Antagonistic microorganisms produce antimicrobials to inhibit the growth of competitors. Although water-soluble antimicrobials are limited to proximal interactions via aqueous diffusion, volatile antimicrobials are able to act at a distance and diffuse through heterogeneous environments. Here, we identify the mechanism of action of *Muscodor albus*, an endophytic fungus known for its volatile antifungal activity toward a wide range of human and plant pathogens and its potential use in mycofumigation. Proposed uses of the *Muscodor* species include protecting crops, produce, and building materials from undesired fungal or bacterial growth. By analyzing a collection of *Muscodor* isolates with varying toxicity, we demonstrate that the volatile mycotoxin, *N*-methyl-*N*-nitrosoisobutyramide, is the dominant factor in *Muscodor* toxicity and acts primarily through DNA methylation. Additionally, *Muscodor* isolates exhibit higher resistance to DNA methylation compared with other fungi. This work contributes to the evaluation of *Muscodor* isolates as potential mycofumigants, provides insight into chemical strategies that organisms use to manipulate their environment, and proffes questions regarding the mechanisms of resistance used to tolerate constitutive, long-term exposure to DNA methylation.

Microorganisms produce numerous secondary metabolites to mediate interactions between organisms in their diverse ecological niches (1). For example, production of antimicrobial agents can be used to inhibit competitors to gain selective advantages, to serve as signaling mechanisms, and to provide protection to symbiotic organisms (2). Antimicrobial agents act by disrupting essential cell functions such as cell wall synthesis, transcription, translation, membrane integrity, and DNA replication (3). Although much focus has been placed on understanding the mechanisms of action of soluble antimicrobials, volatile antimicrobials are relatively understudied (4, 5).

Volatile organic compounds (VOCs)2 are characterized by their low molecular weights and high vapor pressures, features that facilitate evaporation at ambient temperature and pressure (4, 6). Several VOCs from filamentous fungi (7–9), yeast (10, 11), and bacteria (12, 13) have demonstrated antimicrobial activity. Volatile antimicrobials are able to act over a longer distance compared with soluble metabolites, and they can diffuse through heterogeneous environments such as soil and air (12–14). Although volatile antimicrobial activities and VOC profiles of several species have been identified, there are significantly fewer conclusive links between the volatiles responsible for toxicity and their mechanisms of action compared with soluble antimicrobials.

Here we investigate the mechanism of action of fungi in the genus *Muscodor*. *Muscodor* species are typically isolated as endophytes, organisms that reside asymptotically in the inner tissues of plants (15). *Muscodor* isolates produce volatile antimicrobials capable of inhibiting or killing a broad range of plant and human pathogens and certain insect and nematode pests (7, 16, 17). *Muscodor albus* was the first isolate discovered in this genus in the late 1990s. Since then, numerous strains of *M. albus* (18–22) and over 14 additional species of fungi within the *Muscodor* genus have been identified (16, 23–32). These *Muscodor* isolates are widely distributed across the globe and have been found in North and South America, Indonesia, India, East Asia, and Australia (7, 18, 20–22, 32).

*Muscodor* species have been investigated as a promising source for fungal biofumigation, also called “mycofumigation,” to replace chemical biocides, such as methyl bromide, which is being phased out globally due to its role in ozone depletion (33, 34). Furthermore, there is an urgent need for fumigation alternatives as continuous use of certain fungicides such as imazalil, thiabendazole, and *o*-phenylenediamine has resulted in increased resistance to these chemicals (35, 36). Scientific investigations

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2 The abbreviations used are: VOC, volatile organic compound; MNIBA, *N*-methyl-*N*-nitrosoisobutyramide; SPME, solid-phase microextraction; HCA, hierarchical clustering analysis; MNU, *N*-methyl-*N*-nitrosourea; hAAG, human 3-alkyladenine DNA glycosylase; NIST, National Institute of Standards and Technology; ITS, internal transcribed spacer; PDA, potato dextrose agar.
of Muscodor species have demonstrated their potential to prevent soil-borne infection of crops (37, 38), inhibit mold growth on produce after harvest (36), and protect construction materials from biological damage (39).

Despite these applications, little is known about the mechanism of Muscodor toxicity. Understanding how Muscodor species suppress microbial growth using volatile mycotoxins would contribute to a better understanding of the biological strategies fungi use to manipulate their environment and help evaluate their potential use in mycofumigation. M. albus volatile generally consists of a mixture of acids, esters, alcohols, ketones, and aromatic hydrocarbons with a particular abundance of 3-methyl-1-butanol and isobutyric acid derivatives (7, 36). However, no individual compound or class of compounds has been identified as responsible for M. albus toxicity (7). The prevailing hypothesis is that a synergistic effect among multiple VOCs is responsible for toxicity. However, although artificial mixtures can mimic some inhibitory effects of M. albus, the effects are not fully replicated with synthetic mixtures (7). Previous studies by Alpha et al. (40) have suggested that M. albus acts by damaging DNA, with evidence indicating that DNA alklation plays a major role.

Recently, a patent filing by Jimenez et al. (41) observed that M. albus CZ-620 produces N-methyl-N-nitrosoisobutyramide (MNIBA), a VOC that had previously been undetected in standard gas chromatography-mass spectrometry (GC-MS) assays (MNIBA), a VOC that had previously been undetected in standard gas chromatography-mass spectrometry (GC-MS) assays (MNIBA), a VOC that had previously been undetected in standard gas chromatography-mass spectrometry (GC-MS) assays. MNIBA protected produce and plants from pathogenic infection, suggesting that MNIBA plays a role in M. albus toxicity (41). The filing suggests that MNIBA inhibits target organisms through its conversion into an alkylating or nitrosylating agent (41).

