AN ABSTRACT OF THE THESIS OF

Nam-Tae Woo for the degree of Master of Science in Chemistry presented on March 28, 1985

Title: Metabolism of L-α-Arginine in the Biosynthesis of Blasticidin S.

Abstract approved: Redacted for Privacy
Dr. Steven J. Gould

Two labelled arginines, DL-[2,3,3,-$^{2}H_3$]arginine, 8, and DL-[^{13}C, 2-^{15}N]arginine, 9, were synthesized. These were fed to cultures of Streptomyces griseochromogenes in order to probe the mechanism of the apparent arginine-2,3-aminomutase reaction occurring in biosynthesis of blasticidin S, 1. In previous studies of the enzymes lysine-2,3-aminomutase and tyrosine-2,3-aminomutase different mechanisms were demonstrated for each reaction. Our experiments were designed to reveal whether the mechanism of the arginine rearrangement is similar to either one of these or whether it is different from both. Blasticidin S obtained from each feeding was analyzed by high field NMR spectroscopy. We have found that an intramolecular migration of nitrogen from C-2 of α-arginine to C-3 of β-arginine takes place. In addition, our results indicate that when 8 was fed, at least some hydrogen migration from C-3 of α-arginine to C-2 of β-arginine had occurred. These results are qualitatively similar to those previously obtained for the lysine-2,3-aminomutase reaction.

METABOLISM OF L-α-ARGININE IN THE BIOSYNTHESIS OF BLASTICIDIN S

by
Nam-Tae Woo

B.S., Seoul National University, 1975

M.S., Seoul National University, 1977

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of
Master of Science
Completed March 28, 1985
Commencement June 1985

APPROVED:

Redacted for Privacy

Professor of Chemistry in Charge of Major

Redacted for Privacy

Head of Department of Chemistry

Redacted for Privacy

Dean of Graduate School

Date Thesis is presented March 28, 1985

ACKNOWLEDGMENTS

I would like to thank Dr. Steven Gould for encouragement and direction given to me in the last three years. His profound and thorough knowledge always has been a key to solving many problems. The concepts he stressed to me to be a capable scientist and skills he demonstrated shall always be remembered. I am also thankful to Dr. V. A. Palaniswamy for his technical assistance, to Pete Yorgey for solving microbiological problems, to Rodger Kohnert for high field NMR spectra, and all the laboratory members for their helpful discussions and friendship. Finally, I wish to thank the National Institutes of Health for their financial support of this research.

TABLE OF CONTENTS

LIST OF FIGURES

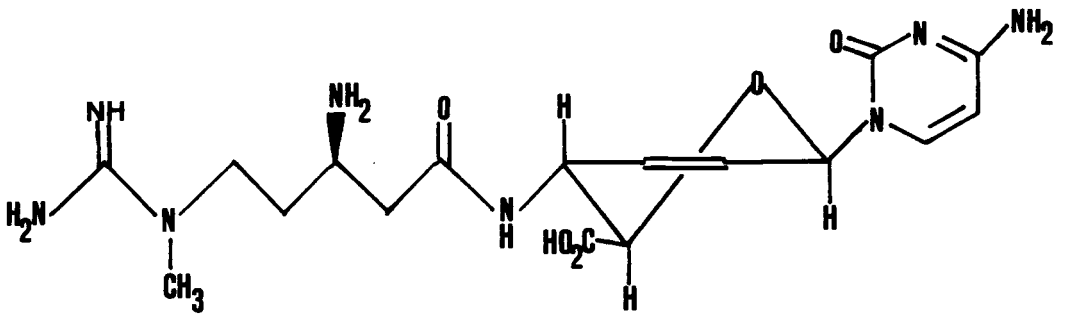
Figure

LIST OF TABLES

METABOLISM OF L-α-ARGININE IN BIOSYTHESIS OF BLASTICIDIN S

I. INTRODUCTION

History and Biological Effects of Blasticidin S. Blasticidin S ($C_{17}H_{26}N_8O_5$), 1, is an antifungal antibiotic first isolated from cultures of Streptomyces griseochromogenes,[1] that also exhibits antitumor[2,3] and antiviral[4] activities. Blasticidin S was reported to exert its antifungal effect by inhibiting the protein synthesis of Piricularia oryzae.[5] This was indicated by the experiments of Misato et al,[5] who showed that blasticidin S inhibited incorporation of (^{14}C)-glutamic acid into the protein fraction in Piricularia oryzae in intact cells.[6] It is also toxic to mice and causes destruction of the cells of the mucous membranes.[7]



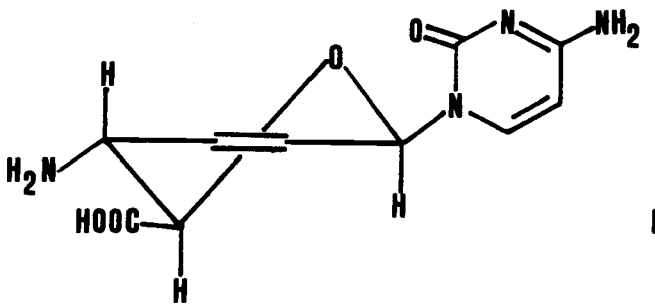
Blasticidin S 1

Chemical and Physical Properties of Blasticidin S.

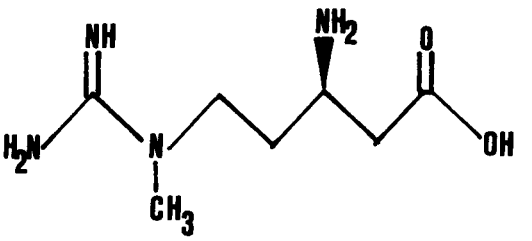
Blasticidin S free base is a white crystalline solid that melts with decomposition at 235-236°C.[1] The free base of 1 easily absorbs CO_2 from the air and forms a carbonate salt, $C_{17}H_{26}N_8O_5 \cdot 1/2\ H_2CO_3$. The free base of blasticidin S is soluble in water and acetic acid, but is insoluble in nearly all other organic solvents.[1] Optical rotation of the free base of 1 is $[\alpha]^D = +108.4°$ (C=1% in H_2O). The absorption maximum in the ultraviolet spectrum is 274nm (ε = 13,400) in 0.1N hydrochloride acid and in 0.1N sodium hydroxide its value is 267 nm (ε = 8,850). This is the same absorption pattern as that of cytidine, suggesting that 1 is an N-1 derivative of cytosine. Blasticidin S contains one acidic group and three basic groups, with pKa's of 2.4 (carboxylic acid), 4.6 (amino group of cytosine), 8.0 (β-amino group) and above 12.5 (guanidine group). Blasticidin S forms crystalline picrate and helianthate salts.[1] It has been shown that blasticidin S (in aqueous solution) is most stable at pH 5.0 - 7.0, is less stable at pH 8.0 - 9.0., and is least stable at pH 4.0.[1]

Degradation and structural elucidation studies of blasticidin S first resulted in an empirical formula of $C_{18}H_{24}N_8O_5 \cdot H_2O$.[7,8] It was later revised to $C_{17}H_{26}N_8O_5$ based on the isolation and characterization of the products cytosinine 2 and blasticidic acid

3, which were obtained from the hydrolysis of 1. It was thus found that this antibiotic is a 1-(substituted dihydropyran-2-yl)-cytosine.[9] This postulation has since been confirmed by an x-ray diffraction study.[10,11] The structure of blasticidin S with the correct absolute configuration is shown in formula 1.[12] It is particularly noteworthy for the current study that 3 is a β-amino acid.



Cytosinine 2



Blastidic acid 3

Biosynthesis of Blasticidin S. The results from feeding experiments with several radioactive precursors are given in Table 1.[12,13] It was found that (U-^{14}C)-D-glucose, (6-^{14}C)-D-glucose, (1-^{14}C)-D-glucose, (2-^{14}C)-cytosine, (U-^{14}C)-cytidine, (methyl-^{14}C)-L-methionine, (guanidino-^{14}C)-L-arginine and (U-^{14}C)-L-arginine were incorporated into blasticidin S in higher yields than were a variety of other compounds.

Table 1. Percent Incorporation of ^{14}C-Labeled Compounds into Blasticidin S.[14]

	(%)
(U-^{14}C)-D-glucose	3.7
(1-^{14}C)-D-glucose	4.0
(6-^{14}C)-D-glucose	4.9
(2-^{14}C)-cytosine	95.1
(U-^{14}C)-cytidine	15.3
(Methyl-^{14}C)-L-Methionine	38.3
(Guanidino-^{14}C)-L-arginine	51.2
(U-^{14}C)-L-arginine	30.3
*(U-^{14}C)-L-aspartic acid	0.5
(1-^{14}C)- -alanine	0.6
(U-^{14}C)-acetic acid	0.5
(U-^{14}C)-glycine	1.1
(U-^{14}C)-alanine	0.5

*unlabeled cytosine was added simultaneously.

The blasticidin S isolated from these feedings was degraded by the scheme presented in Figure 1, and the radioactivity of the degradation products was measured by liquid scintillation counting.

Figure 1. Chemical Degradation of Blasticidin S.

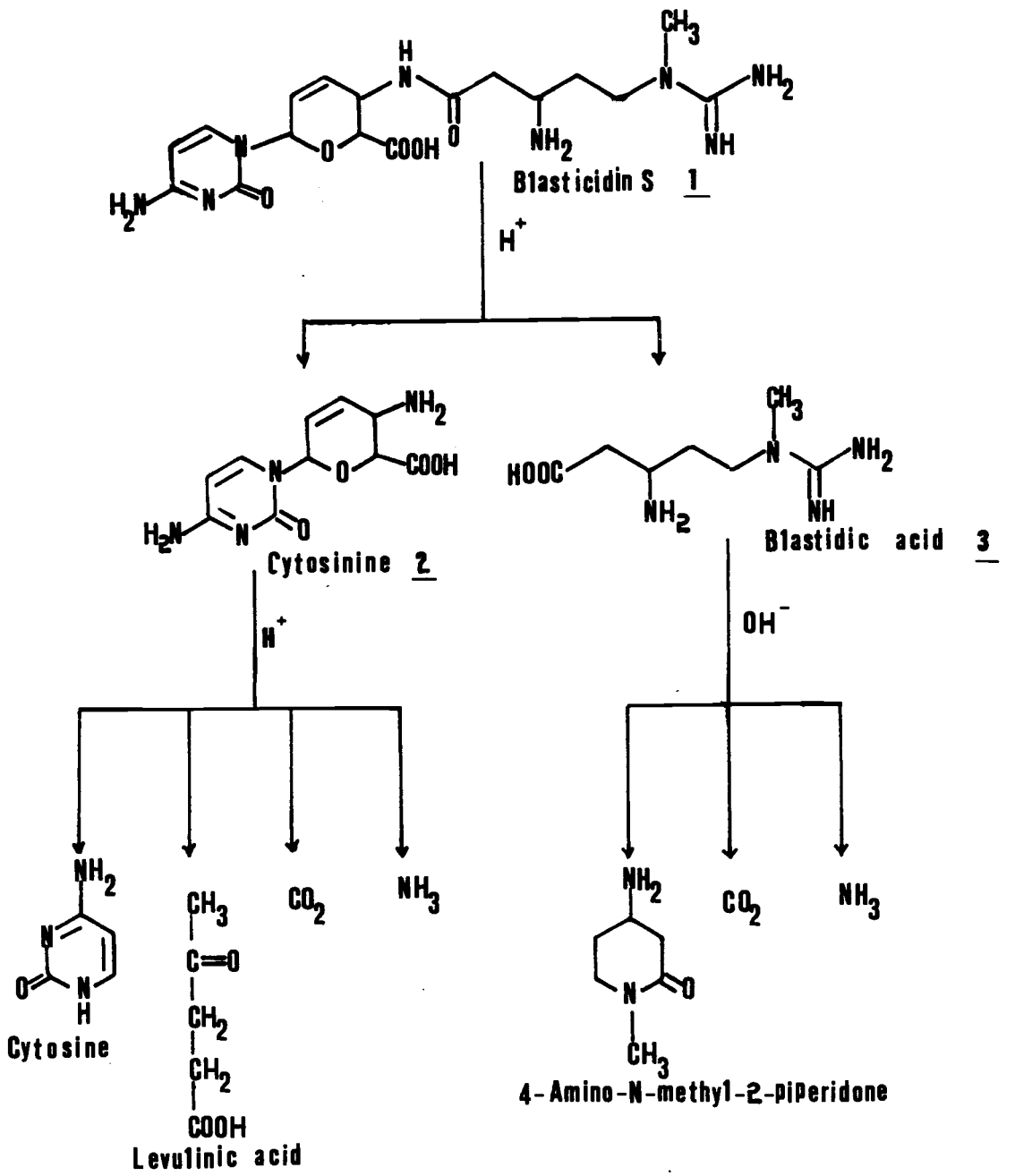


Table 2. Percentage of Radioactivity in the Products of Blasticidin S Degradation.[13]

Precursor / Compound	(2-^{14}C)-Cytosine	(U-^{14}C)-Cytidine	(U-^{14}C)-D-Glucose	(1-^{14}C)-D-Glucose	(6-^{14}C)-D-Glucose	(Methyl-^{14}C)-L-methionine	(U-^{14}C)-L-arginine	(Guanidino-^{14}C)-L-arginine
blasticidin S	100	100	100	100	100	100	100	100
blastidic acid	0	0.5	16.9	14.8	26.6	98.3	98.0	97.9
CO_2	-	-	-	-	-	-	*24.5	99.5
4-amino-N-methyl-2-piperidone	-	-	-	-	-	-	*69.2	0
CH_3I	-	-	-	-	-	101.6	0	0
cytosinine	97.1	98.2	83.6	89.2	74.6	0.1	1.5	2.7
cytosine	96.1	97.9	22.8	21.6	23.8	0.1	1.1	2.6
levulinic acid	0	0.2	53.0	62.9	0	-	-	-
CO_2	0	0.3	11.3	6.7	45.7	-	-	-

*If (U-^{14}C)-L-arginine were incorporated unmodified the ratio of radioactive CO_2 to 4-amino-N-methyl-2-piperidone would be 1:5; nevertheless, the value found in the experiment was approximately 1:3. The deviation from the theoretical value is considered to be caused by transamidination between ^{14}C-labeled arginine and unlabeled ornithine which was produced during cultivation.

The results, presented in Table 2, indicated the following facts:

(i) cytosine was incorporated intact into the cytosine nucleus of blasticidin S.

(ii) the sugar part of cytosinine was derived from D-glucose.

