DOI: 10.1002/cbic.200800080

3-Methylarginine from *Pseudomonas syringae* pv. *syringae* 22d/93 Suppresses the Bacterial Blight Caused by Its Close Relative *Pseudomonas syringae* pv. *glycinea*

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In memoriam Dr. Jonathan B. Spencer.

The epiphyte Pseudomonas syringae *pv.* syringae *22d/93 (Pss22d) produces a toxin that strongly inhibits the growth of its relative, the plant pathogen* P. syringae *pv.* glycinea*. The inhibition can be overcome by supplementing the growth medium with the essential amino acid,* L*-arginine; this suggests that the toxin acts as an inhibitor of the arginine biosynthesis. The highly polar toxin was purified by bioassay-guided fractionation using ion-exchange chromatography and subsequent RP-HPLC fractionation. The structure of the natural product was identified by HR-ESI-MS, HR-ESI-MS/MS, and NMR spectroscopy experiments as 3-methylarginine. This amino acid has previously only been known in nature as a constituent of the peptide lavendomycin from* Streptomyces lavendulae*. Results of experiments in which labeled methionine was fed to Pss22d indicated that the key step in the biosynthesis of 3-methylarginine is the introduction of the methyl group by a S-adenosylmethionine (SAM)-dependent methyltransferase. Transposon mutagenesis of Pss22d allowed the responsible SAM-dependent methyltransferase of the 3-methylarginine biosynthesis to be identified.*

Introduction

Members of the genus *Pseudomonas* are widespread rod-shaped Gram-negative bacteria with remarkable metabolic versatility. The genus not only harbors many plant and human pathogens but also comprises species that promote plant growth, antagonize plant pathogenic microorganisms, or induce resistance in plants.[1] Their ability to produce a variety of secondary metabolites that inhibit the growth of other microorganisms[2] makes fluorescent pseudomonads attractive biological control agents against plant pathogens, in particular, the closely related *Pseudomonas syringae,* which cause severe economic losses worldwide.[3] Like most phytopathogenic bacteria that are not obligate parasites and survive in a wide range of habitats, *P. syringae* pathogens have been identified from many plants and soil.[3,4] *P. syringae* pv. *syringae* is one of the most abundant pathovars of this species in nature.

In recent years, interest in the biological control of bacterial plant diseases by using naturally occurring epiphytic bacteria has increased. The screening for antagonistic epiphytes against *P. syringae* plant pathogens has identified *P. syringae* pv. *syringae* strain 22d/93 (Pss22d) as a promising biocontrol agent. Pss22d was isolated from a soybean leaf that did not show any disease symptoms.[5] The antagonism of Pss22d against bacterial blight of soybean caused by *P. syringae* pv. *glycinea* (Psg; Figure 1A) has been successfully demonstrated in vitro, in planta, and under field conditions.[5,6] As siderophore production of Pss22d was excluded as an active principle of this antagonism,[7] its toxins attracted our attention. In addition to producing the common (among *P. syringae* pv. *syringae*) syringomycin and syringopeptin,[8,9] Pss22d produces a hydrophilic low-molecular-weight toxin the structure of which is so far unknown; this toxin was identified to selectively inhibit Psg but no other pseudomonads (Figure 1B).[5] This inhibition can be compensated for by L-arginine supplementation (Figure 1C) but not by any other essential amino acid; this suggests that the toxin influences arginine biosynthesis.[5] Herein, we describe the isolation, structure elucidation, and biosynthesis of this novel toxin produced by Pss22d isolated from soybean.

Results and Discussion

Toxin production and purification

In order to identify the toxin produced by Pss22d that specifically inhibits Psg, its production was optimized by using vari-

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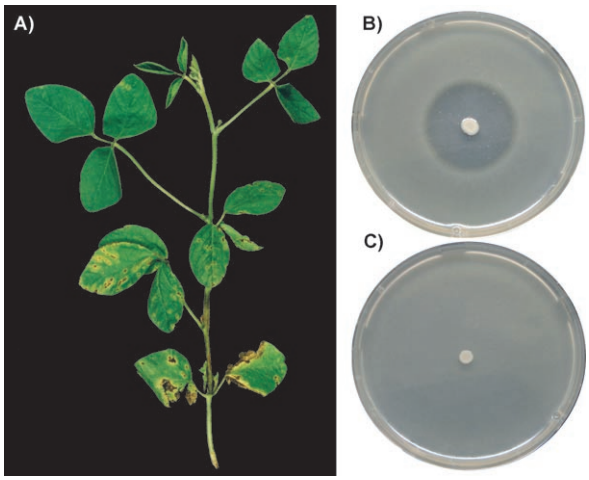


Figure 1. A) Bacterial blight disease caused by the plant pathogen *P. syringae* pv. *glycinea* (Psg) on *Glycine max* (soybean); B) growth inhibition of the plant pathogen Psg by *P. syringae* pv. *syringae* 22d/93 in vitro (white colony in the middle of the plate); C) compensation of Psg growth inhibition by addition of L-arginine (100 mM) to the medium.

ous growth media and temperatures. Highest toxin activity was detected in HSC medium at 28 °C. The growth curve of Pss22d indicated that the toxin is already synthesized during the exponential growth phase (Figure 2).