We sought to investigate the role and mechanism of MNIBA in the toxicity of multiple Muscodor isolates. By utilizing a suite of Muscodor isolates with varying toxicity, we demonstrate that MNIBA is a dominant factor in Muscodor toxicity. The amount of production of MNIBA by the Muscodor isolates correlates with the potency of their toxicity, and a commercially available standard of MNIBA mimics the bioactivity profile for M. albus and causes direct damage to DNA. These data explain the mode of action of Muscodor toxicity and invoke questions of resistance to long-term DNA-damaging agents.

Results

A collection of Muscodor sp. isolates with a broad range of toxicity

Muscodor species isolated from sources around the world demonstrate a wide range of antimicrobial activity. They are, in part, categorized by their different VOC production profiles (42); however, there have been no systematic comparisons assessing the relationship between the potency of Muscodor toxicity and VOC production. By analyzing this relationship across multiple Muscodor isolates under the same conditions, we sought to determine the VOC(s) critical for Muscodor toxicity. Ten Muscodor sp. isolates were collected from the rainforests of Peru and Ecuador as part of the Yale University Rainforest Expedition and Laboratory class (15, 43). Previously reported isolates M. albus CZ-620 (7) and Muscodor crisps B23 (30) were also included in the collection of Muscodor isolates. Information on the 12 Muscodor isolates and their host plants is summarized in Table 1. Sequencing the 5.8S internal transcribed spacer (ITS) region revealed close relation among the Muscodor isolates, although we did not attempt to classify them beyond the genus level. None of the isolates produce spores that would be used for more discriminating mycological assignment.

| Isolate | Host plant | Country | Accession no. |
|---------|------------|---------|--------------|
| P1509A  | Calycopus roraimensis | Peru    | EU977187     |
| P1813B  | Uncaria guianensis   | Peru    | EU977197     |
| P1907B  | Vernomanduca membranacea | Peru | EU977261     |
| P912B   | Psychotria erranti   | Peru    | EU977236     |
| P913A   | Hibiscus adscensionis | Peru    | EU977208     |
| E3801A  | Cavendishia micayensis | Ecuador | KU659024    |
| E3816A  | Balaninia macrostachys | Ecuador | KU659025    |
| E6011C  | Brownea grandice    | Ecuador | KU659026     |
| E6710B  | Tectaria incisa      | Ecuador | HM999898     |
| E8514H  | Besleria quadrangulara | Ecuador | HQ117854    |
| M. albus CZ-620  | Connonumum zeylanicum | Bolivia | EU956129    |
| M. crisps B-23  | Ananas ananassoides | Bolivia | EU195297    |

a Strobel et al. (7).
b Mitchell et al. (17).

The viability of Muscodor isolates was determined by counting CFUs after 7 h of exposure to the Muscodor VOCs compared with a media-only control. The Muscodor isolates demonstrate a range of toxicity against this E. coli strain that varied by 5 orders of magnitude (Fig. 1). Isolate P1907B is the most toxic organism tested (7 × 10−6 fraction viable), whereas isolates E3801A, P1907B, and P1813B display minimal toxicity (∼8 × 10−1 fraction viable). Interestingly, the most widely studied Muscodor isolate (the original M. albus CZ-620) has an intermediate level of toxicity (4 × 10−3 fraction viable) compared with the other isolates. The large variation in bioactivity found in this suite of Muscodor isolates provides a panel to identify potential patterns in Muscodor VOC production profiles and to identify the active agent or agents.

Muscodor isolates produce N-methyl-N-nitrosoisobutyramide commensurate with their bioactivity

Toxicity may be due to a synergistic effect of multiple VOCs, or alternatively, it could be due wholly or primarily to one agent. To identify compounds critical for Muscodor toxicity, we analyzed the VOCs produced by the collection of Muscodor isolates with a broad range of toxicity using hierarchical clustering analysis. First, volatiles were extracted from the headspace of 3-day-old Muscodor cultures using a solid-phase microextraction (SPME) fiber and analyzed via GC-MS. Because of previous reports of thermally labile VOCs produced by M. albus (41), we conducted analyses at both a high injector temperature (240 °C)
DNA methylation by fungi in the genus Muscodor

We used hierarchical clustering analysis (HCA) to compare the Muscodor toxicity profile (generated from the reciprocal of fraction viable E. coli 25922 data in Fig. 1) to VOC production profiles across all Muscodor isolates. HCA of the volatiles revealed that the toxicity profile clusters most closely and exclusively with MNIBA production, indicating that Muscodor isolates are producing MNIBA in a manner that correlates to their toxicity potency (Fig. 3). The clear separation in the HCA-branching structure shows that the toxicity and MNIBA production profiles are closely related to each other and that toxicity does not correlate with any other single compound or cluster of compounds in the VOC dataset.

We also analyzed the relationship of Muscodor toxicity to individual production of key candidate VOCs at their optimal injector temperatures (140 °C for MNIBA and 240 °C for all other VOCs examined). The HCA in Fig. 3 compares the Muscodor toxicity profile to Muscodor volatiles observed at the lower 140 °C injector temperature. The lower injector temperature is necessary for observing MNIBA production; however, it is not an ideal volatilization temperature to observe production of other Muscodor volatiles. Isobutyric acid (17, 25, 26), methyl isobutyrinate (19), 3-methyl-1-butanol (7, 26, 44), 3-methyl-1-butanol acetate (7, 20, 21), and naphthalene (16, 18) have previously been reported to be produced in high abundance in some, but not all, Muscodor species and have been proposed as possible candidates responsible for Muscodor toxicity. We observed a correlation between Muscodor toxicity and MNIBA production (Fig. 4); however, we did not observe any correlation between Muscodor toxicity and production of isobutyric acid, methyl isobutyrinate, 3-methylbutyl acetate, or 3-methyl-1-butanol (supplemental Fig. S1). Naphthalene production was not observed in the VOC production profiles of any Muscodor isolate and therefore was not included in this analysis.

