AN ABSTRACT OF THE THESIS OF

Jincan Guo for the degree of Doctor of Philosophy in Biochemistry and Biophysics presented on June 24, 1992

Title: Biosynthesis of Blasticidin S: Pathway and Enzymes for the Nucleoside Formation and Blastidic Acid Assembly

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Abstract approved:_____

Dr. Steven J. Gould

Blasticidin S (BS) is an antifungal antibiotic produced by *Streptomyces griseochromogenes*. It consists of two unique structural components: a 2',3',4'-trideoxy hexotulose cytosine nucleoside and a β-amino acid.

Biosynthesis of non-ribose nucleoside antibiotics has acquired increasing attention during the searching for new antiviral, antibacterial agents in recent years. The intricate deoxygenation process leading to deoxynucleosides has also been a challenge in the field of bioorganic chemistry.

The objective of this study was to elucidate the pathway for the nucleoside formation and establish the timing of δ-N methylation in the biosynthesis of BS, and search for the responsible enzymes.

Complementing conventional whole-cell feedings of isotopically labelled putative intermediates, alternative approaches were adopted by feeding metabolic inhibitors to block normal biosynthesis. HPLC radiochemical analysis was also used to identify biosynthetic enzymes in cell-free extracts. Feeding metabolic inhibitors resulted in the accumulation of five new metabolites: pentopyranone (**57**), pentopyranone oxime (**59**), 2'-arginine hydroxamate pentopyranone (**60**), 4'-arginine hydroxamate pentopyranone (**61**) and isoblasticidin S (**62**) and dramatically increased the production of three known

metabolites: pentopyranine C (**18**), cytosylglucuronic acid (**20**) and demethylblasticidin S (**14**). These feedings also suggested that pyridoxamine phosphate (PMP) is involved in the biosynthetic pathway. Subsequent work on cell-free extracts identified four biosynthetic enzymes related to BS biosynthesis: UDP-glucose 4'-epimerase, UDP-glucose 6'-oxidoreductase, CGA synthase and DeMeBS δ-N-methyltransferase. These approaches established that BS is derived from UDP-glucose/UDP-galactose primary carbohydrate metabolism with **20** as the first committed intermediate for the nucleoside formation and **14** as the last intermediate in the pathway.

In addition, a whole-cell feeding of [3-^{2}H]glucose revealed that the ^{2}H label was retained in the H-3'a of **18**. The result suggested that the mechanisms of the C-3' deoxygenation in the biosynthesis of **18** and BS is a PMP-mediated deoxygenation similar to that of C-3' deoxygenation in the biosynthesis of ascarylose.

Finally, the first committed enzyme for the nucleoside biosynthesis, CGA synthase, was purified to homogeneity through five steps of chromatography. In contrast to eukaryotic UDP-glucuronosyltransferases, CGA synthase has been found to be soluble, non-phospholipid dependent and to have a strict substrate specificity. However, there are similarities between these two classes of enzymes in that the activities were stimulated by Mg^{++} but no external cofactors were required.

The biosynthesis of BS is the first instance where the immediate precursors and responsible enzyme for the formation of a novel nucleoside has been demonstrated at the cell-free level. In addition, although UDP-glucuronosyl transferases are common in eukaryotes, this was the first discovery of such an enzyme in a prokaryotic organism.

BIOSYNTHESIS OF BLASTICIDIN S: PATHWAY AND ENZYMES FOR THE NUCLEOSIDE FORMATION AND BLASTIDIC ACID ASSEMBLY

by

Jincan Guo

A Thesis

submitted to

Oregon State University

in partial fulfillment of
the requirements for
the degree of

Doctor of Philosophy

Completed June 24, 1992

Commencement June 1993

Approved:

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Date thesis is presented June 24, 1992

Typed by Jincan Guo

This thesis is dedicated to:

my wife and my mother

I want to express my whole-hearted gratitude to
Professor Steven J. Gould
and all the group members,
without their help
I would not have been
so successful in the preparation of this thesis.

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List of Abbreviations

AOAA:	aminooxyacetic acid
ArgA:	argininic acid
ArgH:	L-arginine hydroxamate
BA:	blastidic acid
BH:	blasticidin H
BS:	blasticidin S
CFE:	cell-free extract
CG:	cytosyl glucose
CGA:	cytosyl glucuronic acid
CN:	cytosinine
Cyt:	cytosine
DeMeBS:	demethyl blasticidin S
DON:	6-diazo-5-oxo-L-norleucine
dpm:	disintegretions per minute
DTT:	dithiothreitol
EDTA:	ethyleneldiamine N, N, N', N'-tetraacetic acid
Eth:	ethionine
5-FBS:	5-fluoroblasticidin S
5-FC:	5-fluorocytosine
5-FCGA:	5-fluorocytosyl glucuronic acid
FPLC:	fast protein liquid chromatography
Gal:	galactose
Glu:	glucose
GPC:	gel permeation chromatography
HIC:	hydrophobic interaction chromotography

5-HMBS:	5-hydroxymethyl blasticidin S
5-HMC:	5-hydroxymethyl cytosine
5-HMCGA:	5-hydroxymethyl cytosyl glucuronic acid
HO-BS:	deaminohydroxyblasticidin S
HPLC:	high performance liquid chromatigraphy
5-IC:	5-iodocytosine
5-ICGA:	5-iodocytosyl glucuronic acid
IEC:	ion exchange chromatography
IsoBS:	isoblasticidin S
L-α–Arg:	L-α-arginine
L-β-Arg:	L-β-arginine
L-Eth:	L-ethionine
LeucylBS:	leucyl blasticidin S
LSC:	liquid scintillation counting
Me^{++}:	divalent metal ions
MeArg:	δ-N-methyl arginine
Met:	methionine
min:	minute(s)
MSH:	mercaptoethanol
M_R:	molecular weight
NAD^+:	nicotinamide adenine dinucleotide
NADH:	nicotinamide adenine dinucleotide, reduced form
$NADP^+$:	nicotinamide adenine dinucleotide phosphate
NADPH:	nicotinamide adenine dinucleotide phosphate, reduced form
PC:	phosphatidylcholine
PLP:	pyridoxal phosphate
PMP:	pyridoxamine phosphate

PMSF:	phenylmethylsulfonyl fluoride
2'-PPN-ArgH:	2'-arginine hydroxamate pentopyranone
4'-PPN-ArgH:	4'-arginine hydroxamate pentopyranone
PPN-Oxime:	pentopyranone oxime
PPN:	pentopyranone
PPNA:	pentopyranine A
PPNB:	pentopyranine B
PPNC:	pentopyranine C
PPND:	pentopyranine D
R_f:	retardation factor
rpm:	revolutions per minute
SAM:	S-adenosyl-L-methionine
SDS-PAGE:	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TFA:	trifluoroacetic acid
t_R:	retention time
UDP-Gal:	UDP-galactose
UDP-GalA:	UDP-galacturonic acid
UDP-Glu:	UDP-glucose
UDP-GluA:	UDP-glucuronic acid
UDP:	uridine 5'-diphosphate
UDPGT:	UDP-glucuronosyltransferase
XDP:	purine and purimidine nucleotide diphosphate

BIOSYNTHESIS OF BLASTICIDIN S: PATHWAY AND ENZYMES FOR THE NUCLEOSIDE FORMATION AND BLASTIDIC ACID ASSEMBLY

Chapter I General Introduction

Blasticidin S: A Peptidyl Nucleoside Antibiotic

Blasticidin S (BS), **1**, is a metabolite of *Streptomyces griseochromogenes* that was first isolated in Japan in 1958.[1] It is used in agriculture as a fungicide against rice blast caused by the pathogenic fungus *Piricularia oryzae*. A 5 μg/mL concentration of BS markedly inhibits the spreading of rice blast.[1,2] It is mostly due to the use of this outstanding antibiotic that Japan is no longer compelled to import rice.

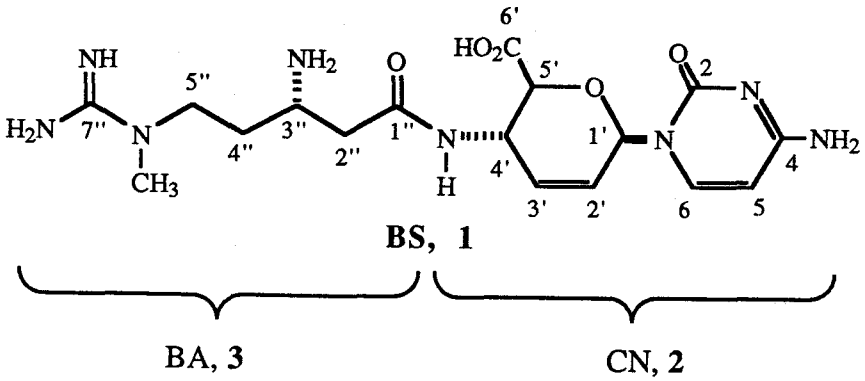


Fig I-1. Structure and components of BS

Structurally, BS consists of an unusual cytosine nucleoside and a new β-amino acid, designated as cytosinine (CN), **2**, and blastidic acid (BA), **3**, respectively (Fig I-1). This structure was deduced from the component products of several chemical degradations[4] and was confirmed by X-ray diffraction studies.[5] Complete assignments of ^{1}H- and ^{13}C NMR spectra of BS have been carried out in our laboratory[6] using one and two dimensional NMR spectroscopy.

Biological Activity of BS

Although BS is most potent as an antifungal agent, it also exhibits antibacterial,[7] antiviral[8] and antitumor activities,[9] and is toxic to mice (LD_{50} ~2.8 mg/Kg).

The mechanism of BS biological activity is believed to involve blockage of protein biosynthesis (Fig I-2).[4a,10,11,12] Studies by Misato et al.[10] showed that BS inhibited the incorporation of ^{14}C-labeled amino acids into proteins. A more detailed study by Yamaguchi[13] and others[14,15] indicated that BS, by binding to the 50S ribosomal RNA, blocked the transfer of amino acids from aminoacyl-tRNA to the elongating peptide and stimulated the release of ribosome subunits.[15] Initially, it had been shown that BS did not inhibit the incorporation of ^{32}P into nucleic acids at concentrations which inhibited protein biosynthesis.[10a,13] However, subsquent work of Griffin et al.[16] indicated that BS did slowly inhibit DNA and RNA synthesis. From their studies they concluded that the mechanism of inhibition of DNA and RNA synthesis was independent of and different from that of inhibition of protein synthesis, and hence BS may possess at least two sites of action.[17] However, no more precise information about its biological activity mechanisms was provided.

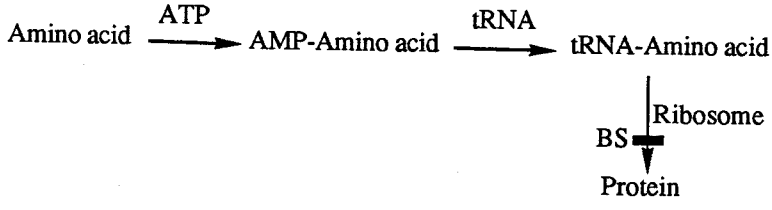
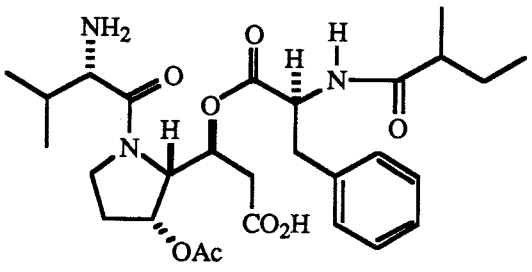


Fig I-2. Site of BS action

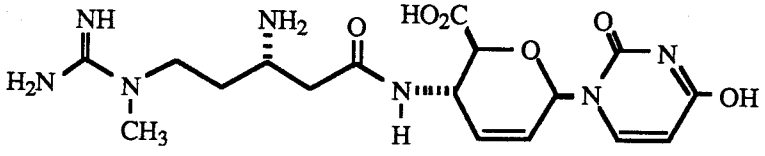
The inhibitory effect of BS against *Bacillus cereus* is negated by the 'detoxin complex' isolated from *S. caespitosus*.[18] The most active component of this complex has been elucidated to be detoxin D_1, **4**.[19] It was reported that detoxin D_1 acted as an antagonist by inhibiting the active transport of BS into cells.[20]



Detoxin D_1, **4**

Biological Transformation of BS

As is common for natural products, BS has been reported to be transformed by several species of fungus and bacteria. An example is deamination to deaminohydroxyblasticidin S (HO-BS), **5**, by the fungi *Aspergillus fumigatus*[21] and *A. terreus*.[22] The biological activity of HO-BS was reduced to one hundredth that of BS.[21] Endo and coworkers[23] have isolated another BS resistant strain, *Bacillus cereus* K55-S1, that also deaminates BS into HO-BS. The structural gene of the BS deaminase was located on a plasmid.[24]



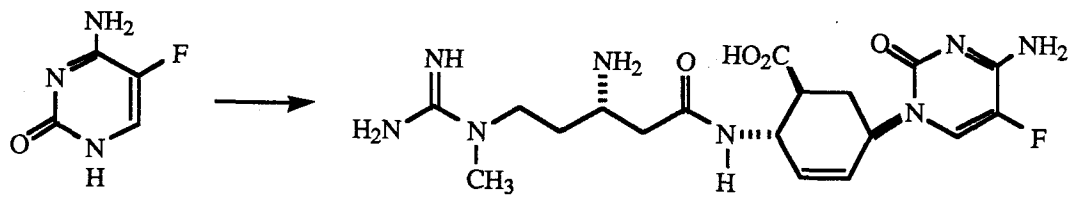
HO-BS, **5**

BS is also known to be inactivated by acetylation *in vitro* by a producing organism, *Streptoverticillium sp.* JCM 4673, in the presence of acetyl coenzyme A[25] and the acetyl transferase has been purified.[26] Another acylated product, leucylblasticidin S (LeucylBS), **6** (see Table I-2, p 6), which has a leucyl group at the β-amino group of BS, was isolated from a *Streptomyces* sp. Sch 36605[27] and from an acidified fermentation broth of *S. griseochromogenes*.[28] However, it was not clear whether LeucylBS was a product of BS acylation or a biosynthetic precursor to BS (see discussion in Chapter IV).

Structurally Related Nucleoside Antibiotics

In addition to BS, there are several other known peptidyl nucleoside antibiotics that have close structural similarities to BS. These include 5-hydroxymethylblasticidin S (5-HMBS), **7**,[29] arginomycin, **8**,[30] mildiomycin, **9**,[31] gougerotin, **10**,[32] bagougeramine A, **11**,[33,34] bagougeramine B, **12**,[33,34] and the polyoxins, **13a-e**.[35] They all consist of a cytosine hexuronic nucleoside unit and a basic amino acid side chain (Table I-1), and some of them exhibit biological activities similar to that of BS.[36] Although they are produced by different species or genera of bacteria, the common structural pattern among these antibiotics suggests that they are biogenetically related.

During the course of screening for new metabolites from *S. griseochromogenes*, Seto et al. have isolated and characterized, in addition to BS, a number of cytosine glycosides (Table I-2). These are demethylblasticidin S (DeMeBS), **14**,[37] blasticidin H (BH), **15**,[38] LeucylBS, **6**,[27,28] pentopyranine A (PPNA), **16**,[39a] pentopyranine B (PPNB), **17**,[39b] pentopyranine C (PPNC), **18**,[39a] pentopyranine D (PPND), **19**,[39b] and pentopyranic acid (cytosylglucuronic acid, CGA), **20**.[40] By supplementing the culture of *S. griseochromogenes* with 5-fluorocytosine (5-FC), **21**, Kawashima et al. have reported the production of 5-fluoroblasticidin S (5-FBS), **22**,[41] as is shown in Figure I-3. Apparently, all these cytosine glycosides are structurally related; however, their biogenetic relationships had not been examined.



21 **22**

Fig I-3. Formation of 5-FBS, **22**

Table I-1. Peptidyl nucleoside antibiotics structurally related to BS

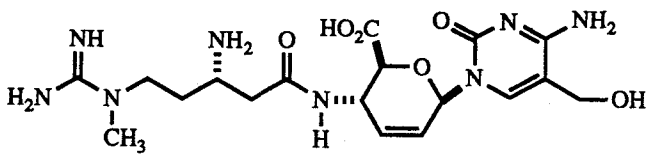
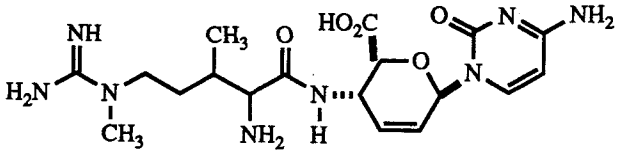
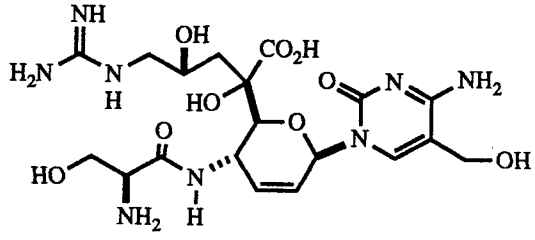
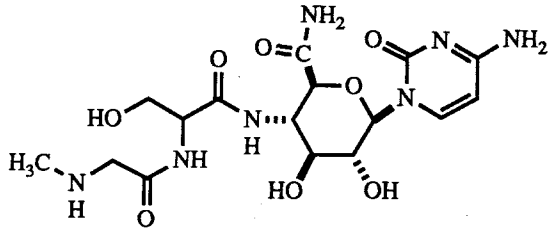
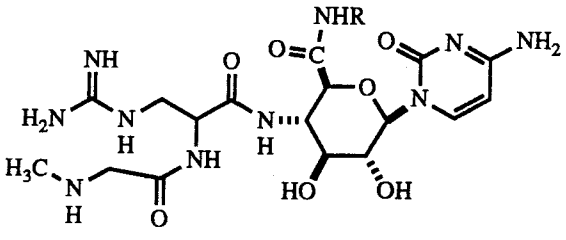
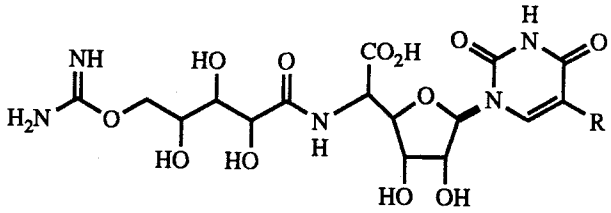
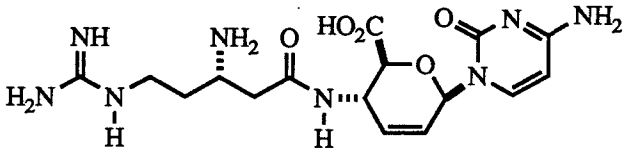
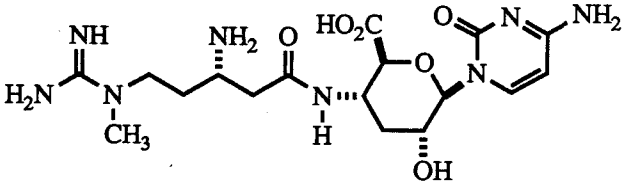
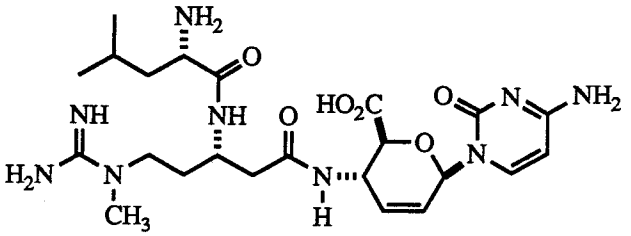
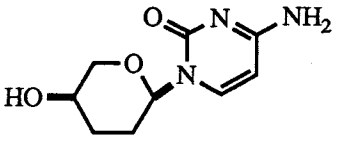
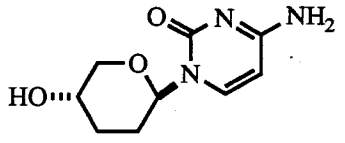
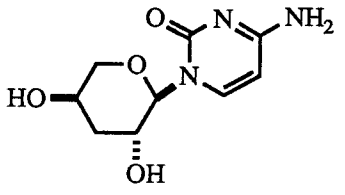
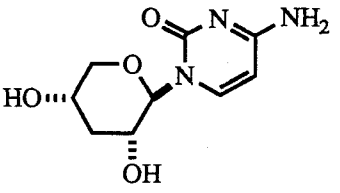
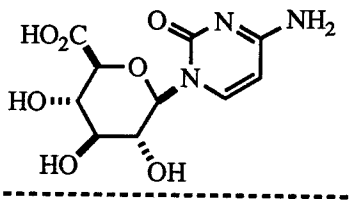
Antibiotic	Structure
5-HMBS, **7**	
Arginomycin, **8**	
Mildiomycin, **9**	
Gougerotin, **10**	
Bagougeramine A, **11**	 (R = H)
Bagougeramine B, **12**	(R = )
Polyoxins, **13a-e** (R = H, CH_3, CH_2OH, CO_2H, F)	

Table I-2. Metabolites from *S. grisechromogenes* structurally related to BS

Antibiotic	Structure
DeMeBS, **14**	
BH, **15**	
LeucylBS, **6**	
PPNA, **16**	
PPNB, **17**	
PPNC, **18**	
PPND, **19**	
CGA, **20**	

Previous Biosynthetic Studies on BS

Although the chemical and biochemical properties of BS have been extensively scrutinized, biosynthetic studies on BS were limited. The first biosynthetic study of BS

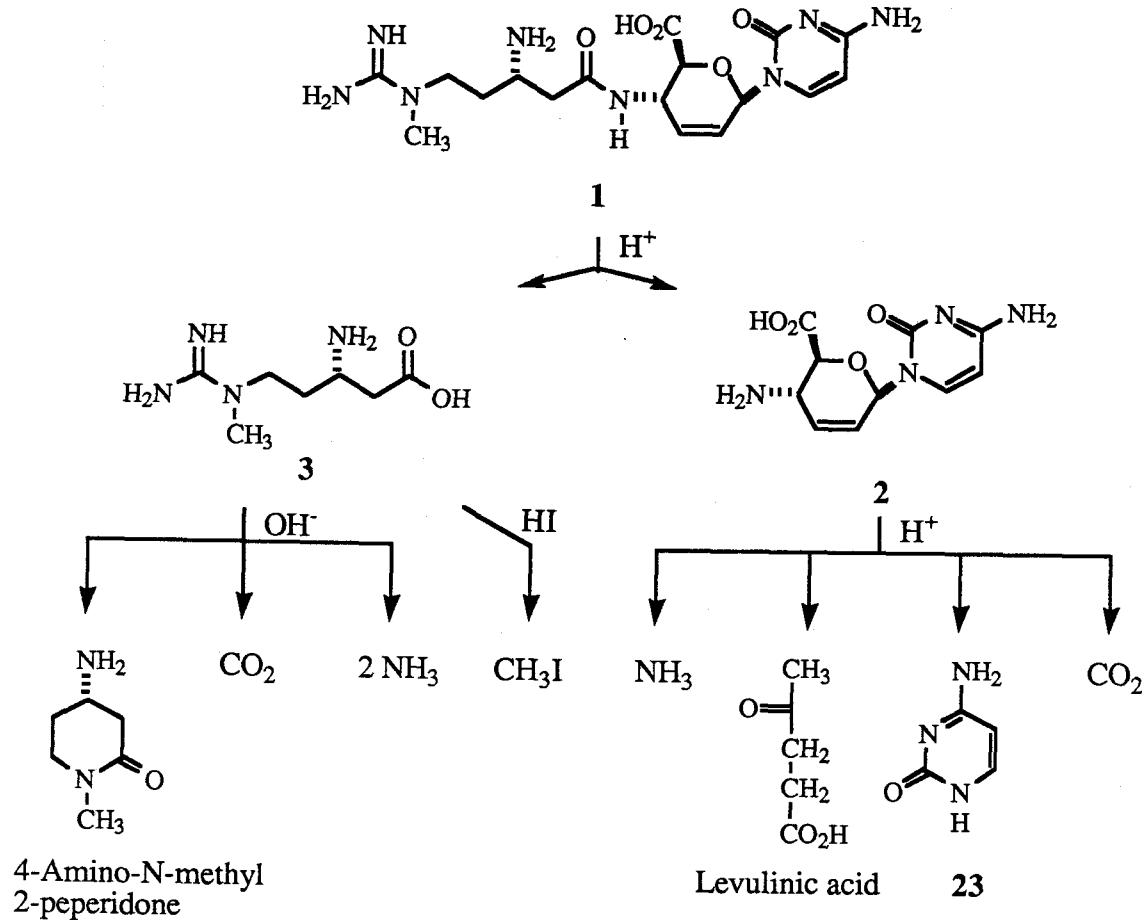


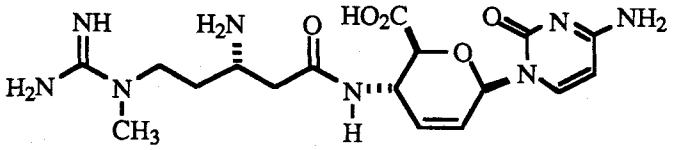
Fig I-4. Degradation of BS from feeding experiments[42]

was carried out by Seto et al. in 1968.[42] Various compounds labeled with ^{14}C were fed to the fermentation cultures of *S. griseochromogenes* and analyzed for their overall percent incorporations into BS (Table I-3). The radioactivity distributions among BS components were determined by classical chemical degradations (Fig I-4). These feedings revealed that the major components of BS are derived from cytosine, **23**, D-glucose, **24**, L-α-arginine (L-α-Arg), **25**, and L-methionine, **26** (Fig I-5).[42]

Table I-3. Percent incorporation of feeding compounds into BS[42]

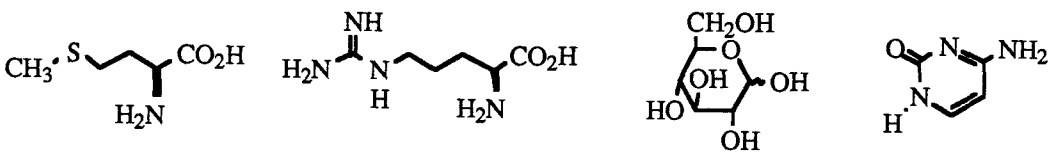
Precursor	% Incorporation
D-[U-^{14}C]Glucose	3.7
D-[1-^{14}C]Glucose	4.0
D-[6-^{14}C]Glucose	4.9
[2-^{14}C]Cytosine	95.1
[U-^{14}C]Cytidine	15.3
L-[Methyl-^{14}C]Methionine	38.3
L-[Guanidino-^{14}C]Arginine	51.2
L-[U-^{14}C]Arginine	30.3
L-[U-^{14}C]Aspartic acid *	0.6
[1-^{14}C]Alanine	0.5
[U-^{14}C]Alanine	0.5
[U-^{14}C]Acetic acid	0.5
[U-^{14}C]Glycine	1.1

* Unlabeled cytosine was added simultaneously



1

S. griseochromogenes



26 **25** **24** **23**

Fig I-5. Primary precursors of BS[42]

Other metabolites isolated from *S. griseochromogenes* listed in Table I-2 seemed to be biogenetically related to BS. With this information, Seto and his coworkers proposed a

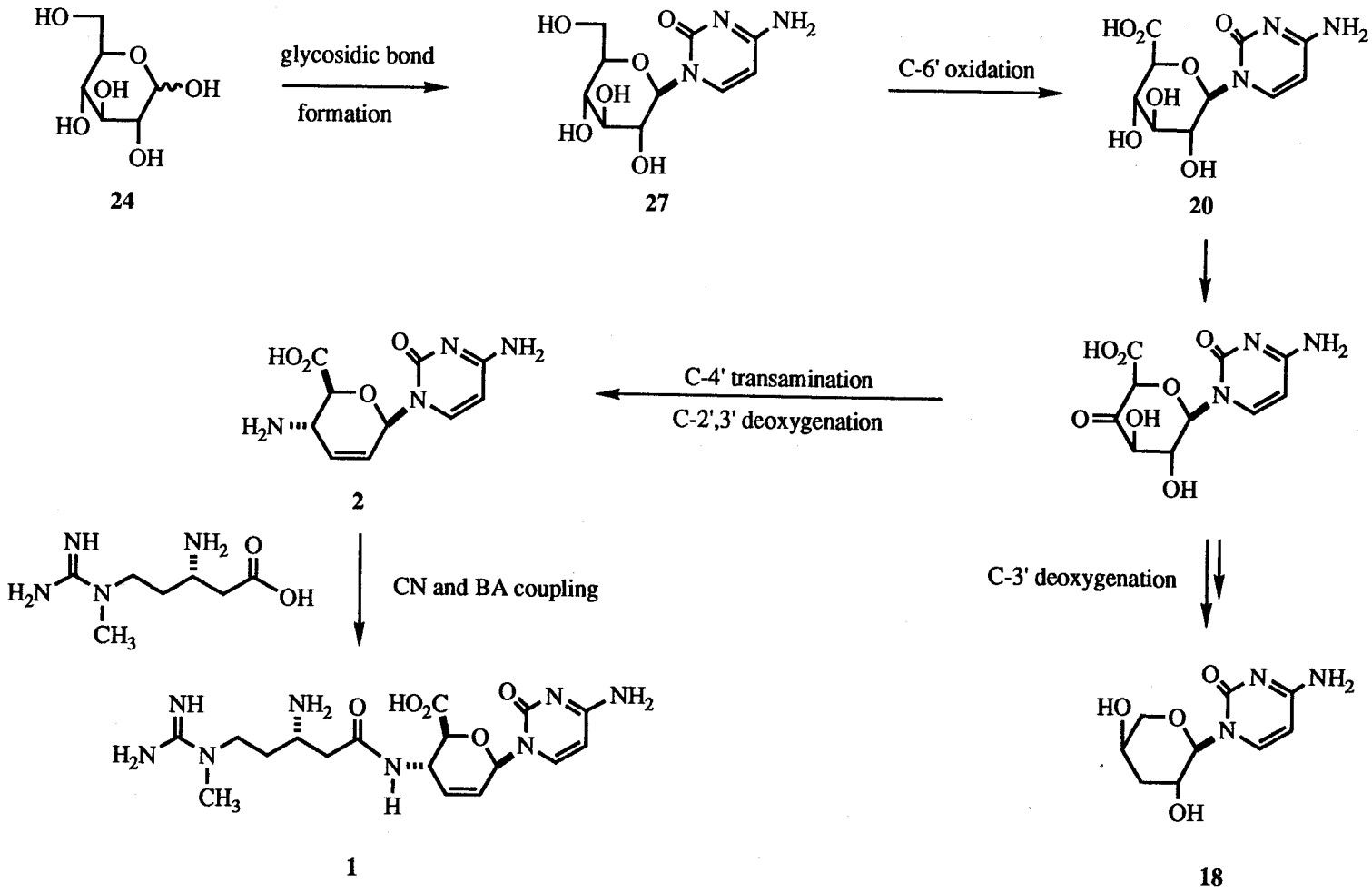


Fig I-6. Seto's proposal for the biosynthesis of BS[39,40]

biosynthetic pathway for BS and those related metabolites (Fig I-6).[39,40] It is noticeable, however, that several important biochemical transformations in their proposal needed to be confirmed, including:

(i) the formation of cytosine glycosidic bond;

(ii) the timing of C-6' oxidation;

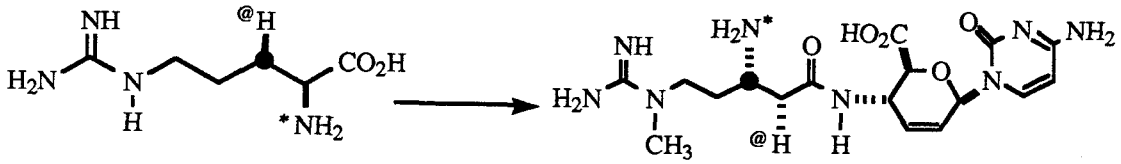
(iii) the C-2',3' deoxygenation and C-4' transamination;

(iv) the coupling of BA and CN;

(v) the δ-N methylation, and the transformation of L-α-Arg into L-β-Arg.

In 1968, Yonehara et al. reported the reconstruction of BS from the component compounds CN and BA by *S. griseochromogenes* .[43] However, this was later shown to be an artifact.[44] Therefore the biochemical transformation in (iv) and the intermediacy of CN and BA needed further study.

Recently, our laboratory has made contributions to clarify the mechanism of the biosynthesis of the L-β-arginine (L-β-Arg), **28**, moiety. [3-^{13}C, 2-^{15}N]Arginine, **25a**, as well as a series of deuterium labeled arginines were synthesized and fed to cultures of *S. griseochromogenes*. The isotopic labelling patterns in the resulting BS samples showed unequivocally that the L-β-Arg is derived from L-α-Arg by an intramolecular exchange of the α-amino group with the β-pro-R hydrogen, as is shown in Figure I-7,[45] resembling the biochemical process of β-lysine biosynthesis.[46]

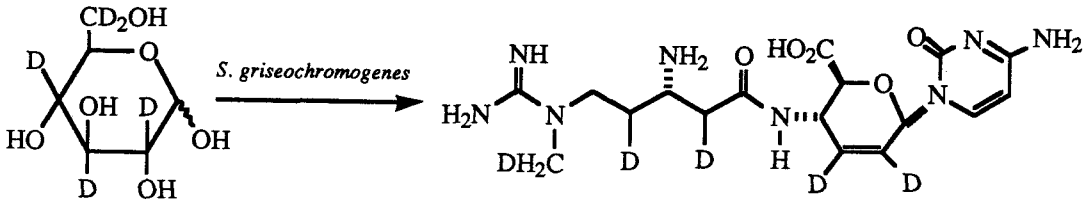


25a **1a**

Fig I-7. Biosynthesis of β-Arg moiety of BS[45]

Our laboratory has also performed the synthesis and feeding of labeled β-Arg and δ-N methylarginine (MeArg), **29**. In these cases, β-Arg was well incorporated, while MeArg was not incorporated to a detectable level.[45] The results indicated that δ-N methylation might occur at a late stage of BA assembly.

In an attempt to elucidate the biosynthetic mechanism of the hexuronic acid moiety, deuterium labeled glucoses were fed. Deuterium from [1-^{2}H]glucose, **24a**, was detected at H-1' of BS, as was expected. When [2,3,4,6,6-$^2H_5$]glucose, **24b**, was fed it was found that both H-2 and H-3 of glucose were also retained in the BS sample, **1b**, whereas H-4 had been lost (Fig I-8).[47] This finding implied a more complicated biochemical transformation than a simple dehydration mechanism for the C-2',3' deoxygenation.



24b **1b**

Fig I-8. Feeding of ^{2}H-glucose[47]

Although some of the above-mentioned biosynthetic studies provided information about BS biogenesis and the mechanisms of β-Arg moiety biosynthesis, others revealed the complexity of BS biosynthesis rather than provided the final delineation of its biosynthetic pathway. Many of those critical steps proposed by Seto et al. remained unelucidated.

Objectives and Significance of Present Studies

One of the original purposes of this study was to isolate and characterize the enzyme for the transformation of L-α-Arg to L-β-Arg. However, after an initial attempt

to isolate the L-α-Arg-2,3-aminomutase failed, the objectives shifted mainly onto the mechanisms and enzymes for the nucleoside formation.

Nucleoside antibiotics represent a class of functionally very diverse natural products.[48] Chemical and biochemical studies on nucleoside antibiotics have increased during the past few years in the search for new antiviral and antibacterial reagents. However, knowledge about nucleoside antibiotic biosyntheses has been rather sparse in respect to the mechanisms of glycosidic bond formation and enzymes involved.[48] Permeability barriers to the uptake of advanced nucleoside intermediates to producing organisms generally render conventional whole-cell feeding experiments inefficient.[48] Information available in the literature only indicated that formation of nucleoside antibiotics are as diverse mechanistically as they are structurally. For example, aristeromycin, **30**, has been proposed to be synthesized from D-glucose via a cyclopentenoid pyrophosphate derivative, **31**, with the original carbon skeleton completely rearranged (Fig I-9),[49] whereas intermediates for polyoxins, **13a-e**,[50]

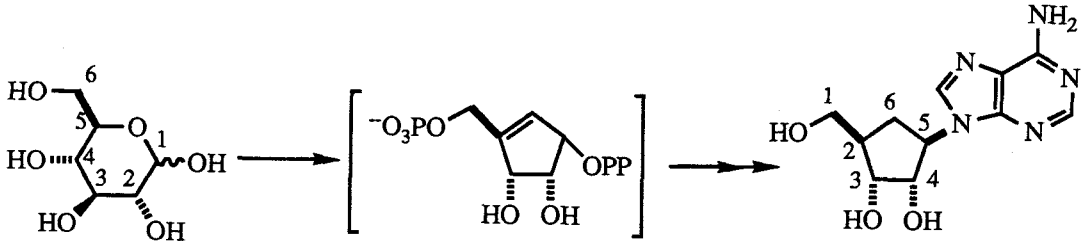


Fig I-9. Proposed intermediate for aristeromycin[49]

and sinefungin, **32**,[51] biosynthesis were proposed to be completely different. In the case of polyoxins, the ribonucleoside, uridine, **33**, was incorporated intact (Fig I-10) while in the case of sinefungin, the ribonucleoside, adenosine, **34**, was first cleaved and then reconnected (Fig I-11). Nevertheless, in none of these cases have the immediate precursors for the corresponding nucleoside formation been confirmed.

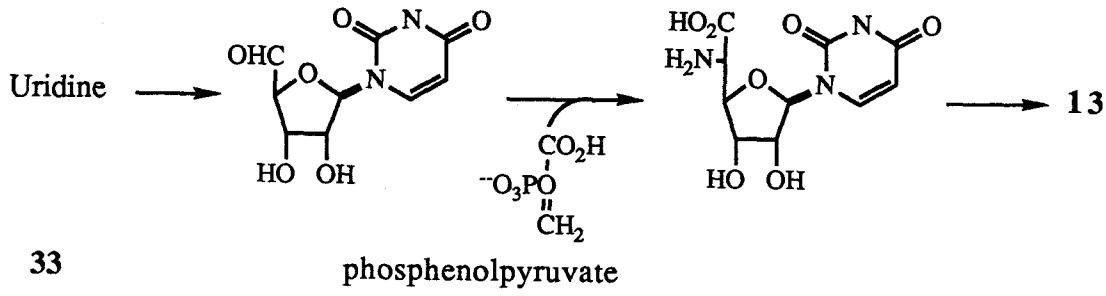


Fig I-10. Proposed intermediates for polyoxins[50]

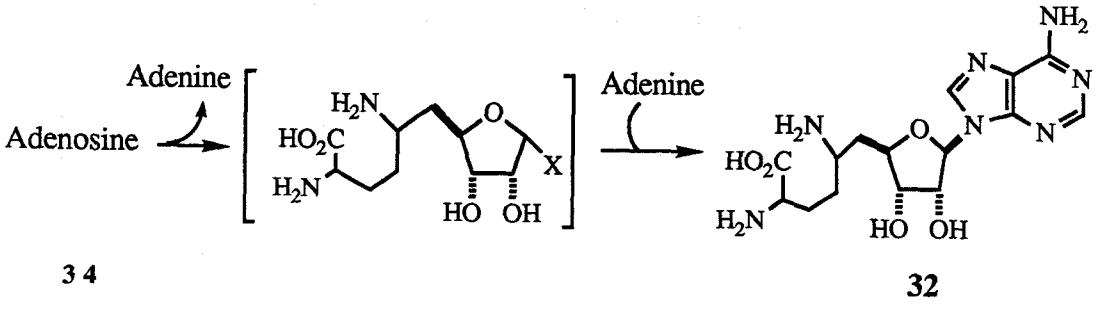


Fig I-11. Proposed intermediate for sinefungin[51b]

The objectives of the present study were to establish how a non-ribose carbohydrate was activated to be precursor for nucleoside antibiotic biosynthesis, to purify and characterize the corresponding enzyme, and to elucidate the stereochemistry and mechanisms of CN biosynthesis. This study was also aimed at determining the sequence in the transformation of L-α-Arg into the BA moiety of BS. The success of this study would be useful for understanding the biosynthesis of other related nucleoside antibiotics and would be helpful in designing and preparing new bioactive nucleoside analogues.

Ultimately, the present study focused on three aspects:

1) To determine the initial precursors for the nucleoside formation and to purify and characterize the responsible nucleoside synthase;

2) To determine the mechanisms of C-2,C-3 deoxygenation and introduction of the C-4 amino group;

3) To determine the timing of δ-N methylation and BA assembly.

The results of these efforts will be described in the following chapters.

Chapter II will describe conventional whole cell feeding experiments. Isotopically-labeled putative biosynthetic intermediates were prepared, fed to the organism and their level of incorporation into BS determined. The work in this chapter primarily focused on the confirmation of key intermediates that had previously been proposed.

Chapter III will describe the results of an alternative approach to identify intermediates by feeding enzyme inhibitors and large quantities of primary precursors to growing cultures, in the hope of forcing them to accumulate intermediates in the fermentation broth.

Chapter IV will present the results of *in vitro* biosynthetic studies. Metabolites accumulated from enzyme inhibitor and primary precursor feedings were examined with cell-free extracts for their intermediary role. The combined approaches described in this chapter and the previous two were expected to provide direct information for BS biosynthesis while overcoming permeability problems and relieving the effort of preparing numerous isotopically labeled putative biosynthetic intermediates.

Chapter V will present the study of the structures and stereochemistries of BS-related metabolites that accumulated during the feeding of metabolic inhibitors. Since those related metabolites were apparently intermediates or aberrant products of the BS biosynthetic pathway, some of the biochemical transformations should be shared in the early steps of their biosynthesis. Understanding the mechanisms for the biosynthesis of aberrant products would be of interest by itself and would give direct information about the biogenetic relationships with BS. Biosynthetic mechanisms leading to the C-3

deoxygenation and to the C-4 transamination during formation of the hexouronic acid moiety of BS were explored.

Chapter VI will present the purification and characterization of the first committed enzyme for the nucleoside formation, cytosine: UDP-glucuronosyl transferase.

Chapter VII will summarize the approaches and results of the present study and the prospects of future work.

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Chapter II

Conventional Whole-Cell Feedings of Putative Intermediates

Introduction

The intriguing structures of secondary metabolites have long provoked scientific curiosity both about their biological origins and about the underlying fascinating biochemical transformations. In the early 1950s, the availability of radioisotopes of carbon and hydrogen opened up the classic era of experimental investigations. Numerous pioneering *in vivo* feeding experiments[1,2,3] largely confirmed early biogenetic speculations[4] on the biological origins of cholesterol, terpenoids and some biologically important alkaloids. More recently, the development of high field NMR techniques, in combination with the application of stable isotopes, brought about a further dramatic increase in the pace and sophistication of biosynthetic investigation and greatly amplified biosynthetic studies to almost every category of natural products. However, the introduction of NMR and stable isotopes did not change the basic experimental approaches. Until recently the vast majority of biosynthetic studies have been carried out with intact cells, whether intact plants, excised organs or suspensions of cells.

Studies with intact cells or living organisms have the advantages that all relevant enzymes and required co-factors remain unaltered. It is generally feasible to carry out a multi-step transformation from early precursors to final products by an intact cell system; therefore, the design and feeding of early intermediates can be performed with little knowledge of the real biosynthetic pathway. On the other hand, *in vitro* experiments at the level of cell-free extracts have encountered tremendous difficulties. The low titers of biosynthetic enzymes and their unstable, diverse nature often make it difficult to develop a unified condition for *in vitro* assays. It is usually necessary to have some basic information about the biosynthetic pathway, about the intermediates, and about types of

reactions that may be involved before starting any enzymatic investigation. Therefore an intact cell feeding approach has been a prerequisite for subsequent successful enzymatic investigation.

For the reasons cited above, the present study of BS biosynthesis was started in a conventional fashion with whole-cell feedings of isotopically labeled putative intermediates. The initial effort focused on identification of key intermediates as well as on gaining an overview about the nucleoside formation.

Result and Discussion

Preparation and Feeding of [1-^{14}C]BA, 3a, and of [1'-^{14}C]CN, 2a

Since the amide bond can be viewed as chemically, and perhaps enzymatically, the most labile linkage in BS, it was reasonable to propose that the CN and BA segments were biosynthesized separately and coupled together at a late or final stage of the biosynthetic pathway.

Yonehara et al. had reported[5] the reconstruction of BS from BA and CN by mycelial suspensions of *S. griseochromogenes*. However, several cautious notes should be recognized. First, it was not clear whether the BS produced was derived from *de novo* biosynthesis or from external administered BA and CN, because the added BA and CN were not labeled. Second, the production of BS was not determined adequately. Using the method of bioassay to quantify the production, as was reported, could be misleading when BS may not have been the only bioactive metabolite in the culture. Finally, the result was not reproducible.[6] Therefore, it was first hoped to repeat Yonehara's experiment, but in our case by feeding ^{14}C labeled BA and CN and then determining their percent incorporation into BS.

Preparations of [1-^{14}C]BA, **3a** and [1'-^{14}C]CN, **2a**, were achieved by 3 N H_2SO_4 hydrolysis of [1''-^{14}C]BS, **1c**, and [1'-^{14}C]BS, **1d**, respectively (Figure II-1).[5] Compounds **1c** and **1d** were prepared by Prabhakaran from the feeding of [1-^{14}C] Arg, **25b**,[7] and [1-^{14}C]glucose, **24c**.[8] The yield for **3a** and **2a** was about 40% in each case. It was later found that hydrolysis of BS with 6 N HCl instead of 3 N H_2SO_4 could double the yield for BA (85%) and the reaction time could be reduced from 40 hours to 4-6 hours. However, under the new conditions some of the CN was cleaved to cytosine, and therefore the revised method was not good for the preparation of CN.

