Diocstatin A is a strong inhibitor of aflatoxin production by *Aspergillus parasiticus*

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Diocstatin A (DotA), a metabolite of *Streptomyces*, is known to be an inhibitor of human dipeptidyl aminopeptidase II. Here, it was found that DotA strongly inhibited aflatoxin production by *Aspergillus parasiticus*, with an IC₅₀ value of 4.0 µM. The mycelial growth of the fungus was not affected by the addition of DotA at a concentration of 50 µM, but inhibition of conidiation was observed at the same concentration. DotA inhibited production of norsolorinic acid, an early biosynthetic intermediate of aflatoxin, and it strongly reduced the mRNA levels of genes encoding aflatoxin biosynthetic enzymes, and significantly decreased the mRNA level of *aflR*, which encodes a key regulatory protein for aflatoxin biosynthesis. In addition to these genes, the mRNA level of *brlA*, which encodes a conidiation-specific transcription factor, was also reduced by the addition of DotA. It was also found that DotA dramatically enhanced kojic acid production by the fungus. Furthermore, DotA inhibited production of sterigmatocystin, which is a toxic aflatoxin biosynthetic intermediate, and it also inhibited conidiation in *Aspergillus nidulans*. These results indicate that DotA has pleiotropic effects on regulatory mechanisms of fungal secondary metabolite production and differentiation, leading to inhibition of aflatoxin production.

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INTRODUCTION

Aflatoxins are a group of mycotoxins with potent toxicity and carcinogenicity toward mammals. They are produced by some strains of *Aspergillus* spp., such as strains of *Aspergillus parasiticus* and *Aspergillus flavus*, and they can be found as contaminants in a wide variety of food and feed commodities. Aflatoxin contamination in agricultural products is a serious problem from the viewpoint of food safety, and also that of economical loss (Payne, 1998; Bennett & Klich, 2003). However, it is difficult to resolve the problem because of the lack of an effective method to control aflatoxin production.

We have been studying specific inhibitors of aflatoxin production by *A. parasiticus*, based on the idea that they may be useful in preventing foods and feeds from contamination by aflatoxin, without incurring a rapid spread of resistant strains. We also wished to determine the mechanism of aflatoxin production by the fungus at the molecular level. We have found that aflastatins A and B (AsA and AsB), and blasticidin A (BcA), are inhibitors of

aflatoxin production, and we have determined their structures; they are produced by *Streptomyces*, and share a similar structure (Sakuda *et al.*, 1996, 2000a; Ono *et al.*, 1997, 1998; Ikeda *et al.*, 2000). They strongly inhibit aflatoxin production by *A. parasiticus* by disturbing the primary metabolism of the fungus, which may regulate a pathway leading to expression of aflatoxin biosynthetic enzymes (Kondo *et al.*, 2001). However, a detailed molecular mechanism of inhibition of aflatoxin production by these compounds has not yet been clarified.

BcA has antibiotic activity toward the yeast *Saccharomyces cerevisiae* (Sakuda *et al.*, 2000b). During the course of a recent investigation into the mode of action of BcA in the yeast, we observed that the amount of a diketopiperazine produced in yeast cells was drastically reduced in the presence of BcA (unpublished data). Since the diketopiperazine, which is composed of methionine and proline, was thought to be synthesized through a dipeptide produced by the action of a dipeptidyl peptidase (DPP) on a certain protein or peptide, it was assumed that there might be a relationship between the action of BcA and the DPP. Thus, the inhibitory activity of BcA toward DPP, and that of several known DPP inhibitors toward aflatoxin

Abbreviations: DAB, 1,4-diamino-2-butanone; DPP, dipeptidyl peptidase; NA, norsolorinic acid; ODC, ornithine decarboxylase.

production, was tested. The results showed that BcA exhibited no inhibition toward human DPP II or DPP IV, but we found that one of the DPP inhibitors, diocatin A (DotA; Fig. 1), strongly inhibited aflatoxin production of *A. parasiticus*, without affecting fungal growth. Although it has not become clear why production of the diketopiperazine is reduced by BcA, we appeared to have discovered a new biological activity of DotA.

