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Reevaluation of the Final Steps in the Biosynthesis of Blasticidin S by *Streptomyces griseochromogenes* and Identification of a Novel Self-Resistance Mechanism

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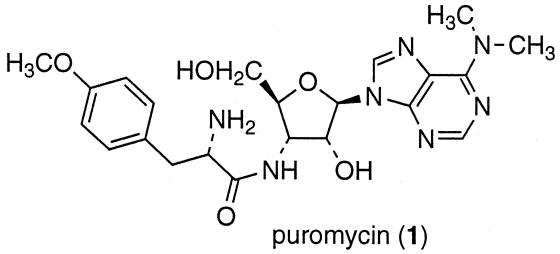
Abstract—The final steps in the biosynthesis of the antifungal peptidyl-nucleoside blasticidin S (**3**) have been revised to include a novel self-resistance mechanism wherein the previously proposed final precursor, demethylblasticidin S (**7**), is modified with a leucine residue yielding leucyldemethylblasticidin S (**10**) which exhibits reduced antibiotic activity. Methylation of **10** yields leucylblasticidin S (**9**) which can be exported from the bacterium and hydrolyzed to **3**. Also disclosed is the finding of a blasticidin S *N*-acetyltransferase activity that may function to detoxify **3** and **7** inadvertently produced prior to export or which gain reentry to the cell.

Introduction

Members of the peptidyl-nucleoside family of antibiotics are characterized by a modified nucleoside possessing an amino sugar coupled via an amide linkage to an amino acid or short peptide.[1,2] Three well-studied examples with potent biological activities are puromycin (**1**), the nikkomycins (e.g. nikkomycin Z (**2**)) and blasticidin S (**3**). Puromycin and blasticidin S block protein biosynthesis in eukaryotic and prokaryotic cells[3–5] while nikkomycins are inhibitors of fungal chitin synthase.[6] Studies on the biosyntheses of **1**–**3** have advanced to the molecular level and gene clusters encoding structural and resistance genes for these three antibiotics have been cloned.[7–9] Investigations of peptidyl-nucleoside biosynthesis in this laboratory have focused primarily on blasticidin S (BS, **3**), a *Streptomyces griseochromogenes* metabolite once used on a large scale in Asia as a fungicide for the prevention of rice blast.[10]



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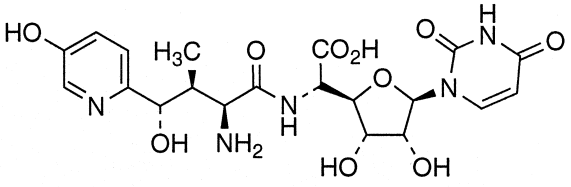
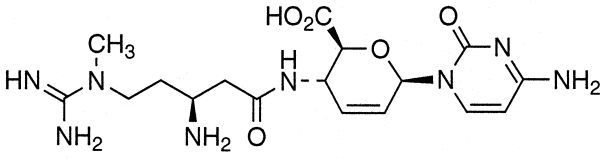


nikkomycin Z (**2**)



blasticidin S (**3**)

Incorporation experiments with isotopically labeled compounds identified the primary precursors to BS as cytosine, D-glucose, L-arginine, and L-methionine.[11] More advanced intermediates identified in the biosynthesis of **3** include β-arginine (**4**),[12] cytosylglucuronic acid (CGA, **5**),[13,14] cytosinine (**6**),[15] and demethylblasticidin S (DBS, **7**)[16] and led to the proposed overall pathway to **3** depicted in Scheme 1.

One of the unanswered questions in BS formation is how *S. griseochromogenes* is protected from the action of BS. Antibiotic producing organisms usually require a self-resistance mechanism to prevent being subject to the lethal

Keywords: amino acids and derivatives; antifungals; nucleosides; biosynthesis.

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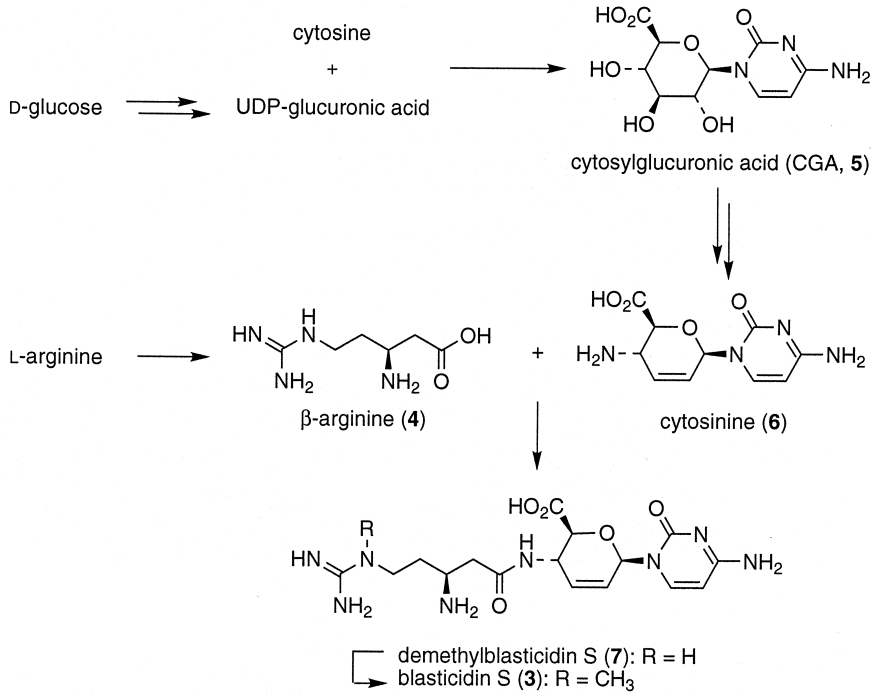