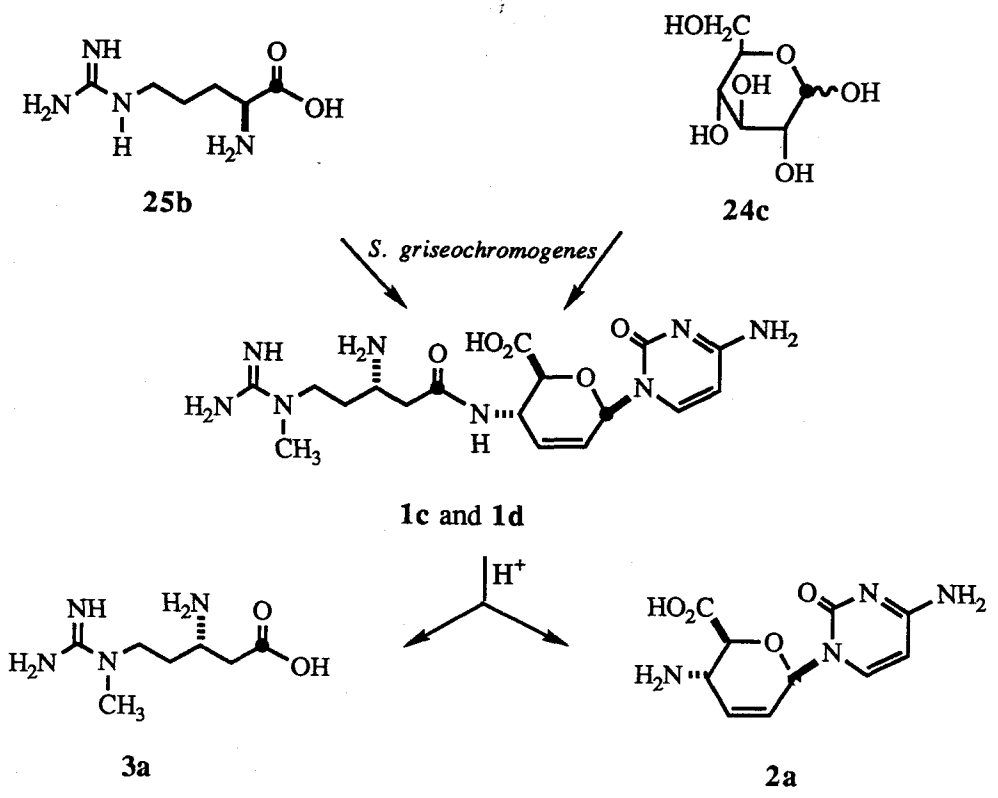


Fig II-1. Preparation of **3a** and **2a** from **1c** and **1d**, respectively. Compound **1c** and **1d** were prepared from two separate fermentation cultures to which **24c** and **25b** were fed.[7,8]

Feeding experiments were subsequently carried out. One μCi of **3a** and 0.45 μCi of **2a** were each fed to two separate 200-mL fermentation cultures of *S. griseochromogenes*. The resulting BS samples were isolated and purified. However, neither of these BS samples retained radioactivity after rigorous purification.

The negative result from the **3a** feeding was considered as a sign that it is not an intermediate. Since a structurally similar compound, β-Arg had been well incorporated,[7] it seemed unlikely that the negative incorporation resulted from BA impermeability. Consistent with the non-incorporation of MeArg,[7] this result also suggested that δ-N-methylation occurred in a late stage or possibly at the last step in the biosynthetic pathway. As is shown in Figure II-2, we proposed that transformation of L-α-Arg to BS

would be through route *a* but not route *b* or *c* . This proposal was later confirmed by converting DeMeBS to BS with a cell-free extract of *S. griseochromogenes* (see Chapter IV).

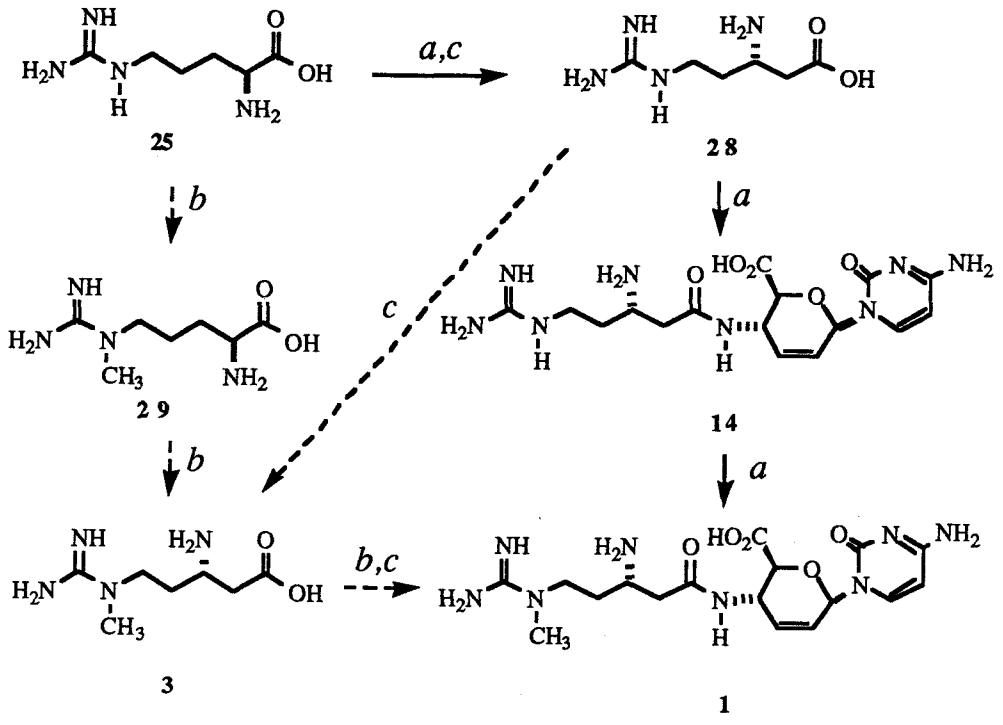


Fig II-2. Proposed pathway for BA assembly in BS biosynthesis

On the other hand, reasons for the negative incorporation of **2a** to BS were more obscure. Impermeability of organisms to the up-take of advanced nucleoside intermediates has been speculated in other biosynthetic studies[8] and has been presumed to be the main reason for the very limited progress in the biosynthesis of nucleoside antibiotics.[9] It was not clear in our feeding experiments whether the negative result arose from the impermeability of CN or from its non-intermediacy.

Isotopic Trapping with CN

As an alternative to the previous experiment, an isotope trapping experiment was carried out to determine whether *de novo* production of CN occurred in *S.*

griseochromogenes. The experiment was performed with 100 mL of synthetic medium with an 8% seed broth inoculation, instead of 2% which was used for typical feeding experiments. Increasing the percentage of seed inoculation had been demonstrated to compress the duration of BS production from 4 - 5 days into 2 - 3 days. Therefore an 8% seed inoculation should synchronize to some extent the production phase of potential intermediates and thus increase the sensitivity of the isotopic trapping experiment.

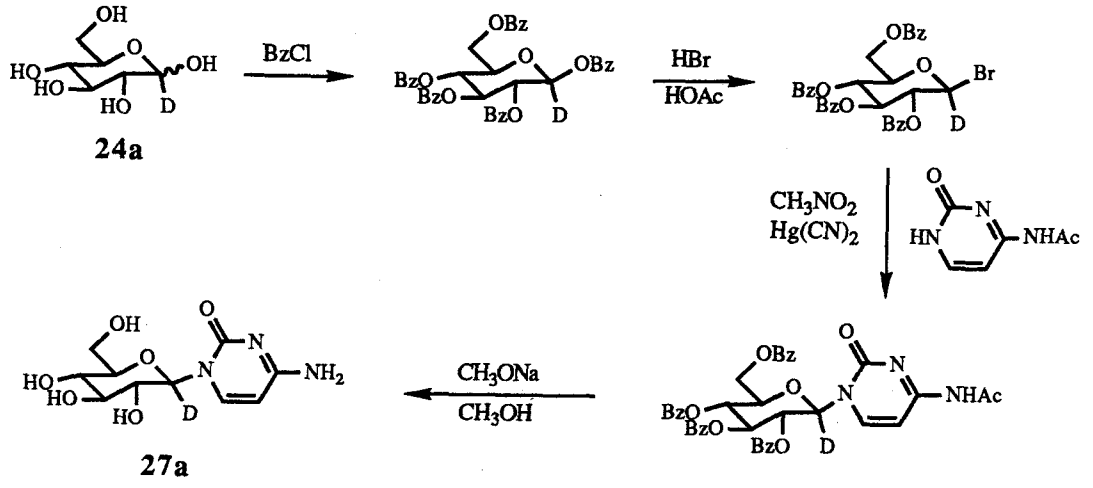
For the trapping experiment, a total of 11 μCi of [2-^{14}C]cytosine, **23a**, was fed in two pulses to a fermentation culture of *S. griseochromogenes* after 46 hours and 48.5 hours of incubation. The mycelia were harvested 4.5 hours later and disrupted by sonication. To the homogenate, 69.4 mg of unlabeled CN was added, re-isolated and then rigorously purified. Liquid scintillation counting (LSC) data indicated that less than 0.02% percent of the radioactivity fed was trapped in the CN. Comparing the percent incorporation of cytosine to BS (95%),[10] the level of 0.02% was considered to be of trivial significance. There were speculations that there might have been a strict substrate channeling system in converting CN to the next step in the biosynthesis or the isotopic trap experiment was not performed in the right biosynthetic time-window. Nevertheless, the potential of CN as an intermediate needs further examination.

Following these unsuccessful feeding experiments, we decided to feed potential intermediates of earlier stages. As cytosylglucose (CG), **27**, was proposed to be the first committed intermediate for the nucleoside portion,[11] [1'-^{2}H]CG, **27a**, was prepared and a feeding experiment was accordingly performed.

Feeding [1'-^{2}H]CG, 27a

[1'-^{2}H]CG had been synthesized by Karl DeJesus by the following procedure, starting with [1-^{2}H]glucose, **24a** (Fig II-3).[12] Deuterium was chosen as the labeling isotope because it could be analyzed by NMR spectroscopy and was easier to prepare

than labeling with tritium or isotopes of carbon. Furthermore, deuterium is non-radioactive.



ig II-3. Synthesis of [1'-^{2}H]CG, **27a**[12]

ment was carried out by adding 100 mg of **27a** to a 200-mL
ıe same manner as in the feeding of BA and CN. The resulting
yzed by ^{2}H NMR spectroscopy. However, no ^{2}H signal was
ıle (Fig II-4). Because H-1 of glucose had previously been
c from a **24a** feeding experiment,[7] H-1' of **27a** should not
quent biosynthetic transformations if it were an intermediate.

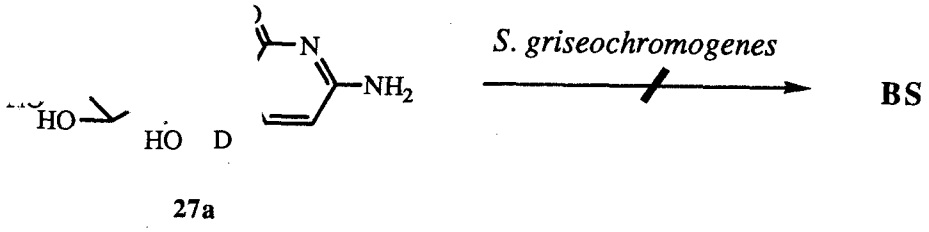


Fig II-4. Feeding of **27a**

The negative result, again, raised our suspicion of a lack of permeability of **27a** to *S. griseochromogenes*. In the meantime, it was also speculated that glucose might not be transformed to the CN moiety as straightforwardly as it was first proposed. Oxidation,

dehydration and epimerization are several biochemical transformations common to carbohydrate metabolism and might have preceded the glycosidic bond formation. This complexity led us to explore the general features of BS biosynthetic pathway.

Feeding [1-^{14}C]Glucose, 24c, and [1-^{14}C]Galactose, 35a

The feeding of [1-^{14}C]galactose, **35a**, was to examine its competence as a precursor in comparison with that of [1-^{14}C]glucose, **24c**. This experiment would determine if glucose was extensively modified before it was connected cytosine and at which point the BS biosynthetic pathway branched off from the primary carbohydrate metabolism. Consistent with previous feeding experiments, feeding **24c** gave a 1.8% incorporation. However, feeding **35a** gave a 15.2% incorporation, which was 8.4-fold higher than that of **24c** (Fig II-5). These results suggested clearly that the CN moiety was derived from one of the intermediates into which both glucose and galactose could easily be transformed.

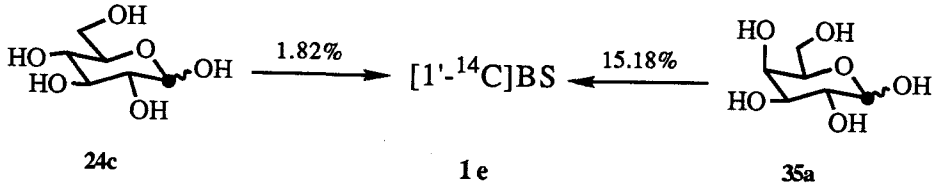


Fig II-5. Percent incorporation of glucose and galactose into BS

These results also suggested that glucose and galactose were not extensively modified before they were committed to the nucleoside formation. Since UDP-glucose, **36**, or UDP-galactose, **37**, is widely used in biological systems as the activated form for glycoside biosynthesis,[13] the nucleoside of BS was expected to be formed in a similar manner, by coupling cytosine to possibly one of the four UDP-glycosides in the glucose-galactose transformation system (Fig II-6).

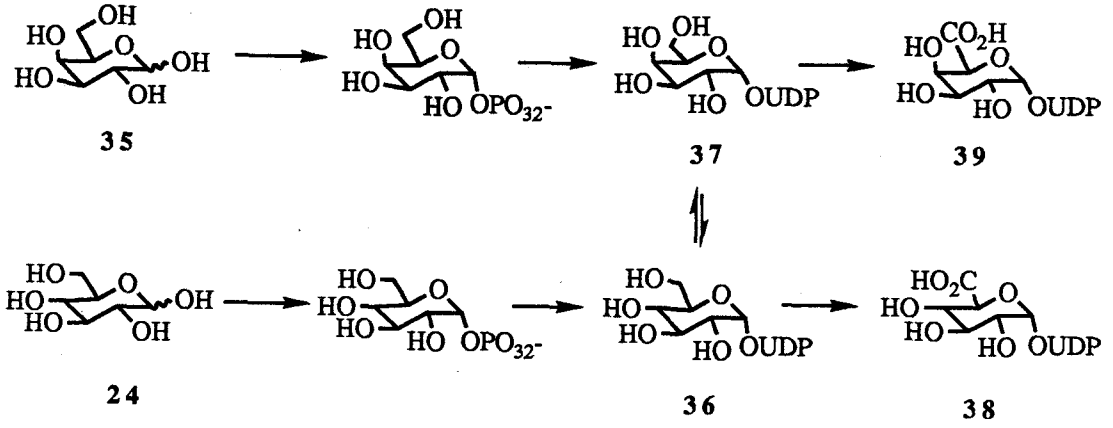


Fig II-6. Interconversion of glucose and galactose[14]

Feeding [1,1-$^2H_2$]Ethanol, 40

In addition to feeding competent precursors, another strategy to explore the general features of the nucleoside formation would be to trace the origins of hydrogens in CN moiety. Knowing whether they were derived from the original carbinol hydrogen of glucose, from an intracellular hydride source such as NADH or from a trapped proton of water would enable us to favor some mechanisms and eliminate others. Such an approach has proven to be very informative in the biosynthetic studies[14] of sarubicin A, streptothricin F and BS, in which[14] it was shown that both H-2' and H-3' of **1b** were deuterium enriched when [2,3,4,6,6-$^2H_5$]glucose, **24b**, was fed, while H-4 was not. This result indicated that a complicated mechanism was involved in the formation of the CN moiety. To complement this experiment, a feeding of [1,1-$^2H_2$]ethanol, **40**, was now performed.

The use of **40** as an *in vivo* NAD^{2}H or NADP^{2}H source to investigate hydride transfers had been reported in the biosynthesis of swainsonine.[15] Since it is known that deuterium at C-1 of **40** can only be utilized through the action of a dehydrogenase (Fig II-7), no direct incorporation of the deuterium into primary precursors could be possible. Any product that is deuterium enriched from such a feeding must have been subjected to a hydride transfer process at some point in its biosynthesis.

Fig II-7. Generation of NAD^2H form [1,1-$^2H_2$]ethanol

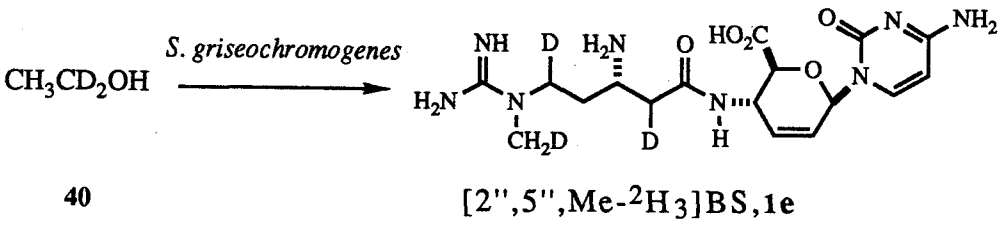


Fig II-8. Feeding [1,1-$^2H_2$]ethanol

The feeding of **40** was carried out in the standard manner. The derived BS, **1e**, was purified and analyzed by ^2H NMR. Three deuterium signals were observed that corresponded to the hydrogens of the δ-N-methyl group, of H-2" and of H-5". No deuterium signals were observed in any other positions of **1e** (Fig II-8). The deuterium enrichment at the positions observed can be explained from the biosynthesis of their precursors (Fig II-9),[16] and in this feeding these enrichments served as internal standards to show that the feeding experiment was performed properly and indeed NAD^2H was generated *in vivo*. The absence of an apparent deuterium enrichment in the nucleoside part of **1e** confirmed that H-2' and H-3' of BS were derived from the original carbinol hydrogens of glucose. It was also noteworthy that H-4' of BS was not labeled by either feeding experiment and therefore it was likely to be derived from a proton source. For such a result a ketonic intermediate must have been involved. Consequently, the introduction of the 4'-NH_2 was likely to have occurred via a classical process of transamination catalyzed by a pyridoxal phosphate (PLP) (or pyridoxamine phosphate, PMP) dependent transaminase.[17] Thus no hydride transfer would be needed for the

formation of the proposed intermediate, **46** (Fig II-10). In contrast, if the transamination were catalyzed by a glutamine-dependent amidotransferase,[18] a hydride transfer would be expected to take place either at C-4' or at C-3' (Fig II-11).

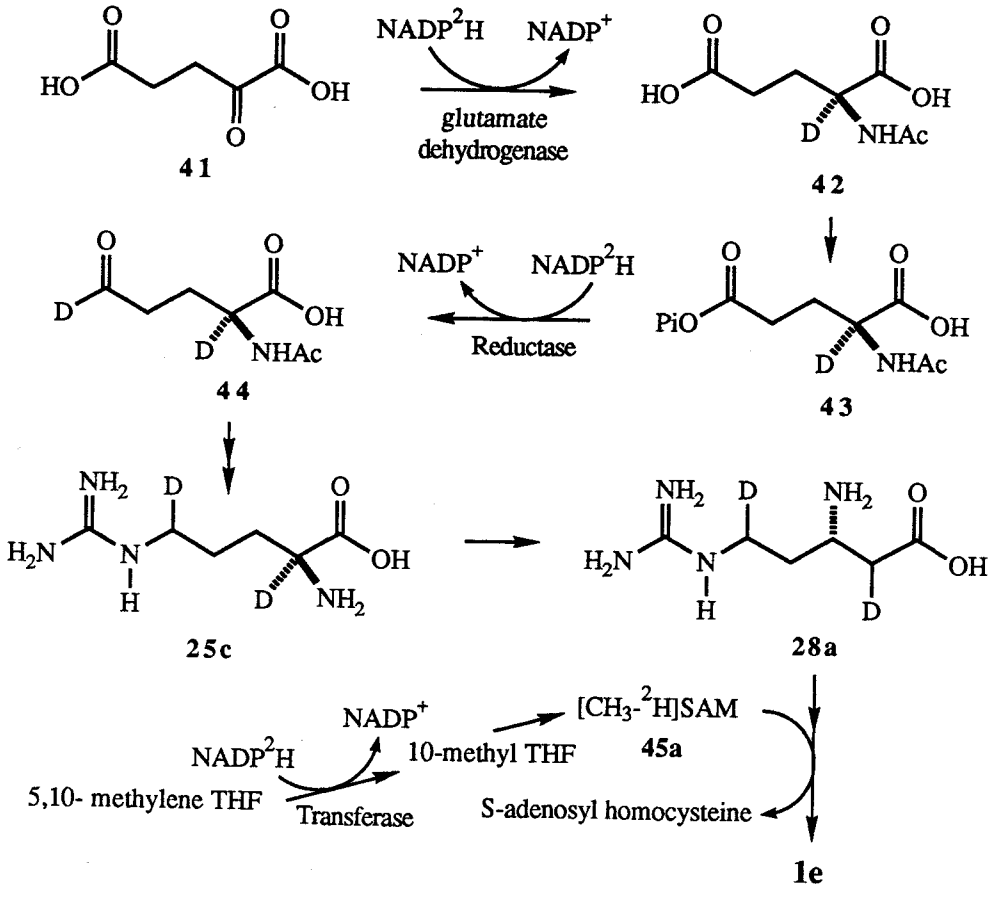


Fig II-9. Involvement of hydride transfer in BA biosynthesis.[16] D-2 and D-5 of Arg must have been introduced in the conversion of **41** to **42**, and in the conversion of **43** to **44**. The deuterium at δ-N methyl should be derived from the biosynthesis of **53a**.

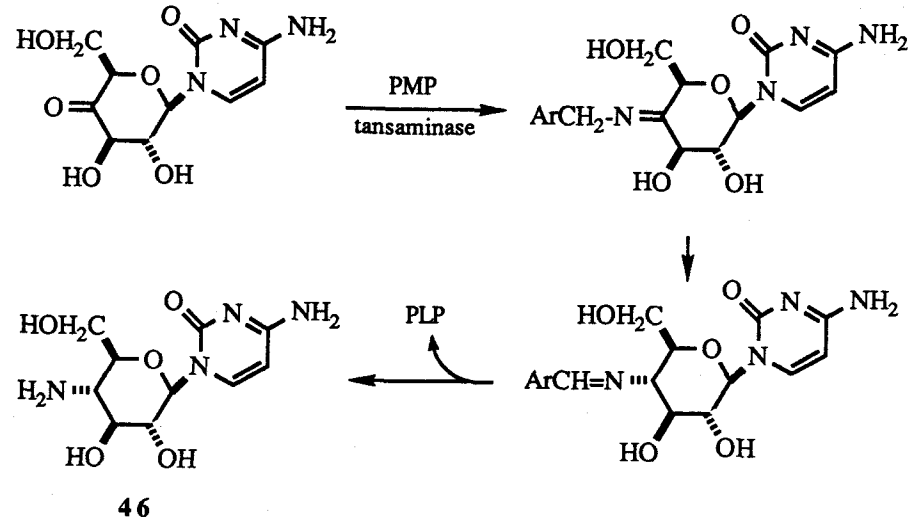


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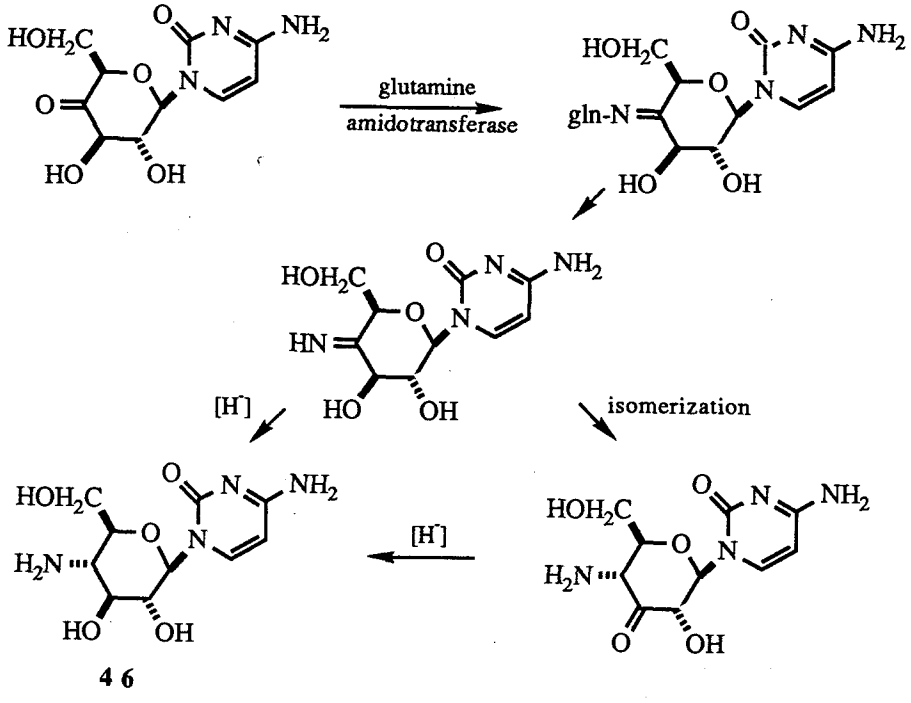
Fig II-10. The proposed PLP-dependent C-4' transamination



46

Fig II-11. The postulated amidotransferase catalyzed C-4' transamination

Summary

So far, the whole-cell feeding experiments had provided circumstantial evidence that the nucleoside portion of BS was derived from one of the intermediates in the glucose to galactose transformation process, and more likely one of the four UDP-glycosides was the immediate precursor. The results of the [1,1-$^2H_2$]ethanol feeding confirmed previous observations that the deoxygenation of C-2' and C-3' resulted in the retention of two original carbinol hydrogens, and suggested that the C-4' amino group was introduced by a conventional transamination. For the assembly of BA, δ-N-methylation seemed to occur after the coupling of β-Arg and CN, and therefore DeMeBS would be the last intermediate in BS biosynthetic pathway. However, due to the apparent permeability problems with the advanced intermediates, achievements to this point were rather limited. It seemed necessary to develop alternative methods to obtain direct evidence for the proposed pathway.

Experimental

General

Organism and Media

S. griseochromogenes ATCC 21024 used in the present studies was that used by Woo and Prabhakaran.[19,20] Soybean flour was purchased from Fred Meyer Stores Inc., Corvallis, and dried brewers yeast was purchased from the First Alternative Corp Store, Corvallis. Bacto agar and yeast extract were purchased from Difco Laboratories (Detroit, MI). Beef extract, polypeptone (trypticase peptone), brain heart infusion agar, brain heart infusion broth and trypticase soy broth were purchased from Baltimore Biological Laboratories (Cockeysville, MD). Wheat embryo was a gift from Kaken Chemical Company (Japan) and malt extract was purchased from Sigma Chemical Co (St. Louis, MO).

Baffled flasks used for fermentation were purchased from Bellco Glass Inc (Vineland, NJ). Sterilization of the culture media was performed in an AMSCO general purpose steam powered sterilizer operating at 121 °C. Fermentations were carried out in a Lab-Line model 3595 gyrotatory incubator shaker at 28-29 °C and 200-225 rpm. Incubations for bioassays and slant preparations were done in VWR model 1520 incubators. All sterile transfers were done in a Edge GARD™ hood manufactured by The Baker Company, Inc. (Sanford, ME). Centrifugations were carried out on an IEC model B-20A centrifuge and cell disruptions were accomplished using a Heat Systems - Ultrasonics Inc, model W-225R cell disrupter (Farmingdale, NY). The paper disks used for bioassay (# 740-E) were purchased from Schleicher and Schuell (Keene, NH).

Chemicals and Product Analysis

Radioactive isotopes used for feeding were purchased from Research Products International Corporation (Mount Prospect, IL) or New England Nuclear (Boston, MA).

Radioactivity measurements were carried out using a Beckman model LS 7800 liquid scintillation counter (LSC) with automatic quench correction through external standardization to yield disintegrations per minute (dpm). A background of 30 dpm (^{14}C) was subtracted from the count rate of each sample. The samples were dissolved in 10 mL each of Beckman Redi-Solve™ HP scintillation cocktail in glass vials. Microgram samples were weighed on a Cahn model 29 Automatic Electrobalance. ^{1}H NMR spectra were recorded either on a Bruker AM 400 (400 MHz) or on a Bruker 300 (300 MHz). All NMR spectra were obtained using 5 mm NMR tubes. ^{1}H samples were prepared in D_2O and the chemical shifts were reported in parts per million relative to an internal standard of t-BuOH (δ ^{1}H, ^{2}H=1.27). ^{2}H NMR Samples of the biosynthetic products were prepared in deuterium depleted water obtained from Aldrich (^{2}H content = natural abundance x 10^{-2}) with 25 μL t-BuOH as an internal reference for chemical shift (δ 1.27) and quantification (0.456 μmol ^{2}H in the methyl groups). The ^{2}H NMR spectra were recorded at 61.4 MHz on the Bruker AM 400 spectrometer. They were proton decoupled and run unlocked.

Ion exchange resins were purchased from either Bio-Rad Laboratories (Richmond, CA) or Sigma Chemical Company (St. Louis, MO) and were converted to the necessary ionic form according to the manufacturer's recommendations.

All fermentation medium and bioassay solutions were prepared with dd H_2O, while HPLC solvents, chromatographic solvents and buffers were made from milli-Q H_2O. Production of BS was quantified by bioassays. The general conditions for analytical HPLC in monitoring the eluates from ion exchange chromatographies were: C_{18} RadialPak^{R} column, 0.8 cm x 10 cm (Waters Assoc. 4 μm packing, eluted with 95% H_2O, 5% CH_3CN, 0.15% TFA, 1.0 mL/min.) with a Waters model 990+ photodiode array detector (displayed at 275 nm).

Bioassay[21]

Preparation of Endospore Suspension of *Bacillus circulans*

The endospores of *Bacilus circulans* were prepared by Peter Yorgey,[21] following procedures provided by Shirley Gerpheride (Upjohn Company, Kalamazoo, MI). All these operations were carried out under sterile conditions.

Preparation of Bioassay Plates

Sterile peptone agar (consisting of 0.5% trypticase peptone (BBL) and 1.5% Bacto agar in dd H_2O and adjusted to pH 9 with 1 M KOH) equilibrated at 50 ^{0}C in a water bath was inoculated with 0.2% v/v of the stock spore suspension of *B. circulans*. To minimize the error during the transfer, 1 mL of the stock spore was initially diluted to 3 mL using sterile saline and 0.6 mL of this suspension was used per 100 mL of peptone agar. Ten milliliter of the resulting agar were dispensed on each Petri plate, allowed to solidify and used for the bioassay. These plates could be stored for a maximum of one week at 4 ^{0}C, after which the bioassay values were found to deviate from the standard.

The Bioassay

Paper bioassay disks or sterile stainless steel wells (up to 4 per plate) were placed evenly on the agar plate prepared as above and 75 μL of the solution to be assayed was placed on each disk or in each well and incubated at 37 ^{0}C for 16 hours. The diameter of the inhibition zone was measured and used to calculate the antibiotic equivalent from the standard curve. Generally the assay was done in duplicate and the standard curve was re-established from time to time. In the case of fermentations using complex medium, the broth was diluted 15 - 20 fold to get the inhibition zone into the proper range.

Fermentation[7,20,21]

Maintenance of *S. griseochromogenes*

Normally, *S. griseochromogenes* was maintained on yeast-malt extract agar slants at 4 °C. However, the production of BS was found to decrease as the slant aged (~18 months). Hence new slants were prepared from time to time, using the *S. griseochromogenes* spores stored on agar plugs at -196 °C (liquid N_2). This was accomplished by transferring the agar plug (using sterile gloves) into 50 mL of YME broth (consisting of 0.4% yeast extract, 1.0% malt extract and 0.4% dextrose in dd H_2O and was adjusted to pH 7.3 with 1 M KOH) and incubating the broth in a shaker at 29 °C, 255 rpm for 48 hours. A portion of the resulting growth (0.1 mL) was used to inoculate each YME agar slant (consisting of 0.4% yeast extract, 1.0% malt extract, 0.4% dextrose and 2% Bacto agar in dd H_2O and adjusted to pH 7.3 with 1 M KOH) which were then incubated at 29 °C. After 7 days, the slants showed dense growth of spores and they were stored at 4 °C. Productivity of the seed could be maintained for up to two years.

Production of BS

Seed medium was composed of 2.0% glucose, 1.0% beef extract, 1.0% polypeptone, and 0.2% NaCl and was adjusted to pH 7.5 with 1 M NaOH. A seed culture was prepared by inoculating a loopful of spores from a seed slant to 50 mL of seed medium in a 250 mL Erlenmyer flask, which was then incubated for 46 - 48 hours at 29 °C and 200 - 225 rpm.

Production broth was prepared by inoculation with 2.0% (v/v) of 46-48 hour seed culture either to complex medium or to synthetic medium[5] as was defined in the text. The complex medium[7,8] was composed of 5.0% sucrose, 1.0% soybean flower, 2.5% wheat embryo, 2.5% dried brewers yeast and 0.6% NaCl and adjusted to pH 7.0 with 1 M NaOH. The synthetic medium was prepared as reported,[5] composed of 10.0%

sucrose and 0.5% glucose in solution A; 1.0% $(NH_4)_2HPO_4$ and 0.3% KCl in solution B; 0.2% $CaCl_2 \cdot 2H_2O$ in solution C; 0.2% $MgSO_4 \cdot 7H_2O$ and 0.004% of each of $FeSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot H_2O$ in solution D. These solutions were prepared and autoclaved separately and then combined right before the seed inoculation. The production broth was incubated under the same conditions as was used for the seed culture. The broth to flask size ratio was 1 : 5 for both complex medium and synthetic medium; however, the complex medium production broth was cultured in baffled flasks but synthetic production medium was cultured in standard Erlenmyer flasks.

Production of BS was determined by bioassay using *B. circulans* as was described in the text. The highest production of BS in the complex medium was at about 120 hours after seed inoculation and in the synthetic medium it was found to be at about 168 hours.

Purification of BS

For a 200-400 mL size fermentation, the production broth was centrifuged at 10,000 *g* for 10 min to remove the solid materials. The pellets were washed with a minimum amount of water, recentrifuged, and the washings were combined with the original supernatants. The total volume of the broth was noted and a sample of ~1 mL was taken for bioassay and radioactivity counting. Similarly, samples were taken after each stage of purification for the same purpose.

The supernatant was carefully adjusted to pH ~2.5 using 6 N HCl and stirred for 20 min. Precipitated colloidal materials were removed by centrifugation at 10,000 x *g* for 10 min, the supernatant was neutralized to pH 5.5 using 1 N NaOH and passed through an anion exchange column (AG 2 x 8, OH^-, 50-100 mesh, 4.0 cm x 30 cm) and the column washed with ~300 mL water. The non-bound fractions and washings containing BS were collected. The flow rate was rather high (6-8 mL/min) and the non-bound fractions and washings were carefully readjusted to pH ~6 using 6 N HCl, *as they were*

collected. These measures were taken to avoid decomposition of BS in the highly basic eluate. The resulting solution was next loaded onto a cation exchange column (Dowex, 50W x 4, H^+, 100-200 mesh, 23 cm x 3.5 cm), at a flow rate of ~4.0 mL/min. The column was washed with ~200 mL water and then with 5% pyridine until highly colored components were eluted. BS was then eluted from the column using about 1500 mL of 1.2% NH_4OH, and fractions of 10 mL were collected. The presence of BS was detected by spotting the solution onto a silica TLC plate, followed by detection with UV light (254 nm) and by ninhydrin spray. On silica gel TLC (*n*-BuOH-MeOH-NH_3-H_2O 5:2:2:1), BS has an R_f of ~0.35. Usually, BS started coming out after ~600 mL of NH_4OH elution. The earliest and latest fractions showed a low concentration of BS and more impurities and hence they were normally omitted. The center fractions were pooled, NH_3 was removed by rotary evaporation at ambient temperature, and the remaining solution lyophilized. Fluffy material was obtained and was taken up in ~10 mL H_2O, adjusted to pH 5.5 using 1 N HCl, and lyophilized again. On recrystallization of the resulting solid from H_2O-MeOH, pure BS was obtained. Radioactive samples were repeatedly recrystallized until constant specific activity was obtained. Samples for radioactivity counting as well as for ^2H NMR spectroscopic analysis were dried under vacuum overnight in an Abderhalden dryer at a temperature of 65 °C (the bp of acetone). BS isolated from synthetic medium fermentation broth was purified in the same way, but the acidic precipitation step was omitted.

Preparation of Compounds for Feeding

Preparation of [1'-^{14}C]CN, 2a

CN was prepared by hydrolysis of BS according to the procedures reported.[5] Briefly, 320 mg of **1d** hydrochloride (3.85 x 10^6 dpm/mmol, prepared from feedings of **24c** and **35a** to *S. griseochromogenes*) was dissolved in 10 mL of 3 N H_2SO_4, and heated to 90 °C for 30 to 40 hours. The extent of the reaction was monitored by TLC

using the same solvent system as was used for BS. CN has an R_f of ~0.66 and BA has an R_f of ~0.40. At the time that most BS had disappeared, the mixture was neutralized with $BaCO_3$ to pH 4.0. $BaSO_4$ precipitate was removed by centrifugation at 3,000 x*g* for 10 min and the supernatant was loaded onto an anion exchange column (Amberlite, IRA-410, 100 mesh, OH^-, 2.5 cm x 30 cm). The effluent was saved for the preparation of BA. The anion column was washed with H_2O (~100 mL) to pH < 9 and then eluted with 0.5 N HCl. Fractions of ~20 mL were collected during the acid elution and were monitored for CN by HPLC (CN has a t_R on HPLC of ~ 3.4 min). Appropriate fractions were lyophilized and the crude product was recrystallized from H_2O - acetone to obtain pure **2a** (69.4 mg, 40% yield, 3.54 x 10^6 dpm/mmol).

Preparation of BA, 3, and of [1-^{14}C]BA, 3a

The effluent from the IRA-410 column was immediately loaded onto a cation exchange column (Amberlite, IRC-50, 50 mesh, H^+, 2.5 cm x 20 cm), which was washed first with H_2O to neutrality and then with 0.5 N HCl until the eluates showed negative for BA (BA was detected by spotting the eluates on a silica gel TLC plate and then spraying with a ninhydrin solution).[20] The HCl eluate was concentrated by rotary evaporation at ambient temperature, and the remaining solution was lyophilized. Recrystallization of the lyophilized residue from EtOH gave BA dihydrochloride (110 mg, ~40% yield).

Hydrolysis of BS using 6 N HCl instead of 3 N H_2SO_4 could be accomplished in 4-6 hours at room temperature and gave a better yield. The radioactivity yield for **3a** from **1c** (prepared by Prabhakaran[8]) was 85% (5.97 x 10^6 dpm/mmol) using the modified conditions. However, using HCl to replace H_2SO_4 was only good for preparation of BA. Significant decomposition of CN in 6 N HCl was observed.

Feeding Protocol

Generally, the compounds to be fed were weighed out, dissolved in 5-10 mL of dd H_2O and the solutions were added sterilely by filtering through a Gelman membrane filter (Product No. 4192, pore size 0.2 μm) to the production medium at the specified time. For the feeding of high radioactivity compounds from commercial sources, the appropriate amount of compound was transferred to a 10 mL volumetric flask and diluted to the exact mark with dd H_2O. Duplicate samples of 50 μL each were taken, each was diluted an additional 100-fold, and then 100 μL of the final dilutions was used for LSC. The remainder of the original 10 mL solution was used for the feeding.

Calculations in Feeding Experiments

Various calculations were performed to determine the % incorporation, % enrichment and specific activity based on the amount of precursor fed, production of metabolites and LSC data. The formulae used for these calculations are given below.

$$\text{Specific activity isolated} = \frac{\text{dpm in BS isolated}}{\text{mmol of BS isolated}}$$

$$\%\ \text{Incorporation} = \frac{\text{specific activity isolated x mmol of BS produced}}{\text{dpm in precursor fed}} \times 100$$

$$\%\ ^2\text{H enrichment} = \frac{\mu\text{mol of } ^2\text{H observed by NMR integration}}{\mu\text{mol of BS used for } ^2\text{H NMR}} \times 100$$

Feeding [1-^{14}C]BA, 3a

The feeding of **3a** was carried out in the standard manner and the results are summarized in Table II-1.

Table II-1. Feeding and LSC data for **3a** feeding

Fermentation size	200 mL
Fermentation medium	Synthetic
Total BS produced (at 144th hour)	122 mg
3a fed at 48th hour	5.6×10^5 dpm
3a fed at 72nd hour	5.8×10^5 dpm
Total dpm fed	1.1×10^6 dpm
Total BS purified	29.4 mg
Recrystallization	dpm/mg
1st recryst .	29
2nd recryst.	27
3rd recryst.	19
4th recryst.	13*

* No statistical significance.

Feeding [1'-^{14}C]CN, 2a

The results of **2a** feeding are summarized in Table II-2.

Table II-2. Feeding **2a**

Broth size	200 mL
Fermentation medium	Synthetic
2a fed (at 42nd hour)	9.3×10^5 dpm
1 produced (at 122nd hour)	95 mg
Crude **1** obtained after anion and cation exchange chromatography	29 mg*

* No radioactivity was detected in the crude product of **1**.

Isotopic Trapping with CN

From a 100 mL, 53-hour-old fermentation broth, the mycelia were pelleted by centrifugation at 10,000 x *g* for 10 min. The mycelial pellet was resuspended in ~ 20 mL of water and 69.2 mg of unlabeled CN was added. The mycelial suspension was sonicated in an ice-bath for 10 min (maximum power, 50% duty cycle) and the

homogenate was centrifuged at 10,000 x *g* for 10 minutes to remove solid material. The supernatant was loaded onto a cation exchange column (Dowex AG 50W x 4, 100 mesh, H^+, 2.5 cm x 20 cm) at a flow rate of ~ 3 mL/min. The column was washed with H_2O to neutral then followed with 5% pyridine. CN eluate was detected by HPLC (see HPLC) and CN-containing fractions from the 5% pyridine elution were loaded onto an anion exchange column (Amberlite IRA-410, 100 mesh, OH^-, 2.5 cm x 20 cm), washed with H_2O to neutral and then with a solution of 0.5 M acetic acid. A mixture of CN and cytosine was eluted in fractions #14 - #18 (20 mL/fraction). The mixture was loaded onto another cation exchange column (S-sepharose, H^+, 3.0 cm x 20 cm) and eluted in a linear gradient of 0 - 0.8 M of KCl in sodium phosphate buffer (30 mM, pH 2.08, 500 mL total), at a flow rate of 2.5 mL/min. Each fraction (20 mL) was analyzed by HPLC. Cytosine was eluted in fractions #6 - #7 and CN was eluted in fractions #9 - #10. Fractions containing CN were diluted with an equal volume of H_2O, and then loaded on to the same S-sepharose column (NH_4^+ form) for desalting. After washing with H_2O, CN was finally eluted off the column with 1.0 M NH_4OH solution. The desalted CN was lyophilized, recrystallized from H_2O - acetone and dried. Part of the CN obtained was used for LSC. Table II-3 summarizes the fermentation, feeding and LSC data of the isotopic trapping experiment.

Table II-3. Isotopic trapping with CN

Broth size	100 mL
Fermentation medium	Synthetic
[2-^{14}C]cytosine, **23a**, fed	5.5 μCi (at 46th hour)
	5.5 μCi (at 48.5th hour)
Broth harvested	At 53th hour
Unlabeled CN added	69.2 mg
Total **2b**, purified	41.3 mg
Specific activity in **2b**	73 dpm/mg
Total dpm trapped in **2b**	5050 dpm*
dpm recovered in **23a** fraction	7.39 x 10^6 dpm (3.36 μCi, 30.55% of fed)

* Represented less than 0.02% of the total radioactivity fed.

Feeding [1'-^{2}H]CG, 27a

The results of the **27a** feeding are summarized in Table II-4.

Table II-4. Feeding **27a**

Broth size	80.0 mL
Fermentation medium	Complex
27a fed (at 44th hour)	100 mg
Total **1** produced (at 120 hour)	160 mg
After acid precip.	144 mg
After anion column	108 mg
After cation column	94 mg
After recryst.	68 mg
Amount for ^{1}H NMR	2 mg
Amount for ^{2}H NMR*	29 mg

* No deuterium signal was observed in the ^{2}H NMR spectrum

Feeding [1,1-$^2H_2$]Ethanol, 40

The results of the **40** feeding are summarized in Table II-5.

Table II-5. Feeding [1,1-$^2H_2$]ethanol, **40**

Fermentation size	200 mL
Fermentation Medium	Complex
Total **40** fed	3.6 mL (1.0 mL at 38th and 48th hour, 0.8 mL at 60th and 72nd hour)
Total **1e** produced (at 120 hour)	340 mg
Pure **1e** isolated	144 mg
Amount for ^2H NMR*	64 mg
(acquired both at 24 and 67 °C)	(0.14 mmol)

* ^2H signals were observed at 3.52 ppm, 3.08 ppm and 2.75 ppm, corresponding to H-5", δ-N-CH_3 and H-2" of **1e**. The deuterium enrichments were 0.26% (0.363 μmol), 1.30% (1.814 μmol) and 0.16% (0.223 μmol), respectively.

Feeding [1-^{14}C]Glucose, 24c, and [1-^{14}C]Galactose, 35a

The results of the **24c** and **35a** feedings are summarized in Table II-6.

Table II-6. Fermentation and LSC data for **24c** and **35a** feedings

Flask	#2	#3
Fermentation size	300 mL	300 mL
Fermentation medium	Complex	Complex
Compound fed	**24c**	**35a**
Total dpm fed	4.25×10^7	4.00×10^7
1e produced (120 hour)	390 mg	390 mg
1e isolated	175 mg	156 mg
Specific activity in **1e**	1.99×10^3 dpm/mg	1.56×10^4 dpm/mg
% Incorporation	1.8% (from **24C**)	15.2% (from **35a**)

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Chapter III

Feeding Metabolic Inhibitors and Purifying New Metabolites

Introduction

Although several interesting results were obtained from the whole-cell feedings with putative intermediates, none of the results were conclusive. Theoretically, biochemical transformations at C-1, C-2, C-3, C-4 and C-6 of glucose in BS biosynthesis could be executed in a variety of sequences and many hypothetical intermediates could be proposed. It was clear that in order to identify the true biosynthetic intermediates, alternative approaches to the conventional feeding experiments were needed to overcome potential impermeability problems and to avoid the synthesis of a large number of putative intermediates. A novel approach was adopted by feeding specific enzyme inhibitors and large quantities of primary precursors. Feeding metabolic inhibitors to block the *de novo* biosynthesis of some essential biosynthetic precursors while at the same time supplementing large quantities of others was expected to distort the biosynthetic precursor pools and force the accumulation of biosynthetic intermediates or new metabolites in the fermentation broth.