DotA was isolated from the culture broth of *Streptomyces* sp. SA-2581, as an inhibitor of human dipeptidyl peptidase II (DPP II), in 1991 (Takeuchi *et al.*, 1991). DPP II acts at acidic pH, and releases an N-terminal dipeptide from a protein with proline or alanine in the penultimate position. In mammals, DPP II is known to be an intracellular protease, and is thought to be associated with cell death of human leukocytes (Maes *et al.*, 2006), but a detailed function of DPP II, including its substrate protein in the cells, has not yet been clarified. There has been no published information on the biological activity of fungal DPP II. A homology search based on known fungal

genome sequences of *Aspergillus oryzae* (Machida *et al.*, 2005), *Aspergillus nidulans* and *Aspergillus fumigatus* has not found any gene encoding a protein with high similarity to human DPP II in these fungal genomes, although a gene encoding an unknown serine-protease-like protein, with about 25 % similarity to DPP II, is present in each genome.

In this paper, we describe the detailed biological activity of DotA as a strong inhibitor of aflatoxin production by *A. parasiticus*. Our results indicate that DotA has pleiotropic effects on regulatory mechanisms of fungal secondary metabolite production and conidiation.

METHODS

Strains, growth media and chemicals. *Aspergillus parasiticus* strains NRRL 2999 (ATCC 26691) and ATCC 24690 were used as producers of aflatoxin and norsolorinic acid (NA), respectively. Aflatoxins B₁ and G₁ are the main aflatoxins produced by the NRRL 2999 strain. *Aspergillus nidulans* FGSC A4 was used as a sterigmatocystin producer. The strains were maintained on potato dextrose agar (PDA; Difco), and subcultured monthly. A spore suspension was prepared from a 2-week-old culture, at a concentration of 2.5×10^3 c.f.u. μl^{-1} , and used as inoculum for this study. All incubations were done at 28 °C. DotA was obtained from the culture broth of *Streptomyces* sp. SA-2581 (Takeuchi *et al.*, 1991). Orn-Pip and Dab-Pip were synthesized according to the method of Senten *et al.* (2002).

Analysis of growth and aflatoxin production. DotA was dissolved in a solution of methanol/HCl (100:0.01, v/v) at appropriate concentrations, and the solution was added to potato dextrose broth (PD; Difco) at a final concentration of 0.1 % (v/v). As a control culture, methanol/HCl (100:0.01, v/v) solution was added to a medium at 0.1 %. A 3 ml volume of medium was put into each well of a six-well microplate. The spore suspension of *A. parasiticus* NRRL 2999 (10 μl) was inoculated into each well, and the plate was incubated statically for 4 days. The culture broth was separated into mycelium and culture filtrate by filtration. After washing with 5 ml distilled water, the mycelium was collected into a 1.5 ml microtube, and dried at 100 °C for 3 h. Mycelial weight was calculated by subtracting the weight of a 1.5 ml microtube without the mycelium from the total weight. The culture filtrate was subjected to HPLC analysis of aflatoxin [COSMOSIL 5-ph column, 4.6 mm $\times$ 150 mm (Nacalai Co.); isocratic elution of tetrahydrofuran-H₂O (20:80, v/v); flow rate, 1.0 ml min^{-1} ; detection at 365 nm]. The retention times of aflatoxins B₁ and G₁ were 5.0 and 6.3 min, respectively. When the amount of aflatoxin in the mycelium had been analysed, the mycelial cake that had been separated from the culture broth by filtration was extracted with chloroform, and the chloroform extracts were subjected to HPLC analysis of aflatoxin under the conditions described above.

Analysis of NA production. DotA was added to PD, according to the method used for analysis of aflatoxin. A 1 ml volume of the medium was put into each well of a 24-well microplate. The spore suspension of *A. parasiticus* ATCC 24690 (10 μl) was inoculated into the plate, and it was incubated statically for 5 days. The mycelial cake was harvested by filtration, and extracted with chloroform (1 ml). The chloroform extract was evaporated to dryness. The residue was dissolved in methanol (100 μl), and subjected to HPLC analysis [Capcell-Pak C₁₈ column, 4.6 mm $\times$ 250 mm (Shiseido); gradient elution of 70–100 % methanol in water containing 0.1 % TFA for 15 min, followed by isocratic elution of 100 % methanol containing

Fig. 1. Structures of DotA, Orn-Pip, Dab-Pip and cyclo(L-leucyl-L-prolyl).

0.1% TFA; flow rate, 0.8 ml min⁻¹; detection at 315 nm]. The retention time of NA was 21 min.

