Cytosinine: Pyridoxal Phosphate Tautomerase, a New Enzyme in the Blasticidin S Biosynthetic Pathway†

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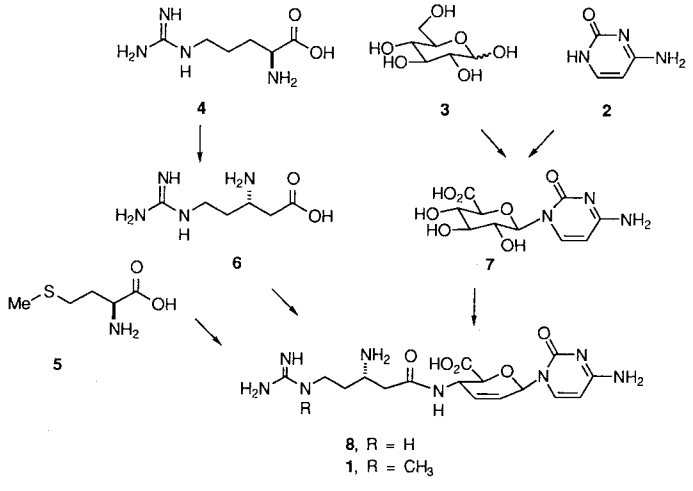
Cytosinine—the nucleoside portion of blasticidin S—and pyridoxal phosphate were incubated with cell-free extracts of *Streptomyces griseochromogenes* prepared in D_2O. ^2H NMR analysis of recovered cytosinine showed it to contain deuterium enrichments at H-4′ and H-2′. No exchange was observed with either boiled extract or from cytosinine and pyridoxal phosphate alone. These results reveal the presence of a tautomerase activity that contributes to the net transamination at C-4′ in the conversion of cytosylglucuronic acid to blasticidin S, and its discovery supports the role of cytosinine as a biosynthetic intermediate.

Blasticidin S, **1**, was isolated from *Streptomyces griseochromogenes*[1] as part of a major effort to replace mercury-based compounds for the prevention of *Piricularia oryzae* infection of rice plants in Japan.[2] This fungus causes rice blast disease, a major rice pathogen in Japan and other parts of Asia. The structure of blasticidin S was elucidated by chemical degradation,[3] and the absolute configuration was assigned on the basis of the work of WATANABE[4] and of YONEHARA.[5] X-ray diffraction studies have supported these results.[6,7]

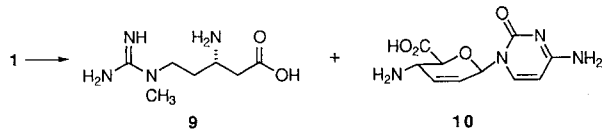
Biosynthetic studies identified the primary precursors as cytosine, **2**, D-glucose, **3**, L-α-arginine, **4**, and L-methionine, **5**,[8] *via* β-arginine, **6**,[9] cytosylglucuronic acid, **7**,[10,11] and demethylblasticidin S, **8**.[12] A number of enzymes from the blasticidin S and related pathways have been isolated from *S. griseochromogenes*.[11,12] Numerous other cytosine-based, peptidyl nucleoside antibiotics have also been characterized from various Actinomycetes (*Streptomyces*, *Nocardia*, *Streptoverticillium*) and from at least one *Bacillus* species.[13]

Retrobiogenetic analysis indicated that **1** might result from coupling products from two hypothetical subpathways. Thus, blastidic acid, **9**, and cytosinine, **10**, which have been obtained by hydrolysis of **1**,[14] were considered potential biosynthetic intermediates. The reconstitution of **1** from **9** and **10** by a mycelial suspension of *S. griseochromogenes* had been reported,[15] but was not reproducible,[16] and had been tested without the benefit of isotope labels. When radiolabeled samples of **9** and **10** were prepared and tested, however, neither was incorporated into **1** by cultures of *S. griseochromogenes*.[17]

Scheme 1.



† This paper is a special contribution in honor of Professor SATOSHI ŌMURA's 60th birthday.



9 10

Except for cytosylglucuronic acid synthase (cytosine: UDP-glucuronic acid transferase),[11] and demethylblasticidin S *N*-methyltransferase,[12] we had been unable to detect any enzymes beyond CGA synthase on the main pathway leading directly to **1**. We now report the detection of one such enzyme, cytosinine: pyridoxal phosphate tautomerase, that for the first time confirms cytosinine as an intermediate in blasticidin S biosynthesis.