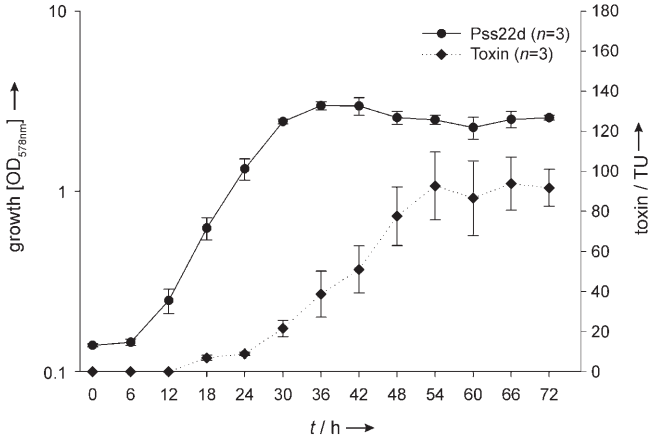


Figure 2. Growth of Pss22d in HSC liquid media at 28 °C for 72 h ($n = 3$) and toxin production ($n = 3$; TU: toxin units).

Toxin production at the beginning of the log phase is rather untypical for microbial secondary metabolite formation, such as antibiotics or toxins. Their production is often initiated in response to quorum sensing signals at the end of the stationary growth phase.[10] As we have observed in Pss22d toxin formation, other secondary metabolites from *P. syringae* pathovars, for example, coronatine, are known to be produced during the growth phase.[11] Pss22d reached its maximum toxic activity after 48 h and maintained a constant level (*t*-test, $p < 0.05$). The toxic activity of the cell-free filtrates remained despite exposure to drastic pH values (pH 3–12) and temperatures of up to 121 °C.

Initial purification experiments revealed that the toxin is highly polar and cannot be extracted with organic solvents. Nevertheless, it was soluble in methanol and binds to ion-exchange resins such as CM-Sephadex, C-25. In order to elucidate the toxin structure, Pss22d was grown in HSC liquid medium (1 L) for 48 h at 28 °C. The culture supernatant was extracted with ethyl acetate and the bioactive water phase was freeze-dried, taken up in methanol, and purified by ion-exchange chromatography. The obtained fractions were analyzed in agar-diffusion assays for bioactivity against the plant pathogen Psg. Active fractions were eluted with ammonium hydrogen carbonate (0.3 M) from the ion-exchange resin. HPLC separation on a Phenomenex Synergy polar RP in combination with ESI-MS detection allowed us to collect the fractions that contained the pure toxin.

Structure elucidation

The toxin eluted at 3.7 min from the Phenomenex polar RP HPLC column and exhibited a quasimolecular ion at *m/z* 189. High-resolution ESI-MS suggested the molecular formula $C_7H_{17}N_4O_2$, which indicates the presence of two double bond units. Considering the physicochemical behavior of the toxin together with its HR-ESI-MS, and the observation that the toxicity of the compound could be compensated for when L-arginine (**2**) is supplied to the medium,[5] an amino-acid-like structure was suspected (Figure 3).

Comparison of the ESI-MS/MS spectrum of the toxin with that of arginine (**2**) revealed a highly similar fragmentation pattern. The shift of 14 amu for most of the fragments (175→189, 157→171, 158→172, 130→144, 116→130, 112→126, 70→84) pointed to an arginine derivative with an extra methyl group (Figure 3). In order to prove the deductions and to identify the position of the extra methyl group, 1D and 2D NMR spectra were recorded from the purified toxin (1 L; yield about 1 mg L^{-1}). In the ^1H NMR spectrum (MeOD, 500 MHz, 300 K) a doublet signal that accounted for a 3-CH_3 group at 0.91 ppm was observed, and showed a cross-signal in the H,H-COSY to the 3-CH group adjacent to the 2-CH–NH_2 moiety of an α-amino acid. Moreover, signals corresponding to a –CH_2–CH_2– chain were observed from the 3-CH group in the direction of the guanidine residue. In addition, the APT and HSQC spectra supported the deductions and proved the presence of a carboxyl group (signal at 171.7 ppm) as well as the imino group (signal at 158.8 ppm) of the expected guanidine moiety (see the Supporting Information).

By combining the obtained spectral information, the toxin from Pss22d was identified to be 3-methylarginine (**1**). This amino acid has so far not been found to occur as a free amino acid in nature but it has been characterized previously as an amino acid component of the peptide antibiotic lavendomycin from *Streptomyces lavendulae*.[12]

Attempts to address the stereochemistry of 3-methylarginine (**1**) by NMR spectroscopy and derivatization methods failed because of the limited sample material. However, treatment of

