A New Cytosine Glycoside from *Streptomyces griseochromogenes* Produced by the Use in Vivo of Enzyme Inhibitors

Qibo Zhang,[†] Steven J. Gould,[†,‡] and T. Mark Zabriskie*[,§]

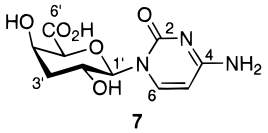
Department of Chemistry, Oregon State University, Corvallis, Oregon 97331, Merck Research Laboratories-Basic Research, Rahway, New Jersey 07065, and College of Pharmacy, Oregon State University, Corvallis, Oregon 97331

Received October 15, 1997

The identification of new cytosine glycosides and intermediates in the biosynthetic pathway of the antifungal antibiotic blasticidin S (**1**) was investigated using in vivo enzyme inhibition. Fermentations of *Streptomyces griseochromogenes*, the organism that produces **1**, supplemented with the arginine analogue argininic acid or the argininosuccinate synthase inhibitor 2-methylaspartic acid were found to produce a new metabolite (**7**).

Blasticidin S (BS, **1**) is a *Streptomyces griseochromogenes* metabolite first isolated in 1958.[1] It has been used commercially on a large scale to replace mercury-based antifungal compounds for the prevention of rice blast caused by the fungus *Piricularia oryzae*, a major rice disease in much of Asia.[2,3] The structure of **1** was originally elucidated from the products of chemical degradation studies[4−6] and was later confirmed by X-ray diffraction analysis.[7,8] Early biosynthetic experiments identified the primary precursors as cytosine, D-glucose, L-arginine, and L-methionine.[9] Subsequent studies established the identities of several later intermediates in the pathway to **1**, including β-arginine (**2**),[10] cytosylglucuronic acid (CGA, **3**),[11,12] cytosinine (**4**),[13] and demethylblasticidin S (**5**)[14] (Scheme 1).

Many other cytosine glycosides have been identified from *S. griseochromogenes* including blasticidin H (**6**)[15] and the decarboxylated nucleoside pentopyranines A−F.[16−18] Conclusive biosynthetic relationships between these metabolites and **1** remain to be established. As part of our work to elucidate the intermediates between CGA (**3**) and cytosinine (**4**), and to explore the relationships between the various cytosinyl metabolites, the in vivo utilization of known inhibitors of enzymes expected to function in the biosynthesis of **1** was investigated. This proved to be an effective approach to study the biosynthesis of **1**, resulting in accumulated intermediates and the enhanced production of other cytosinyl metabolites.[19,20] Here, we report additional in vivo inhibitor studies leading to the production of a new cytosine nucleoside, **7**.



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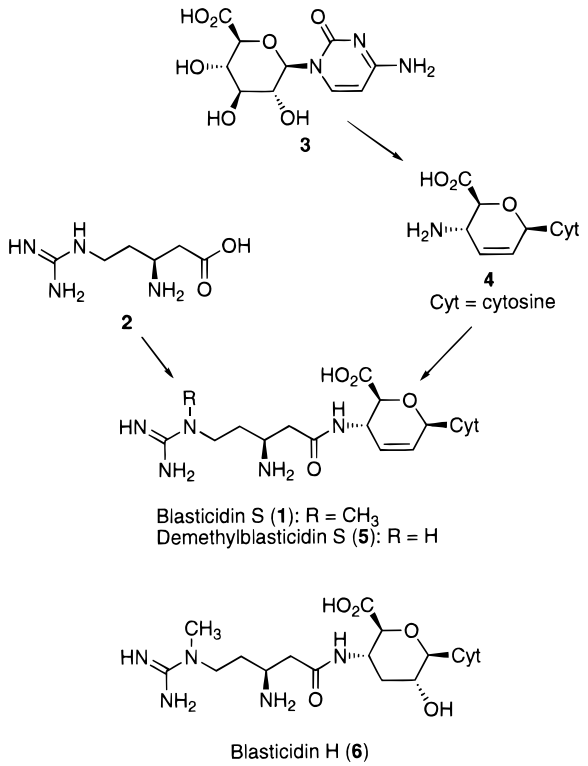
Enzyme inhibitors tested in this latest study included several that should affect arginine production. These were γ-aminobutyric acid and 2,4-diaminobutyric acid