Scheme 1. Proposed pathway for the biosynthesis of blasticidin S (**3**) by *S. griseochromogenes*.

effect of the substances they make. Several self-resistance mechanisms have been described in antibiotic-producing *Streptomyces* including antibiotic inactivation, target modification, efflux pumps, and combinations of these mechanisms.[17] Antibiotic inactivation is generally achieved by *N*-acetylation or *O*-phosphorylation. In some instances, intermediates generated during the biosynthesis are toxic and must be converted to an inactive form. Examples of such cases are found in puromycin[8,18–22] and bialaphos[23–25] formation. The fully assembled antibiotics are then deprotected when they no longer present a threat to the producer. In the example of puromycin, three methylation steps proceed after *N*-acetylation of an active intermediate.[8] The last step of the biosynthesis involves active transport of *N*-acetylpuromycin from the cell and hydrolysis of the acetamide on the exterior to release the active substance into the surroundings.[26] Here we report results of recent experiments to determine the mechanism of self-resistance towards blasticidin S in the producing bacterium *S. griseochromogenes* and the findings that led us to further examine and redefine the final steps in the biosynthetic pathway.

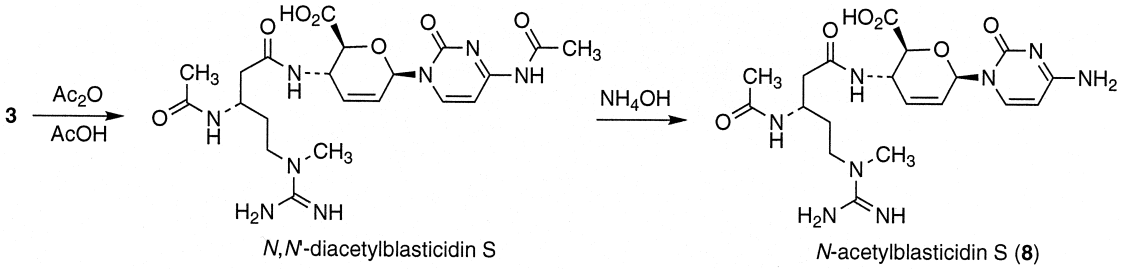
Results and Discussion

Mechanisms of blasticidin S self-resistance in *S. griseochromogenes*

Antibiotic modification. Some peptidyl-nucleoside antibiotics such as puromycin and streptothricin are *N*-acetylated by the producing organism to prevent binding to its own ribosomes.[20,27] In the case of puromycin, the biologically inactive *N*-acetylpuromycin is exported via a proton-dependent electrochemical gradient-driven efflux pump and deacetylated by an amidohydrolase to generate the active antibiotic.[8,26,28] *N*-Acetylblasticidin S (AcBS) has not been previously identified in *S. griseochromogenes* but a blasticidin S acetyltransferase has been detected, isolated and characterized from the BS-producing actinomycete *Streptoverticillium* sp. JCM 4673.[29,30] The enzyme has an M_r of 15 kDa and exhibits K_ms of 2 and 3 mM for BS and acetyl CoA, respectively. The inhibition of protein synthesis by BS could be relieved in a cell-free system when the enzyme was added along with acetyl CoA. Other nucleoside antibiotics including puromycin and streptothricin were not substrates for the purified BS *N*-acetyltransferase.[30] A gene encoding the acetyltransferase, *bls*, has been cloned and expressed in *Streptomyces lividans*.[31] The *bls* gene is now a commercially available resistance marker used in generating stable mammalian cell lines (Invitrogen Corp., Carlsbad, CA).

The only other documented mechanism of resistance towards BS is deamination of the cytosine moiety to produce the corresponding inactive uracil analog. Blasticidin S deaminase has been characterized in the non-producing bacterium *Bacillus cereus*.[32] The most extensively studied deaminase is from the fungus *Aspergillus terreus*. The fungal *bsd* gene has been cloned and expressed, and crystals of the enzyme suitable for X-ray diffraction studies have been produced.[33] *S. lividans* 66 was shown to inactivate BS by a means other than acetylation but the product of the degradation/derivatization was not characterized.[34]

An unanswered issue pertaining to BS acetylation is which site on the molecule becomes modified. In the earlier studies on AcBS formation the evidence for acetylation was based on the consumption of acetyl CoA or the loss of antibacterial activity.[30,31] The product of the acetyltransferase reaction was not characterized and the actual function that AcBS plays in the cell was not addressed.



Scheme 2. Preparation of *N*-acetylblasticidin S (**8**).

Synthesis of N-acetylblasticidin S and detection in fermentation broth. Blasticidin S (**3**) was treated with acetic anhydride in acetic acid to give *N*,*N*′-diacetylblasticidin S. Selective basic hydrolysis of the cytosine acetamide in dilute ammonium hydroxide gave *N*-acetylblasticidin S specifically acetylated at the β-arginine amine (AcBS, **8**, Scheme 2). Cation-exchange HPLC analysis of the synthetic material revealed a peak with a retention time and UV chromophore closely matching a peak observed in a chromatogram of wild-type *S. griseochromogenes* complex medium fermentation broth. Confirmation that the fermentation broth contained **8** came from HPLC coinjection analysis on both ion exchange and C_{18} reverse phase columns and FAB mass spectrometry. This finding is consistent with the report that small amounts of *N*-acetylpuromycin are found in the culture medium of the producing organism.[26] AcBS was not detected when *S. griseochromogenes* was grown in a chemically defined medium.