Analysis of sterigmatocystin production. The wells of a 24-well microplate, each containing 1 ml PDA, were inoculated with the spore suspension of *A. nidulans* FGSC A4 (10 µl), and incubated for 6 days. The agar medium in each well, together with the mycelial cake grown on it, was extracted with methanol (10 ml). The methanol extract was filtered, and evaporated to dryness. Water and chloroform (1 ml each) were added to the residue, and the mixture was vortexed. The chloroform layer was collected, air-dried, dissolved in chloroform (100 µl), and subjected to HPLC analysis using the same conditions as those described for NA. The retention time of sterigmatocystin was 16 min.

RT-PCR analysis of the genes encoding proteins involved in aflatoxin biosynthesis and conidiation. The wells of a 24-well plate, each containing 1 ml PD, were inoculated with the spore suspension of *A. parasiticus* NRRL 2999 (10 µl), and incubated for 3 days. The mycelial cake was harvested by filtration. Total RNA was isolated from the mycelial cake by using TRIzol Reagent (Invitrogen). First-strand cDNA was prepared with the SuperScript III First Strand Synthesis System (Invitrogen), using random hexamer primers, according to the protocol. The cDNA derived from 0.005 µg total RNA was used as a template. Real-time quantitative RT-PCR was carried out using the SYBR Green Master Mix (Applied Biosystems), in a final volume of 25 µl for each reaction, and an ABI PRISM 7300 thermal cycler (Applied Biosystems). Two-step PCR conditions were as follows: after an initial incubation at 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min were performed. The PCR primers for each gene were as follows: *pksA*, 5'-TGCATGGCGATGTGGT-AGTT-3' and 5'-GTAAGGCCGCGAAGAAAG-3'; *omtA*, 5'-GGC-CATATCCGCGAGCTT-3' and 5'-CGCATGACCACATCCCAAT-3'; *ver-1*, 5'-CCAATGCGGCCGTTGT-3' and 5'-TGAGAAAAACGAC-GCAATGAA-3'; *aflR*, 5'-GGCTGGTCAGGAGCAAAGC-3' and 5'-CCCCGAATTCGAATCG-3'; *brlA*, 5'-CCCTCTATGGTTTCGCA-GTAAC and 5'-GGCGGCTCTGGCAGATAC; *β-actin* (control gene), 5'-TGCTCTCGTTATCGACAATGGT-3' and 5'-CATCGTCA-CCGGCGAAA-3'.

Determination of spore numbers. The spore suspensions of *A. parasiticus* NRRL 2999 (50 µl) and *A. nidulans* (50 µl) were inoculated onto PDA, with and without 50 µM DotA. After 7 days cultivation, 3 ml distilled water containing 0.1% Triton X-100 was added to the plate. Conidia were harvested by scraping the surface of the agar with a bent glass rod. This procedure was repeated twice. The spore suspensions obtained were combined, and the number of conidia was counted using a haemocytometer.

Analysis of the production of yellow pigment and kojic acid. The spore suspension of *A. parasiticus* NRRL 2999 (50 µl) was inoculated onto PDA plates, with and without 50 µM DotA. After 7 days cultivation, the agar and the mycelial cake were extracted with methanol. The methanol extract was filtered, and evaporated to dryness. The residue was dissolved in 1 ml water, and fractionated by a Sep-Pak C₁₈ Cartridge (Waters). A yellow pigment and kojic acid were eluted from the cartridge by 20% acetonitrile in water. The fraction was subjected to HPLC [Capcell-Pak C₁₈ column, 10 mm × 250 mm (Shiseido); gradient elution of 0–100% acetonitrile in water containing 0.1% TFA for 30 min; 3.0 ml min⁻¹ flow rate; detection at 315 nm]. The yellow pigment was eluted from the column at 18 min, but after lyophilization it could not be rechromatographed under the same HPLC conditions, possibly because of its instability. The following HPLC data were obtained for kojic acid: retention time, 10 min; FAB-MS *m/z* 165 (M+Na)⁺; δ_H (CD₃OD, 500 MHz): 6.50 (H-3, s), 7.96 (H-6, s), 4.41 (H-7); δ_C (CD₃OD, 500 MHz): 177.4 (C-4), 170.9 (C-2), 147.9 (C-5), 141.6

(C-6), 111.3 (C3), 61.7 (C-7). Kojic acid was quantified by the colorimetric method of Bentley (1957): a 20 µl volume of sample was mixed with 200 µl freshly prepared 1% (w/v) FeCl₃, and the absorbance was measured at 500 nm.