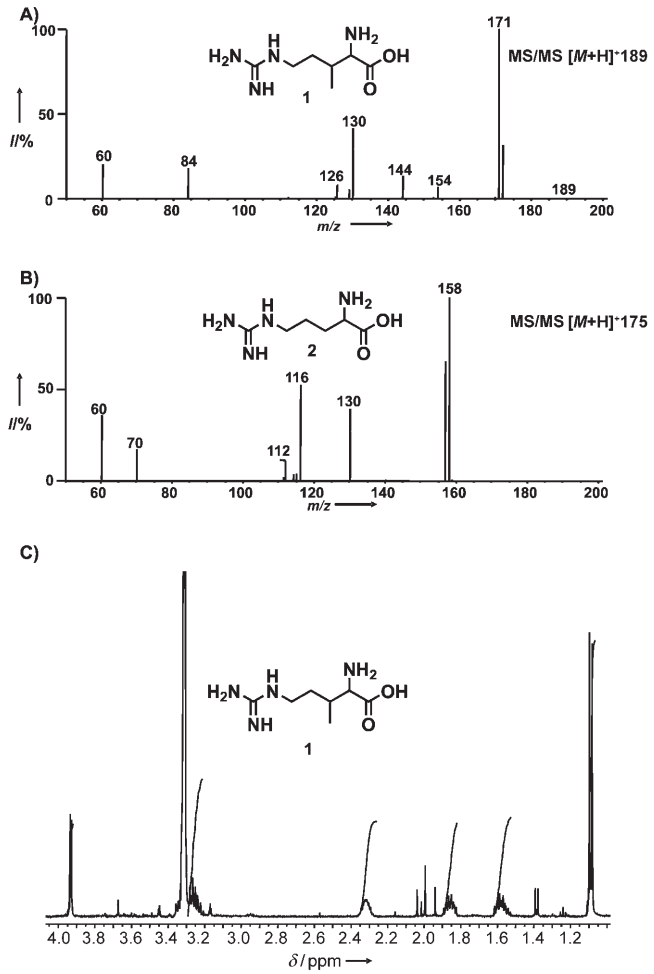


Figure 3. A) LC-ESI MS/MS of the $[M+H]^+$ 189 of 3-methylarginine (**1**); B) LC-ESI MS/MS of the $[M+H]^+$ 175 of arginine (**2**); and C) ^1H NMR spectrum (500 MHz, MeOD, 300 K) of **1**.

3-methylarginine (**1**) with both the D-amino acid oxidase[14] from porcine kidney and the L-amino acid oxidase[13] from *Crotalus atrox* yielded only the expected oxo acid **7** with the L-specific amino oxidase. Therefore, the amino group of **1** appears to be L configured.

Notably, the *syn*- and *anti*-isomers of the pentafluorobenzylhydroxyloxime derivatives of **7**, which were used for the sensitive LC-MS detection of **7**, exhibited strong differences in the peak intensities of their ESI-MS/MS spectra. The separation of the *syn*- and *anti*-isomers of the pentafluorobenzyloximes of **7** was more pronounced than that of the corresponding pentafluorobenzylhydroxyloximes derived from the transamination of arginine (**2**; see the Supporting Information). The latter effect can be attributed to the 3-methyl group of **1**.

Biosynthesis of 3-methylarginine

Regarding the biosynthesis of 3-methylarginine (**1**), we focused on the origin of the 3-methyl group, as it is responsible for the toxic effect of **1**. The methyl group of **1** might stem from a small-molecule precursor that undergoes condensation with another precursor molecule to form **1**. However, methyl groups are often introduced into molecules by *S*-adenosylmethionine (SAM)-dependent methyltransferases.[15] The methyl transfer from *S*-adenosylmethionine can be mediated by either a nucleophilic attack to the protonated methyl group of SAM or a radical reaction mechanism.[16] Alternatively, the methyl group of SAM could be used to methylate the cobalamin cofactor of a vitamin B12-dependent enzyme. In cobalamin-dependent enzymes, the methylcobalamin catalyzes the methyl group transfer.[17]

In order to address the origin of the methyl group, we cultured Pss22d in the presence of labeled [$^2H_3$-CH_3-S]-methionine, the precursor of SAM (**5**). As the [$^2H_3$]-methyl group was found to be incorporated into [3-$^2H_3$]-3-methylarginine (**1 b**) in high yields (95%), there is no doubt that the methyl group of 3-methylarginine (**1**) originates from SAM. However, the feeding experiment did not clarify whether 5-amino-2-oxopentanoic acid (**6**) or 5-guanidino-2-oxopentanoic acid (**7**) serves as precursor for the introduction of the methyl group into **1**. Moreover, this feeding experiment did not address any mechanistic details of the methyl group transfer.

Random Tn5 transposon mutagenesis was used to identify the methyltransferase gene of Pss22d that catalyzes the methyl group transfer in 3-methylarginine biosynthesis. One of the obtained mutants, Pss22d.1 (Table 1), was identified as a SAM-dependent methyltransferase mutant by sequencing the flanking regions of the miniTn5 insertion. We analyzed the supernatant of mutant Pss22d.1 that was grown in the presence of spectinomycin as a selection marker and detected no trace of **1** in the LC-MS analysis (Figure 4). As a control, to see whether spectinomycin itself might affect 3-methylarginine formation, two nonmethyltransferase Tn5 mutants (Pss22d.2, Pss22d.3) were analyzed; these were also grown in the presence of spectinomycin, and both produced **1**, as did the wild type.

Table 1. Bacterial strains and plasmids used in this study.

