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BIOSYNTHESIS OF BLASTICIDIN S. CELL-FREE DEMONSTRATION OF N-METHYLATION AS THE LAST STEP

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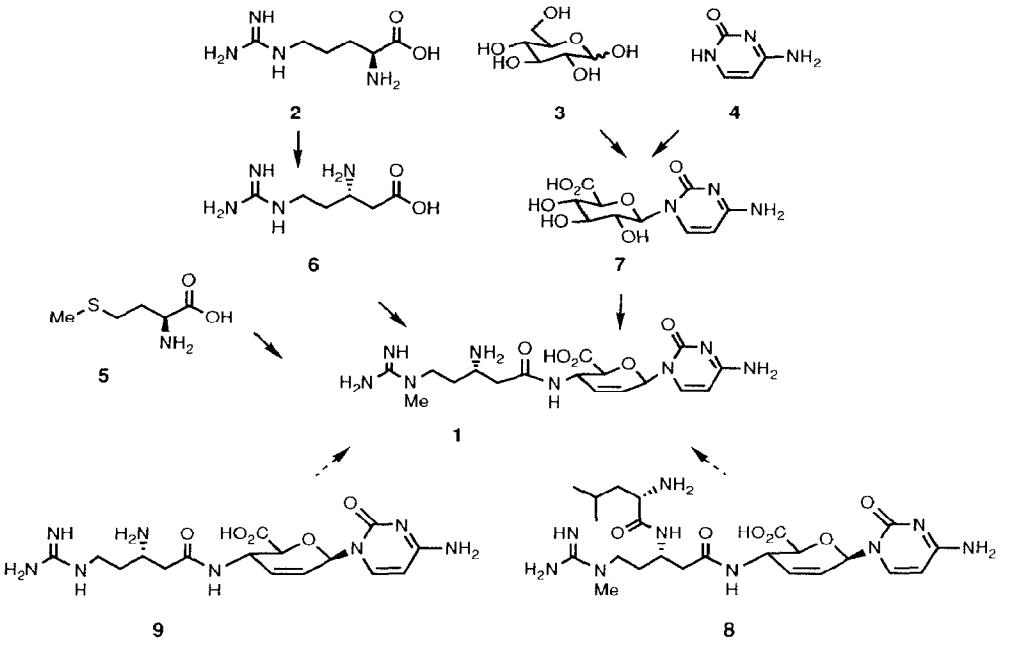
Abstract: In continuing studies on the biosynthesis of the antifungal antibiotic blasticidin S, **1**, a crude cell-free extract was prepared from *Streptomyces griseochromogenes* that converted demethylblasticidin S and *S*-adenosyl-L-[$^{14}CH_3$]methionine to **1a** Hydrolysis of **1a** to blastidic acid (δ-N-methyl-L-β-arginine) and cytosinine and radiochemical analysis of the products confirmed that the former contained all the ^{14}C radioactivity. Thus, N-methylation is the last step in the biosynthesis of **1**

Blasticidin S, **1**, an antifungal antibiotic produced by *Streptomyces griseochromogenes* used commercially for the control of *Piricularia oryzae* (rice blast), was first isolated by Takeuchi et al. in 1958 [1] Its structure and absolute stereochemistry were elucidated by chemical means, [2-5] and confirmed by x-ray diffraction.[6,7] Seto et al.[8] established that **1** is biosynthesized from L-α-arginine, **2**, D-glucose, **3**, cytosine, **4** and L-methionine, **5** We showed that **2** is converted to L-β-arginine, **6**, and have established the stereochemistry of the arginine-2,3-aminomutase reaction,[9] and recently demonstrated that cytosylglucuronic acid, **7**, is the first sugar intermediate committed to the pathway.[10] Of other metabolites structurally related to **1** that are also produced by *S. griseochromogenes*,[11-15] both leucylblasticidin S, **8**,[11] and demethylblasticidin S, **9**,[15] had been suggested for the final intermediate in the biosynthetic pathway We now report that the latter compound fulfills this role.

Initially we obtained HPLC evidence for the formation of small amounts of **8** when fermentation[11] was maintained below pH 4 We also observed probable leucyl aminopeptidase activity with washed mycelia in neutral buffer[11] since over a period of several hours authentic **8** was consumed (HPLC assay), although the direct conversion to **1** could not be established since the mycelia produced a measurable amount of **1** under these conditions without the addition of **8**. Subsequently, in the course of evaluating the addition of specific enzyme inhibitors and non-physiological quantities of biosynthetic precursors as a way to block potential biosynthetic steps and accumulate intermediates,[16] we found that addition of a mixture of cytosine (0.3 g/L) and arginine (2.0 g/L) led to a four-fold increase in the concentration of **6** (to 1 mM) when *S. griseochromogenes* was grown in a chemically-defined medium.[11] We then were able to prepare a cell-free extract that could convert **9** to **1**.