Antibacterial activity of AcBS. The ability of **8** to inhibit the growth of *Bacillus circulans*, a BS-sensitive bacterium, was evaluated by disk-diffusion bioassay on solid medium. No activity was observed with levels of **8** as high as 410 μg/disk. Control assays using **3** at 3.8 μg/disk resulted in a 24 mm zone of inhibition. Thus, the antibiotic activity of **3** is effectively eliminated by acetylation at the β-arginine amine and implicates this as a possible self-protection process in the producing organism.

Detection of blasticidin S N-acetyltransferase activity. To determine if *S. griseochromogenes* possesses detectable BS acetyltransferase activity, BS and acetyl CoA were incubated with aliquots of a *S. griseochromogenes* cell-free extract (CFE) and analyzed for AcBS production. Cation-exchange HPLC analysis confirmed the formation of **8** and this finding was further supported by coinjection. AcBS was not detected in control experiments containing boiled CFE or in which acetyl CoA was omitted. Similar activity was also detected in the supernatant of a CFE sample centrifuged at 100,000×*g* for 30 min. These results indicate that *S. griseochromogenes* possesses a soluble *N*-acetyltransferase similar to that found in *Streptoverticillium* sp. JCM 4673.

Role of *N*-acetylblasticidin S. The actual function of AcBS in the blasticidin biosynthetic pathway has never been fully addressed. If AcBS is a true intermediate in the pathway, Scheme 1 must be amended to accommodate points where the acetyl group is added and removed. We therefore proceeded to determine at which stage acetylation occurs and identify a AcBS amidohydrolase activity.

Antibacterial activity of DBS and AcDBS. Studies on puromycin illustrated that the first biosynthetic intermediate in a pathway with potentially lethal activity is the logical target for self-resistance through derivatization. Demethylblasticidin S (DBS, **7**) was reported to exhibit antibiotic activity slightly less than that of **3** and it is reasonable to assume that acetylation might be required to block this action.[35] DBS was isolated from a complex medium fermentation of *S. griseochromogenes* supplemented with methionine hydroxamate, a methionine adenosyltransferase inhibitor.[36] A standard of *N*-acetyldemethylblasticidin S (AcDBS) was synthesized from **7** using the same procedure described for the preparation of AcBS (Scheme 2). At 3.8 μg/disk DBS produced an 18 mm clear zone of inhibition in the disk diffusion assay versus *B. circulans*. No antibiotic activity was observed with AcDBS at levels up to 190 μg/disk.

Acetylation of demethylblasticin S in a cell-free extract. If AcBS is a true pathway intermediate, the previous result suggests that DBS is the more likely target for inactivation by acetylation. To test this, DBS and acetyl CoA were incubated with an aliquot of *S. griseochromogenes* CFE and analyzed for the presence of AcDBS. HPLC analysis revealed a peak identical in retention time and UV profile to that of authentic AcDBS. Coinjection confirmed this finding. FABMS analysis showed a molecular ion at *m*/*z* 451, consistent with the expected $[M+H]^+$ ion for AcDBS. Control assays using either boiled CFE or lacking added acetyl CoA contained no detectable AcDBS.

Table 1. Apparent kinetic parameters for the acetylation of BS (**3**) and DBS (**7**)

Substrate	K_m^{app} (mM)	V_{max}^{app} (nmol min^{-1} mg^{-1})	V_{max}/K_m
BS (**3**)	4.6±0.2	0.60±0.01	0.13
DBS (**7**)	14±2	1.0±0.1	0.07

Identification of the preferred substrate for the N-acetyltransferase. To determine if either BS or DBS is preferentially modified by the *N*-acetyltransferase(s) in *S. griseochromogenes*, the kinetic parameters K_m and V_{max} were measured for both substrates at a constant acetyl CoA concentration (Table 1). Using V_{max}/K_m as a measure of specificity, these data suggest BS should be the preferred substrate at low concentrations in vivo. A direct competition experiment was also conducted in which equimolar quantities of BS and DBS were incubated with the CFE and acetyl CoA and the rate of production of each product was monitored (Fig. 1). The competition study revealed that AcDBS accumulated more rapidly and in greater quantity than AcBS. The amount of the AcDBS was 20% greater than

that of AcBS after 1 h and 30% greater after 3 h. The results of these specificity experiments suggest neither compound is the overwhelmingly preferred substrate for the acetyltransferase. It should be noted that a crude preparation from *Streptoverticillium* sp. JCM 4673 was found to acetylate BS as well as puromycin and streptothricin while the purified enzyme exhibited specificity for BS.[30] The possibility that more than one enzyme is acting on these compounds prevents a conclusion to be made about substrate specificity in this case. It is plausible that the results are consistent with the action of a general detoxifying enzyme.

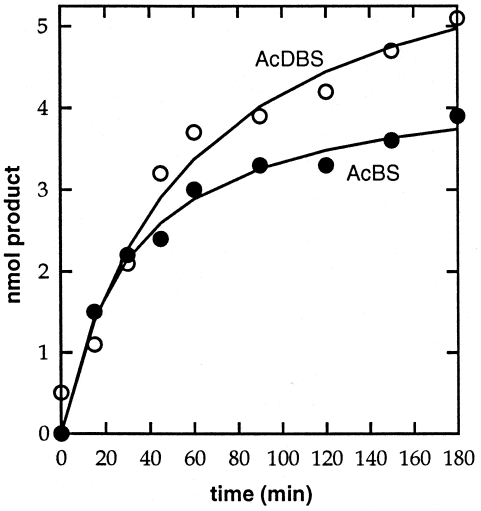
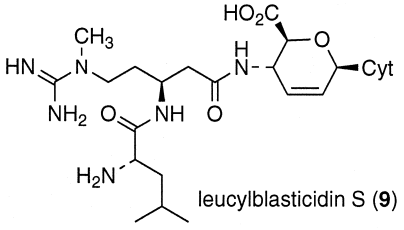


Figure 1. Progress curves for the formation of AcBS and AcDBS.