Strains	Relevant characteristics[a]	Source
P. syringae pv. *syringae*		
Pss22d	wild type from soybean	[5]
Pss22d.1	transposon mutant, 3-methyl-arginine negative, Sp^r	this study
Pss22d.2	transposon mutant, 3-methyl-arginine positive, Sp^r	this study
Pss22d.3	transposon mutant, 3-methyl-arginine positive, Sp^r	this study
P. syringae pv. *glycinea*		
Psg1a	wild type from soybean	[6]
E. coli		
DH5α	*rec*A *lac*ZΔM15	[34]
S17λpir	*rec*A, *thi*, *pro*, *hsd*R-M+, RP4:2-Tc:Mu:Km Tn7, *λpir*, Tp^r, Sm^r	[35]
Plasmids		
pCAM-Not	Sp^r, Amp^r, mTn5SS40 transposon, pUT/mini-Tn5 Sm/Sp	[35]
pBBR1 MSC	cloning vector, broad host range, IncP IncQ, Cm^r	[36]

[a] Cm^r, Sp^r, Km^r, Amp^r, Tp^r, Sm^r resistance to chloramphenicol, spectinomycin, kanamycin, ampicillin, trimethoprim, streptomycin, respectively.

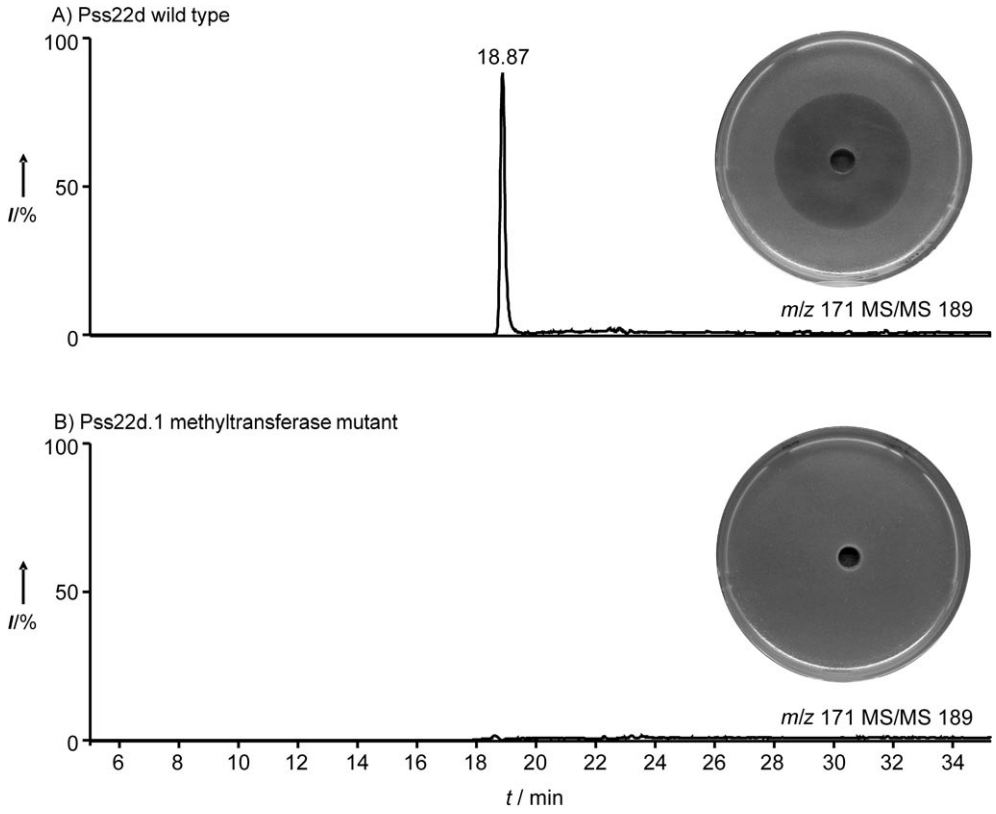


Figure 4. A) Wild-type Pss22d and B) SAM-methyltransferase mutant Pss22d.1. The ESI-MS/MS traces $[M+H-18]^+$ *m/z* 171 of 3-methylarginine (**1**) together with the agar-diffusion assays against Psg are shown (HPLC: HILIC conditions, Phenomenex Luna NH_2).

As the test organism, Psg, is sensitive to spectinomycin for the agar-diffusion assay, the mutant Pss22d.1 had to be cultured without spectinomycin in order to assess the supernatant of the mutant Pss22d.1 in the bioassay (see the Supporting Information). No growth inhibition of Psg by the mutant extract was observed in this bioassay (Figure 4B).

The obtained sequence for Pss22d was 95% identical to the hypothetical protein YP_233230 (putative methyltransferase) of the fully sequenced strain *Pseudomonas syringae* pv. *syringae* B728a (PssB728a does not produce **1**).[18] In order to distinguish between a SAM-dependent, a radical SAM-dependent, or a cobalamin-dependent methyl transfer mechanism, the conserved domains of the putative methyltansferase protein of Pss22d were compared to representatives of each class (Figure 5).

Methyltransferases share characteristic sequence motifs that allow them to be classified together. For example, motif 1 (DXGXGXG) is characteristic for a SAM binding site.[19] For radical SAM methyltransferases, a CXXXCXXC motif that is crucial for Fe–S cluster binding and a SAM-binding site DXHXXG motif (motif 1) are typical. Cobalamin-dependent enzymes have motif 1 but also comprise motif 2 (DXXGXS…GG), which is involved in cobalamin binding.[20]

The methyltransferase of Pss22d comprises a SAM-binding site (motif I) but is missing the characteristic motifs of radical SAM enzymes as well as those of cobalamin-dependent enzymes (motif 1 and 2, Figure 5). Also no Fe–S cluster binding site (CXXCXXC) motif was found in the sequence of the Pss22d methyltransferase (unpublished results). Protein BLAST search analysis revealed a conserved domain similar to that of methyltransferases type 12[21] (InterPro: IPR013217), the members of which are known to catalyze O-, N-, and C-methylation and have a Rossman-like α/β fold in common.

