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New Metabolic Pathway for Converting Blasticidin S in *Aspergillus flavus* and Inhibitory Activity of Aflatoxin Production by Blasticidin S Metabolites

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Supporting Information

ABSTRACT: Blasticidin S, a protein synthesis inhibitor, inhibits aflatoxin production of *Aspergillus flavus* without affecting fungal growth. Analysis of metabolites in blasticidin S-treated *A. flavus* using quadrupole time-of-flight liquid chromatography−mass spectrometry showed that blasticidin S was metabolized into a novel metabolite, *N*-acetyldeaminohydroxyblasticidin S. Conversion of blasticidin S to *N*-acetyldeaminohydroxyblasticidin S via deaminohydroxyblasticidin S or *N*-acetylblasticidin S was observed in in vivo and in vitro *A. flavus* systems. Blasticidin S and *N*-acetylblasticidin S inhibited the growth of *Aspergillus niger* strongly and weakly, respectively, but deaminohydroxyblasticidin S and *N*-acetyldeaminohydroxyblasticidin S did not inhibit its growth. On the other hand, deaminohydroxyblasticidin S sustained the inhibition of aflatoxin production whereas *N*-acetylblasticidin S and *N*-acetyldeaminohydroxyblasticidin S did not. These results suggest that the free amino group at C-13 of blasticidin S and deaminohydroxyblasticidin S may be important for the inhibitory activity of aflatoxin production.

KEYWORDS: *aflatoxin, blasticidin S, metabolome*

INTRODUCTION

Aflatoxins are toxic and carcinogenic metabolites produced by some *Aspergillus* species, such as *Aspergillus flavus* and *Aspergillus parasiticus*.[1] They are frequently detected in a wide variety of food and feed commodities such as groundnuts, tree nuts, dried fruit, spices, and cereals. The contamination of aflatoxins in agricultural products causes huge economic loss and is a serious health threat to both humans and domestic animals.[2,3] There are few practical methods to control aflatoxin contamination except for the use of atoxigenic strains of Aspergilli that can competitively exclude toxigenic strains from crops.[4] Therefore, it is critical to develop effective methods to prevent aflatoxin contamination. We have been studying specific inhibitors of aflatoxin production that may be useful for aflatoxin control without incurring a rapid spread of resistant strains.[5,6]

Blasticidin S (**1**) is a metabolite of *Streptomyces griseochromogenes* and was formerly used in practice as a fungicide against a phytopathogenic fungus, *Pyricularia oryzae*.[7,8] Blasticidin S inhibits protein synthesis of both prokaryotic and eukaryotic organisms by interacting with their ribosomes.[9,10] Enzymes for the inactivation of blasticidin S have been well studied. Blasticidin S *N*-acetyltransferase is produced by blasticidin S-producing *Streptomyces* for self-protection. It produces *N*-acetylblasticidin S (**2**) by acetylating an amino group at the C-13 of blasticidin S (Figure 1).[11,12] Blasticidin S deaminase transforms blasticidin S into a nontoxic derivative, deaminohydroxyblasticidin S (**3**), by deaminating an amino group in the cytosine moiety (Figure 1). Blasticidin S deaminase activity was found in a variety of *Aspergillus* species, including *A. flavus*.[13]

We previously reported that blasticidin S inhibited aflatoxin production of *A. flavus* without affecting fungal growth.[14] Blasticidin S could much more selectively inhibit aflatoxin production in *A. flavus* than other protein synthesis inhibitors tested, including cycloheximide. Because *A. flavus* is able to detoxify blasticidin S, the mode of action of blasticidin S in the inhibition of aflatoxin production in this fungus cannot be fully explained by the activity of blasticidin S on protein synthesis. Therefore, we aimed to investigate the mode of action by performing metabolomic analysis to identify compounds for which amounts were altered by the addition of blasticidin S using quadrupole time-of-flight liquid chromatography−mass spectrometry (Q-TOF LC-MS). The results of metabolomic analysis as well as new insight into the inhibitory activity of aflatoxin production by blasticidin S are described.

MATERIALS AND METHODS

Strains, Growth Media, and Chemicals. *A. flavus* strain IMF 47798 was used as an aflatoxin producer. Aflatoxin B_1 is mainly produced by this strain. *Aspergillus niger* strain KJ16 was used for the antifungal assay. These strains were maintained on potato dextrose agar (Difco, Sparks, MD, USA) and subcultured monthly. A spore

