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Isolation and Identification of *Aspergillus fumigatus* Mycotoxins on Growth Medium and Some Building Materials

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Genotoxic and cytotoxic compounds were isolated and purified from the culture medium of an indoor air mold, *Aspergillus fumigatus*. One of these compounds was identified as gliotoxin, a known fungal secondary metabolite. Growth of *A. fumigatus* and gliotoxin production on some building materials were also studied. Strong growth of the mold and the presence of gliotoxin were detected on spruce wood, gypsum board, and chipboard under saturation conditions.

Molds and actinomycetes are some of the microbial types that are associated with moisture- and mold-damaged buildings (10, 17). Exposure to dampness and different indoor air microorganisms is now accepted as harmful in both occupational and home environments. These microorganisms produce a diverse range of health effects including many irritative symptoms in the respiratory tract and eyes, allergies, asthma, and respiratory infections (9, 13, 14, 20, 27, 28). There are also many hazardous agents responsible for these health effects, i.e., immunogenic, physiologically reactive, and toxic cell wall components or metabolic products produced by microorganisms. Molds and actinomycetes are known to produce bioactive secondary metabolites, though the contribution of these metabolites to the human symptoms associated with microbial aerosols is still largely unknown. Trichothecene mycotoxins produced by *Stachybotrys chartarum* have been associated with mycotoxicosis in people living or working in buildings infested with this fungus (4, 11).

Actinomycetes and molds isolated from soil or indoor air are known to produce genotoxic secondary metabolites (8, 21). As long as the chemical nature of these compounds remains obscure, it is difficult to evaluate their toxicological significance. Therefore, our objective has been to detect and isolate genotoxic compounds from different indoor air molds and actinomycetes. The production of microbial toxins on water-damaged building materials (2, 18, 19, 25) has been assessed, but there is no knowledge about the production of *Aspergillus fumigatus* specific secondary metabolites under similar conditions. However, *A. fumigatus* belongs to the group of indicator microorganisms typical of moisture-damaged buildings (23).

In this study, we describe the isolation of three genotoxic and cytotoxic fractions from the cultivation medium of *A. fumigatus* isolated from indoor air and the identification of one of these fractions as gliotoxin. We also investigated the growth of, and gliotoxin production by, *A. fumigatus* on some building materials.

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MATERIALS AND METHODS

The tester strain, *Aspergillus* sp. strain HT30, was among the strains isolated from the indoor air of buildings with a history of moisture problems producing symptoms in the inhabitants of the building. These symptoms included those of respiratory irritation, such as rhinitis, rising phlegm, night cough, and hoarseness; irritation of eyes and skin; fatigue; and subfebrile temperatures and also included common respiratory infections, such as sinusitis and bronchitis. Subsequently, the isolate was typed as *A. fumigatus* by the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

For the isolation of genotoxic compounds from cultivation medium, HT30 was cultured at 30°C for 6 to 7 days in a medium containing glycerol-arginine-yeast extract (8). The cultivation broth was made acidic and extracted with chloroform. The dry extraction residue was separated by thin-layer chromatography (silica 60 F254; Merck, Darmstadt, Germany; mobile phase, methanol-chloroform [9:1, vol/vol]). The active fraction was eluted with methanol and evaporated to dryness. The residue was purified by high-performance liquid chromatography (HPLC) with a Kromasil C_8 (5 μm, 150 by 4.6 mm; Higgins Analytical, Mountain View, Calif.) column, isocratic elution at a flow rate of 1 ml/min, and acetonitrile and water (25:75) as the mobile phase. The eluent was monitored in a UV spectrometer at $\lambda = 264$ nm, and active compounds were collected. For nuclear magnetic resonance (NMR) studies, the purification of compounds was performed by column chromatography with a 2.5- by 40-cm glass column containing silica phase (Kieselgel 60, 0.063 to 0.200 mm; Merck) and a mixture of dichloromethane and methanol (99.25:0.75, vol/vol) to elute active compounds. The column fractions were monitored by thin-layer chromatography with thin-layer chromatography silica plates (silica 60 F254) and a mixture of dichloromethane and methanol (96:4, vol/vol) as the mobile phase. The active fractions were further purified with a Kromasil C_8 (5 μm, 250 by 10 mm; Eka Nobel AB, Surte, Sweden) column, isocratic elution at a flow rate of 3 ml/min, and acetonitrile and water (40:60) as the mobile phase. For the identification of isolated fractions, ^{1}H and ^{13}C NMR spectra were recorded with a Bruker Avance DRX 500 (500-MHz) spectrometer (Rheinstetten, Germany) in $CDCl_3$. Electron ionization mass spectra were obtained with a VG 70-250SE magnetic sector mass spectrometer (VG, Manchester, United Kingdom), and the UV spectrum was measured with a Varian Cary 50 Bio spectrophotometer (Mulgrave, Victoria, Australia).