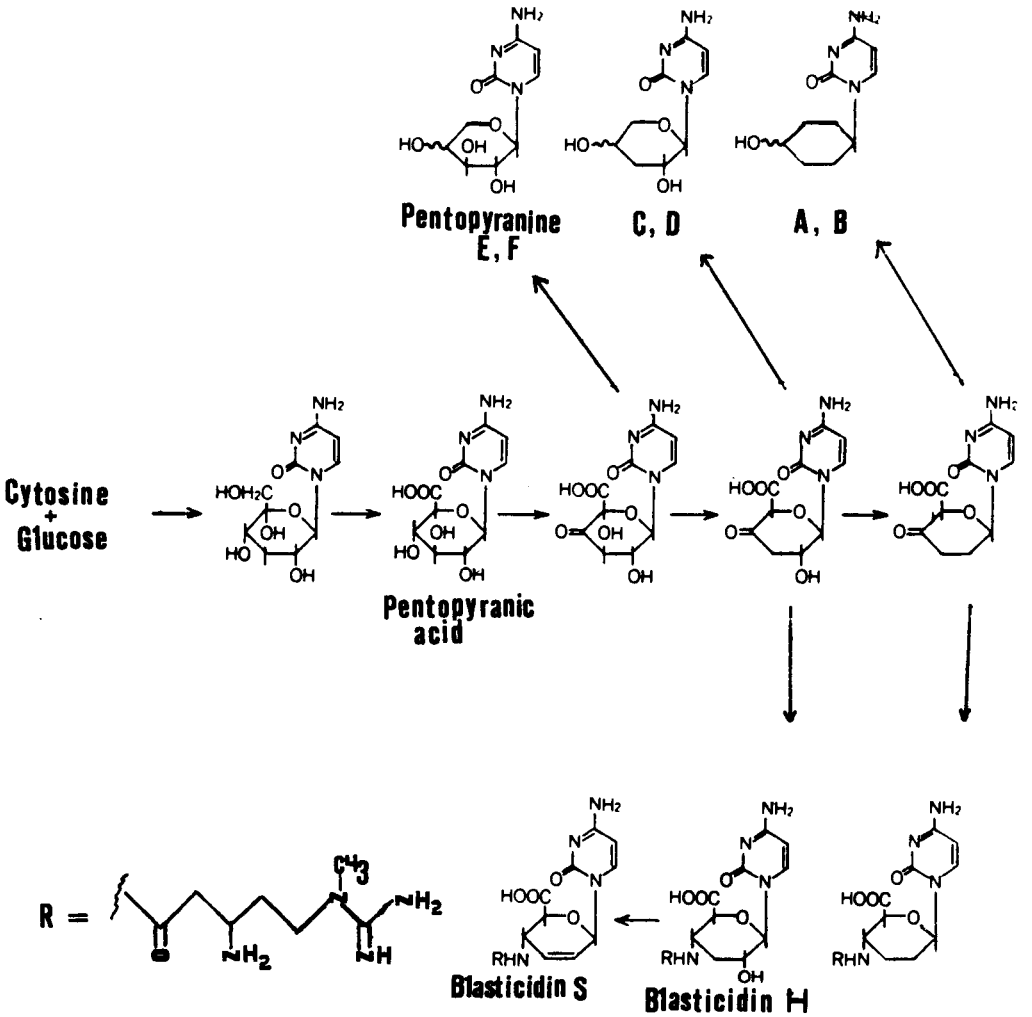
(iii) when (U-^{14}C)-cytidine was added, almost all of the radioactivity existed in the cytosine nucleus of blasticidin S. Therefore, only the cytosine nucleus was incorporated into blasticidin S, after cleavage of the C-N linkage of cytidine.

(iv) the N-methyl group of blastidic acid was derived from methionine.

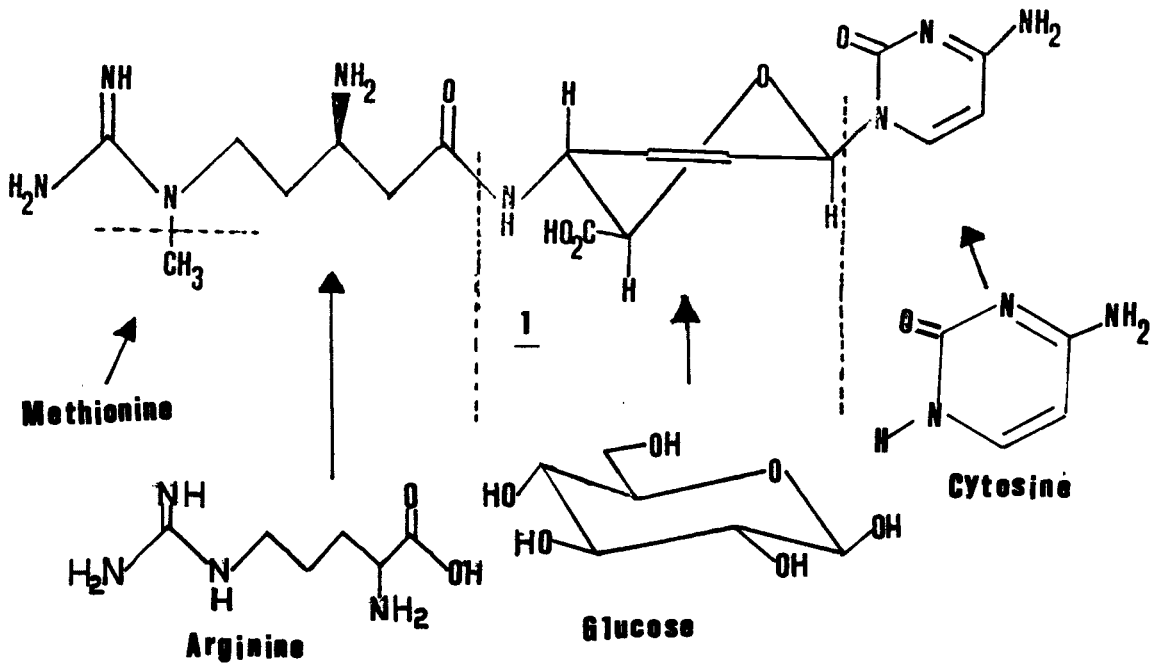
(v) the whole carbon skeleton of L-arginine was probably incorporated into the skeleton of blastidic acid.

Seto et al.[14] further studied the biosynthesis of the unusual nucleoside cytosinine. They isolated a number of additional metabolites from cultures of S. griseochromogenes. These are pentopyranic acid,[14] pentopyranines A to F,[32,33,34] and blasticidin H.[19] Figure 2 shows the possible biosynthetic pathway of blasticidin S formation involving these additional metabolites.

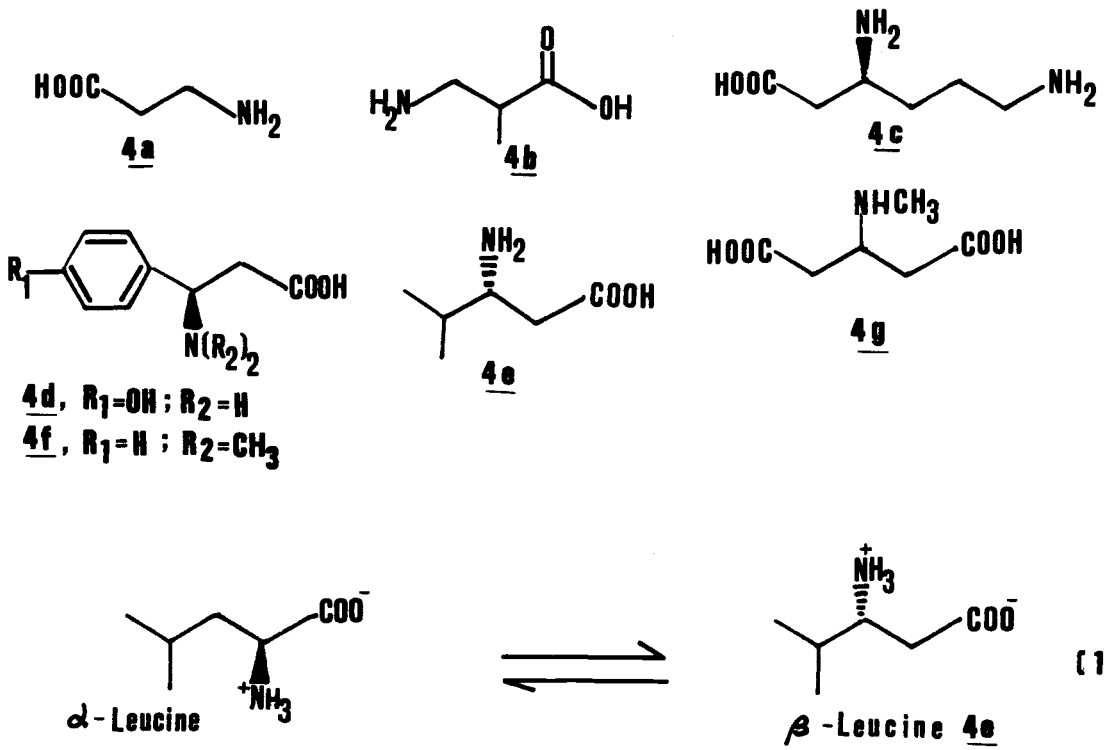
Figure 2. Proposed Pathway for the Biogenesis of Blasticidin S.



From the results mentioned above the primary precursors of blasticidin S can be depicted as follows.



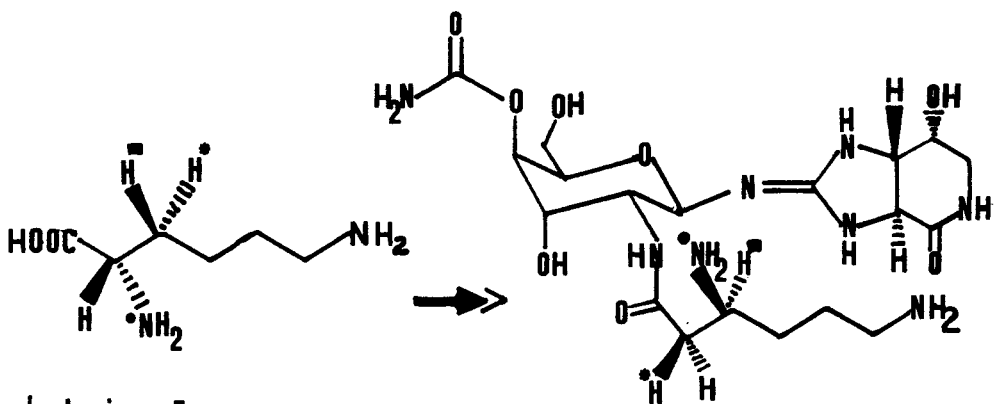
Biosynthesis of β-Amino Acids. The structures of other naturally occurring β-amino acids are: β-alanine, 4a,[29] β-aminoisobutyric acid, 4b,[29] β-lysine, 4c,[20,21] β-tyrosine, 4d,[35] β-leucine, 4e,[36] (dimethylamino)-β-phenylalanine, 4f,[37] and N-methyl-β-glutamic acid, 4g.[38]



[1]

It would be a reasonable assumption that β-amino acids would be derived from the corresponding α-amino acids. However it is known that 4a is formed by degradation of uracil[28] or by the decarboxylation of aspartic acid,[28] and 4b is formed by degradation of thymine.[29] Work on β-leucine, 4e,[31] did indicate that (2S)-α-leucine is the metabolically active substrate for leucine-2,3-aminomutase, which converts it reversibly to (3R)-β-leucine, as shown in (1). No details from isotope labeling experiments to elucidate the stereochemical features of the isomerization of α-leucine have been reported.

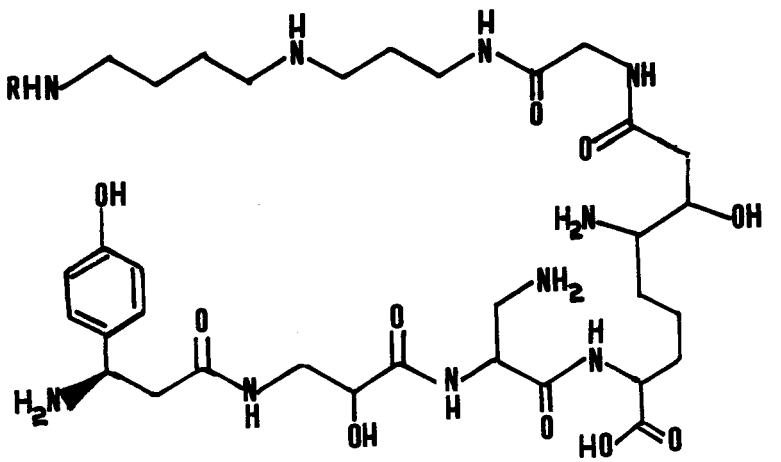
The studies of streptothricin F, 6, biosynthesis performed in this laboratory[15,21] and stereochemical studies of lysine-2,3-aminomutase isolated from Clostridium subterminale SB4[21] revealed that the β-lysine moiety is derived from α-lysine, 5. The α-amino group migrates to C-3 in an intramolecular fashion while the 3R-hydrogen migrates to C-2 in an intermolecular fashion. In each case the migration results in inversion of stereochemistry at the respective center.



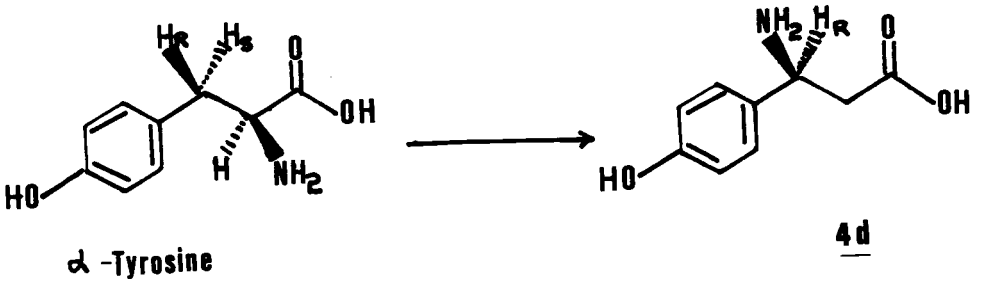
α-Lysine 5

Streptothricin F 6

The biosynthesis of β-tyrosine, 4d, which occurs in the linear oligopeptide antibiotic edeine, 7, isolated from the cultures of Bacillus brevis,[30,35] results from the isomerization of L-α-tyrosine and this reaction is catalyzed by the enzyme tyrosine-2,3-aminomutase.[35] An