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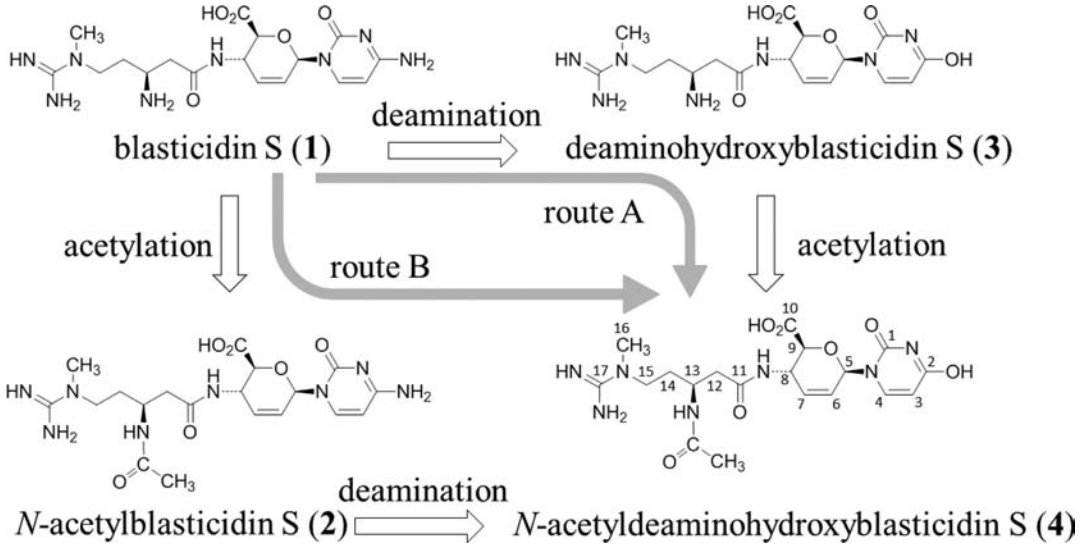

Figure 1. Structures and metabolic pathway of blasticidin S and its derivatives.

Table 1. NMR Spectroscopic Data for Blasticidin S Metabolites

	N-acetylblasticidin S		deaminohydroxyblasticidin S		*N*-acetyldeaminohydroxyblasticidin S		
position	δ_C[a]	δ_H[a] (*J* in Hz)	δ_C[a]	δ_H[a] (*J* in Hz)	δ_C[a]	δ_H[a] (*J* in Hz)	HMBC[b]
1	148.8		151.4		151.5		
2	159.5		165.8		166.0		
3	95.0	6.20, d (8.0)	102.2	5.90, d (8.0)	102.1	5.70, d (8.0)	2, 4
4	145.2	7.82, d (8.0)	142.3	7.64, d (8.0)	142.8	7.47, d (8.0)	1, 2, 3, 5
5	78.8	6.53, s, br	78.4	6.54, s, br	78.4	6.30, s, br	1, 4, 6, 7
6	124.0	6.24, dt (10.0, 2.5)	124.8	6.23, dt (10.0, 2.5)	124.7	6.00, dt (10.0, 2.5)	5
7	133.5	5.91, dt (10.0, 2.5)	132.4	5.94, dt (10.0, 2.5)	133.4	5.70, dt (10.0, 2.5)	5, 8
8	44.6	4.75, ddd (8.0, 2.5, 2.5)	44.2	4.86[c]	45.1	4.52, ddd (8.5, 2.5, 2.5)	7, 9
9	76.3	4.29, d (8.0)	74.6	4.47, d (8.5)	77.3	4.02, d (8.5)	5, 7, 8, 10
10	173.5		171.6		174.4		
11	172.2		170.8		172.1		
12	41.1	2.48, m	36.0	2.09, m	41.0	2.31, m	11, 13, 14
13	44.4	4.16, m	46.0	3.70, m	44.5	3.97, m	11, 12, 14, 15, $\underline{C}OCH_3$
14	30.1	1.81, m; 1.97, m	28.7	2.09, m	30.0	1.65, m; 1.80, m	12, 13, 15
15	46.7	3.36, m; 3.46, m	46.2	3.51, m	46.8	3.17, m; 3.29, m	13, 14, 16, 17
16	35.2	3.01, s	35.4	3.07, s	35.2	2.84, s	15, 17
17	156.3		156.2		156.3		
$\underline{C}OCH_3$	173.3				173.4		
$CO\underline{C}H_3$	21.7	2.00, s			21.7	1.84, s	$\underline{C}OCH_3$

[a]150 MHz (δ_C) and 500 MHz (δ_H) in D_2O. [b]From protons stated to the indicated carbon. [c]Overlapped with H_2O signal.

suspension was prepared from a 2-week-old culture, at a concentration of 2.5 × 10^3 cfu/μL, and used as inoculum for this study. All incubations were done at 30 °C.

Blasticidin S HCl (Invitrogen, Carlsbad, CA, USA), aflatoxin B_1 (Sigma-Aldrich, St. Louis, MO, USA), and cyclopiazonic acid (Sigma-Aldrich) were dissolved in distilled water, acetonitrile, and dimethyl sulfoxide, respectively. They were stored at −20 °C.

Deaminohydroxyblasticidin S was prepared by microbial transformation of blasticidin S according to the method of Seto et al.[15] *Aspergillus terreus* strain TSY 578 was cultured in YE liquid medium (25 g/L yeast extract) containing 300 μM blasticidin S for 6 days. The culture filtrate was applied to an InertSep C18 cartridge (GL Sciences Inc., Tokyo, Japan), which was preequilibrated with 0.1% acetic acid in water. The eluate of 10% acetonitrile in water containing 0.1% acetic acid from the cartridge was subjected to an LC-20A series HPLC system (Shimadzu Corp., Kyoto, Japan) [column, 250 mm × 4.6 mm i.d., 3 μm, Inertsil ODS-3 (GL Sciences Inc.); isocratic elution of 10% acetonitrile in water containing 0.1% trifluoroacetic acid; flow rate, 1.0 mL/min; detection at 260 nm] and 24 mg (yield, 48%) of deaminohydroxyblasticidin S (HRESI-TOF/MS *m/z* 423.1879; calcd for $C_{17}H_{25}N_7O_6$, 423.1866) was obtained from 50 mg of blasticidin S.