We also collected a VOC production data set at 240 °C for the 12 Muscodor isolates and performed HCA. We did not observe strong discernible trends based on HCA of the volatiles detected at 240 °C; however, we noted that toxicity clustered in closest proximity with a C_5H_11NO compound and isobutyric acid (supplemental Fig. S2). The chemical identity of the C_5H_11NO was not defined, but top candidates include N-methyl-isobutryamide and N-(1-methylpropyl)-formamide. M. cris- pans had been previously reported to produce a C_5H_11NO compound that was tentatively identified as N-(1-methylpropyl)-formamide, but the assignment was not confirmed with an authentic standard (17). Isobutyric acid has been reported as the most abundant compound in several Muscodor species and is one of the main compounds attributed to Muscodor toxicity (17, 25, 26, 38, 39). It is noteworthy that isobutyric acid and N-methyl-isobutyramide are degradation products of MNIBA (41) suggesting the possibility that these compounds are not the active agents but are the breakdown products of the active agent.

MNIBA mimics M. albus bioactivity profile

Previous studies have shown that different strains of E. coli have different sensitivities to M. albus exposure (40). E. coli HB101, DH10B, and 25922 are sensitive to M. albus volatiles, whereas E. coli BW25113 and 35218 show higher levels of resis-
To determine whether MNIBA was capable of mimicking the bioactivity profile of *M. albus*, the five *E. coli* test strains mentioned above were exposed to the MNIBA standard and *M. albus*. Commercial MNIBA was added to premium quality pump oil in a sterilized microcentrifuge cap for steady release as a VOC from a non-volatile, inert solvent. When *E. coli* 25922 cultures were exposed to moderate amounts of MNIBA (0.08–0.1 mg), MNIBA inhibited the same test strains.
DNA methylation by fungi in the genus Muscodor

Previously, a screen of E. coli single-gene knock-outs revealed that E. coli containing gene deletions in DNA repair, DNA metabolism, and stress-response pathways demonstrated high sensitivity to M. albus volatiles (40). The E. coli Keio collection, which consists of single-gene knock-outs of nonessential genes in E. coli K12 strain BW25113 was used for this analysis. In particular, sensitivity of gene knock-outs involved in repair of alkylated DNA suggested that M. albus VOCs may induce alkylcylation (40). MNIBA has structural similarity to the well studied model N-nitrosamide, N-methyl-N-nitrosourea (MNU), which is a well characterized DNA-methylating agent (45). Based on the mechanism of DNA methylation by MNU, we postulated that MNIBA exposure would similarly result in DNA methylation.

To test whether DNA methylation occurs as a result of exposure to Muscodor VOCs, we adapted a strategy described by Wu et al. (46) for detecting DNA methylation using DNA repair enzymes and changes in DNA superhelicity (Fig. 6). We exposed supercoiled plasmid DNA to Muscodor VOCs or the MNIBA standard, and DNA was incubated with two repair enzymes, human 3-alkyladenine DNA glycosylase (hAAG) and human apurinic/apyrimidin endonuclease (APE1). If the DNA contains a methylated adduct recognized by hAAG, the enzyme removes the base via N-glycosidic bond cleavage and creates an abasic site. APE1 subsequently nicks the plasmid at the abasic site, releasing the superhelicity of the plasmid and converting supercoiled DNA into relaxed nicked DNA. The relaxed DNA can be distinguished from the supercoiled DNA via agarose gel electrophoresis.

hAAG is a DNA glycosylase that excises several alkylated DNA base sites, including N3-methyladenine, N7-methyladenine, and N7-methylguanine (47). hAAG also recognizes 1,6-ethenoadenine and hypoxanthine (47); however, given that N7-methylguanine is the main DNA adduct generated by N-methyl-N-nitroso compounds (48), we expect that hAAG will primarily recognize sites of DNA methylation. As a negative control, DNA was incubated with human 8-oxoguanine DNA N-glycosylase 1 (hOGG1), a DNA glycosylase that only recognizes oxidation adducts, in place of hAAG.

Using this nicking assay, DNA methylation can be indirectly detected (Fig. 6A). All samples are compared with a nicked control (Fig. 6A, lane 1). Upon addition of both hAAG and APE1, significantly increased amounts of nicked DNA were observed after exposure to 1 mg of MNIBA (Fig. 6A, lane 1; 97% nicked) and the most toxic Muscodor isolate P912B (lane 16; 61% nicked) compared with a media-only control (lane 6; 10% nicked). Controls that contained no modifying enzymes did not yield significant amounts of nicked DNA (Fig. 6A, lanes 2, 7, and 12). There was no effect upon incubation with hAAG (Fig. 6A, lanes 3, 8, and 13) or hOGG1 (lanes 5, 10, and 15) in the absence of APE1.