In their recent study, Mahlert et al. investigated a SAM-dependent methylation mechanism similar to that of 3-methylarginine biosynthesis by Pss22d. Their methyltransferases (GlmT, Dptl, Lptl) convert α-ketoglutatarate to 3-methylglutamate, which is part of acidic lipopeptides, such as calcium-dependent antibiotic (CDA), daptomycin, and A54145.[22,23] The SAM-dependent methyltransferases of 3-methylglutamate biosynthesis, however, show similarity to the ubiquinone methyltransferase (UbiE).[22] The results of the comparison of the conserved domains of type 12 SAM methyltransferases and UbiE SAM methyltransferase indicate that motif I but not motifs II and III are conserved between both types (Figure 5). These differences may also suggest some differences in the catalytic mechanism.

Given the protein sequence alignment, we suggest the following mechanism for the formation of 3-methylarginine by the methyltransferase from Pss22d (Scheme 1).

The electrophilic protonated methyl thioether of SAM (**5**) is attacked by a nucleophilic enol of a 2-oxo acid precursor (**3** or **4**); this yields the 3-methyl-2-oxo-acid **6** or **7**. Subsequently, the 3-methyl-2-oxo-acid **6** or **7** is converted to 3-methyl-2-amino acid **8** or **1** by a transaminase. Furthermore, radical methylation mechanisms are usually only favored to perform reactions at non-nucleophilic sites of the target molecule. Clearly, the ease of the formation of a nucleophilic enol **3** or **4** that is prone to react with the electrophilic protonated methyl group of SAM favors the suggested mechanism over a radical that requires a more elaborate catalytic mechanism.[16]

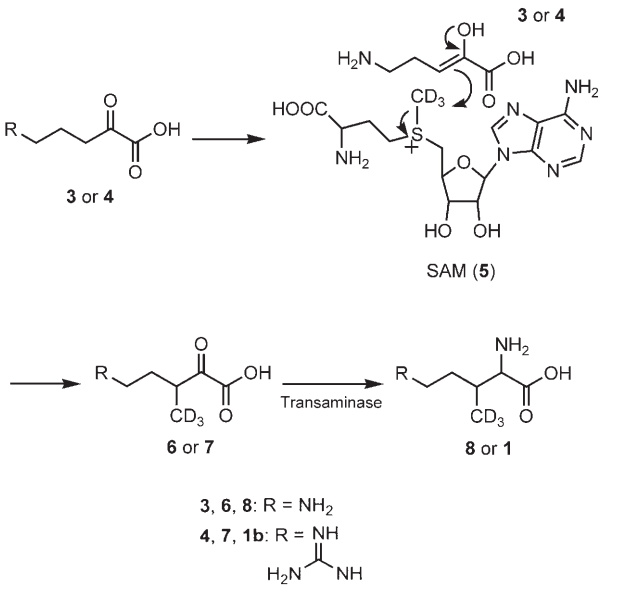
Ecological role of 3-methylarginine and suggested mode of action

3-Methylarginine (**1**) from Pss22d constitutes a powerful and selective toxin against Psg, and approximately 20 nmol of **1** cause a 33 mm inhibition zone in the agar-diffusion assay against Psg (Figure 4A). Thus it is likely that the epiphytic

Figure 5. A) Multiple protein sequence alignment of SAM-dependent methyltransferases. The alignment of the N terminus of type 12 and UbiE methyltransferases illustrates that motif I but not motifs II and III are highly conserved between these two groups. The type 12 SAM methyltransferase proteins are 22d (*P. syringae* pv. *syringae* 22d/93, putative methyltransferase, this study), B728a (*P. syringae* pv. *syringae* B728a, hypothetical protein, GI: 66043389), AAC (*Acidovorax avenae* subsp. *citrulli*, methyltransferase type 12, GI: 120612348), GNMT (*Rattus norvegicus*, glycine *N*-methyltransferase, GI: 8567354). The UbiE SAM methyltransferases are SCO[22] (*Streptomyces coelicolor*, hypothetical protein SCO3215, GI: 2122650), BS (*Bacillus subtilis*, menaquinone biosynthesis methyltransferase UbiE, GI: 399775), LL (*Lactococcus lactis* subsp. *lactis*, menaquinone biosynthesis methyltransferase UbiE, GI: 1346175), SCE (*Saccharomyces cerevisiae*, ubiquinone biosynthesis methyltransferase COQ5, GI: 1353083). B) Multiple protein sequence alignment of the N terminus of radical SAM and cobalamin-dependent methyltransferases show the highly conserved motifs 1 and 2, which are not present in type 12 and UbiE SAM methyltransferases. The radical SAM methyltransferases and cobalamin-dependent proteins are the bacteriochlorophyll biosynthesis protein BchE (GI: 114858), a fortimicin methyltransferase (GI: 1125024), a fosfomycin methyltransferase (GI: 2144248), the oxetanocin biosynthesis protein OxsB (GI: 7474372), CMT (corrinoid methyltransferase, GI: 7483270), MGM (2-methyleneglutarate transferase, GI: 543481), MS (methionine synthase, GI: 40024), GM (glutamate synthase, GI: 7245512).