N-Acetylblasticidin S was prepared according to the method of Zhang et al.[16] Briefly, a blasticidin S-free base (200 mg) was dissolved in a solution of acetic acid (20 mL) and acetic anhydride (20 mL), and the reaction mixture was stirred overnight at room temperature. The resulting mixture was evaporated to a small volume (2 mL) and stored at 4 °C overnight. The resulting crystals were collected to give 63 mg of diacetylblasticidin S. Diacetylblasticidin S was stirred in 1 M aqueous ammonium hydroxide (15 mL) at room temperature for 30 min. After the solution was evaporated to dryness, the residue was resolved in water (3 mL) and subjected to an LC-20A series HPLC system. Under the conditions used for purification of deaminohydroxyblasticidin S mentioned above, 30 mg (yield, 15%) of *N*-acetylblasticidin S (HRESI-TOF/MS *m/z* 464.2135; calcd for $C_{17}H_{25}N_7O_6$, 464.2132) was obtained. ^1H NMR and ^{13}C NMR data of deaminohydroxyblasticidin S and *N*-acetylblasticidin S are shown in Table 1.

Sample Preparation for Q-TOF LC-MS Analysis. A spore suspension of *A. flavus* IMF 47798 (5 μL) was added to potato dextrose (PD) (Becton, Dickinson and Co., Sparks, MD, USA) medium (2 mL) with or without blasticidin S (300 μM) in a well of a 12-well microplate and incubated statically for 2 days. The culture broth was rapidly separated into mycelium and culture filtrate by filtration. To prepare the mycelial extract, mycelium was placed into a mortar containing 3 mL of an extraction solvent (acetonitrile/methanol, 50:50) precooled at −40 °C. After homogenization with a pestle, the mixture was transferred in a 15 mL tube and incubated at 4 °C for 15 min. After centrifugation (3000*g*, 10 min), the supernatant was collected in a new 15 mL tube. The pellet was extracted again with 3 mL of an extraction solvent and finally extracted with 3 mL of water at 4 °C for 15 min. The collected supernatants were pooled and made up to 10 mL with water. This solution was subjected to LC-MS analysis. Culture filtrate was diluted 50 times with water before LC-MS analysis.

Liquid Chromatographic Conditions. The LC system consisted of an Agilent 1200 series (Agilent Technologies, Palo Alto, CA, USA). Mycelial extract solutions and culture filtrates prepared as described above were analyzed using the following gradient LC conditions: mobile phase, 10 mM ammonium acetate/acetonitrile; hold at 5% acetonitrile for 3 min, linear gradient of 5−90% acetonitrile in 10 min, hold at 90% acetonitrile for 6 min, linear gradient of 90−5% acetonitrile in 1 min, followed by equilibration in 5% acetonitrile for 20 min before the next injection. The flow rate was 0.2 mL/min, and the column used was a 150 mm × 2.1 mm i.d., 3 μm, InertSustain C18 (GL Sciences). The injection volume was 0.2 μL for mycelial extract solution and 1 μL for culture filtrate. The column oven was held at 40 °C, and the autosampler tray was maintained at 4 °C. The HPLC system was connected to an Agilent 6530 Q-TOF mass spectrometer.

Mass Spectrometry. An Agilent 6530 Q-TOF mass spectrometer with an electrospray ionization (ESI) interface with Agilent Jet Stream Technology was used. The instrument was calibrated in the high-resolution mode (4 GHz, high res mode) with a standard mass range (m/z < 3200). Data were collected in a centroid format. Reference masses at m/z 121.0508 and 922.0098 in the positive ion mode and at m/z 119.0363 and 980.0164 in the negative mode were continually introduced along with the LC stream for accurate mass calibration. The drying gas (nitrogen) temperature was set at 325 °C, drying gas flow at 10 L/min, nebulizer pressure at 30 psi, and capillary voltage (Vcap) at 3500 V. A centroid data within the mass range m/z 100−1000 was acquired at a 1 spectrum/s rate with a Mass Hunter workstation (Agilent). Peak identification and quantitation were performed using Qualitative Analysis software version B.04.00 (Agilent). The extractions of compounds for which the amount had increased >2-fold or fallen >50% following the addition of blasticidin S were selected using Mass Profiler Professional version 2.1 (Agilent).

Isolation of *N*-Acetyldeaminohydroxyblasticidin S from Blasticidin S-Treated *A. flavus*. *A. flavus* IMF 47798 was cultured statically in 300 mL of YE liquid medium with 50 mg of blasticidin S for 6 days. The mycelial cake was harvested by filtration and extracted with methanol (300 mL). The methanol solution was evaporated to dryness. The residue was dissolved in water (10 mL) and centrifuged (3000*g*, 10 min). The supernatant was subjected to an InertSep C18 cartridge (GL Sciences), which was preequilibrated with 0.1% acetic acid. The eluate (10% acetonitrile in water containing 0.1% acetic acid) from the cartridge was subjected to an LC-20A series HPLC system [column, 250 mm × 10 mm i.d., 5 μm, Inertsil ODS-3 (GL Sciences); mobile phase, 10 mM ammonium acetate/acetonitrile, hold at 5% acetonitrile for 4 min, linear gradient of 5−50% acetonitrile in 10 min, hold at 50% acetonitrile for 5 min; flow rate, 1.0 mL/min; detection at 260 nm] to obtain 18.1 mg of *N*-acetyldeaminohydroxyblasticidin S (retention time, 8.8 min; yield, 36%): HRESI-TOF/MS m/z 465.1977 (calcd for $C_{19}H_{27}N_7O_7$, 465.1972); ^1H and ^{13}C NMR, see Table 1.