Methylation of acetyldemethylblasticidin S. For AcDBS to be a true biosynthetic intermediate to BS it must first be methylated at the guanidine followed by acetamide hydrolysis. Previous isotope trapping experiments using ^{14}C-labeled *S*-adenosylmethionine (AdoMet) demonstrated that DBS is a substrate for a methyltransferase present in a CFE.[16] However, only 0.85% of the added DBS was converted to BS after 20 h. When we incubated AcDBS and AdoMet with *S. griseochromogenes* CFE, HPLC analysis revealed a 21% conversion of AcDBS to AcBS in 1 h. The identity of AcBS was confirmed by mass spectrometry. In a control experiment using boiled CFE, AcBS was not detected. When DBS and AdoMet were incubated with CFE the amount of BS detected was insignificantly different from the low background level of BS present in the boiled control. This establishes AcDBS as the superior substrate for the guanidine methyltransferase.

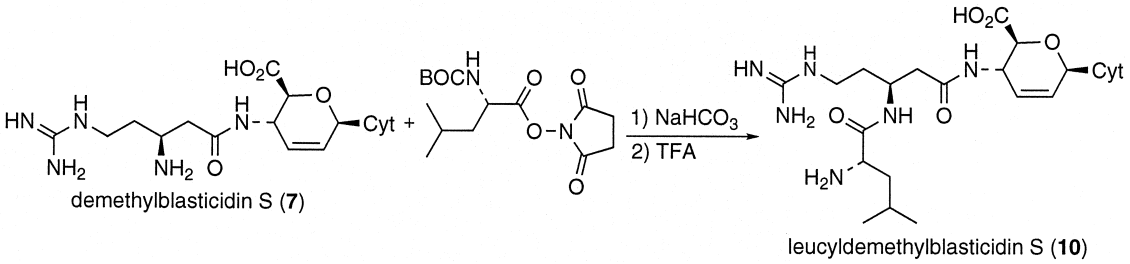
Deacetylation of N-acetylblasticidin S. With strong evidence suggesting *S. griseochromogenes* acetylates DBS as a self-resistance mechanism, it remained to demonstrate the final step—removal of the acetyl group from AcBS. There are no reports on detection of an amidohydrolase activity that can convert AcBS to BS and all our attempts to directly detect such an activity in either fermentation broth or CFE have failed. In another experiment to see if AcBS is converted to BS, AcBS was added to a fermentation in chemically defined medium at the beginning of antibiotic production (48 h after inoculation) and AcBS and BS levels were periodically monitored by HPLC over 48 h. The amount of AcBS remained constant during the fermentation while a substantial quantity of BS was produced. The amount of BS produced was negligibly different than a control fermentation that did not contain added AcBS and AcBS was not detected in this control. These findings indicate that AcBS is unlikely to serve as an intermediate of BS biosynthesis. The absence of an enzyme to deacetylate AcBS suggests the *N*-acetyltransferase may act as a detoxifying enzyme used by *S. griseochromogenes* when free DBS or BS is inadvertently formed or produced in excess under artificial culture conditions.

Intermediacy and role of leucylblasticidin S in the BS pathway. In 1968, Seto et al. isolated two compounds that were viewed as potential precursors to BS. One was demethylblasticidin S (DBS **7**) discussed above, the second was leucylblasticidin S (LBS, **9**).[35,37] Both compounds require a single transformation for conversion to **3**. LBS was originally identified as a minor metabolite of *S. griseochromogenes* when culture conditions were kept below pH 4. LBS was later isolated from *Streptomyces* sp. SCC 1785 along with Sch36605, a LBS analog possessing a 5-hydroxymethylcytosine.[38] Similar to AcBS, these compounds are acylated at the β-arginine amine. LBS was suggested to be a direct precursor to BS when it was observed that incubating LBS with washed *S. griseochromogenes* cells resulted in the formation of BS.[37] To determine if the leucyl modification serves as a self-resistance mechanism we conducted similar experiments to those used to evaluate the role of AcBS.



Antibacterial activity of leucylblasticidin S and leucyldemethylblasticidin S. LBS was isolated from the fermentation broth of *S. griseochromogenes* grown in a chemically defined medium using ion exchange chromatography followed by reverse phase HPLC. In our studies LBS production did not require the fermentation be kept below pH 4.[37] The production of LBS by *Streptomyces* sp. SCC 1785 was also reported under normal fermentation conditions.[38] If LBS is the last intermediate in the biosynthetic pathway to **3**, the earlier finding that AcDBS is a better methyltransferase substrate than DBS suggests that LBS should be formed by methylation of leucyldemethylblacticidin S (LDBS, **10**). LDBS was synthesized by coupling **7** with *N-t*-BOC-L-leucine-*N*-hydroxysuccinimide followed directly by removal of the BOC group in trifluoroacetic acid without purification (Scheme 3). LDBS was purified by reverse phase HPLC and characterized spectroscopically.