Pss22d benefits from the toxin **1** in its natural habitat and gains a competitive advantage against the closely related plant pathogen Psg, which lives in the same environment. Consequently, since **1** functions ecologically as a toxin makes it an attractive biocontrol agent to protect soybean against bacterial blight.

Although the mode of action of 3-methylarginine (**1**) against the plant pathogen Psg remains to be established, the structure of the toxin provokes several hypothetical scenarios. The incorporation of **1** instead of L-arginine (**2**) into proteins might seriously affect their function and result in metabolic defects and finally lead to cell death. However, the rather low amount of **1** produced by Pss22d could point instead to its function as an enzyme inhibitor. As arginine analogues act as potent inhibitors of nitric oxide synthase (NOS),[24] it could be that **1** interferes with this enzyme. NOS is common among most animals, plants, and bacteria;[25] it uses L-arginine to generate nitric oxide (NO), which plays an important role as a signal in plant defense, and it might influence the virulence of pathogens and contribute to OxyR-mediated antioxidant defense.[26] Furthermore, hypothetical NOS genes are annotated for some *P. syringae* pathovars: for example, there are two genes in the genome of the fully sequenced strain *P. syringae* pv. *syringae* B728a.[18] Alternatively, **1** might competitively inhibit an enzyme of the arginine biosynthesis pathway in Psg. Inhibition of the plant pathogen Psg is reversed by **2**, but not by other precursors of arginine biosynthesis. Therefore, we suspect argininosuccinate lyase, which cleaves L-argininosuccinate to **2** and fumarate, to be a possible target enzyme of **1**. A similar mechanism is well known from phaseolotoxin produced by *P. syringae* pv. *phaseolicola*; this inhibits the ornithine carbamoyl transferase (OCTase).[8] Recently, Arrebola et al.[27] demonstrated that *P. syringae* pv. *syringae* strains isolated from mango trees produce a novel antimetabolite toxin named mangotoxin. It inhibits ornithine acetyl transferase—a key enzyme in the syn-



Scheme 1. Suggested biosynthesis of [3-$^2H_3$]-3-methylarginine (**1b**) deduced from administration of [$^2H_3$-CH_3-*S*]-methionine to the growth medium and the protein sequence alignment (Figure 5).

thesis of ornithine and arginine. Even though mangotoxin is not structurally related to **1**, it is interesting that both compounds seem to target arginine biosynthesis.

Conclusions

In summary, we have established the structure of the toxin from Pss22d to be the rare nonproteinogenic amino acid 3-methylarginine (**1**), which is highly active against the closely related plant pathogen Psg. The key step in its biosynthesis is the introduction of a 3-methyl group—very likely into a 2-oxo acid precursor—by the action of a SAM-dependent methyltransferase, which has been identified by transposon mutagenesis. Strikingly, the small modification of the additional methyl group, in comparison to the proteinogenic amino acid arginine (**2**), turns **1** into a potent and selective toxin against Psg.

Future experiments are needed to fully characterize the biosynthetic genes. The enzymes involved in 3-methyl amino acid biosynthesis are also attractive for the biotechnological production of 3-methyl amino acids, because such amino acids are not easily accessible in an enantiopure form by synthetic approaches.[26] The identification of 3-methylarginine (**1**) now allows further detailed studies of the chemoecological role of this toxin for the epiphyte Pss22d and its habitat to be carried out. Moreover, its potential as a biocontrol substance against the plant pathogen Psg can be evaluated in detail.

Experimental Section

General: ESI-MS measurements were performed by using a Thermoelectron LTQ or LCQ hooked to a HP-1100 HPLC that was fitted with a Phenomenex Synergy polar RP (250 mm × 2 mm, 4 μm) column, Phenomenex Luna NH_2 (250 mm × 2 mm, 5 μm) column, or an Alltech Grom-Sil ODS-7 PH (125 mm × 2 mm, 4 μm) column. Alternatively, the HPLC system was hooked to a Gilson 206 fraction collector in order to collect pure samples for NMR spectroscopy analysis. HR-ESI-MS measurements were conducted by direct insertion of the purified sample via a syringe pump by using a Thermoelectron Orbitrap; NMR spectroscopy was performed with a Bruker DRX 500 MHz NMR spectrometer. The chemical shifts of ^1H NMR and ^{13}C spectra are given in ppm (δ) and were referenced to the solvent signal CD_3OD 3.31 and 49.00 ppm, respectively.

Strains and culture conditions: The bacterial strains used in this study are listed in Table 1. *P. syringae* pv. *syringae* 22d/93 (Pss22d) wild type was previously isolated from soybean leaves.[5] Pss22d, the mutants of Pss22d, and the indicator strain *P. syringae* pv. *glycinea* 1a/96 (Psg1a) were cultured and maintained on King's B[28] agar plates at 28 °C. Cultures of Pss22d and its mutants were cultured in 1 L HSC (Hoitink–Sinden medium optimized for coronatine production) liquid medium[29] on a shaker with 200 rpm at 28 °C for 48 h. Then spectinomycin (25 μg mL^{-1}) was added to the medium of the mutants. *E. coli* DH5α were cultured on Standard 1 (Merck) agar plates and used for DNA manipulation.