RESULTS

Effects of DotA on aflatoxin production and fungal growth

We tested the effects of DotA on aflatoxin production and growth of *A. parasiticus* NRRL 2999. The fungus was cultured in PD, with and without DotA, at 28 °C for 4 days; in the control culture without DotA, it is known that both the amount of aflatoxin and the mycelial weight reach a maximum under these culture conditions (Kondo *et al.*, 2001). Fig. 2(a) shows the amount of aflatoxin produced in each culture filtrate of the fungus. DotA produced dose-dependent inhibition of aflatoxin production by the NRRL 2999, with an IC₅₀ value of 4.0 µM. However, the mycelial weight of the fungus was not affected by DotA, even at a concentration of 50 µM (Fig. 2b). A small amount of aflatoxin (less than 3% of the total amount in the culture broth) was obtained from the mycelium of the fungus in the control culture, but aflatoxin was not detected in the mycelium cultured in the presence of DotA at a concentration of 0.2 µM, indicating that DotA inhibited aflatoxin production without causing accumulation of aflatoxin in the mycelium. Unlike AsA and BcA, DotA showed no antibiotic activity toward *S. cerevisiae* at a concentration of 50 µM (data not shown) (Sakuda *et al.*, 2000b).

Effect of DotA on NA production

NA is a very early intermediate in the aflatoxin biosynthetic pathway. *A. parasiticus* ATCC 24690, a mutant blocked in aflatoxin biosynthesis, can accumulate NA in the mycelium (Lee *et al.*, 1971). When the ATCC 24690 strain was cultured in PD with DotA, NA production in the mycelium was inhibited more strongly than aflatoxin production by the NRRL 2999 strain, and showed an IC₅₀ value of 0.8 µM (Fig. 2c). This result shows that DotA inhibits an early step of aflatoxin biosynthesis, before the synthesis of NA.

Effects of DotA on the transcription of genes encoding proteins involved in aflatoxin biosynthesis

Next, we examined whether DotA affects the expression of genes encoding proteins involved in aflatoxin biosynthesis. The NRRL 2999 strain was cultured in PD, with and without DotA, for 3 days, when mRNA levels of aflatoxin biosynthetic genes are known to be maximal (Kondo *et al.*, 2001). The mycelium was obtained by filtration, and total RNA was extracted. We analysed the mRNA levels of *pksA*, *ver-1*, *omtA* and *aflR* by quantitative PCR. The genes *pksA*, *ver-1* and *omtA* encode enzyme proteins involved in the

Fig. 2. Effects of DotA on aflatoxin production, fungal growth and NA production. (a, b) *A. parasiticus* NRRL 2999 was cultured in PD, with and without DotA, at 28 °C for 4 days. (a) The amount of aflatoxin (total amount of aflatoxins B₁ and G₁) in the culture filtrate was analysed by HPLC. (b) The mycelial cake was harvested by filtration, dried at 100 °C for 3 h, and weighed. Data are means $\pm$ SD ($n=6$). (c) *A. parasiticus* ATCC 24690 was cultured under the same conditions used in the cultivation of the NRRL 2999 strain. NA was extracted from the mycelial cake with chloroform, and analysed by HPLC. Data are means $\pm$ SD ($n=4$).

aflatoxin biosynthetic pathway (Yabe & Nakajima, 2004), and *aflR* is a regulatory gene whose product regulates transcription of some genes, including *pksA*, *ver-1* and *omtA* (Woloshuk *et al.*, 1994). When DotA was added to the culture, there was a strong dose-dependent repression of the transcription of *pksA*, *ver-1* and *omtA* (Fig. 3a–c), and a significant repression of *aflR* (Fig. 3d).

Effects of DotA on conidiation

When NRRL 2999 was cultured in a liquid medium, DotA did not influence the morphology of the fungus or the mycelial weight. However, on solid medium, DotA strongly affected differentiation and morphology of the fungus. Seven days after cultivation, the control culture without DotA germinated green conidia (Fig. 4a), while inhibition of conidium formation (Fig. 4b) and induction of fluffy morphology (Fig. 4c) were observed in the culture with DotA (50 µM). The inhibition of conidiation was confirmed by counting conidia numbers, and these were strongly reduced by the addition of DotA (Fig. 4d). The mRNA level of *brlA*, which encodes one of three conidiation-specific transcription factors (Boylan *et al.*, 1987), was significantly reduced by the addition of DotA: mRNA levels were (mean $\pm$ SD) 63 ± 24 , 59 ± 10 , 55 ± 21 and 45 ± 20 % in 0.8, 3.2, 12.5 and 50 µM DotA, respectively, and they all showed a significant difference ($P < 0.01$, $n=7-9$) from the control that did not contain DotA (100 % mRNA).