Inhibition of some step in a biosynthetic pathway by growing an organism in the presence of an enzyme inhibitor has been used in the literature to provide evidence for the involvement of a particular enzymatic activity *in vivo*,[1] or to show which kind of biochemical system has to be functioning for the biosynthesis of the natural product.[2] Numerous successful examples of feeding enzyme inhibitor to study biosynthetic pathways or biosynthetic mechanisms have recently been published.[3] In the present study, this approach was used primarily as an alternative to the method of generating blocked mutants that facilitate the identification of biosynthetic intermediates. Even though this approach suffers from the inability to control the effects an inhibitor may

have at other points in the cellular metabolism, selective inhibition to the secondary metabolism with little stress on the primary counterpart is possible because in most cases the timings of bacterial growth and antibiotic production are well differentiated.[4] In comparison with the method of developing blocked mutants, this approach offers greater flexibilities in respect to the selection of inhibition points. Moreover, inhibitors could be applied in combination with the manipulation of nutritional conditions to complement or amplify inhibitory effects.

The rationale for inhibitors selected in the following studies came from earlier feeding results and three attending assumptions:

a) the 4'-amino was introduced after the formation of the cytosine glycosidic bond and was formed by a mechanism of transamination;

b) the nucleoside part was coupled to the β-amino acid part at a relatively late stage and the formation of the nucleoside part was independent of the biosynthesis of the β-amino acid part;

c) δ-N-methylation occurred at very late stage.

Based on these assumptions, there would be three potential biosynthetic points (Fig III-1) that, if blocked, might result in the accumulation of useful metabolites. These were:

a) blocking at the point of transamination;

b) blocking at the availability of L-α-Arg;

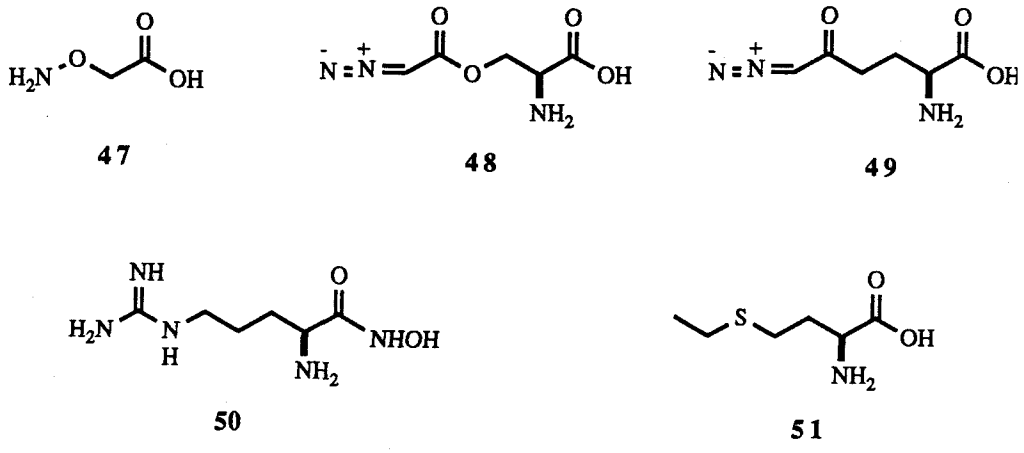
c) blocking at the point of δ-N-methylation.

Accordingly, the effects of three types of enzyme inhibitors were examined. They were:

a) transaminase inhibitors: aminooxyacetic acid (AOAA), **47**,[5] azaserine, **48**, [6] and 6-diazo-5-oxo-L-norleucine (DON), **49**.[7]

b) an L-α-Arg biosynthetic inhibitor: L-arginine hydroxamate (ArgH), **50**.[8]

c) a methyltransferase inhibitor: L-ethionine (L-Eth), **51**.[9]



47 48 49

50 51

In addition, several primary BS biosynthetic precursors (cytosine, arginine and glutamine) were also included.

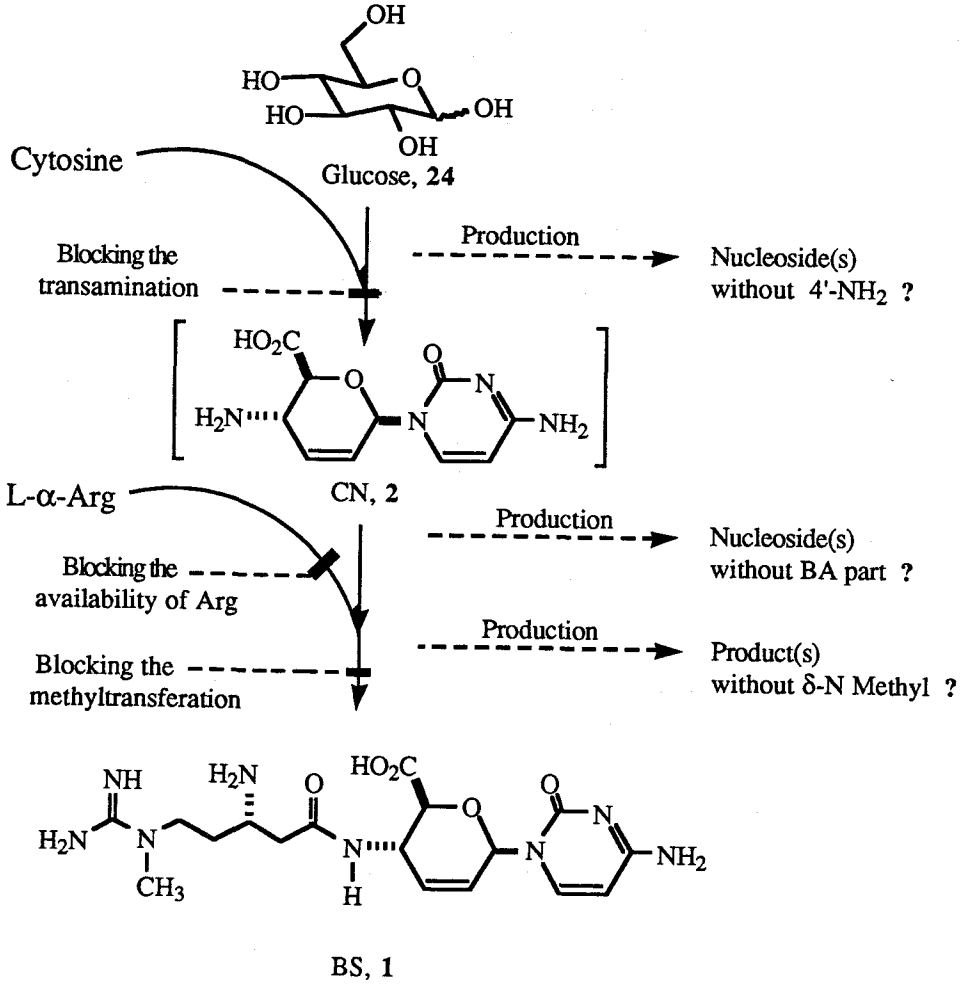
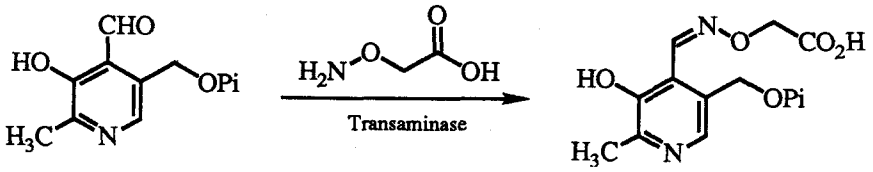


Fig III-1. Potential blocking sites and expected metabolites

These inhibitors selected have been extensively used in biochemical studies. AOAA has been demonstrated to inhibit a broad spectrum of transaminases, presumably by reacting with pyridoxal phosphate (PLP), **52**, and forming a PLP-AOAA adduct, **53**, in transaminase active sites (Fig III-2).[5,10] The hydroxylamine functionality in AOAA is a strong nucleophile and the reaction equilibrium is driven to the right. However, the adduct, **53**, can not be further processed by transaminases. Feeding AOAA to the culture would thereby inhibit normal PLP-dependent transaminations reactions.[5,10]



52 **53**

Fig III-2. Reaction of AOAA with PLP[5,10]

DON and azaserine, on the other hand, are two irreversible inhibitors of glutamine dependent nitrogen transfer reactions, including amidotransferases which are enzymes normally responsible for amino sugar biosynthesis.[6,7] In the reaction of azaserine, and possibly also of DON, with amidotransferases an essential thiol group of a cysteine residue in the catalytic site is acylated to form an inactive thioether derivative.[11] The feedings of azaserine and DON were to test whether amidotransferase system was involved for the introducing of C-4' amino group in the biosynthesis of BS.

To limit the availability of Arg for BS biosynthesis, ArgH was selected. It is known that ArgH functions like Arg as a feedback inhibitor to the first two biosynthetic enzymes in Arg biosynthesis (Fig III-3).[12] ArgH has also been shown to be more effective than other Arg analogues.[12] If *S. griseochromogenes* were grown in a chemically defined medium in which no amino acid was externally added, inhibition of *de novo* Arg biosynthesis would hopefully halt the final assembly of BS and lead to the accumulation of metabolites that bear no BA part.

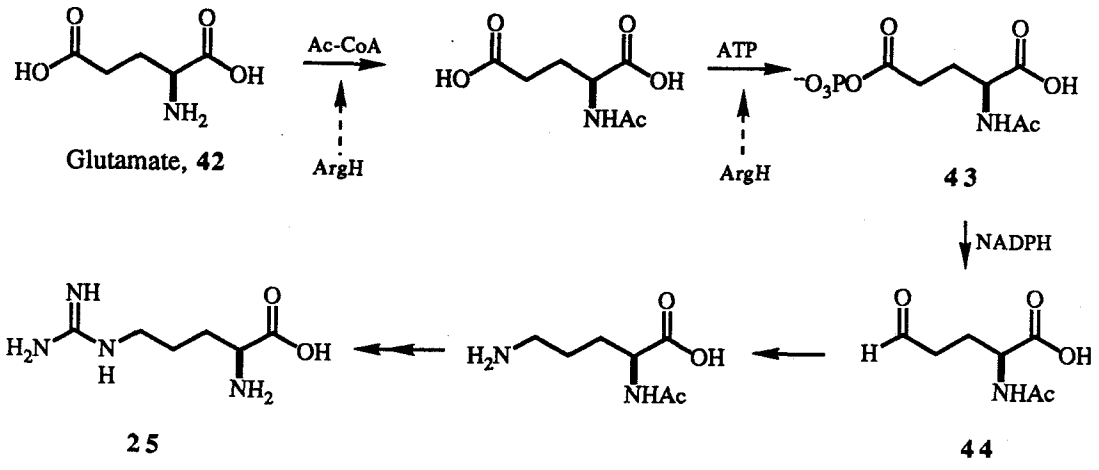
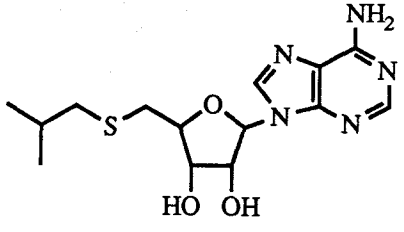


Fig III-3. Sites of ArgH, **50**, action[12a]

With the same notion, Eth was also fed. Although the exact mechanism of *in vivo* Eth action is not completely clear and its effect seems to be relatively non-specific, there is evidence that some of its biological effects are manifested through the inhibition of SAM, **45**, dependent methyltransferases.[9] Since it has been proven that the δ-N-methyl of BS is derived from methionine,[13] feeding Eth should stimulate the production of unmethylated metabolites and thus provide information on the timing of δ-N-methylation. There are other adenosine derivatives of methyltransferase inhibitors that have been developed, such as, sinefungin, **32**,[3h,14] and 5'-deoxy-5'-S-isobutyl thioadenosine, **54**,[15] which confer the advantage that they specifically inhibit SAM dependent methyltransferases. However, since the antibiotics **32** and **54** are nucleoside derivatives, their biological activities might be hampered by the limited permeability of *S. griseochromogenes* to nucleoside analogues. For this reason Eth was selected in this study, though the effect of Eth is generally mild.[9]



54

In summary, any accumulation of a metabolic intermediate from these inhibitor feeding experiments could be of manifold significance, such as:

a) in facilitating the identification of biosynthetic intermediates;

b) in clarifying the involvement of a particular enzymatic system in the biosynthetic pathway;

c) in serving as a means to prepare biosynthetic intermediates for further studies.

Result and Discussion

Feeding Biosynthetic Inhibitors

In order to maximize both the effect and selectivity of the inhibitors, it was necessary first to determine the relative timing of bacterial growth and BS production so as to feed inhibitors at the onset of BS production. A synthetic medium (see Chapter II) was adopted since it would give a defined nutritional condition, which would not only be expected to enhance the inhibitory effects but also be expected to simplify the interpretation of the inhibitory results. It was found that the bacteria entered log phase right after seed inoculation and reached stationary phase generally at the 84th to 96th hour. BS began to be detected during the 50-60th hour of incubation and the accumulation lasted for about five days. Therefore, inhibitor feedings were typically commenced at the 52nd hour after seed inoculation. Production of BS and its related metabolites was assayed at the 170th hour. The amounts of inhibitors used were comparable to those used in the literature. Some of them were adjusted according to the information obtained during the feeding experiments, although the amount fed was not completely optimized.

Accumulation of CGA, PPNC and DeMeBS

In several preliminary feedings of AOAA and ArgH, we noticed that the production of two components was moderately stimulated, up to 200 - 300% higher than that in the control broth, while the production of BS was inhibited by 50 - 70%. One of the increased components had a retention time on HPLC identical to that of authentic PPNC. The other was more polar and its UV spectrum clearly showed it to be a cytosine derivative. It was also found that the later component could be bound to both anion exchange and cation exchange resins. Therefore, it seemed to be a cytosine glycoside that contained a carboxyl group. This amphoteric property fit perfectly with the properties of

Fig III-4. HPLC analysis of inhibitor feedings.

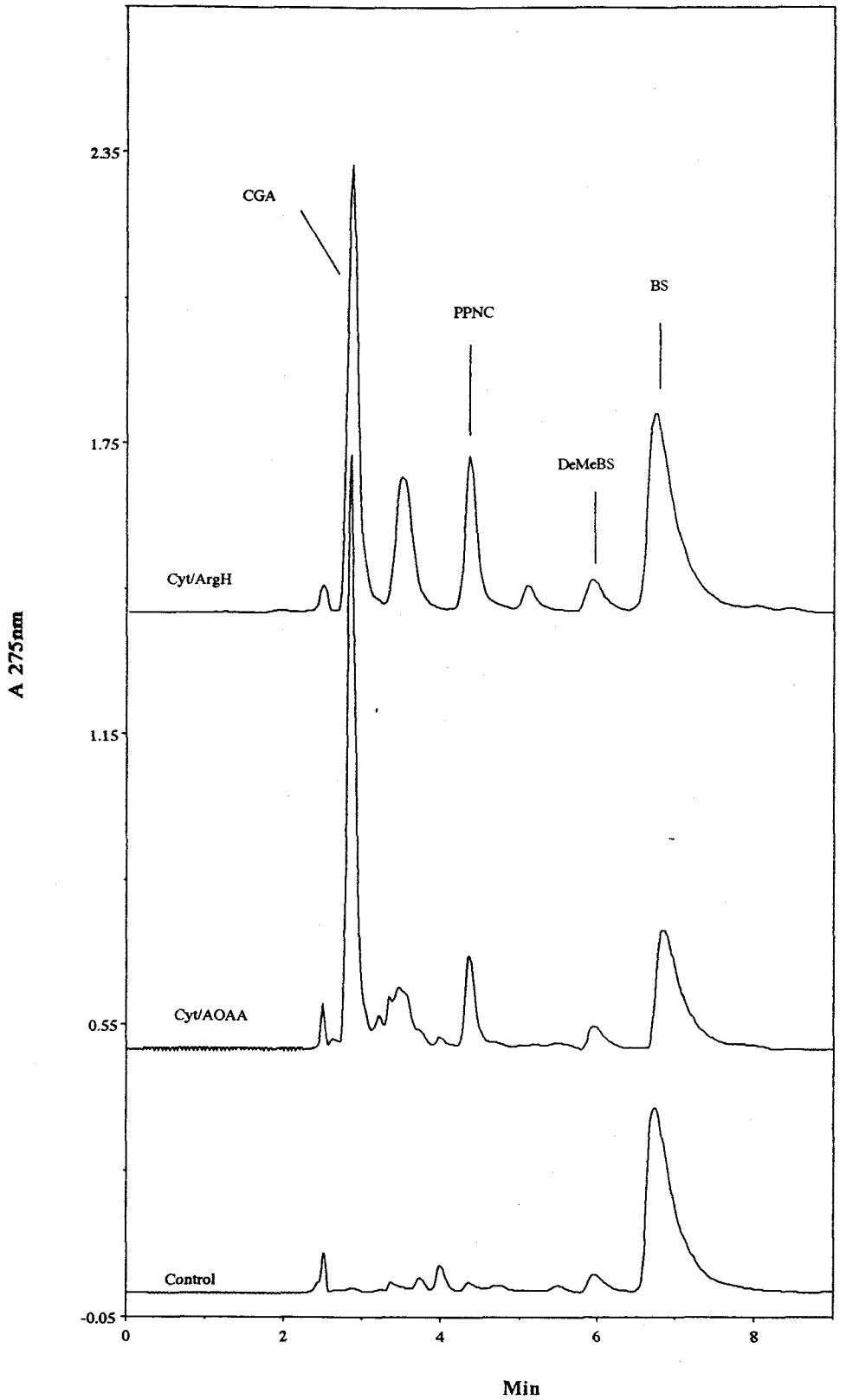
The fermentation was done with the synthetic medium, 100-mL broth size. Feedings of cytosine and AOAA or ArgH were commenced at 52nd hour of incubation and the production was analyzed at 170th hour by injecting 20 μL of the broth supernatants to HPLC. HPLC conditions are described in the Experimental section. Flow rate was 1.1 mL/min.

Control: no additives;

Cyt/AOAA: cytosine, 30 mg and AOAA, 50 mg were fed;

Cyt/ArgH: cytosine, 30 mg and ArgH, 200 mg were fed.

Fig III-4. HPLC analysis of inhibitor feedings



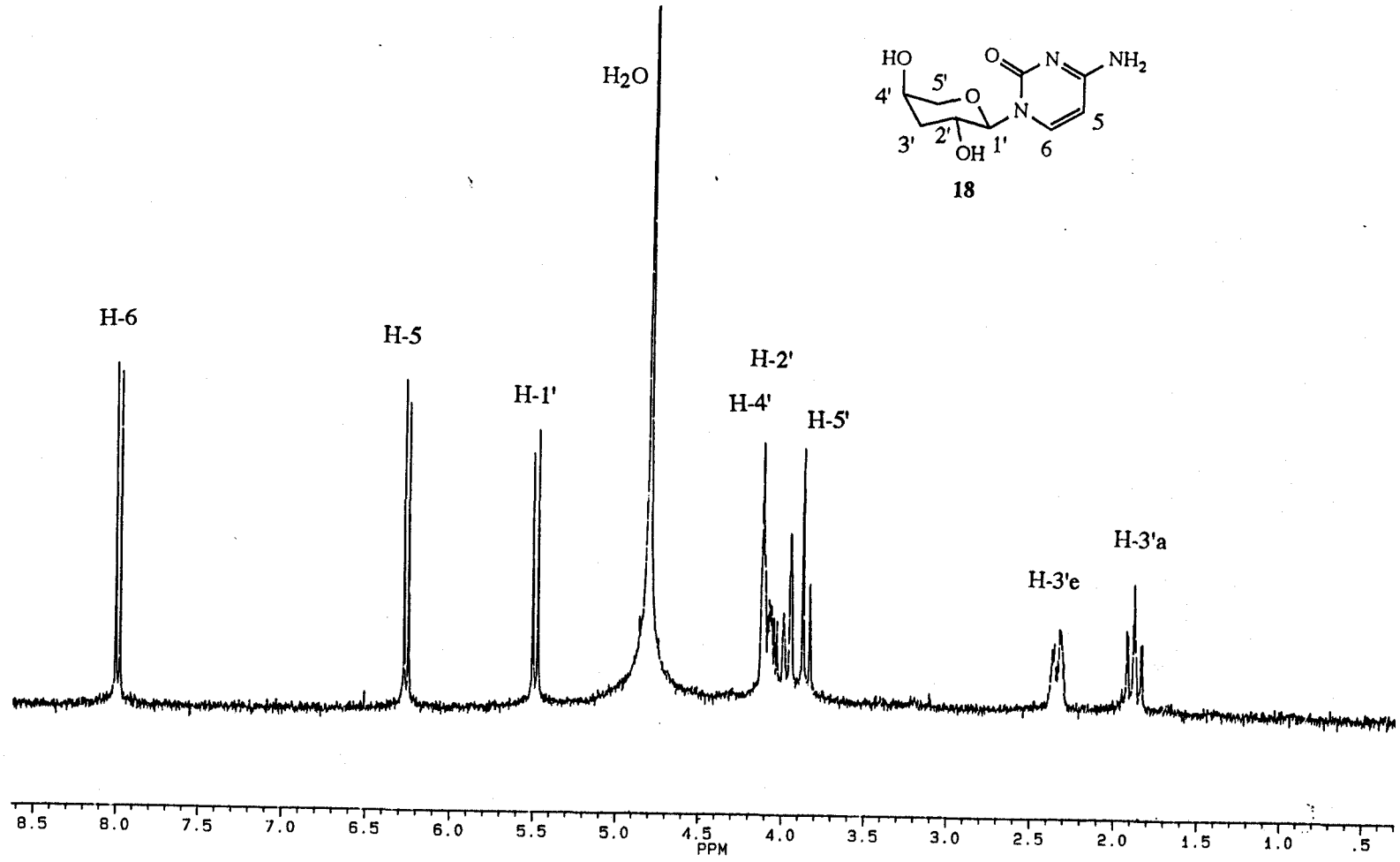


Fig III-5. ^1H NMR spectrum of PPNC, **18**

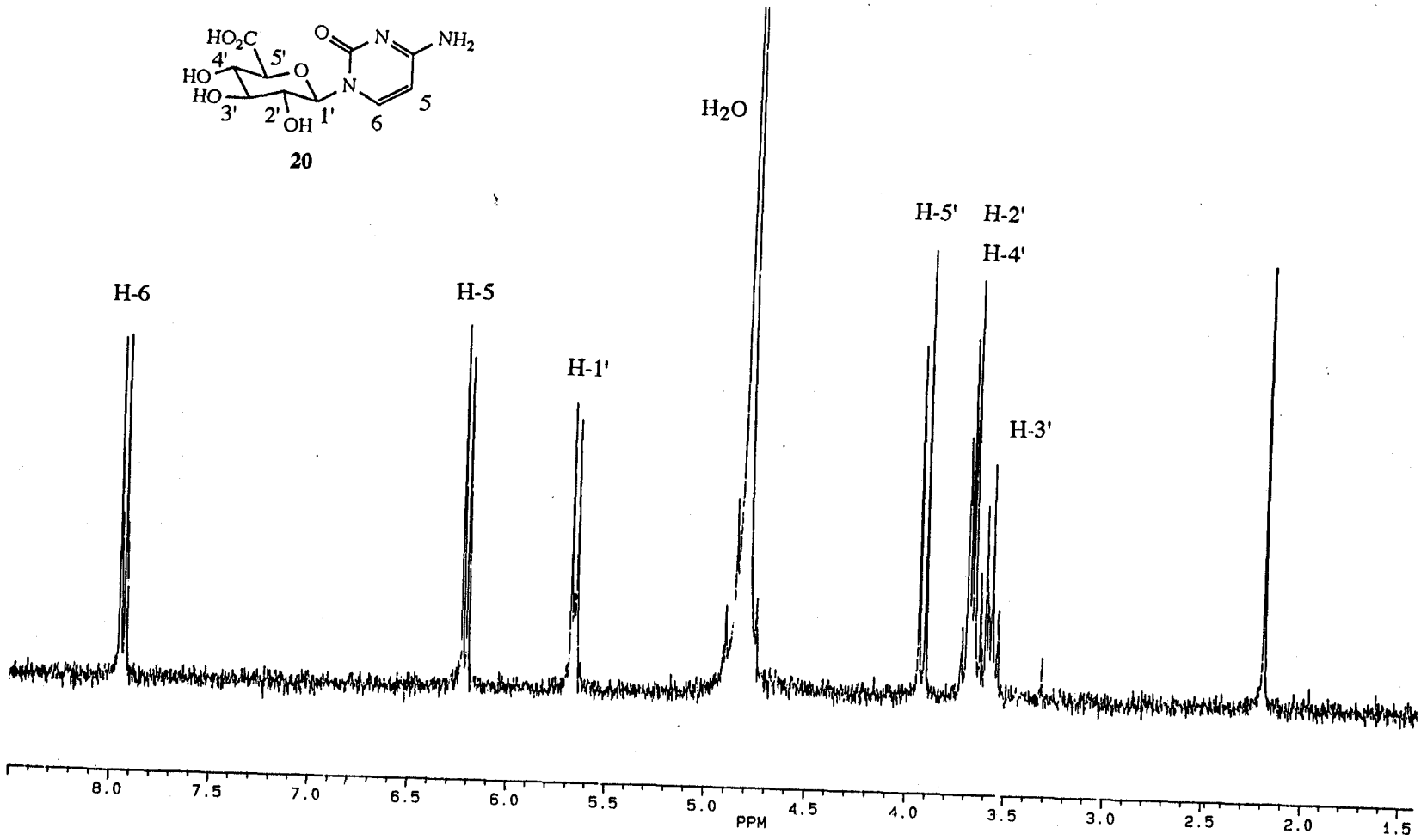


Fig III-6. ^{1}H NMR spectrum of CGA, **20**

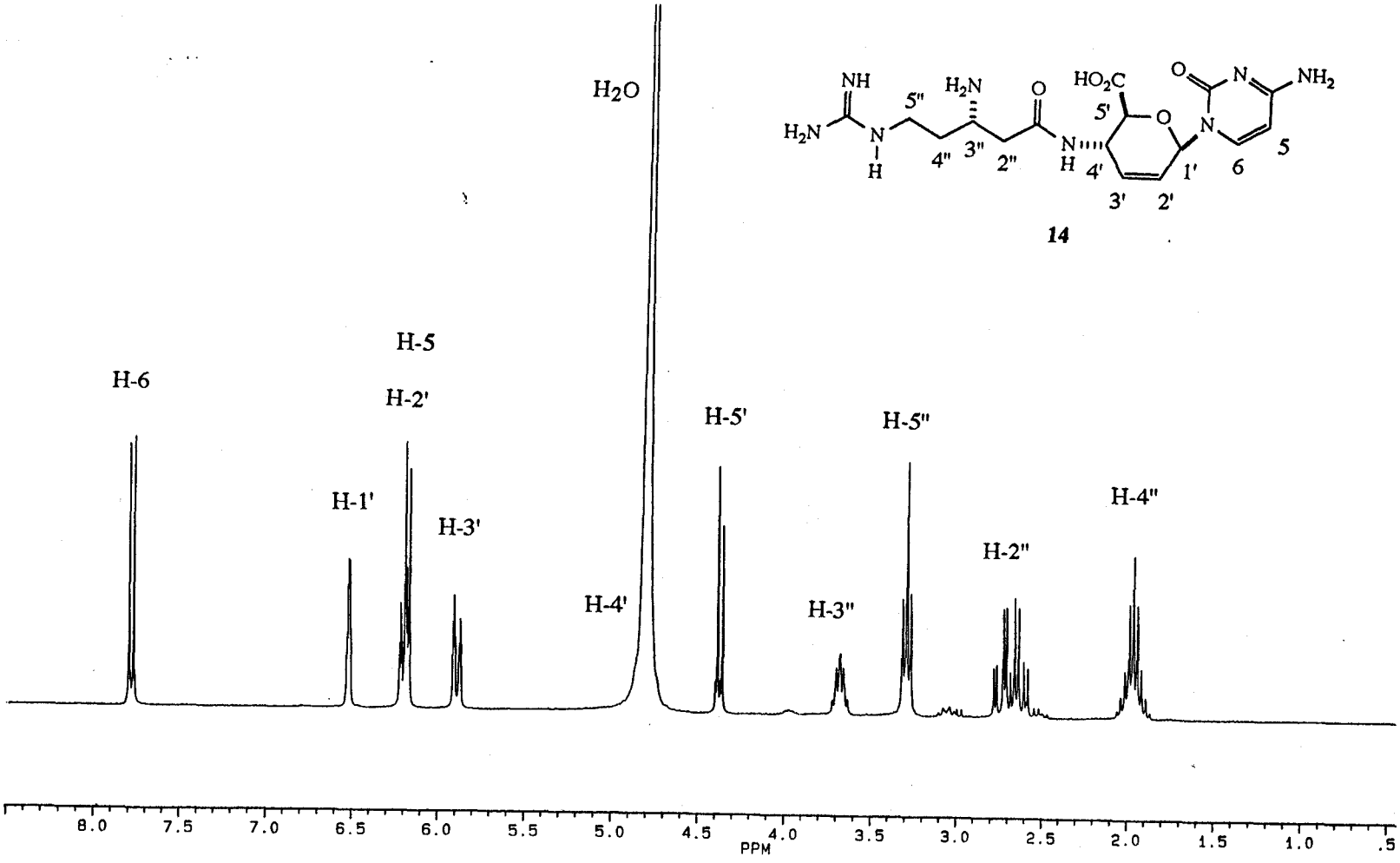


Fig III-7. ^{1}H NMR spectrum of DeMeBS, **14**

intermediates we proposed earlier. Further studies found that when AOAA or ArgH was co-fed with cytosine, the production of those two components was dramatically increased, as is shown in Figure III-4. In these feedings, the PPNC-like component was increased to about 10-fold, and the other over 100-fold. These two components were then purified and identified by NMR spectroscopy (Fig III-5, III-6). One of them was indeed PPNC, **18**,[16] and the other found to be CGA, **20** (previously isolated by Seto and named pentopyranic acid).[17] In those feedings, a third component, which was similar to BS in its ability to bind to cation exchange resins, was also slightly stimulated to about 2-fold. Purified by HPLC, this component was identified by ^{1}H NMR spectroscopy (Fig III-7) to be DeMeBS, **14**.[18]

These preliminary studies demonstrated that feeding metabolic inhibitors could be a potentially powerful approach to the identification of biosynthetic intermediates.

Normalization of Inhibitor and Primary Precursor Feedings

With the technical assistant of Anja Geitmann, effects of the inhibitors AOAA, ArgH and Eth and the primary precursors, cytosine and Arg, were subsequently systematically examined. Their effects on CGA, PPNC, DeMeBS and BS production are presented in Table III-1 and Table III-2. These data were formulated from several independent sets of feeding experiments and were normalized to the production in each control broth. Although the data varied somewhat among different experiments, several trends of effects were apparent and are summarized as follows:

a) Feeding primary precursors, cytosine, Arg and Met, as well as potential amino-donors glutamate and glutamine had a stimulatory effect on the production of all four compounds assayed (Table I-1, entry 2-6). Cytosine had a stronger effect than the other precursors, especially on the production of CGA, which had been increased by 71-fold (Table III-1, entry 2). Cofeeding the precursors of cytosine and Arg had additive effect

Table III-1. Effect of inhibitors and primary precursors on CGA, PPNC, DeMeBS and BS production[a]

NO. of Sample	Compounds Fed	Amount Fed (mg)	CGA (units)	PPNC (units)	DeMeBS (units)	BS (units)
1	None		1.00	1.00	1.00	1.00
2	Cytosine	30mg	70.58	6.38	3.47	1.62
3	Arg	200mg	2.18	1.45	1.17	1.32
4	Met	100mg	0.83	1.27	1.15	1.11
5	Glu	180mg	1.09	0.98	1.22	1.22
6	Gln	200mg	2.25	1.67	2.29	1.78
7	AOAA	50mg	2.94	3.17	0.77	0.62
8	AOAA	40mg	3.75	2.07	0.67	0.74
9	AOAA	140mg	23.25	2.40	0.49	0.18
10	DON	5mg	1.50	1.07	1.00	0.90
11	DON	17mg	1.50	1.40	0.99	0.93
12	Azaserine	10mg	1.25	1.07	1.08	0.89
13	Azaserine	31mg	0.50	1.27	0.94	0.86
14	MeGlu	50mg	3.21	2.00	1.06	0.79
15	MeGlu	50mg	2.45	2.76	1.03	0.67
16	MeGlu	50mg	16.75	1.67	1.07	0.61

Table III-1 (continued)

No. of Sample	Compounds Fed	Amount Fed (mg)	CGA (units)	PPNC (units)	DeMeBS (units)	BS (units)
17	ArgH	200mg	2.21	6.86	0.89	0.85
18	ArgH	200mg	1.08	10.07	0.06	0.46
19	Eth	50mg	6.79	1.12	1.31[b]	0.58
20	Eth	50mg	3.08	2.07	1.01[c]	0.95
21	Cyt/Arg	30/200mg	68.15	5.33	5.69	1.96
22	Cyt/Arg	30/200mg	33.75	4.80	7.74	2.17
23	Cyt/AOAA	30/50mg	61:12	6.60	2.23	1.14
24	Cyt/ArgH	30/200mg	48.85	16.93	1.70	1.07
25	Cyt/MeGlu	30/50mg	30.58	11.73	2.05	0.86
26	ArgH/MeGlu	200/50mg	5.94	12.21	0.68	0.63
27	ArgH/MeGlu	200/50mg	2.21	12.07	0.88	0.31
28	ArgH/Cyt/Glu	200/180/30mg	71.79	13.12	1.51	2.46

a Feedings were commenced at 50th hour of incubation and production was assayed at 170th hour. All productions were normalized to a control broth which was arbitrarily assigned as 1.00 unit. Production of CGA, PPNC, DeMeBS and BS in the control broth ranged from 1.0 - 9.5 mg.L, 3.5 - 9.5 mg/L, 30 - 95 mg/L and 300 - 756 mg/L, respectively.

b Production of DeMeBS was detected as early as 74th hour of incubation, while it was not detectable in the control broth until 146th hour.

c DeMeBS production was 246% as high as that in the control broth if assayed at 146th hour of incubation.

Table III-2. Effect of inhibitors and primary precursors on CGA, PPNC, DeMeBS and BS production[a]

No. of Sample	Compounds Fed	Amount Fed (mg)	CGA (units)	PPNC (units)	DeMeBS (units)	BS (units)
1	None		1.00	1.00	1.00	1.00
2	AOAA	35mg	3.61	1.99		0.69
3	AOAA/Cyt/Arg/ Met/Leu	35/50/50/20/20 mg	44.66	8.88		0.37
4	AOAA/Cyt/ β-Arg/Met/Leu	35/50/50/20/20 mg	45.65	8.14		0.55
4	ArgH	120mg	1.74	8.97		0.85
6	ArgH	120mg	2.08	8.13		0.86
7	ArgH/BA	120/13mg	1.25	4.11		0.57
8[b]	ArgH/Arg/Pro	120/22/22mg	14.63	8.69		0.626
9	ArgH/Arg	200/100mg	2.00	10.20	0.77	0.50
10[c]	ArgA	120mg	0.74	0.83		1.50

a All compounds were fed at 52th hour of incubation except specified and production was assyed at 168th hour. All productions were normalized to a control broth which were arbitrarily assigned as 1.00 unit. The absolute amounts of CGA, PPNC, DeMeBS and BS were not quantitated.

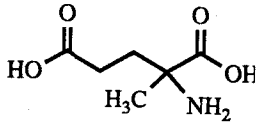
b Compounds were fed at 0th hour of incubation.

c The same broth, but #10 was assayed at 168th hour of incubation and #11 at 116th hour.

and was shown to be a good condition for the production of DeMeBS (Table I-1, entry 21, 22).

b) The transaminase inhibitor, AOAA, substantially inhibited the production of BS and DeMeBS while stimulating the production of CGA and PPNC (Table III-1, entry 7, 8, 9). In contrast, DON and azaserine showed a much milder effect. These results suggested that a transamination reaction was involved in the transformation of CGA to DeMeBS or to BS, presumably by introduction of the C-4' amino group, and these results were consistent with the results from [1,1-$^2H_2$]ethanol feeding experiment. This earlier experiment had suggested that a PLP-dependent transaminase rather than a glutamine-dependent amidotransferase was involved in the BS biosynthetic pathway. The effect of AOAA also appeared to be quite specific. As shown in Table III-2 (entry 2, 3, 4), the inhibitory effect of AOAA on the production of BS could not be relieved by Met, Leu, Arg or β-Arg. Apparently, inhibition of BS production by AOAA was not due to its non-specific effect on the precursor pools of amino acid metabolisms.

α-Methyl glutamate (MeGlu, **55**),[19] a glutamate-dependent transaminase inhibitor, had a similar effect as that of AOAA (Table III-1, entry 14, 15 and 16). This result could indicate that BS biosynthesis was tightly connected to general transamination processes. However, the inhibitory effect of α-methyl glutamate could also be due to the inhibition of the Arg biosynthesis because α-methyl glutamate has been shown to inhibit the biosynthesis of glutamate,[19] which is a primary precursor for the biosynthesis of Arg (Fig III-3).[12] Therefore, the result of α-methyl glutamate feedings was not able to be interpreted clearly.



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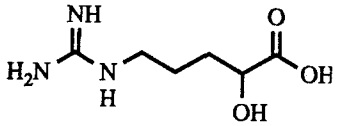

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c) The effect of ArgH was very much similar to that of AOAA (Table III-1, entry 17, 18) in inhibiting the production of metabolites in the late part of biosynthetic pathway (DeMeBS and BS) and stimulating the production of metabolites in the early part (CGA and PPNC). ArgH was shown to be particularly efficient in the stimulation of PPNC production. The concomitant decrease in BS and DeMeBS production and increase in CGA and PPNC production in feedings with either AOAA or ArgH confirmed that these four metabolites were metabolically related. Thus, CGA and DeMeBS should be intermediates to BS, while PPNC would obviously be an aberrant product from the main pathway. It was also interesting to note that the inhibitory effect of ArgH was not negated by Arg or by β-Arg (Table III-2, entry 7, 8, 9). This might be because the need for Arg was quite high and the amount of Arg fed was not enough to relieve the ArgH effect. There were also possibilities that ArgH not only functioned as an inhibitor in Arg biosynthesis but also acted as an β-Arg analogue that inhibited the formation of β-Arg or the coupling of β-Arg with CN.

Feeding argininic acid (ArgA), **56**,[12] another Arg analogue, did not have an inhibitory effect. In contrast, ArgA seemed to stimulate BS production, possibly by being converted to Arg, thus serving as an pre-precursor.

d) As was expected, Eth inhibited BS production only. The production of all three other products were slightly stimulated. A closer examination showed that Eth had only a transitory effect. The production of DeMeBS was highest at the 96th hour of incubation and decreased afterwards (Fig III-8). It seemed that either the bacteria became resistant to Eth inhibition or Eth was degraded after longer periods of incubation. Nevertheless, the Eth effect was consistent with the previous proposal that δ-N-methylation occur at very late stage.

e) The combination of primary precursors (Cyt, Arg) and inhibitors had an additive effect. Co-feeding primary precursors (Cyt, Arg) with inhibitors (ArgH, AOAA, MeGlu) counteracted the effect of the inhibitors on DeMeBS and BS production but

potentiated their effect on CGA and PPNC production (Table III-1, entry 23,24,25,28). These additive effects very much reflected our predictions and strongly supported our hypotheses.

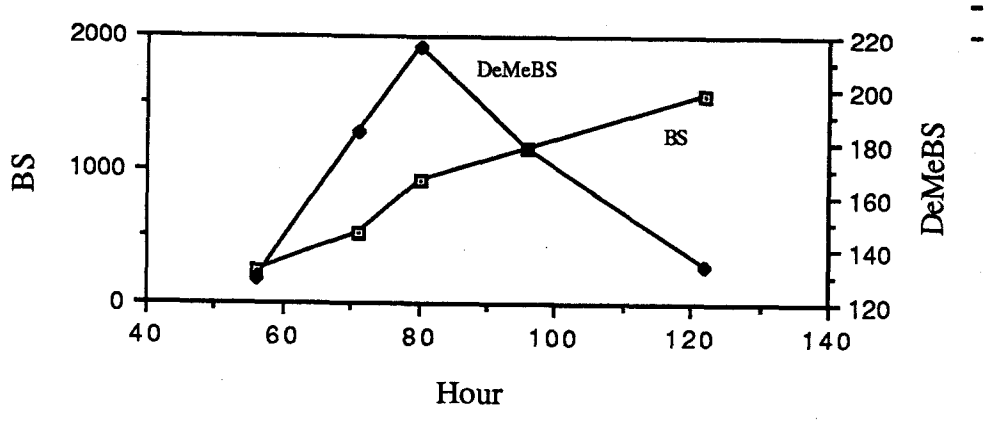


Fig III-8. Concentration of DeMeBS in Eth-fed broth from 56th to 120th hour of incubation.

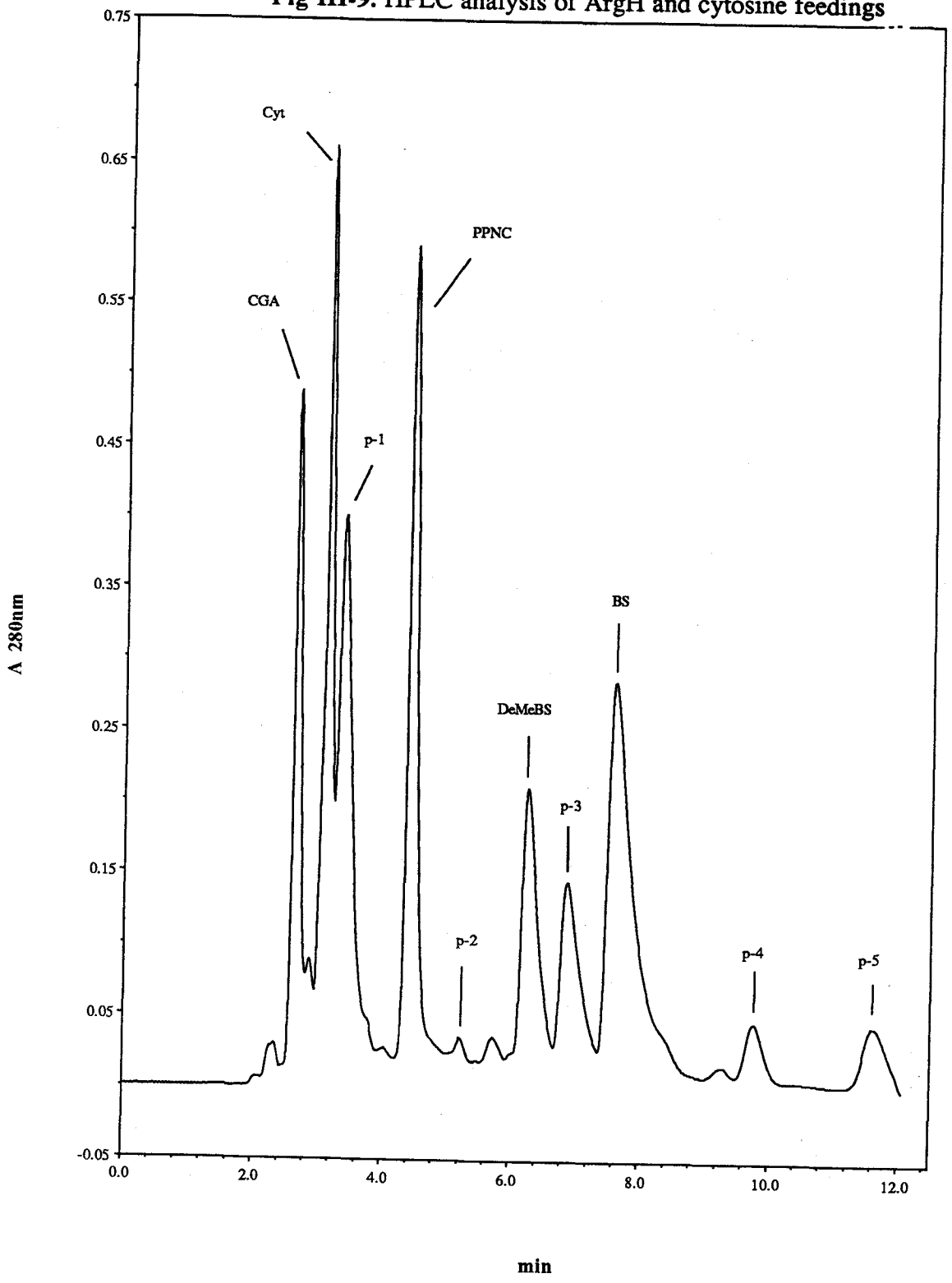
The examination of metabolic inhibitor and primary precursor effects were further extended to fermentation in the complex medium. Although higher dosages of inhibitors were needed to bring about a comparable extent of inhibition, the same patterns of inhibition-stimulation effects were observed on the production of CGA, PPNC, DeMeBS and BS. Figure III-9 shows the production analysis of one of such feeding in which the production of CGA, PPNC and DeMeBS were estimated to be 100, 170 and 120 mg/L respectively. As a result, CGA, PPNC and DeMeBS, which were originally produced only in trace amounts, could now be purified from a relatively small fermentation (typically 1 L).

In addition to the four known compounds obtained by feeding 1.0 g/L of cytosine and 2.0 g/L ArgH to a complex medium fermentation, HPLC analysis of the crude fermentation broth revealed several new components (Fig III-9). Photodiode array detection showed that at least five of those new components had UV spectra exhibiting maxima at 215 and 275 nm, which are characteristic of cytosine glycosides. In Figure III-

Fig III-9. HPLC analysis of ArgH and cytosine feeding.