The antibiotic activity of LDBS and LBS was evaluated against *B. circulans*. At levels of 75 μg/disk, 15 and 20 mm zones of inhibition were observed, respectively, for **9** and **10**. For comparison, 3.8 μg of BS produced a 24 mm zone of inhibition on the same agar plate. Formation



Scheme 3. Preparation of leucyldemethylblasticidin S (**10**).

of the leucyl derivative does not abolish activity towards the test bacterium as does acetylation, but activity is decreased by 20-fold and this supports modification of the toxic DBS with a leucine as a plausible mechanism of self protection.

Conversion of leucyldemethylblasticidin S to leucylblasticidin S. To determine if LDBS is a substrate for the previously detected guanidine methyltransferase, LDBS was incubated with *S. griseochromogenes* CFE and AdoMet for 3 h at 30°C. HPLC analysis showed that all the LDBS had disappeared but only a small amount of LBS was produced. The major peak formed was DBS, which should arise from hydrolysis of LDBS. The BS peak increased three-fold compared to the small amount always present in the boiled controls. Increasing the incubation time to 8 h resulted in complete disappearance of the LBS peak. In control assays using boiled CFE, amounts of LDBS remained unchanged and no DBS or LBS was detected. In a second control experiment that did not contain AdoMet, LBS was not detected and the BS peak was at the background level, whereas all the LDBS was converted to DBS. The identities of peaks in the chromatograms were confirmed by coinjecting standard samples.

HPLC analysis of the assay mixtures indicated 19% of the added LDBS was methylated and subsequently converted to BS. If the competing hydrolysis of LDBS to DBS is considered (see below), LDBS appears to be a very good substrate for the methyltransferase. Because DBS was shown to be a poor substrate, the sequence where LDBS is transformed to DBS followed by methylation to yield BS should not contribute significantly to the production of BS. To verify this, a parallel experiment was carried out where DBS was included in the assay mixture. The total amount of BS present increased by only 2%, confirming the minor contribution of this route.

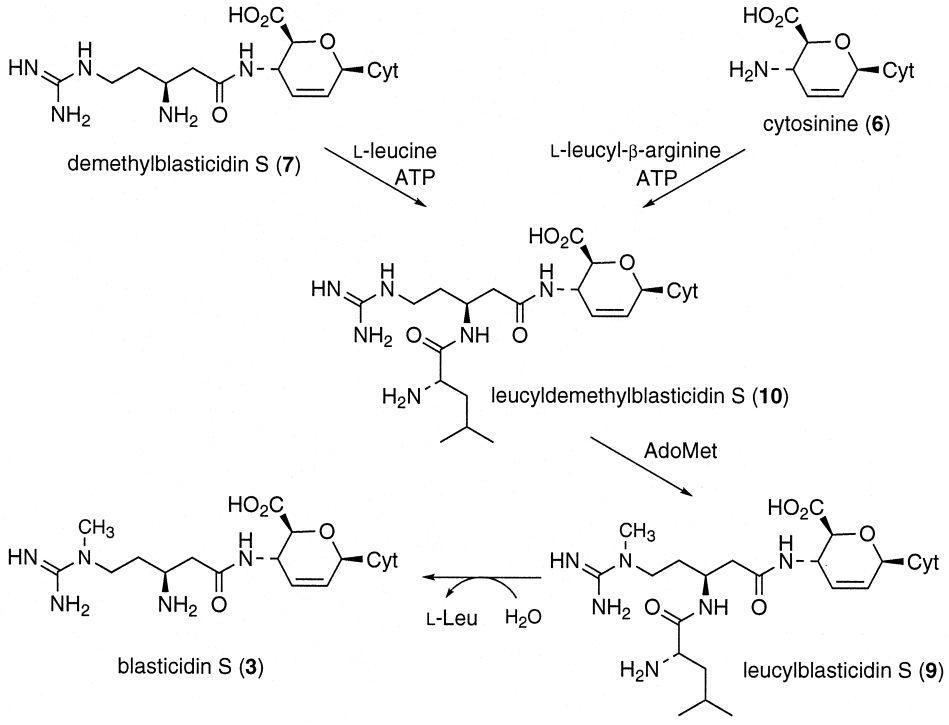
Conversion of leucylblasticidin S to blasticidin S. Seto et al. reported that incubating LBS with washed *S. griseochromogenes* cells resulted in the formation of BS.[37] We also identified this leucylpeptidase activity in the CFE. Incubating LBS overnight at 30°C with *S. griseochromogenes* CFE resulted in complete conversion to BS. The level of LBS was unchanged in the boiled control. Centrifuging the CFE and assaying the pellet and supernatant revealed nearly equal hydrolysis activity in both fractions. This may indicate the enzyme is only loosely associated with membrane but more extensive solubility studies are required. There was no detectable activity in the fermentation broth. Attempts to block LBS hydrolysis with protease inhibitors, such as benzamidine, pepstatin A, leupeptin, and phenylmethylsulfonyl fluoride were unsuccessful.

Formation of leucylblasticidin S in cell-free extracts. Seto et al. also described efforts to detect LBS formation from BS and L-leucine.[37] This was addressed because of concerns that low pH fermentation conditions could promote the reversal of the hydrolysis reaction. The authors provide evidence that, under the conditions used, radiolabeled BS is not a precursor in vivo to LBS. Because BS might not readily be transported into *S. griseochromogenes* cells we attempted to detect synthesis of LDBS from DBS and L-leucine in a cell-free system. ATP was included because the leucine carboxyl is most likely activated as an acyl-adenylate or a phosphate mixed anhydride. We observed no detectable LDBS formation using HPLC analysis. One obvious explanation for the failure to detect LDBS is the facile hydrolysis to DBS. It is also conceivable that a β-arginyl–leucine dipeptide is coupled to cytosinine (**6**) to yield LDBS. The investigation of this possibility will have to await preparation of a properly labeled precursor.