We also observed increased nicked DNA upon addition of only APE1 in samples exposed to MNIBA (Fig. 6A, lane 9; 66%
DNA methylation by fungi in the genus Muscodor

Muscodor species are known to produce different VOC profiles, but it is unclear whether they utilize similar mechanisms of action. To test whether Muscodor isolates in our collection act similar to M. albus, we screened a partial set of the Keio single-gene knock-out collection against VOCs of four Muscodor isolates (E6710B, M. albus, M. crispans, and P1509A) representing different levels of toxicity (Fig. 7). This partial collection consisted of 48 E. coli BW25113 knock-outs with gene deletions representing pathways in DNA repair, membrane integrity and stress, and oxidative response, categories that had been sensitive to M. albus volatiles in the original screen (40). After exposure to Muscodor VOCs, the viability of each knock-out was qualitatively assessed in comparison with knock-out controls that had not been exposed to volatiles. Results were individually scored on a scale of 0 to 2 (0 = no growth; 1 = reduced growth; 2 = no change from control).

Common features between Muscodor mechanisms of toxicity

Muscodor species are known to produce different VOC profiles, but it is unclear whether they utilize similar mechanisms of action. To test whether Muscodor isolates in our collection act similar to M. albus, we screened a partial set of the Keio single-gene knock-out collection against VOCs of four Muscodor isolates (E6710B, M. albus, M. crispans, and P1509A) representing different levels of toxicity (Fig. 7). This partial collection consisted of 48 E. coli BW25113 knock-outs with gene deletions representing pathways in DNA repair, membrane integrity and stress, and oxidative response, categories that had been sensitive to M. albus volatiles in the original screen (40). After exposure to Muscodor VOCs, the viability of each knock-out was qualitatively assessed in comparison with knock-out controls that had not been exposed to volatiles. Results were individually scored on a scale of 0 to 2 (0 = no growth; 1 = reduced growth; 2 = no change from control).
Scores of each set of triplicates were consistent throughout the experiment. The parental wild-type strain, BW25113, was resistant to the four *Muscodor* isolates (supplemental Fig. S7).

VOCs from all *Muscodor* isolates strongly affect *E. coli* cultures that have defects in DNA repair, suggesting that this is a common feature across the *Muscodor* isolates. In particular, several of the gene knock-outs suggest DNA alkylation is occurring upon VOC exposure to the toxic *Muscodor* isolates. For example, knock-outs of AlkA, also known as 3-methyladenine DNA glycosylase II, are completely inhibited by VOCs from all *Muscodor* isolates tested. AlkA primarily excises nucleobases containing alkylation adducts such as N3-methyladenine, N7-methylguanine, and N7-methyladenine, although it also recognizes O2-alkylpyrimidines, hypoxanthine, 1,N6-etheno- adenine, and 5-formyluracil (51). Additionally, knock-outs of the DNA methyltransferase Ada are completely inhibited upon exposure to VOCs from *M. albus* and P1509A and sensitive upon exposure to *M. crispans* and E6710B volatiles. Ada is an O6-methylguanine-DNA methyltransferase that also acts as a transcriptional regulator in the adaptive response to alkylating agents (52).

Knock-outs that maintained resistance to *Muscodor* VOCs also provide insight into the *Muscodor* mechanism of action. For example, *E. coli* containing mutM, nfo, and nei deletions are not affected by any *Muscodor* VOCs. MutM is a DNA glycosylase that specifically removes 8-oxo-guanine adducts (53), and Nfo and Nei are endonucleases that respond to oxidative damage (54, 55). The sensitivities and insensitivities of gene knock-outs observed in this genetic screen, in combination with the observation that *Muscodor* isolates are producing a volatile methylating agent, suggest that multiple *Muscodor* isolates are similarly inhibiting organisms via DNA methylation.

Curiously, *E. coli* cultures that contain gene deletions related to membrane integrity and oxidative response are significantly more sensitive to *M. albus* VOCs than VOCs from other *Muscodor* isolates. Knock-outs of glmM, minC, yraP, hycc, ducC, rseA, yegQ, yggT, alsB, yfaV, and mdtK genes are solely inhibited by *M. albus* volatiles. Therefore, although *Muscodor* isolates seem to utilize similar mechanisms of action in terms of their effect on DNA repair pathways, the original *M. albus* isolate appears to also target the cell membrane. This is consistent with previous studies that observed increased membrane permeability in *E. coli* upon exposure to *M. albus* volatiles (40). *M. albus* may be utilizing a combination of DNA damage and membrane permeability not seen in the other isolates tested. This could potentially explain why *M. albus* is more toxic than other isolates, such as E3816A, despite having lower MNIBA production (Fig. 4). Additionally, compared with the other isolates, *M. albus* also produced the highest amount of iodomethane, which is also known to be a weakly mutagenic DNA-methylating agent (56, 57). These data suggest that MNIBA production is the dominant factor, but possibly not the sole factor, in *M. albus* toxicity.

**Fungal resistance to MNIBA**

In this study, we have demonstrated that many *Muscodor* isolates are actively producing and excreting a DNA-methylating agent. This suggests that *Muscodor* isolates are able to coun-
ter the damaging effects of MNIBA. To determine whether *Muscodor* isolates have an unusual resistance to MNIBA exposure compared with other fungi, we incubated seven fungi in the presence of increasing amounts of MNIBA (0–1 mg) for 3 days and measured mycelial growth in comparison with the growth of fungal controls that were not exposed to volatiles (Fig. 8). Fungi in this analysis included the most and least toxic *Muscodor* isolates (P912B and P1813B, respectively), fungi from genera that are known to be susceptible to *M. albus* volatiles (*Colletotrichum* sp., *Aspergillus* sp., and *Geotrichum* sp.), and fungi from genera that are reported to be resistant to *M. albus* volatiles (*Trichoderma* sp. and *Fusarium* sp.) (7, 18–21).