The fermentation was done with 200-mL of the complex medium in a 1-L baffled flask. Cytosine (1.0 g/L) and ArgH (2.0 g/L) were fed at the 52nd hour of incubation. The production was analyzed at 160th hour by injecting 10 μL of the broth supernatant onto HPLC. Conditions for HPLC are described in the Experimental section (flow rate, 1.0 mL/min).

Fig III-9. HPLC analysis of ArgH and cytosine feedings



9, these new components are labeled as p-1, p-2, p-3, p-4, p-5, in order of their elution. Based on the assumption that all of them had approximately the same molar extinction coefficient as that of PPNC, the production of these would be about 200 mg/L, 10 mg/L, 60 mg/L, 35 mg/L and 40 mg/L, respectively. Such levels of production were considered to be practical for a laboratory-scale purification.

Purification of New Metabolites

The purification procedures are depicted in Figure III-10. The basis for developing such a purification protocol took into account the fact that these new metabolites accumulated only when ArgH and Cyt were fed. Consequently, they seemed either to be cytosine glycosides that lacked a BA part or to be BS analogues with a modified BA residue. Accordingly, these two groups of metabolites were separated simply by eluting the ion-exchange column first with 5% aqueous pyridine and then with a solution of 1.2% ammonium hydroxide. The former group of metabolites would be eluted by the pyridine solution and the later by the ammonium hydroxide solution. Metabolites in each group were then subjected to a more sophisticated fractionation through a preparative HPLC. By using this purification scheme, nine compounds, including CGA, PPNC, DeMeBS and BS could be purified in a relatively short period of time. Typically, 1 L of fermentation broth would give adequate amounts of each of those metabolites for characterization, except p-2 which was purified from a 2.5 L fermentation. Although apparently large quantities of p-1 were produced, it proved exceedingly difficult to separate from the large amount of residual cytosine, and the recovery of pure compound was modest. Recovery of p-4 was also hampered, in this case apparently due to its facile hydrolysis. The ^{1}H NMR and ^{13}C NMR spectroscopic data for the five new metabolites are listed in Table III-3 and III-4, with the data for PPNC and BS included for comparison.

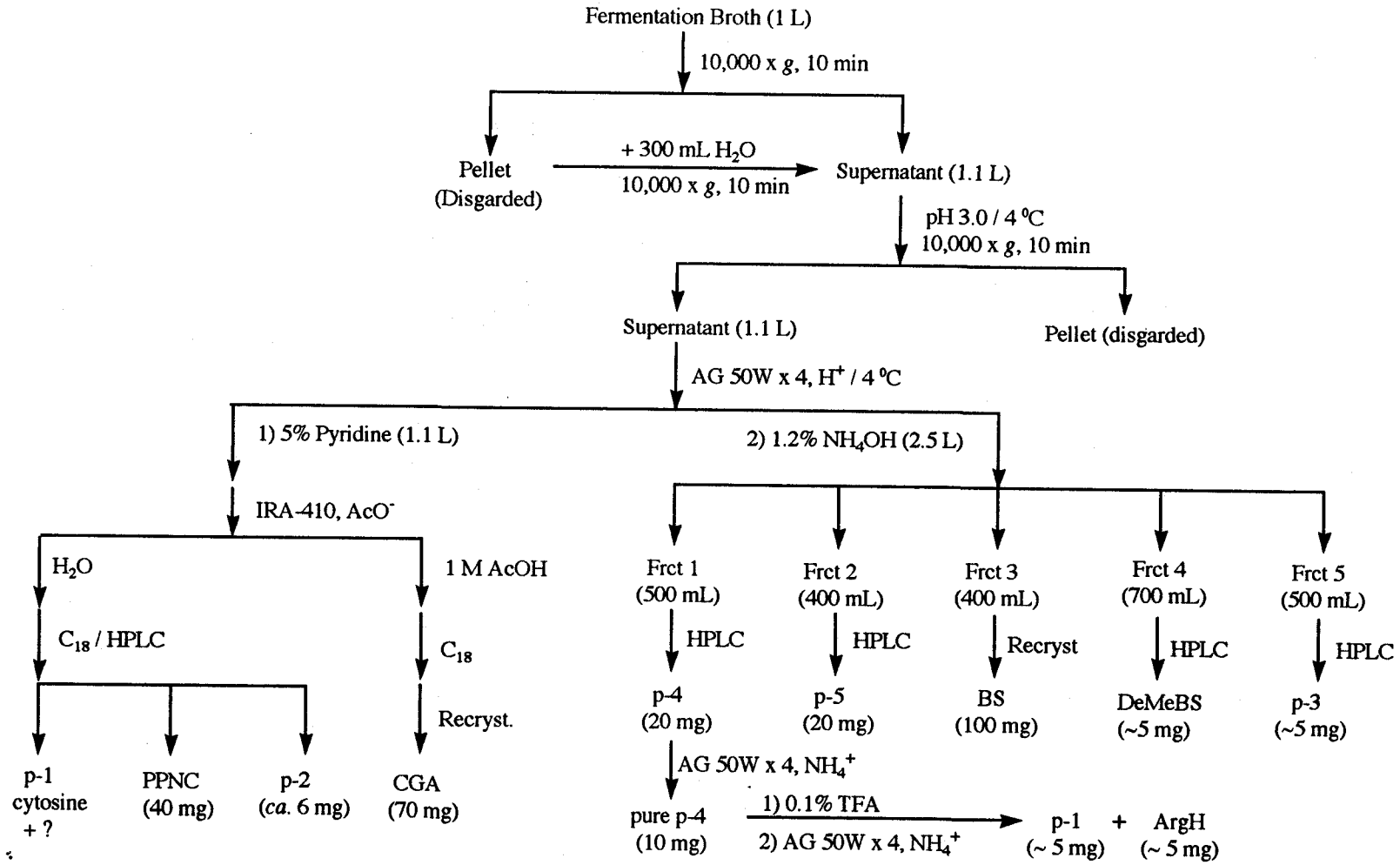


Fig III-10. Purification scheme for BS and related metabolites

Table III-3. ^{1}H NMR data for new metabolites

	PPNC	p-1	p-2	p-4	p-5	BS	p-3
5	6.33 (1H, d, J=7.8)	6.11 (1H, d, J=7.6)	6.04 (1H, d, J=7.5)	6.14 (1H, d, J=7.7)	6.31 (1H, d, J=7.9)	6.02 (1H, d, J=7.5)	6.24-6.28 (2H, m)
6	8.05 (1H, d, J= 8.0)	7.72 (1H, d, J=7.6)	7.64 (1H, d, J=7.5)	7.68 (1H, d, J=7.7)	8.00 (1H, d, J=7.7)	7.59 (1H, d, J=7.5)	7.84 (1H, d, J=7.9)
1'	5.53 (1H, d, J=9.2)	5.60 (1H, d, J=9.4)	5.69 (1H, d, J=9.2)	5.62 (1H, d, J=9.3	5.60 (1H, d, J=9.0)	6.46 (1H, d, J=0.9)	6.58 (1H, br. S)
2'	4.13 (1H, m)	4.06 (1H, m)	3.98 (1H, m)	4.57 (1H, m)	4.16 (2H, m)	6.09 (1H, ddd, J=10.2, 1.8, 0.9)	in 5
3'e	2.39 (1H, m)	2.48 (1H, m)	3.71 (1H, dd, J=14.7, 5.3)	2.54 (1H, ddd, J=13.3, 4.6, 2.6)	2.38 (2H, br. s)	5.85 (1H, dd, J=10.2, 2.4)	5.96 (1H, d, J=10.3)
3'a	1.93 (1H, m)	1.98 (1H, dd, J=12.9, 11.4)	2.26 (1H, dd, J=14.7, 11.0)	2.14 (1H, dd, J=13.0, 11.6)	in 3'e		
4'	4.17 (1H br. s)					4.73 (1H, ddd, J=9.3, 2.4, 1.8)	under D2O
5'e	3.92 (1H, d, J=12.7)	3.81 (1H, dd, J=11.9, 2.8)	4.36 (1H, 2H, br. s)	4.22 (1H, dd, J=12.5, 2.6)	in 2'	4.10 (1H, d, J=9.3)	4.33 (1H, d, J=8.2)
5'a	3.88 (1H, d, J=12.7)	3.72 (1H, d, J=12.0)	in 5'e	3.88 (1H, d, J=12.5)	3.88 (2H, m)		
2"a				3.70 (1H, dd, J=8.3, 4.1)	in 5'a	2.74 (1H, dd, J=16.2, 4.8)	2.69 (1H, dd, J=15.3, 5.0)
2"b						2.62 (1H, dd, J=16.2, 8.1)	2.58 (1H, dd, J=15.4, 8.5)
3"a				1.88 (1H, m)	1.90 (1H, m)	3.64 (1H, m)	4.03 (1H, m)
3"b				1.62 (1H, m)			
4"				1.72 (2H, m)	1.67-1.77 (3H, m)	2.03 (2H, m)	2.06 (2H, m)
5"				3.24 (2H, t, J=6.7)	3.24 (2H, t, J=5.5)	3.46 (2H, t, J=7.8)	3.15 (2H, t, J=8.1)
δ-N-CH_3						3.02 (3H, s)	2.75 (3H, s)

Table III-4. ^{13}C NMR data for new metabolites

C	PPNC	p-1	p-2	p-4	p-5	BS	p-3
2	158.8	160.2	167.8	168.4	160.5	158.5	161.0
4	148.4	149.7	159.4	160.5	150.0	159.6	150.1
5	95.4	96.6	99.1	99.4	97.4	98.7	97.0
6	144.0	145.0	144.0	144.2	145.8	144.8	147.0
1'	85.3	86.2	87.9	87.4	86.8	81.8	80.7
2'	64.9	66.3	68.2	66.9	65.9	135.0	135.1
3'	36.7	43.1	32.1	44.3	38.2	128.2	125.9
4'	71.7	91.1	155.5	78.2	77.4	47.6	46.2
5'	63.5	73.9	70.1	76.3	73.2	79.8	77.1
6'						177.1	174.0
1"				166.8	172.3	172.9	173.5
2"				58.1	56.2	39.1	41.9
3"				31.2	29.8	48.6	48.8
4"				26.4	25.8	31.2	31.9
5"				43.1	42.0	48.6	47.1
7"				159.1	158.3	168.3	156.2
δ-N-CH_3						37.8	34.4

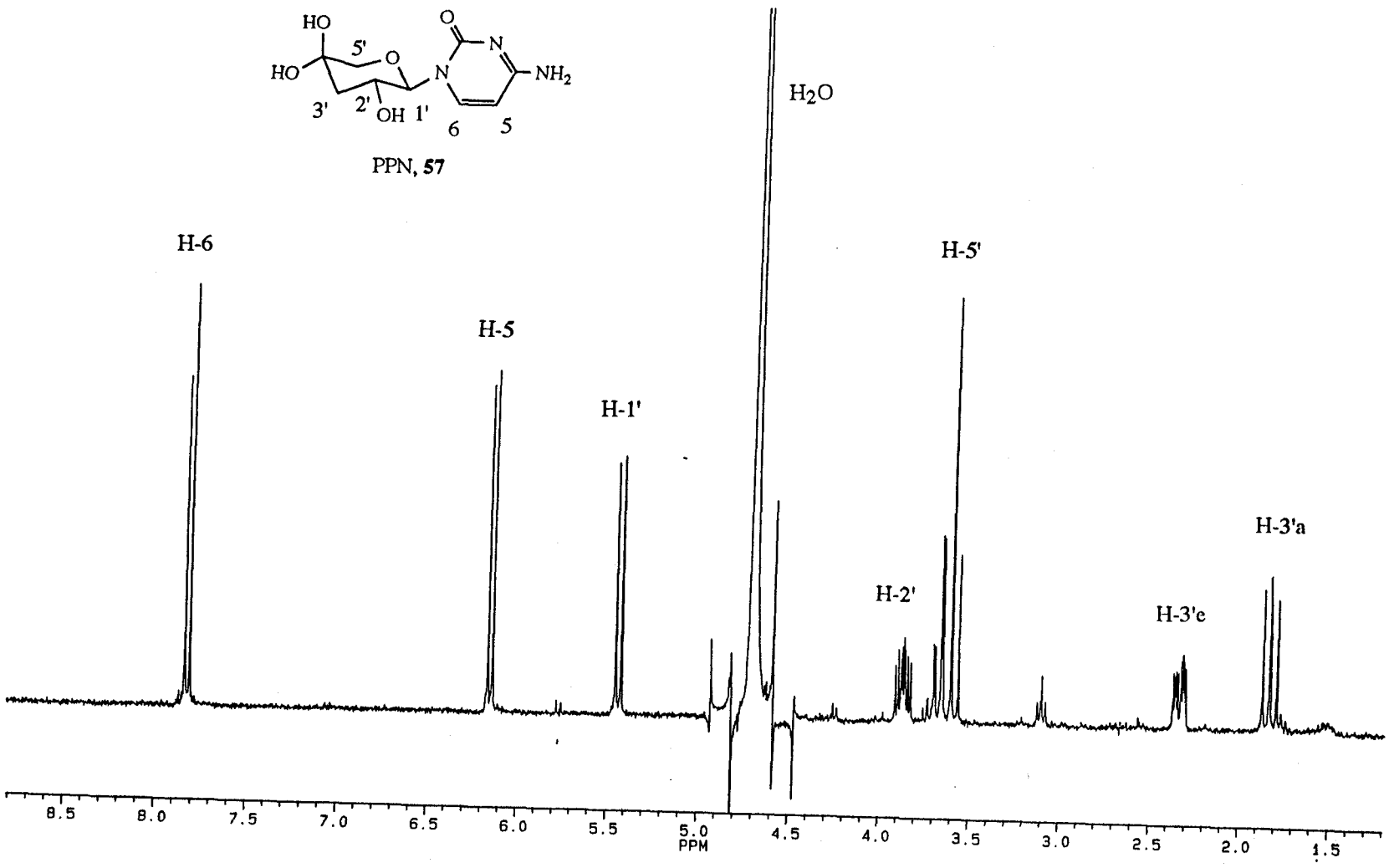


Fig III-11. ^{1}H NMR spectrum of p-1, **57**

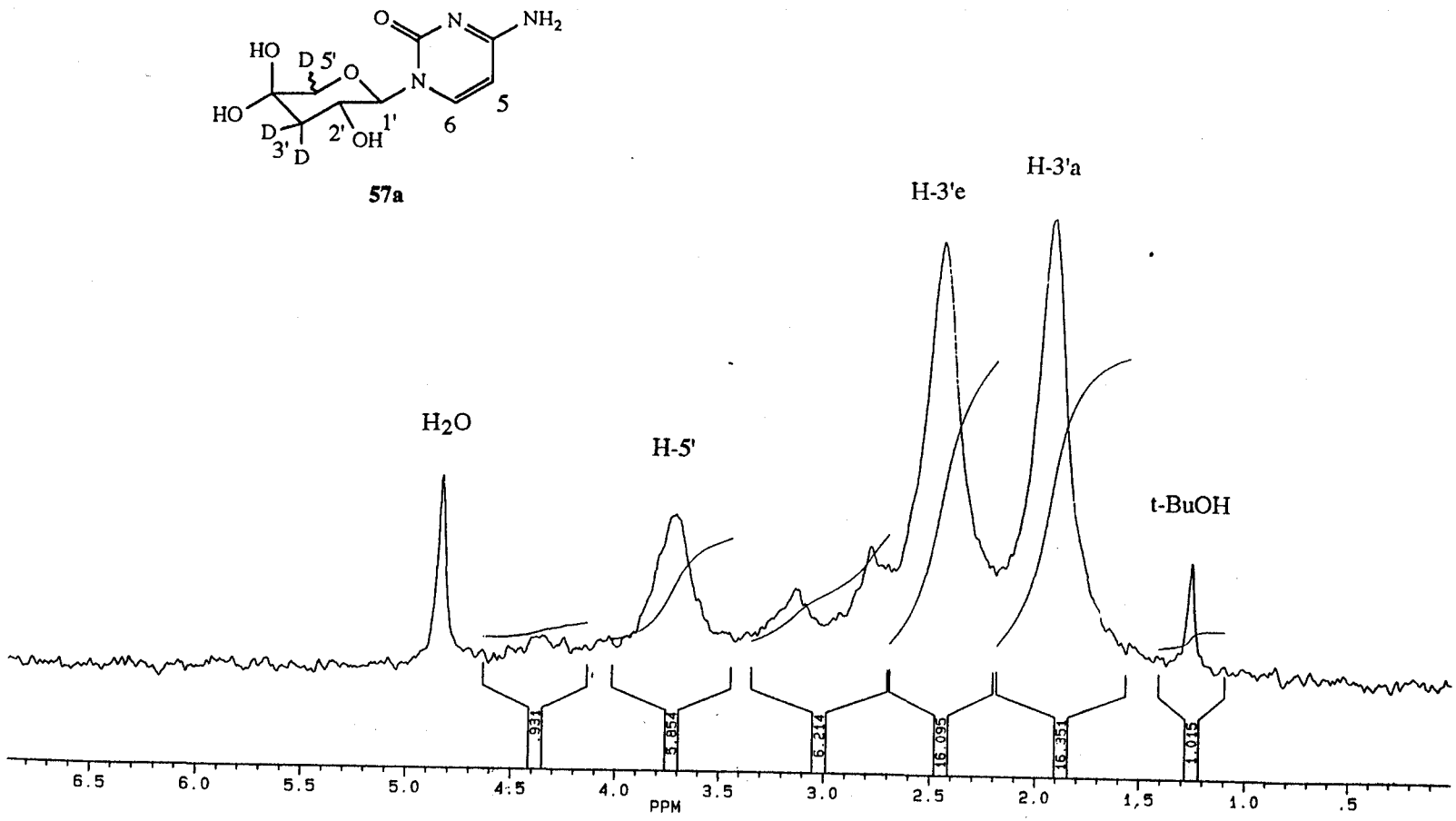
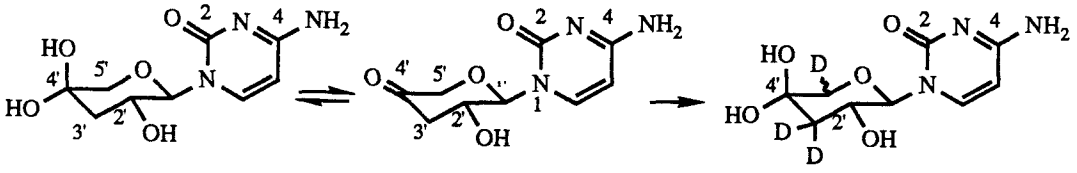


Fig III-12. ^{2}H NMR spectrum of **57a**

The Structure of p-1 (57)

High resolution mass spectrometry showed that p-1 had a molecular formula of $C_9H_{13}N_3O_5$, which has one more oxygen than the formula of PPNC. Examination of the ^1H NMR, ^1H COSY and HETCOR spectra showed that p-1 possessed an identical pattern to that of PPNC, except for the absence of an H-4' resonance at approximately 4.1 ppm (Fig III-11). The ^{13}C NMR spectrum was also similar to that of PPNC, but in addition included a signal at 91.1 ppm. These data suggested the presence of a keto-pyranoside, with the ketone existing in the hydrated form, **57**.[20] In base the ketonic form **58** probably exists because the C-3' hydrogens of **57** were completely exchanged with D_2O at pD 9.3 in 45 min. Under the same condition, the C-5' hydrogens were exchanged 18%. The ^2H NMR spectrum of **57a** is shown in Figure III-12. The chemical process leading to **57a** from **57** is proposed in Figure III-13.



57 **58** **57a**

Fig III-13. Deuterium exchange of PPN, **57**

Recently, with the technical assistance of Martin Klumpp, we have observed that a cell-free extract of *S. griseochromogenes* could convert p-1 to PPNC in the presence of NADH. Therefore, the structure and biogenetic role of **57** (named as pentopyranone, PPN) seems firmly established.

The Structure of p-2 (59)

With this metabolite, the high resolution mass spectrum provided a molecular formula of $C_9H_{12}N_4O_4$. ^1H NMR (Fig III-14), ^1H COSY and HETCOR spectra of p-2 were similar to that of PPN and, again, had a pattern similar to that of PPNC. However,

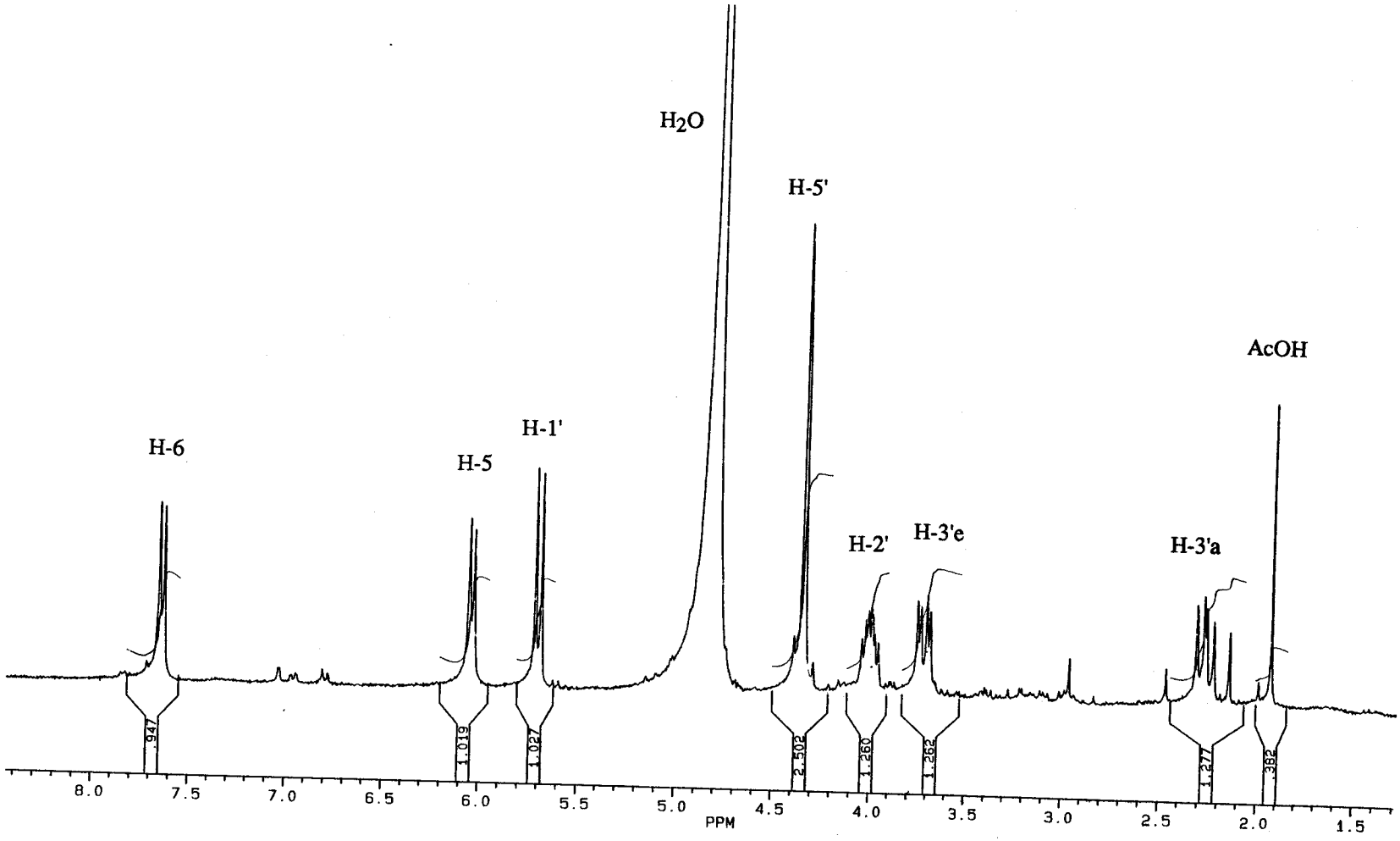


Fig III-14. ^{1}H NMR spectrum of p-2, **59**

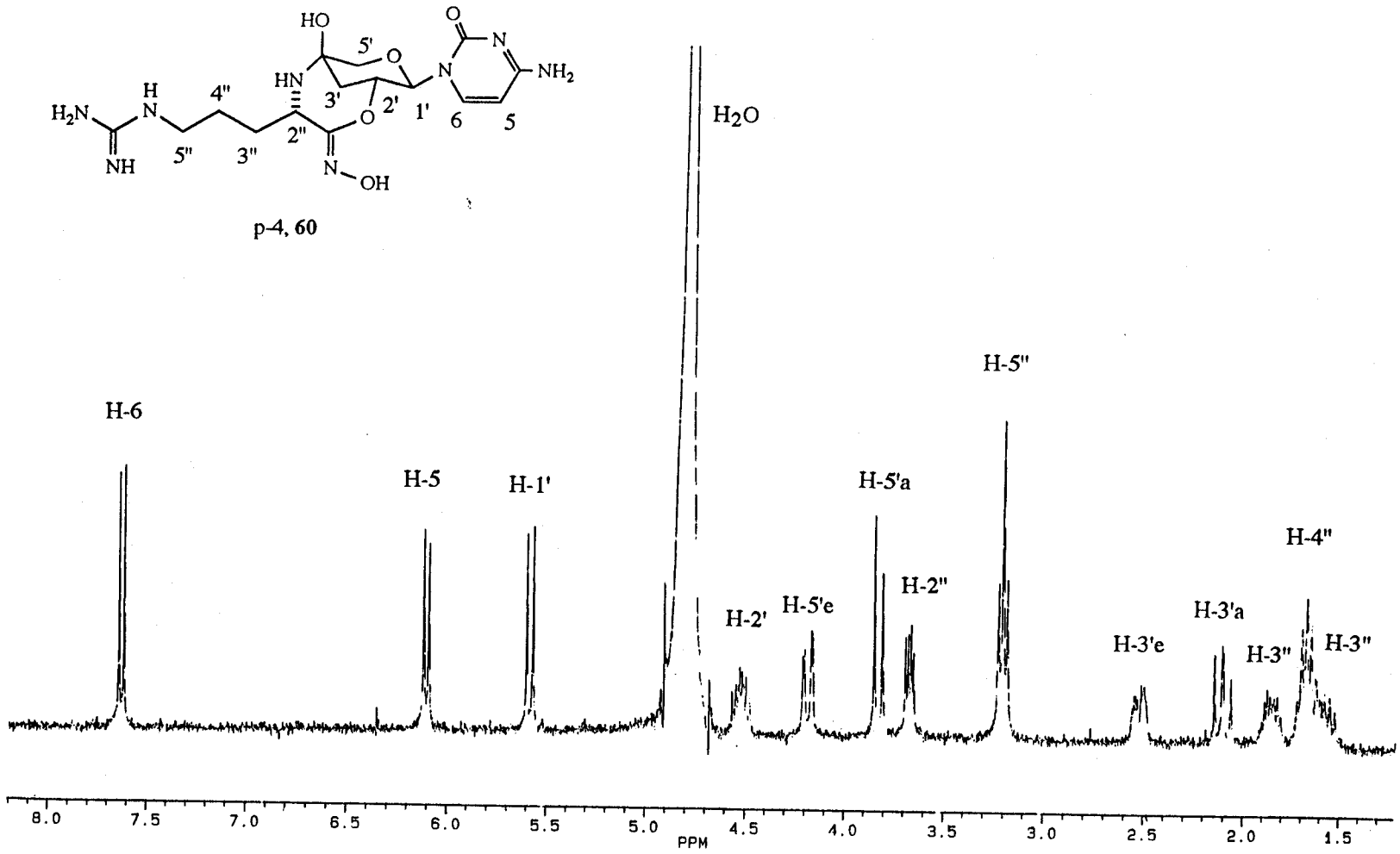
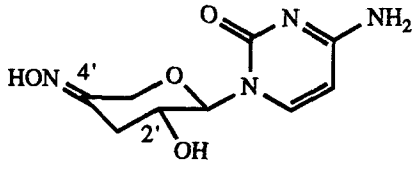


Fig III-15. ^{1}H NMR spectrum of p-4, **60**

the ^{13}C NMR spectrum now showed a resonance at 155.5 ppm, but not at 91.1 ppm. This was too far upfield for a ketone carbonyl, and treatment of p-2 with 1% aqueous TFA readily converted p-2 to PPN. Since the formula differs from PPN (ketonic form, **58**) by NH, these experiments suggested p-2 to be the oxime, **59**.[21] This could have been formed from hydroxylamine derived from the hydrolysis of the ArgH that had been added to the fermentation and the production of **59** indicated that free PPN was generated in the fermentation.



59

The Structure of p-4 (60)

Much of the ^{1}H NMR spectrum of p-4 was also similar to that of PPN. However, there were additional signals that corresponded to those of an arginine residue (Fig III-15). HR-MS revealed that p-4 had a molecular formula of $C_{15}H_{24}N_8O_5$. Hydrolysis of p-4 with 1% aqueous TFA produced PPN and ArgH. From this, it was clear that p-4 is a conjugate of PPN and ArgH and p-4 was proposed to have the structure of **60** (Fig III-16). Because the H-2' resonance of p-4 was shifted 0.5 ppm downfield (Fig III-15) in comparison with that of H-2' in PPN (Fig III-5), it suggested that the ArgH residue was connected to the 2'-hydroxyl. A positive $FeCl_3$ test[22] suggested that a free hydroxylamine moiety was present. This, combined with the NMR data and its easy cleavage with acid suggested that the linkage between the PPN residue and the arginine hydroxamate residue was by an ester rather than an amide bond.

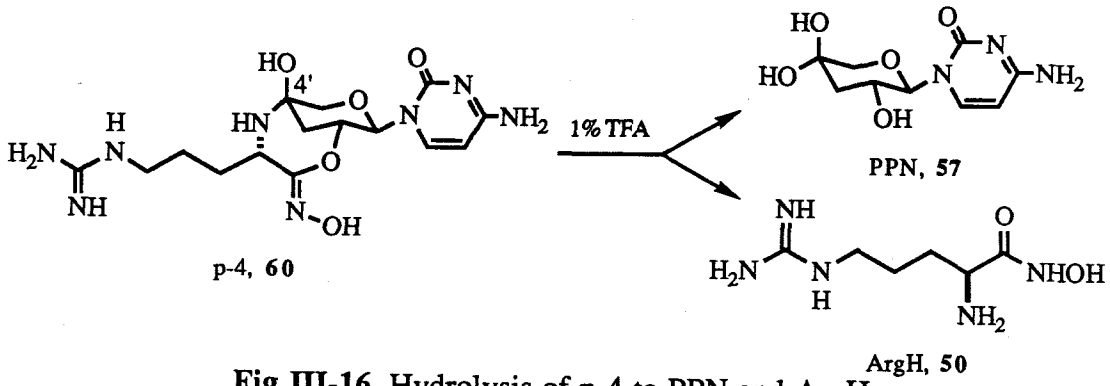


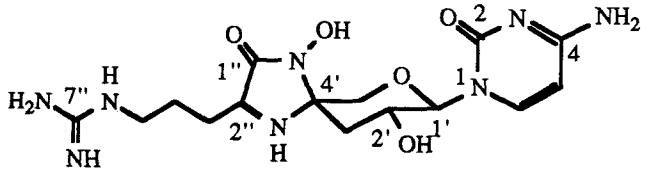
Fig III-16. Hydrolysis of p-4 to PPN and ArgH

A resonance at 78.2 ppm in the ^{13}CNMR spectrum of p-4 was assigned to C-4'. Since this was 12.9 ppm upfield from the corresponding carbon in PPN, it would be consistent with an aminal rather than a ketone hydrate. This could be explained by addition of the α-amino group of the arginyl residue to the C-4' carbonyl. The derived structure **60** would account for the observed molecular formula. Unlike PPN or **58**, the hydrogens at C-3' of p-4 were not exchanged to a noticeable level under the same conditions, even for a prolonged period (3-4 days), consistent with C-4' being blocked. Structure **60** also explains why p-4 had eluted earlier than BS from the cation exchange column and why it appeared to be less polar than BS on reverse phase HPLC. Compound **60** is now named 2'-arginine hydroxamate pentopyranone (2'-ArgH-PPN).

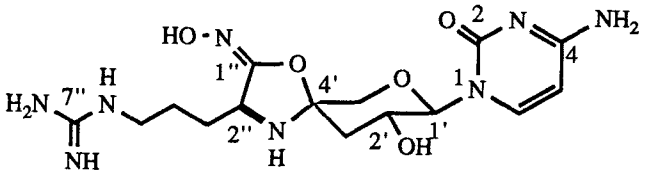
The Structure of p-5 (61)

Interestingly, p-5 was found to have the same molecular formula as p-4, and their ^{1}H NMR and ^{13}C NMR spectra were very similar. However, several differences were noticeable. First, the chemical shift of the H-2' resonance in p-5 (Fig III-17) was nearly the same as in PPN (Fig III-5) and was 0.41 ppm upfield from that of H-2' in p-4 (Fig III-15). Clearly, in this case the arginine hydroxamate residue is not connected to the 2'-hydroxyl. At the same time, the C-4' resonance of p-5 (δ 77.4) was very close to the value in p-4 (δ 78.2), suggesting a similar semi-aminal structure at C-4'. This notion was further supported by a D_2O exchange experiment, which indicated that C-4' was blocked

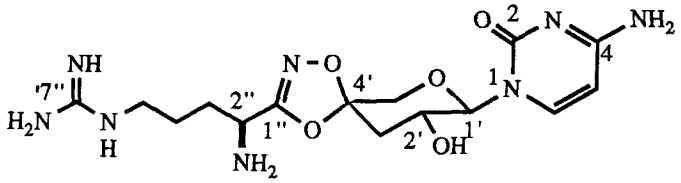
in a non-ketonic form, since no measurable deuterium exchange could be observed. P-5 was much more stable than p-4, and no measurable hydrolysis occurred when p-5 was treated with 1% aqueous TFA at room temperature overnight. It was also noticeable that the chemical shifts of the 3'-hydrogens in p-4 were 0.40 ppm apart, while in p-5 the two 3'-hydrogens were not resolved. These different chemical shift patterns suggested that the pyranose moiety in p-5 might have been distorted away from a regular chair conformation. p-5 was also $FeCl_3$ test positive. Therefore, there seemed to be a free hydroxylamine or hydroxamate group in the molecule. From these data we concluded that out of the possible connections of ArgH to PPN, p-5 has the structure of either **61a** or **61b** (Should **61a** be proven to be the right structure for p-5, then the PPN-oxime, **59**, might be derived from the hydrolysis of **61a**). The structure of **61c** should be excluded because there is no free hydroxylamine or hydroxamate moiety, and the chemical shift of C-4' should have been similar to that of the ketal carbon of C-4' in PPN, **57**.



61a



61b



61c

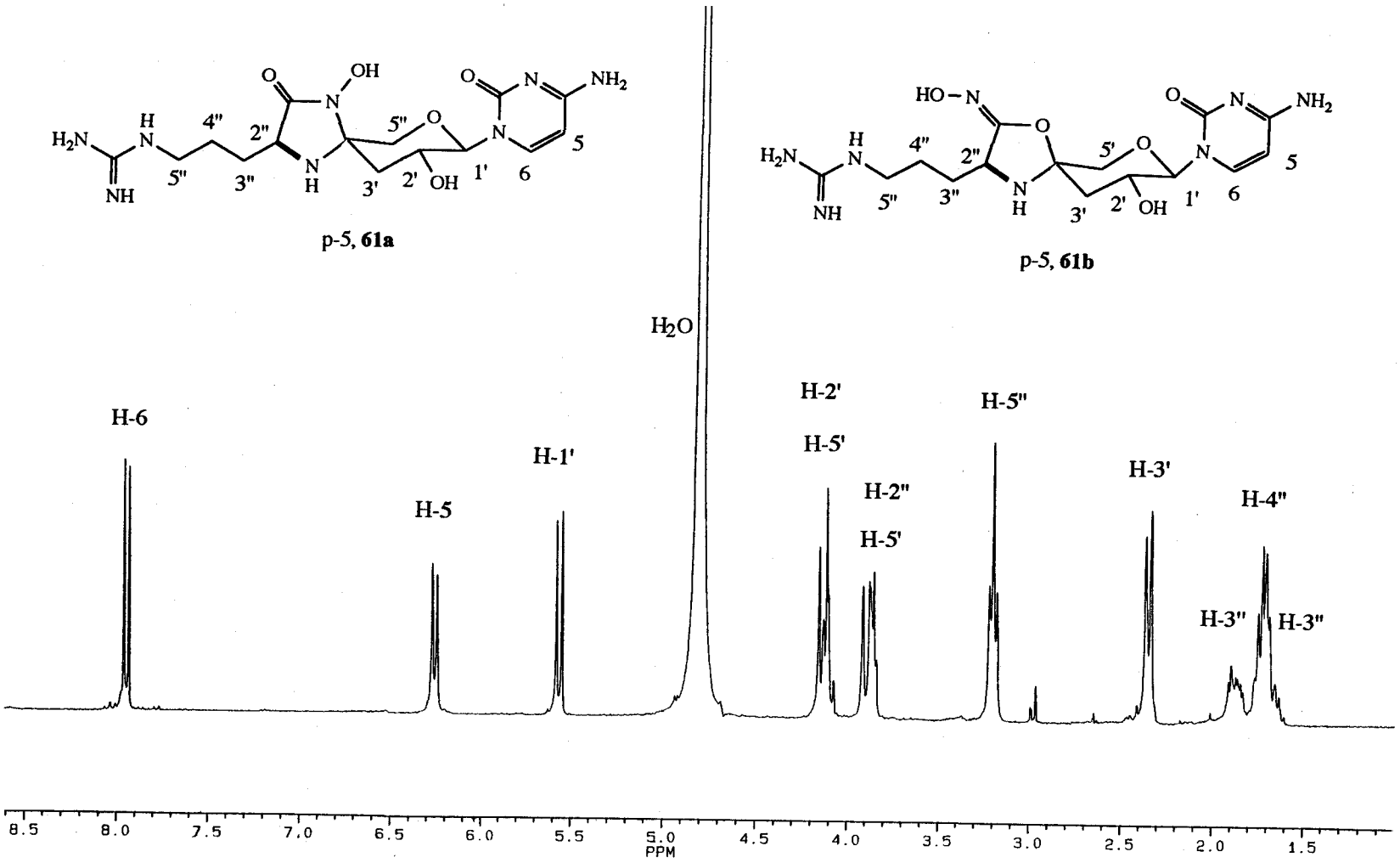
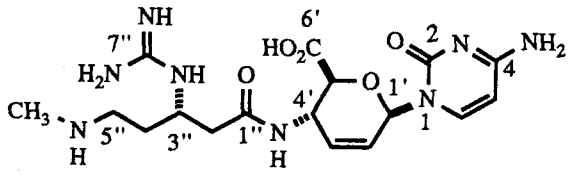


Fig III-17. ^{1}H NMR spectrum of p-5, **61**

The Structure of p-3 (62)

P-3 has a molecular formula, $C_{17}H_{25}N_8O_5$, identical to that of BS. Moreover, their ^1H and ^{13}C NMR spectra were nearly identical. The significant differences were the H-3" and H-5" resonances of p-3. The H-3" resonance shifted 0.39 ppm downfield relative to H-3" in BS, and the H-5" shifted 0.31 ppm upfield (Fig III-18). These shifts indicated that either the guanidino group or the methyl group had migrated to the β-amino group, and the correct one was easily distinguished by irradiation of the δ-N-methyl group in the ^1H NMR spectrum of p-3, which generated an NOE for the H-5" resonance. Thus, the guanidino group was the one to have migrated, and the structure, **62**, has been named iso-blasticidin S (Iso-BS).



Iso-BS, **62**

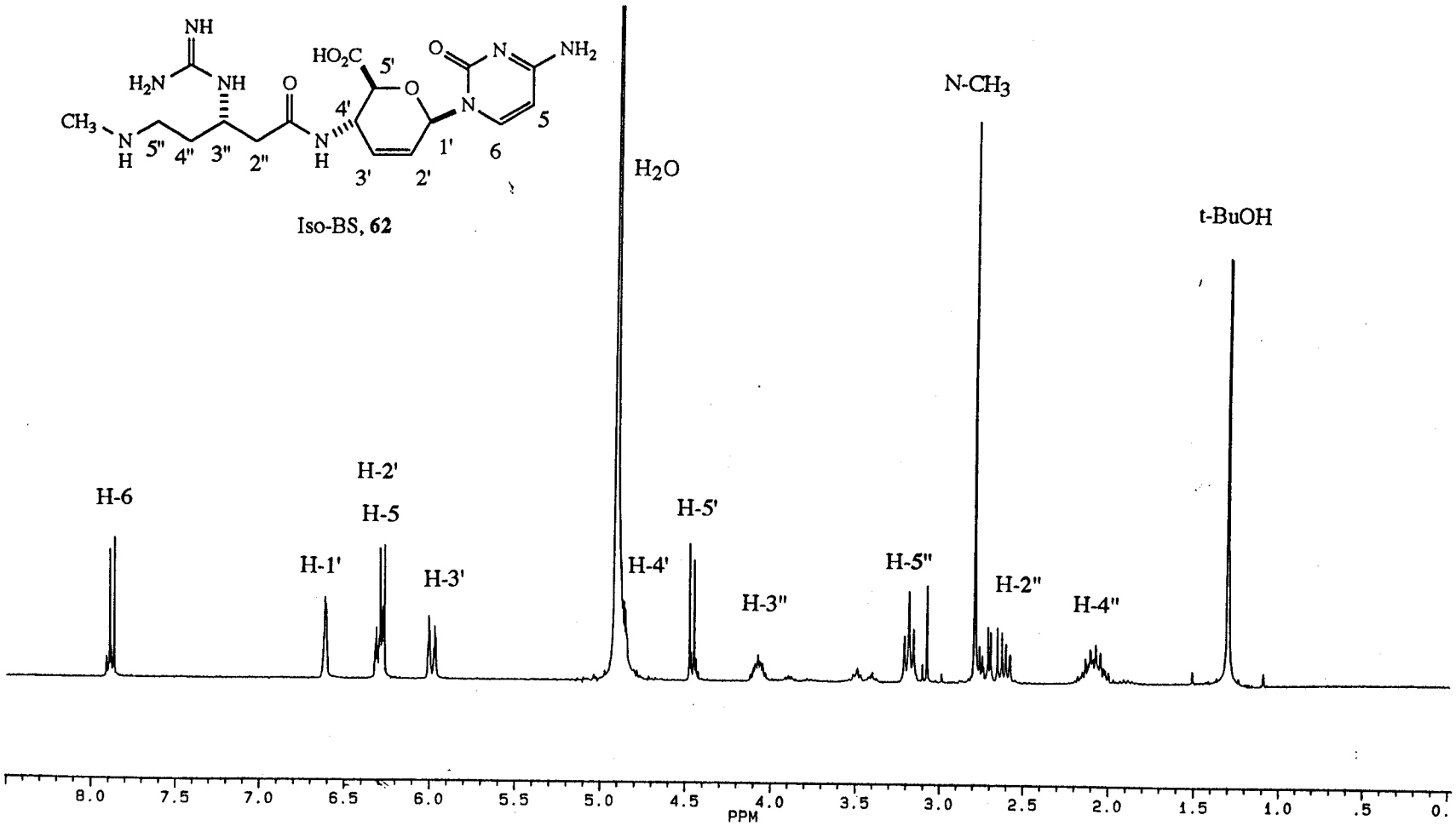


Fig III-18. ^{1}H NMR spectrum of p-3, **62**

Summary

Biosynthetic pathways for microbial metabolites have most often been determined by *in vivo* feedings of presumed precursors, sometimes abetted by isolation and characterization of co-metabolites that fortuitously also accumulate in the fermentation broth. A complementary approach has been to develop mutants blocked at various steps in a particular pathway, in the hope that they will accumulate intermediates that can no longer be processed, but it is likely to take considerable time to obtain the desired mutants. Less frequently, specific inhibitors of reactions thought to be essential have been fed to cultures, again in the hope of accumulating otherwise unobserved intermediates. We have found this latter approach to be exceedingly productive for understanding the biosynthesis of the blasticidins. The present study extended this success in providing CGA, potentially the first intermediate for the nucleoside formation; PPN and PPNC, the first metabolite and the final product in one of the prominent branches of this metabolic matrix; DeMeBS, potentially the last intermediate in BS biosynthetic pathway; and finally, in identifying Iso-BS, which revealed a new branch.

Surprisingly, we also encountered new metabolites that were derived from the pathway originally under study combined with the enzyme inhibitor, ArgH, that had been added to alter the natural product pool sizes. The occurrence of p-5, **61**, is perhaps the easiest to rationalize, since, given the large amount of ArgH that had been added to the fermentation, p-5 could be generated by a purely chemical reaction between ArgH and PPN, although we have not had enough of PPN or **58** available to test this independently. The oxime **59** could be formed from PPN and hydroxylamine generated from the hydrolysis of ArgH (or from the hydrolysis of **61a** as was noted above). Formation of p-4, **60**, would seem to require enzyme intervention in order to activate the hydroxamic acid, as shown in Figure III-18. Closure to the hemi-aminal might then take place spontaneously. The production of **60** revitalized our earlier speculation that ArgH

might have interacted with BS biosynthetic enzyme(s) in addition to the inhibition of *de novo* Arg biosynthesis. Nonetheless, this approach has provided a number of interesting new compounds. It would be interesting to see if any of them has a similar biological activity to that of BS.