Growth curve, physical, and chemical characteristics: In order to optimize the toxin production of Pss22d, different media (HSC,[29] 5b,[30] Pipes[31]) and temperatures (18, 28 °C) were tested. The growth curve of Pss22d was determined in three parallel experiments by using a Pss22d culture grown in HSC (100 mL) medium that was shaken (200 rpm, 28 °C) for 72 h. From an overnight preculture, the main cultures were inoculated with approximately 7×10^7 cfu mL^{-1}. Every 6 h samples were withdrawn and absorbance at 578 nm was measured (Amersham Bioscience, Ultraspec 2100 pro). The toxic activity of each sample was determined by agar-diffusion assay (see below). Physical parameters of the toxin were assessed by using a cell-free filtrate from Pss22d (heat stability at 65, 80, 100, and 121 °C for 15 min, and at 100 °C for 60 min; pH stability at pH 3, 9, and 12 for 15 min, and then readjusted to the original pH 6).

Agar-diffusion assay: Pss22d, its mutants, and all fractions of the purification were screened for toxin activity by using agar-diffusion assays with Psg1a as indicator strain. Psg1a was cultured on King's B agar plates, overnight, at 28 °C. Single colonies of Psg1a were scraped from the plate and resuspended in sterile water. A sample of this suspension (2 mL, about 4×10^8 cfu mL^{-1}) was added to 50 mL of melted 5b agar medium (50 °C) and poured onto plates (130 mm); samples (50 μL) were added into wells (9 mm) in the agar plates. The plates were incubated at 28 °C and analyzed after 24 h. To determine the relative toxin concentration, a standard curve was prepared by using the culture filtrate after the ion-exchange purification step (Supporting Information).

Isolation and identification of 3-methylarginine (1): Preliminary experiments with anion- and cation-exchange resins and different potassium buffers indicated that the best binding activity was on a cation exchange matrix (CM-Sephadex C-25) with HPLC water (pH 8.0). In order to remove hydrophobic substances from the crude extract it was extracted with ethyl acetate. The subsequent agar-diffusion assay indicated the presence of toxic activity in the water phase. The toxin extract was freeze-dried. Despite the hydrophilic properties of the toxin, it was easily soluble in methanol (recovery about 90% of toxic activity). The methanol phase was concentrated in vacuo and resuspended in water. These samples were directly applied onto a CM-Sephadex column (GE Healthcare Tricorn™, diameter 10 cm, length 300 cm). Preliminary gradient analysis on CM-Sephadex column resulted in a stringent elution of the toxin at 0.3 M ammonium hydrogen carbonate (pH 7.8). To simplify

the purification, HPLC water and ammonium hydrogen carbonate (0.2 M, pH 7.8) were used for loading and washing, respectively. A 0.3 M ammonium hydrogen carbonate solution served for elution and a 1.0 M ammonium hydrogen carbonate solution (pH 7.9) was used to clean the column. Active fractions were pooled and concentrated in vacuo. Further purification was performed by using HPLC separation (Phenomenex Synergy polar RP column); flow rate 0.25 mL min^{-1}, solvent A: H_2O, 0.1% TFA; solvent B: MeCN, 0.1 TFA; gradient: 5 min 100% A, in 27 min to 100% B, 100% B 5 min; injection volume 10–100 μL. Alternatively, samples were assayed by using a Phenomenex Luna NH_2 under HILIC conditions (solvent A: H_2O, 0.1% AcOH; B MeCN, 0.1% AcOH; gradient: 3 min 100% B, in 27 min 100% A, 100% A 5 min, injection volume 10–100 μL).

*3-Methylarginine (**1**)*: t_R = 3.7 min (Phenomenex polar RP); t_R = 18.9 min (HILIC Phenomenex Luna NH_2); ESI-MS: $[M+H]^+$ 189 (100); HR-ESI-MS: $C_7H_{17}N_4O_2$ found 189.1344, calcd 189.1345; ESI-HR-MS/MS of 189: 189.1344 (9), 172 (25), 171.1239 (100, $C_7H_{15}ON_4$), 154 (4), 144.1130 (12, $C_6H_{14}ON_3$), 130.0861 (35, $C_6H_{12}O_2N$), 84.0807 (6, $C_5H_{10}N$), 60.0557 (18, $C_1H_6N_3$); ^{1}H NMR (500 MHz, MeOD, 300 K): δ = 1.09 (d, J = 7.1, 3H, CH_3), 1.53–1.63 (m, 1H, C4), 1.81–1.91 (m, 1H, C4), 2.27–2.37 (m, 1H, C3), 3.21–3.37 (m, 2H, C5), 3.93 (d, J = 3.7 1H, C2); APT-NMR (125 MHz, MeOD, 300 K): δ = 14.70 (CH_3, C7), 32.58 (CH_2, C4), 32.96 (CH, C3), 40.12 (CH_2–N, C5), 58.62 (CH–NH_2, C2), 158.79 (C=NH, C6), 171.67 (C=O, C1).

*Arginine (**2**)*: ESI-MS: $[M+H]^+$ 175 (100); ESI-MS/MS of 175: 175 (23), 158 (100), 157 (73), 130 (37), 116 (51), 70 (7), 60 (41).