Scheme 1



(inhibitors of ornithine carbamoyltransferase),[21] α-methyl aspartate (inhibitor of argininosuccinate synthase),[22] and 2-fluorofumarate (inhibitor of argininosuccinate lyase).[23] Assuming that β-arginine is formed by an *S*-adenosylmethionine-dependent enzyme similar to lysine 2,3-aminomutase, we tested the methionine adenosyltransferase inhibitors *S*-carbamoylcysteine,[24] methionine hydroxamate, and sinefungin.[25] The lysine-2,3-aminomutase and *N*-methyltransferase inhibitor *S*-adenosylhomocysteine[26] was also evaluated. Finally, several arginine analogues were included in fermentations to see if they would have inhibitory effects or serve as alternate substrates. These included nitro-L-arginine, N^G-methyl-L-arginine, α-chloro-δ-guanidino-*n*-valeric acid, and L-argininic acid.[27]

With the exception of sinefungin, which was tested only at 0.13 mM, all of the enzyme inhibitors were added

* To whom correspondence should be addressed. Tel.: (541) 737-5774. Fax: (541) 737-3999. E-mail: zabriskm@ccmail.orst.edu.
[†] Department of Chemistry, Oregon State University.
[‡] Merck Research Laboratories.
[§] College of Pharmacy, Oregon State University.

S0163-3864(97)00468-0 CCC: $15.00
Published on Web 04/21/1998

Table 1. NMR Assignments for Compound **7**[a]

position	^{13}C (δ)	^{1}H (d) (mult, *J* (Hz))	HMBC
2	148.7		6, 1′
4	160.1		5, 6
5	94.9	6.12 (d, 7.8)	6
6	144.6	7.90 (d, 7.8)	5, 1′
1′	82.2	5.96 (d, 9.2)	5′
2′	62.9	3.87 (ddd, 11.2, 8.9, 4.4)	1′, 4′
3′	35.5	1.54 (H_{ax} ddd, 13.6, 11.1, 2.5)	1′, 5′
		2.08 (H_{eq} bd, 13.3)	
4′	65.8	4.21 (bs)	5′
5′	78.8	4.42 (s)	
6′	170.9		5′
exchangeable		9.33 (s), 8.69 (s), 5.35 (bs)	

[a] ^{1}H and ^{13}C NMR spectra were obtained in DMSO-d_6 at 300 and 75 MHz, respectively.

at concentrations ranging from 2.0 to 10 mM. The inhibitors were administered both with and without added cytosine (2.7 mM). Pyridoxamine and pyridoxamine 5′-phosphate (PMP), at final concentrations of 2.0 and 4.0 mM, respectively, were also added to fermentations with many of the above compounds in an attempt to accumulate intermediates possessing a 4′-nitrogen. The metabolite production in the fermentations was monitored by HPLC beginning 2 days after the addition of the inhibitors. In most fermentations, no new cytosine-containing metabolites were detected when compared to the controls. Pentopyranine C (**8**; cf. Scheme 2), CGA (**3**), and demethylblasticidin S (**5**) were detected when appropriate inhibitors were incubated.[19] One new metabolite, however, having a cytosine chromophore was reproducibly detected in fermentations containing either argininic acid or α-methylaspartate along with added cytosine. This unidentified metabolite was purified from a preparative-scale fermentation of *S. griseochromogenes* using ion-exchange, size-exclusion, and reversed-phase chromatography.

The new metabolite was assigned structure **7** on the basis of spectroscopic data and comparison to related compounds. The ^{1}H and ^{13}C NMR spectroscopic data are presented in Table 1. High-resolution mass spectrometry provided a molecular formula of $C_{10}H_{13}N_3O_6$ and suggested that the new glycoside was a deoxysugar analogue of CGA (**3**). The presence of a methylene carbon in **7** was supported by an HMQC experiment revealing a coupling between the carbon at δ 35.5 and diastereotopic protons at δ 1.54 and 2.08. Assignment of the deoxygenated carbon as C-3′ was based on interpretation of coupling patterns in a COSY spectrum as well as HMBC couplings of the methylene carbon to H-1′ and H-5′. Irradiation of H-1′ in a difference NOE experiment produced enhancement of the H-5′ and H-3_{ax}′ signals and established that the carboxyl group was equatorial. The small coupling constant with H-5′ suggested that H-4′ was also equatorial.