Both *Muscodor* isolates P912B and P1813B were more resistant to MNIBA exposure compared with the other fungi tested. Isolate P912B was the least affected by the presence of MNIBA and showed only a 13% decrease in growth at the highest MNIBA exposure (1 mg). Isolate P1813B also demonstrated MNIBA resistance, and we observed a 24% decrease in growth upon exposure to 1 mg of MNIBA. Interestingly, P1813B exhibited resistance despite its lack of detectable production of MNIBA, indicating that MNIBA resistance is not necessarily correlated to its production. *Trichoderma* sp. displayed moderate resistance to MNIBA, consistent with the observation that *Trichoderma* species show some resistance to *Muscodor* volatiles (19, 21). This could be related to the fact that fungi in this genus also produce antimicrobial volatiles (58) and may have developed their own mechanisms of resistance. *Colletotrichum* sp. was completely inhibited upon addition of 1 mg of MNIBA. *Fusarium* sp., *Aspergillus* sp., and *Geotrichum* sp. were completely inhibited upon addition of 0.5 mg of MNIBA and higher. These data suggest that *Muscodor* isolates are unusually resistant to MNIBA and serve as a first step toward understanding the mechanisms of *Muscodor* resistance to MNIBA.

**Discussion**

Fungi in the *Muscodor* genus have demonstrated the ability to inhibit a broad spectrum of organisms including bacteria, fungi, oomycetes, nematodes, and insects (7, 16, 59, 60). Here we identified that *Muscodor* isolates produce a volatile DNA-methylating agent, N-methyl-N-nitrosobutyramide, and we determined that this mycotoxin is the main contributing factor to their antimicrobial activity using hierarchical clustering analysis and bioactivity assays. *In vivo* genetic and *in vitro* biochemical data are all consistent with a toxicity mechanism involving DNA methylation.

Antimicrobial activity by *Muscodor* species has previously been attributed to a synergistic effect of a combination of VOCs because individually tested VOC standards did not replicate the lethal effects seen by *M. albus* volatiles, and artificial mixtures at high concentrations could partially mimic the *M. albus* bioactivity profile (7). Additionally, artificial mixtures of *M. cris pans* volatiles were shown to inhibit test organisms, although these effects were not compared with the *M. cris pans* bioactivity profile (17). It is important to note that MNIBA was not detected or tested in those reports (7, 17). Although artificial mixtures exhibit inhibitory effects, we have observed that inhibition is greatly influenced by the concentration of chemical exposure, and it is difficult to determine whether artificial mixtures represent physiologically relevant compositions. However, MNIBA is produced in amounts commensurate with *Muscodor* toxicity, shows the same selectivity profile of *M. albus*, and is chemically reactive with DNA.

Based on its chemical structure, MNIBA is classified as an N-nitroso compound, and many chemicals in this class are known to exhibit carcinogenic, mutagenic, and genotoxic properties (48). Specifically, N-alkyl-N-nitroso compounds are known alkylating agents, with the type of alklylation damage being dictated by the agent’s chemical structure (61). Because MNIBA is an N-methyl-N-nitrosamide, it is likely that its mechanism of action is similar to that of the well characterized compound, MNU. MNU undergoes spontaneous decomposition to form a highly reactive methyl diazonium ion capable of methylating DNA (62, 63). There is also a possibility that nitrosoamides such as MNU can undergo thiol- or CYP oxidoreductase-catalyzed denitrosation to form an NO radical capable of nitrosylation chemistry and the corresponding amide; however, this pathway is unlikely to contribute to genotoxic effects (62).

The data suggest that *Muscodor* isolates primarily use DNA methylation to inhibit target organisms. Based on the mechanism of action of model N-methyl-N-nitrosamides, the predicted mechanism of action for MNIBA is illustrated in Fig. 9A. MNIBA is expected to spontaneously decompose to form isobutyric acid and methyl diazohydroxide, and the methyl diazohydroxide would subsequently convert into a methyl diazonium ion. The methyl diazonium ion can further interact with nucleophilic sites on DNA, and methylation at common nucleophilic sites are shown in Fig. 9B. Consistent with this proposal, we observed methylation primarily at guanosine bases. Furthermore, knock-out of AlkA, which repairs N7-methylguanosine, rendered *E. coli* susceptible to *M. albus*.

Notably, isobutyric acid is expected to be a by-product of MNIBA decomposition and has been previously reported in numerous analyses of *M. albus* volatiles (7, 18–21). Additionally, denitrosylation of MNIBA may yield N-methyl-isobutyramide, which has been previously observed as an *M. albus* volatile (41).
Although DNA is commonly accepted as the primary target of alkylating agents, other macromolecules such as RNA and proteins can also be targeted by these agents (48). Such non-DNA alkylation would explain the multitarget effects observed in previous single-gene knock-out screens against *M. albus* volatiles (40). As described previously, *E. coli* lacking enzymes related to DNA repair represented the highest proportion of sensitive knock-outs; however, sensitivity was also observed in other gene knock-outs related to cellular stress, cell division, chemotaxis, and RNA and protein degradation (40).

Microbes produce antimicrobial agents that can cause DNA damage through a variety of mechanisms, including DNA methylation by methyl halides (64), induction of single- or double-strand breaks by bleomycin (65), inhibition of topoisomerase IV and DNA gyrase by quinolone derivatives (66), and induction of reactive oxygen species that can damage DNA by numerous antibiotics (67) and antifungals (68). Production of MNIBA by *Muscodor* isolates is most analogous to production of streptozotocin, a D-glucopyranose derivative of MNU, by *Streptomyces achromogenes* (69). Streptozotocin is a known methylating agent that is utilized as an anticancer drug that primarily targets pancreatic tumors (70).