For genotoxicity screening purposes a simple bioassay was used based on the differential response of DNA repair-deficient and repair-proficient *Escherichia coli* strains (7). Cytotoxic activity was screened by using a mouse hepatoma cell line (Hepa-1, subclone Hepa-1c1c7) as described by von Wright et al. (26).

Building material tests were performed with unused spruce wood, gypsum board, chipboard, and mineral wool as building material samples. Materials were cut into disks 90 mm in diameter, sterilized, and wetted with sterile water before

the inoculation. Identically treated materials without inoculation were used as material controls. From the spore suspension of *A. fumigatus*, 2×10^8 spores in 0.5 ml were inoculated onto the building material pieces. The pieces were incubated under saturated water conditions in sterilized 4-liter glass vessels at 20 to 23°C for 12 weeks. The vessels were aerated once a day for 10 min at a flow rate of 400 ml min^{-1}. Microbial growth and sporulation were observed visually and with swab samples.

The spores were collected from the building material pieces (spruce, gypsum board, and chipboard) with a scalpel. Following this, 1 to 2 mm of the material surface, weighing from 0.7 g (wood) to 1.5 g (chipboard), was scraped off with a knife. Mineral wool samples were cut into pieces and extracted with 100 ml of methanol as such (2.5 h, 180 rpm). Spores from other materials were extracted with methanol for 2.5 h on a rotary shaker (180 rpm), and surface pieces were extracted for 1 day (100 rpm). After the extraction, the samples were filtered and evaporated to dryness. Surface samples were further fractionated by thin-layer chromatography with silica plates (silica 60 F254), a mixture of dichloromethane and methanol (9:1, vol/vol) for the development of plates, and gliotoxin as a standard compound. The gliotoxin-containing fractions were eluted with methanol and evaporated to dryness.

For analysis of the samples by HPLC combined with mass spectrometry (MS), samples were dissolved in acetonitrile-water and filtered through an 0.45-μm-pore-size filter (Millipore SA, Molsheim, France). The HPLC system consisted of a Rheos 4000 pump (Flux Instruments, Danderyd, Sweden) and a LaChrom L-7200 autosampler (Merck-Hitachi, Tokyo, Japan). The compounds were separated with a Genesis C_{18} column, 2 by 50 mm (Jones Chromatography, Lakewood, Colo.), with a 5 to 90% acetonitrile gradient in 8 min, the flow was set to 200 μl/min, and the injection volume was either 20 or 40 μl. Mass spectra were recorded with an LCQ quadrupole ion trap mass spectrometer (Finnigan, San Jose, Calif.) by atmospheric pressure chemical ionization. The source voltage was set to 4 kV in the positive ion mode. The spray was stabilized by a nitrogen sheath flow, and the value was set to 90 (arbitrary units). The vaporizer temperature was set to 400°C, and the inlet capillary temperature was 150°C. The HPLC-MS system was used to first measure the full-scan mass spectrum and collision-induced dissociation mass spectrum of standard gliotoxin. The identification of gliotoxin in samples was based on selected reaction monitoring of the fragment ions at *m/z* 245 and *m/z* 263, which were formed by collision-induced dissociation from the MH^+ ion of the gliotoxin at *m/z* 327. Helium was used as the target gas, and collision energy was set to 30% (arbitrary units).