The above findings provide evidence for a revision of the final steps in blasticidin S formation as outlined in Scheme 4. The results indicate that modification of BS and DBS with an acetyl group may serve as a detoxification process while the actual self-resistance mechanism is the previously unreported formation of a leucyl derivative. To our knowledge, this is the first example of a bioactive secondary metabolite being derivatized with an amino acid as a means of self-resistance.

Efflux mechanism. As previously mentioned, active removal of an antibiotic or a precursor from a producing bacterium via an efflux pump is a common mechanism of self-resistance. We recently reported the cloning of two DNA fragments (2.6 and 4.8 kb) from *S. griseochromogenes* which confer increased resistance to BS upon *S. lividans*.[9] Further sequence analysis of the two fragments has revealed the presence of genes characteristic of ATP-binding cassette transporters (ABC-transporters) associated with antibiotic efflux pumps in some *Streptomycetes*.[39] The 2.6 kb resistance fragment was found to carry a gene encoding a protein with two ATP-binding motifs while a gene on the 4.8 kb fragment of DNA appears to encode a transmembrane protein.[40] Thus, a type I ABC-transporter, where the hydrophilic and hydrophobic components are encoded by different genes, appears to function in the mechanism of resistance to BS in *S. griseochromogenes*.

The resistance-encoding DNA fragments were used to probe a *S. griseochromogenes* cosmid library to identify biosynthetic genes clustered with the resistance determinants.[9] Several cosmids hybridizing with the larger resistance



Scheme 4. Revised final steps for the biosynthesis of blasticidin S.

fragment were identified which, when expressed in *S. lividans*, resulted in production of CGA (**5**) and other BS-related metabolites including DBS (**7**), but not **3** itself (cf. Scheme 1). There was no evidence for the presence of AcBS and BS *N*-acetyltransferase activity was not detectable in *S. lividans* harboring cosmids carrying the BS gene cluster or which were transformed with plasmids carrying the 4.8 kb resistance fragment. Since the initial report, we have determined that next to CGA (**5**), the most abundant blasticidin-related metabolite produced by these transformants is LBS (**9**) (Fig. 2). These findings further support the revised pathway in Scheme 4 and help exclude AcBS as a biosynthetic intermediate to BS.

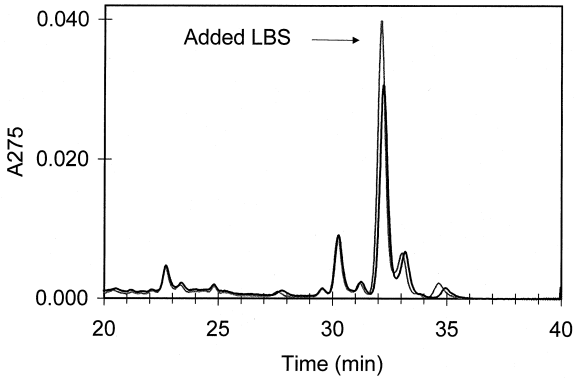


Figure 2. HPLC chromatograms of *S. lividans* fermentation broth extract containing blasticidins. The broad line corresponds to the extract from a transformant expressing the blasticidin gene cluster. The thinner line represents the same extract to which authentic LBS was added.

Conclusion

The overall process of self-protection by *S. griseochromogenes* towards BS shares many similarities with the mechanism used by *S. alboniger* to escape the effects of puromycin. Both organisms appear to acylate early intermediates and export the final products in protected form out of the cell where liberation of the active species occurs. The detection of LDBS synthetase activity remains to be demonstrated but the steps elaborating LDBS to BS have all been established. Our findings require the revision of the earlier proposal that DBS is the last precursor to BS and do not support acetylation as the self-resistance mechanism used under normal conditions. Currently, efforts are underway in our laboratory to complete the sequencing of the BS biosynthetic gene cluster. This information will allow us to rationally target specific genes for disruption and subcloning, permitting the full characterization of each enzyme and chemical transformation in the blasticidin S biosynthetic pathway.

Experimental

General experimental procedures

Many of the general procedures have been previously described.[9,41] NMR spectra were obtained on either a Bruker AC 300 or AM 400 spectrometer. For NMR spectra recorded in D_2O, *t*-BuOH was added as an internal reference; δ 1.27 ppm and 31.2 ppm for ^1H and ^{13}C, respectively.

HPLC analysis

The conditions for cation-exchange analytical HPLC were as follows: polysulfoethylaspartamide cation exchange

column (The Nest Group, Southboro, MA); 4.6×200 mm; mobile phase, (A) 5 mM potassium phosphate in 25% aqueous MeCN, pH 3.0, (B) 5 mM potassium phosphate in 25% aqueous MeCN containing 0.25 M KCl, pH 3.0, 30 min linear gradient from 0 to 100% B followed by 10 min at 100% B; flow rate 1.0 mL/min. Conditions for reverse phase HPLC were: C_{18} column (Microsorb-MV, Varian) 4.6×250 mm; mobile phase, 5% aqueous MeCN, 0.1% TFA, isocratic, flow rate 1.0 mL/min. The UV region from 200 to 300 nm was scanned with the photodiode array detector.