Analysis of Aflatoxin B_1. *A. flavus* IMF 47798 was cultured for 4 days under the culture conditions described above. The culture broth was separated into mycelium and culture filtrate by filtration. The culture filtrate (200 μL) was extracted with 200 μL of chloroform, and the chloroform solution was evaporated to dryness using a CVE-3100 centrifugal concentrator (Tokyo Rikakikai Co., Tokyo, Japan) at 40 °C. The residue obtained was dissolved in 1 mL of 10% acetonitrile water. Aflatoxin B_1 was quantitated by a 3200 Q Trap LC-MS/MS system (AB Sciex, Foster City, CA, USA), equipped with an ESI source and an LC-20A series HPLC system. HPLC conditions were as follows: column, 150 mm × 2.1 mm i.d., 3 μm, InertSustain C18 (GL Sciences); mobile phase, 10 mM ammonium acetate/methanol; linear gradient of 40−80% methanol in 8 min, hold at 80% methanol for 1 min; flow rate, 0.2 mL/min; retention time, 7.8 min.

Analysis of Blasticidin S Metabolites. *A. flavus* IMF 47798 was cultured for 4 days under the culture conditions mentioned under Sample Preparation for Q-TOF LC/MS Analysis. The culture broth was separated into mycelium and culture filtrate by filtration. The mycelium was collected into a 5.0 mL microtube and lyophilized for 12 h. Blasticidin S metabolites in the mycelium extract and the culture filtrate were quantitated separately. To quantitate blasticidin S metabolites in the mycelium extract, the lyophilized mycelium was extracted with 3.0 mL of methanol. After centrifugation (12000*g*, 10 min), the supernatant was evaporated to dryness. The residue was dissolved in 1.0 mL of water containing 0.1% acetic acid. Blasticidin S metabolites were partially purified by using an InertSep C18 cartridge as described previously. Deaminohydroxyblasticidin S, *N*-acetylblasticidin S, and *N*-acetyldeaminohydroxyblasticidin S were all eluted by 10% acetonitrile in water containing 0.1% acetic acid. To quantitate blasticidin S metabolites in the culture filtrate, partial purification using an InertSep C18 cartridge was performed as described above. Blasticidin S metabolites were quantitated by a 3200 Q Trap LC-MS/MS system equipped with an ESI source and an LC-20A series HPLC system. HPLC conditions were as follows: column, 150 mm × 2.1 mm i.d., 3 μm, InertSustain C18; mobile phase, 0.1% formic acid/acetonitrile; linear gradient, 5−90% acetonitrile in 7 min, hold at 90% acetonitrile for 1 min; flow rate, 0.2 mL/min; retention times, deaminohydroxyblasticidin S, 3.5 min; *N*-acetylblasticidin S, 3.7 min; *N*-acetyldeaminohydroxyblasticidin S, 4.9 min.

Assay of Blasticidin S *N*-Acetyltransferase activity in Vitro. *A. flavus* IMF 47798 was cultured for 40 h in YE liquid medium under the culture conditions mentioned above. The mycelium was collected by filtration. After a washing with 10 mL of distilled water, total proteins of the mycelia were extracted using CelLytic Y Yeast Cell Lysis/Extraction Reagent (Sigma-Aldrich) with a protease inhibitor cocktail for use with fungal and yeast extracts (Sigma-Aldrich) and 10 mM dithiothreitol. One milliliter of the reagent was used for the mycelia collected from three wells of a 12-well microplate. For enzyme assays, blasticidin S (final concn = 0.73 mM) and acetyl-CoA (final concn = 0.2 mM) were incubated with 60 μL of protein extracts in phosphate-buffered saline (total volume of 300 μL) in a 30 °C water bath. The reaction was stopped by adding 600 μL of acetonitrile. After diluting five times with water, the mixture was centrifuged (12000*g*, 10 min). The supernatant was analyzed directly by LC-MS/MS. Control experiments lacked acetyl-CoA or protein extracts.

Analysis of Antifungal Activity of Blasticidin S and Its Metabolites. A spore suspension of *A. niger* KJ16 (5 μL) was added to PD medium (2 mL) in each well of a 12-well microplate. Blasticidin S, deaminohydroxyblasticidin S, *N*-acetylblasticidin S, or *N*-acetyldeaminohydroxyblasticidin S was added to the medium and incubated statically for 4 days. The culture broth was separated into mycelium and culture filtrate by filtration. The mycelium was collected into a 1.5 mL microtube and lyophilized for 12 h. Mycelial weight was calculated by subtracting the weight of a 1.5 mL microtube without the mycelium from the total weight.