It is important to consider two factors that contribute to the efficacy of an antimicrobial agent: (i) its active moiety and (ii) the ability of the compound to access its target. The active component of both streptozotocin and MNIBA is the N-methyl-N-nitroso moiety. In streptozotocin, this moiety is attached to a glucose derivative, and streptozotocin is preferentially uptaken by pancreatic cells by the glucose transporter GLUT2 (71). In MNIBA, the active moiety is attached to a derivative of isobutyric acid, a low molecular weight volatile, and *Muscodor* isolates utilize volatility as their delivery mechanism.

Antimicrobial agents are often thought to be used as mechanisms of defense to reduce competition or manipulate the composition of the surrounding microbial community. Because *Muscodor* sp. are being pursued as potential biofumigants, it is essential to consider the limitations of using fungi that produce a broad-spectrum DNA-methylating agent, particularly in evaluating the effects of *Muscodor* sp. on the microbiome of their environment and their toxicity to humans and animals. Additionally, given that *Muscodor* isolates reside inside the inner tissues of plants, MNIBA production could provide protection from pathogenic infection and provide benefit to the host. The behavior of *Muscodor* species within the context of their host plants has not been investigated. Because MNIBA can target any DNA, it is interesting to consider how these mycotoxin-producing endophytes use MNIBA inside plants without seriously affecting their hosts. The extent to which MNIBA is produced, if at all, and whether production is constitutive or induced in the host plant is currently unknown. *Muscodor* species may maintain this balance by regulating the amount of MNIBA produced or the host plant may have its own mechanisms of resistance to MNIBA.

*M. albus* has been passaged in laboratory culture for over 15 years, and the cultures still maintain robust antimicrobial activity. The amount of MNIBA that vaporizes represents a fraction of the total amount produced, and the local concentration of MNIBA that *Muscodor* isolates are exposed to is certain to be higher. It stands to reason that *Muscodor* isolates must have a mechanism of resistance that enables them to thrive in this toxic environment. We observed that *Muscodor* isolates exhibited higher levels of resistance to MNIBA compared with other fungi regardless of whether or not the isolate was a significant producer of MNIBA. Possible methods of resistance could include maintaining a robust DNA repair pathway, restricting uptake or increasing efflux of MNIBA, detoxifying MNIBA via modification of its chemical structure, or eliminating affected pathways that trigger cell death. Additionally, an adaptive response mechanism to DNA alkylation has been described in the filamentous fungus *Aspergillus fumigatus* and is suspected to exist in other fungi in the Ascomycota lineage, including *Muscodor* species (72). Further investigation is needed to determine the mechanism of resistance used by *Muscodor* isolates.

Figure 9. Proposed mechanism of action of MNIBA. A, nitrosamides spontaneously decompose to form reactive diazonium ions. B, examples of interactions between diazonium ions and nucleobases. Methylation at common nucleophilic sites (N’meG, O’meG, and N’meA) are shown.
DNA methylation by fungi in the genus Muscodor

In conclusion, we report that fungi in the *Muscodor* genus produce a volatile DNA-methylating mycotoxin as a form of microbial fumigation, which provides insight into strategies that organisms use to mediate interactions in their environment and provokes questions regarding the mechanisms of resistance organisms use to survive in toxic environments.

**Experimental procedures**

**Media**

All fungi were grown on potato dextrose agar (PDA) consisting of 24 g/liter potato dextrose broth (EMD Chemicals, Gibbstown, NJ) and 15 g/liter agar (Affymetrix, Santa Clara, CA). Bacteria were grown in either liquid Luria-Broth (LB) made with Lennox tablets (Sigma) or on plates containing LB and 15 g/liter agar.

**Strains**

*M. albus* CZ-620 and *M. crispsans* B-23 were provided by Dr. Gary Strobel at Montana State University. *E. coli* 25922 (ATCC 25922) and *E. coli* 35218 (ATCC 35218) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The Yale Coli Genetic Stock Center provided *E. coli* BW25113 and HB101 (Yale University, New Haven, CT). *E. coli* ElectroMax DH10B was purchased from Invitrogen.

**Muscodor isolation and identification**

Ten *Muscodor* isolates were collected by students or instructors of the Yale University Rainforest Expedition and Laboratory over the course of several trips to the rainforests of Peru and Ecuador (2007–2010). The isolation and identification of fungal endophytes obtained in Peru (P912B, P913A, P1509A, P1813B, and P1907B) have been previously described (15), and a similar procedure was used to obtain the Ecuadorian endophytes (E8514I, E6011C, E6710B, E3801A, and E3816A). Fungal identification was performed by amplifying the nuclear ribosomal ITS region using ITS1 and ITS4 primers and comparing consensus sequences to GenBank™ using the National Center for Biotechnology Information (NCBI) nucleotide Basic Local Alignment Search Tool (nBLAST) as described previously (15). *Muscodor* isolates were deposited into the Yale Peabody Cryo Collection (New Haven, CT) with the following catalog numbers: YU.101684 (P1509A); YU.101685 (P1813B); YU.101686 (P1907B); YU.101687 (P912B); YU.101688 (P913A); YU.101689 (E8501A); YU.101690 (E8316A); YU.101691 (E6011C); YU.101692 (E6710B); and YU.101693 (E8514I).