The stereochemistry at H-5′ was confirmed by formation of a β-lactone involving the 4′ hydroxyl and the 5′ carboxyl group. When HPLC fractions containing **7** were concentrated to dryness in a centrifugal concentrator instead of by lyophilization, a ^{1}H NMR spectrum of the resulting material in DMSO-d_6 showed the presence of two related compounds in a 1:1 ratio. One set of signals was readily assigned to **7**, while the other set had a similar pattern but different chemical shifts. Upon standing for several days, the amount of the unknown compound decreased while the percent of **7** increased, suggesting the compounds could be interconverted. Acquiring ^{1}H NMR spectra at higher temperature (70 °C), or in the presence of trifluoroacetic acid, did not change the ratio of the two sets of signals. Thus, the two compounds did not appear to be conformers or tautomers. The resonances for H-4′ and H-5′ of the second compound were shifted downfield 1.4 and 0.5 ppm, respectively, relative to the corresponding signals in **7**. In addition, the ^{13}C NMR signal for the new carbonyl came at δ 168.7. These data, along with connectivity information from a COSY spectrum of the mixture, were consistent with the β-lactone derived from **7**. When the ^{1}H NMR was measured again after 25 days, all of the β-lactone had been converted to **7**. The interrelationship was confirmed when the solvent was removed under vacuum and an ^{1}H NMR spectrum was acquired in fresh DMSO-d_6, revealing approximately 10% of the sample was again in the β-lactone form.

Scheme 2 depicts a possible pathway leading to the formation of **7** and also illustrates the probable biogenetic relationship of **7** with other known *S. griseochromogenes* cytosine glycosides. Compound **7** appears to be most closely related to pentopyranine C (**12**) and pentopyranamine D, the nucleoside portion of blasticidin H (**6**).[28]

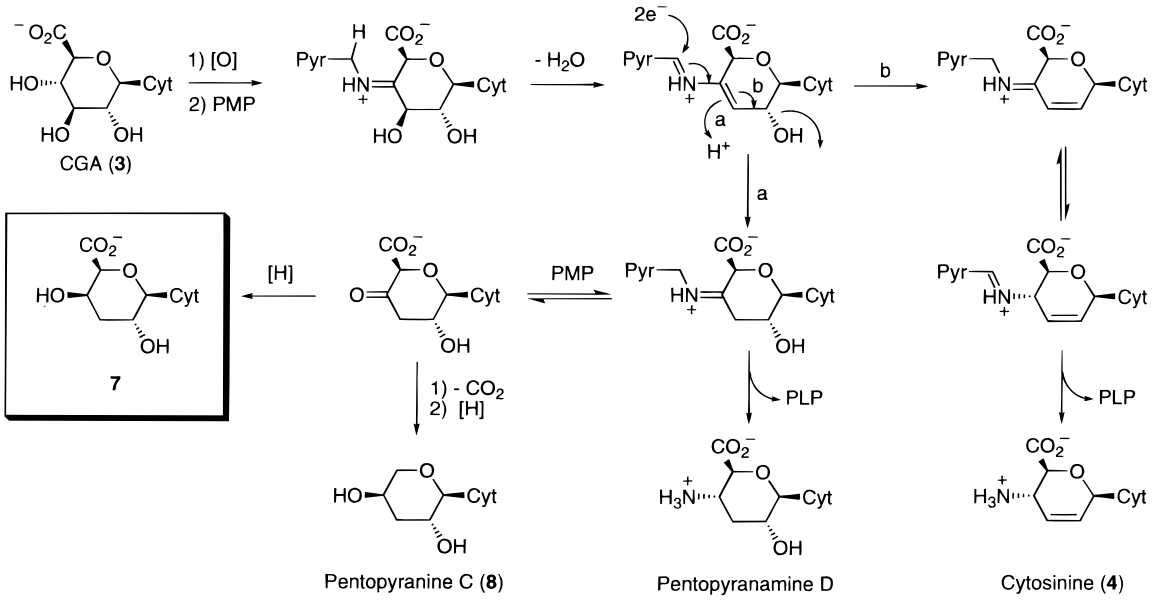
The series of transformations in Scheme 2 is consistent with that proposed by Liu et al. for the C-3 deoxygenation of 3,6-dideoxyhexoses such as ascarylose.[29] In *Yersinia pseudotuberculosis*, this deoxygenation proceeds through a unique single electron-transfer mechanism requiring two enzymes: a PMP-dependent dehydrase and a NADH-dependent, FAD-containing reductase. Both enzymes also possess an iron−sulfur cluster.[29] Genes homologous to that for the *Yersinia* dehydrase have also been identified in *Streptomyces* spp. (Liu, H.-w., personal communication to T.M.Z.), and the involvement of pyridoxal cofactors in the formation of blasticidin S was previously demonstrated with the identification of cytosinine:pyridoxal phosphate tautomerase activity in a cell-free extract.[13] The reason for the observed formation of **7** in the presence of argininic acid or α-methyl aspartate is unclear and will be explored further when the sugar-modifying enzymes of *S. griseochromogenes* are studied individually. We have recently cloned and expressed the blasticidin biosynthetic gene cluster and are in the process of characterizing these enzymes.[30]