RT-PCR Analysis of the Genes Encoding Proteins Required for Aflatoxin Biosynthesis. *A. flavus* IMF 47798 was cultured for 32 h under the culture conditions mentioned above with blasticidin S or deaminohydroxyblasticidin S, and the culture broth was filtered to obtain the mycelia. The mycelial cake was homogenized in liquid nitrogen, and total RNA was obtained from the homogenates by using a TRIzol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was prepared with SuperScript VILO Master Mix (Invitrogen) according to the protocol as follows. A solution (20 μL)

Table 2. In Vivo Conversion Experiments of Blasticidin S, Deaminohydroxyblasticidin S, and *N*-Acetylblasticidin S[a]

additive	product			additive	product	additive	product
blasticidin S (μM)	deaminohydroxy blasticidin S (μM)	*N*-acetyl blasticidin S (μM)	*N*-acetyldeaminohydroxy blasticidin S (μM)	deaminohydroxy blasticidin S (μM)	*N*-acetyldeaminohydroxy blasticidin S (μM)	*N*-acetyl blasticidin S (μM)	*N*-acetyldeaminohydroxy blasticidin S (μM)
30	0.20 ± 0.08	nd	0.34 ± 0.03	30	1.12 ± 0.26	30	nd
100	0.93 ± 0.05	nd	0.94 ± 0.06	100	2.70 ± 0.22	100	0.09 ± 0.02
300	4.19 ± 0.50	nd	3.08 ± 0.21	300	4.62 ± 0.06	300	0.31 ± 0.04
1000	19.22 ± 2.59	nd	8.06 ± 0.63	1000	6.74 ± 0.52	1000	1.56 ± 0.06

[a]Data are presented as the mean ± SD (n = 4). nd, not detected (<0.05 μM).

containing 1.5 μg of the extracted RNA and SuperScript VILO MasterMix was incubated stepwise at 25 °C for 10 min, at 42 °C for 60 min, and at 85 °C for 5 min. The cDNA derived from 0.01 μg of total RNA was used as a template. Real-time quantitative RT-PCR was carried out using Thunderbird qPCR Mix (Toyobo Co., Ltd., Osaka, Japan), in a final volume of 20 μL for each reaction, and an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The two-step PCR conditions were as follows: after an initial incubation at 95 °C for 1 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min were performed. All primers were designed with Primer Express software version 3.0 (Applied Biosystems). The specificity of each primer was checked by analyzing a melting curve of the PCR product. A clear single peak was observed for each PCR product. The PCR primers for each gene were as follows: *aflR*, 5′-GGCTGGTCAGGAGCAAAGC-3′ and 5′- CCCCGAATTCCG-AATCG-3′; *pksA*, 5′-TGCATGGCGATGTGGTAGTT-3′ and 5′-GTAAGGCCGCGGAAGAAAG-3′; *omtA*, 5′-ACGGGTTTCGCA-AAAGCAT-3′ and 5′-GCAGGCAGGTCCTGTACGA-3′; *β-actin* (control gene), 5′-TGCTCTCGTCATTGACAATGGT-3′ and 5′-CATCGTCACCGGCGAAA-3′.

RESULTS AND DISCUSSION

Effects of Blasticidin S on *A. flavus* Metabolome. *A. flavus* was cultured with blasticidin S (300 μM) for 2 days, and the metabolites involved in each mycelial extract and culture filtrate were analyzed by Q-TOF LC-MS. Molecular formulas and compound names were estimated from molecular masses. The fold change was calculated by dividing the peak area of a blasticidin S-treated sample by that of the control sample. Production of aflatoxin B_1 and cyclopiazonic acid was observed both in the mycelial extract and in the culture filtrate. They were identified by comparing retention times with commercially available standards. Blasticidin S inhibited aflatoxin B_1 production by 95%, but inhibition of cyclopiazonic acid production was partial and the fold change was about 0.3. A remarkable increase in the peak area of a compound having an estimated molecular formula of $C_{19}H_{27}N_7O_7$ (compound **4**) was observed in the mycelial extract following the addition of blasticidin S. As a result of MS/MS fragmentation analysis, there were common fragments between blasticidin S and **4** (data not shown).

Identification of Compound 4. Because the production of **4** was expected to be related to an unknown action of blasticidin S against *A. flavus*, identification of **4** was performed. *A. flavus* was cultured in YE medium containing blasticidin S. YE medium was used for the production of **4** because much more **4** was produced in YE medium than in PD medium. Methanol extracts of the obtained mycelia were purified by a C_{18} cartridge and reverse-phase HPLC to isolate **4**. The molecular formula of **4** was larger than that of deaminohydroxyblasticidin S ($C_{17}H_{25}N_7O_6$) by C_2H_2O, suggesting that **4** was a monoacetate of deaminohydroxyblasticidin S. By comparison of ^{1}H NMR and ^{13}C NMR spectra of **4** with those of *N*-acetylblasticidin S and deaminohydroxyblasticidin S, and analysis of the HMBC spectrum of **4**, compound **4** was identified as *N*-acetyldeaminohydroxyblasticidin S (Table 1 and Figure 1). Compound **4** was a new metabolite of blasticidin S although it has been chemically prepared from deaminohydroxyblasticidin S.[17]