Preparation of cell-free extracts

Standard culture maintenance and fermentation conditions have been described previously.[12] *S. griseochromogenes* grown in a chemically defined medium was generally harvested at 72 h after inoculation. Mycelia were pelleted by centrifugation at 10,000×*g* for 10 min at 4°C and washed twice by resuspending in five volumes of 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM dithiothreitol and centrifuging. The pellet was either used directly or stored at −80°C. In general, pellets were suspended in two volumes of buffer, cooled in an ice/ethanol bath, and disrupted by sonication (Heat Systems Ultrasonic, Inc. model W-225, power level 8, 40% duty cycle). Sonication was for 3×1 min with 1 min cooling intervals. Boiled CFE was obtained by immersion in boiling water for 10 min.

Bioassay

Sterile peptone agar solution (50°C) was mixed with 0.2% v/v of a stock spore suspension of *B. circulans* and aliquots (10 mL) were dispensed into Petri dishes and allowed to solidify. Sterile paper bioassay disks were treated with the solution to be assayed and distributed evenly on the agar plates. The diameter of the inhibition zone was measured after incubation at 37°C for 16 h.

Detection of *N*-acetyltransferase activity in *S. griseochromogenes* CFE

For typical assays, 3 mM BS or DBS and 6 mM acetyl CoA were incubated with 0.4 mL CFE in a total volume of 0.5 mL in a 30°C water bath. After 8–12 h, 0.5 mL cold EtOH was added and the mixture was centrifuged. The supernatant was analyzed directly by ion-exchange HPLC or evaporated under reduced pressure and the residue redissolved in water for reverse phase HPLC analysis. Control experiments contained boiled CFE or lacked acetyl CoA.

Determining kinetic parameters for the acetylation of blasticidin S (3) and demethylblasticidin S (7)

For BS: 0.5 mL incubation mixtures contained 0.4 mL CFE, 3 mM acetyl CoA and one of the following concentrations of **3**: 0.25, 0.31, 0.40, 0.56, 1.07 or 6.0 mM. For DBS: 0.25 mL incubation mixtures contained 0.2 mL CFE, 3 mM acetyl CoA and one of the following concentrations of **7**: 5.0, 6.1, 7.8, 10.9, 17.8, or 50 mM. The assay mixtures were incubated at 30°C for 30 min and an equal volume of cold EtOH was added to each incubation to terminate the reaction. After centrifugation the resulting supernatant (50 μL) was analyzed by ion-exchange HPLC.

N-Acetyltransferase competition experiment

A 1.0 mL assay mixture containing 0.7 mL CFE, 5 mM BS, 5 mM DBS and 8 mM acetyl CoA was incubated in a 30°C water bath and aliquots (100 μL) were removed at 0, 15, 30, 45, 60, 90, 120, 150 and 180 min. Each sample was immediately mixed with an equal volume of cold EtOH, centrifuged, and the resulting supernatant (50 μL) analyzed by HPLC. A control experiment lacking acetyl CoA was carried out under identical conditions. The rate of production of AcBS and AcDBS was calculated from the differences of the two incubations.

Methylation of acetyldemethylblasticidin S

In a total volume of 0.5 mL, 0.35 mL CFE, 1 mM AcDBS and 23 mM AdoMet were combined and incubated at 30°C water for 10 h. The reaction was stopped with the addition of 0.5 mL cold EtOH and the mixture was centrifuged. The supernatant was analyzed by ion-exchange HPLC. Control experiments contained boiled CFE or lacked AdoMet.

Diacetylblasticidin S. Blasticidin S hydrochloride (800 mg) was dissolved in water (50 mL) and the pH adjusted to 11.0 with Amberlite 410 resin (OH^- form). Removal of the resin and lyophilization of the filtrate gave 630 mg of BS as free base. The free base (500 mg) was dissolved in AcOH (50 mL) and Ac_2O (50 mL) and the reaction mixture was stirred overnight at room temperature. The resulting mixture was evaporated to a small volume (15 mL) and stored at 4°C overnight. The resulting crystals were collected and recrystallized from H_2O (5 mL) to give 260 mg (43%) of diacetylblasticidin S. ^{1}H NMR (D_2O) δ 1.84 (m, 1H), 1.96 (m, 1H), 2.03 (s, 3H), 2,27 (s, 3H), 2.50 (d, J=6.6 Hz, 2H), 3.03 (s, 3H), 3.37 (m, 1H), 3.47 (m, 1H), 4.15 (m, 1H), 4.23 (d, J=8.5 Hz, 1H), 4.78 (1H, under HOD), 5.94 (d, J=10.3 Hz, 1H), 6.19 (d, J=10.2 Hz, 1H), 6.58 (s, 1H), 7.35 (d, J=7.5 Hz, 1H), 8.07 (d, J=7.5 Hz, 1H). ^{13}C NMR (D_2O) δ 23.6, 25.7, 31.8, 37.1, 42.8, 46.3, 46.9, 48.6, 79.2, 81.8, 100.1, 126.7, 134.7, 148.8, 158.2, 158.9, 164.8, 173.9, 175.2, 175.6, 176.2. HRFABMS calcd for $C_{21}H_{31}N_8O_7$ $[M+H]^+$ 507.2316, found *m/z* 507.2317.