Feeding of [$^2H_3$]-L-methionine: [$^2H_3$]-L-methionine (1 mg) was added to HSC liquid medium (100 mL) with a cell density of approximately 7x10^7 cfu mL^{-1} of Pss22d. After 48 h at 28 °C, the culture was harvested and the samples were worked-up by ion-exchange chromatography as described above. The incorporation of the label into 3-methylarginine (**1**) was monitored by LC-ESI-MS/MS analysis.

*[$^2H_3$]-3-Methylarginine (**1b**)*: t_R = 3.7 min; ESI-MS: $[M+H]^+$ 192 (100); ESI-MS/MS of 192: 192 (8), 175 (27), 174 (100), 157 (4), 147 (14), 133 (37), 87 (6), 60 (17).

Tn5 mutagenesis: Transposon mutagenesis of Pss22d was carried out by mating experiments on Standard 1 agar at 28 °C, overnight, by using *E. coli* S17λpir containing the plasmid pCAM-Not with the Tn5 minitransposon mTn5SS40 as donor strain (Table 1). Derivative Pss22d mutants were isolated on MG medium[32] with spectinomycin (25 μg L^{-1}) as selection agent. Toxin negative Pss22d mutants were analyzed by shotgun sequencing. Briefly, isolated genomic DNA of toxin negative Pss22d mutants and the cloning vector pBBR1MCS were digested, overnight, with SalI and then ligated by using T4 DNA ligase (Fermentas EL0015). Derived plasmids were transformed by electroporation into *E. coli* DH5α. Transformed *E. coli* cells were screened on selective standard 1 medium containing spectinomycin for antibiotic selection. The primer "miniTn5-out" (5′-CTCACAGCCAAACTATCAGG-3′) was used for sequence analysis of the region that flanked the miniTn5 insertions. For the alignment the program DNAStar Megalign was used. Conserved domains were analyzed by using the protein BLAST search engine of NCBI.[21]

LC-ESI-MS/MS analysis of the mutants Pss22d.1, Pss22d.2, Pss22d.3, and the wild-type Pss22d was performed by using a 100 mL culture. For the mutants two cultures were analyzed. The first culture, which was without spectinomycin, was used to evaluate toxin activity in the agar-diffusion assay. The second culture contained spectinomycin (25 μg L^{-1}) as a selective agent and was used for the LC-MS analysis of the mutant's 3-methylarginine (**1**) content. To quantify **1** production by the mutants and wild-type, L-arginine (5 μg mL^{-1}, **2**), which is not present in the Pss22d culture medium was added to the supernatant before the sample was worked-up as internal standard. After ion-exchange chromatography (see above), the samples were injected into the LC-MS system. Wild-type samples were worked-up, assayed, and analyzed in the same way as the samples of mutants.

Stereochemistry of 3-methylarginine (1): 3-Methylarginine (ca. 100 μg) was added to either D-amino acid oxidase (3 units) from porcine kidney (Sigma A5222) and catalase from bovine liver (2950 units; Sigma C1345) in Tris buffer (300 μL, 30 mM, pH 8.3), or to L-amino acid oxidase type VI (0.2 units) from *Crotalus atrox* (Sigma A5147) in Tris buffer (pH 6.7, 30 mM). The mixtures were incubated at 37 °C for 1 h. The enzymes were precipitated by being vortexed for 2 min after the addition of HCl (6 N, 50 μL) and CH_2Cl_2 (500 μL). After centrifugation, the H_2O layer was collected, dried in an argon stream and redissolved in methanol (500 μL). After derivatization with pentafluorobenzylhydroxylamine hydrochloride (200 μg; 37 °C, 1 h) the samples were analyzed by LC-MS/MS.[33] As controls L- and D-arginine were each subjected to both amino acid oxidases and analyzed in the same way as **1**. HPLC column: Grom-Sil ODS-7 PH (125 mm × 2 mm, 4 μm) HPLC program: 3 min 0% B, in 27 min 100% B, 10 min 100% B; A: H_2O, 0.5% AcOH; B: MeCN, 0.5% AcOH; flow rate 0.2 mL min^{-1}.

*Pentafluorobenzyloxime of 2-oxo-3-methylarginine (**7**)*: t_R = 16.6 and 16.9 min (*syn*- and *anti*-isomers, respectively); ESI-MS: $[M+H]^+$ 383; HR-ESI-MS: $C_{14}H_{16}O_3N_4F_5$ found 383.11368, calcd 383.11371; ESI-MS/MS of 383 at 17.7 min: 339 (4), 141 (100), 126 (1), 124 (9); ESI-MS/MS of 383 at 18.4 min: 339 (100), 141 (11), 126 (18), 124 (1).

Acknowledgements

S.D.B. and B.V. are grateful for financial support by the Deutsche Forschungsgemeinschaft (FO 558/6-3) and further, we thank Prof. Erika Kothe and colleagues for their generous support. D.S. thanks Prof. Wilhelm Boland for his generous support and the Deutsche Forschungsgemeinschaft for funding by the Emmy Noether programme (SP110631) as well as the Verband der Chemischen Industrie and the Max Planck Society for financial support.

Keywords: biosynthesis · methylarginine · methyltransferase · natural products · *Pseudomonas syringae*

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Received: February 3, 2008
Published online on July 24, 2008