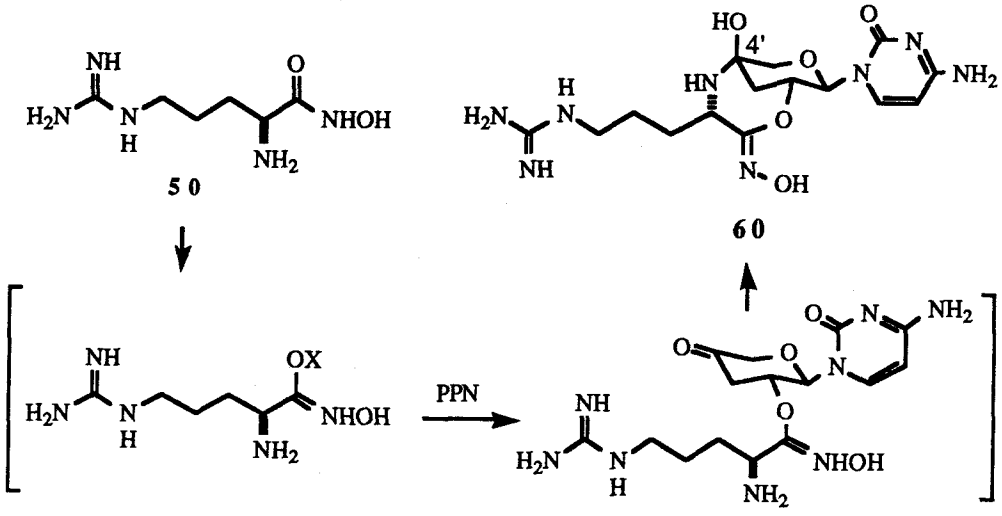


Fig III-19. Proposed mechanism for the formation of p-4, **60**

Experimental

General

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AM 400 (400.13 MHz and 100.61 MHz respectively). All ^{13}C NMR spectra were broadband decoupled. ^{1}H and ^{13}C samples were prepared in D_2O and the chemical shifts are reported in parts per million downfield relative to an internal standard of t-BuOH (δ 1.27) or TFA (δ 164). ^{2}H NMR Samples of the biosynthetic products were prepared in deuterium depleted water obtained from Aldrich (^{2}H content = natural abundance x 10^{-2}) with 25 µL t-BuOH or 25 µL of 1,4-dioxane (δ 3.54) as an internal reference for chemical shift and quantification (0.456 µmol ^{2}H in the methyl groups in t-BuOH or 0.34 µmol ^{2}H in dioxane). The ^{2}H NMR spectra were recorded at 61.4 MHz on the Bruker AM 400 spectrometer. They were proton decoupled and run unlocked. D_2O and ND_4OD were purchased from Aldrich Chemical Co (Milwaukee, WI). TFA and t-BuOH were from Sigma Chemical Co (St. Louis, MO). Low-resolution mass spectra were recorded on a Varian MAT CH-7 instrument and high-resolution mass spectra were recorded on a Kratos MS 50 TC spectrometer. Ion exchange resins were purchased from either Bio-Rad Laboratories (Richmond, CA) or Sigma Chemical Co and were converted to the necessary ionic form according to the manufacturer's recommendations. Other general chemicals were from Sigma Chemical Company (St. Louis, MO). Authentic PPNC was a gift from Professor Haruo Seto.

Production of metabolites was quantified by HPLC. For the assay, 0.5 mL of broth was taken, centrifuged (Eppendorf, Model 5414) for 10 min, and 10 to 20 µL of the supernatant was injected to HPLC.

The general conditions for analytical HPLC, except those specified, were: Nova pack, C_{18} RadialPak^{R} column, 0.8 cm x 10 cm (Waters Assoc. 4 µm packing), eluted with 95% H_2O, 5% CH_3CN, 0.15% TFA at a flow rate of 1.2 mL/min. The effluent was

detected with a Waters Model 990+ photodiode array detector. The conditions for preparative HPLC were: Nova pack, C_{18} RadialPak^R column, 2.5 cm x 10 cm (Waters Assoc. 6 μm packing), eluted with 97% H_2O, 3% CH_3CN, 0.05% AcOH at a flow rate of 6 mL/min. The effluent was detected with a Linear UVIS 200 detector at 275 nm.

Fermentation media and feeding solutions were prepared from double distilled H_2O, while HPLC and chromatographic solvents were prepared from milli-Q H_2O.

$FeCl_3$ Test

$FeCl_3$ test was conducted following the procedures reported.[22] A drop of 5% $FeCl_3$ (in 0.5 M HCl) and a drop of sample solution (in H_2O) were spotted close to each other on a piece of Beckman No 1 filter paper. Samples containing free hydroxamate group show a red color in the interface of the spotted solutions.

Deuterium Exchange Experiment

For deuterium exchange studies, samples of generally a few mg were dissolved in ~400 μL of D_2O in an NMR tube. After collecting the ^1H NMR spectrum for each sample, 4.0 μL of ND_4OD (28%) was added, mixed and a new ^1H NMR spectrum was obtained 45 min later. These solutions had a pD ~9.0 - 9.3.

Fermentation Conditions and Feeding Protocol

Standard culture maintenance, fermentation conditions and feeding procedures have been described previously in Chapter II. Inhibitors and primary precursors were fed by adding the solid compounds directly at the 52nd hour of incubation except those specified. Cytosine was fed by adding 3% sterilized solution. For metabolite isolation and purification, fermentation broths were harvested at the 144th hour of incubation.

Isolation

The Initial Step

Procedures for the isolation of CGA, PPNC, DeMeBS, BS and other new metabolites are as follows (see also Fig III-11).

The broth (1 L) was centrifuged at 10,000 x *g* for 10 min to remove solids. The pellets were washed with a minimum amount of water, recentrifuged and the washings were combined with the original supernatants. A sample (0.5 mL) was saved for production analysis by an analytical HPLC, the remainder was cooled to 4 °C, carefully adjusted to pH ~3.0 using 6 N HCl, and allowed to stand for 10 min. After removal of precipitated colloidal materials by centrifugation at 0-4 °C, the supernatant was neutralized to pH 6.0 using 1 N NaOH and loaded onto a cation exchange column (Dowex 50W x 4, H^+, 100-200 mesh, 5.0 cm x 50 cm) at a flow rate of ~6.0 mL/min. The column was washed with ~300 mL water and then with 5% aqueous pyridine. The first 500 mL were discarded, and the next ca. 1 L was collected and saved ("pyridine eluate", see below). The cation exchange column was next eluted with 1.2% NH_4OH (2.5 L). The eluate was monitored regularly by HPLC and five fractions were collected according to the appearance of the major components. All fractions were then lyophilized.

CGA (20)

The "pyridine eluate" was loaded onto an Amberlite IRA-410 anion exchange column (100 mesh, AcO^-, 5.0 cm x 30 cm), at ~ 3 mL/min. After loading, the column was washed with H_2O until pH in the effluent was neutral and then with 1.0 M acetic acid. The eluates of the acetic acid washings were monitored regularly for the presence of CGA with the analytical HPLC. Most CGA was eluted in the late part of acetic acid washings. The appropriate eluates were lyophilized, which gave crude CGA. This was dissolved in ~10 mL of 1% aqueous pyridine, divided into two parts, filtered through two C_{18} sep^R disposable columns (SepPak columns, J.T.Baker Inc., 3 mL bed volume),

and washed with two column volumes of H_2O. The effluent and H_2O washings were combined and lyophilized. Recrystallization twice from hot H_2O yielded 70 mg of pure CGA, **20**: ^1H NMR (D_2O, 400 MHz) δ 7.92 (H-5), 6.22 (H-6), 5.66 (H-1'), 3.91 (H-5'), 3.64 (H-2' and H-3'), 3.57 (H-4'); ^{13}C NMR (D_2O, 400 MHz) δ 175.2 (C-6'), 160.6 (C-2), 150.4 (C-4), 144.6 (C-6), 96.6 (C-5), 83.4 (C-1'), 78.9 (C-5'), 76.2 (C-4') 71.8 (C2', C-3'); Fab-MS (H_2O-glycerol) calculated for $C_{10}H_{13}O_7N_3$ ($M+H^+$): 288.1, found: 288.1.

PPNC (18) and p-2 (59)

The effluent and early acetic acid eluate from the anion exchange column was lyophilized, taken up in ~50 mL of H_2O and filtered through a C_{18} silica gel pad (J.T.Baker, 40 μm, 10 cm x 5 cm in a filtration funnel connected to an aspirator). The C_{18} pad was washed thoroughly with H_2O and the effluent was monitored by HPLC continually. The effluents containing different major components were collected and lyophilized separately and each was subjected to further purification by preparative HPLC. Pure PPNC, **18** (40 mg), and p-2, **59** (15 mg, from a seperated 2.5 L fermentation), were obtained after the preparative HPLC.

PPNC, **18**: The ^1H NMR and ^{13}C NMR data are listed in Table III-3 and III-4. The ^1H NMR data were identical to those obtained from an authentic sample.

p-2, **59**: The ^1H NMR and ^{13}C NMR data are listed in Table III-3 and III-4. FAB-MS (glycerol/H_2O) m/z (relative intensity) 241.1 ($M+H^+$, 30%), 207.1 (40%), 115.1 (100%), 112.0 (55%); HR FAB-MS (glycerol/H_2O) exact mass calculated for $C9H_{13}N_4O4$: 241.09368 ($M+H^+$), found: 241.09370.

BS (1), DeMeBS (14), p-3 (62), and p-5 (61)

Fractions from the cation exchange column obtained by 1.2% NH_4OH elution were each further purified. Fraction 3 gave BS (30 mg), which was further purified by

recrystallization from H_2O-MeOH. Fraction 1, 2, 4 and 5 were each redissolved in a minimum amount of H_2O and purified by preparative HPLC.

Fraction 2 gave pure p-5, **61** (~5 mg): The ^1H NMR and ^{13}C NMR data are listed in Table III-3 and III-4. FAB-MS (Thioglycerol-glycerol) exact mass calculated for $C_{15}H_{25}N_8O_5$: 397.19479 ($M+H^+$), found 397.19470. $FeCl_3$ test positive.

Fraction 4 gave pure DeMeBS, **14** (2-5 mg). ^1H NMR spectrum was identical to that reported.[18]

Fraction 5 gave pure p-3, **62**: The ^1H NMR and ^{13}C NMR data are listed in Table III-3 and III-4. Neg-MS (thioglycerol-glycerol) m/z (relative intensity) 421.1 ($M-H^+$, 65%), 113.0 (100%); HR Negative-MS (thioglycerol-glycerol) exact mass calculated for $C_{17}H_{25}N_8O_5$ 421.19479 ($M-H^+$), found: 421.1928.

p-4 (60)

Fraction 1 gave a crude product of p-4, **60**. The crude product of p-4 from preparative HPLC was lyophilized, redissolved in ~3 mL of H_2O and loaded onto a cation exchange column (Bio-Rad, AG 50W x 4, NH_4^+, 1.5 cm x 10 cm) and washed with 30 mL of H_2O. Pure p-4, **60** (~5 mg)[a] , was next eluted from the column with 30 mL of 1.2% NH_4OH. The ^1H NMR and ^{13}C NMR data are listed in Table III-3 and III-4. FAB-MS (3-NBA) m/z (relative intensity) 397.2 ($M+H^+$, 100%),112.0 (20%); HR FAB-MS (3-NBA) exact mass calculated for $C_{15}H_{25}N_8O_5$: 397.19479 ($M+H^+$), found: 397.19490. $FeCl_3$ test positive.

Preparation of p-1 (57) and ArgH (50) by hydrolysis of p-4 (60)

Pure p-1, **57**, was prepared from the hydrolysis of p-4, **60**, (~20 mg) with 1% aqueous TFA (5 mL) at room temperature overnight. The reaction mixture was then evaporated to dryness under reduced pressure with a warm water bath (~ 50 °C). The

[a] In a seperated 2.5 L fermentation, 15 mg of **60** was obtained with the same purification sheme.

residue was loaded onto a cation exchange column (AG 50W x 4, NH_4^+, 1.5 cm x 10 cm). The column was washed sequentially with H_2O (~ 20 mL), 5% aqueous pyridine (30 mL) and 1.2% NH_4OH (30 mL). Pyridine elution gave p-1, **57**, and ammonium hydroxide elution gave ArgH, **50**. Each product was then lyophilized and yielded about 10 mg of **57** and 5 mg of **50**.

p-1, **57**: The ^1H NMR and ^{13}C NMR data are listed in Table III-3 and III-4. FAB-MS (glycerol) m/z (relative intensity) 246.1 ($M+H^+$,45%), 228.1 (25%), 207.1 (50%), 204.1(100%), 195.2 (35%); HR FAB-MS (glycerol) exact mass calculated for $C_9H_{12}D_2N_3O_5$: 246.10590 ($M+H^+$),[b] found: 246.10588.

ArgH, **50**: The ^1H NMR of **50** that was prepared from the hydrolysis of **60** was identical to an authentic sample from Sigma Chemical Co. FAB- MS (glycerol-H_2O) m/z (relative intensity) 190.1 ($M+H^+$, 100%), 174.2 (25%), 159.0(20%), 70.1 (85%); HR FAB-MS (glycerol-H_2O) exact mass calculated for $C_6H_{16}N_5O_2$: 190.13040 ($M+H^+$), found: 190.13030. $FeCl_3$ test positive.

Preparation of [3',3'-$^2H_2$]PPN, 57a

Compound **57a** was prepared by dissolving **57** (2-5 mg) in D_2O (400 μL) in an NMR tube. Fifteen μL of 28% ND_4OD (~ 1% final concentration, this solution had a pD of 9.3) was added and the sample was left at room temperature for 45 min. The mixture was lyophilized, triturated with H_2O and lyophilized for two times. This lyophilized powder was used for ^1H NMR, ^2H NMR and MS spectral analysis. In the ^1H NMR spectrum, resonances of H-3'a and H-3'e had disappeared; however, in the ^2H NMR spectrum H-3'a and H-3'e were observed at δ 1.98 and at 2.48, respectively (100% enrichment for both); HR FAB-MS (glycerol) exact mass calculated for $C_9H_{12}D_2N_3O_5$: 246.10590 ($M+H^+$), found: 246.10588.

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[b] The MS data were collected from the sample of **57a**

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Chapter IV

Identifying Biosynthetic Enzymes in Cell-free Extracts of *S. griseochromogenes*

Introduction

Although an impressive array of methodologies has been established for the biosynthesis of secondary metabolites, the most commonly used approach is an *in vivo* isotopic tracer experiment. Such studies are performed with intact cells and consequently suffer from several inherent disadvantages. First, the externally administered precursors must traverse cellular barriers in order to reach the active biosynthetic site at which they can join the mainstream of the biosynthetic pathway along with the *de novo* produced intermediates. As was true in the previous two chapters describing *in vivo* feeding of [1'-^{2}H]cytosylglucose ([1-^{2}H]CG), **27a**, and [1-^{14}C]cytosinine, **2a**, uncertainties imposed by permeability barriers have rendered reliable feeding experiments unachievable in the present study. Second, compounds fed may be degraded *en route* or distributed among other catabolic or anabolic pathways; therefore, precursor incorporation is generally low and isotopic labelling patterns in the product may sometimes be complicated. Finally, with intact cell feedings, for all of their conceptual and methodological elegance, the experimental approach and interpretation of results remain an indirect science.

As a complement to intact cell feedings, biosynthetic investigations have recently turned with increasing frequency to studies with enzyme preparations. One of the most obvious advantages of such a study is the elimination of permeability barriers both to the incorporation of added substrates and to the detection and isolation of transitory products.[1] Investigations with cell-free enzyme preparations also have the advantage that each step can be examined independently from the metabolic grid of the producer cell without interference from other transformations of the administered precursor or of the

products formed.[1] This shift in emphasis has allowed the most direct insights into the structures of intermediates, the sequences, cofactor requirements and mechanisms of individual biosynthetic reactions, and into the structures and properties of the protein catalysts themselves.

In light of the information obtained from previous whole-cell studies, regarding the possible biochemical transformations involved, and the ready availability of potential intermediates that had been accumulated in the fermentation cultures of inhibitor and primary precursor feedings, studies with cell-free preparations became a desirable and possible approach. This chapter describes studies with cell-free extracts of *S. griseochromogenes* for identification of biosynthetic intermediates and biosynthetic enzymes.

Result and Discussion

Isolation of CGA Synthase and Preparation of [^{3}H,^{14}C]CGA, 20b

Although CGA had been isolated previously from *S. griseochromogenes* by Seto et. al.,[2] its role in the biosynthesis of BS had largely been ignored, in part due to the fact that only a very small amount of CGA was normally produced (~1 mg/L) and perhaps in part because no XDP-glucuronosyltransferase type of enzyme had been isolated from a prokaryotic organism. Instead, many glycoside antibiotics have been found to be derived from XDP-glucose or other XDP-hexoses.[3] Therefore, studies of BS biosynthesis had focused on testing CG as the first committed intermediate for the nucleoside formation.

The accumulation of CGA in the inhibitor-primary precursor feedings suggested its cardinal role in the biosynthesis of BS. A conventional approach to clarify this possibility would have been to do a whole-cell tracer experiment. However, a preliminary feeding experiment and HPLC analysis indicated that CGA could not be significantly consumed (or adsorbed) when added to a mycelial suspension of *S. griseochromogenes*. Neither treating the mycelia with an organic solvent (toluene, DMSO) nor converting them into protoplasts (by digesting the mycelial cell wall with chicken lysozyme), the methods that have been reported to improve bacterial permeability,[4,5] was successful. We next attempted to isolate from crude cell-free extracts (CFE's) of *S. griseochromogenes* metabolic enzymes capable of converting CGA to other metabolites that are related to BS. However, these were not able to give a clear conclusion. Because crude cell-free preparations gave too many peaks in the HPLC, we were not able to identify with certainty products derived from CGA. After the failure of these initial efforts, we decided to isolate the enzyme that catalyzes the formation of CGA. This experiment had two purposes. First, finding the enzymatic activity would clarify the timing of C-6' oxidation and the activation group for CGA synthesis (Fig IV-1). Second, finding the enzymatic

activity would simplify the procedure to prepare radiolabeled CGA for a tracer feeding experiment.

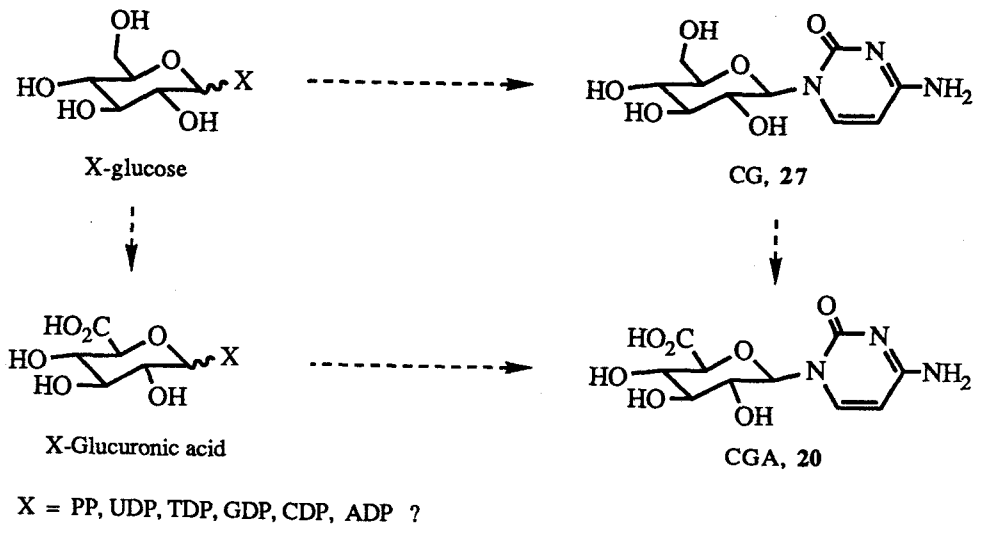


Fig IV-1. Possible pathways for CGA formation

A CFE of *S. griseochromogenes* was prepared by sonication of the mycelial suspension. The CFE was then incubated with cytosine and either UDP-glucuronic acid, **36**, or UDP-glucose, **37**. Using an HPLC assay, it was found that CGA was synthesized from UDP-glucuronic acid and cytosine (Figure IV-2). The formation of CGA was later confirmed by a preparative scale synthesis starting with 100 mg of UDP-glucuronic acid and 20 mg of cytosine in 100 mL of CFE obtained from ~ 5 g of cells (wet weight). The preparation generated enough CGA to be characterized by ^{1}H NMR spectroscopy. This enzyme was designated as CGA synthase. The synthase seemed to be a soluble protein and showed similar activity both in a potassium phosphate buffer at pH 7.2 and in a Tris buffer at pH 8.0.

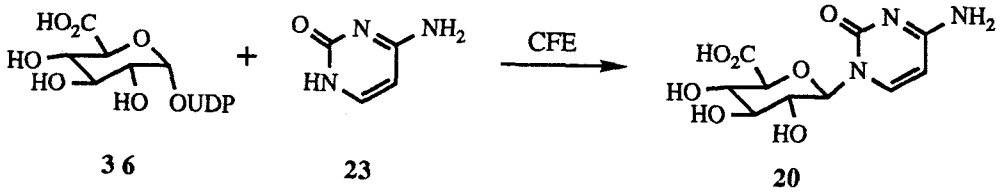


Fig IV-2. Formation of CGA

Fig IV-3. HPLC UV- and radioactivity traces of CGA synthase specificity analysis.

Conditions for the preparation of CFE and for HPLC are described in the Experimental Section. Each sample was composed of 1.0 μCi [2-^{14}C]cytosine, **23a**, in 500 μL of CFE and 1.0 mg of the nucleotide substrate was added to: A: none; B: UDP-glucuronic acid, **38**; C: UDP-glucose, **36**; D: UDP-galactose, **37**; and E: UDP-galacturonic acid, **39**. The samples were incubated at 30 °C overnight and 25 μL of the supernatant of each sample was used for the HPLC analysis. The upper-panels are UV-traces detected at 275 nm and the lower-panels are ^{14}C-radioactivity traces.

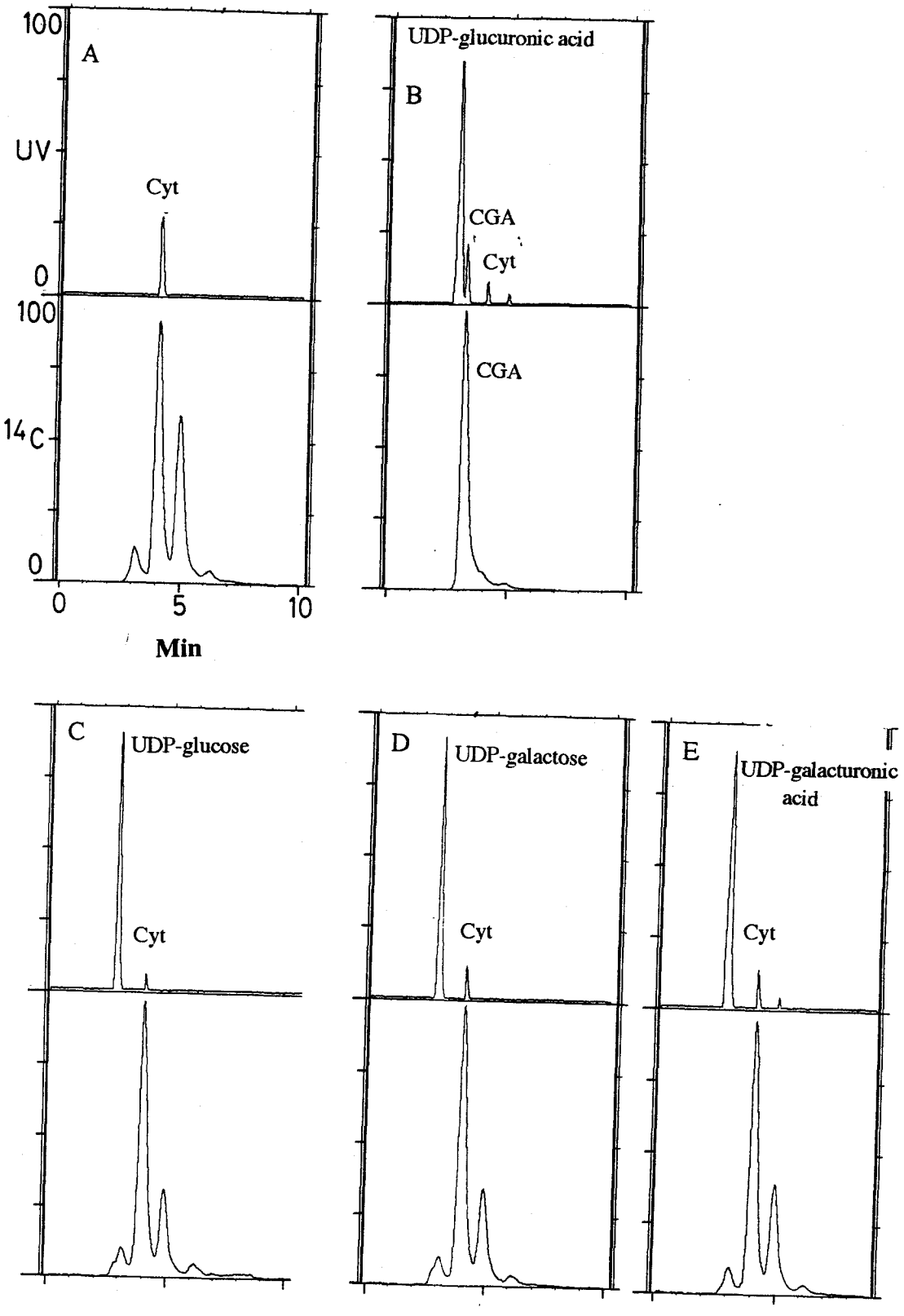
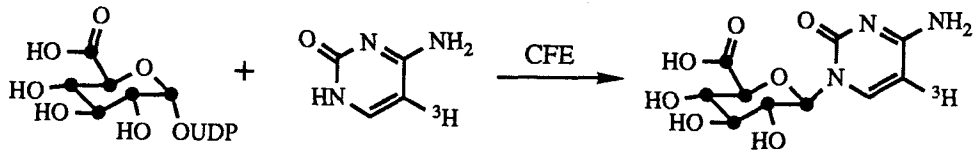


Fig IV-3. HPLC UV- and radioactivity traces of CGA synthase specificity analysis

It was also found that CGA synthase has a strict substrate specificity. This conclusion was drawn from HPLC-radiochemical analyses of the CFE incubation of [2-^{14}C]cytosine, **23a**, and each of the four potential nucleotide substrates (UDP-glucose, UDP-galactose, UDP-glucuronic acid and UDP-galacturonic acid, **36** - **39**, respectively). By connecting a radiochemical detector to the HPLC system, only the products that were derived from the radioactive substrate, **23a**, were detectable. Thus the HPLC profiles were greatly simplified and the identities of the products could be assigned unambiguously. Figure IV-3 shows the HPLC analysis profiles. In the incubation of A, in which no nucleotide substrate was added, most of the **23a** remained unchanged and no significant amount of CGA was produced. The incubation of C, D and E had essentially the same profiles as that of A, indicating no reaction of the added nucleotides. However, in the incubation in which UDP-glucuronic acid was included (B) over 90% of the **23a** was converted to a new radioactive component which had the same t_R as that of CGA.

With the aid of a crude CFE preparation, 25 mg of [^{3}H,^{14}C]CGA, **20b**, was prepared from 54.0 μCi of [5-^{3}H]cytosine, **23b**, and 10.9 μCi of UDP-glucuronic acid ([U-^{14}C] at ulonate part), **38a** (Fig IV-4). The radioactive conversion of substrates to product was 80.37% for ^{14}C and 77.81% for ^{3}H. The enzymatically prepared **20b** had a specific radioactivity of 4.49 x 10^5 dpm/mg of ^{14}C and 1.91 x 10^6 dpm/mg of ^{3}H (^{3}H/^{14}C = 4.25). This level of radioactivity was estimated to be 100-500 times higher than obtainable if **20b** were prepared by whole-cell fermentation.



38a **23b** **20b**

Fig IV-4. Preparation of [^{3}H,^{14}C]CGA, **20b**

The increase in specific radioactivity was important because it would proportionally increase the sensitivity of the following isotopic tracer experiment.

Feeding [^{3}H,^{14}C]CGA, 20b

In order to test CGA as an intermediate to BS, a feeding experiment was performed by adding 10 mg of **20b** to a 200-mL complex medium fermentation at the 39th hour of incubation. Ninety-eight hours later, the broth was workedup as usual, which yielded 455 mg (1.0 mmol) of labelled [^{3}H,^{14}C]BS, **1f**, (1.01 x 10^4 dpm/mmol ^{14}C, ^{3}H/^{14}C = 6.75), as shown in Figure IV-5. Most of the **20b** fed (88.4%) was recovered from the broth unchanged. On the basis of the unrecovered material, the ^{14}C incorporation was 1.80%, and ^{3}H incorporation was 2.86%.

The overall low percent incorporation of **20b** was believed to be due to the lack of good permeability of the bacteria to **20b**. A higher percent of ^{3}H incorporation compared to that of ^{14}C was explained due to the additional incorporation of a trivial amount of **23b**, generated from the degradation of **20b** during the incubation. Since **23a** had been showed to incorporate almost quantitatively (95%) into BS,[7] if only 0.14% of the **20b** fed had been hydrolyzed to **23b**, the ratio of ^{3}H/^{14}C in **1f** would have been changed from 4.25 to 6.75. In order to test whether the incorporation of **20b** was specific, a portion of **1f** (300 mg) was hydrolyzed with 3 N H_2SO_4.[8] This gave a mixture of [^{3}H,^{14}C]CN, **2c**, [^{3}H]cytosine, **23b**, and BA, **3**. The mixture was separated by anion exchange chromatography and further separated by S-sepharose cation exchange chromatography, which gave as pure products **2c** (9.67 x 10^3 dpm/mmol of ^{14}C, ^{3}H/^{14}C = 6.26) and **23b** (5.84 x 10^4 dpm/mmol, ^{3}H/^{14}C = 99.5). These data proved that 95% of ^{14}C activity in **1f** was located only in the ulonate moiety and that at least 85% of ^{3}H in **1f** was distributed only in the cytosine residue. The other 15% of ^{3}H was likely also initially distributed in the cytosine part but was probably lost during the hydrolysis process (Fig IV-6). The exchange of H-5 of cytosine with solvent under

acidic conditions had been observed by Karl DeJesus in our group in the preparation of [5-^{2}H]CG, **27b**.[9]

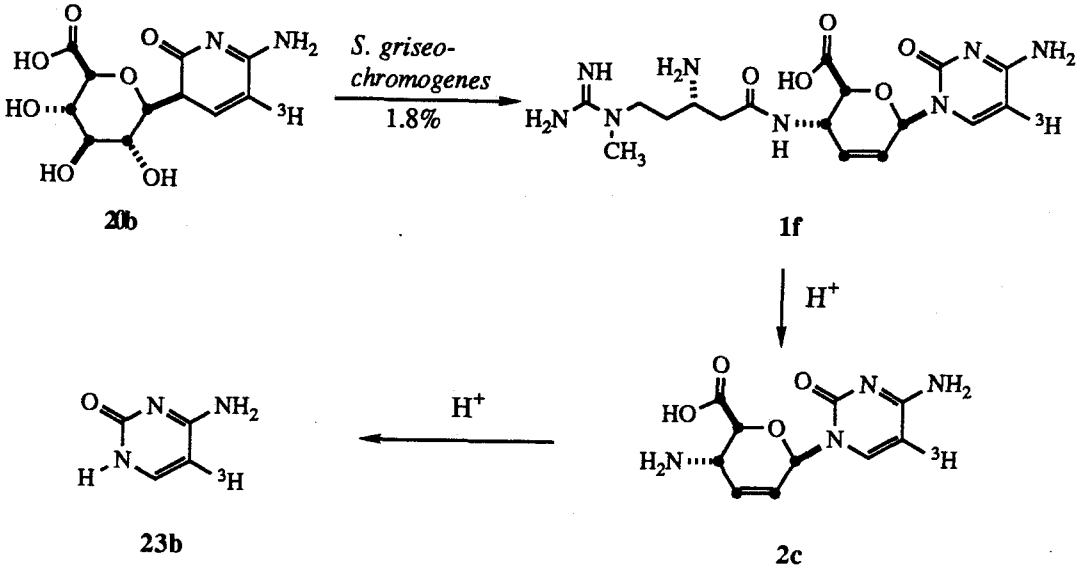


Fig IV-5. Intact incorporation of **20b** into BS, **1f**

Nevertheless, it was clear that **20b** was incorporated into **1f** almost completely intact, indicating that CGA is the first committed intermediate for nucleoside formation in the BS biosynthetic pathway.

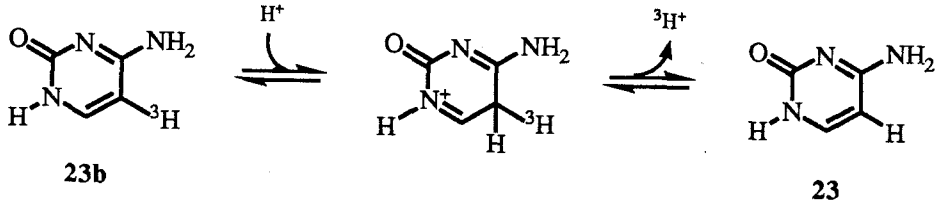


Fig IV-6. Proposed mechanism of losing ^{3}H in **23b**

Isolation of UDP-glucose 4'-Epimerase and 6'-Oxidoreductase

Finding CGA synthase has clarified the timing of C-6' oxidation and the immediate precursor for CGA biosynthesis. This finding also indicated that incorporation of galactose into BS must have been through the well-characterized primary carbohydrate metabolism of the UDP-glucose/UDP-galactose biotransformation pathway. In this case,

both UDP-glucose 4'-epimerase and UDP-glucose 6'-oxidoreductase must have been involved. A higher percentage incorporation of galactose into BS than of glucose could have been due to fewer metabolic pathways available for the utilization of galactose by the organism. Consequently, a higher percentage of the galactose fed channeled into the BS biosynthetic pathway.

The notion that UDP-glucose 4'-epimerase and 6'-oxidoreductase are involved in the biosynthesis of BS was later supported by HPLC-radiochemical analyses that a crude CFE of *S. griseochromogenes* was able to synthesize **20a** from **23a** and either UDP-glucose or UDP-galactose when the required cofactor (NAD^+) for the two enzymes was included (Fig IV-7, incubation D and E). In these incubations, the CGA peak constituted about 50% of the total radioactivity in the samples. As has been noted earlier (Figure IV-3), in the absence of NAD^+, such incubations produced only a very little amount of what might have been **20a**, which could have been derived from residual UDP-glucuronic acid in the crude CFE. In the absence of UDP-glucose or UDP-galactose (incubation B), a small amount of possible CGA was also generated. This small amount of possible CGA might have been derived from the residual UDP-glucose or UDP-galactose in the crude CFE. Including **23a** and UDP-glucuronic acid in the incubation (incubation C), again, over 90% of the **23a** was converted to **20a**, indicating the CFE was prepared properly.

The percent conversion of substrate to product, **20a**, in the CFE incubation of UDP-galactose, NAD^+ and **23a** was quantified by a standard isotopic trapping experiment. In this experiment, 190 mg of unlabeled **20** was added as a carrier 24 hours after the incubation and then the compound **20** was reisolated and purified. Liquid scintillation counting (LSC) analysis on the re-purified **20** indicated that the percent conversion of **23a** to **20a** was 42.1%. In the CFE incubation of UDP-glucose, NAD^+ and **23a**, the percent conversion of **23a** to **20a** was estimated to be similar (from the HPLC analysis). The actions of UDP-glucose 4'-epimerase and UDP-glucose 6'-oxidoreductase in the crude enzyme preparations are presented in Figure IV-8.

Fig IV-7. HPLC radioactivity traces of UDP-glucose 4'-epimerase and UDP-glucose 6'-oxidoreductase analysis.

Conditions for the preparation of CFE and for the HPLC are described in the Experimental Section. Each sample was composed of 0.5 μCi of [2-^{14}C]cytosine in 250 μL of CFE and the following compounds were added: none (A); 1.1 mg of NAD^+ (B); 0.6 mg of UDP-glucuronic acid (C); 1.1 mg of NAD^+ and 0.5 mg of UDP-glucose (D); 1.1 mg of NAD^+ and 0.5 mg of UDP-galactose (E). The reaction mixtures were incubated at 30 ^oC overnight and 25 μL of the supernatant of each was used for the HPLC analysis.

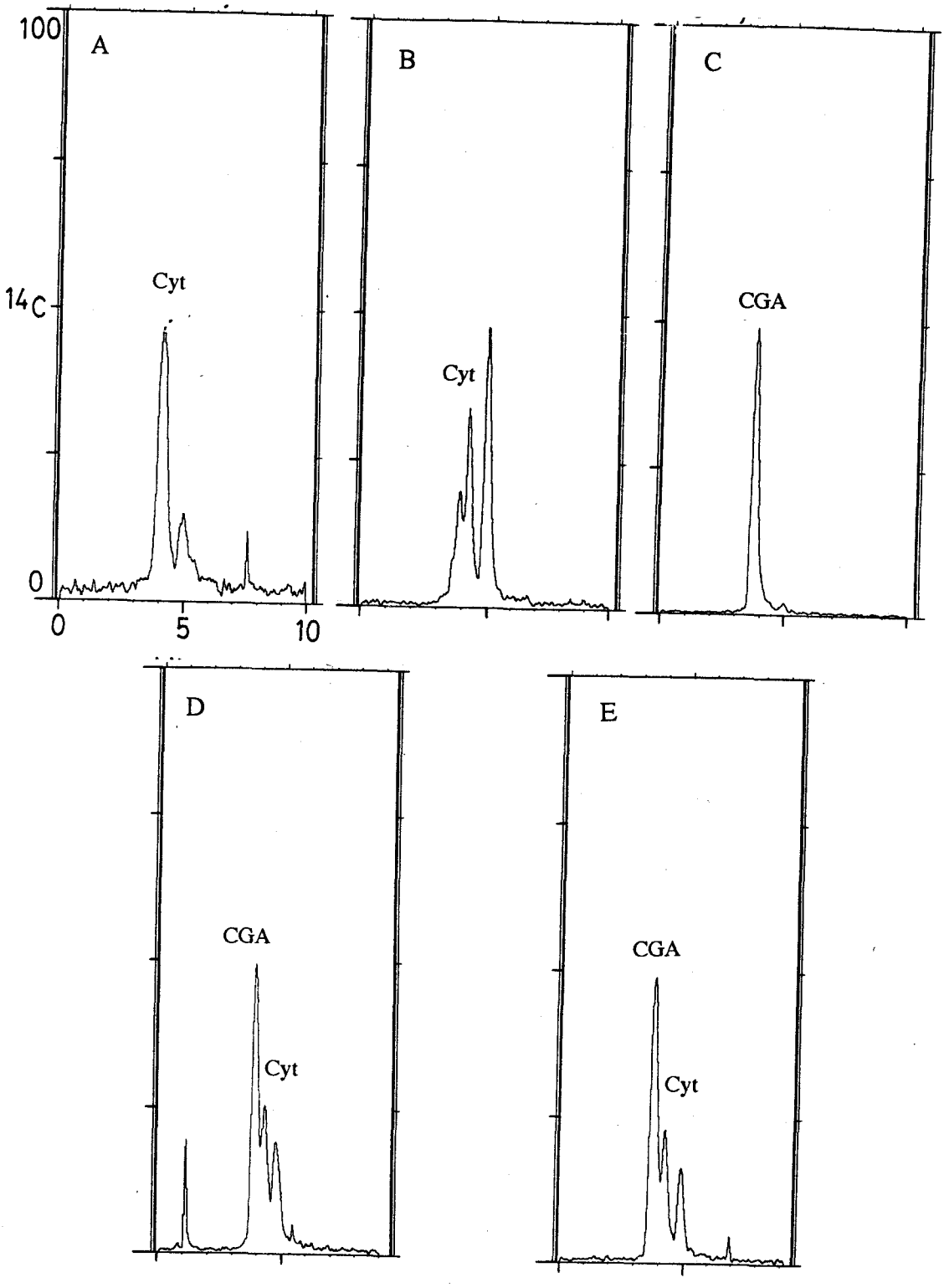


Fig IV-7. HPLC radioactivity traces of UDP-glucose 4'-epimerase and 6'-oxidoreductase analysis

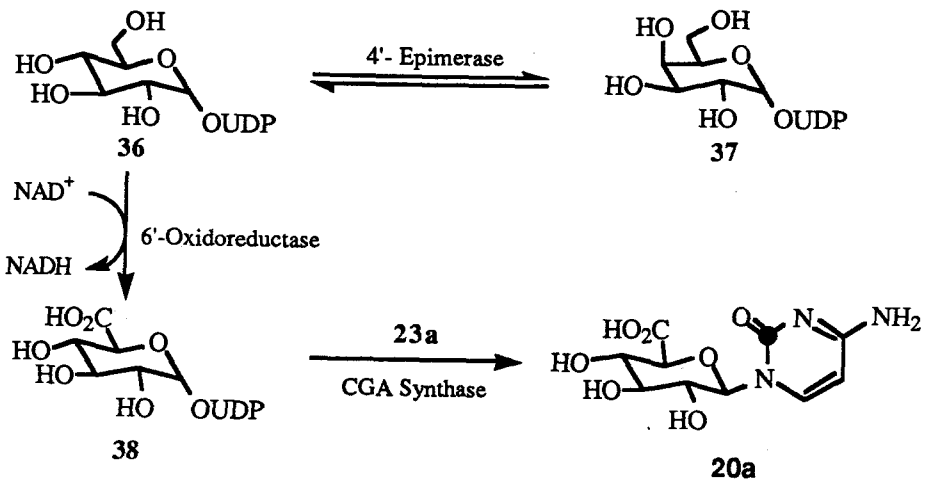


Fig IV-8. Involvement of UDP-glucose 4'-epimerase and 6'-oxidoreductase in the biosynthesis of BS.

Isolation of DeMeBS δ-N-Methyltransferase

The timing of δ-N-methylation was an important aspect in determining the sequence of assembly leading to the BA moiety of BS. However, earlier reports were contradictory. Seto et. al. had reported the isolation of LeucylBS, **6**, from *S. griseochromogenes* and had claimed that leucylBS was the last precursor in BS biosynthetic pathway.[10] In our laboratory, we had found that β-Arg could be incorporated into BS, but MeArg[11] or [1-^{14}C]BA was not (see Chapter II). Results from these whole-cell feedings, along with the stimulatory effect of Eth on DeMeBS production (see Chapter III), had suggested that δ-N-methylation occurred as the last step and DeMeBS should be the immediate precursor to BS.

Initially, we obtained HPLC evidence for the formation of a small quantity of LeucylBS when a fermentation was maintained below pH 4 as previously reported.[10] The leucyl aminopeptidase activity was also probably observed with mycelial suspensions in a neutral buffer,[10] since over a period of several hours authentic LeucylBS was consumed (HPLC assay), although the direct conversion of LeucylBS to

BS could not be established since the mycelia produced a measurable amount of BS under these conditions without the addition of LeucylBS.

With the ready availability of DeMeBS (Chapter III), it was possible to examine the possibility of converting DeMeBS to BS in a fairly convenient fashion.

DeMeBS and the standard methylation agent, [Me-^{14}C]S-adenosyl-L-methionine, [Me-^{14}C]SAM, **45b**, were incubated with the CFE of *S. griseochromogenes*. Monitoring the reaction mixture several hours later by HPLC with again both UV-detection and radiochemical detection revealed a new peak with the approximate retention time of BS (Fig IV-9). No formation of the BS-like peak was detected in the absence of DeMeBS. In order to test if radioactive BS was indeed produced in the incubation, a few milligrams of unlabeled BS were added to the remainder of the reaction mixture and then re-isolated by HPLC. This was further purified by cation exchange chromatography. After recrystallization to constant specific radioactivity, this BS sample, **1g**, contained ^{14}C activity corresponding to a 0.82% of conversion. The experiment was later repeated. In the second incubation, it was found that at least 1.22% of the ^{14}C had been converted to **1g**.

Although the incorporation of radioactivity with the crude CFE was low, it was clearly reproducible. Hydrolysis of the derived **1g** to [CH_3-^{14}C]BA, **3b**, and CN, **2**, was next carried out and radiochemical analysis of the products, **2** and **3b**, revealed that 97.4% of the ^{14}C in **1g** was retained in **3b** (Fig IV-10). The result confirmed that the labeling had been completely specific. Therefore, methylation is the last step in the biosynthesis of BS. While it was probable that LeucylBS could be converted to BS, it was likely to be the result of a non-specific leucylaminopeptidase.[12] BS producing organisms are known to make other acylated derivatives, as well.[13]

Fig IV-9. HPLC UV- and radioactivity traces of DeMeBS δ-N-methyltransferase analysis.

Conditions for the preparation of CFE and for the HPLC are described in the Experimental Section. Each sample was composed of 1.0 μCi of [Me-^{14}C]SAM in 500 μL of CFE. Sample B also contained 1.5 mg of DeMeBS. The samples were incubated at 30 ^{o}C overnight and 45 μL of the supernatant of each was used for the HPLC analysis. The upper-panel in sample B is a UV-trace detected at 275 nm and the lower-panel is a ^{14}C-radioactivity trace.