Conversion Experiments of Blasticidin S, *N*-Acetylblasticidin S, and Deaminohydroxyblasticidin S. There may be two possible routes to produce *N*-acetyldeaminohydroxyblasticidin S from blasticidin S: through deaminohydroxyblasticidin S (Figure 1, route A) or through *N*-acetylblasticidin S (Figure 1, route B). To investigate the route by which *N*-acetyldeaminohydroxyblasticidin S was produced in *A. flavus*, in vivo and in vitro conversion experiments of blasticidin S, *N*-acetylblasticidin S, and deaminohydroxyblasticidin S were carried out. First, in vivo conversion experiments were performed. *A. flavus* was cultured in PD liquid medium containing blasticidin S, deaminohydroxyblasticidin S, or *N*-acetylblasticidin S for 4 days, and blasticidin S metabolites were quantitated (Table 2). In the conversion experiment with blasticidin S, deaminohydroxyblasticidin S and *N*-acetyldeaminohydroxyblasticidin S were produced but *N*-acetylblasticidin S was not. About 1% of blasticidin S was transformed into *N*-acetyldeaminohydroxyblasticidin S, and when 1000 μM blasticidin S was added to the culture, 914 μM blasticidin S remained in the culture filtrate. In the conversion of deaminohydroxyblasticidin S, *N*-acetyldeaminohydroxyblasticidin S was produced. A nearly equal amount of *N*-acetyldeaminohydroxyblasticidin S was produced in the conversion experiments of blasticidin S and deaminohydroxyblasticidin S. A small amount of *N*-acetyldeaminohydroxyblasticidin S was also produced in the *N*-acetylblasticidin S conversion experiment. Next, an in vitro conversion experiment of blasticidin S using a cell-free extract of *A. flavus* was performed. It has been reported that *A. terreus* produced far more blasticidin S deaminase when grown in the presence of blasticidin S.[18] Therefore, cell-free extracts were prepared from the fungal mycelia cultured in the presence and absence of 30 μM blasticidin S, and the enzyme activities were compared. Blasticidin S was incubated with the cell-free extract with acetyl-CoA, and deaminohydroxyblasticidin S, *N*-acetylblasticidin S, and *N*-acetyldeaminohydroxyblasticidin S were quantitated by LC-MS/MS. As previously reported, blasticidin S deaminase activity was increased after the addition of blasticidin S. Blasticidin S, when added (0.73 mM), was almost completely transformed into deaminohydroxyblasticidin S within 60 min when the cell-free extract prepared from the blasticidin S-treated fungal mycelium was used (Figure 2A), but only 7 μM blasticidin S was transformed into deaminohydroxyblasticidin S when the cell-free extract prepared from the control fungal mycelium was used (Figure 2B). *N*-Acetylblasticidin S was

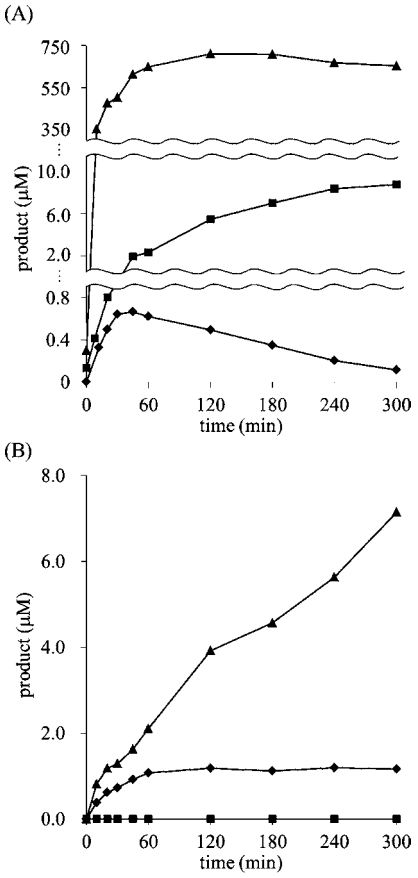

Figure 2. In vitro conversion experiments of blasticidin S in (A) treated and (B) control *A. flavus*. Cell-free extracts were incubated with blasticidin S (MW 422.2, 0.73 mM) and acetyl CoA (0.2 mM). Produced deaminohydroxyblasticidin S (▲, MW 423.2), *N*-acetylblasticidin S (◆, MW 464.2), and *N*-acetyldeaminohydroxyblasticidin S (■, MW 465.2) were quantitated.

detected in both of the cell-free extracts, but its amount decreased from 60 min onward when the blasticidin S-treated fungal mycelium was used (Figure 2A). *N*-Acetyldeaminohydroxyblasticidin S was produced only when the blasticidin S-treated fungal mycelium was used. When the cell-free extract was incubated at 95 °C for 10 min before addition of the substrates, both blasticidin S deaminase and *N*-acetyltransferase activities were remarkably attenuated.

