with a conidial suspension of *Aspergillus carbonarius* and incubated, in the dark, for 40 days at 28°C. Following incubation, the sclerotia were separated from the kernels as described by Wicklow et al. (1988). The dried ground sclerotia (93 g) were suspended in methanol and stirred at room temperature for 12 h. This process was repeated four times (4 × 2 L). The combined organic extracts were evaporated under reduced pressure to afford a brown oily residue (6.6 g), which was tested for antiinsectan activity. Assays for antiinsectan activity, as described by Wicklow et al. (1988) and Dowd (1988*a*, 1988*b*), assessed the feeding response (pinto bean diet) of adults and

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[1] Names are necessary to report factually on available data; however, the U.S. Department of Agriculture (USDA) neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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second-instar larvae of the dried fruit beetle *Carpophilus hemipterus* (Nitidulidae) and neonate larvae of the corn earworm *Helicoverpa zea* (Noctuidae). A portion of the methanol extract (20.6 mg) was incorporated into 5000 mg of the pinto bean diet. High performance liquid chromatographic (HPLC) separations employed a binary gradient system composed of two Beckman 110A pumps, a Beckman model 166 variable-wavelength UV detector, a Rheodyne model 7125 injector, and column sizes and types as specified below. Details of nuclear magnetic resonance (NMR) and mass spectrometry (MS) studies and other instrumentation used in this work have been reported elsewhere (TePaske et al. 1989; Belofsky et al. 1995).

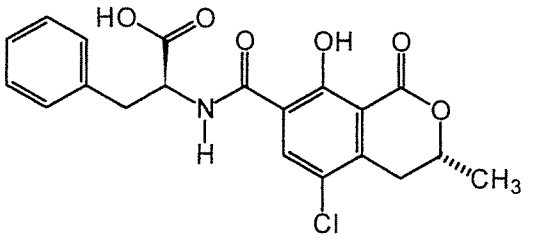
Results and discussion

Aspergillus carbonarius NRRL 369 was isolated from an unknown source by A. F. Blakeslee at Harvard University and sent to Dr. Charles Thom, USDA, for identification in 1915. Because Bainier's type strain had been lost, Al-Musallam (1980) designated CBS 556.65, received as WB 369 from K.B. Raper in 1965, as neotype culture and specimen. The source of Blakeslee's isolate was not "paper," as is commonly cited. Inspection of Thom's notebook (ARS Culture Collection, Peoria, Ill.) shows that the initial entry No. 4030-1, in black pencil, gives no source for Blakeslee's isolate. At some later date, Thom used a blue pencil to enter the species name "*A. carbonarius*—see paper." This probably referred to the paper describing this isolate of *Aspergillus carbonarius* (Thom and Currie 1916) and was erroneously interpreted to mean that the strain was "isolated from paper" (Raper and Fennell 1965).

The methanol extract of sclerotia produced by *Aspergillus carbonarius* NRRL 369 showed limited antiinsectan activity against *Helicoverpa zea* larvae (29% reduction in weight gain (RWG)), and *Carpophilus hemipterus* adults (27% reduction in feeding rate (RFR)), but much greater activity against *Carpophilus hemipterus* larvae (58% RFR). The methanol extract was partitioned between H_2O (1 L) and $CHCl_3$ (4 × 1 L). The resulting organic extracts were combined, evaporated under reduced pressure, and redissolved in 10% H_2O–methanol. This mixture was extracted with hexane (4 × 2 L) to remove nonpolar inactive material. The aqueous methanol fraction was evaporated under reduced pressure to afford a yellowish residue (1.14 g), which was fractionated on a Si gel flash column. Fractionation of the methanol extract afforded two fractions showing potent activity to *Helicoverpa zea* larvae (50 and 53% mortality, respectively, with 99% RWG among surviving larvae), *Carpophilus hemipterus* adults (23 and 62% RFR), and *Carpophilus hemipterus* larvae (75% RFR). The major metabolite accounting for this activity against *Carpophilus hemipterus* and *Helicoverpa zea* was a known compound, ochratoxin A, a dihydroisocoumarin derivative linked through a carboxy group to L-phenylalanine by an amide group (**1**).

Final purification of **1** was carried out by reversed-phase HPLC. The electron ionization (EI) mass spectrum of **1** did not show a molecular ion, but exhibited intense fragment ions at *m/z* 239 and 255. The molecular weight of **1** was determined by analysis of its fast atom bombardment spectrum, which displayed a pseudomolecular ion at *m/z* 404 $(M + H)^+$ with an $(M + H + 2)^+$ isotope peak characteristic of the presence of a chlorine atom in the molecule. These results, in conjunction with data obtained from ^{13}C-NMR and experiments of distortionless enhancement by polarization transfer, suggested the molecular formula $C_{20}H_{18}ClNO_6$. The ^1H- and ^{13}C-NMR spectra revealed the presence of mono- and 1,2,3,4,5-pentasubstituted aromatic rings, one aliphatic methyl substituent, two methylenes, one oxymethine, and one hydrogen-bonded hydroxy group. Comparison of UV and melting point data for the isolated compound with literature values implied that this metabolite might be identical to ochratoxin A. High-resolution EI-MS data revealed that fragment **a** has the molecular formula $C_{11}H_{10}ClNO_4$ (255.0303), which verified the amide linkage in **1** and eliminated the possibility of the ester linkage. Thus the compound was assigned structure **1** (ochratoxin A). The methanol extract of 93 g of *Aspergillus carbonarius* sclerotia afforded 4.7 mg of ochratoxin A (= about 50 ppm natural level); however the isolated yield of this compound underrepresents its true concentration in the sclerotia. In another study, sclerotia produced by *Aspergillus ochraceus* Wilhelm NRRL 3174 (= *Aspergillus alutaceus* Berk. & Curt.), when grown on a synthetic culture medium with xylose as the sole carbon source, were reported to contain 2 ppm ochratoxin A (Paster et al. 1985).