7, Edeine A; R=H
" B; R = $H_2N-\overset{\overset{NH}{\|}}{C}-$



α-Tyrosine

4d

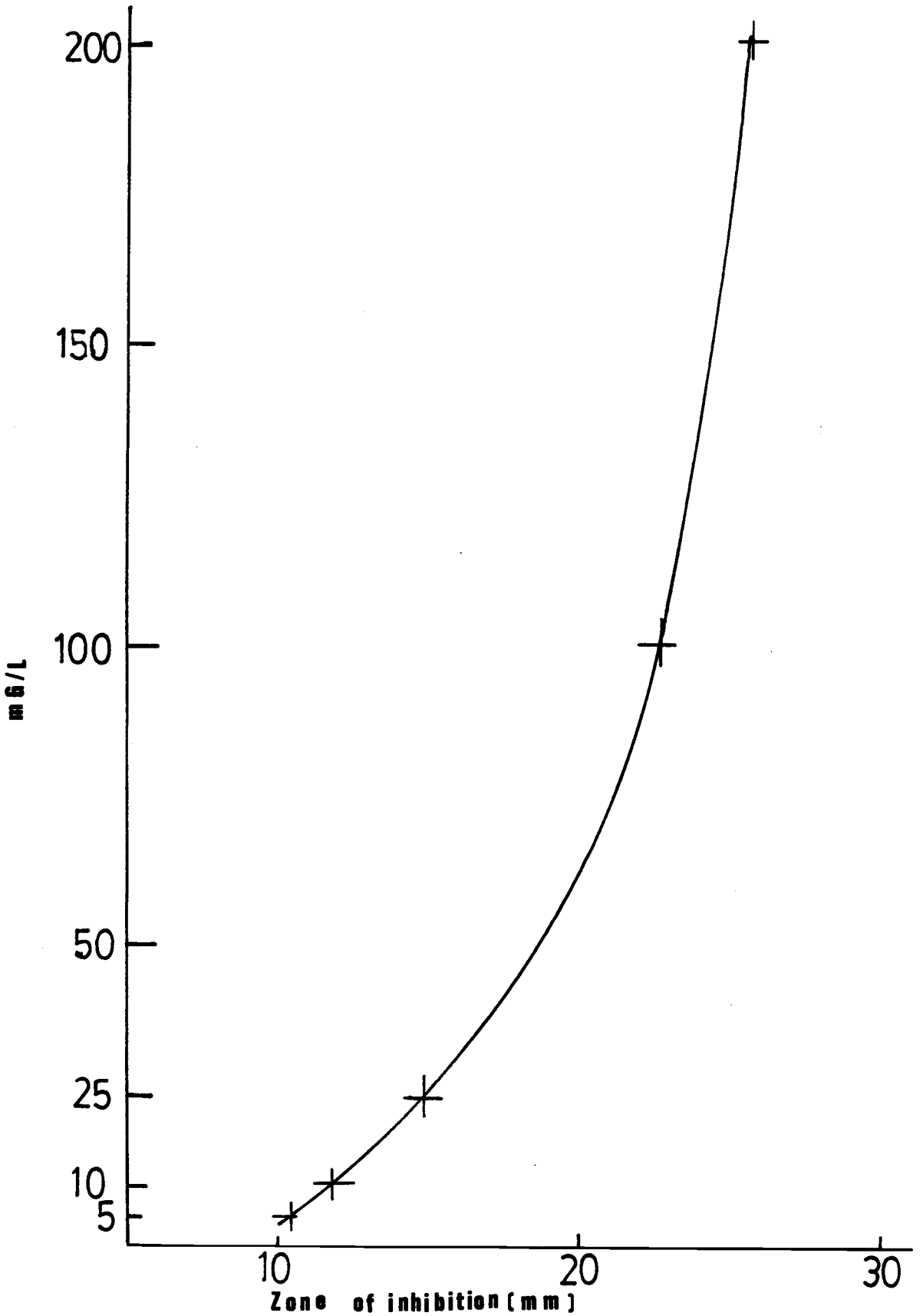
enzyme preparation obtained from B. brevis[35] transformed L-α-tyrosine to L-β-tyrosine with loss of the 3-pro-S hydrogen.[30] In addition, much of the original nitrogen was lost when L-α-[^{15}N]-tyrosine was the substrate (the data appears to indicate an 80 % loss), and when an equimolar amount of $^{14}NH_4Cl$ was included the retention of ^{15}N was reduced by half. The data suggest that the original nitrogen is eliminated and mixes with an ammonium pool, although some direct transfer from C-2 to C-3 cannot be ruled out by the experiments reported. Nonetheless, the labeling data clearly indicate a different mechanism for this enzyme than that of the lysine-2,3-aminomutase.

Purpose of the Present Study. Preliminary studies reported by H. Seto et al.[12,13] established that arginine, glucose, and cytosine are precursors to 1. However, much remained to be done to understand the biosynthetic pathway and the mechanisms of the key reactions. The specific objective of this thesis work was to examine whether the mechanism of the apparent arginine-2,3-aminomutase reaction in blasticidin S biosynthesis is similar to the lysine-2,-3-aminomutase or to the tyrosine-2,3-aminomutase, or whether it is different from both. Experiments were designed and executed to determine whether there are migrations of nitrogen and hydrogen (inter- or intramolecular) involved in the conversion of α-arginine to β-arginine.

II. RESULTS AND DISCUSSION

Bioassay. The goal of this research was to study aspects of the biosynthesis of blasticidin S relating to arginine metabolism. Since this antibiotic was to be produced by fermentation of the producing organism, Streptomyces griseochromogenes, it was necessary to monitor the quantity and timing of blasticidin S production. The approach chosen was a microbiological assay for inhibition of the growth of a blasticidin-sensitive bacterium. The most commonly used methods of microbiological assay are the plate (or agar diffusion) method and the cup-plate method.[40] The bioassay of blasticidin S using the cup-plate method had been initially reported by Takeuchi et al.,[1] and they used Bacillus subtilis PCI 219 as the test organism. The procedure reported covered the concentration range of approximately 50-1000 µg/mL. Subsequently, Koyama[16] found that Bacillus cereus gave a better defined clear zone with blasticidin S than had Bacillus subtilis PCI 219. Shomura et al.[17] found Bacillus circulans IAM-112 to be the most favorable test organism for the bioassay of blastidicin S. Using a strain of Bacillus circulans obtained from the microbiology department of Oregon State University, we have followed Shomura's [17] procedure. The cup-plate method was used, employing stainless steel cylinders (5 mm x 7 mm), and a standard curve was prepared

Figure 3. Bioassay of Blasticidin S with Bacillus circulans.



using concentrations varying from 5 to 200 μg/mL. The results are shown in Figure 3.

Fermentation of Blasticidin S. Fermentation studies were started at the University of Connecticut using a synthetic medium developed by H. Seto et al.[12] for their biosynthetic studies. Although a substantial amount of antibiotic activity was produced by S. griseochromogenes ATCC 21024 (180 mg/L) from this synthetic medium,* it was not clear that it was due to blasticidin S. Much of the bioactivity was lost during the isolation procedure and the small amount of material recovered was not positively identified. A further concern regarding our early work was that the synthetic medium contained a significantly large amount of sucrose (100 g/L). This potentially could interfere with the success of certain feeding experiments planned to investigate other aspects of blasticidin S biosynthesis where hydrolysis of sucrose could result in a

---

*The synthetic medium used for the production of 1 contained the following: (A) sucrose, 100 g, and glucose, 5 g, in 800 mL of distilled water; (B) $(NH_4)_2HPO_4$, 10 g, and KCl, 3 g, in 100 mL of distilled water; (C) $CaCl_2 \cdot 2H_2O$, 3 g, in 50 mL of distilled water; (D) $MgSO_4 \cdot 7H_2O$, 2 g, $FeSO_4 \cdot 7H_2O$, 40 mg, and $ZnSO_4 \cdot 7H_2O$, 40 mg, in 50 mL of distilled water. Parts A, B, C, and D were sterilized separately and combined after cooling to avoid precipitation of the mineral complex.

large *in vivo* pool of unlabelled glucose and unacceptable dilution of the labelled material to be fed. In order to standarize our procedures for all anticipated feeding experiments, the protocol was changed shortly after moving to Oregon State University.

The fermentation procedure adopted in feeding experiments was developed by P. Yorgey in this laboratory. He examined blasticidin S production using three strains of *S. griseochromogenes* (a strain obtained from the Kaken Chemical Co. in Japan, strain ATCC 14511, and strain ATCC 21024). The seed and fermenation media that were tested with each of these organisms were those suggested by the Kaken microbiologists and the synthetic fermentation media used by Seto et al.[12] We found that ATCC 21024 was the highest producer in both media, showing antibiotic activities equivalent to about 150 μg/mL in samples assayed directly from the fermentations. Our resulting standardized procedure used ATCC 21024 grown in the Kaken seed medium as an inoculum for the Kaken production medium for all subsequent studies. The contents of these media are listed below.

Kaken Seed Medium

Glucose	1.0 g
Beef extract	0.5 g
Polypeptone (BBL)	0.5 g
NaCl	0.1 g
dd H_2O	50 mL (in a 250 mL Erlenmeyer Flask)

Adjust the dissolved solution to pH 7.5 prior to autoclaving.

After autoclaving pH is approximately 7.0.

Kaken Production Medium

Sucrose	12.50 g
Soybean meal	2.50 g
Wheat embryo	6.25 g
Dried brewer's yeast	6.25 g
NaCl	1.50 g
ddH_2O	250 mL (in a 1 liter Erlenmeyer flask).

Adjust pH of the solution to 7.0 prior to autoclaving.

After autoclaving the pH of the medium is approximately 6.5.

The fermentation studies were carried out using a two-stage protocol. A sterilized solution of seed broth (100 mL in a 500 mL Erlenmeyer flask) was inoculated with a 1 cm x 1 cm square of spores grown on a yeast malt agar slant. After a two-day incubation period at 29°C and 225 rpm, a 2% (V/V) inoculum of this used to inoculate a production broth (250 mL in a 1 L Erlenmeyer flask). A time-course study of blasticidin S production was performed and the results are shown in Figure 4. Based on this curve, appropriate times for feeding and harvesting in the labeling

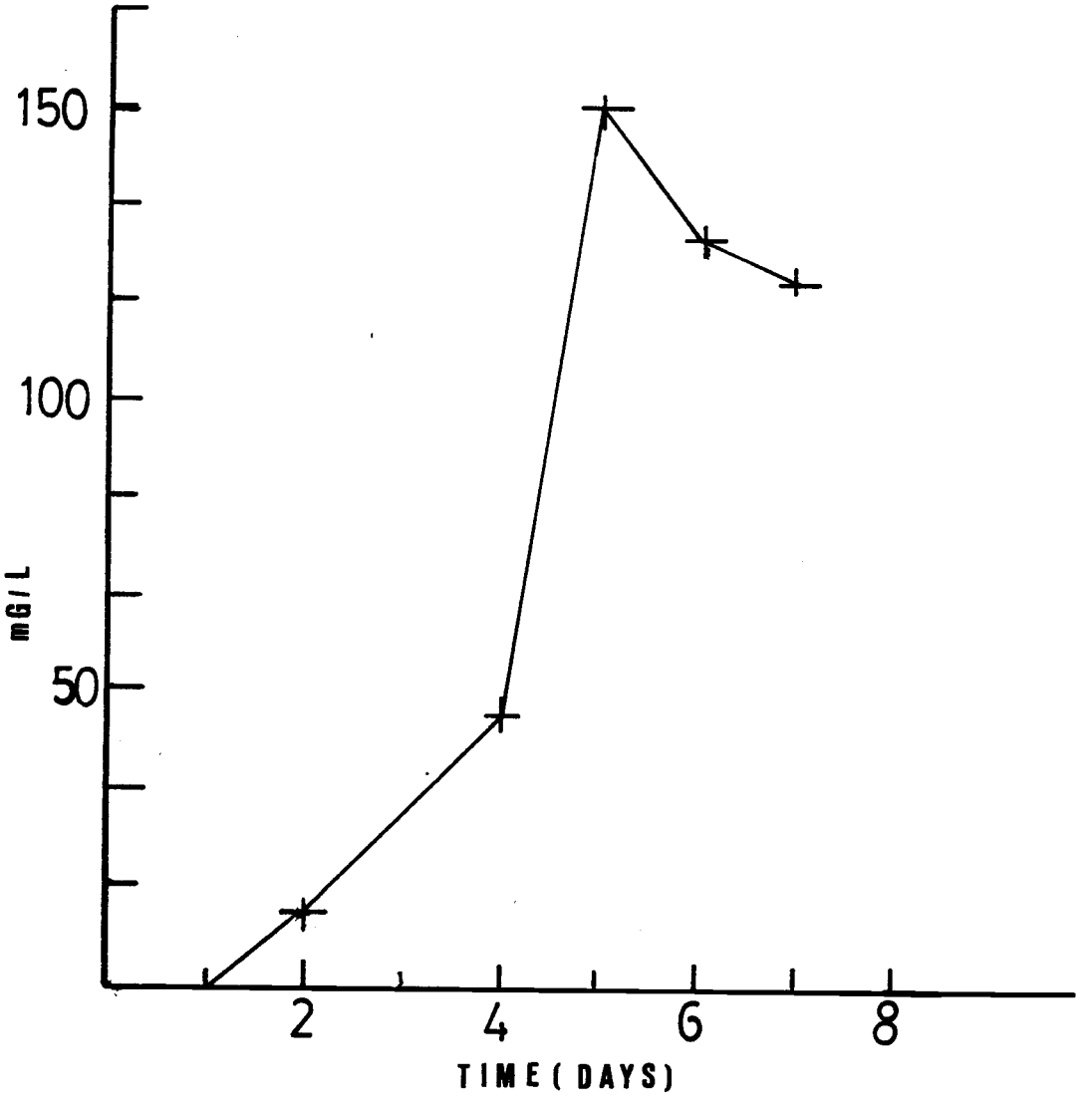


Figure 4. Time Course of Blasticidin S Production.

studies were chosen. These are described in conjunction with those studies.

Isolation of Blasticidin S. A typical experiment used two 250 mL broths and they were inoculated with a two-day seed culture. The fermentation was stopped after a total of 120 hours (five days). Bioassay typically indicated production of 300 mg/L of blasticidin S equivalents. At this point, attempted removal of the mycelia and other solids by vacuum filtration through cheese cloth and a celite bed in a Buchner funnel was extremely slow due to the viscosity of the Kaken fermentation medium. This problem was overcome by acidifying the filtrate to pH 3 after harvesting the fermentation and subsequently removing the precipitate by centrifugation.

The supernatant was neutralized to pH 6-7 and the clear brown solution was loaded onto a column of AG2-X8(OH-) anion exchange resin (5 cm x 13 cm). After loading, the column was washed with distilled water. These washings were mixed with the initial non-bound fraction and the pH of these combined fractions was adjusted to 6.0. Initially, it was found that at least one-third of the antibiotic activity was lost as a result of the anion exchange chromatography. This was probably due to the high basicity (pH >12.5) of the eluate since blasticidin S is unstable in strongly basic

solutions. Maintaining the eluate at pH 6.0 - 7.0 by simultaneous titration with acid eliminated much of this problem. The sample was next loaded onto an AG 50W-X2 (H^+) cation exchange column (3.3 cm x 23.3 cm), and the column was washed with distilled water, then with 5% pyridine, and finally with 1.2% NH_4OH at a flow rate of 1.5-2.0 mL/min. Fractions of 6-7 mL were collected and blasticidin S started eluting after approximately 600 mL of the NH_4OH wash. Samples of the fractions were spotted on silica gel TLC plates, and then checked by UV absorption and by reaction with ninhydrin. The fractions showing a ninhydrin positive reaction were further tested specifically for UV absorption at 266 nm.

Since pyridine absorbs strongly in the absorption range 240-280 nm, it would have been impossible to see the blasticidin S absorption clearly if blasticidin S it had come off at an early stage of the chromatography. Fortunately, blasticidin S did not come off until the pyridine concentration was greatly reduced. Once this was recognized, only fractions that did not have a strong pyridine smell were subsequently checked.