RESULTS

The genotoxic compounds were recovered in three successive HPLC fractions with retention times of 10, 18, and 20 min. In the repair assay all of these fractions were more active against *E. coli* CM781 (repair deficient) than against *E. coli* WP2 (repair proficient), indicating some form of genotoxic damage (Fig. 1). All three fractions were also toxic to mouse hepatoma cells. The 50% effective dose of the most active fraction (retention time, 10 min) was approximately 0.3 μg/ml (data not shown).

The most genotoxic and cytotoxic fraction, with a retention time of 10 min, was identified as gliotoxin on the basis of its ^1H and ^{13}C NMR spectra combined with electron ionization MS and UV spectral analysis. Identification of gliotoxin was also confirmed by using a database search (AntiBase 1.3; Chemical Concepts, Weinheim, Germany). Also the 18-min fraction was pure enough for tentative identification, and it turned out to be a mixture of gliotoxin (two sulfurs), gliotoxin E (three sulfurs), and gliotoxin G (four sulfurs). Although the poor recovery of the 20-min fraction prevented its further analysis, it most likely also represents a gliotoxin or a gliotoxin derivative.

In the analysis of gliotoxin from building materials the atmospheric pressure chemical ionization mass spectrum of gliotoxin shows a protonated molecule at *m/z* 327 and no major fragment ions (Fig. 2). The collision-induced dissociation MS-MS spectrum of the gliotoxin standard (Fig. 3A) shows a major fragment ion at *m/z* 263, which is presumably formed by loss of S_2 from the protonated molecule. The other main fragment is seen at *m/z* 245, which indicates additional loss of water.

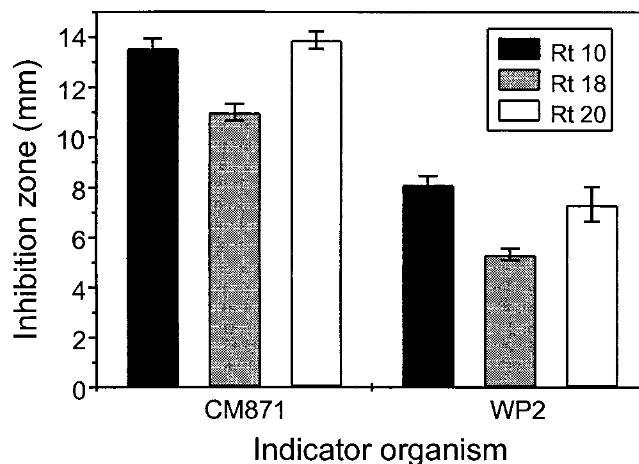

FIG. 1. Inhibition zones for *E. coli* CM871 *trpE65 urvA155 recA56 lex*A (repair-deficient) and WP2 *trpE56* (repair-proficient) strains caused by HPLC fractions with retention times of 10 (identified as gliotoxin), 18, and 20 min. The concentration of each fraction was 200 μg/ml, and the sample volume was 100 μl. The well diameter (10 mm) has been subtracted. A difference of 5 mm or more in the diameter of inhibition zones between repair-proficient and -deficient strains is considered a positive response. For the solvent control, the test typically yielded an inhibition zone of 0 to 2 mm.

With the HPLC system used, the gliotoxin standard gave a chromatographic peak with a retention time of 5.3 min. The identification of gliotoxin in samples was based on identical retention time and identical fragment ions found for the gliotoxin standard (Fig. 3 and 4). Figures 3 and 4 present spectra and chromatograms of the gypsum board sample, the gypsum board control sample, and the gliotoxin standard as an example of gliotoxin analysis.

Data for growth of, and gliotoxin production by, *A. fumigatus* on building materials are presented in Table 1. Production of gliotoxin was detected on spruce wood, gypsum board, and chipboard surfaces but not in spores grown on materials. No growth or gliotoxin production was observed for the control samples.

DISCUSSION

Gliotoxin, a known fungal secondary metabolite, is produced by various *Penicillium* and *Aspergillus* species (22) but also by *Gliocladium* (12), *Thermoascus* (30), and *Candida* (24) species. Gliotoxin belongs to the epipolythiodioxopiperazine class of secondary metabolites. The properties of gliotoxin have been investigated in a number of studies, which have revealed its many biological actions (29). Cytotoxicity is a well-known property of gliotoxin, confirmed in the present study.