**Comparison of Muscodor VOC production profiles**

VOCs were analyzed via GC-MS as described previously (73). The 5-mm *Muscodor* plugs were inoculated onto 5-ml PDA slants in 20-ml Supelco vials and grown for 3 days at room temperature in triplicate. Volatiles in the headspace were analyzed via SPME by exposing a pre-conditioned 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane StableFlex SPME fiber (Agilent) to the culture headspace for 1 min and then to the headspace of an internal standard of octane (Sigma) for 30 s at 30 °C. The internal standard consisted of 0.1 μl of octane (Sigma) in 5 ml of pump oil. The SPME fiber was injected into an Agilent 7890 gas chromatograph coupled to Waters GCT Premier time of flight mass spectrometer (Milford, MA) with a DB-5MS column (30 m × 0.25-mm inner diameter × 0.25-μm film thickness; Agilent). VOCs were desorbed from the SPME fiber for 30 s at 140 °C, an injector temperature that was intentionally tailored for the identification of thermally unstable compounds, with a split ratio of 50:0. The oven temperature programming was as follows: the initial temperature of 40 °C was held for 5 min; temperature increased to 105 °C at a rate of 7 °C per min; temperature increased at a rate of 20 °C per min until a final temperature of 325 °C was reached; temperature was held at 325 °C for 5 min. VOCs are tentatively identified according to the best match in the NIST database (NIST Mass Spectral Search Program Version 2.0, built 2005) or are marked as unknown. Contamination peaks from the fiber or inlet septum or compounds identified in media-only blanks were excluded from this analysis. The commercial MNIBA standard (98% purity, ChemPacific, Baltimore, MD) was used to verify the retention time and fragmentation pattern of this compound. To quantify relative MNIBA production, the peak area of MNIBA was normalized to the peak area of the internal standard.

Similar GC-MS parameters were used for the analysis of *Muscodor* VOCs at the higher GC inlet injector temperature of 240 °C. For this analysis, the SPME fiber was exposed to *Muscodor* VOCs for 5 min and to an internal standard of 2-nonenone (Sigma) for 30 s. The internal standard consisted of 2 μl of 2-nonenone in 10 ml of pump oil. For this analysis, a DB-WAX (30 m × 0.25 mm inner diameter × 0.5-μm film thickness; Phenomenex) and a splitless injection port were used. The oven temperature programming was as follows: the initial temperature of 40 °C was held for 5 min; temperature increased to 215 °C at a rate of 5 °C per min; temperature increased at a rate of 20 °C per min until a final temperature of 325 °C was reached; temperature was held at 325 °C for 5 min.

Hierarchical clustering analysis of VOC production and toxicity profiles across species was performed using Matlab R2015a. Each profile was first normalized to its maximum measured value. Distances between profiles were calculated using the “pdist” function with the euclidian distance. Clustering was performed using the “linkage” function using the average linkage method. The tree was reorganized to show toxicity and VOC profiles, we compared datasets that were collected at similar time points.

**Growth inhibition assays**

The viability of *E. coli* 25922 upon *Muscodor* volatile exposure was determined as described previously (40). Briefly, 5-mm plugs of *Muscodor* isolates were inoculated onto PDA slants on one side of a split vial system and grown for 3 days at room temperature. Split vials consisted of two identical clear 20-ml screw cap vials (Supelco, St. Louis, MO) fused together by a glass bridge near the top of the vials. *E. coli* 25922 cells were grown overnight at a constant agitation rate of 150 rpm at 23 °C.
DNA methylation by fungi in the genus Muscodor

E. coli cultures were diluted to an optical density of 0.05 and added to the opposite side of the split vial. E. coli 25922 was incubated with the 3-day-old Muscodor for 7 h at 23 °C at 150 rpm. The E. coli 25922 cultures were then serially diluted and plated onto LB agar plates. Plates were incubated at 37 °C overnight, and the number of colony-forming units (CFUs) were quantified. The fraction of viable CFUs is the ratio of the number of CFUs after exposure to a Muscodor isolate to the number of CFUs after exposure to a PDA-only control. Minitab 17.3.1 was used to conduct a 2-sample t test without assumption of equal variance. We tested whether the average fraction viable for the untreated control was greater than the average fraction viable for the samples exposed to each Muscodor sp. (p value <0.05).

The toxicity of M. albus and MNIBA to E. coli test strains was determined as follows. 5-mm plugs of M. albus were grown for 3 days on PDA plates at room temperature. Commercial N-methyl-N-nitrosobutiramid (ChemPacific) was added to Ultra grade 19 premium quality mechanical vacuum pump oil (Edwards, Sanborn, NY) for steady release from a non-volatile inert solvent. Varying amounts of MNIBA (0.07–0.12 mg) were added to sterilized caps of 1.5-ml microcentrifuge tubes (USA Scientific; Ocala, FL) in an empty Petri dish. E. coli test strains BW25113, HB101, DH10B, 25922, and 35218 were streaked onto five sections of LB plates. The LB plates were placed on top of the plates containing the following: (i) M. albus on PDA; (ii) different concentrations of MNIBA in pump oil; (iii) controls containing caps of pump oil on PDA; and (iv) PDA-only controls in a sandwich plate assay and sealed with Petri seal. The test strains were exposed to M. albus or MNIBA for 2 days. Plates were separated and imaged using a Bio-Rad ChemiDoc MP imaging system. Controls included E. coli cultures that were not exposed to any volatiles and E. coli cultures that were exposed to pump oil volatiles that did not contain MNIBA. This experiment was performed in triplicate.