The fractions that were ninhydrin positive and that showed the correct UV absorption were finally checked by TLC before combining and lyophilizing those containing relatively pure antibiotic. Each step of the purification was monitored by bioassay. Usually, the antibiotic could be

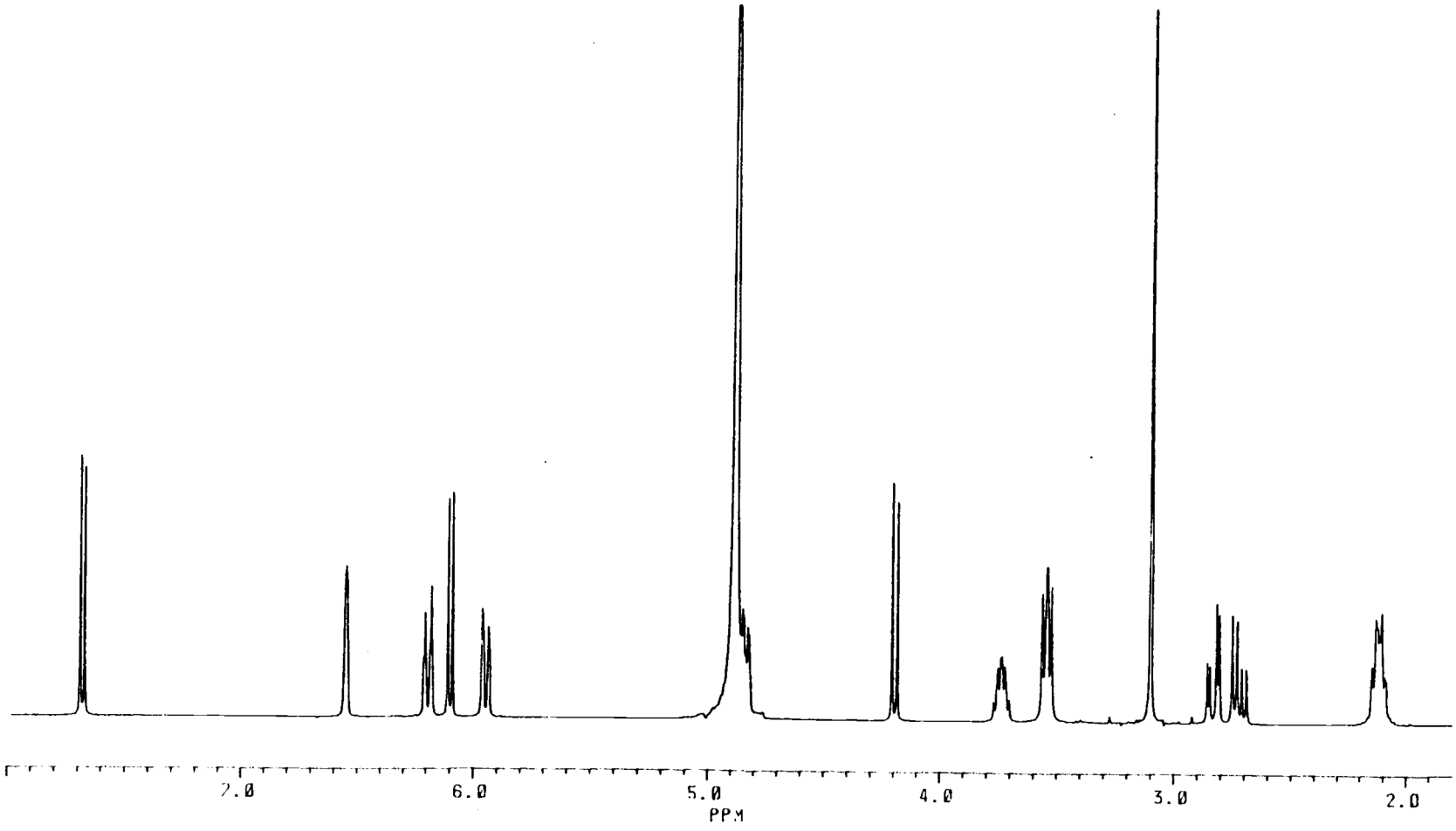
successfully recrystallized from methanol-water at this point, although failure was sometimes encountered at this late stage.

Proton and ^{13}C NMR Assignments of Blasticidin S.

Low-field ^{1}H NMR spectra of 1 had been reported and the protons were partially assigned by N. Otake et al.,[18] and H. Seto et al.[19] However, no carbon spectrum of 1 had been reported. The high field proton and carbon spectra of 1 were obtained from Varian Associates and were assigned unequivocally before any feeding experiments were conducted. A more recent proton spectrum, taken on the Bruker AM 400 is shown in Figure 5. However, no carbon spectrum of 1 had been reported.

1) ^{1}H NMR Assignments. A doublet at δ 7.59 (J = 7.5 Hz) was assigned to H-4 and the doublet at δ 6.02 (J = 7.5 Hz) was assigned to H-3. These assignments were quite well matched with the proton chemical shifts of authentic cytosine,[41] where the chemical shift of H-3 was at δ 6.2 (d, J = 7.5 Hz) and H-4 was at δ 7.8 (d, J = 7.5 Hz). H-5, deshielded by nitrogen, oxygen, and a double bond, was assigned to δ 6.46 (d, J = 0.9 Hz), this being the next furthest downfield resonance. The small coupling constant could be explained by the torsional angle between H-5, C-5, C-6 and H-6 being close to 90°, since no other methine proton in the molecule would have such a small coupling

Figure 5. ^{1}H NMR Spectrum of Blasticidin S.



constant. H-6, which is the only proton coupled with H-5 was assigned to δ 6.09 (ddd, J = 10.2, 1.8 and 0.9 Hz). These assignments were clearly confirmed from a two-dimensional COSY NMR experiment correlating proton and proton chemical shift resonances (Figure 6)

The resonance at δ 5.85, which has a coupling constant (J = 10.2 Hz) typical of a cis olefinic proton and another fine coupling (J = 2.4 Hz) was assigned to H-7. The clean doublet (J = 9.3 Hz) at δ 4.10 was assigned then to H-9. This in turn led to the assignment of H-8 to δ 4.73 from the COSY experiment. The H-8 proton was partly hidden under the big HOD peak in the one-dimensional spectrum.

The proton chemical shift assignments of the β-arginine portion of 1 were reasonably obvious. The two diastereotopic protons of H-12 were expected to have a doublet of doublet splitting pattern so were assigned to δ 2.62 (dd, J = 16.2, 8.1 Hz) and δ 2.74 (dd, J = 16.2, 4.8 Hz). H-16 (N-methyl) was assigned to δ 3.02. The chemical shift of the H-15 methylene protons, which are coupled to H-14, were assigned to δ 3.46 (t, J = 7.8 Hz). From the COSY experiment the H-14 methylene and the H-13 methine were then assigned to δ 2.03 (dd, J = 9.3, 6.6 Hz) and δ 3.64 ppm, respectively. The complete proton chemical shift assignments of blasticidin S are given in Table 3.

Figure 6. ^{1}H COSY Spectrum of Blasticidin S.

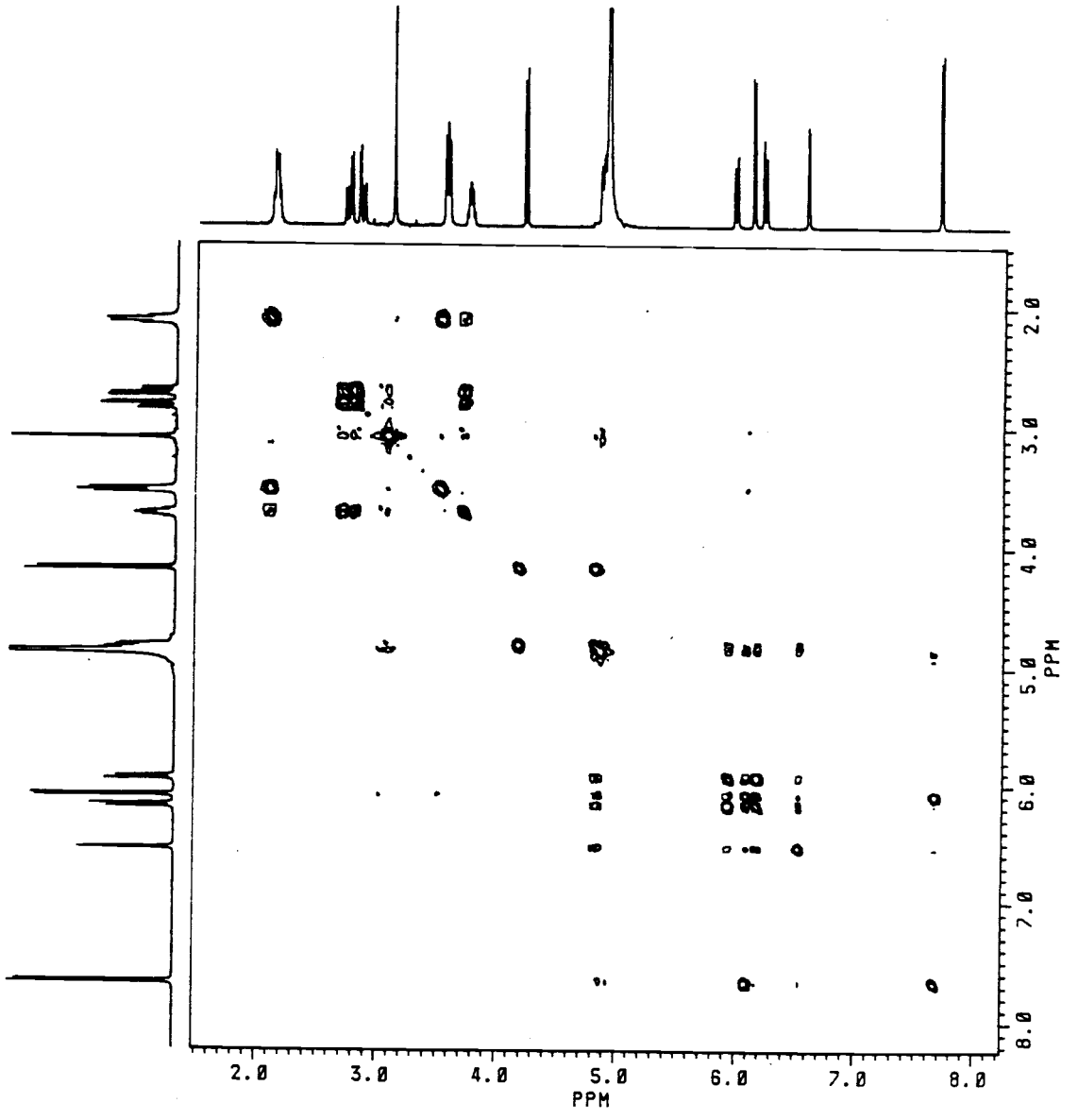
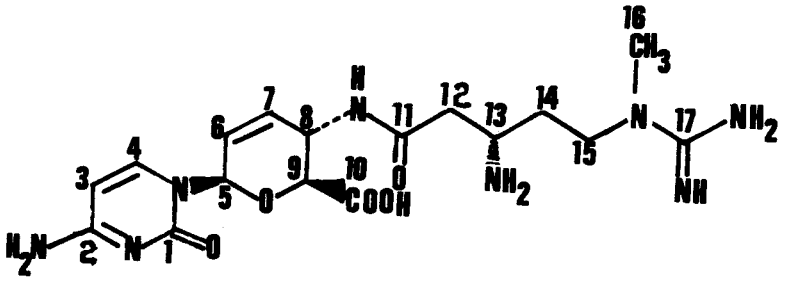


Table 3. ^{1}H NMR Assignments of Blasticidin S.



^{1}H Resonance #	Chemical Shift	J_{HH} (Hz)
3	6.02	$J_{3,4}=7.5$
4	7.59	$J_{3,4}=7.5$
5	6.46	$J_{5,6}=0.9$
6	6.09	$J_{6,7}=10.2$; $J_{6,8}=1.8$, $J_{5,6}=0.9$
7	5.85	$J_{6,7}=10.2$; $J_{6,8}=1.8$; $J_{7,8}=2.4$
8	4.73	$J_{8,9}=9.3$; $J_{7,8}=2.4$
9	4.10	$J_{8,9}=9.3$
12a	2.74	$J_{12a,12b}=16.2$;$J_{12a,13}=4.8$;$J_{12b,13}=8.$
12b	2.62	
13	3.64	$J_{12,13}=6.9$; $J_{13,14}=6.9$
14	2.03	$J_{14a,14b}=9.3$;$J_{13,14}$;$J_{14,15}=6.6$
15	3.46	$J_{14,15}=7.8$
16	3.02	———

2) ^{13}C NMR Assignments. The carbon-13 NMR spectrum was taken on the Bruker AM 400 at 100.6 MHz and is shown in Figure 7.

Referring to the ^{13}C NMR spectrum of authentic cytosine[42] and the HETCOR[43] (Heteroscalar Correlated) ^{1}H-^{13}C 2D-NMR spectrum of 1 (Figure 8), C-1 was assigned to δ 158.5, and δ 144.9 ppm should be C-4. C-3 was assigned to δ 98.7 and C-2 was assigned to δ 159.6. The carbons bearing three hydrogens (methyl), two hydrogens (methylene) and one hydrogen (methine) were characterized by a DEPT[44] (Distortionless Enhancement by Polarization Transfer) experiment. As can be seen in the DEPT spectra (Figure 9) the resonances at δ 98.7, δ 128.2, δ 135.0, and δ 144.9 are among those due to methine carbons.

Figure 7. ^{13}C NMR Spectrum of Blasticidin S.