The mechanism of gliotoxin toxicity is believed to be based on its ability to produce reactive oxygen species (5). These reactive oxygen species or other radicals generated through redox cycling are also capable of causing DNA damage as demonstrated elsewhere in in vitro studies (3, 5, 6). In this study, we found that bacterial DNA repair systems appear to

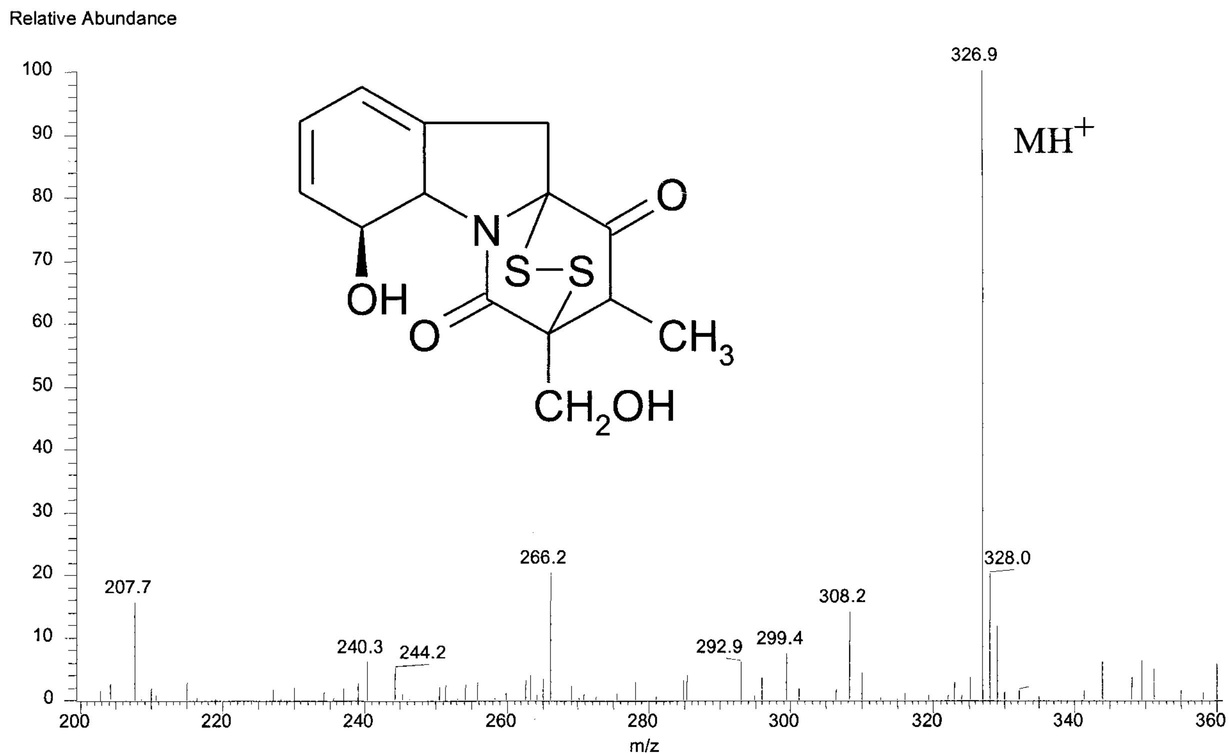

FIG. 2. Structure and full-scan electrospray ionization mass spectrum of gliotoxin.

protect cells against the effects of gliotoxin. The implication is that, in accordance with the studies cited above, one of the targets of gliotoxin in bacterial cells is DNA. However, it is not yet possible to determine to what extent the cytotoxic activity of gliotoxins in mammalian cells reflects their DNA reactivity in view of their many other toxic effects.

Aspergillus species can colonize the respiratory mucosa in the normal respiratory tract, especially if it is damaged (15, 16).