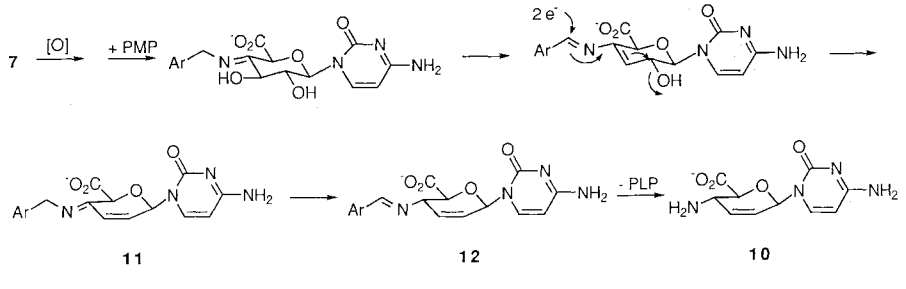
Results and Discussion

The reason for lack of incorporation of [^{14}C]cytosinine was ambiguous because bacterial cells are generally not readily permeable to nucleosides.[18~20] Interfering with the secondary metabolism of *S. griseochromogenes* by feeding specific enzyme inhibitors led to substantial accumlations of a number of blasticidin intermediates and other metabolites related to the pathway.[17,21] However, cytosinine was not amongst these. Believing that cytosinine was still a likely intermediate, we therefore approached the problem from the reverse direction. We had determined that the first sugar deoxygenation in the pathway occurs with retention of configuration in what appeared to be a pyridoxamine phosphate-dependent process.[22] If the first sugar deoxygenation were, indeed, a pyridoxamine phosphate-dependent 1,4-elimination, the second deoxygenation would be expected to follow as a consequence, as shown in Scheme 2. In such a situation, the tautomerization of imine **11** to imine **12** should be reversible.

Cytosinine was obtained by hydrolysis of blasticidin S. *S. griseochromogenes* mycelia from a 72-hour fermentation were concentrated by centrifugation, resupended in D_2O, and sonicated with cooling to prepare a cell-free extract (CFE). A sample of **10** was incubated with pyridoxal phosphate (PLP) and portions of the CFE. Cytosinine was recovered from this mixture by ion exchange chromatography, and recrystallized. A portion was analyzed by ^{1}H NMR and the resonance for H-4′ was noticeably diminished in comparison to those for the rest of the molecule. The ^{2}H NMR spectrum of a second portion confirmed this with a substantial signal at δ 4.25 for H-4′ and, surprisingly, also contained a resonance at δ 6.42 for H-2′. By normalization to the resonance for *t*-butanol included as a chemical shift and deuterium quantitation reference, the enrichments were 31% and 8%, respectively. Since the incubation mixture had been prepared from mycelia and D_2O in a 1 : 2 ratio (therefore 67% deuterated), these enrichments would correspond to 46% and 12%, respectively, had the incubation solvent been 100% D_2O.

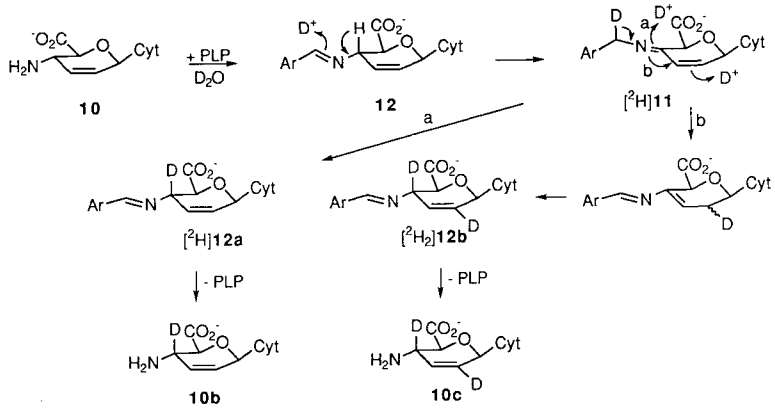
This experiment was repeated with CFE that was 80% deuterated, and in this case half the CFE was boiled and then also incubated with cytosinine and PLP. The incubation with active CFE yielded cytosinine with deuterium enrichments of 36% and 10% for H-4′ and H-2′, respectively, and these were normalized to 45% and 13% for incubation in 100% D_2O. A molecular ion could not be obtained by either EI-MS or by CI-MS, but could be obtained by positive FAB-MS. Mass spectrometric analysis of the molecular ion region revealed a distribution of 66% d_0, 28% d_1, and 6% d_2. The observed natural isotope abundances, $[(M+1)+1]^+$ and $[(M+1)+2]^+$, differed from calculated values by a few percent. While this introduced some error in the calculated enrichment distribution, the trend is clearly in line with the NMR results. Each d_2 molecule contributed to both the H-4′ and H-2′ resonance. The cytosinine recovered from the boiled enzyme control showed no deuterium enrichment by ^{2}H NMR or MS. Similarly, a mixture of cytosinine and PLP in D_2O failed to result in any exchange of the relevant cytosinine hydrogens.

Scheme 2.



11 12 10

Scheme 3.