Enzymatic assay for DNA methylation

The plasmid pUC19 (Invitrogen) was propagated in E. coli DH5α (Invitrogen), and a Qiagen Plasmid MaxiPrep kit was used to obtain a sufficient amount of plasmid. 2 ml of 25 ng/µl plasmid pUC19 in 10 mM HEPES, pH 7.5, 50 mM NaCl were incubated on one side of a split vial system and exposed to volatiles from a 3-day-old Muscodor isolate (P912B, M. albus, or P1813B) or 1.5-ml microcentrifuge tube caps containing varying amounts of MNIBA (0.01 to 1 mg) in pump oil with a final volume of 100 µl. Additionally, controls containing only PDA or PDA with a cap of pump oil were included. Aliquots of the plasmid were withdrawn from the vial every day for 6 days by piercing the rubber septum cap with a syringe to minimize VOC dispersal. 100 ng (4 µl) of the exposed pUC19 plasmid were incubated with a DNA glycosylase (hAAG or hOGG1) and APE1 for 1 h at 37 °C. Controls included pUC19 samples that were either (i) untreated, (ii) incubated with a single DNA glycosylase, or (iii) incubated with APE1 alone. Samples were run on 1% agarose gels (Affymetrix) stained with ethidium bromide (Sigma) Gels were visualized with the Bio-Rad ChemiDoc MP imaging system. Nicked pUC19 controls were acquired by incubating pUC19 with Nt.BsmA1 in New England Biolabs Buffer 4 for 1 h at 37 °C. hAAG (10,000 units/ml), APE1 (10,000 units/ml), and hOGG1 (1600 units/ml) were purchased from New England Biolabs (Ipswich, MA). Reaction buffers with hAAG and hOGG1 were Buffer 4 and Buffer 2 with 1% BSA, respectively (New England Biolabs).

Mass spectrometry assay for DNA methylation

DNA oligonucleotides were purchased from Keck Oligonucleotide Synthesis Facility at Yale University. The DNA sequence CGTCAGACTAAGGTTCC and its reverse complement were annealed by mixing 300 µM of each strand, heating to 90 °C for 1 min, and then cooling to room temperature. 30 µM duplex was incubated with 0, 4, or 8 mM MNIBA for 24 h. For studies of the dependence on nucleobase content, the guanosines at positions 13, 6, 2, and 12 were successively changed to cytidine. The five DNA strands were separately annealed to their reverse complements, mixed together in a single tube, and then exposed to MNIBA.

Samples were diluted 8:1 with 50:50 methanol/water containing 50 mM ammonium hydroxide and directly injected into an Orbitrap XL LC/MS/MS instrument. Spectra were collected at 60,000 resolving power for 1.5 min in negative mode. Averaged m/z spectra were deconvoluted using Xtract software in Xcalibur.

The intensities of single peaks corresponding to the mono-isotopic mass plus 2 daltons were used in quantitation. Each singly methylated species was compared with the non-methylated peak to determine the fraction methylated. Analyses using other isotopic peaks gave similar results.

Library screen of E. coli single-gene knock-outs against representatives of Muscodor collection

A partial set of the Keio collection, consisting of single-gene knock-outs of enzymes in E. coli DNA repair, membrane integrity and stress, and oxidative stress, was assessed against volatiles produced by Muscodor isolates that represented a broad range of toxicity. Assessment of Muscodor VOC effects on knock-out viability was performed as described previously (40). Briefly, the knock-out collection was grown at 37 °C overnight in LB-kanamycin (25 µg/ml) in a 96-well microtiter plate. Cultures from each well were pinned onto a fresh LB-kanamycin plate, which was placed on top of a plate containing 3-day-old Muscodor on PDA. The select Keio collection was either exposed to Muscodor isolates or to a PDA control for 24 h. E. coli growth was qualitatively assessed by visually comparing knock-outs exposed to Muscodor VOCs in relation to the knock-outs that were not exposed to Muscodor VOCs. Each knock-out was categorized as inhibited (no growth upon Muscodor VOC exposure), sensitive (decreased growth upon Muscodor VOC exposure), or not affected (growth upon Muscodor VOC exposure was equivalent to control). This experiment was performed in triplicate.

Fungal resistance to MNIBA

5-mm plugs of seven fungal isolates (Muscodor sp. P912B, Muscodor sp. P1813B, Aspergillus sp., Colletotrichum sp., Geotrichum sp., Trichoderma sp., and Fusarium sp.) were inoculated onto PDA plates. Inoculated PDA plates were immediately placed on top of Petri dish plates containing varying
amounts of MNIBA (0.05–1 mg) in microcentrifuge tube caps and sealed with Petri seal. Fungi were incubated with MNIBA for 4 days at room temperature, with the exception of Trichoderma sp. which was incubated for 2 days due to the fast-growing nature of this fungus. Radial growth from the agar plug was recorded (in millimeters). Percent growth was determined by dividing the distance of mycelial growth after MNIBA exposure by the distance of fungal growth with no volatile exposure and multiplying by 100. This experiment was performed in triplicate.

Accession numbers

Muscodor ITS consensus sequences obtained in this study were submitted to the GenBank database with the following accession numbers: P1509A (GenBank accession number EU977187); P1813B (GenBank accession number EU977197); P1907B (GenBank accession number EU977281); P1812B (GenBank accession number EU977236); P1901A (GenBank accession number EU977208); E3801A (GenBank accession number EU977281); E3816A (GenBank accession number KE659025); E6011C (GenBank accession number KE659026); E6710B (GenBank accession number HM999898); and E8541l (GenBank accession number HQ117854).

Author contributions—M. L. H. and S. A. S. discussed experimental ideas and wrote the manuscript together. M. L. H. also conducted most of the experiments and data analysis. C. J. A. -C. collected Muscodor VOC datasets collected at the low and high injector temperatures. J. B. performed HCA on the data. D. A. H. performed MS experiments on DNA oligonucleotides exposed to MNIBA and helped write the paper. S. C. (2005) Identification and use of potential bacterial organic antifungal volatiles in biocontrol. Soil Biol. Biochem. 37, 955–964.

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