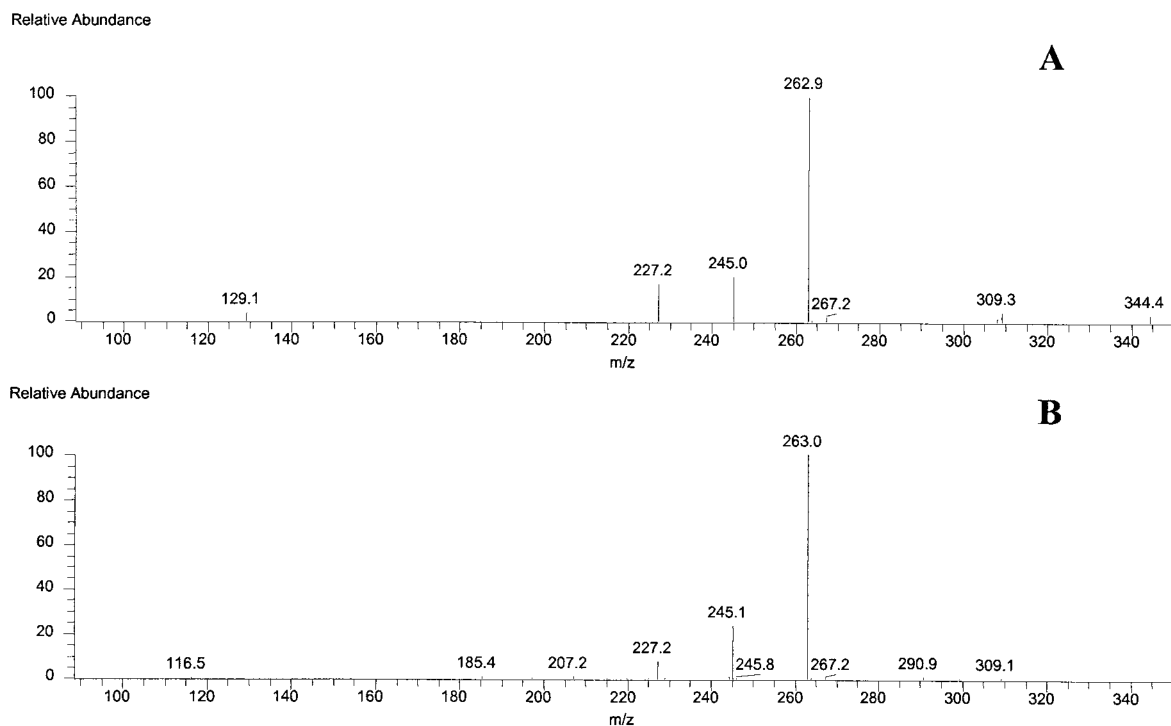

FIG. 3. MS-MS spectra of gliotoxin standard (A) and gliotoxin found in gypsum board sample (B).