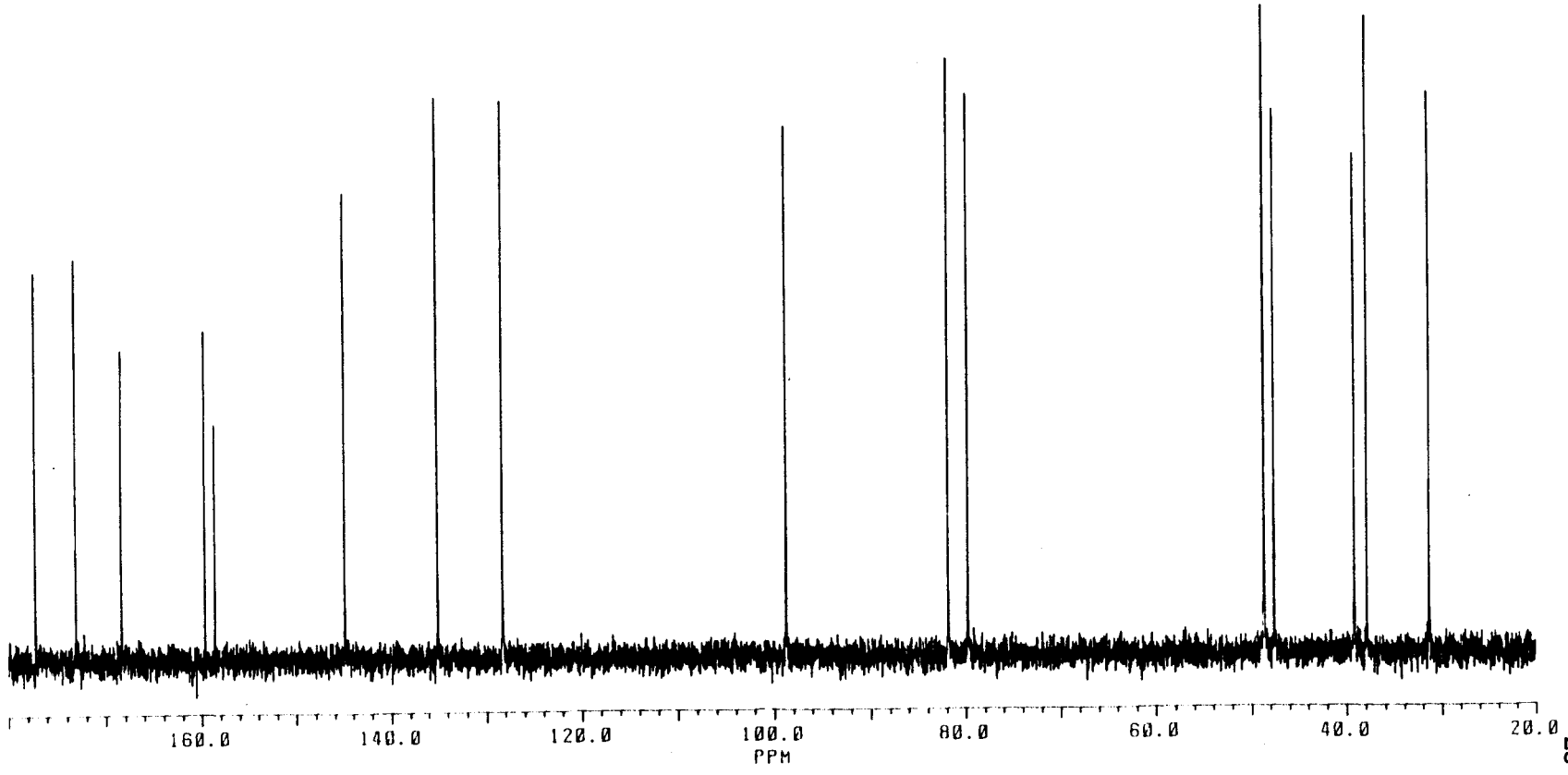


Figure 8. ^{1}H-^{13}C Chemical Shift Correlation Spectrum of Blasticidin S.

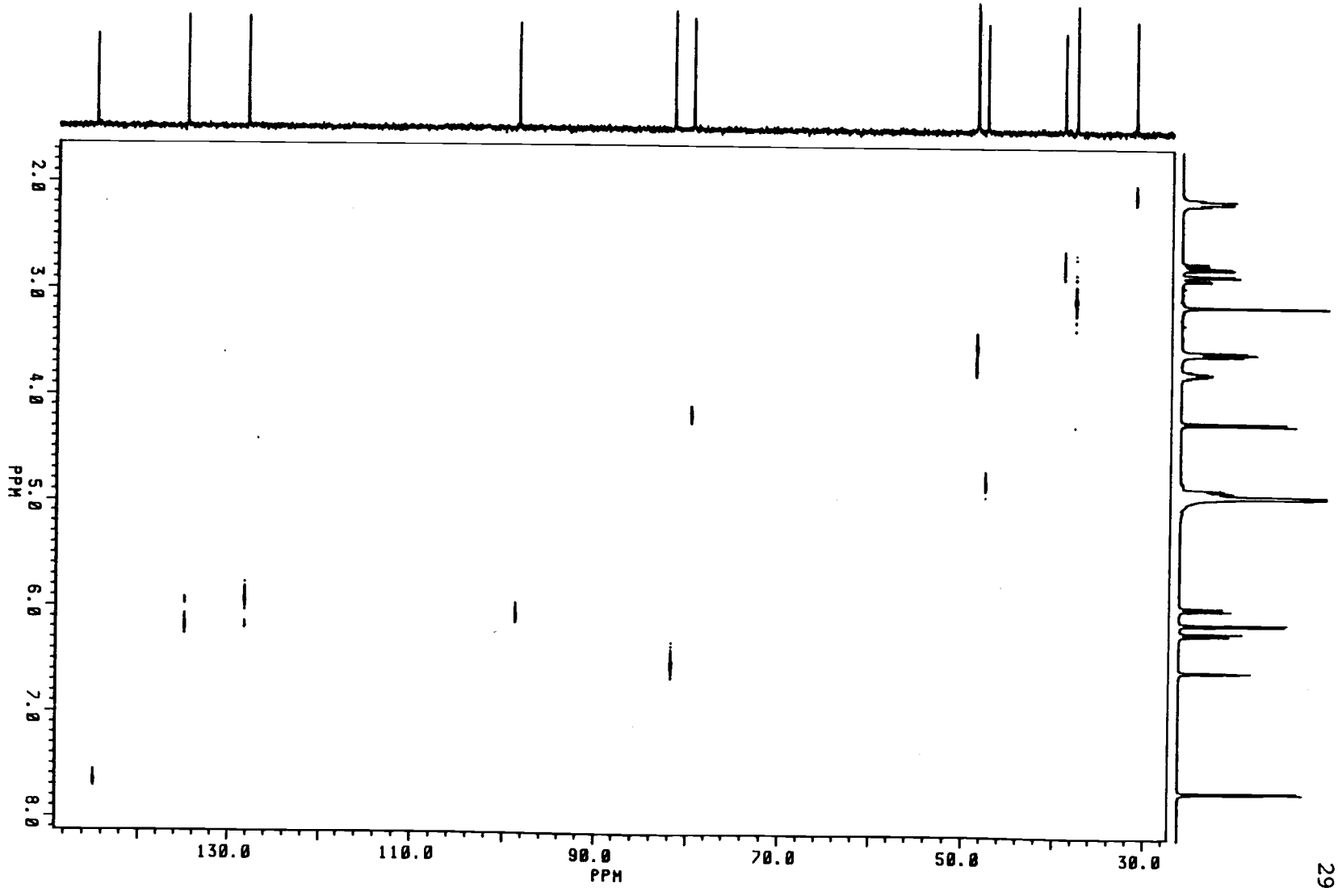
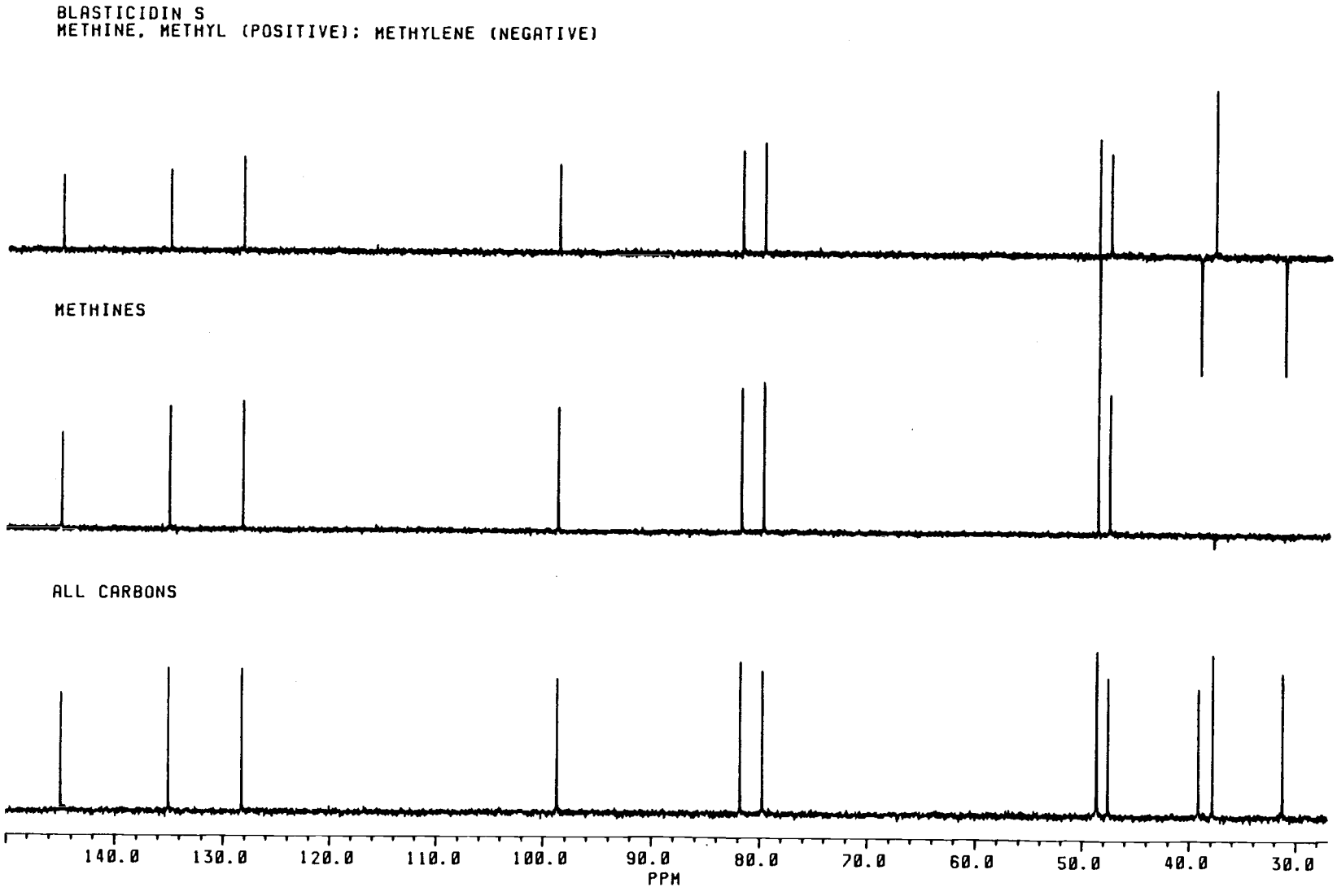


Figure 9. DEPT Spectra of Blasticidin S.



The two olefinic carbons of the enopyranuronic acid portion, C-6 which bears H-6 and C-7 which bears H-7, were clearly assigned to δ 135.0 and δ 128.2, respectively, from the HETCOR spectrum. Methine carbons C-5 (H-5), C-8 (H-8) and C-9 (H-9) were also assigned to δ 81.8, δ 47.6 and δ 79.7, respectively, from the ^{13}C-^{1}H chemical shift correlations and the DEPT experiment. In the same way, the sp^3 carbons of the β-arginine portion of blasticidin S - C-12, C-13, C-14, C-15, and the N-methyl - were assigned to δ 39.1, δ 48.7, δ 31.3, δ 48.6 and δ 37.8, respectively. This only left three sp^2 carbons - C-10, C-11, and C-17, - and these were assigned to δ 177.1, δ 172.9, and δ 168.3 by comparison with chemical shifts of authentic arginine.[44] The complete ^{13}C NMR spectral assignments are listed in Table 4.

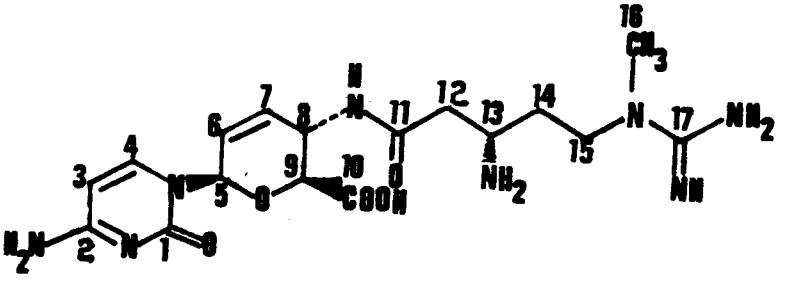
Synthesis of Labelled Precursors.

1) Synthesis of DL-[2,3,3,-$^{2}H_3$]Arginine, 8.

This compound was prepared by the procedure of LeMaster and Richards.[23] The synthesis of 8 involved a pyridoxal catalyzed exchange with $^{2}H_2O$ in the presence of aluminum ions. The mechanism is suggested in Figure 10.

This exchange reaction was tried three times. Initially the reaction flask was placed inside an oven at 125°C for two days. Later, this reaction was performed in the oil bath with stirring, and it was found that efficient stirring

Table 4. ^{13}C NMR Assignments of Blasticidin S.



^{13}C Resonance	Chemical Shifts (δ)
1	158.5
2	159.6
3	98.7
4	144.9
5	81.8
6	135.0
7	128.2
8	47.6
9	79.7
10	177.1
11	172.9
12	39.1
13	48.7
14	31.3
15	48.6
16	37.8
17	168.3