Scheme 1

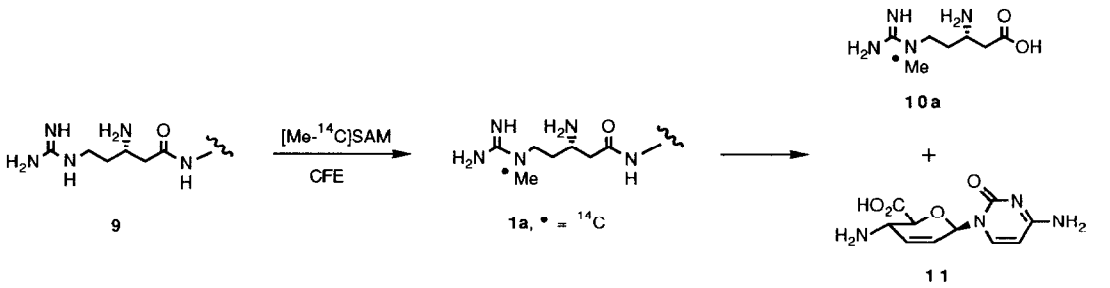


Cells (5 g) from 77 h fermentations[8] were harvested by centrifugation, washed twice with buffer (100 mM Tris pH 8.0, 1.2 mM DTT), suspended in 10 mL of the buffer and sonicated (Heat Systems Ultrasonics, Inc., model W-225R, maximum power, 40% duty cycle, 2 x 1 min with cooling in an ice-ethanol bath) to yield the cell free extract (CFE). In a first incubation, 1.5 mg of **9** and 500 μL CFE were incubated for 20 h at 30 °C with 10 μL *S*-adenosyl-L-[$^{14}CH_3$]methionine (ICN Radiochemicals, Inc., 1 μCi, 46 mCi/mMol, 10 μL). An aliquot (100 μL) was centrifuged (15,000 rpm, 10 min) and 45 μL assayed by HPLC[17] with radiochemical detection,[18] revealing a new peak with the approximate retention time of **1**. To the remaining mixture, 3.0 mg of authentic **1** was added, followed by centrifugation and purification by repeated injections of 40 μL aliquots onto the HPLC column, now monitored by UV (275 nm) detection. The fractions containing **1** were combined (total volume 25 mL), 350 mg more authentic **1** added, the mixture lyophilized, and the residue taken up in a few mL of distilled water and chromatographed on Dowex 50Wx4 (H^+, 100 mesh, 3.0 x 30 cm) eluting with 5% aq pyridine (200 mL) and then with 1.2% aq NH_4OH. This yielded 236 mg of **1a** after lyophilization, which was taken up in distilled water, adjusted to pH 5 and re-lyophilzed, and then recrystallized from H_2O/MeOH to constant specific activity (1.83 x 10^4 dpm/mmol), which corresponded to at least 0.85% conversion.[19]

In a second incubation 0.65 mg of **9** and 2 μCi of [$^{14}CH_3$]SAM were used. Two milligrams of **1** were added to the completed incubation, before HPLC fractionation, and 238 mg were added afterwards. This time the ion exchange chromatography was unnecessary and recrystallization to constant specific activity yielded 8.48 x 10^4 dpm/mmol. The apparent minimum conversion this time was 1.2%.

The remaining **1a** from the second experiment (125.0 mg) and a portion from the first experiment (70.0 mg) were combined (net specific activity 6.08 x 10^4 dpm/mmol) and hydrolyzed in 6N HCl for 5 h at reflux to yield labeled blastidic acid **10a** and unlabeled cytosinine **11**. The mixture was concentrated *in vacuo*, taken up in distilled water and chromatographed on Amberlite IRA 410 (OH^-, 100 mesh, 3.5 x 15 cm) eluting with water. The eluate was adjusted to pH 1, lyophilized, taken up in 2 mL water and filtered through a Waters Assoc. C_{18} SepPak. After lyophilization of the filtrate the blastidic acid was recyrstallized from ethanol to a constant specific activity of 5.92 x 10^4 dpm/mmol (Scheme 2) This was 97 4% of the theoretical value and confirmed the enzymatic methylation of **9** to yield **1a**.

Scheme 2



No formation of **1a** was obtained either in the absence of CFE or in the absence of **9**. Although the conversion of **9** and [$^{14}CH_3$]SAM with the crude cell-free extract was low, it was clearly reproducible. Hydrolysis of the derived **1a** to blastidic acid and cytosinine and radiochemical analysis of the products confirmed that the labeling had been completely specific Therefore, methylation is the last step in the biosynthesis of blasticidin S. While it is probable that **8** can be converted to **1**, it is likely to be the result of a *non-specific* leucylaminopeptidase.[20] Blasticidin S producing organisms are known to make other acylated derivatives as well.[21,22]

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16 A full description of this work will be published in detail elsewhere
17 Waters Assoc. 4m C_{18} RadialPak^R column, 8 mm x 10 cm, 97% H_2O, 3% CH_3CN, 0.1% TFA, 1 mL/min
18. Flow-One/Beta Radioactive Flow Detector, Radiomatic Instruments and Chemical Co , Inc , Flow-Scint I, 4 mL/min.
19 Total radioactivity in the diluted sample (14,120 dpm), divided by [$^{14}CH_3$]SAM used (2.2 x 10^6 dpm), corrected for volume of incubation used (420 out of 520 µL), and assuming 100% recovery of labeled **1a** from the HPLC purification
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