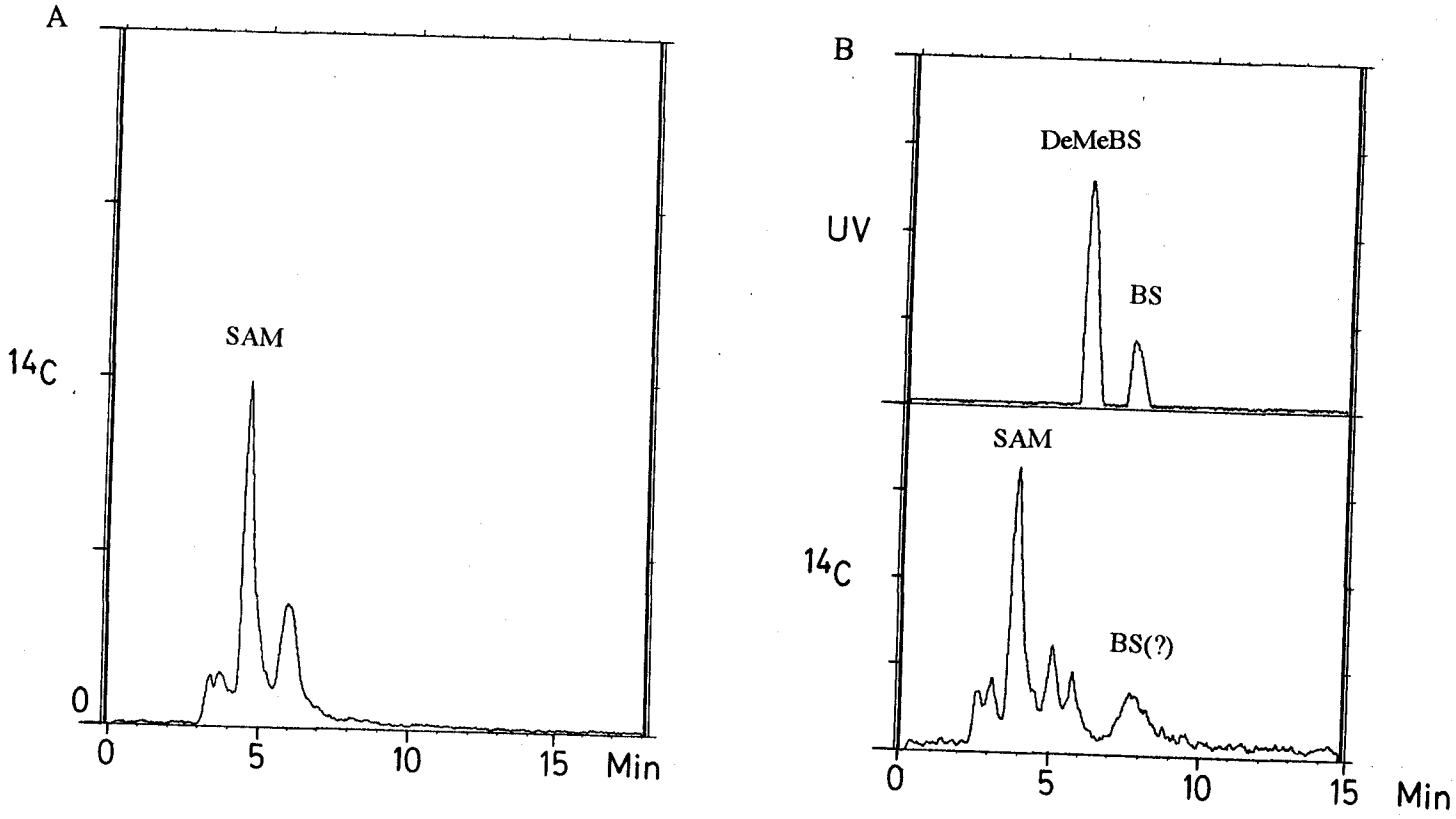


Fig IV-9. HPLC radioactivity traces of DeMeBS δ-N-methyltransferase analysis

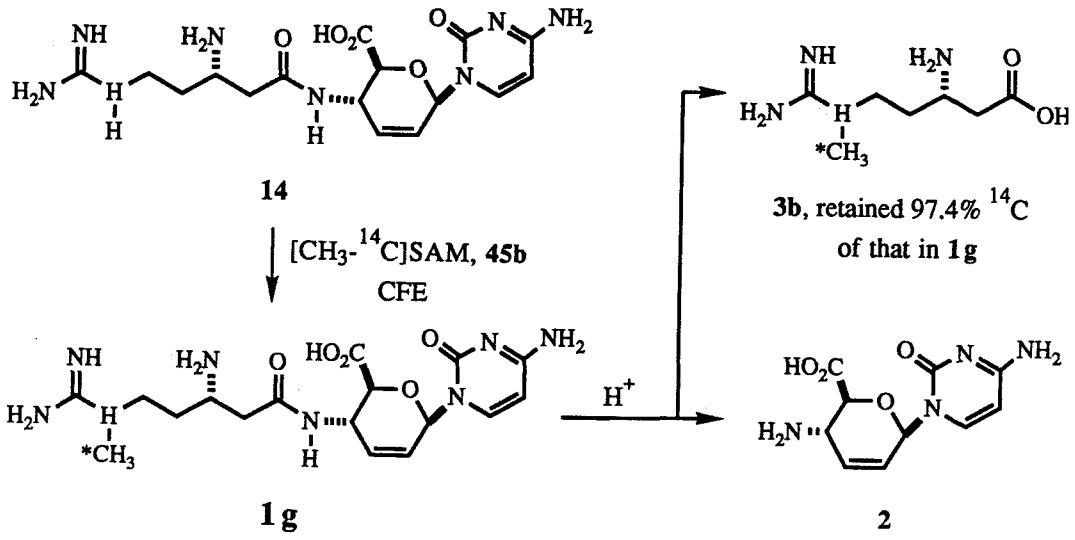


Fig IV-10. Conversion of DeMeBS to BS by CFE

Summary

Studies with CFE have provided the most direct information about the biosynthesis of BS. The isolation of UDP-glucose 4'-epimerase, UDP-glucose 6'-oxidoreductase, CGA synthase and the incorporation of CGA into BS has clarified the early steps of biosynthesis and the first committed intermediate for the nucleoside portion, while the identification of an δ-N-methyltransferase clarified the last step.

The biosynthesis of a number of nucleoside antibiotics have been studied.[14] The biosynthesis of BS appears to be the first instance where the formation of a novel nucleoside has been demonstrated at the cell-free level to be the first committed step in the biosynthetic pathway. In addition, although UDP-glucuronosyl transferases are common in mammalian xenobiotic metabolism[15] and some have also been reported in fungi,[16] this was the first discovery of such an enzyme from a prokaryotic organism.

Experimental

General

All compounds used were reagent grade and used directly without further purification. [U-^{14}C]UDP-glucuronate disodium salt, **36a**, and [CH_3-^{14}C]SAM, **45b**, were purchased from ICN. [2-^{14}C]cytosine, **23a**, was from Research Products International Corp. [5-^{3}H]cytosine, **23b**, and other general chemicals were from Sigma Chemical Company (St. Louis, MO). LeucylBS was a gift from Prof. Haruo Seto. Conditions for the fermentation and for the preparation of synthetic and complex fermentation media were described previously in Chapter II. An IEC B-20A refrigerated centrifuge was used for harvesting fermentation broths, both for collecting mycelia and for collecting the fermentation supernatant for product purification. An Eppendorf model 5414 centrifuge was used for the preparation of samples of small volume, if a centrifugation was needed. Radioactivity measurements were carried out using a Beckman Model LS7800 liquid scintillation counter, as were described in Chapter II. Radiochemical HPLC detector, Flo-One/Beta Radioactive Detector, borrowed from John Westall's Laboratory (Oregon State University) was manufactured by Radiomatic Instruments and Chemical Co, Inc (Tampla, FL), and the LSC cocktails used with the detector were from Research Products International Corp (Mount Prospect, IL). A Waters 600E HPLC system connected to a 990+ photodiode array detector was used for routine analysis. HPLC columns were purchased also from Waters Assoc. Spe^{R} disposable columns (SepPak column) were from J. T. Baker (Philisburg, NJ). Sonicator (Heat-system Ultrasonic Inc, Model W-225R) was used for the disruption of mycelia in CFE preparations.

HPLC Conditions and Enzyme Analysis

Conditions for HPLC analysis were: Nova pack, C_{18}, RadialPak^R cartridge column, 8 mm x 10 cm, 4 μ; 96% H_2O, 4% CH_3CN, 0.1% TFA; 1.0 mL/min. Enzyme activities were analyzed by monitoring the formation of the corresponding product. In analyses where radioactive products were monitored, a Flo-One HPLC radiochemical detector was connected to the outlet. The scintillation cocktail was used at a flow ratio of 4.0 mL/min.

Buffers

Buffer A: 100 mM Tris, 25 mM $MgCl_2$, 25 mM $CaCl_2$, 8.0% sucrose (w/v), pH 7.4;

Buffer B: 50 mM sodium phosphate, 1.0 mM DTT, pH 7.2;

Buffer C: 100 mM Tris, 1.0 mM DTT, pH 8.0.

Preparation of *S. griseochromogenes* Mycelia

Mycelia of *S. griseochromogenes* were collected from synthetic medium fermentation broths at 72-77th hour of incubation by centrifugation of the broths at 10,000 x *g* for 10 min. The pelleted mycelia were washed two times with buffer C and then re-pelleted at 10,000 x *g* for 10 min. Some of the "broth supernatant" was also kept for the following experiments (see below).

Preparation of *S. griseochromogenes* Protoplasts

Protoplasts were prepared according to the method reported.[4] One volume of mycelia was suspended in two volumes of Buffer A (protoplast buffer). To the suspension, solid chicken lysozyme (24,000 units/mg) was added to a final concentration of 1.5 mg/mL and the mixture was incubated at 30 °C for about two hours with periodic shaking. During the incubation, the generation of protoplasts was examined with a phase

contrast microscope. At the end of incubation, the suspension was filtered through a tube (2.0 cm x 10 cm) with loosely packed cotton, and protoplasts in the effluent were collected by centrifugation at 3,000 rpm (Eppendorf, Model 5414) for 7 min. The pellet was washed two times with buffer A (~ 5 mL each) and recentrifuged each time. The protoplasts thus prepared were used for the following experiments.

Testing the Production of BS by Protoplasts

Suspensions of protoplasts either in buffer A or in the broth supernatant in one-tenth of original broth volume were incubated in a rotatory shaker at 180 rpm and 30 °C. Production of BS or any other related compounds was assayed by HPLC.

Feeding CGA to Protoplasts

To examine the absorption of CGA by protoplasts, CGA was added to the above mentioned protoplast suspension to a final concentration of 1.0 mg/mL. The suspensions were incubated at 30 °C and 180 rpm in a rotatory shaker. At regular intervals (30 min, 1 hour, 2 hours, 4 hours and overnight of incubation), about 0.5 mL of the suspension was taken, centrifuged briefly, and 1-5 μL of the supernatant was injected into the HPLC for CGA quantification.

Conversion of LeucylBS by Mycelia Suspensions

One volume of mycelia (~ 5 g, wet weight) was suspended in about five volumes of buffer B. To the mycelia suspension, about 5 mg of LeucylBS was added and the suspension was then incubated in a rotatory shaker at 30 °C, 180 rpm. Supernatant of 1-5 μL of the suspension was used for HPLC analysis (90% H_2O, 10% CH_3CN and 0.1% TFA, 1.0 mL/min). Eluted with this solvent system, LeucylBS had a t_R of ~ 3.5 min and BS, of ~ 2.8 min. LeucylBS disappeared completely generally after 2 hours of incubation.

Preparation of the CFE

CFE's of *S. griseochromogenes* that were used for enzyme assays were prepared either in buffer B or buffer C. Typically, one volume of mycelia was suspended in two volumes of pre-cooled buffer and disrupted by sonication. Disruption was conducted at power level 8 and 40% duty cycle for 3 min in an ice-ethanol bath, with one minute break after each minute of sonication. The CFE thus prepared was generally used directly without centrifugation. In the case of preparing doubly-labelled CGA, **20b**, after sonication the CFE (5.0 mL) was briefly centrifuged with an Eppendorf model 5414 centrifuge for 10 min at room temperature and then dialysed against a 100-fold volume of buffer at 4 °C for 4 hours (changing the buffer once after about 2 hours of dialysis). The dialysis procedure was to remove small molecules in the CFE and thus to simplify the procedures for product purification.

CGA Synthase Activity Assays on an Analytical Scale

Reaction samples were prepared by taking 300 μL of CFE mixed with solid substrates, UDP-glucuronic acid and cytosine (to a final concentration of 0.2 mg/mL of each) and bringing the total volume to 500 μL with the same buffer as was used in preparing the CFE. The reaction mixture was then incubated at 30 °C. The production of CGA was assayed by withdrawing 50 μL of the reaction mixture, mixing it with 100 μL of methanol, vortexing thoroughly and then centrifuging for 10 min. Two microliters of the supernatant were injected for HPLC analysis. The percent conversion of a reaction was estimated from the decrease of the cytosine peak and was about 30% after 4 hours incubation.

Preparation of CGA, 20, with CFE

CGA was prepared enzymatically by mixing 50 mL of CFE (prepared in buffer B), 50 mL of buffer B, 100 mg (0.16 mmol) of UDP-glucuronate trisodium salt, **36**, and

20 mg (0.18 mmol) of cytosine, **23**. The reaction mixture was incubated at 30 °C for about 20 hours (shaken periodically in the early period of incubation). The reaction was monitored by HPLC at the 0th, 9th and 20th hour of incubation. The pH of the reaction was decreased to 6.6 after 9 hours incubation and thus was readjusted to pH 7.2 with 1.0 N KOH. At the 20th hour of incubation, the conversion was estimated to be 50% based on the integration area of UDP-glucuronic acid peak. TCA (30%, 50 mL) was added and the mixture was stirred thoroughly for several minutes. Precipitates were removed by centrifugation (with IEC B-20A) at 10,000 x*g* for 10 min. The resulting supernatant was adjusted to pH 6.0 with 1 N HCl and loaded onto a cation exchange column (AG 50W x 4, H^+, 100-200 mesh, 3 cm x 10 cm). The column was first washed with water to neutral and then with 5% pyridine. The eluate was monitored for CGA by HPLC. Fractions containing CGA were combined and loaded directly onto an anion exchange column (IRA-410, AcO^-, 3 cm x 10 cm) without removing the pyridine. The anion column was first washed with water (100 mL) and then eluted by a stepwise increase of AcOH concentration (1 mM, 100 mL; 5 mM, 50 mL; 20 mM, 50 mL and finally 1.0 M, 150 mL). The eluates were also monitored by HPLC. Most CGA was eluted in the 1.0 M AcOH solution. CGA fractions were pooled and evaporated to dryness at ambient temperature under reduced pressure (with an aspirator), taken-up in ~4 mL of H_2O and filtered through a C_{18} SepPak column (3.0 mL bed volume). Effluent from the C_{18} SepPak column was lyophilized and then recrystallized from hot H_2O to give about 10 mg of CGA, which was analyzed by ^1H NMR spectroscopy. The ^1H NMR spectrum showed the product to be identical to CGA that was prepared from a fermentation broth (see Chapter III).

Specificity Analysis of CGA Synthase

To test the specificity of CGA synthase, 1.0 μCi of [2-^{14}C]cytosine, **23a**, (43 mCi/mmol in 0.01 N HCl) was incubated with 1.0 mg of one of the four UDP-

glycosides, **36**, **37**, **38**, **39**, (UDP-glucose, UDP-galactose, UDP-glucuronic acid and UDP-galacturonic acid, respectively) and 500 μL of CFE (prepared in buffer C) of *S. griseochromogenes* (collected from 74 hour old fermentation). The reaction was incubated at 30 °C for 20 hours. At the end of the incubation, 10 μL of saturated cytosine solution was added to each vial as a reference peak for identifying components in the HPLC traces. The reaction samples were centrifuged (Eppendorf) for 10 min and 25 μL of each supernatant was analyzed by HPLC with both UV- and radiochemical detection. Only the UDP-glucuronic acid incubation yielded [^{14}C]CGA, **20a** (Figure IV-3, panel B).

Preparation of [^{3}H,^{14}C]CGA, 20b, with CFE

To a vial containing 10.95 μCi of UDP-glucuronic acid trisodium salt ([U-^{14}C] in the ulonate part, 250 μCi/μmol in 500 μL of 20% ethanol), **38a**, 52.45 μCi of [5-^{3}H]cytosine, **23b**, (19.9 mCi/μmol in 500 μL of 0.01 N HCl) and 400 μL of CFE (prepared in buffer B) were added. The CFE had been dialysed for 4 hours as was previously described. The reaction mixture was incubated at 30 °C and gently shaken periodically. After 40 min of incubation, 1.0 μL of a saturated cytosine solution (contained about 8 μg cytosine) was added. The addition of unlabeled substrates was further repeated every 30 min of incubation as follows: 20 μL UDP-glucuronic acid (1.0 mg/mL of H_2O solution), 20 μL UDP-glucuronic acid, and 20 μL UDP-glucuronic acid with 1.0 μL of saturated cytosine solution. One hour after the last addition, 40 μL more UDP-glucuronic acid was added and the reaction mixture was incubated overnight. The adding of unlabeled substrates was to push the reaction to give higher percent conversion of the radioactive substrates. After 13 hours, the reaction mixture was centrifuged (IEC-B20A) at 10,000 x*g* for 10 min, and the pellet was washed with 400 μL of CGA solution (2.0 mg/mL) and centrifuged again at 15,000 x*g* for 10 min. The supernatants were combined and lyophilized, redissolved in ~1.0 mL of water and filtered through a

C_{18} SepPak column. The column was washed with several milliliters of CGA solution (3 mg/mL). The eluates were combined and authentic CGA (45 mg) was added. The resulting solution was designated as Sol-A (6.20 mL). From Sol-A, 5.0 μL was taken and combined with 21.73 mg of authentic CGA and was designated as Sol-B. Sol-A and Sol-B were recrystallized separately three times and Sol-A gave 25.3 mg of CGA and Sol-B gave 10.0 mg. However, the ^{3}H to ^{14}C activity ratio obtained from Sol-A was different from that obtained from Sol-B (4.07 and 2.99 respectively). To rectify this discrepancy, CGA obtained from Sol-A (after recrystallizations) was redissolved in 4.060 mL of water, 10 μL was combined with 2.764 mL CGA solution (17.5 mg of authentic CGA), and was designated as Sol-C. Recrystallization one time yielded 13.0 mg CGA. LSC showed that the ^{3}H to ^{14}C ratio of CGA from Sol-C was 4.25 and showed that the CGA had the same specific activity before and after recrystallization. Thus it confirmed that Sol-A was radiochemically pure and had a ^{3}H to ^{14}C ratio of 4.25. The specific activity for **20b** thus prepared was 4.49×10^{5} dpm/mg (1.26×10^{8} dpm/mmol) of ^{14}C and 1.91×10^{6} dpm/mg (5.37×10^{8} dpm/mmol) of ^{3}H.

Feeding [^{3}H,^{14}C]CGA, 20b

To a 200-mL fermentation (complex medium), 10 mg of **20b** was fed at the 39th hour of incubation. The fermentation was continued for 98 hours and worked up as usual. The broth produced about 400 mg of BS, to which 55 mg of unlabeled BS was added. After standard anion and cation chromatography, 360 mg of BS was obtained. The BS was recrystallized repeatedly to give a constant specific radioactivity. LSC data are summarized in Table IV-1 and Table IV-2.

Table IV-1. Fermentation data of **20b** feeding

Size of broth	200 mL
Fermentation medium	Complex
20b fed (at 39th hour)	10 mg (4.49×10^6 dpm of ^{14}C, 1.91×10^7 dpm of ^{3}H)
BS, **1f**, produced	400 mg
Unlabeled BS added	55 mg
BS, **1f**, purified	373 mg
20b recovered	88.4% based on ^{14}C

Table IV-2. LSC data of **20b** feeding

Recrystallization	dpm/mg*	$^{3}H/^{14}C$	incorporation% (based on ^{14}C)
Before recryst.	22.4	6.74	
1st	22.1	6.72	
2nd	22.3	6.73	
3rd	22.2	6.84	
Average	22.2	6.75	1.9%**

* A background of 30 dpm for ^{14}C and 19 dpm for ^{3}H was subtracted from each counting data before used for calculation. In each LSC, enough material was used to obtain at least 150 dpm.
** Based on **20b** consumed.

Hydrolysis of BS, 1f, to CN, 2c

BS, 1g (300 mg from the third recrystallization) was dissolved in 8.0 mL of 3 N H_2SO_4 and heated to 90 °C (the temperature was accidentally increased to over 110 °C for an unknown period of time). After 24 hours, it was estimated that more than 75% of **1f** was hydrolyzed. The reaction mixture was cooled to room temperature, loaded onto an anion exchange column (Amberlite, IRA-410, OH^-, 2.5 cm x 30 cm), washed thoroughly with water (to pH < 8) and then with 1.0 M AcOH (500 mL). Products **2c** and **23b**, were co-eluted in the AcOH fraction. The mixture was lyophilized, redissolved in ~4.0 mL of H_2O, and filtered through a C_{18} SepPak column to remove colored impurities and undissolved particles. The filtrate was lyophilized again and recrystallized from H_2O-acetone once which gave 65 mg of a mixture of **2b** and **23b**.

Separation 2c from 23b

The mixture of **2c/23b** (45 mg) was redissolved in a few milliliters of water and loaded onto a cation exchange column (S-sepharose, 2.5 cm x 10 cm, pre-equilibrated with potassium phosphate buffer, 30 mM, pH 2.0), washed with water and then with a KCl gradient (0 to 1.0 M, 500 mL total) in the same phosphate buffer. Fractions of 3 mL were collected. Cytosine, **23b**, eluted at about 0.30 M KCl and **2c** at about 0.45 M. Each solution was lyophilized, taken up in a few milliliters of water, and desalted with the same S-sepharose column (NH_4^+ form) by eluting with a gradient of 0-0.1 M of NH_4OH solution (500 mL total). The appropriate fractions were lyophilized. Product **2c** was recrystallized from water-acetone to yield 27.4 mg. Cytosine, **23b** (21.4 mg), was used for LSC directly. LSC data of **2c** and **23b** are tabulated in Table IV-3.

Table IV-3. LSC data of **2b** and **23b**

Product	dpm/mg*	dpm/mmol	$^3H/^{14}C$
2c	38.5 dpm/mg (^{14}C)	9.67 x 10^3 dpm/mmol	6.25
23b	525.0 dpm/mg (^3H)	5.84 x 10^4 dpm/mmol	99.5

* The same note "*" in Table IV-2 apply.

Isolation of UDP-glucose 4'-Epimerase and 6'-Oxidoreductase

CFE used for this experiment was prepared from a 77-hour-old mycelia in a standard manner (prepared in buffer C). Reaction samples were prepared as follows: to each vial, the corresponding substrates (solid) were added followed by adding 250 μL of CFE (see Table IV-4 for sample compositions). The reaction mixture was incubated at 30 °C for 24 hours. Enzyme activities were assayed by taking 100 μL of the mixture, centrifuging (Eppendorf model 5414) for 10 min and injecting 5 μL of the supernatant onto the HPLC, which was connected with both a UV-detector and a radiochemical HPLC detector. The identity of [2-^{14}C]CGA, **20a**, produced in sample #5 was proved

by adding 190 mg of unlabeled CGA and 10 mL of water to the supernatant of the remainder of the reaction mixture (~230 μL, centrifuged with Eppendorf model 5414 for 10 min), and re-isolating the CGA by anion exchange chromatography (Amberlite, IRA-410, AcO^-, 3.0 cm x 10 cm). After the column had been washed thoroughly with water (to neutral pH), CGA was eluted by 0.5 N HCl. The eluate was lyophilized, filtered through a C_{18} SepPak column (washed with 10-15 mL of water) and then rechromatographed with the same anion exchange column (but in the OH^- form) with the same procedures. CGA fractions were combined, lyophilized and recrystallized from hot H_2O for LSC. LSC data are listed in Table IV-5.

Table IV-4. Sample compositions in UDP-glucose 4'-epimerase and UDP-glucose 6'-oxidoreductase analysis

Substrate (mg)	1	2	3	4	5
23a (0.5 μCi)	+	+	+	+	+
38 (0.6 mg)	-	-	+	-	-
36 (0.5 mg)	-	-	-	+	-
37 (0.5 mg)	-	-	-	-	+
NAD^+ (1.1 mg)	-	+	-	+	+
CFE (250 μL)	+	+	+	+	+

Table IV-5. LSC data of **20a** from UDP-galactose

Recrystallization	dpm/mg
1st	2238
2nd	2235
3rd	2254
Average	2242

Percent conversion of **23a** to **20a** in sample #5 was calculated as follows:

$$\% \text{ conversion} = \frac{2242 \text{ dpm/mg} \times 190 \text{ mg}}{0.5 \text{ μCi} \times 2.2 \times 10^6 \text{ dpm/μCi}} \times \frac{250 \text{ μL}}{230 \text{ μL}} = 42.1\%$$

Isolation of DeMeBS δ-N-Methyltransferase

To a vial containing 1.5 mg of DeMeBS, **14**, was added 500 μL of CFE (prepared from 74 hour old mycelia) and 10 μL (1.0 μCi) of stock [CH_3-^{14}C]SAM, **45b**, (45 mCi/mmol, in 0.01 N H_2SO_4, 10% ethanol). The reaction mixture was vortexed gently and incubated at 30 °C for 20 hours. A control sample was also prepared without adding **14**. After incubation, 100 μL of the sample was taken, centrifuged (Eppendorf model 5414) for 10 min, and 45 μL was injected onto the HPLC (UV-detection and radiochemical detection).

To the remaining 390 μL of the reaction mixture, 3 mg of BS was added and then re-isolated with HPLC (with UV detection only) by repeatedly injecting 40 μL aliquots. The BS containing fractions were combined (~25 mL). Additional unlabeled BS (350 mg) was added, and the combined material was re-purified by cation exchange chromatography (Dowex 50W x 4, H^+, 3.0 cm x 30 cm). The column was first washed with 5% pyridine and then with 1.2% NH_4OH. BS eluted with the NH_4OH was lyophilized and recrystallized from H_2O-MeOH to constant specific radioactivity. This gave 236 mg of BS, **1g**, (40 dpm/mg of ^{14}C).

The percent conversion of **45b** to **1g** was calculated as follows:

$$\% \text{ conversion} = \frac{40 \text{ dpm/mg} \times 353 \text{ mg}}{1.0 \text{ μCi} \times 2.2 \times 10^6 \text{ dpm/μCi}} \times \frac{500 \text{ μL}}{390 \text{ μL}} = 0.82\%$$

In a second incubation, 0.65 mg of DeMeBS and 2.0 μCi of **45b** were used in a total incubation volume of 500 μL. After 20 hours incubation at 30 °C, analysis by HPLC again revealed a new peak. Two milligrams of BS was added to the remainder of the reaction mixture (420 μL), fractionated by HPLC, lyophilized and redissolved in ~4 mL of H_2O. To the solution, 238 mg of BS was added and then recrystallized from H_2O-

MeOH to constant specific radioactivity. The product **1g** obtained had an activity of 185 dpm/mg.

The percent conversion of **45b** to **1g** in the second incubation was calculated as follows:

$$\% \text{ conversion} = \frac{185 \text{ dpm/mg} \times 240 \text{ mg}}{2 \ \mu\text{Ci} \times 2.2 \times 10^6 \text{ dpm/}\mu\text{Ci}} \times \frac{500 \ \mu\text{L}}{420 \ \mu\text{L}} = 1.2\%$$

Hydrolysis of 1g to 2 and 3b

Hydrolysis of **1g** was carried out with the revised method (see Chapter II), i. e. with 6 N HCl instead of 3 N H_2SO_4. Seventy milligrams of **1g** from the first incubation (40 dpm/mg) and 125 mg from the second incubation (185 dpm/mg) were combined (133 dpm/mg, 6.1 x 10^4 dpm/mmol) and hydrolyzed at 110 °C for 5 hours. The hydrolyzate was evaporated to remove HCl and then redissolved in 10 mL of H_2O and loaded onto a anion exchange column (Amberlite IRA-410, OH^-, 3.5 cm x 15 cm). The column was washed with H_2O. The effluent and H_2O eluate were combined, adjusted to pH 1.0 with 6 N HCl, lyophilized and then filtered through a C_{18} SepPak column. Product [CH_3-^{14}C]BA, **3c**, from the SepPak column (119 mg) was again lyophilized and recrystallized from ethanol to constant specific radioactivity of 227 dpm/mg (5.9 x 10^4 dpm/mmol, 97.2% of expected).

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Chapter V

Stereochemistry of the C-3' Deoxygenation

Introduction

C-3' deoxygenation leading to the nucleosides in BS and its related metabolites could be a mechanistically very interesting biochemical transformation. Our earlier whole-cell feedings of [2,3,4,6,6-$^2H_5$]glucose, **24a**,[1] and [1,1-$^2H_2$]ethanol had revealed a more complicated mechanism than a simple dehydration process. Having elucidated the mechanism of CGA formation and shown CGA to be the first committed intermediate to BS, the question could now be addressed as to how CGA is transformed into BS and PPNC (Fig V-1).

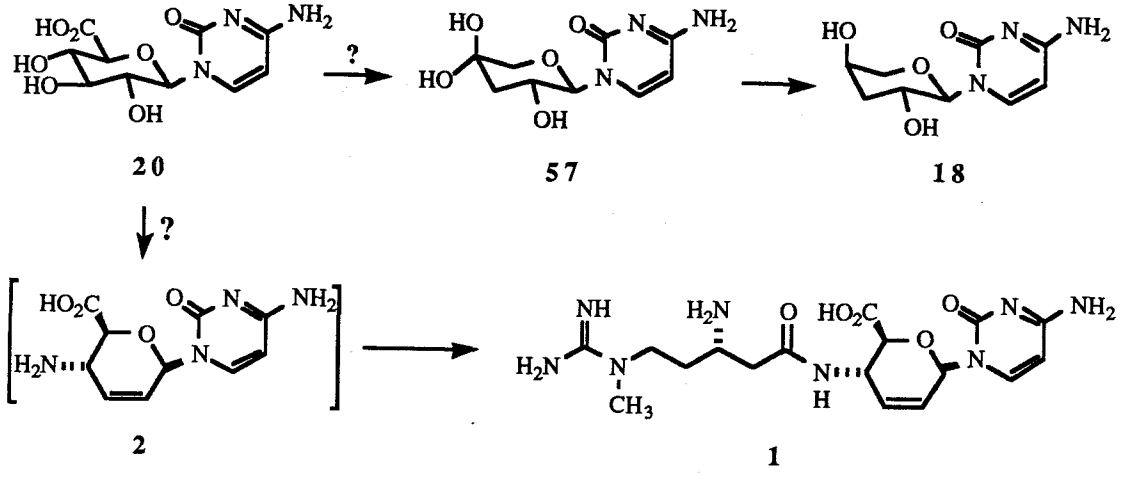

Fig V-1. Transformation of CGA

Unfortunately, in our earlier effort towards the identification of biosynthetic enzymes beyond CGA synthase for the nucleoside transformations, we were not able to identify any enzymatic activity (see Chapter IV). Inhibitor feedings did not result in the accumulation of intermediates that could possibly sit between the conversion of CGA to CN either, although the production of an apparent shunt pathway product, PPNC, was significantly increased. Nonetheless, the accumulation of PPNC provided an alternative

approach to exploring the mechanism(s) of C-3' deoxygenation in the biosynthesis of BS and PPNC. Since from their structures it was fairly clear that PPNC and BS should be derived from the same biosynthetic precursor, presumably the same deoxygenation mechanism would apply for the formation of both of them.

C-3' deoxygenation is also an important biochemical transformtion in the biosynthesis of a class of 3',6'-dideoxy carbohydrates which are found solely in the lipopolysaccharide components of a number of Gram-negative bacterial cell envelopes.[2] These unusual sugars have been shown to contribute to the serological specificity of many immunologically active polysaccharides.[3] In the past two decades, substantial efforts have been devoted to explore their biosynthesis. The identity of the biosynthetic precursor for four of the five known 3',6'-dideoxyhexoses - paratose, abequose, tyvelose and ascarylose - has been shown to be CDP-glucose,[4] while the fifth, colitose, is derived from GDP-glucose.[5]

These 3',6'-dideoxyhexoses are formed by the action of three consecutive enzymes, two of which are NAD^+-dependent oxidoreductases,[6,7] while the third one is a PMP-linked enzyme. The most interesting aspect is that no net transamination process is involved in the reactions,[8] in spite of the requirement for a PMP-dependent enzyme. Recently, enzymes that catalyze the biosynthesis of ascarylose, **63**, have been purified[9,10] and through a detailed mechanistic study with the pure enzymes, the processes of 3',6'-dideoxygenation from CDP-glucose, **64**, have been proposed to be through the sequential reactions of an NAD^+-mediated oxidoreduction, a novel PMP-mediated β-elimination,[9] and a novel NAD^+-dependent electron transfer reaction,[10] as shown in Figure V-2. By these processes, CDP-glucose, **64**, is first converted to CDP-4'-keto-6'-deoxyglucose, **65**, and then to ascarylose. However, in these proposed transformations, the postulated intermediates, **66**, **67** and **68** have not yet been found, and the fate and the stereochemistry of the original carbinol hydrogen at C-3' remains unknown.[11]

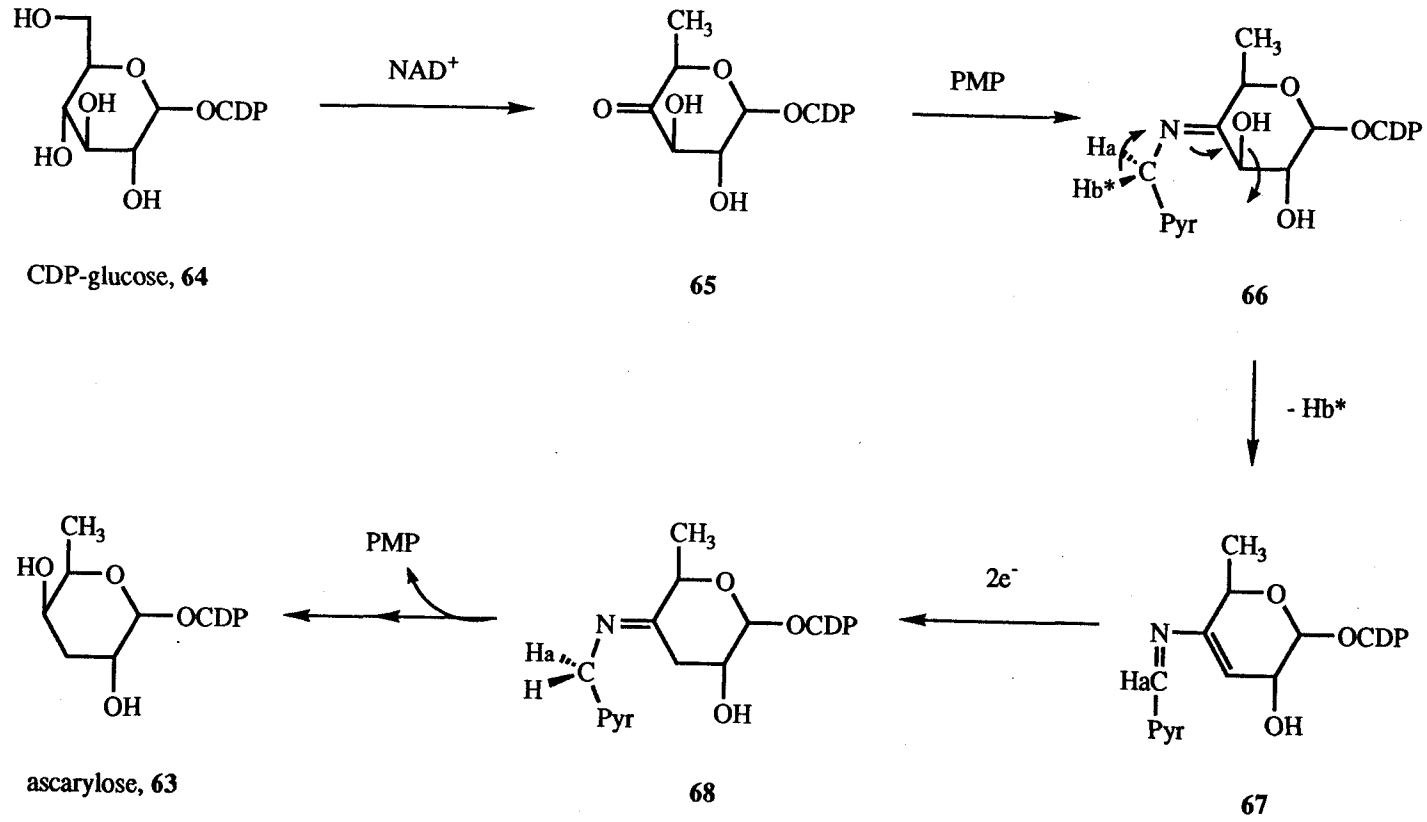

Fig V-2. Proposed mechanisms for C-3' deoxygenation in the biosynthesis of ascarylose[9]

Our earlier whole-cell feedings of metabolic inhibitors had also suggested a PMP(PLP)-dependent catalysis in the biosynthesis of BS and, presumably, also in the formation of PPNC. The remarkable structural similarities of C-3' deoxygenation in PPNC and ascarylose suggested a potential similarity in the mechanisms of their formation.

It now appeared possible to elucidate part of the mechanism of C-3' deoxygenation by tracing the fate of the original C-3 carbinol hydrogen of glucose in PPNC biosynthesis. A feeding experiment with [3-^{2}H]glucose, **24d**, would reveal the fate of the H-3 during the C-3' deoxygenation.

Feeding [3-^{2}H]Glucose, 24d

The deuterium labeled glucose, **24d**, was previously synthesized in our group by Dr. V. A. Palaniswamy following the literature precedure.[12] [3-^{2}H]Glucose was fed to a complex medium fermentation (1 L) with the protocols that had been established.[1] In order to promote the production of CGA and PPNC, large quantities of cytosine and ArgH were also fed (see Chapter III). This fermentation produced 112 mg of CGA, **20c**, and 170 mg of PPNC, **18a**, after 160 hours of incubation. Products **20c** and **18a** were purified by ion exchange chromotography and HPLC as described in Chapter III.

Deuterium NMR analysis of **18a** revealed that the deuterium label had been retained preferentially (85%) at H-3'$_{axial}$. A small amount of deuterium (15%) was present at H-3'$_{equatorial}$ (Fig V-4). As expected, the deuterium label was still found at H-3' of **20c** (Fig V-5). The deuterium enrichment in **18a** was 3.3% and in **20c** it was 3.7%. In combination with the result previously obtained from feeding **24a**,[1] the fate of the H-3 of glucose can be displayed as shown in Figure V-3.

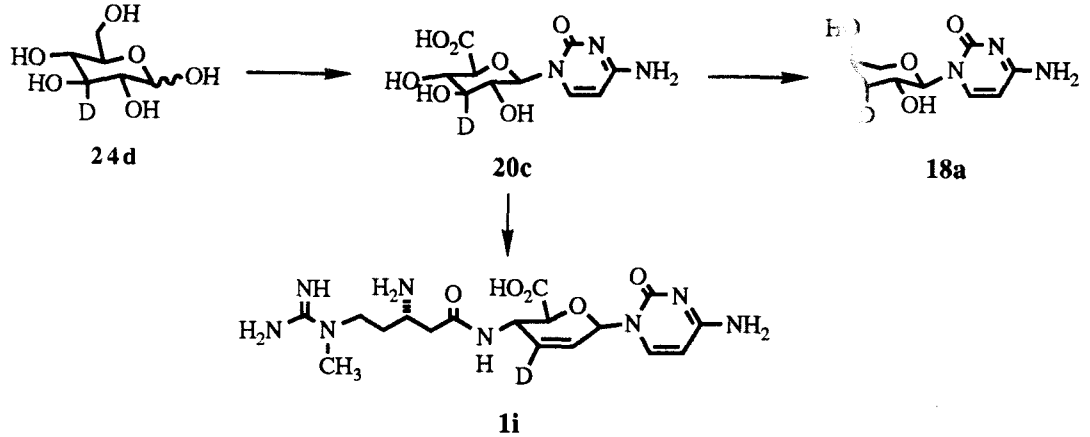

Fig V-3. Stereochemistry of the C-3' deoxygenation

The presence of 15% of deuterium at H-3'$_{equatorial}$ presented a problem since enzyme catalyzed reactions are usually stereospecific. However, in the same

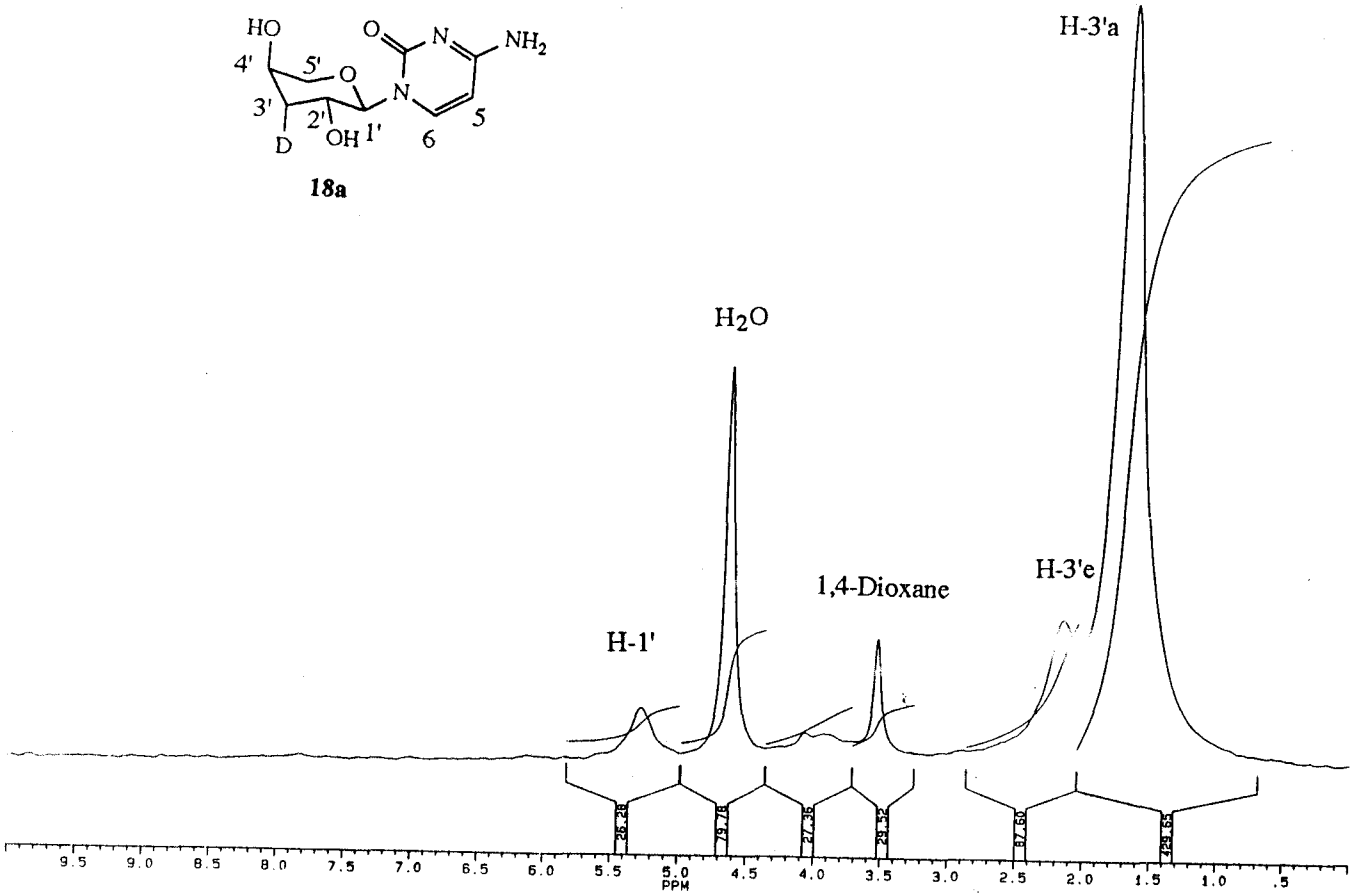

Fig V-4. ^{2}H NMR spectrum of PPNC, **18a**

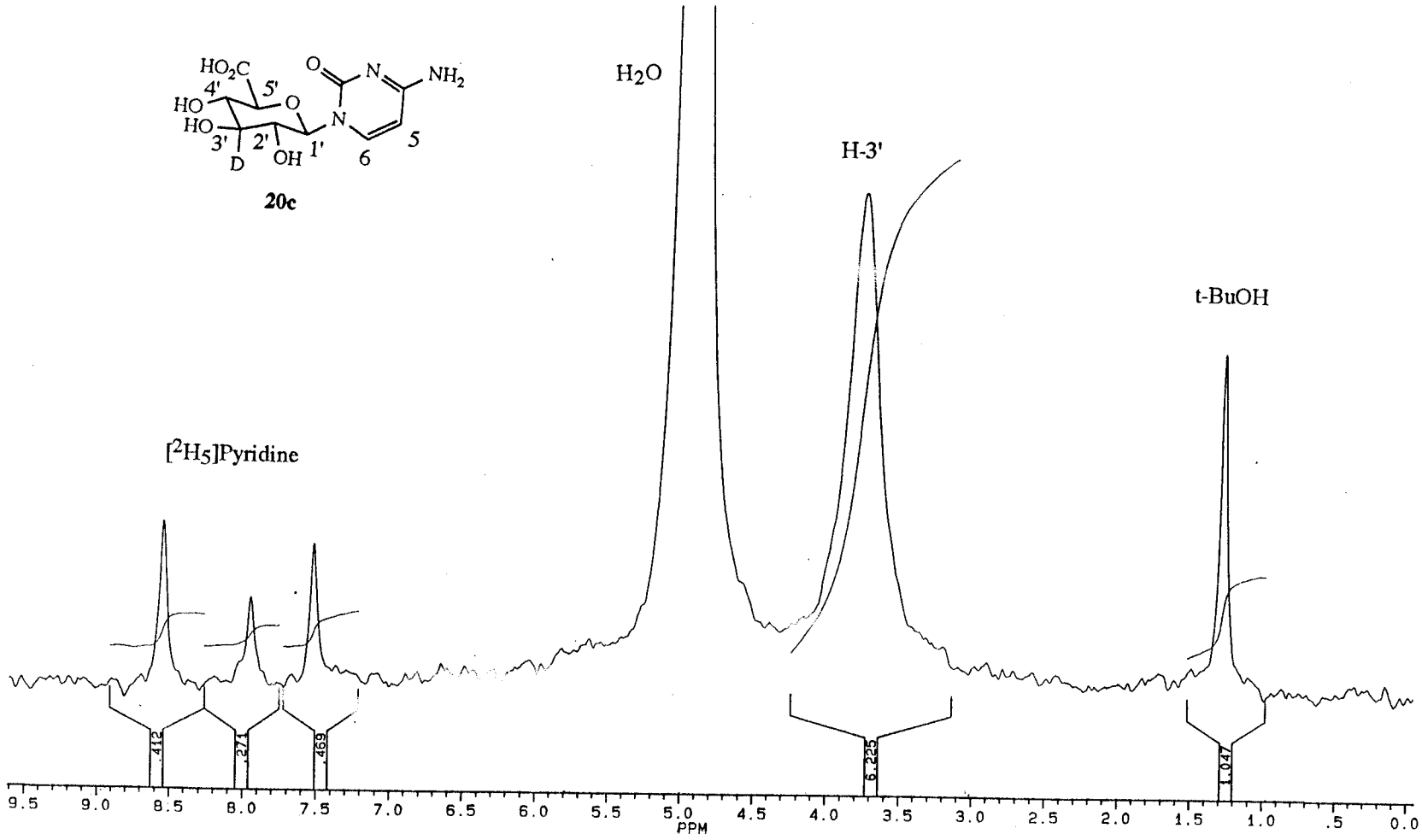

Fig V-5. ^{2}H NMR spectrum of CGA, **20c**

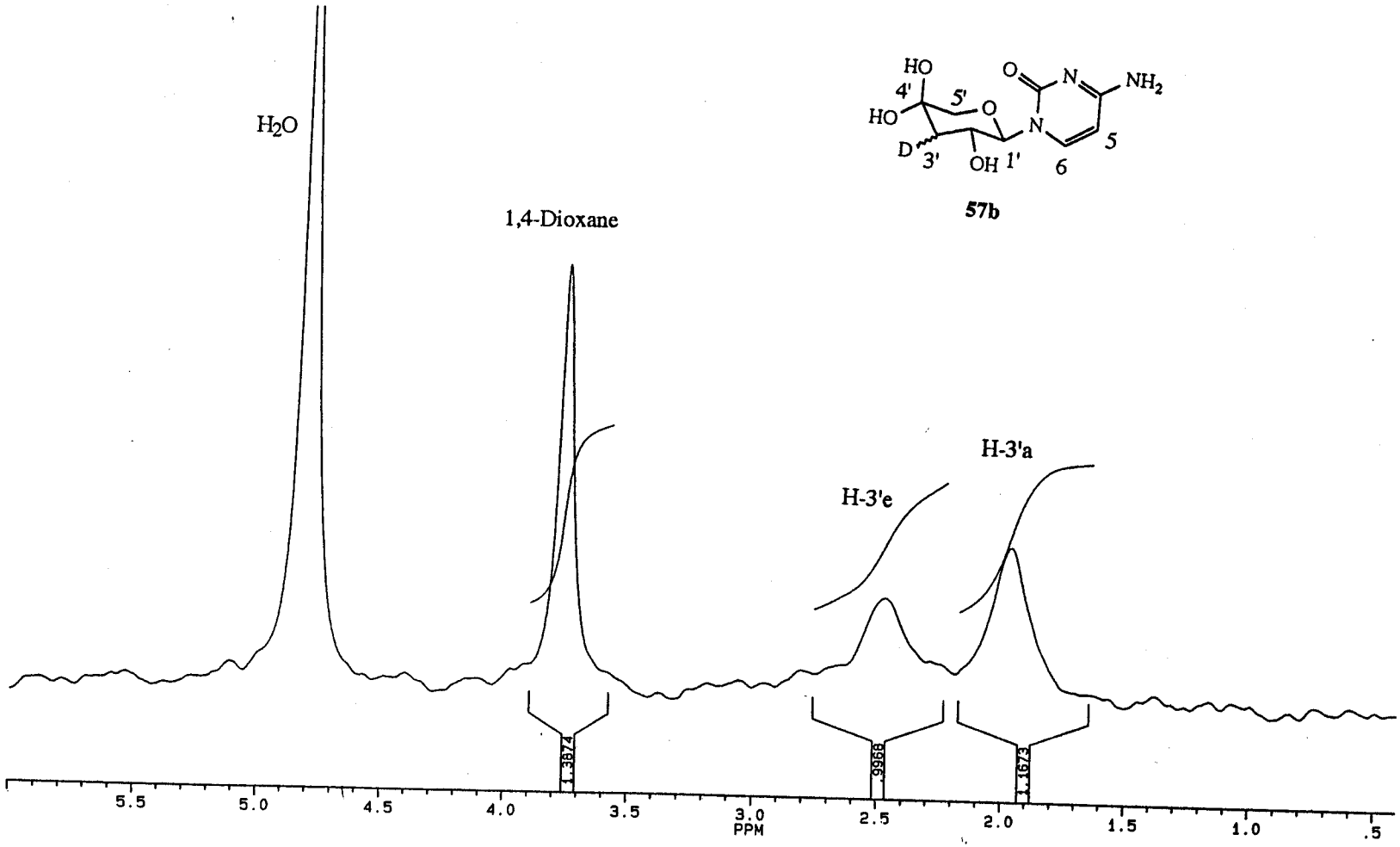

Fig V-6. ^{2}H NMR spectrum of PPN, **57b**

fermentation, we also isolated a few milligrams of [3'-^{2}H]PPN, **57b**, which had deuterium enrichment both at the H-3'$_{axial}$ and the H-3'$_{equatorial}$ to almost the same extent (54 : 46, relatively) as shown in Figure V-6. This suggested that before PPN was converted to PPNC, the H-3'$_{axial}$ could be epimerized to the H-3'$_{equatorial}$. Therefore, the 15% ^{2}H at the H-3'$_{equarorial}$ of **18a** seemed to result from a chemical epimerization rather than from a lack of stereospecificity of the deoxygenation enzyme(s). In order to confirm the possibility of H-3 epimerization, a deuterium-exchange experiment was carried out. It was revealed that both of the H-3' hydrogens in PPN, **57**, were completely exchanged when PPN was treated with D_2O at pD 9.3 at room temperature for 45 min (see Fig III-12 for the ^{2}H NMR spectrum of the deuterium exchange product, **57a**, p 74).