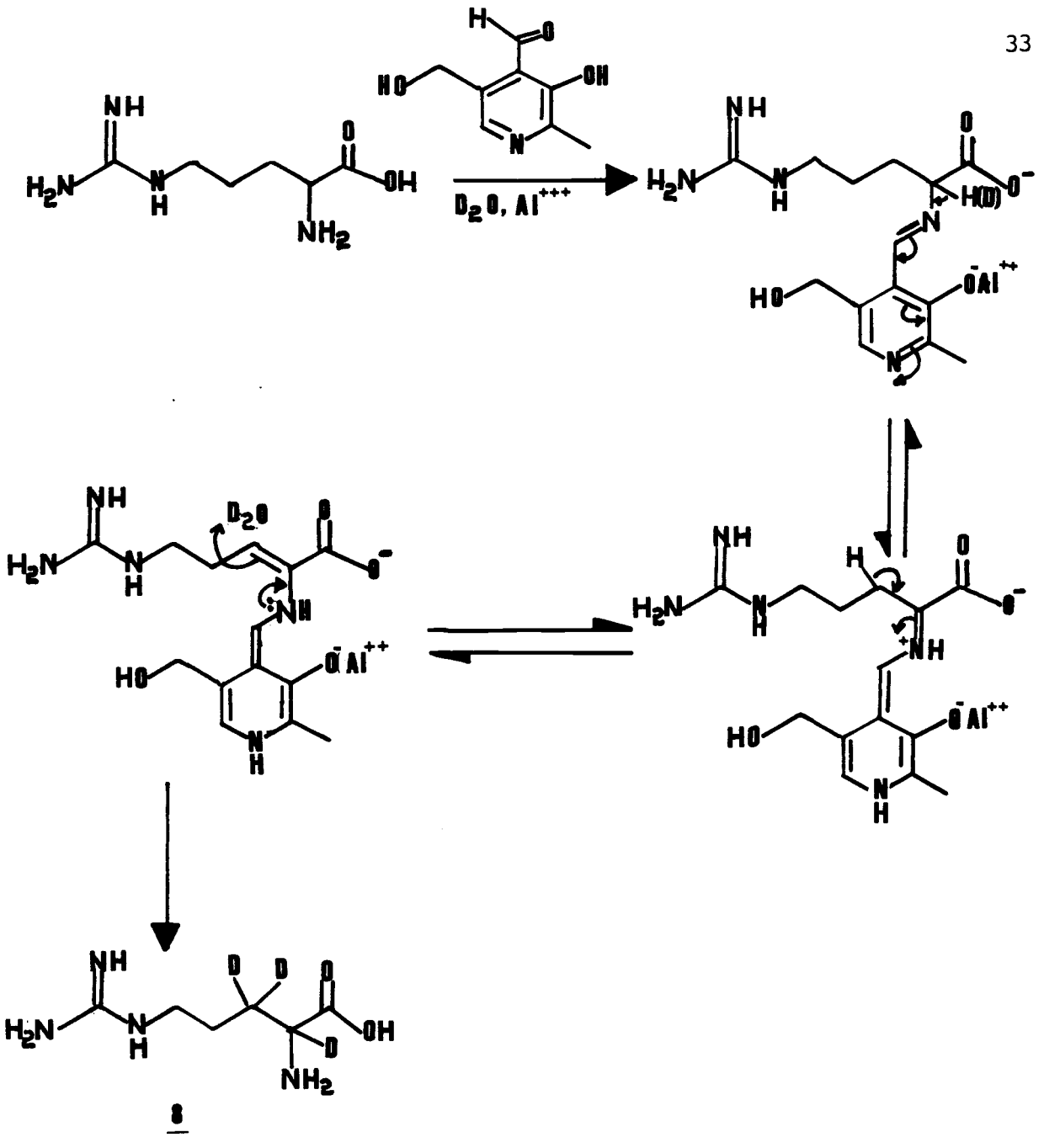
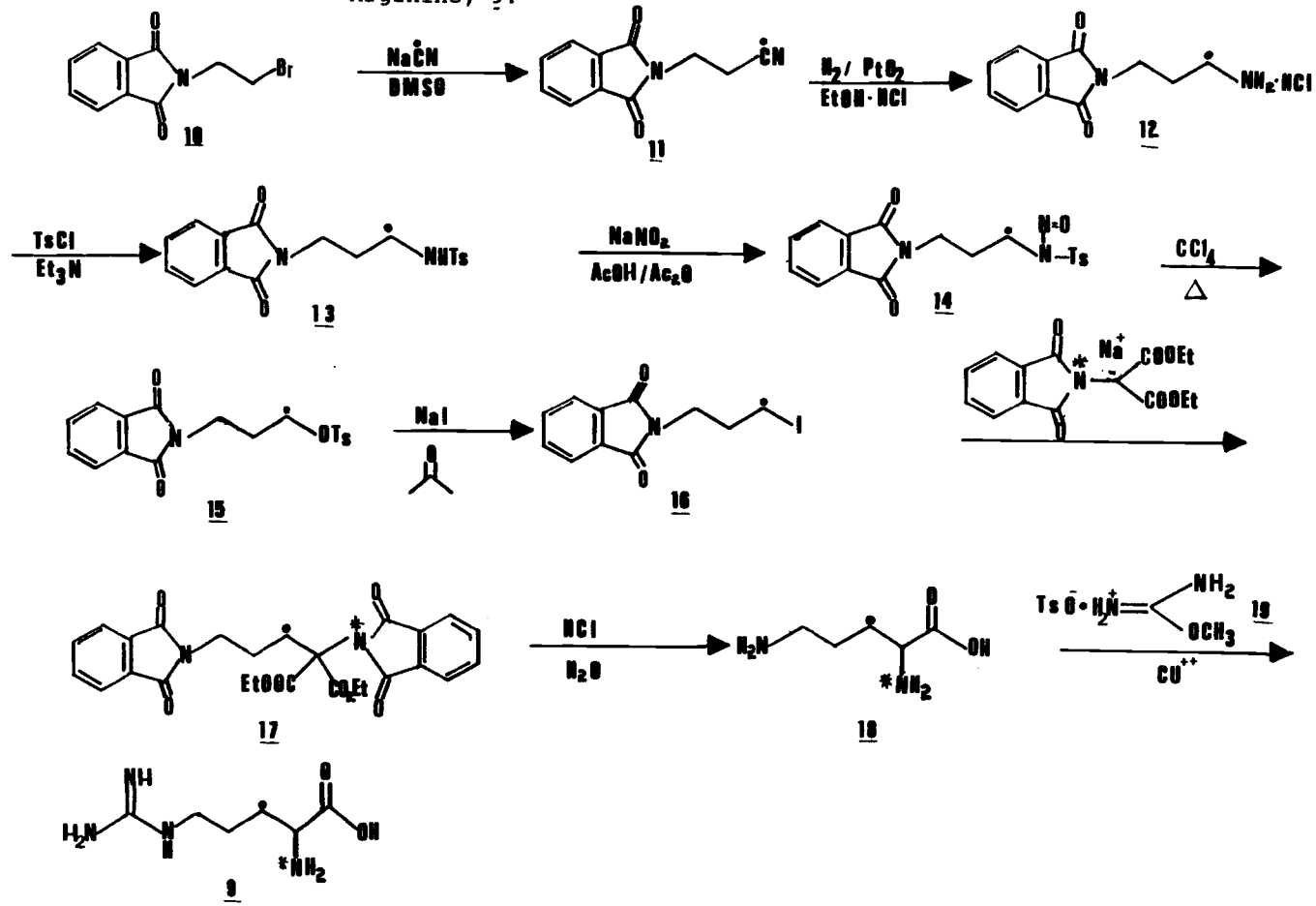


Figure 10. Synthetic Scheme for DL-[2,3,3-$^2H_3$]-Arginine, 8.

during the deuterium exchange reaction gave much better results. The deuterated arginine was next purified by ion exchange chromatography. For the first two experiments a 0.25N NaOH solution was used to elute the arginine and several ninhydrin positive frations were obtained. It was extremely difficult to separate co-generated inorganic salts from the labelled arginine. This problem was overcome by changing to a 0.25N NH_4OH solution to elute the sample from the ion exchange resin. Recrystallization was then done from a water-ethyl alcohol mixture at pH 6-7, and the yield after final purification was 31%. A proton NMR analysis of the trideuterated arginine indicated the C-2 and C-3 protons of L-arginine were completely exchanged.

2) Synthesis of Dl-[3-^{13}C,2-^{15}N]Arginine 9. The synthesis of 9 was performed in the manner shown in Figure 11 and is mainly based on the reactions done in this laboratory by Drs. T.K. Thiruvengadam and C.H. Tann for their DL-[^{13}C, 2-^{15}N]lysine synthesis[15,21] and by K. Martinkus for a number of his labeled arginine syntheses.[52] Each reaction was carried out with unlabelled materials in order to optimize reaction conditions. The ^{13}C label was then introduced via Na^{13}CN (>90% atom % enriched) and the ^{15}N label was derived from potassium [^{15}N]phthalimide (98% atom enriched).

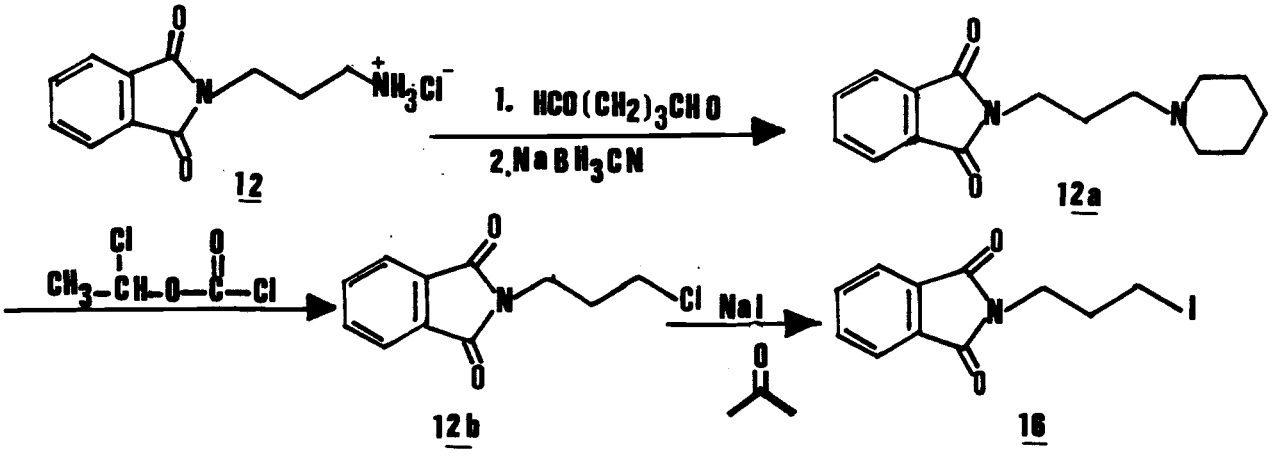
Figure 11. Synthetic Scheme for DL-[3-^{13}C, 2-^{15}N]-Arginine, 9.



N-(Bromoethyl)Phthalimide, 10, was converted to the nitrile 11 by reaction with sodium cyanide in DMSO. The average yield of this conversion was 84%. The nitrile 11 was reduced by Adams' catalyst and hydrogen gas in ethanolic HCl to give the unstable amine salt 12, which was reacted immediately with tosyl chloride to generate the stable sulfonamide 13. The overall yield obtained was 47.4% from starting nitrile 11. Diazotization of 13 yielded the N-nitroso derivative 14 (87% from 13).

The most difficult step for the synthesis of 9b was the thermal rearrangement of 14 to generate tosylate 15. The thermal rearrangement reaction of 14 to 15 was attempted several times using one equivalent of sodium carbonate in carbon tetrachloride at reflux under an argon atmosphere. Each time more than three spots appeared on a TLC of the reaction mixture. In addition to some tosylate, a significant amount of sulfonamide 13 was formed. Changing some of the reaction conditions (using dioxane/N_2 or dioxane/argon) did not improve this situation. Therefore, we briefly attempted an alternative reaction route, shown in Figure 12, to synthesize the corresponding iodide 16.

Figure 12. New Approach for the Synthesis of 16.



Olofson[47] had obtained several chlorides in very high yield by reacting the corresponding tertiary amines with α-chloroethylchloroformate. Since it is known that cyclic tertiary amines could be generated in relatively high yield from the reaction of primary amines with glutaraldehyde and subsequent reduction by sodium cyanoborohydride,[25,26,45] we had hoped that this would lead to a shorter route to 16. If the Olofson procedure worked with 12a the Finkelstein reaction, converting 12b to 16 would give the iodide in nearly quantitative yield.[48] We first

attempted to synthesize 12a by following the procedure of Gribble,[26] where 12 was reacted for seven days with a 25% aqueous solution of glutaraldehyde in the presence of phosphate buffer (pH 6.5). The reaction was monitored by TLC. No noticeable changes occurred either according to TLC, or in the proton NMR spectrum of the material taken after work-up. Since the starting material 12 was only sparingly soluble in the aqueous system, a minimum amount of methanol was added as a cosolvent and the reaction was otherwise run as described before. Again, no product was formed. Another attempt to synthesize 12a was made by following the procedure of Borch[25] where the bisulfite addition product of glutaraldehyde was used. Again, only starting materials were recovered after work-up.

At this point we returned to the initial route shown in Figure 11. This time, without purification the unstable tosylate mixture (15) was treated with sodium iodide in acetone and this generated 16. The average overall yield from sulfonamide 13 was 27%.

The iodide 16 was then coupled with the sodium salt of diethyl [^{15}N]phthalimidomalonate to generate the bis-phthalimido compound 17.[50,51] It had been found that this coupling reaction only worked in the absence of solvent and when the reaction mixture was in a molten state (temperature range from 160° to 175°C). However, solvent

was needed initially to generate the carbanion reagent and to ensure intimate mixing of the two reactants. Hydrolysis of the bisphthalimide 17 afforded DL-[3-^{13}C,-2-^{15}N]ornithine, 18. Ornithine 18, following conversion to the Cu++ salt, was coupled with O-methyl isourea tosylate, 19, to form the desired arginine 9. This was purified by chromatography on Dowex 50W x 8 - 100 (H+). The yield obtained after the final purification was 44% from 18.

Feeding Experiments.

1) DL-[2,3,3-$^{2}H_3$]Arginine, 8, Feeding. It was possible that the arginine-2,3-amino mutase reaction was mechanistically similar to either the tyrosine or the lysine reaction, although it was also possible that it proceeded by yet a third mechanism. Our first experiment was designed to determine which - if any - of the hydrogens at C-2 and C-3 of arginine were retained. We had synthesized DL-[2,3,3-$^{2}H_3$]arginine, 8, for this purpose.

In the initial feeding using DL-[2,3,3-$^{2}H_3$]arginine, DL-(1-^{14}C)arginine was added to allow rapid monitoring of the fermentation and of the work up by liquid scintillation counting. Two 250 mL broths were used. The results of this experiment are summarized in Table 5. An aqueous solution of the deuterated and radioactive arginines was divided into

Table 5. First DL-[2,3,3,-$^{2}H_{3}$]Arginine, 8, Feeding.

Fractions	Estimated Amount of Blasticidin S (mg)	Total Radioactivity (dpm x 10^{6})	Total Radioactivity Remaining (%)
Fermentation Filtrate	159	3.64	37.18
Anion Exchange Column Nonbound	NA	0.34	3.47
Cation Exchange Column Nonbound	NA	NA	NA
Cation Exchange Column Water Washings	NA	NA	NA
5% Pyridine Washings	NA	NA	NA
1.2% NH_4OH eluate Fraction 29-58	138	0.84	8.58

Summary

Length of Fermentation	120 hours	Specific Activity of 8 fed (dpm/mM x 10^{6})	13.95
DL(1-^{14}C)arginine fed (dpm x 10^{6})	9.79	Blasticidin S (dpm/mM x 10^{6})	3.59
DL-[2,3,3-$^{2}H_{3}$]arginine added (mMol)	0.70	dilution value	3.78

NA: The analysis was not performed.

two equal portions and each divided equally amongst the two broths at 48 hours and 60 hours after inoculation. This two-pulse feeding was done to improve our chances of retaining the deuterium label at C-2 of arginine in case there were an unrelated transaminase or racemase activity present at some point in the fermentation. The fermentation was stopped after an additional sixty hours, and work-up was done as previously described.

Bioassay results showed the blasticidin S production was 159 mg per 500 mL fermentation. Although the chromatographic purifications seemed to work well, an error was made in the fractions from the second column that were finally combined and lyophilized. It proved impossible to recrystallize the blasticidin S and during these efforts the antibiotic decomposed.

A second feeding experiment was carried out in the same manner as the previous one. Again, 159 mg was produced. The isolation and purification was successful, and 20 mg of pure 1a was obtained. A 16.4% incorporation of ^{14}C had resulted from the fermentation. The results of this feeding experiment are summarized in Table 6.