Fig. 1. Structure of ochratoxin A (compound **1**).



Ochratoxin A, being nephrotoxic, teratogenic, and carcinogenic, is considered an important mycotoxin in temperate countries (Kuiper-Goodman and Scott 1989; Castagnero et al. 1991). In tests of oral toxicity to larvae of *Helicoverpa zea* and larvae of the fall army worm *Spodoptera frugiperda*, ochratoxin A caused significant larval mortality at 25 ppm and significant reduction in larval weight gain at 2.5 ppm (Dowd 1989). Ochratoxin A also caused a 78% RGR of *Carpophilus hemipterus* larvae (Dowd 1992). Our primary methanol extract of sclerotia containing at least 50 ppm ochratoxin A did not show potent activity against *Helicoverpa zea* larvae because the actual concentration of ochratoxin A in the diet was only 0.2 ppm. Earlier reports on the toxicity of ochratoxin A to insects include the following: Mediterranean flour moth *Anagasta kuhniella* (Wright and Harein 1982); yellow mealworm *Tenebrio molitor* (Davis 1982); fruit fly *Drosophila melanogaster* and Egyptian cotton leafworm *Spodoptera littoralis* (Patterson et al. 1987); *Heliothis virescens* and *Spodoptera frugiperda* (Patterson et al. 1990). Species of *Aspergillus* produce kojic acid, which has been shown to synergize the toxicity of aflatoxin in tests with insects (Dowd 1988*a*). Penicillic acid, a metabolite produced by some species of *Penicillium* and *Aspergillus* (Ciegler 1972; Frisvad 1989), synergized the oral toxicity of ochratoxin A to *Helicoverpa zea* (Dowd 1989). Kojic acid may synergize the toxicity of ochratoxin A when coproduced by species of *Aspergillus* classified in section *Nigri*. The production of multiple secondary metabolites in fungal sclerotia could thus enhance the toxicity of

ochratoxin A to insect fungivores, a biosynthetically efficient strategy for survival.

Aspergillus carbonarius is classified in *Aspergillus* section *Nigri* (= *Aspergillus niger* group Thom & Church). The subgenus *Nigri* also includes kuro-koji strains used to produce shou-chuu and awamori, traditional alcoholic liquors in Japan. The production of ochratoxin A by two isolates of *Aspergillus foetidus* (Nakazawa) Thom & Raper, a fungus used in the production of shou-chuu, is the first report (Ueno et al. 1991) of this mycotoxin being produced by a species in subgenus *Nigri*. Ono et al. (1995) reported ochratoxin A production on milled rice grains by *Aspergillus awamori* var. *fumeus* Nakazawa et al. IFO 4122 and *Aspergillus usamii* Sakaguchi et al. ex Iizuka & Sugiyama IFO 8875 (= 40 ppb), IFO 8876 (= 239 ppb), IFO 8877 (= 145 ppb), and IFO 6082 (= 9 ppb). However, the three *Aspergillus usamii* strains that produced the highest levels of ochratoxin A are not from koji but were isolated in 1963 from core samples of a deep lithosphere stratigraphic drilling near Kambara, Niigata, Japan (Sugiyama 1967). Abarca et al. (1994) reported ochratoxin A production by strains of *Aspergillus niger* var. *niger* van Tieghem and Horie (1995) showed that *Aspergillus carbonarius* produces ochratoxin. Ochratoxin A is better known as a mycotoxin produced by *Penicillium verrucosum* Dierckx and members of *Aspergillus* section *Circumdati* (= *Aspergillus ochraceus* group Thom & Church) (Frisvad 1989, 1994). The latter included strains of *Aspergillus alutaceus*, *Aspergillus fresenii* Subram. (= *Aspergillus sulphureus* (Fres.) Wehmer), *Aspergillus sclerotiorum* Huber, *Aspergillus alliaceus* Thom & Church, *Aspergillus melleus* Yukawa, *Aspergillus ostianus* Wehmer, and *Aspergillus petrakii* Voros (Ciegler 1972; Hesseltine et al. 1972). Each of these species also produce sclerotia. Sclerotia produced by *Aspergillus* spp. have proven to be a rich source of antiinsectan compounds with activity against *Helicoverpa zea* and *Carpophilus hemipterus* (Wicklow et al. 1994). Fungal sclerotia can be important to the long-term survival of a fungus and ochratoxin A may contribute to the survival of *Aspergillus carbonarius* sclerotia by functioning an effective chemical defense against fungivorous insects.

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