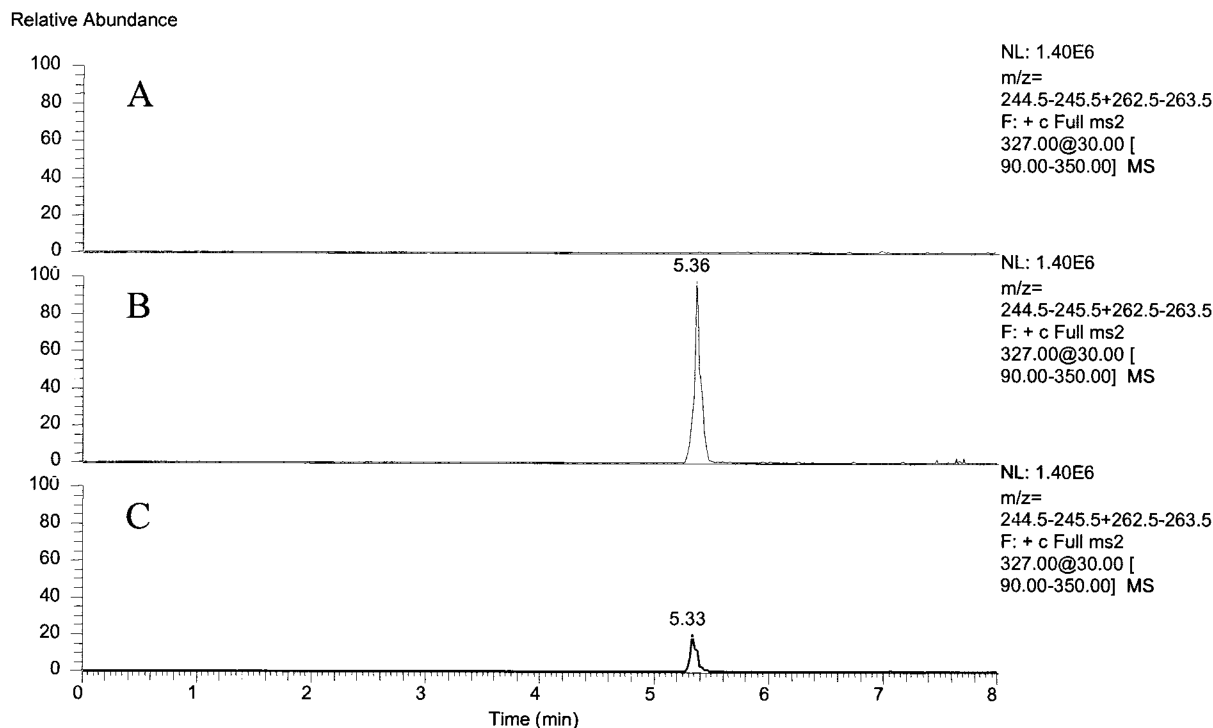

FIG. 4. Selected reaction monitoring chromatograms of gypsum board control (A), gypsum board sample (B), and gliotoxin standard (0.5 μg/ml) (C).

The mechanism of colonization is still poorly understood, but the ability of culture filtrates of *A. fumigatus* to decrease ciliary beating frequency (gliotoxin has been identified as a cilioinhibitory factor) and to damage human respiratory epithelium in vitro (R. Amitani, A. Sato, Y. Matsui, A. Niimi, K. Yamada, E. Tanaka, T. Murayama, K. Maeda, and F. Kuze, American Thoracic Society meeting, Am. Rev. Respir. Dis. **145:**A548, 1992) may have some role. It is also possible that epithelial damage caused by *Aspergillus* could release nutrients for fungal growth. In view of the bioproperties of gliotoxin, the possibility that respiratory epithelium is exposed to this toxin represents a health hazard not only because of the cilioinhibitory activities of this agent but also because of its genotoxic and cytotoxic properties.

As far as we are aware, this is the first time that *A. fumigatus* has been shown to produce toxic substances when grown on common building materials. In this specific case, the toxins were not associated with spores or mycelium but rather were absorbed into the materials themselves. The concentrations of gliotoxin known to lower ciliary beating frequency or cause DNA adduct formation were above 0.2 and 32.6 μg/ml, respectively (1, 6). These can be compared to the levels detected in the experimentally mold-damaged building materials reported here, from 1 to 40 ng of gliotoxin per cm^2 depending on the building material tested. According to these results, the amount of gliotoxin detected, for example, from an area of 100 cm^2 of gypsum board could lower the ciliary beating frequency, provided, of course, that there is a quantitative release of gliotoxin from contaminated materials. However, formation of DNA adducts would require a greater amount of gliotoxin to be released from building materials. Although it is not yet known how common the production of gliotoxin is in actual moisture-damaged buildings (materials from the specific building from which *A. fumigatus* HT30 was isolated are not available for analysis any more), or to what extent and by what kind of mechanism gliotoxin could be mobilized from building materials, contaminated building materials may present a risk to the health of occupants. Also, the present observation certainly highlights the need for further research into the metabolic activities of indoor air fungi.

TABLE 1. *A. fumigatus* growth and gliotoxin production on various building materials after a 12-week incubation

Sample material	Growth[a]	Gliotoxin concn[b]	
		Spores	Surface
Control samples			
Wood	−	ND	ND
Gypsum board	−	ND	ND
Chipboard	−	ND	ND
Mineral wool	−	ND	ND
Exptl samples			
Wood	++	ND	1
Gypsum board	+++	ND	20
Chipboard[c]	+++	ND	4
Mineral wool	+	ND	ND

[a] −, no growth; +, slight growth; ++, 50% covered by mycelium; +++, material totally covered by mycelium.
[b] The average concentration of two samples (nanograms per square centimeter); ND, not detected.
[c] Results of one sample.

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