***N*-Acetylblasticidin S (8).** Diacetyl BS (100 mg) was stirred in 1 M aqueous ammonium hydroxide at room temperature for 5 h. The solution was evaporated to dryness and the residue was crystallized from water to give 82 mg (89%) of **8**. ^{1}H NMR (D_2O) δ 1.83 (m, 1H), 1.98 (m, 1H), 2.03 (s, 3H), 2.49 (d, J=6.7 Hz, 2H), 3.03 (s, 3H), 3.37 (m, 1H), 3.46 (m, 1H), 4.16 (m, 1H), 4.19 (d, J=8.9 Hz, 1H), 4.70 (ddd, J=8.8, 3.0, 2.4 Hz, 1H), 5.88 (dt, J=10.3, 1.8 Hz, 1H), 6.06 (d, J=7.5 Hz, 1H), 6.13 (dt, J=10.3, 2.0 Hz, 1H), 6.50 (s, br, 1H), 7.63 (d, J=7.5 Hz, 1H). ^{13}C NMR (D_2O) δ 23.6, 31.8, 37.1, 42.8, 46.3, 47.1, 48.6, 79.3, 81.2, 98.2, 127.4, 134.5, 144.5, 158.2, 159.3, 168.0, 174.0, 175.2, 176.4. HRFABMS calcd for $C_{19}H_{29}N_8O_6$ $[M+H]^+$ 465.2210, found *m/z* 465.2209.

***N*-Acetyldemethylblasticidin S.** DBS free base (**7**)

(110 mg) in AcOH (6 mL) and Ac_2O (6 mL) was stirred for 2 h at room temperature and then evaporated to dryness. Spectroscopic analysis showed the residue contained a mixture of mono- and di-acetylated **7**. The residue was then dissolved in 5 mL of 1 M aqueous ammonium hydroxide and stirred for 2 h at room temperature. After lyophilization and recrystallization from MeOH/H_2O, 56 mg of AcDBS was obtained from two crops of crystals (46%). ^{1}H NMR (D_2O) δ 1.79 (m, 1H), 1.89 (m, 1H), 2.02 (s, 3H), 2.50 (d, J=6.8 Hz, 2H), 3.25 (m, 2H), 4.20 (d, J=8.9 Hz, 1H), 4.24 (m, 1H), 4.72 (ddd, J=8.8, 3.4, 2.1 Hz, 1H), 5.88 (d, J=10.3 Hz, 1H), 6.07 (d, J=7.5 Hz, 1H), 6.13 (d, J=10.2 Hz, 1H), 6.51 (s, 1H), 7.64 (d, J=7.5 Hz, 1H). ^{13}C NMR (D_2O) δ 23.6, 33.7, 39.5, 42.7, 46.2, 47.1, 79.4, 81.2, 98.1, 127.3, 134.6, 144.6, 158.4, 158.8, 167.6, 174.0, 175.3, 176.4. HRFABMS calcd for $C_{18}H_{27}N_8O_6$ $[M+H]^+$ 451.2054, found m/z 451.2054.

Leucyldemethylblasticidin S (10). To a solution of DBS hydrochloride (**7**, 50 mg) and $NaHCO_3$ (15 mg) in H_2O (2 mL), was added a solution of BOC–L-leucine–*N*-hydroxysuccinimide (72 mg) in EtOH (3 mL). The reaction mixture was stirred at room temperature for 2 h and then acidified to pH 3 with 1N HCl. The solution was evaporated to dryness under vacuum, the residue dissolved in a 1:1 mixture of TFA/CH_2Cl_2 (10 mL), stirred for 2 h at room temperature and evaporated to dryness. The residue was dissolved in H_2O and purified by reverse phase HPLC (Varian C_{18} Microsorb-MV, 4.6×250 mm, mobile phase: 7% aqueous CH_3CN containing 0.1% TFA, flow rate 1.0 mL/min). Fractions containing **10** were pooled and evaporated to yield 54 mg (92%). ^{1}H NMR (D_2O) δ 0.98 (d, J=6.0 Hz, 3H), 0.99 (d, J=5.9 Hz, 3H), 1.70 (m, 3H), 1.87 (m, 2H), 2.52 (dd, J=14.8, 7.9 Hz, 1H), 2.63 (dd, J=14.9, 5.6 Hz, 1H), 3.26 (m, 2H), 4.00 (t, J=7.3 Hz, 1H), 4.33 (m, 1H), 4.49 (d, J=7.6 Hz, 1H), 4.8 (1H under HOD), 5.98 (d, J=10.3 Hz, 1H), 6.24 (d, J=7.8 Hz, 1H), 6.27 (dt, J=10.3, 2.3 Hz, 1H), 6.57 (d, J=2.0 Hz, 1H), 7.82 (d, J=7.8 Hz, 1H). ^{13}C NMR (D_2O) δ 22.5, 23.3, 25.4, 34.1, 39.5, 41.7, 42.0, 45.9, 46.6, 53.5, 76.5, 80.6, 96.9, 125.8, 134.8, 147.1, 150.2, 158.4, 161.0, 171.6, 173.5, 173.9. HRFABMS calcd for $C_{22}H_{36}O_6N_9$ $[M+H]^+$ 522.2789, found m/z 522.2782.

Conversion of leucylblasticidin S (9) to blasticidin S (3)

S. griseochromogenes CFE (0.2 mL) was incubated with 1 mM **9** in a total volume of 0.25 mL at 30°C bath overnight. Cold ethanol (0.25 mL) was added and the mixture was centrifuged. The supernatant was analyzed by ion-exchange HPLC. Control experiments were prepared the same way using boiled CFE.

Methylation of leucyldemethylblasticidin S (10)

Assay mixtures (0.25 mL) containing 0.20 mL CFE, 10 mM AdoMet and 1 mM LDBS (**10**) were incubated in a 30°C water bath overnight. After 0.25 mL cold ethanol was added, the mixture was centrifuged and the supernatant analyzed by ion-exchange HPLC. Control experiments contained boiled CFE, lacked AdoMet or contained 1 mM DBS (**7**).

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