Thus, the exchange observed with active CFE must be enzyme catalyzed (Scheme 3), and provides *prima facie* evidence that cytosinine is a metabolite of *S. griseochromogenes* and an intermediate in the biosynthesis of **1**. These experiments also corroborate the prediction that the sugar deoxygenations in this pathway involve pyridoxamine as a coenzyme,[22] confirming the biogenetic relationship to the formation of 3,6-dideoxycarbohydrates (*e.g.*, the CDP-4-keto-6-deoxy-D-glucose-3-dehydrase leading to ascarylose,[23~25]) found in the lipopolysaccharide components of a number of Gram-negative bacterial cell envelopes.[26]

While exchange at H-4′ was expected, exchange at H-2′ was not. Nonetheless, it is a logical consequence of the apparent reaction mechanism (Scheme 3). Tautomerization of intermediate imine **12** results in formation of [^{2}H] **11** and, if deuterons are accessible at either end, ultimately leads to deuteration at both sites. Thus, the observed exchange at two positions is not an indication of "sloppiness" of the tautomerase. Rather, it probably reveals topological information about the active site. The difference in the degree of exchange may reflect either different electron densities at the two sites or different accessibilities to one or more protonated active-site residues or to solvent. In any event, exchange at either site reveals turn-over for the tautomerase reaction. Since the solvent was either 67% or 80% deuterated, formation of **12b** would yield doubly-deuterated product (*i.e.* also deuterated at H-4′) only 67% or 80% percent of the time, respectively.

Whether the tautomerase is an independent protein or an additional activity of (one of) the dehydrase(s) remains to be determined. However, the results reported herein reveal a new biochemical role for pyridoxamine phosphate.

Experimental

Preparation of Cell-free Extracts

Streptomyces griseochromogenes was grown for 72 hours in a chemically defined medium[15] (200 ml in 1-liter Erlenmeyer flasks). The mycelia were pelleted by centrifugation at 10,000 × *g* for 10 minutes at 4°C, and washed twice by resuspending in 50 mM pH 7.0 KP_i buffer (100 ml) containing 1 mM dithiothreitol and centrifuging as described. The pellet was then resuspended in buffer made from D_2O, 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 minute with 1 minute cooling intervals.

Standard Enzyme Reaction Work-up

An aliquot of trichloroacetic acid (TCA, 30~40%) was added and the mixture centrifuged (10,000 × *g*, 10 minutes). The supernatant was applied to a column of Dowex 50W × 4 (100~200 mesh, H^+, 3.0 × 17.0 cm), and the column washed with water to neutrality and then eluted with 5% pyridine (5 ml/minute). Cytosinine eluted in the earliest pyridine-containing fractions. These were combined and lyophilized to give a fluffy solid that was dissolved in a small volume of H_2O, adjusted to pH 3.0 (1 N HCl), filtered, and lyophilized again. The residue was recrystallized from H_2O/acetone.

Enzyme Incubation #1

Mycelia (25 ml) from two 200-ml fermentations were suspended in D_2O (50 ml) to yield CFE (75 ml). Cytosinine (15 mg), PLP (0.52 mg), and CFE (25 ml) were incubated for 1 hour at 30°C in a 125 ml Erlenmeyer flask with occassional gentle swirling. Another aliquot of CFE (25 ml) was added and the mixture treated in the same manner for an additional 2 hours, at which time the third 25-ml portion of CFE was added and the mixture incubated overnight. TCA (25 ml, 40%) was added to the mixture. Chromatography twice on the Dowex column yielded 111 mg of crude cytosinine con-

taining a substantial amount of salt. Recrystallization afforded 11 mg of cytosinine. An aliquot was dissolved in 0.5 ml deuterium-depleted water containing 25 μl (0.36 μmole ^2H) *t*-BuOH, and analyzed by ^2H NMR (61.4 MHz, acq. time 1.430 seconds, 90° pulse angle, 28,437 scans, 3 Hz line-broadening). Integration of the resonances at δ 1.27 (*t*-BuOH), 4.25 (H-4′), and 6.42 (H-2′) yielded a 31% enrichment at the former site and a 8% enrichment at the latter.

Enzyme Incubation #2

Mycelia (20 ml) from four 200-ml fermentations were suspended in D_2O (80 ml) to yield CFE (100 ml). Half the CFE was boiled for 10 minutes and then cooled on ice. Cytosinine (13.5 mg), PLP (0.54 mg) were added to each of two flasks. One flask received CFE (25 ml) and the other received boiled CFE (25 ml). These were incubated for 2 hours at 30°C with occassional gentle swirling. The remaining aliquot of CFE or boiled CFE was added and the mixtures incubated overnight. TCA (25 ml, 30%) was added to each and worked up in standard fashion. The active CFE experiment yielded 5.2 mg of cytosinine, pure by ^1H NMR, while the boiled control yielded 19 mg of impure cytosinine that was further purified by chromatograhy on S-Sepharose (H^+, 1.3 × 30.0 cm), washing first with H_2O and then eluting with 3% pyridine. Impure fractions were rechromatographed and ultimately 4.5 mg of pure cytosinine were obtained. ^2H NMR (23,950 scans) showed resonances at δ 4.25 and 6.42 only for the sample from incubation with active enzyme. Integration of these relative to the *t*-BuOH indicated enrichments of 36% and 10%, respectively. Positive ion FAB-MS: $[M+1]^+$ 53.4%, $[(M+1)+1]^+$ 34.1%, $[(M+2)+1]^+$ 12.5%; observed for a sample at natural abundance: $[M+1]^+$ 78.3%, $[(M+1)+1]^+$ 16.5%, $[(M+2)+1]^+$ 5.2%.

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