Antifungal Activity of Blasticidin S, Deaminohydroxyblasticidin S, *N*-Acetylblasticidin S, and *N*-Acetyldeaminohydroxyblasticidin S. *A. niger* was used to analyze the antifungal activity of blasticidin S, deaminohydroxyblasticidin S, *N*-acetylblasticidin S, and *N*-acetyldeaminohydroxyblasticidin S. *A. niger* KJ16 was cultured in PD liquid medium containing each compound for 4 days and the dry weight of mycelia was measured (Figure 3). Blasticidin S significantly decreased the dry weight dose-dependently with an IC_{50} value of 66 μM, and *N*-acetylblasticidin S partially inhibited fungal growth (IC_{50} = 760 μM), whereas deaminohydroxyblasticidin S and *N*-acetyldeaminohydroxyblasticidin S did not affect fungal growth even at 1000 μM.

Inhibitory Activity of Blasticidin S, Deaminohydroxyblasticidin S, *N*-Acetylblasticidin S, and *N*-Acetyldeaminohydroxyblasticidin S on Aflatoxin Production. The inhibitory activity of blasticidin S, deaminohydroxyblasticidin S, *N*-acetylblasticidin S, and *N*-acetyldeaminohydroxyblasticidin S

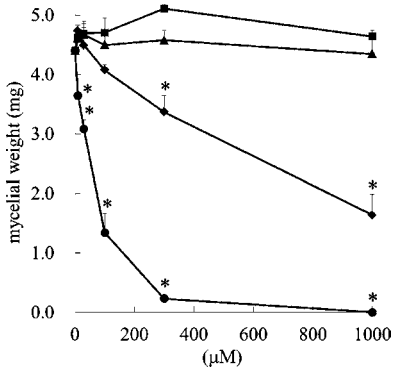

Figure 3. Antifungal activity of blasticidin S (●) and three of its metabolites, deaminohydroxyblasticidin S (▲), *N*-acetylblasticidin S (◆), and *N*-acetyldeaminohydroxyblasticidin S (■), against *A. niger*. Data are presented as the mean ± SD (n = 4). The differences between the dry weights of mycelia were assessed by one-way ANOVA, followed by the Dunnett test. ∗,$P < 0.05$, versus the control.

on aflatoxin production was examined (Figure 4). Blasticidin S and deaminohydroxyblasticidin S inhibited aflatoxin production

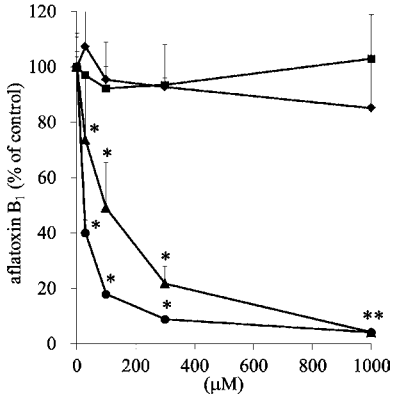

Figure 4. Aflatoxin inhibitory activity of blasticidin S (●) and three of its metabolites, deaminohydroxyblasticidin S (▲), *N*-acetylblasticidin S (◆), and *N*-acetyldeaminohydroxyblasticidin S (■). Data are presented as the mean ± SD (n = 4). The differences between the amount of aflatoxin B_1 were assessed by one-way ANOVA, followed by the Dunnett test. ∗, $P < 0.05$, versus the control.

in a dose-dependent manner with IC_{50} values of 24 and 109 μM, respectively, whereas *N*-acetylblasticidin S and *N*-acetyldeaminohydroxyblasticidin S did not show inhibitory activity. None of the compounds tested affected fungal growth even at 1000 μM.

Effects of Blasticidin S and Deaminohydroxyblasticidin S on the Transcription of Genes Encoding Proteins Required for Aflatoxin Biosynthesis. *A. flavus* was cultured in PD liquid medium with blasticidin S or deaminohydroxyblasticidin S for 32 h. The mycelium was obtained by filtration, and total RNA was extracted. We analyzed the mRNA levels of *pksA*, *omtA*, and *aflR* by quantitative PCR. The genes *pksA* and *omtA* encode enzyme proteins involved in the aflatoxin biosynthetic pathway, and *aflR* encodes a transcriptional factor that regulates transcription of some genes, including *pksA* and *omtA*. Both blasticidin S and deaminohydroxyblasticidin S repressed the transcription of *pksA*, *omtA*, and *aflR* dose-dependently (Figure 5). The repression level of blasticidin S

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was much more than that of deaminohydroxyblasticidin S, and this correlated with the aflatoxin production inhibitory activity.

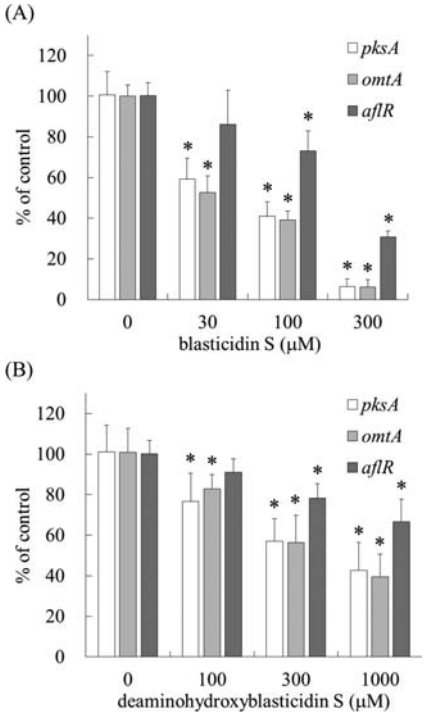

Figure 5. Effects of blasticidin S (A) and deaminohydroxyblasticidin S (B) on the transcription of genes encoding aflatoxin biosynthesis enzymes. Data are means ± SD (n = 6). The differences between the amounts of mRNA were assessed by one-way ANOVA, followed by the Dunnett test. *, $P < 0.05$, versus the control.