Promotion of kojic acid production by DotA

We noticed that production of two compounds was clearly increased by addition of DotA in the solid culture of the NRRL 2999 strain. To identify the compounds, the strain was cultured on PDA for 7 days, and the compounds were extracted with methanol from the agar, and quantified by a colorimetric method. One compound, a yellow pigment observed in Fig. 4(b), was not identified because of its instability, but the other was identified as kojic acid. The effect of DotA on the increase of kojic acid production was very marked: kojic acid levels (mean $\pm$ SD, $n=3$) were 0.06 ± 0.03 , 0.5 ± 0.1 , 2.2 ± 0.5 , 4.1 ± 0.4 and 4.6 ± 0.4 mg ml⁻¹ in 0, 0.8 3.2 12.5 and 50 µM DotA, respectively.

Effect of DotA on sterigmatocystin production and conidiation in *A. nidulans*

Sterigmatocystin is known to be a biosynthetic intermediate of aflatoxin, and also a mycotoxin produced by many fungi, including *A. nidulans*. Since *A. nidulans* is a genetic model organism whose whole genome has been sequenced, and there is more information available on the regulatory mechanism of sterigmatocystin production in *A. nidulans* than information on aflatoxin production in *A. parasiticus*, the effect of DotA on the sterigmatocystin production was tested to investigate the mode of action of DotA. When *A. nidulans* FGSC A4 was cultured on PDA with DotA, production of sterigmatocystin was strongly inhibited, with an IC₅₀ value of 0.3 µM: sterigmatocystin levels (mean $\pm$ SD, $n=3$) were 17.2 ± 7.0 , 8.9 ± 3.0 , 5.7 ± 1.3 , 1.1 ± 0.7 , 0.6 ± 0.6 and 0.2 ± 0.5 mg ml⁻¹ in 0, 0.2, 0.8 3.2 12.5 and 50 µM DotA, respectively. DotA also inhibited conidiation of *A. nidulans*: $1.0 \times 10^9 \pm 3.0 \times 10^8$ conidia per plate were produced in 50 µM DotA, compared with

Fig. 3. Effects of DotA on the transcription of genes encoding aflatoxin biosynthesis enzymes. Total RNA was prepared from the mycelium of *A. parasiticus* NRRL 2999 cultured in PD, with and without DotA, for 3 days. Transcription of *pksA* (a), *ver-1* (b), *omtA* (c), and *aflR* (d), was analysed by real-time quantitative PCR. We used β -actin mRNA as an endogenous reference because its copy number is not influenced by DotA. The amount of each mRNA was normalized to the amount of β -actin mRNA in each sample. Data are means $\pm$ SD ($n=7-9$). The differences between the amounts of mRNA were assessed by one-way ANOVA, followed by the Dunnett test. * $P<0.05$, ** $P<0.01$, versus the control.

$3.3 \times 10^9 \pm 2.9 \times 10^8$ conidia per plate in the control containing no DotA (mean $\pm$ SD, $n=3$). This effect on conidiation was weaker than that observed for *A. parasiticus* (Fig. 4d).

DISCUSSION

The discovery of the aflatoxin production inhibitory activity of DotA was a surprise finding of our study on the mode of action of BcA (unpublished data). Since DotA is a strong inhibitor of human DPP II, it is possible to speculate that the activity of DotA toward aflatoxin production could be attributed to inhibition of a function of a DPP-II-like protein present in *A. parasiticus*. Some synthetic compounds, such as Orn-Pip and Dab-Pip (Fig. 1), are known to be human DPP II inhibitors (Senten *et al.*, 2002), and they can inhibit the enzyme to the same extent as DotA. We tested the effects of Orn-Pip and Dab-Pip on aflatoxin production of the NRRL 2999 strain, but they showed no inhibition, even at concentrations as

high as 500 μ M (data not shown). At present, it is not clear if the mode of action of DotA in *A. parasiticus* is related to a function of a DPP-II-like protein that has low homology to human DPP II, and which may be present in the fungus, as it is in other *Aspergillus* spp. mentioned in the Introduction. It has been reported that cyclo(L-leucyl-L-prolyl) (Fig. 1) can inhibit aflatoxin production weakly, with an IC_{50} value of 0.2 mg ml $^{-1}$ (Yan *et al.*, 2004). Because this diketopiperazine contains a proline residue, and DPP II releases an N-terminal dipeptide from a protein with a proline residue in the penultimate position, it is possible that some DPP-II-like proteins of the fungus are concerned with the action of the diketopiperazine compound.