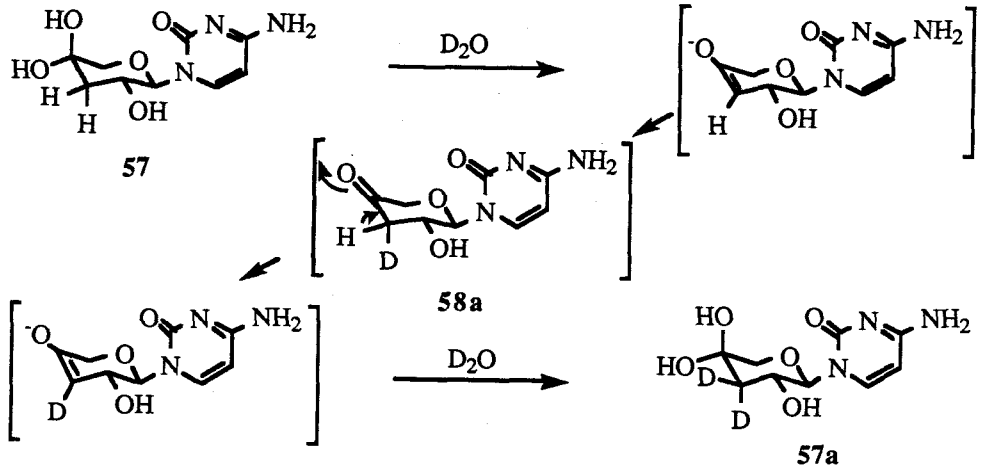

Fig V-7. Formation of [3',3'-$^2H_2$]PPN, **57a**, from PPN, **57**

This deuterium exchange experiment proved that the chemical enolation-reprotonation process could convert **57** to **57a** (Fig V-7) and, in the same way, should be able to convert **57c** to **57d** (Fig V-8). Therefore, the product, **18a**, was really a mixture of **18b** and **18c** that was generated from the process shown in Figure V-9.

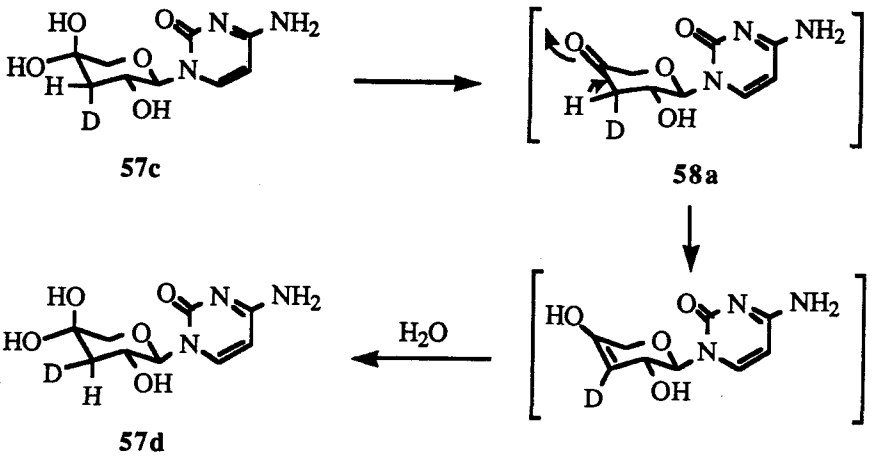

Fig V-8. Epimerization of ^{2}H-3'axial to ^{2}H-3'equatorial

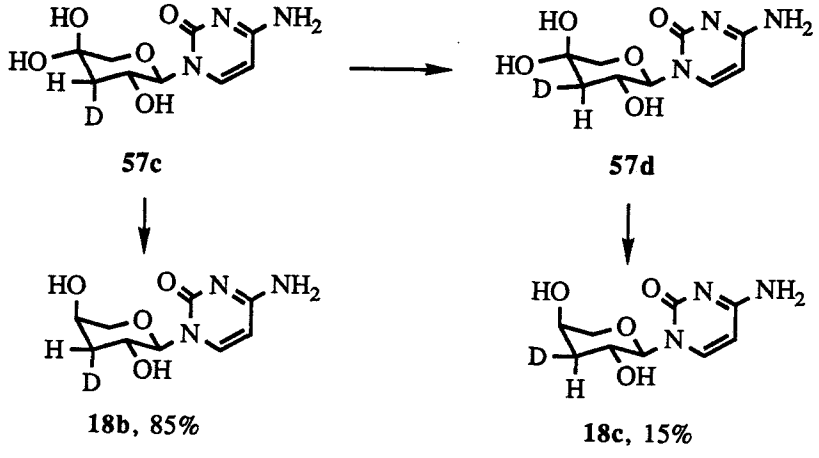

Fig V-9. Explanation for the existence of ^{2}H-3'*e* in **18a** isolated from **24d** feeding

The net retention of configuration at C-3' of **18a**, the implied involvement of PMP, and the earlier result[1] that H-3 of glucose **24a** is retained in **1a** leads us to propose the mechanism to that of ascarylose biosynthesis[10] as shown in (Fig V-10) for the formation of **1h** and **18a** from **20d**. In the proposed mechanism, C-6' decarboxylation leading to **18a** should be similar to that in UDP-xylose biosynthesis.[13] The mechanism for C-2'/C-3' dideoxygenation in **1h** biosynthesis could be accommodated by elimination of the 2'-OH from the proposed intermediate, **69**, rather than protonation at C-3', now leading to the proposed intermediate, **70**, and finally to BS (Fig V-10).

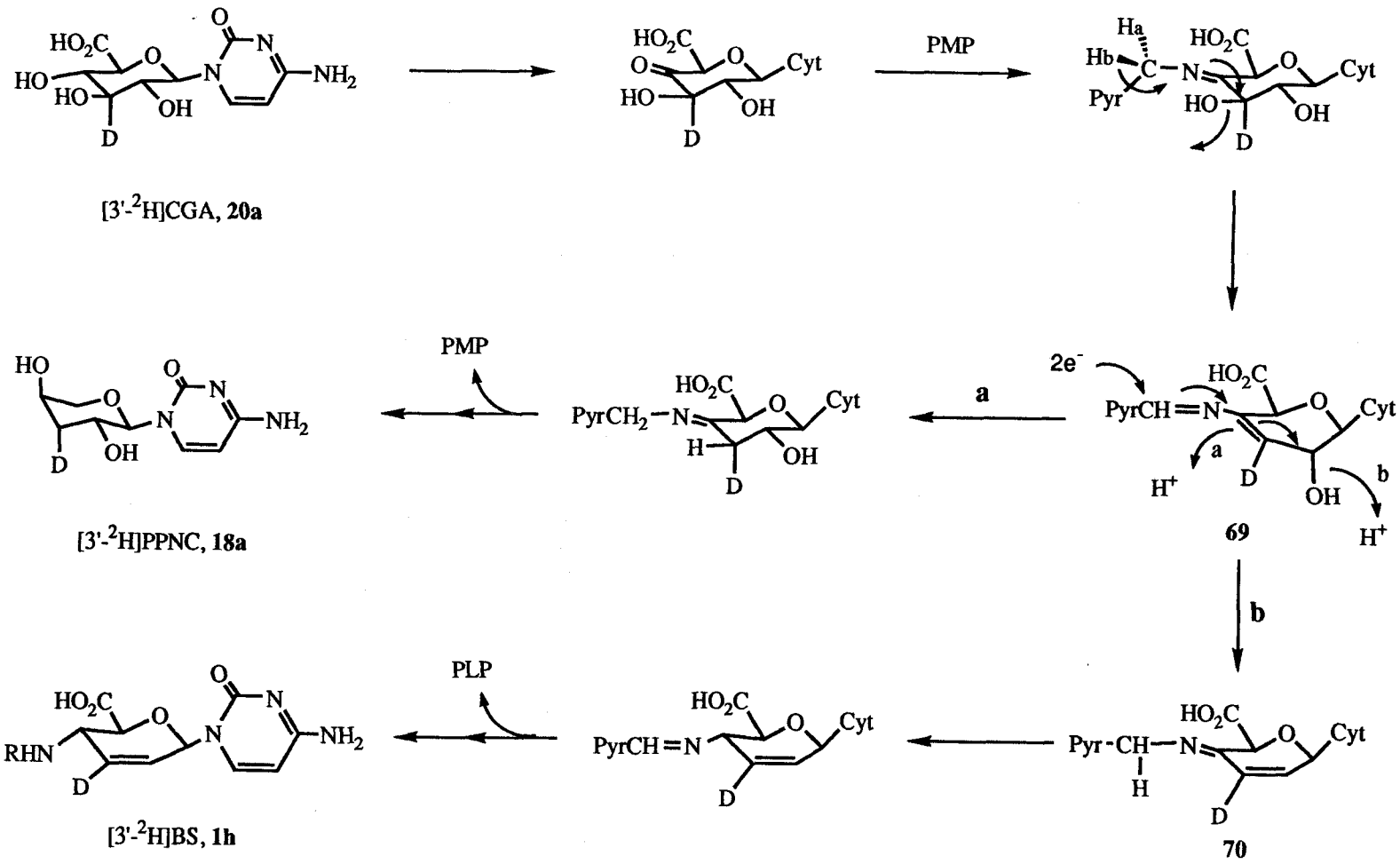

Fig V-10. Proposed mechanisms for C-3' deoxygenation in the biosynthesis of PPNC and BS

A fundamental stereochemical concept of PLP (or PMP) mediated reaction was first proposed by Dunathan[14] that the bond that is ultimately broken in the C-α position of a PLP-imine is oriented perpendicular to the plane of the π-electron system of the cofactor. In this conformation, bond cleavage is favored by the maximal orbital overlap between the σ-bond at the C-α and the extended π-system, and resulted in the formation of a coplanar orientation complex of the pyridinium ring and the imine double bond.[15]

Another key stereochemical principle of the PLP/PMP mediated reaction is that reaction occurs on only one face of the planar enzyme-bound PLP-substrate complex and that the reactive face of the complex is exposed to solvent and accessible to substrates and external reagents whereas the opposite face is shielded by enzyme.[16] Therefore, in PLP/PMP mediated reactions, bond breaking and forming take place on one face of the complex. In the case of a PLP/PMP mediated β-replacement reaction, the reaction results in the retention of the stereochemistry of the C-β position.[16,17] Moreover, the exposed face is the *si* face relative to C-4' in the PLP-substrate imine and the *re* face in the internal Schiff's base.[16]

It is interesting to note that the overall process of the C-3' deoxygenation of PPNC biosynthesis, if directly analogous to the formation of the 3',6'-dideoxysugars of Gram-negative bacterial lipopolysaccharides, would be consistent with the stereochemical course of nearly all PLP/PMP-dependent enzyme catalyzed β-elimination reactions.[17] The only exception known so far is the ω-amino acid:pyruvate aminotransferase of *Pseudomonas* sp. F-126, which abstracts the *pro-R* hydrogen from the C-4 carbon of γ-aminobutyrate.[18]

Summary

From the rather arduous accumulation of isolated facts concerning the mechanistic details of C-3' deoxygenation in PPNC, BS and 3',6'-dideoxyhexoses, it seems to suggest that a group of enzymes, apparently metabolically unrelated, have probably an identical mechanism of catalysis. However, determing the stereochemical course of the C-3' deoxygenation was only one step in elucidating the mechanisms of PPNC, and also BS, biosynthesis. A futher systematic investigation will be needed to complete this study.

Experimental

General

Fermentation conditions and medium preparation used in the following experiment were described in Chapter II. Conditions for analytical HPLC were listed in Chapter III.

Feeding and Product Purification

To a 1.0 L (five flasks with 200-mL broth in each) complex medium fermentation, [3-^{2}H]-D-glucose (**24d**), ArgH and cytosine were fed with the following protocol: 500 mg of **24d** at the 54th hour of incubation and additional 256 mg at the 68th hour; 1.0 g of ArgH and 2.0 g of cytosine at the 52nd of hour incubation and additional 0.5 g of ArgH and 0.2 g of cytosine at the 66th hour. The broth was worked-up at the 160th hour of incubation. Using the same purification procedures as described in Chapter III, 70 mg of **20c**, 40 mg of **18a** and ~ 5 mg of **57b** were obtained.

Conditions for ^{2}H NMR Spectra

Deuterium NMR samples were prepared in ~400 μL deuterium depleted water obtained from Aldrich (^{2}H content = natural abundance x 10^{-2}) with either 25 μL t-BuOH (0.456 μmol ^{2}H in the methyl groups) or 25 μL of 1,4-dioxane (0.409 μmol ^{2}H) as an internal reference for chemical shift and quantification. The ^{2}H NMR spectra were recorded at 61.4 MHz on the Bruker AM 400 spectrometer. They were proton decoupled and run unlocked under the following conditions: sweep width, 1433 Hz; data points, 4096 zero filled to 16384; acquisition time, 1.43 sec; pulse width, 90^0; line broadening, 1 or 3 Hz.

Samples for ^{2}H NMR were prepared as follows:

CGA, **20c**: 20 mg **20c** in 400 μL H_2O, 25 μL t-BuOH (as internal reference) and 20 μL pyridine (to improve the solubility of **20c**). The scan number was 35837 and the line broadening (LB), 3.0 Hz.

PPNC, **18a**[a]: 40 mg **18a** was dissolved in 400 μL H_2O, 25 μL 1,4-dioxane (as internal reference). The scan number was 99226 and the LB was 3.0 Hz.

PPN, **57b**: ~5 mg **57b** was dissolved in 400 μL H_2O, 25 μL 1,4-dioxane (as internal reference). The scan number was 43367 and the LB was 3.0 Hz.

PPN, **57a**: Preparation of **57a** has been described in Chapter III, p 90. About 5 mg of **57a** was used for ^2H NMR with 25 μL t-BuOH as internal reference. The scan number was 2774 and the LB was 1.0 Hz.

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[a] About 0.406 μmol ^2H (0.23% enrichment) was obseaved at δ 5.27, corresponding to the H-1' of **18a**. This ^2H enrichment was found to be derived from the small amount of ^2H enrichment at H-1 of the **24d** fed.

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Chapter VI

Purification and Characterization of CGA Synthase

Introduction

CGA synthase isolated from *S. griseochromogenes* catalyses the formation of CGA from UDP-glucuronic acid and cytosine. Functionally, it belongs to the family of UDP-glucuronosyltransferases (UDP-GTs).

Interest in the purification and characterization of CGA synthase came from several aspects of consideration. First, since it has been demonstrated that CGA synthase is the first committed enzyme to the nucleoside formation in BS biosynthesis, full characterization of this enzyme would enable a more detailed investigation into the mechanisms of nucleoside biosynthesis. Obtaining the sequence information of CGA synthase would allow the designing of genetic probes for searching for the gene clusters of the BS biosynthetic pathway because genes for biosynthetic enzymes of secondary metabolisms have been found to cluster together in all *Streptomyces* studied.[1]

Second, our previous studies have shown that the production of CGA could be stimulated over 100-fold in the fermentation broth when cytosine was fed. The final production of CGA could be as high as 1.4 g/L, along with 1-2 g/L of other related metabolites, which were biogenetically derived from CGA. Such a high production for a secondary metabolite has rarely been encountered in a wild type species of *Streptomyces*. These results indicated that either the CGA synthase has a very high catalytic coefficient or there may be a strong promoter for the expression of CGA synthase gene. A strong promoter from a *Streptomyces* species could have a practical application for the cloning and expression of secondary metabolite genes of *Streptomyces* origin.

Finally, UDP-GTs have been so far isolated only from eukaryotic organisms. Those UDP-GTs are membrane associated,[2] and when purified, need phospholipids for

maximal activity.[2] They also have broad substrate specificities.[2,3b] In contrast, in our preliminary characterizations, CGA synthase was shown to be a soluble enzyme and to have a strict substrate specificity (see Chapter IV). Hydrophobic interaction chromatography (HIC) with pentyl agarose of a crude ammonium sulfate pellet recovered over 80% of the enzymatic activity, suggesting that the enzyme is not phospholipid dependent. These differences in the apparent physicochemical properties have prompted us to purify CGA synthase for comparative studies and for exploring the genetic relationships of these two classes of UDP-GTs.

Eukaryotic UDP-GTs are a family of enzymes that catalyze the glucuronidation of a wide variety of lipophilic endogenous metabolites and xenobiotics.[3a,3b] Recently, it has been proven that glucuronidation, by quantity, is the most important detoxification system in the elimination of carcinogen metabolites, such as phenols, quinols and amine oxides of polycylic aromatic compounds and prevents their reaction with DNA, RNA, and proteins.[3a,3b] Several lines of evidence had suggested that there may be more than one type of UDP-GT,[2d,2e,2f,3,4] with some of them more specifically responsible for transforming endogenous substrates and others for xenobiotic substrates.[3a,3b] However, the study of physicochemical and molecular properties of UDP-GT has not hitherto enjoyed the same development and success as has the characterization of the closely related detoxification enzyme system, the cytochrome P-450-dependent monooxygenase complex.[2a] The scarcity of knowledge about the UDP-GT system has been attributed to several factors: first, the difficulty in purifying these enzymes, mainly due their phospholipid-dependent character and also the weak immunogenicity of the purified proteins;[2a] second, a lack of specific inhibitors for kinetic and mechanistic investigation;[2a] and third, the overlap of substrate specificities among UDP-GTs.[2a,3] In order to discover the metabolic fate of a drug or the risk of toxicity of a xenobiotic, it is essential to know which UDP-GT isoenzyme conjugates it, and how and where this isoenzyme is specifically regulated.[2a]

A full characterization of CGA synthase, in addition to being an essential step towards the elucidation of BS biosynthesis, could potentially help to understand several crucial properties of UDP-GTs, such as which part of their structure confers them to be membrane associated; what kind of structural arrangement contributes to the substrate specificity; and what is the genetic origin for evolution of this pharmaceutically important family of enzymes.

Result and Discussion

Collection of the Mycelia of *S. griseochromogenes*

Mycelia used for the purification of CGA synthase were collected from the synthetic medium fermentation (see Chapter II), to which a large quantity of cytosine (300 mg/L) was fed in the middle of the bacterial growing phase. Cytosine was fed originally to try to induce the generation of the enzyme. However, it was later found that there is no apparent inducibility for CGA synthase (see below). It has been generally accepted that the production of metabolic enzymes has a parallel relationship with the production of the metabolites. Therefore, the timing of CGA production was monitored during the fermentation and the result is shown in Figure VI-1. CGA began to accumulate after about 50 hours of incubation and the accumulation continued to increase for more than 5 days. Based on this result, the mycelia were collected at the late stationary phase of the fermentation (96-120 hours of incubation), at which time the accumulation of CGA was fast while enzymes for the primary metabolisms should have been largely degraded. In general, a 4-L fermentation would generate 50-60 g of mycelia (wet weight), which could be stored at -80 °C for up to three months.

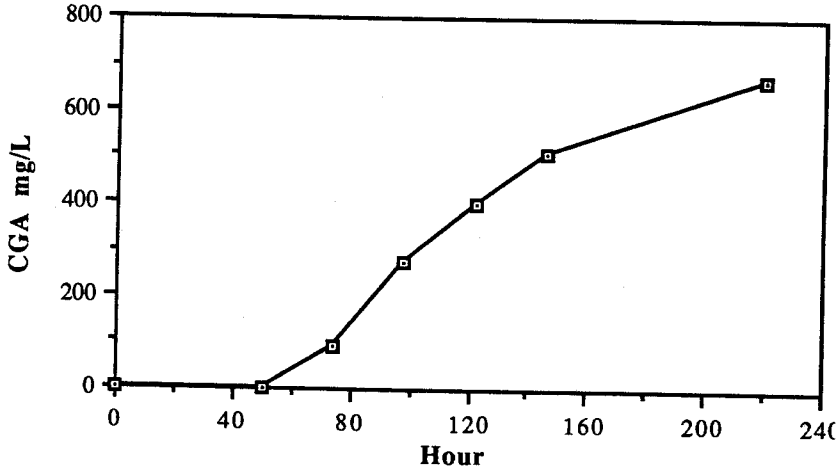

Fig VI-1. Production of CGA in cytosine fed broth. The fermentation was carried out with 100-mL synthetic medium, to which 30 mg of cytosine was fed at 50th hour of incubation (see the experiment for more details).

Preparation the CFE of *S. griseochromogenes*

For convenience and flexibility in scaling up the preparation, sonication was adopted as the method for cell disruption. This method has been shown to be efficient and gives good CGA synthase activity in the CFE. Sonication of a mycelial suspension for three minutes released most of the enzyme activity (~75%). As a general practice in enzyme purification,[5] several protective reagents were included in the extraction buffer, such as PMSF (0.0125%, w/v), EDTA (2.0 mM) and DTT (4.0 mM). The extraction buffer also contained 0.5 M ammonium sulfate (AS) which increased the ionic strength of the buffer and thus could facilitate the extraction of soluble enzymes.[6] The sonicated CFE was then centrifuged to give a gum-like supernatant (S-1), in which CGA synthase was readily detected. It was necessary to use S-1 immediately.

Ammonium Sulfate (AS) Precipitation

AS precipitation is a commonly used technique in protein purifications. It offers one great advantage over virtually all other techniques in stabilizing enzymes.[7] High concentrations of AS also prohibit proteolysis and bacterial actions.[7a] However, a sample of S-1 was shown to have a similar absorbance value at 280 nm and 260 nm, indicating that S-1 contained a lot of nucleic acid materials. In the presence of these materials, our initial trials to fractionate CGA synthase with a stepwise increase of AS were rather unsatisfactory. CGA synthase activity distributed substantially in all fractions of precipitates. One commonly used approach to improve the efficiency of AS fractionation is to remove DNA/RNA material with polycation reagents such as protamine sulfate or streptomycin sulfate.[8] However, in our case, neither reagent could precipitate a significant amount of DNA/RNA material, nor did they provide any help in removing other gum-like materials in the S-1. For these reasons, S-1 was fractionated by two broad AS cuts. The first cut was at 25% AS saturation, and the supernatant (S-2) was next cut at 100% AS saturation to give a crude protein pellet (P-3). The first cut helped to remove

pre-aggregated or highly hydrophobic materials, and the second cut precipitated virtually all proteins. Although such broad fractionation resulted in little purification, it had two desirable features of speed and good activity recovery (>90% of S-1). The fractionation was essentially a cleaning-up operation to get a messy extract into a suitable state for the following more sophisticated chromatographies.

Hydrophobic Interaction Chromatography (HIC)

Since the development of HIC, its scope of application in protein purification has been greatly extended.[9] The nature of HIC is similar to that of salting-out fractionations by promoting the binding of proteins to a HIC medium in the presence of high concentration of salt and then eluting the proteins by decreasing the salt concentration in the elution buffer. HIC possesses the same advantages as those of salting-out fractionations in having high capacities and good activity recovery. Furthermore, we found that HIC had several additional advantages. The behavior of HIC was not so sensitively effected by the presence of nucleic acids or gum-like materials as was the AS fractionation, and a sophisticated gradient elution generated a sharper cut of separation than AS fractionation.

In the initial trials, HIC was performed with a set of small alkyl agarose test columns. The alkyl ligands ranged from ethyl to dodecyl. The pellet, P-3, obtained from 100% AS saturation precipitation was dissolved in a minimal volume of Tris buffer (20 mM, pH 8.0). To each of the test columns that had been pre-equilibrated with a beginning buffer (Tris, 20 mM, pH 8.0, 1.6 M AS), 0.75 mL (7.5 mg protein) of the redissolved sample was applied and the columns were washed with the beginning buffer. These were then eluted with plain buffer that did not contain the added salt. CGA synthase activity in the initial washes and the eluates were assayed for activity recovered. The results, shown in Table VI-1, indicated that butyl or pentyl agarose was the desirable packing medium for CGA synthase purification, since with these media the adsorption

was complete and the activity could readily be eluted. Although only 46% of the activity was eluted from the pentyl agarose column, it was believed that a greater amount of activity would be obtained if a lager volume of elution buffer was used.

Table VI-1. HIC with test columns of alkyl agarose

Alkyl	Activity in the effluent	Activity in the eluate
Ethyl	15%	61%
Propyl	8%	88%
Butyl	~2%	75%
Pentyl	0%	46%
Hexyl	0%	0%
Octyl	0%	0%
Decyl	0%	0%
Dodecyl	0%	0%

The test columns (Sigma Chemical Co, prepacked, 2.5 mL bed volume) were pre-equilibrated with 5 bed volume of beginning buffer. After loading the sample, the columns were washed with 5.0 mL of beginning buffer and then eluted with 4 mL of elution buffer (beginning buffer without AS). CGA synthase activity was assayed by HPLC (see the experimental section for HPLC conditions).

In preparative purifications, HIC with a butyl agarose column gave 6 - 10 fold purification and ~80% recovery of activity. The detailed elution gradient and a typical profile of the chromatography are shown in Figure VI-2. HIC with pentyl agarose gave a similar result, except that CGA synthase was eluted with a lower concentration of AS (0.2 - 0.3 M). The latter column was used for subsequent work.

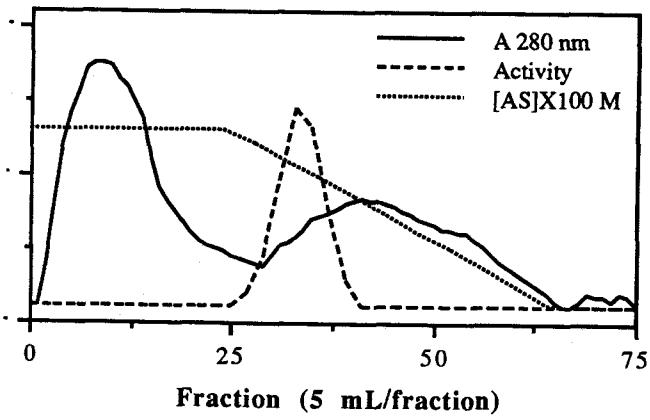

Fig VI-2. HIC with butyl agarose. $(NH_4)_2SO_4$ gradient shown was 1.2 M to 0 M; absorbance scale: 0-2.0 AU; activity profile represents relative activity in each fraction (CGA peak area on HPLC traces)

FPLC HIC with Phenyl Agarose

Active fractions from the pentyl agarose column were brought to an AS concentration of 1.0 M with solid AS and half of the sample (~ 35 mg protein) was applied to an FPLC phenyl agarose column (8 mm x 75 mm, Waters Assoc. Inc). It was eluted in the same fashion as the previous column by decreasing the concentration of AS in the buffer. Although the principle of phenyl agarose HIC was the same as that of the butyl or pentyl agarose column, phenyl ligands seemed to have a special affinity for binding CGA synthase. As shown in Figure VI-3, enzyme activity was eluted later than most of the other components, and a 3-4 fold purification was achieved. The phenyl agarose HIC also resulted in the reduction of sample volume to only 20-30% of that from the previous step. A smaller sample volume is an important factor for stabilizing the enzyme and for obtaining a better resolution with the gel permeation chromatography (GPC) used next. The major disadvantage of this step was the low capacity of the FPLC column. Only half of the active fraction from the previous step could be applied in each run. An attempt to scale up the step with a larger column (2.0 cm x 40 cm) resulted in complete loss of CGA activity (no CGA synthase activity could be eluted). It seemed that the phenyl sepharose (Pharmacia) used to pack the large column had a higher hydrophobicity than that of the phenyl agarose packed in the FPLC column.

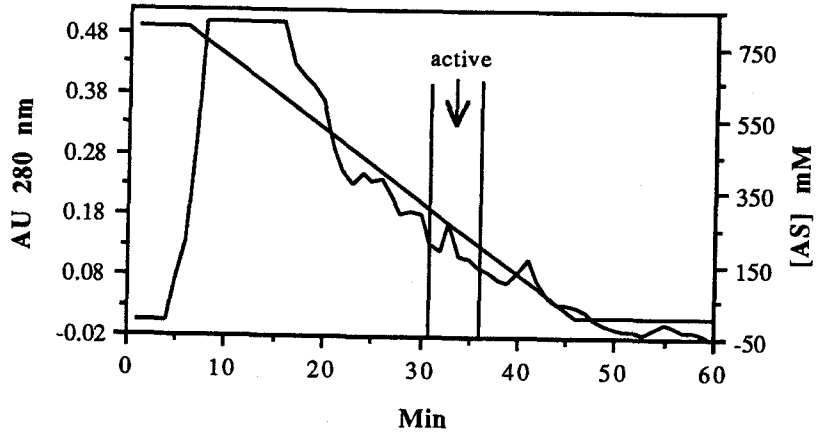

Fig VI-3. Chromatographic profile of FPLC HIC with phenyl agarose

It needs to be mentioned that in the HIC with phenyl agarose, we repeatedly found two peaks of CGA synthase activity. One activity peak centered at fractions that had an AS concentration of 0.2 M and the other minor peak (~ 20% of the first one) emerged at the end of the elution gradient (i.e. at 0 M AS). They might be isoenzymes of CGA synthase. However, occurrence of peaks for the same enzyme in adsorption chromatography is not uncommon because of the heterogeneity of binding sites of the packing materials.[10]

Dialysis

The step of dialysis was to further concentrate the sample for GPC. Sample size is the most important factor for a successful GPC and the volume should not be greater than 3% of the bed volume of a GPC column.[11] In addition, it was essential to have a step where the sample could be left overnight without attention.

There are many ways to concentrate enzyme preparations.[12] Dialysis of a sample against a buffer containing high concentrations of salt or glycerol is a convenient one, given that the sample volume is not too big to start with and the high concentrations of salt or glycerol in samples after dialysis would not effect the operation of the following steps. Active fractions from the phenyl agarose column had a protein concentrations of ~ 1.0 mg/mL and, after being combined, had a total volume of 10-20 mL. Dialysis of the sample overnight against a Tris buffer containing 50% glycerol reduced the sample volume to one-third of the original. Due to the stabilizing influence of glycerol, the dialyzed sample could also be stored at -20 ^{o}C for up to one week without any loss of enzyme activity.

GPC with S-200HR

GPC separates macromolecules on the basis of their differences in molecular size. SDS-PAGE analysis on samples after the dialysis displayed many protein bands with a

wide range of molecular weight distribution. GPC, therefore, was expected to give some degree of purification.

The GPC column was packed with fine particles (high resolution version) of Sephacryl 200 (S-200HR) in a medium pressure column (2.5 cm x 60 cm). An FPLC pump was employed to deliver solvent. The column was run in an ascending mode to obtain a better resolution than if it were run in the descending mode. In order to get a high recovery of activity, 20% (v/v) glycerol was included in the buffer and the column was run relatively fast, at a flow rate of 1.0 mL/min. With a flow rate this high, the power of the resolution was largely dependent on proper packing of the column and on maintaining a tight band of the sample when it was applied. About 80% of CGA synthase activity was typically eluted in a volume of 20-24 mL. Figure VI-4 shows the profile of the chromatography. By comparing the elution volume of CGA synthase with those of calibration proteins (Fig VI-5), the molecular weight of CGA synthase was estimated to be 35,000-43,000.

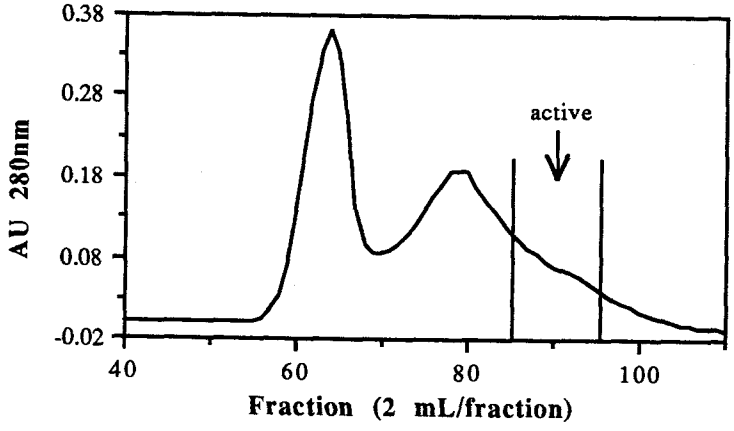

Fig VI-4. Chromatographic profile of GPC with S-200HR

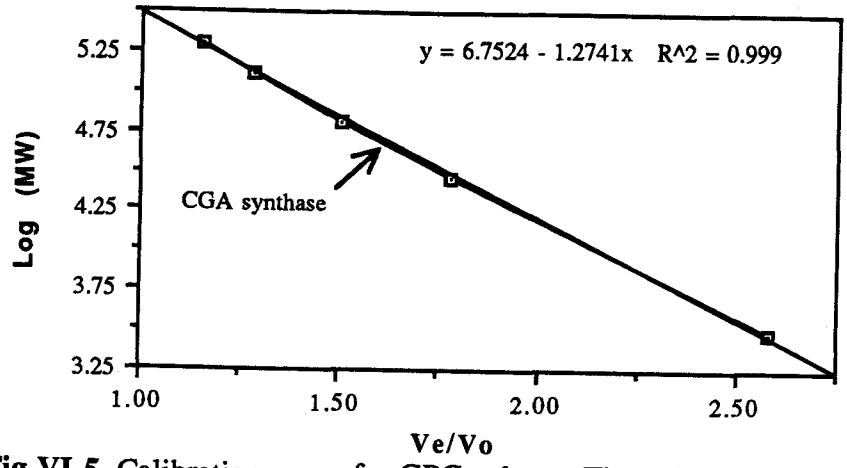

Fig VI-5. Calibration curve for GPC column. The column was run with an identical condition as that used for CGA synthase GPC. MR standards used were: β-amylase (200,000); BSA (132,000, dimmer; 66,000, monomer); carbonic anhydrase (29,000). The total (V_t) column volume was determined by cytidine to be 285 mL and the void column volume (V_o) was determined by dextran blue to be 105 mL.

FPLC Ion Exchange Chromatography (IEC) with DEAE-8HR

The purification of CGA synthase was continued with an FPLC DEAE column. IEC is one of the most commonly used methods in protein purification. IEC media in general have high binding capacities for proteins and a high power of resolution.[13] The volume of sample applied in most cases does not have much effect on the behavior of the chromatography as long as the concentration of salts is low to ensure the binding of required proteins.[13] An additional advantage of IEC is that enzyme activities can generally be eluted into a small volume of buffer with an increasing gradient of salts.[18] Therefore, IEC is a method appropriate in any stage of a purification scheme. It is especially valuable as a step following one that dilutes the sample.

CGA synthase preparations after the S-200HR column had a low protein concentration (~0.2 mg/mL) and large volume (> 20 mL). However, for IEC the sample could be applied directly to the IEC column (DEAE-8HR, 1.0 cm x 10.0 cm, Millipore-Waters Assoc.). The column was eluted with an increasing gradient of NaCl shown in Fig VI-6. CGA synthase was eluted at a NaCl concentration of ~0.14 M as a symmetrical

peak well separated from other major proteins. The preparation was designated as S-8. The detailed chromatographic profile is shown in Figure VI-6. This IEC resulted in an 11-14 fold purification. Analyzed by SDS-PAGE, the active fraction from this step displayed one predominant band. Only after being concentrated 11-fold could other minor contaminants be detected by SDS-PAGE (Fig VI-7). One further advantage of IEC as the last step was that it enabled the preparation of pure CGA synthase at a sufficient concentration for immediate use.

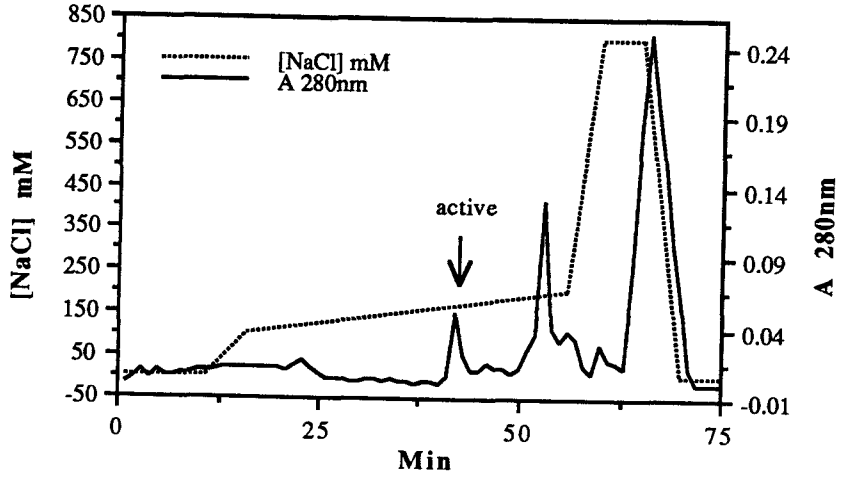

Fig VI-6. Chromatographic profile of FPLC-IEC with DEAE-8HR

Affinity Chromatography with UDP-glucuronate Agarose

In order to remove a few minor components in the preparation S-8, an affinity chromatography step with UDP-glucuronate agarose was applied. UDP-glucuronate agarose has been reported to be a useful medium for the purification of UDP-GT.[2d] However, in the purification of CGA synthase, this affinity chromatography resulted in only ~30% recovery of activity with essentially no increase, if not decrease, in specific activity. The active fraction (S-9) from the affinity chromatography was analyzed by SDS-PAGE, which shows only one band of protein (Fig VI-8).

Fig VI-7. SDS-PAGE of crude and IEC purified CGA synthase.

Conditions for the SDS-PAGE are described in the Experimental section. The following samples were applied:

lane 1: S-8 (~4 μg)*

lane 2 and 8: Bio-Rad M_R standards (1 μg each band)

lane 3: P-3 (~20 μg)

lane 4: S-5 (~20 μg)

lane 5: S-6 (~20 μg)

lane 6: S-7 (~4 μg)

lane 7: S-8 (~15 μg)*

* Samples for lane 1 and 7 were from two separate runs.

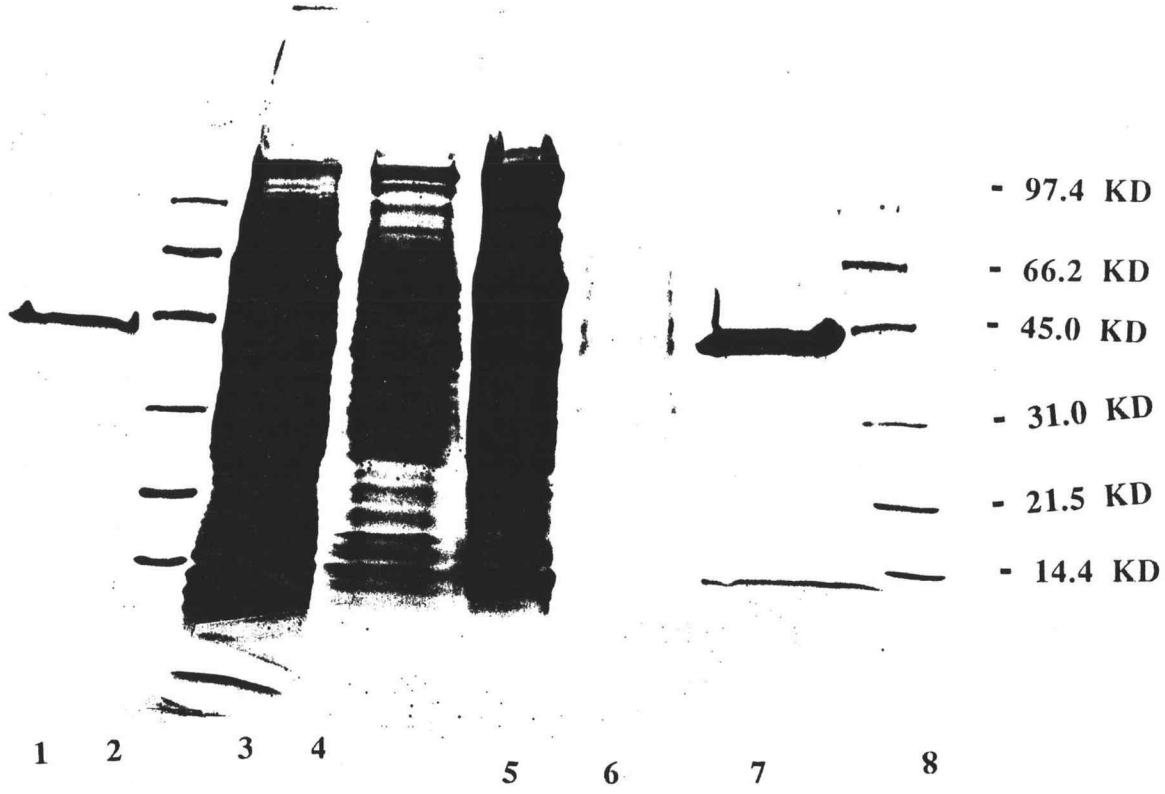

Fig VI-7. SDS-PAGE of crude and ion exchange chromatography purified CGA synthase

Fig VI-8. SDS-PAGE of crude and affinity chromatography purified CGA synthase.*

Conditions for the SDS-PAGE are described in the Experimental section. The following samples[a] were applied:

lane 1 and 10: Bio-Rad M_R standards (2 μg each band)

lane 2: P-3 (~5 μg)

lane 3: S-7 (~1 μg)

lane 4 and 5 : two active fractions of S-8 (concentrated by 10-fold, ~5 μg)**

lane 6 and 7: two active fractions of S-8 (before being concentrated, ~0.4 μg)

lane 8 and 9: S-9 (concentrated by 10-fold, ~4 μg and ~1 μg, respectively)

* Samples for Fig VI-7 and Fig VI-8 were from differen runs of purification.