Table 6. Second DL-[2,3,3-$^{2}H_{3}$]Arginine, 8, Feeding

Fractions		Estimated Amount of Blasticidin S (mg)	Total Radioactivity dpm x 10^{6}	Total Radioactivity Remaining (%)
Fermentation Filtrate		159	8.18	35.0
Anion Exchange Column Nonbound		115	4.46	19.5
Cation Exchange Column Nonbound		none	low	--
Cation Exchange Column Water Washing		none	low	--
5% Pyridine Washings		none	low	--
1.2% NH_4OH elutate	Fraction 54-63	2	0.09	0.38
	" 64-75	16	0.41	1.75
	" 76-78	NA	NA	NA
	" 79-87	8	0.35	1.49
	" 88-100	20	0.48	2.05

Summary

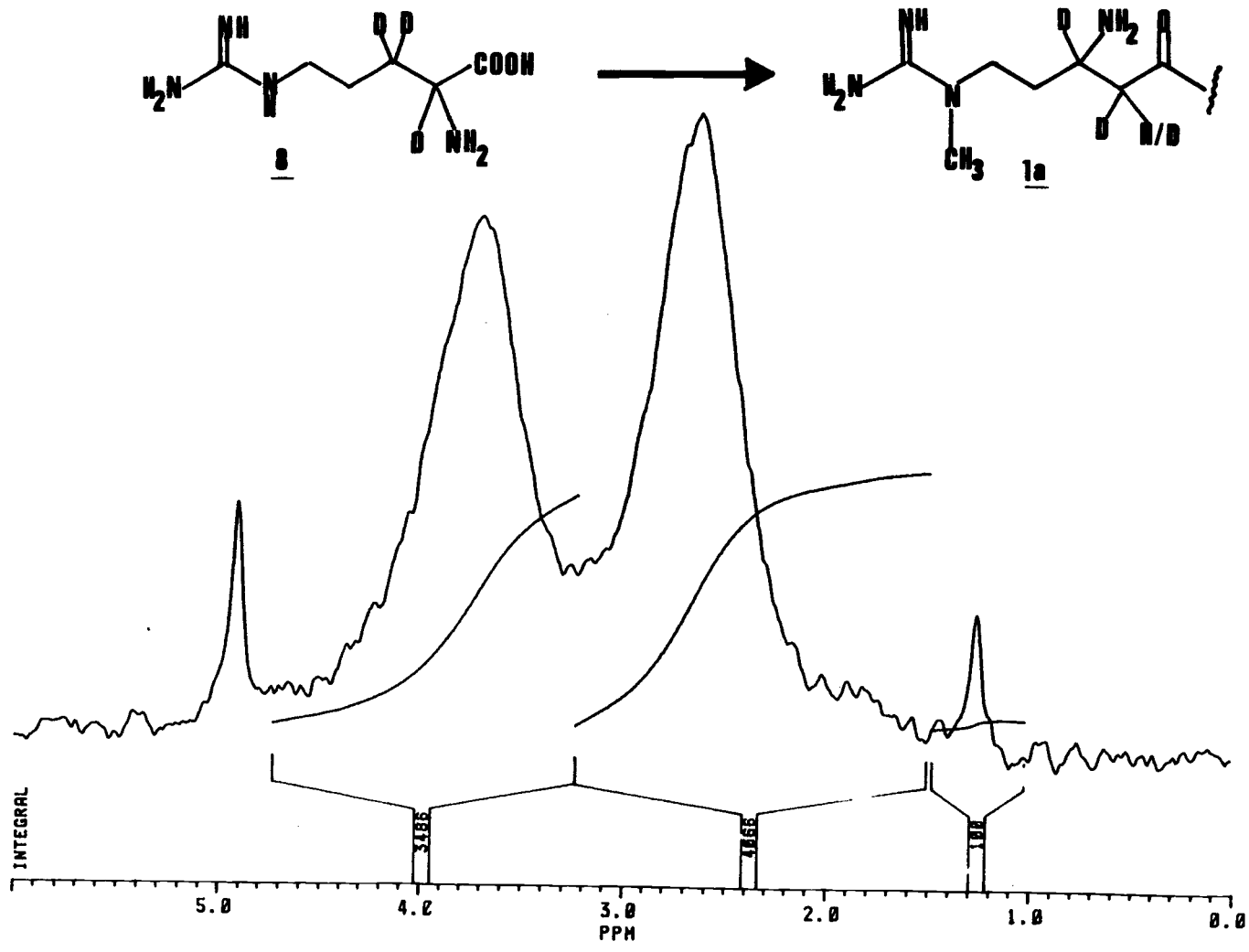
Length of Fermentation	120 hours	Specific Activity of 8 fed (dpm/mMol 10^{6})	25.00
Total DL-(1-^{14}C)arginine fed (dpm x 10^{6})	23.40	Specific activity of Blasticidin S 1a (dpm/mMol x 10^{6}) calc. based on fraction 88-100	10.13
DL-[2,3,4-$^{2}H_{3}$]arginine added (mMol)	0.94	Dilution value	2.47

NA: The analysis was not performed.

A 20 mg sample of pure 1a, previously adjusted to pH 6.5 and then lyophilized, was dissolved in 0.3 mL of deuterium depleted water. Twenty-five µL of t-butanol was added for an internal chemical shift reference (δ 1.28) and for measuring the deuterium content (0.4 µmole of ^{2}H natural abundance). A ^{2}H NMR spectrum was obtained at 61.43 MHz (Figure 13). In addition to resonances at δ 4.90 (residual HOD) and at δ 1.28 (t-butanol), resonances at δ 3.74 and 2.67 were observed demonstrating enrichments at H-13 and H-12, respectively. Based on the 0.4 µmole deuterium present in the t-butanol, the deuterium content in the blasticidin S sample could be calculated as 16 µmole (H-12) and 13 µmole (H-13), for a ratio of 1.2:1. This is significantly less than the 2:1 expected if all three deuterium labels had been retained.

DL-[3-^{13}C,2-^{15}N]Arginine, 9, Feeding. The next objective was to examine whether or not the original amino group of arginine was retained and, if so, whether the migration was an intramolecular or intermolecular process. For this DL-[3-^{13}C,2-^{15}N]arginine, 9, was synthesized. If migration were intramolecular, there would

Figure 13. ^2H NMR Spectrum of Blasticidin S from DL-[2,3,3-$^2H_3$]Arginine Feeding.



be a ^{13}C-^{15}N coupling in the ^{13}C NMR spectrum indicating a direct carbon-nitrogen bond had been formed. Compound 9 HCl and DL-(1-^{14}C)arginine were mixed and added aseptically to two 250 mL production broths as before. Again, equal portions of the labelled arginine was fed at 48 hours and 60 hours after inoculation of the production broths in order two improve our chances of retaining the ^{15}N label if it were mechanistically relevant. The production of 1b was 55 mg based on the bioassay result, and the results of this feeding experiment are shown in Table 7.

The ^{13}C NMR spectrum (Figure 14) of the most pure fraction obtained directly from the second ion exchange column (9 mg) showed an intense doublet (J_{CN} = 5.1 Hz) at the correct chemical shift for C-13, indicating an apparent intramolecular nitrogen immigration in blasticidin S biosynthesis; this doublet was sufficiently large to encompass the natural abundance signals of C-13, and C-15. However, perhaps due to the poor signal to noise of this spectrum, peaks were missing at δ 168 (guanido carbon) and at δ 159 (pyrimidone carbon). In addition, two small extra peaks at δ 150.2 and 47.7 were present, suggesting the presence of impurities.

In order to be sure of the biosynthetic result, and taking advantage of the apparently large enrichment (ca, 6.4%), a sample was diluted four-fold with authentic

Table 7. DL-[3-^{13}C, 2-^{15}N]Arginine, 9, Feeding

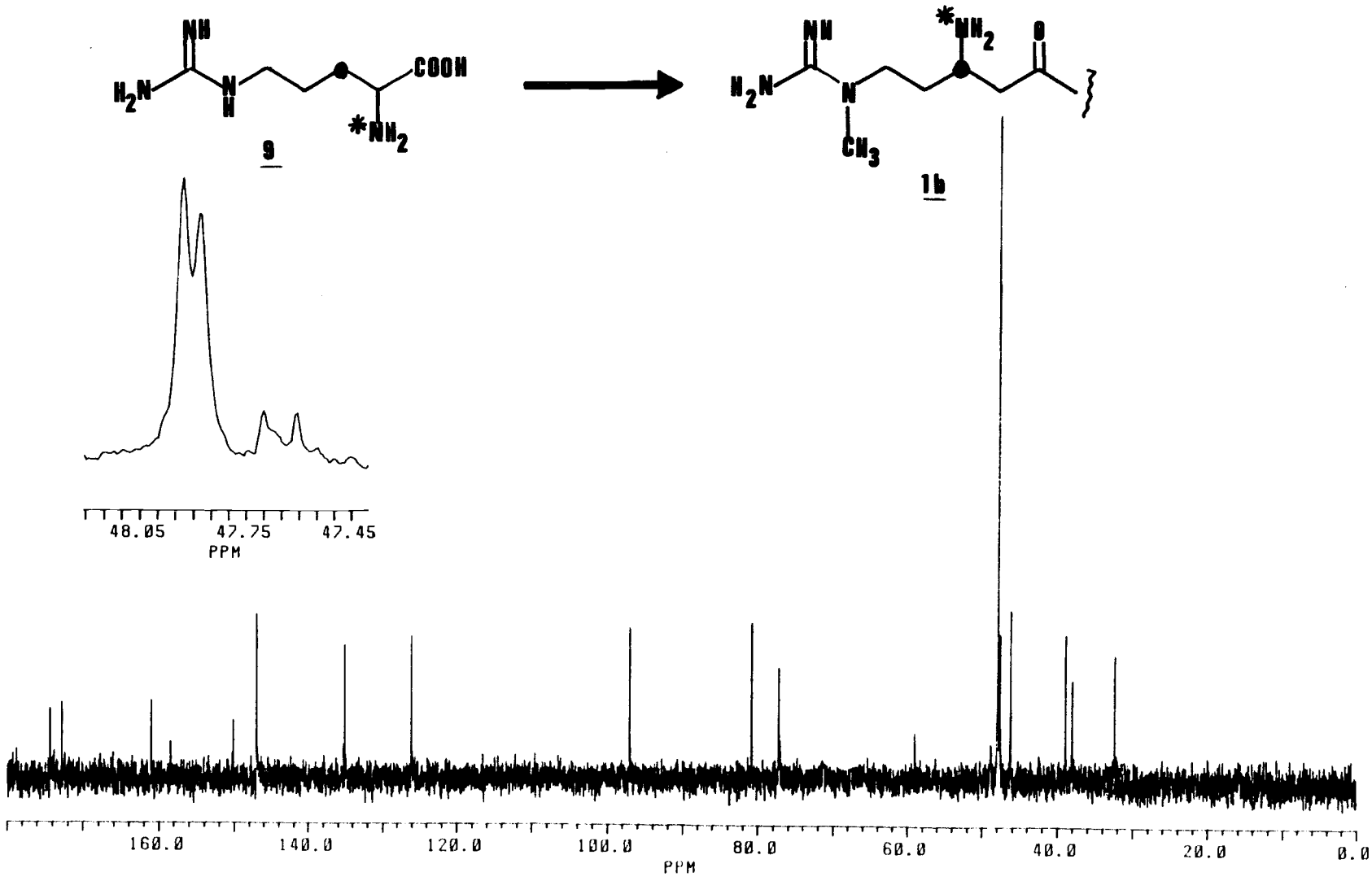
Fractions		Estimated Amount of Blasticidin S (mg)	Total Radioactivity dpm x 10^6	Total Radioactivity Remaining (%)
Fermentation Filtrate		55	18.2	34.3
Anion Exchange Column Nonbound		39.6	9.3	17.5
Cation Exchange Column Nonbound		None	low	--
Cation Exchange Column Water Washing		None	low	--
5% Pyridine Washing		None	low	--
1.2% NH_4OH eluate	Fraction 65-74	0.5	0.05	0.09
	" 75-79	NA	NA	NA
	" 80-95	6.5	0.52	0.98
	" 96-104	5.6	0.42	0.79
	" 105-120	4.7	0.34	0.64
	" 121-140	0.8	0.06	0.11

Summary

Length of Fermentation	120 hours	Specific Activity of 4b fed (dpm/mMole 10^6)	204.90
Total DL-(1-^{14}C)arginine fed (dpm x 10^6)	53.0	Specific activity of Blasticidin S 1b (dpm/mMol x 10^6) calc. based on fraction 105-120	30.56
DL-[3-^{13}C,2-^{15}N]arginine added (mMol)	0.26	Dilution value	6.71

NA: The analysis was not performed.

Figure 14. First ^{13}C NMR Spectrum of Blasticidin S from DL-[3-^{13}C,2-^{15}N]Arginine Feeding.

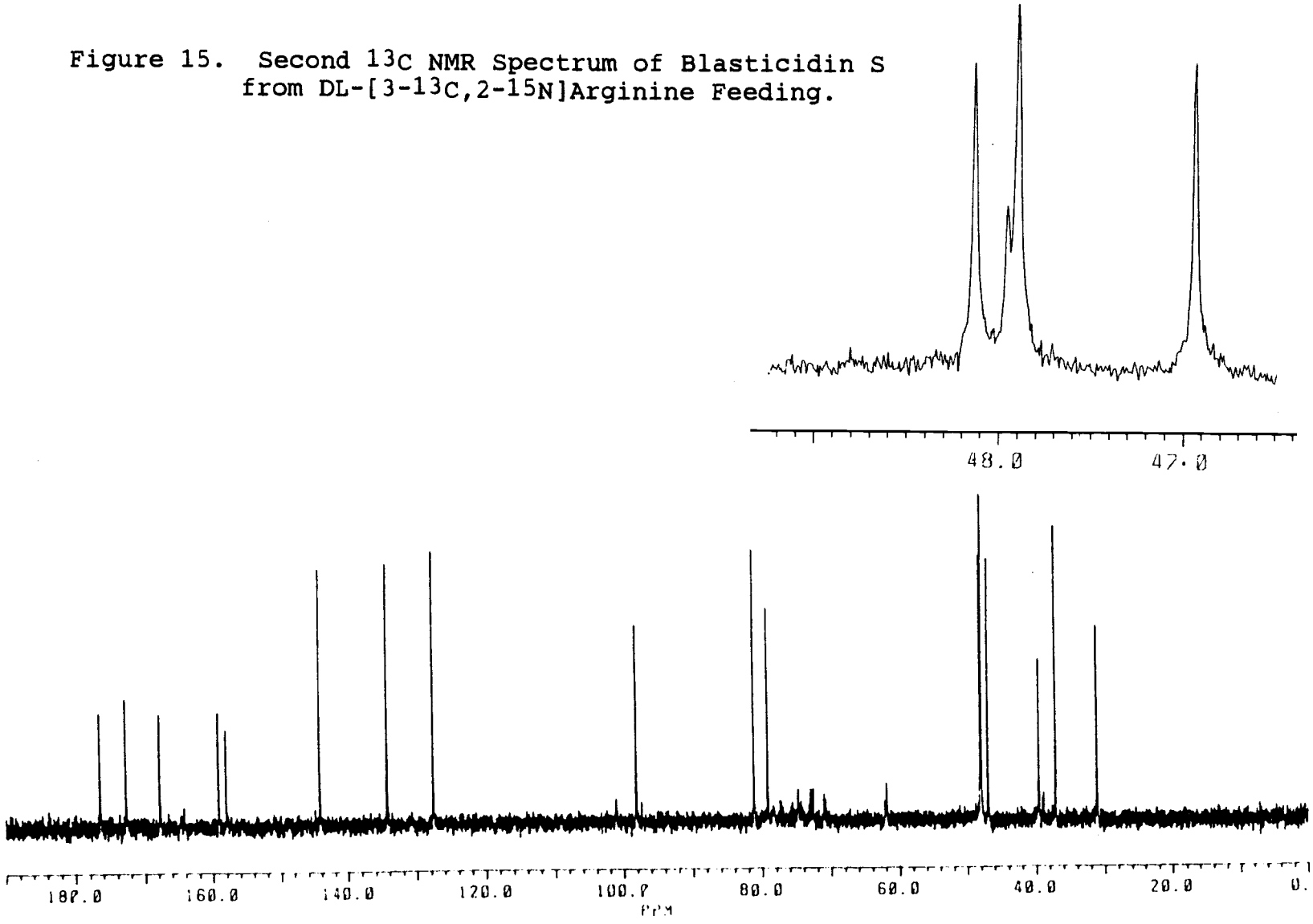


blasticidin S and recrystallized. In this manner a purer sample was obtained and in sufficient quantity to provide better signal to noise ratio, but still with enough enrichment to observe the spin-coupling. All expected resonances - and no unexpected resonances - were clearly observable in the ^{13}C NMR (Figure 15) of this sample. Perhaps due to an effect of different pH, the C-15 resonance was now fortuitously shifted 0.2 ppm from the C-13 resonance. The natural abundance singlet of the C-13 resonance was at δ 47.90, and a doublet having the low field satellite at δ 47.95 (J_{CN} = 5.1 Hz) was visible due to the enriching ^{13}C spin-coupled to the enriching ^{15}N. Apparently, a small (ca. 2.5 Hz) isotope induced downfield shift resulted in overlap of the upfield satellite with the natural abundance singlet.