A new blasticidin S metabolite, *N*-acetyldeaminohydroxyblasticidin S, was discovered by metabolomic analysis during the investigation of the mode of action of blasticidin S in the inhibition of aflatoxin production. Conversion experiments suggested the existence of two metabolic routes to produce *N*-acetyldeaminohydroxyblasticidin S (Figure 1, routes A and B). However, the result of an in vitro conversion experiment using the blasticidin S-treated *A. flavus* extract showed that blasticidin S deaminase activity was increased far more than *N*-acetyltransferase activity, and blasticidin S was preferentially transformed into deaminohydroxyblasticidin S, after which acetylation occurred. A small amount of *N*-acetylblasticidin S was produced, but it was rapidly transformed into *N*-acetyldeaminohydroxyblasticidin S by the strengthened deaminase activity. This may be why *N*-acetylblasticidin S was not detected in the in vivo blasticidin S conversion experiment. These results suggest that *N*-acetyldeaminohydroxyblasticidin S was mainly produced from blasticidin S via deaminohydroxyblasticidin S by *A. flavus* (Figure 1, route A), and the pathway via *N*-acetylblasticidin S (Figure 1, route B) was hardly used. The antifungal activity of deaminohydroxyblasticidin S and *N*-acetylblasticidin S against *A. niger* (both of IC_{50} > 500 μM) was much lower than that of blasticidin S (IC_{50} = 66 μM). This indicates that either deamination of the amino group in the cytosine moiety or acetylation of the amino group at C-13 seems to be enough to reduce the antifungal activity of blasticidin S. *N*-Acetylblasticidin S inhibited the growth of *A. niger* slightly (IC_{50} = 760 μM) in this study. Weak polypeptide synthesis inhibitory activity and antimicrobial activity of deaminohydroxyblastidicin S have been reported.[15,18] Therefore, the reason why both amino groups were modified and why *N*-acetyldeaminohydroxyblasticidin S was produced by *A. flavus* may be due to the complete inactivation of antifungal activity of blasticidin S.

Among the three blasticidin S derivatives, only deaminohydroxyblasticidin S inhibited aflatoxin production by *A. flavus*, although it lost its antifungal activity against *A. niger*. This result indicates that an amino group at the C-13 moiety is important for inhibiting aflatoxin production and that the mode of action of deaminohydroxyblasticidin S in the inhibition of aflatoxin production was different from antifungal activity. Because the toxicity of deaminohydroxyblasticidin S against mammal cells is quite low[19] and because its specificity for aflatoxin production inhibition is higher than that of blasticidin S, deaminohydroxyblasticidin S may be a good lead compound for developing practically effective aflatoxin production inhibitors.

Blasticidin S and deaminohydroxyblasticidin S repressed the mRNA levels of *pksA*, *omtA*, and *aflR*. It has been reported that the expression of AflR was important for initiation of aflatoxin biosynthesis and was regulated by various environmental and nutrition conditions, including temperature, air supply, carbon source, nitrogen source, and zinc availability.[20] This result suggested that blasticidin S and deaminohydroxyblasticidin S might inhibit aflatoxin production by affecting an early step prior to *aflR* expression.

Acetyl-CoA was needed to produce *N*-acetyldeaminohydroxyblasticidin S in the in vitro conversion experiment, and the starter material for aflatoxin biosynthesis is also acetyl-CoA. When 1000 μM deaminohydroxyblasticidin S was added to the culture medium, 6.7 μM *N*-acetyldeaminohydroxyblasticidin S was produced (Table 2). Because one molecule of acetyl-CoA is used to produce one molecule of *N*-acetyldeaminohydroxyblasticidin S, 6.7 μM acetyl-CoA was consumed. On the other hand, 0.95 μM aflatoxin B_1 was reduced by the addition of 1000 μM deaminohydroxyblasticidin S (Figure 4). The amounts of aflatoxin B_1 in control and 1000 μM deaminohydroxyblasticidin S-treated sample were 0.99 and 0.04 μM, respectively. This meant that 9.5 μM acetyl-CoA was saved because 10 molecules of acetyl-CoA are consumed to biosynthesize 1 molecule of aflatoxin B_1.[21] The amount of acetyl-CoA consumed by acetylation of deaminohydroxyblasticidin S and that of acetyl-CoA saved by reducing aflatoxin B_1 production were approximately equal. It is speculated that intracellular acetyl-CoA was consumed by acetylation of deaminohydroxyblasticidin S, and this caused a decrease in aflatoxin biosynthesis. To verify this hypothesis, functional analysis of blasticidin S *N*-acetyltransferase may be essential. Work to purify blasticidin S *N*-acetyltransferase from *A. flavus* is now in progress.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Time course analysis of the effects of blasticidin S on aflatoxin and *N*-acetyldeaminohydroxyblasticidin S production by *A. flavus*; MS parameters for individual analytes; compounds whose amounts were altered by blasticidin S. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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