** Only the sample of lane 5 was used for next purification.

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[a] Samples for Figure VI-7 and Figure VI-8 were from two different preparations.

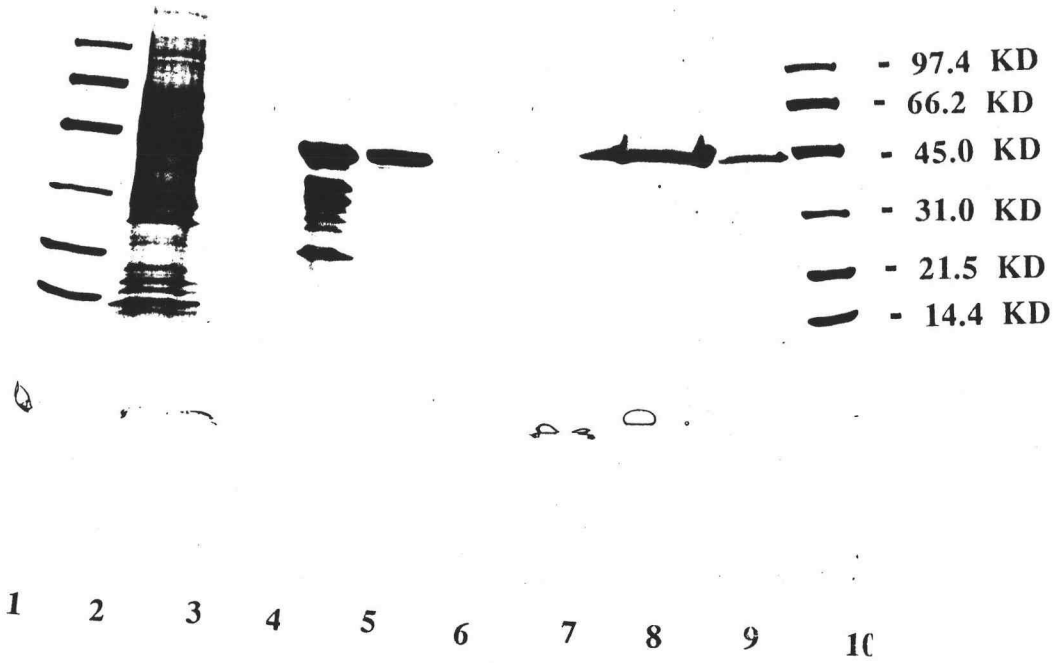

Fig VI-8. SDS-PAGE of crude and affinity chromatography purified CGA synthase

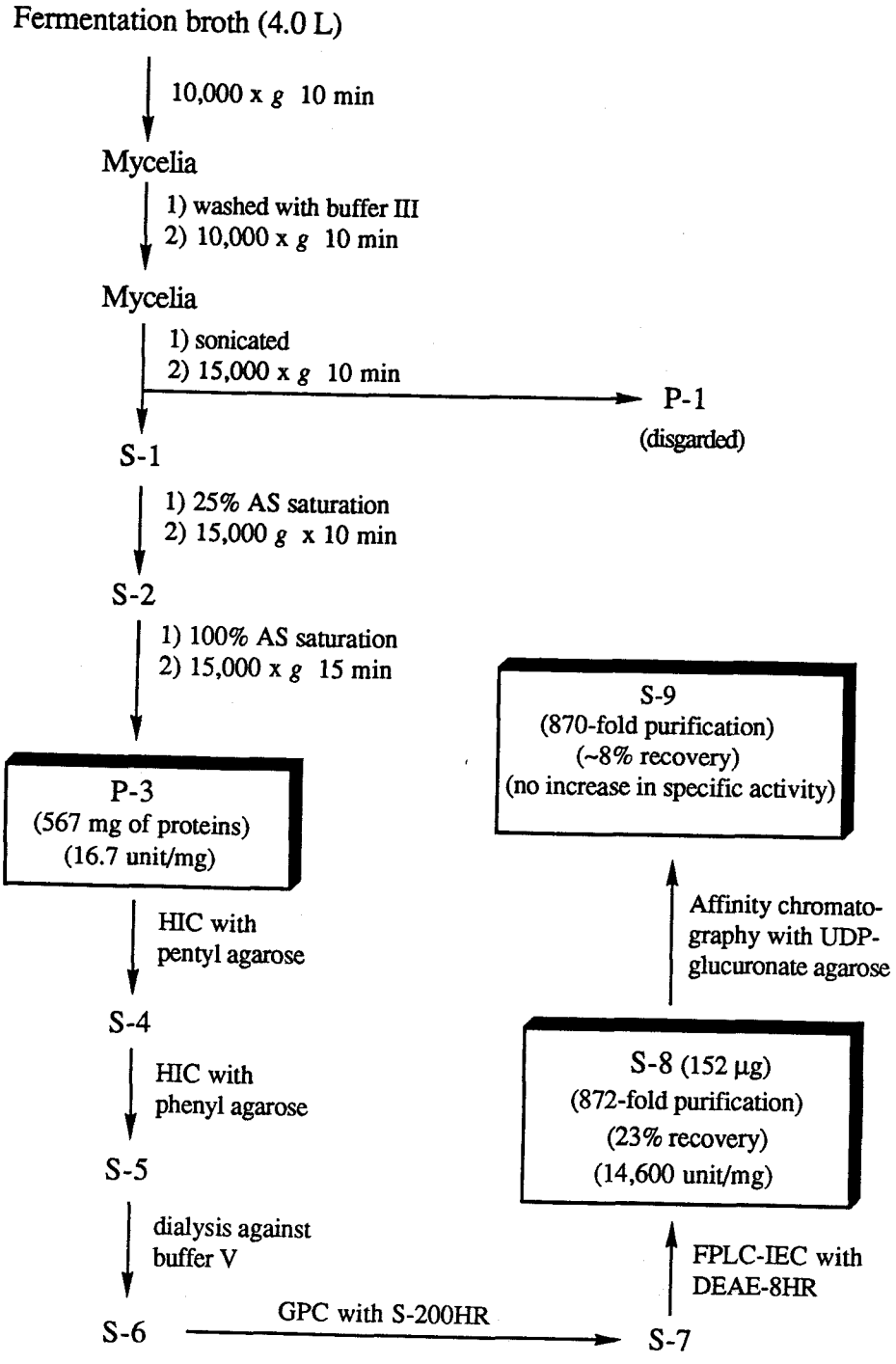

Fig VI-9. Purification scheme for CGA synthase

Table VI-2. CGA synthase purification table

Steps	Volume (mL)	Protein (mg)	Specific Activity (unit)[a]	Total Activity (unit)	Activity Recovery (%)	Overall Recovery (%)	Step Purificat. Fold	Overall Purificat. Fold
25-100% $(NH_4)_2SO_4$ Saturation Pellet	35.0	566.7	6.8	3853	100	100	1	1
HIC (Pentyl agarose)	49.0	67.5	43	2894	75.0	75.0	6.3	6.3
FPLC-HIC (Phenyl agarose)	9.0[b]	15.54	150	2337	80.6	60.5	3.5	22
GPC (S-200HR)	20.0	3.30	541	1785	76.4	46.2	3.6	79
FPLC-IEC (DEAE-8HR)	2.0	0.15	5,950	904	50.6	23.4	11.0	870

a. Specific activity was defined as the production of 1.0 nmol CGA per mg of protein per min under optimal condition (the same conditions as used for kinetic studies).

b. The volume was reduced to 3.1 mL after dialysis.

In summary, the whole purification process is depicted in Figure VI-9. With this purification scheme, CGA synthase was purified from a crude AS pellet by four chromatography steps and the process could be finished in about two days. Table VI-2 shows the progress of a representative purification, in which CGA synthase was purified by a factor of 870 with an overall recovery of ~8%. For comparison, eukaryotic UDP-GTs have been purified by 5-6 steps of chromatography with an overall recovery of 0.23-4.4%.[2d,3d,3e,14]

Characterization

Molecular Weight (M_R)

The subunit of CGA synthase was estimated to be ~43,000 by comparison with a set of protein standards in the SDS-PAGE analysis (Fig VI-7 and VI-8). On GPC its native M_R was estimated to be 35,000-43,000 (Fig VI-5). Therefore we concluded that CGA synthase thus purified is a monopeptide protein with M_R of ~43,000. This is comparable to that of UDP-GTs which have been reported to have M_R of 50,00-57,000.2b,[2d,3d,3e,14]

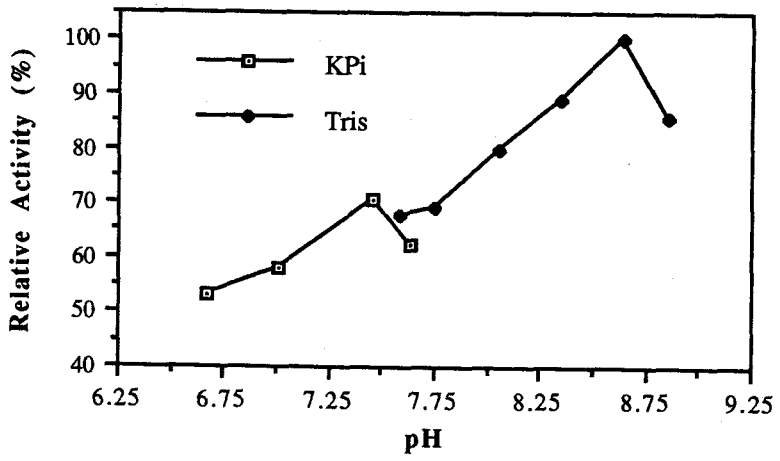

Fig VI-10. pH dependence of CGA synthase

Optimal pH and Temperature

Examined in Tris and phosphate buffer, CGA synthase exhibited highest activity at pH 8.45-8.65 and a temperature of 30-37 °C, as are shown in Figure VI-10 and Table VI-3, respectively. The shape of the pH dependency curve showed that CGA synthase activity was essentially linearly correlated to the increase of pH in the range of pH 6.5 to 8.5. This may suggest that an ionizable group with a pKa in the range of 7.0-8.0, such as histine or tyrosine, is important for the enzyme activity. Mechanistically, this ionizable group could be involved in facilitating the leaving of UDP group by attacking at C-1' or in promoting the deprotonation of cytosine.

Table VI-3. Temperature dependency of CGA synthase activity

T (°C)	4	22	30	37	50
Activity (%)	~5	70	94	100	51

Cofactor Requirements

The purified CGA synthase did not appear to require any external cofactors or metal ions for catalysis, although it was observed that the activity was slightly stimulated by Mg^{++} and Ca^{++} (increased 20 - 30%) (Table VI-4). Interestingly, UDP-GT of eukaryotic sources displayed the same characteristics.[15] However, the mechanistic role of divalent cations in the reaction, while intriguing, may not be an important issue. The evidence was that CGA synthase activity was not inhibited by EDTA (Table VI-4). Therefore, divalent cations might simply act to neutralize the charged UDP-glucuronic acid and facilitate its binding by a similar mechanism to that of forming $ATPMg^{2-}$ in a ATP kinase catalyzed reaction.[16] CGA synthase was inhibited significantly by the cations Fe^{++}, Co^{++} and Zn^{++}, and was not much effected by Mn^{++} and Cu^{++}. It was likely that these oxidative cations oxidized some catalytically or structurally essential thiol groups. However, eukaryotic UDP-GTs are not affected by these metal ions.[15b,15c]

Table VI-4. Effect of metal ions and phospholipid on CGA synthase activity[a]

Sample	1	2	3	4	5	6	7
Compounds added (5 mM)	-	$MgSO_4$	$CaCl_2$	$MnCl_2$	$ZnCl_2$	$FeCl_2$	$CoCl_2$
Relative activity (%)	100	128	123	90	0	~5	~5

Table VI-4. (continued)[a]

Sample	8	9	10	11	12	13	14
Compounds added	$CuCl_2$ (5 mM)	EDTA (4 mM)	PC[b] (0.1%, w/v)	PC[b] (0.4%, w/v)	2,3-butane dione (25 mM)	UDP (8 mM) $MgCl_2$ (5 mM)	UDP-GT (0.1 unit) $MgCl_2$ (5 mM)
Relative activity (%)	94	98	119	119	85	15	0[c]

a. All samples were composed of 0.5 mM cytosine, 0.25 mM UDP-glucuronic acid, 0.31 μg of enzyme (preparation S-9) in 500 μL of buffer I (see the Experimental). The reactions were incubated at 37 °C for 60 min and terminated by freezing to -80 °C, rethawed and analysed by HPLC (see the Experimental for conditions). % conversion in the control sample was 52% based on the consumption of cytosine.

b. PC: egg yolk phosphatidylcholine.

c. Eukaryotic UDP-glucuronosyltransferase was added instead of CGA synthase. No product (CGA) was detected.

Being similar to other UDP-GTs,[17] CGA synthase was also inhibited to ~85% by 4 mM UDP. CGA synthase was slightly inhibited (by 15%) by 2,3-butanedione at the concentration of 25 mM, at which concentration UDP-GTs are significantly inhibited.[18] The compound has been shown to react with an essential arginine residue in the active site of eukarytic UDP-GT and irreversibly inactivate the enzymes. However, for direct comparison we should have done a side-by-side study of the effect of 2,3-butanedione on CGA synthase and eukaryotic UDP-GTs.

Remarkably, the activity of CGA synthase was not much affected by the phospholipid of phosphatidylcholine (PC), without which eukaryotic UDP-GTs would lose all catalytic activities.[2,3] PC was selected in our study because it has been shown to be the most effective phospholipid in reconstitution eukaryotic UDP-GT activity.[2d,15c]

Substitution of CGA synthase with a crude microsomal UDP-GT (prepared from rat liver) did not result in any production of CGA. This result indicated that catalyzing the formation of CGA is not one of the general functions of UDP-GT.

The K_m and V_{max}

With the pseudo-first order approach,[19] the catalysis of CGA synthase followed the classical Michaelis-Menten kinetics (Fig VI-11 and VI-12). The V_{max} of the enzyme was calculated to be 14.6 μmol/mg·min and the K_m values for UDP-glucuronic acid and cytosine were 6.0 μM and 243 μM, respectively. Apparently, CGA synthase has a much higher affinity for UDP-glucuronic acid than for cytosine. In contrast, eukaryotic UDP-GTs have higher affinities for the aglycone substrates than for UDP-glucuronic acid.[3e,20] We believed that the distinct patterns of substrate affinities could reflect the differences of their metabolic demands. UDP-GTs may need to have a high affinity for aglycone substrates to ensure the glucuronidation of potentially toxic substances even at low concentration. For CGA synthase, a lower affinity for the aglycone substrate, cytosine, might be beneficial to the organism in that the biosynthesis of primary nucleotides is not

interfered by the biosynthesis of BS. It is conceivable that the same kinetics would also be found in other biosynthetic enzymes of nucleoside antibiotics.

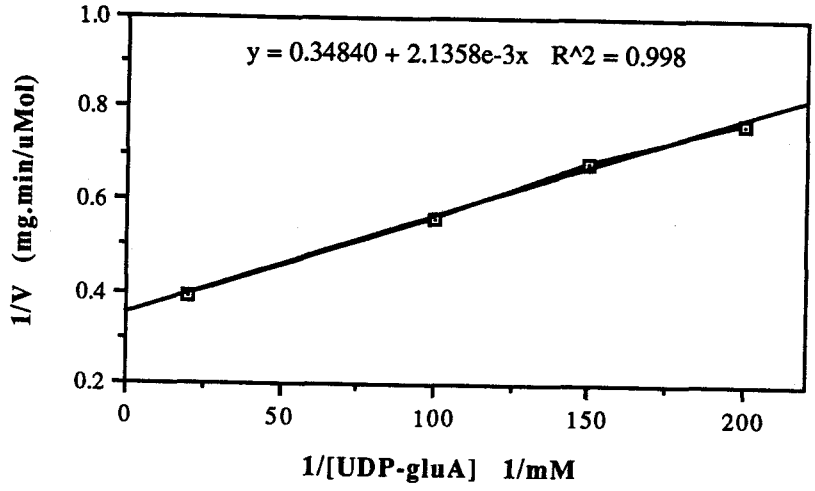

Fig VI-11. Lineweaver-Burk Plot of 1/V vs 1/[UDP-GluA]. Details of the reaction are described in the experimental section. The production of CGA was quantified by HPLC, calibrated with authentic samples.

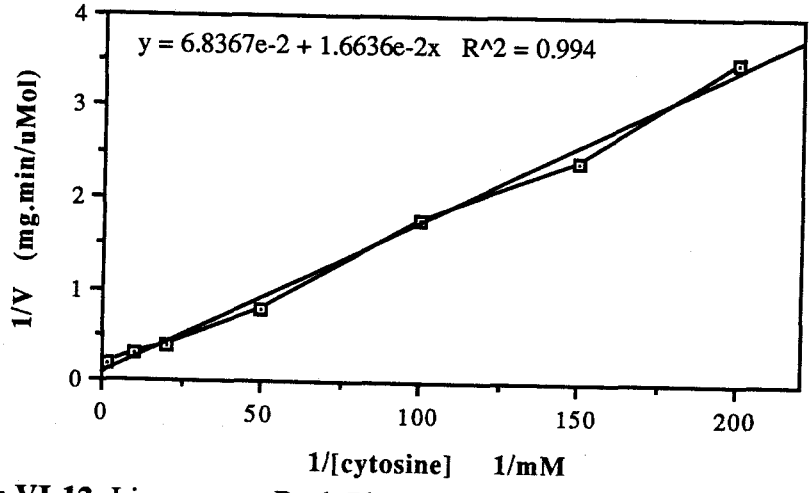

Fig VI-12. Lineweaver-Burk Plot of 1/V vs 1/[cytosine]. Details of the reaction are described in the experimental section. The production of CGA was quantified by HPLC calibrated with authentic samples.

Substrate Specificities

The substrate specificity of CGA synthase for the nucleotide part has been examined previously in Chapter IV (Fig IV-2). As was also true for eukaryotic UDP-

GT,[3e] only UDP-glucuronic acid reacted. For the aglycone substrate, in addition to cytosine several 5-substituted cytosine derivatives, such as 5-fluorocytosine, **71**, 5-iodocytosine, **72**, 5-hydroxymethylcytosine, **73**, and 5-methylcytosine, **74**, were also utilized by CGA synthase and the corresponding products, 5-fluoro-CGA (5-FCGA), 5-iodo-CGA (5-ICGA), 5-hydroxymethyl-CGA (5-HMCGA), 5-methyl-CGA (5-MCGA), **75-78**, respectively were apparently produced. One of these CGA analogues, 5-HMCGA, **77**, has been purified and characterized by ^{1}H NMR and by high resolution mass spectroscopy. The relative reaction rates with cytosine and those 5-x-cytosines are shown in Table VI-5. However, 4-nitrophenol and α-naphthol, the two most commonly used substrates for the eukaryotic UDP-GT,[2,3,17] were not utilized by CGA synthase. Adenine or uracil was not utilized either. Apparently, CGA synthase has a more strict substrate specificity than that of eukaryotic UDP-GT.

Table VI-5. Relative rate of 5-x-cytosine as a substrate

X	H	F	I	$HOCH_2$	CH_3
Rate (%)	100	130	110	95	65

Inducibility

It was noticed in earlier inhibitor-primary precursor feeding studies that cytosine enhanced dramatically the production of BS and its related metabolites, especially, the production of CGA. Originally, it was thought that CGA synthase might be an inducible enzyme, as has been found for eukaryotic UDP-GT.[3d,3e,14a,21] However, a comparative experiment revealed that growing the bacteria in the presence of cytosine did not result in any increase in the specific activity (or total activity) of CGA synthase in crude CFE's (Table VI-6). This revealed that feeding cytosine had no effect. However, it should be noted that, being an essential moiety in primary metabolite, cytosine has already existed in the bacterium. The external supplemented cytosine might not bring about a further

induction of the enzyme. Therefore, more studies may be needed to draw a conclusion about the inducibility.

Table VI-6. Inducibility of CGA synthase

Sample	1	2	3	4
Cytosine fed to fermentation[a]	no	no	yes	yes
Protein in CFE (mg/mL)	2.98	4.12	3.45	3.20
Specific activity (unit/mg)[b]	2.50	3.28	2.22	3.83

a. Cytosine was fed at 54th hour of incubation (300 mg/L).
b. One unit was defined as the production of 1 nmol CGA per minute under the assay conditions.

Amino Acid Composition and N-Terminal Sequence

Attempts to obtain the amino acid composition and N-terminal sequence have not been successful. Samples prepared for the sequencing analysis were prepared by blotting about 30 μg of the enzyme preparation (S-8) onto a piece of immobilon-P or a piece of immobilon-P^{SQ} hydrophobic membrane, following procedures described by Sheer and Legendre.[22] However, neither sample gave amino acid signals in the sequencing analysis. At this point, it is not clear whether the N-terminus is blocked or the blotting process was not performed properly.

After having been subjected to ten cycles of sequencing analysis without obtaining any signal, one of the samples was rescued for amino acid composition analysis and the result is listed in Table VI-7. Since the rescued sample contained only about 5 μg of protein, these data may not have enough accuracy to reflect the true composition of CGA synthase. However, the composition analysis did show that some protein was blotted onto the immobilon membrane. Therefore, the experiment supported the notion that the N-terminal of CGA synthase is blocked. On the other hand, eukaryotic

UDP-GT isolated from kidney microsomes of β-naphthoflavone-treated rat has been shown to be open and the first 20 amino acids were sequenced.[14a]

Table VI-7. Amino acid compositions of CGA synthase

a.a.	res.	a.a.	res.	a.a.	res.
Ala.	53.0	His.	6.9	Pro.	13.6
Arg.	27.7	Ile.	14.1	Ser.	21.6
Aspx	32.0	Leu.	40.9	Trp.	-
Cys.	-	Lys.	4.7	Tyr.	7.0
Glx	28.4	Met.	1.0	Val.	31.4
Gly.	35.1	Phe.	11.0	Thr.	22.4

Summary

CGA synthase, a distinct member of the UDP-GT family of enzymes was purified ~870 fold from a crude AS pellet by 5 steps of chromatography with an overall recovery of about 8%. Each step of the purification utilized a different aspect of the enzyme properties, such as hydrophobicity, molecular size, charges and functionality. In the whole process, samples from one step could be applied to the next without any treatment.

Characterization of CGA synthase revealed that many aspects of its properties were similar to those of eukaryotic UDP-GT, although CGA synthase displayed more strict substrate specificity, was soluble and was not non-phospholipid dependent. Table VI-8 summarizes the properties of CGA synthase and eukaryotic UDP-GT. The genetic relationships of these two classes of enzyme await the sequencing information of CGA synthase.

Table VI-8. Characteristics of CGA synthase from *S. griseochromogenes* and UDP-GT from eukaryotes

	CGA synthase	Eukaryotic UDP-GT	Ref.
substrate	UDP-glucuronic acid & cytosine	UDP-glucuronic acid & phenols, quinols, steroids, billlirubin, morphyins, etc.	2,3
solubility	soluble	membrane associated	2
MW	43,000	50,000-57,000	3d,3e,4b, 4c,4e,14
subunit	monopeptide	monopeptide	3,4,14
substrate specificity:			
(UDP-glucuronic acid)	absolute	absolute	3e
(aglycone)	cytosine & 5-x-cytosine	broad	3
V_{max} (μmol/mg/min)	14.6	4.35[a]	3d,3e
K_m (μM)			
(nucleotide)	6.0	5040	3d,3e
(aglycone)	243 (cytosine)	60 (1-naphthol)	20
		200 (4-nitrophenol)	20
optimal pH	8.45-8.65	7.4[b]	
optimal T (°C)	30-37	37[c]	
cofactor required	none	none	15a
activity stimulated by:	Mg^{++}/Ca^{++}	Mg^{++}/Mn^{++}	15a
activity inhibited by:	UDP	UDP	17
	2,3-butanedione	2,3-butanedione	18
	Fe^{++}, Co^{++}, Zn^{++}		
N-terminal	blocked (?)	open	14a
inducibility	no (?)	1-5 fold by barbituates	3d,3e 14a,21

a. Determined in the presence of 0.1% phosphatidylcholine, without which there is little activity.
b. Not examined although enzyme activity has always been assayed at the physiological pH of 7.4.
c. Not determined although enzyme activity has always been assayed at the physiological temperature of 37 °C.

Experimental

General

Chemicals and Equipment

All procedures for the enzyme purification were performed at 0-4 °C. HIC media, Sephacryl S-200HR, molecular weight standards for gel filtration, Coomassie Brilliant blue R-250 and G-250 and general chemicals used for the purification and characterization were purchased from Sigma Chemical Co. PMSF was purchased from Aldrich. Reagents and the Mini-protein II slab cell used for SDS-PAGE, including the precast SDS-slab gels, molecular weight standards and electrode buffers were purchased from Bio-Rad. Ultrafree MC filtration unit (has a M_R cut at 10,000 Da.) and the Immobilon-P and Immobilon-P^{SQ} hydrophobic membrane were from Waters Millipore Corp. Dialysis tubing (cellulose membrane, 4.7 mm) which has a M_R cut of 12,400 Da. was from Sigma Chemical Co. The tubing had been boiled in 0.2 M $NaHCO_3$ and 5 mM EDTA for 30 min and was stored at 4 °C in the same solution. It was rinsed thoroughly with milli-Q water before use.

An IEC B-20A refrigerated centrifuge was used for routine centrifugation. Cell disruption was performed on a Sonicator Model W-225R made by Heat Systems-Ultrasonic Inc. The flow rate for all purification chromatographies was controlled by Waters 650E FPLC Pump and the fractions were monitored by a Lambda-Max Model 481 LC spectrophotometer as the detector and an HP 3396A integrator as the recorder. IBM 9420 UV-Visible spectraphotometer was used for protein determination. The FPLC columns were purchased from Millipore-Waters Assoc. An IBM 9550 Heating/Cooling Fluid Circulator (± 0.1 °C) was used for temperature control in the kinetic studies.

Buffers:

I: 100 mM Tris, pH 8.0, 4 mM DTT, 0.2 mM EDTA.

II: 0.5 M $(NH_4)_2SO_4$ in buffer I.

III: 0.8 M $(NH_4)_2SO_4$ in buffer I.

IV: 1.0 M $(NH_4)_2SO_4$ in buffer I.

V: 50% glycerol in buffer I.

VI: 50 mM Tris, pH 8.0, 20% glycerol, 0.2 mM EDTA, 4 mM DTT.

VII: 25 mM Tris, pH 8.0, 4 mM DTT.

VIII: 1.0 M NaCl in buffer VII.

IX: 10 mM Tris, pH 8.0, 4 mM DTT, 0.2 mM EDTA, 2 mM $MgSO_4$.

X: buffer IX without $MgSO_4$.

XI: 50 mM Tris, pH 8.50, 4.0 mM DTT, 2.0 mM $MgSO_4$, 0.2 mM EDTA, 62.5 mM NaCl.

Protein Determination

Protein concentrations were determined by the method of Coomassie Blue Binding,[23] using bovine serum albumin (BSA) as the calibration standard. The dye reagent was prepared[23a] by dissolving 60 mg of Coomassie Brilliant Blue G-250 in 1 L of 3% perchloric acid and then filtering the solution to remove undissolved material. This reagent was stable indefinitely at room temperature. Samples for analysis were prepared as follows: to a 1.5-mL cuvette, 450 μL of the dye reagent, 450 μL of milli-Q H_2O and then 100 μL of protein solution was combined. After mixed for 3 min, the samples were read at 595 nm by a UV-spectrophotometer. Concentrated protein samples were diluted to 0.05-0.15 mg/mL before use.

Enzyme Activity Assays

CGA synthase activity was assayed by monitoring the production of CGA by HPLC on a Waters 600E HPLC instrument connected to a Waters 990+ Photodiode Array detector. The HPLC conditions were: RadialPak^R C_{18} column (Novapak, 4 μ, 8 x

100 mm, Waters Assoc.), eluted with 95% H_2O, 5% CH_3CN and 0.1% TFA at a flow rate of 1.5 mL/min for routine assays (see below) and 1.2 mL/min for other assays (kinetic, cofactor requirement, specificity, specific activity and pH dependency assays). The effluent was monitored at 275 nm.

For routine determination of activity after each chromatography, samples were prepared by mixing 10 μL of pre-cooled substrate solution (0.5 mM UDP-glucuronic acid, 0.5 mM cytosine and 2.0 mM $MgSO_4$ in buffer I, 4 °C) with 5 μL of enzyme preparation (potentially active fractions from each column, 4 °C) and incubated at 37 °C for 15 min. Reactions were terminated by putting samples into an ice bath. Five μL of each sample was injected onto the HPLC for analysis.

For kinetic assays, samples of 500 μL (in buffer XI) containing 500 μM cytosine and 5-50 μM UDP-glucuronic acid; or containing 50 μM UDP-glucuronic acid and 5 - 500 μM cytosine were incubated at 37 °C for 2-5 min. The assay was initiated by the addition of 10 μL (0.38 μg of protein) of enzyme preparation (S-8) to each sample and incubated for 6 min at 37 °C. The enzyme reaction was terminated by putting the whole sample rack into a deep freezer (-80 °C). The frozen samples were thawed and 50 μL from each was analyzed by HPLC while the samples were still ice-cold. The HPLC system was calibrated with authentic CGA.

For specific activity assays, samples were composed of 0.5 mM cytosine, 0.25 mM UDP-glucuronic acid in 500 μL buffer XI. Reactions were initiated by adding 20 μL of enzyme preparation (from each step of purification), incubated at 37 °C for 15 min and terminated by freezing at -80 °C and analyzed as above.

For the pH dependence study, complete assay solutions composed of 200 μM UDP-glucuronic acid, 200 μM cytosine, 4.0 mM DTT, 2.0 mM $MgSO_4$ and 0.38 μg enzyme (S-8) in 200 μL of 40 mM potassium phosphate (pH 6.60-7.64) or 40 mM Tris buffer (pH 7.59 to 8.85), were incubated at 37 °C for 30 min. The assay mixtures were terminated by freezing at -80 °C and 10 μL from each sample was analyzed by HPLC

For cofactor requirement assays, 500 μL of sample solutions containing 0.5 mM cytosine, 0.25 mM UDP-glucuronic acid and specified concentrations of cofactors (Table VI-4) in buffer X were incubated at 37 °C for 2-5 min. The reaction was initiated by adding 10 μL (0.31 μg) of the enzyme preparation (S-9) to each sample, incubated at 37 °C for 60 min and then terminated and analyzed in similar way. Only the activities relative to the control sample were measured.

Specificities towards nucleotide substrates have been described in Chapter IV. Assays for aglycone substrate specificity were prepared in the same way as those used for routine activity assays (with 0.5 mM of specified aglycone substrate instead of cytosine).

Solutions for SDS-PAGE

Sample buffer: 1 g SDS, 2 mL glycerol, 2 mL bromophenol blue (0.1% aqueous solution, w/v), 1.25 mL Tris buffer (1.0 M, pH 6.8), 2 mL mercaptoethanol diluted to 10 mL with H_2O.

Staining and fixation solution: 1g Coomassie Brilliant Blue R-250, 450 mL methanol, 100 mL acetic acid diluted to 1 L with H_2O.

Destaining solution: 100 mL methanol, 70 mL acetic acid diluted to 1 L with H_2O.

All these solutions were stored at 4 °C and could be used for 6 months.

SDS-PAGE

Denaturing gels were run according to the procedures reported[24] on precast 4-20% gradient gels (Bio-Rad mini-gel) with a constant current of 10 mA/gel, and run at 4 °C until the tracking dye had reached the bottom of the gel. Samples from early steps of the purification were diluted while from late steps of purification were concentrated as necessary. Concentration was performed by ultrafiltration with Ultra-free MC filtration

units which have M_R cut at 10,000. Twenty-μL samples were combined with 20 μL of sample buffer and boiled for 4 min. 20 μL of each was loaded onto the slab gel. The M_R and the purity of the final CGA synthase preparation was assayed by SDS-PAGE. The low M_R standards from Bio-Rad were used as markers: phosphorylase B (97,400), bovine serum albumin (66,200), chicken egg albumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400). After electrophoresis, gels were rinsed two times with water, stained/fixed overnight and destained overnight.

GPC Calibration Curve for M_R Determination

The M_R of CGA synthase was determined according to its elution position on the S-200HR column, which had been calibrated by a set of standard proteins. Conditions for calibration of the column were identical to those used for the GPC of CGA synthase. Briefly, the column was eluted with buffer VII at a flow rate of 1.0 mL/min. The following standards from Sigma Chemical Co. were used: β-amylase (200,000), bovine serum albumin (monomer and dimer, 66,000 and 132,000, respectively), carbonic anhydrase (29,000) and cytidine (used to determine the V_t of the column).

Preliminary HIC with Alkyl Agarose Columns

A portion of a crude AS pellet (30-85% saturation cut) was dissolved in Tris buffer (20 mM, pH 8.0 and 1.6 M AS, designated as beginning buffer) to a protein concentration of ~10 mg/mL. To each alkyl agarose testing column (prepacked, 2.5 mL bed volume, Sigma Chemical Co., pre-equilibrated with the beginning buffer), 0.75 mL of the redissolved sample was loaded and the columns were washed with 5.0 mL of beginning buffer and then eluted with 3.0 mL of elution buffer (20 mM Tris, pH 8.0). Three fractions (3.75 mL, 2.0 mL and 3.0 mL, respectively) were collected for each

column and fraction #2 and #3 from each column were assayed for CGA synthase activity.

Purification

Step 1: Growing Bacteria

Mycelia of *S. griseochromogenes* used for the purification of CGA synthase were collected from typically 4.0 L of synthetic medium fermentation (see Chapter II), harvested at the 120th hour of incubation by centrifugation (10,000 x *g*, 10 min) and washed one time with buffer III and then repelleted as above. The washed mycelia (44.3 g, wet wt.) could be used immediately or stored at -80 °C for up to three months without the loss of enzyme activity.

Step 2: Preparation CFE

About 44 g of mycelia (wet wt.) was suspended in buffer II (120 mL) cooled in an ice bath, and disrupted by sonication (power level 8, 50% duty cycle of pulse, for 3 x 60 sec, with a one min stop after every 60 sec sonication). The CFE was centrifuged (15,000 x *g*, 10 min) and the supernatant (S-1) was used in the following ammonium sulfate (AS) precipitation fractionation.

Step 3: AS Fractionation

The S-1 was brought to 25% AS saturation by slowly adding solid AS. The suspension was stirred for 30 min and then centrifuged (15,000 x *g*, 10 min). The resulting supernatant (S-2) was brought to 100% saturation with more A.S. (until no more AS could be dissolved. The final AS concentration was usually ~ 3.7 M), stirred for an additional hour, and then centrifuged at 15,000 x *g* for 15 min. The pellet (P-3) from the last centrifugation (about 730 mg of protein) was used for the following

purification. P-3 could also be stored at - 80 °C for a month without effect on the following purification.

Step 4: HIC with Pentyl Agarose

P-3 prepared above was dissolved in 35 mL of buffer I (567 mg of protein, 16.2 mg/mL) and applied to a pentyl agarose column (2.5 cm x 20 cm) which had been pre-equilibrated with buffer IV. After loading, the column was washed with 100 mL of buffer IV and then eluted with a linear gradient of buffer IV to buffer I (totally 270 mL). The flow rate was 0.7 mL/min[a] and fractions of 4.9 mL/fraction were collected. CGA synthase was eluted in fractions which had an AS concentration of 0.15-0.25 M.

Step 5: FPLC HIC with Phenyl Agraose

Fractions with high CGA synthase activity were combined (10 fractions, 49 mL, 67.5 mg protein), adjusted to an AS concentration of 1.0 M (it was assumed that AS in the combined fraction was 0.2 M) and it was then divided into two parts (28 ml and 23 ml). Each part was loaded onto a phenyl agarose FPLC column (8 mm x 75 mm) that had been pre-equilibrated in buffer III. The column was eluted (1.0 mL/min, 3.0 mL/fraction) with a gradient of buffer III to buffer I in 22 min. Active fractions from two runs (9.0 mL, two fractions from the first run and one fraction from the second run) were combined and dialyzed against 400 mL of buffer V overnight at 4 °C and then stored at - 20 °C until the commencement of the next step of purification (up to one week without the loss of activity). Sample volume was reduced to 3.1 mL after the overnight dialysis.

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[a] The pressure built up when run at a higher flow rate. A newly packed column could run at a flow rate of 2.0 mL/min without reducing the power of resolution.

Step 6: GPC with Sephacryl S-200HR

The dialyzed sample (2.8 mL out of 3.1 mL, 67.5 mg protein) was applied to a Sephacryl S-200HR column (Packed in a medium pressure glass column from Kontex, 2.5 cm x 60 cm, pre-equilibrated with Buffer VI). The column was run in the ascending mode. At the end of the sample loading, ~ 3 mL of the dialysis buffer was loaded to push all of the sample onto the column before starting the elution with buffer VI (1.0 mL/min, 4.0 mL/fraction). High CGA synthase activity was eluted in Ve = 166-186 mL with highest activity in Ve = 174 mL, which corresponded to M_R of 43,000. Five fractions were pooled (20 mL). In a separate experiment, the highest activity was eluted in Ve = 182 mL, corresponded to M_R of 35,000.

Step 7: FPLC IEC with DEAE-8HR

Active fractions (20 mL, 3.3 mg protein) from the S-200HR column were applied immediately to a DEAE-8HR FPLC column (1.0 cm x 10 cm) equilibrated in Buffer VII. The column (run in the ascending mode, flow rate 1.0 mL/min) was first washed with buffer VII for 10 min and then eluted with a gradient of NaCl (0-50 mM in 5 min, and then 50-200 mM in 45 min) in buffer. Fractions of 2.0 mL were collected and assayed for activity. The chromatographic profile is shown in Fig VI-6. The most active fraction (fraction 14) had a protein concentration of 76 μg/mL, which was high enough for directly use in kinetic and other studies. Part of this (220 μL) was concentrated to 20 μL with an Ultrafree MC filtration unit and the concentrated sample was used for the SDS-PAGE analysis (Figure VI-7). To the rest of the final preparation, an equal volume of glycerol was added (final glycerol concentration should be 50%), shaken gently and then stored at - 20 °C (the solution was used for kinetic studies).

Affinity Chromatography with UDP-glucuronate Agarose

One of the two active fractions (Fraction 5 in Fig VI-8, 2 mL, ~0.09 mg/mL protein) from the IEC of a different preparation purification were combined, diluted with

2.0 eq volume of buffer IX and loaded onto an affinity column (1.5 cm x 6 cm, ~10 mL of packing medium, pre-equilibrated with buffer IX) with a flow rate of 0.2 mL/min. After loading, the column was washed with 10 mL of buffer X and then with a linear gradient of 0-200 mM of NaCl in 30 mL of buffer X (flow rate 0.5 mL/min). CGA synthase was eluted at an NaCl concentration of ~ 80 mM. Two fractions (1 mL/fraction) were most active and were combined (designated as S-9, 2 mL, 0.031 mg/mL) and used for cofactor requirement analysis. Half of the preparation (1 mL) was concentrated to 100 μL with Ultrafree MC filtration unit (M_R cut at 10,000) and was saved for SDS-PAGE analysis (Fig VI-8).

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Chapter VII

Conclusions and Prospects

Through a combined approach with conventional whole-cell feedings of isotopically labeled putative intermediates, feeding metabolic inhibitors, and identifying biosynthetic enzymes in the CFE, many key features of the biosynthesis of BS have been elucidated as summarized in Fig VII-1.

BS is now known to be derived from the primary hexose metabolism via the action of UDP-glucose 4'-epimerase, UDP-glucose 6'-oxidoreductase and CGA synthase leading to the first committed intermediate, CGA. The nucleoside intermediate is then modified through several as yet unidentified reactions, but they are likely to be similar to those in the 3',6'-dideoxy liposaccharrides biosynthesis, to the last intermediate, DeMeBS. DeMeBS is then converted to BS by DeMeBS δ-N-methyltansferase. CGA synthase, the first committed enzyme to the nucleoside portion in the pathway, has many similar features of catalysis to that of UDP-GTs isolated from eukaryotic organisms. However, CGA synthase has some distinctly different physicochemical properties: it is non-membrane associated, non-phospholipid dependent and it has a strict substrate specificity.

From the feeding of metabolic inhibitors, several new metabolites were isolated: PPN, PPN-oxime, 2'-ArgH-PPN, 4'-ArgH-PPN and Iso-BS. Inhibitor feedings also resulted in the accumulation of important intermediates: CGA, PPNC and DeMeBS. It was the readily availability of these accumulated intermediates that greatly facilitated the identification of the biosynthetic enzymes. We propose that the approach of feeding metabolic inhibitors to facilitate the identification of biosynthetic intermediates could be extended to the study of other nucleoside antibiotics as listed in Table I-1 (p 5).

Although the first committed step towards the nucleoside formation has been elucidated, mechanisms related to C-2', C-3' deoxygenation and C-4' transamination

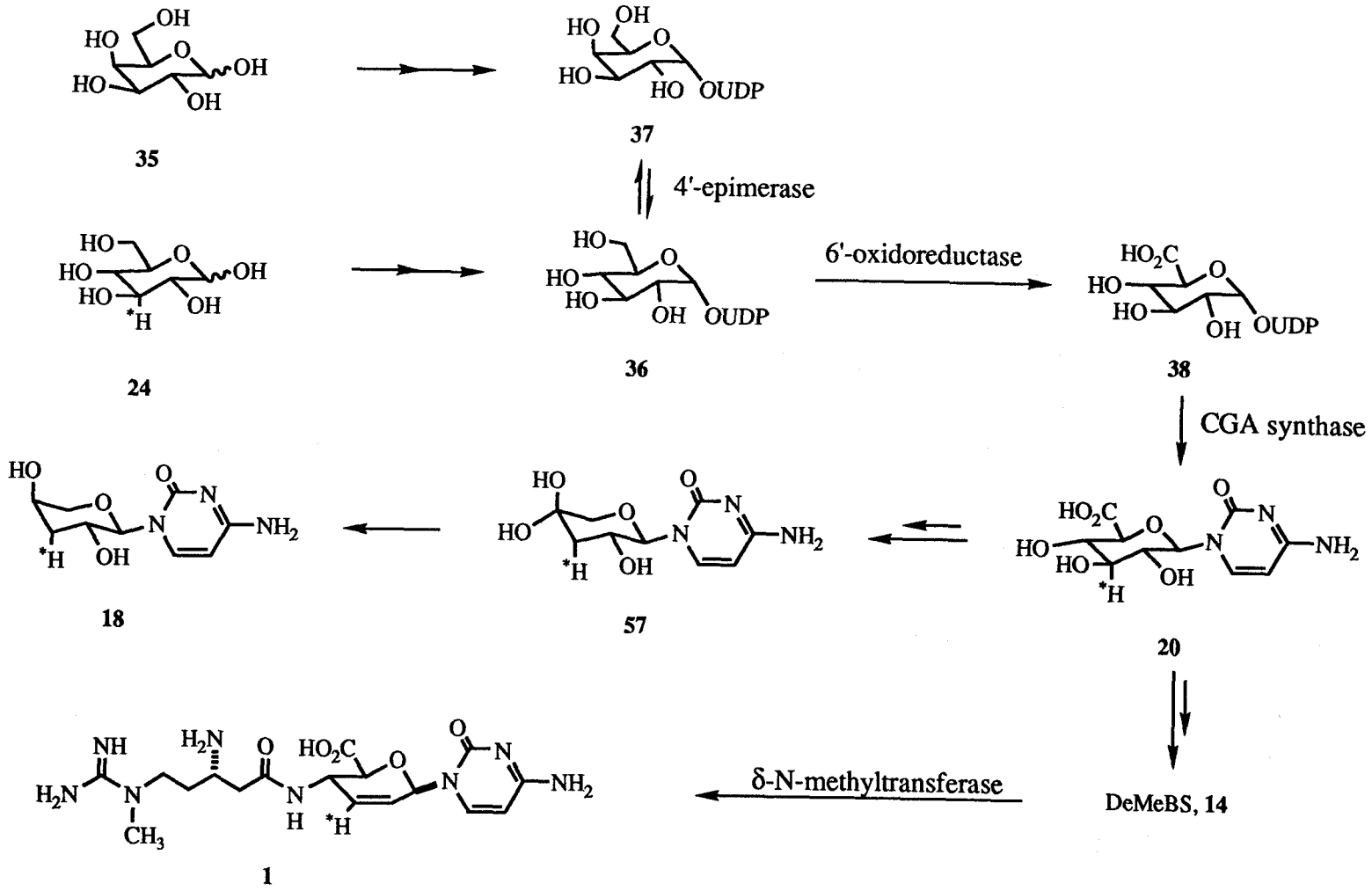

Fig VII-1. Summary of present studies on the biosynthesis of BS

need further study. Our initial efforts to find the arginine-2,3-aminomutase activity in crude CFE of *S. griseochromogenes* were not been successful. This could be attributed to one or more of the following reasons, such as, instability of the enzyme, low sensitivity of the assay procedure, and the inappropriate reaction conditions. The future effort on the biosynthesis of BS should focus on the following aspects.

a) Identifying biosynthetic enzymes or intermediates for the transformation of CGA to CN.

b) Obtaining sequence information from the purified CGA synthase, and then searching for the synthase gene and the genes for the whole biosynthetic pathway.

c) Developing a more sensitive method for activity assay in the isolation of arginine 2,3-aminomutase.

d) Extending the present study to other peptidyl nucleoside antibiotics.

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