DotA inhibited aflatoxin production, and also conidiation of *A. parasiticus*. Similar inhibition by DotA was also observed in sterigmatocystin production and conidiation of *A. nidulans*. Some phenomena have been observed that suggest that there is a relationship between conidiation and mycotoxin production in *Aspergillus* spp. (Calvo *et al.*,

Fig. 4. Effect of DotA on conidiation. *A. parasiticus* NRRL 2999 was cultured on PDA without DotA (a), and with 50 μ M DotA (b, c), for 7 days. Part of (b) is magnified in (c). Bars, 1 cm (a, b); 0.2 cm (c). (d) Conidia numbers were counted with a haemocytometer. Data are means $\pm$ SD ($n=3$).

2002). For example, many of the fluffy mutant strains of *A. nidulans* that are unable to form conidia cannot produce sterigmatocystin either (Wieser *et al.*, 1997). It is also known that conidiation-inhibiting compounds can sometimes decrease mycotoxin production. For example, ethylene can inhibit both conidiation and sterigmatocystin accumulation in *A. nidulans* (Roze *et al.*, 2004a), and 1,4-diamino-2-butanone (DAB), a competitive inhibitor of ornithine decarboxylase (ODC), can repress both conidiation and aflatoxin biosynthesis of *A. parasiticus*, at a concentration of 50 mM. This effect of DAB is counteracted by addition of putrescine, which is a product of the action of ODC, and spermidine, which is a polyamine biosynthesized from putrescine (Guzman-de-Pena & Ruiz-Herrera, 1997). We tested the effect of spermidine on the activity of DotA, but the activity of DotA on both aflatoxin production and conidiation was not counteracted at all (data not shown), suggesting that the mode of action of DotA may be different from that of DAB.

Because DotA inhibited NA production, and reduced the mRNA levels of *aflR* and *brlA*, it may affect a regulatory system that controls both aflatoxin production and conidiation. Recently, an adenylyl cyclase/cAMP/cAMP-dependent protein kinase cascade has been suggested to be important for both sterigmatocystin production and sporulation in *A. nidulans* (Shimizu *et al.*, 2003; Roze *et al.*, 2004b). In the cascade, G-protein-mediated signalling regulates the activity of adenylyl cyclase, and mutation of a protein, e.g. FadA or FlbA, involved in the signalling pathway causes the fluffy phenotype. The effects of DotA observed in *A. nidulans* suggest that this G-protein-signalling pathway may be a possible target for DotA.

Aflatoxin production by *A. parasiticus* was inhibited by DotA, but production of kojic acid and a yellow pigment was markedly increased. This suggests that DotA may influence secondary metabolism of the fungus. If DotA can affect secondary metabolism of other fungi in a similar way to that for *A. parasiticus*, DotA may have the potential to reduce the production of unwanted compounds, and also to increase production of useful compounds.

DotA strongly inhibits aflatoxin production and conidiation, without affecting fungal growth. These features are very advantageous in prevention of aflatoxin contamination of foods and feeds, without a rapid spread of resistant strains, and also without wide diffusion of conidia. DotA inhibited aflatoxin production in a model infection system consisting of *A. parasiticus* on raw peanuts (Sakuda *et al.*, 2006), suggesting that it may be effective in preventing aflatoxin contamination of peanuts during storage. We are currently testing the effect of DotA on the whole peanut plant by using a method that was previously used for confirming the effect of aflastatin A in prevention of aflatoxin contamination (Sakuda *et al.*, 1999). DotA has a relatively simple structure, and shows no toxicity to mammals. Therefore, DotA may be a good lead compound for developing practically effective drugs. From the viewpoint

of basic research, DotA is a very important probe to use in the investigation of the regulatory mechanism of fungal secondary metabolite production and differentiation. Work to clarify the mode of action of DotA with a photo-affinity probe, and to develop more effective DotA derivatives, is now in progress.

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