Experimental Section

General Experimental Procedures. With the exception of 2-fluorofumarate, enzyme inhibitors were purchased from either Aldrich or Sigma. The 2-fluorofumarate was prepared as described in the literature.[31] NMR spectra were recorded on either a Bruker AC 300 or a Bruker AM 400 spectrometer. HPLC was performed on a Waters model 600E system connected to a Waters model 990+ photodiode array detector. Mass spectra were obtained on a Kratos MS 50 TC spectrometer, and IR spectra were recorded on a Nicolet 5DXB FT-IR spectrophotometer. Ion-exchange and size-exclusion resins were purchased from Sigma. Double-deionized water (Milli-Q, Millipore) was used for all fermentations and HPLC.

HPLC Analysis. The conditions for analytical HPLC were as follows: polysulfoethylaspartamide strong cation-

Scheme 2



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; flow rate, 1.0 mL/min. The UV region from 200 to 300 nm was scanned with the photodiode array detector.

Culture Maintenance and Fermentation Conditions. Standard culture maintenance and fermentation conditions have been described previously.[10] In these studies, the fermentation broth was harvested at 168 h after inoculation.

Feeding Protocol (Preparative Scale). Argininic acid (750 mg) was dissolved in approximately 15 mL of H_2O, and the solution was sterile filtered. The solution was added in equal portions to five 200 mL production broths 50 h after seed inoculation. At the same time, a sterile solution of cytosine (150 mg) was similarly fed.

Isolation. The fermentation broth (1 L) was centrifuged at 10000*g* for 10 min. Pellets were washed with a minimum amount of water and recentrifuged, and the washings were combined with the original supernatants. The supernatant fractions were loaded onto a Dowex 50W-X4 cation-exchange column (H^+ form, 2.5 × 30 cm) at a flow rate of 5 mL/min. The column was washed with water until the pH of the effluent was neutral, and then bound material was eluted with 5% aqueous pyridine. Compound **7** was found in the dark eluate of the early pyridine fractions. Lyophilization yielded a fluffy solid (1.5 g) that was triturated with methanol to remove insoluble material including most of the argininic acid. The methanol extract was dissolved in a small amount of MeOH/H_2O (1:1), applied to a Sephadex LH-20 column (2 × 100 cm), and eluted with MeOH/H_2O (1:1). Fractions of 15 mL were collected and analyzed for **7** by HPLC. Fractions containing **7** were combined and evaporated to dryness, yielding 420 mg of material still contaminated with argininic acid. The sample was dissolved in a small amount of water, loaded onto a S-Sepharose column (H^+ form, 1.5 × 20 cm), and eluted with water. Under these conditions, argininic acid was retained on the column while **7** passed through. Fractions containing **7** were combined and evaporated to dryness, yielding 45 mg of solid. To remove contaminating CGA, one-half of this residue was dissolved in water and purified by reversed-phase HPLC (Rainin Microsorb-MV C_{18}), 4.6 × 250 mm; mobile phase, 1% MeCN in H_2O containing 0.15% TFA; flow rate, 1.0 mL/min. Fractions containing **7** were combined and lyophilized to give 7 mg of a fluffy solid. This sample was used for the structure elucidation. The remaining residue from the S-Sepharose column was also purified by HPLC, and fractions containing **7** were concentrated in a centrifugal vacuum concentrator to give an 8 mg sample that contained the β-lactone.

The cytosine glycoside **7** was obtained as a white fluffy solid: FTIR (KBr) ν_{max} 3500−2500, 1730, 1682, 1273, 1200 cm^{-1}; ^1H- and ^{13}C NMR data, see Table 1; FABMS (positive ion, thioglycerol−glycerol) *m/z* 272 (70) $[M + H]^+$, 237 (30), 131 (70), 112 (100). HRFABMS *m/z* 272.0883, calcd for $C_{10}H_{14}N_3O_6$, 272.0883.

Acknowledgment. Dr. Yukio Miyazaki, Kaken Pharmaceutical Co., Ltd., is thanked for providing a culture of *S. griseochromogenes*. The helpful advice of Dr. John Carney is gratefully acknowledged as is the critical reading of the manuscript by Prof. Philip J. Proteau. This work was supported by No. Grant GM 32110 from the National Institutes of Health.

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NP970468O