The results from these two experiments are qualitatively similar to those obtained for the lysine-2,3-amino mutase reaction. In particular, an intramolecular migration of nitrogen from C-2 of α-arginine to C-3 of β-arginine has been demonstrated. Furthermore, the observation of approximately 1.2 deuterium atoms at C-2 of arginine indicates that at least some hydrogen migration from C-3 had also occurred.

The value of 1.2 deuteriums at C-3 can be explained two ways. Intervention of an arginine transaminase or racemase

Figure 15. Second ^{13}C NMR Spectrum of Blasticidin S from DL-[3-^{13}C,2-^{15}N]Arginine Feeding.



reaction unrelated to the actual subsequent rearrangement may have led to 80% exchange of the C-2 deuterium label, while rearrangement by the amino mutase enzyme would then have transferred a full deuterium equivalent. Although a transaminase can be eliminated since all of the ^{15}N label was retained in the second experiment, a racemase would still be possible, and the observation by Aberhart[20] of lysine racemase activity in crude preparations of the Clostridium lysine-2,3-amino mutase makes this a quite probable explanation. An alternative explanation would be that hydrogen is transferred incompletely, either as a proton abstracted by a base and undergoing partial exchange before returning to the arginine intermediate, or as hydride or hydrogen radical transferred to a coenzyme that mixes with an in vivo pool before hydrogen is returned.

The extent of hydrogen migration must be clarified in the future, and the simplest tests would be to feed [2-^{2}H]arginine and [3,3-$^{2}H_2$]arginine in separate experiments. Nonetheless, the data obtained so far are consistent with the two mechanisms shown below, analogous to those proposed for the lysine rearrangement.[21,48,49] One possibility (pathway a) is somewhat like an ammonia-lyase reaction where the α,β-unsaturated acid 21 would be generated as an intermediate and the originally eliminated

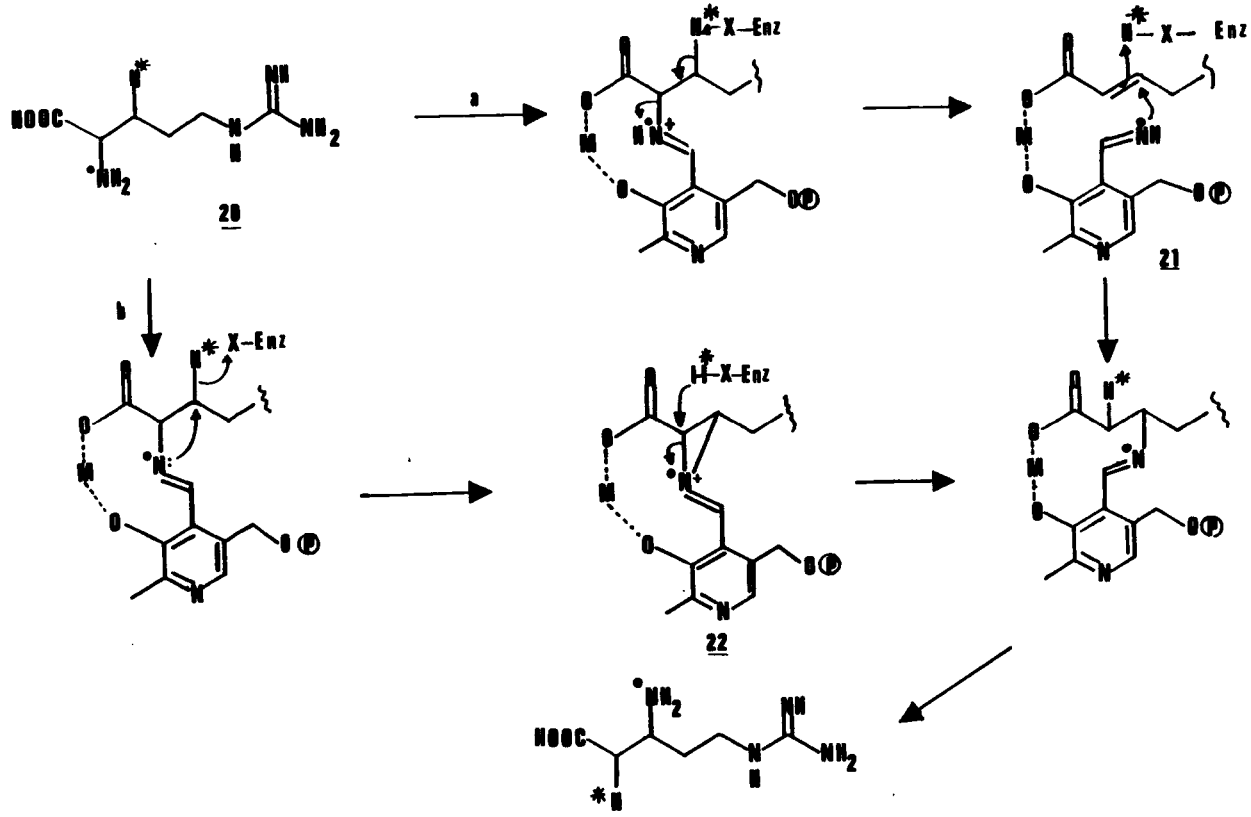


Figure 16. Possible Mechanisms for the Conversion of α-Arginine to δ-Arginine.

nitrogen and hydrogen would be returned in a conjugate addition.[21] The other possibility (pathway b) would involve an aziridine intermediate 22 as has been suggested for other aminomutase reactions (Figure 16).[21,22,49,50]

III. EXPERIMENTAL

Materials and Methods

Radioactive Isotopes. DL-(1-^{14}C)Arginine (specific activity = 20 mCi/mMol) was purchased from Research Products International Corporation, Mount Prospect, Illinois.

Stable Isotopes. [^{13}C]NaCN (90 atom % enriched, lot 1963-E) was obtained from Merck, Sharp and Dohme Canada Limited, Montreal, Canada distributed in the U.S.A. by Merck and Co., Inc. Rahway, New Jersey. [^{13}C]NaCN (99 atom % enriched, lot CLM-297) was purchased from Cambridge Isotope Laboratories, Cambridge, Massachusettts. Diethylmalonyl [^{15}N]phthalimide was a gift from the late Dr. K.J. Martinkus who synthesized this compound by reacting bromodiethyl malonate and potassium [^{15}N]phthalimide (98 atom % enriched) which was provided by the Los Alamos Stable Isotopes Resources, Los Alamos Scientific Laboratories, Los Alamos, New Mexico.[53] The deuterium oxide (99.8% atom % ^{2}H, lot 4613 LLLL) and deuterium-depleted water (natural abundance x 0.0046 %, lot 4602 CJML) were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wisconsin.

General. Authentic blasticidin S and a strain of blasticidin S-producing streptomyces were generous gifts from

Kaken Chemical Company, Japan. Other blasticidin S-producing strains, Streptomyces griseochromogenes ATCC 21024 and S. griseochromogenes ATCC 14511, were purchased from the American Type Culture Collection, Rockville, Maryland. Soybean meal was obtained from Waremart Store, Corvallis, Oregon. Agar and yeast extract were purchased from Difco Laboratories, Detroit, Michigan. Beef extract and polypeptone were purchased from Baltimore Biological Laboratories, Cockeysville, Maryland. Wheat embryo and dried brewer's yeast were gifts from Kaken Chemical Company, Japan, and malt extract was purchased from Sigma Chemical Company, St. Louis, Missouri.

All remaining supplies for microbiological and fermentation work were obtained from either VWR (Boston, MA) or Fisher Scientific (Medford, MA) chemical companies. All other chemicals were purchased from commercial sources, primarily Aldrich Chemical Company, J.T. Baker Chemical Company, and Allied Chemical Corporation, and were of reagent grade. All solvents for chromatography were distilled prior to use. The adsorbent for preparative (20 x 20 cm, 2.0 mm thickness) and analytical (0.25 mm thickness) silica (60F-254) and cellulose (13254) thin layer chromatography (TLC) sheets (20cm x 20 cm) were purchased from VWR Scientific, Boston, MA. Flash chromatography grade silica gel 60 (230-400 mesh) was purchased from MC/B Manufacturing

Chemists, Inc., Cincinnati, Ohio. All cation and anion exchange resins were obtained from Bio-Rad Laboratories, Rockville Centre, New York and were converted to a suitable counter-ion form according to manufacturer's directions. Absolute toluene was obtained by distillation from sodium metal. Dioxane was purified according to the method of Hess.[54] In vacuo refers to water aspirator pressure (14-18 mm Hg) and under vacuum refers to vacuum pump pressure (2 mm Hg or lower). Rotary evaporations were conducted at 25-35°C unless otherwise specfified.

Instrumentation. All radioactivity measurements were carried out in a Beckman model LS 7800 liquid scintillation counter. All measurements were done in duplicate to a + 1.5% standard deviation. Micro samples were weighed on a Cahn Model 29 electrobalance. Centrifugations were carried out in an IEC model B-20A Centrifuge. UV absorbances were determined with an IBM Model 9420 UV-Visible spectrophotometer. IR spectra were obtained on Perkin Elmer Model 727 B spectrometer. Melting points were taken in unsealed capillaries in a Buchi melting point apparatus and were uncorrected.

^{1}H NMR spectra (pulsed Fourier Transform Model) were taken on either a Varian FT 80A (80 MHz) or Bruker AM 400 (400 MHz) spectrometer. Chemical shifts are given in parts

per million relative to internal TMS (for organic soluble compounds) or t-butanol (1.28 ppm relative to TMS) for water soluble compounds). All ^{1}H NMR spectra were obtained at 298 $\pm$ 2°K unless otherwise specified. Splitting patterns are designated s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Coupling constants are given in Hertz (Hz). ^{13}C NMR Measurements were recorded on one of two FT NMR spectrometers, either a Varian FT 80A (20 MHz) or a Bruker AM 400 (100 MHz). All ^{13}C NMR spectra were broad-band proton decoupled and were obtained at an ambient temperature of 298 $\pm$ 2°K in 5 mm tubes. Chemical shifts were measured in parts per million downfield from internal TMS (organic solvents) or relative to acetonitrile (C-2 at 117.0 ppm, downfield from TMS) for aqueous samples.

A ^{2}H NMR spectrum was obtained on a Bruker AM 400 spectrometer operating at 61.4 MHz and 283 $\pm$°K. The ^{2}H NMR spectrum was obtained employing a 5 mm tube and chemical shifts were referenced to natural abundance of the internal t-butanol (1.28 ppm). The solvent used for the deuterium spectrum was deuterium-depleted water. UV absorbances were determined with an IBM Model 9420 UV-Visible spectrophotometer. IR spectra were obtained on Perkin Elmer Model 727 B spectrometer. Melting points were taken in unsealed capillaries in a Buchi melting point apparatus and were uncorrected. In vacuo refers to water aspirator pressure

(14-18 mm Hg) and under vacuum refers to vacuum pump pressure (2 mm Hg or lower). Rotary evaporations were conducted at 25-35°C unless specified otherwise.

Bioassay

Preparation of Bioassay Plates. Peptone Agar was used for the bioassay base. This was prepared by dissolving 0.5 g peptone (Trypticase peptone, BBL) in 100 mL double distilled water. This solution was adjusted to pH 9.0 and 1.5 g of agar was then added. After heating this solution with stirring to dissolve the agar, it was then dispensed into Wheaton bottles or other autoclavable containers. Finally, the solutions were autoclaved for 15 minutes at 121°C. The agar solution was then dispensed to sterile petri dishes (20 mL each) with a sterile 25 mL pipet.

Growth of Bioassay Test Organism. The test organism used was a strain of Bacillus circulans obtained from stock cultures of the Department of Microbiology, Oregon State University. This organism was maintained on Nutrient Agar (Difco) slants at 4°C. Bacillus circulans was transfered from a slant culture with an inoculating loop into 25 mL 2% peptone (Trypticase peptone in double distilled water) in a 125 mL Erlenmeyer flask. Incubation of the flask was performed at 37°C

in an orbital incubator shaker at 225 rpm for 8-10 hours.

Preparation of the Agar Overlay. When the OD_{660} of the growing B. circulans broth culture equaled 0.10 (Bausch and Lomb spectronic 20), the appropriate amount of this culture was transferred to a peptone agar solution to make a 7% inoculum. Six aliquots (6 mL each) of the suspension were pipetted onto the surface of the 20 mL agar base. The overlay agar plates were available for bioassay after 30 minutes.

The Bioassay. After overlay agar plates were prepared, the bioassay was performed by putting sterile, stainless steel wells (5 mm x 7 mm) onto the agar and then adding 150 µL of the sample to each appropriate well using a micropipet. After adding the samples to the wells, the plates were incubated at room temperature for 1 hour, then placed in a 37°C incubator for 16 hours. The diameter of each inhibition zone was checked after 16 hours of incubation.

Fermentation of Blasticidin S. A 250 mL flask containing Kaken seed medium (50 mL) was inoculated with spores of S. griseochromogenes ATCC 21021 grown and maintained on Yeast Malt Extract agar slants. Approximately 1 cm^2 of the slant was scraped off using a sterile inoculating loop. Incubation of the seed medium was done at 29°C and 225 rpm in incubator shaker (Lab-line Model No. 3590) for 48 hours. Using a sterile pipet, 5 mL of this seed culture (2% inoculum) was

added to 250 mL of Kaken fermentation medium in a 1L flask and the mixture was incubated at 29°C and 225 rpm in an incubator shaker for 120 hours. Feedings of the labelled precursors were done 48 hours and 60 hours after inoculation of the production medium.

Isolation, Purification, and Quantification of Blasticidin S. After 120 hours of fermentation, the product was harvested by centrifugation at 10,000 x g for 20 minutes to remove solids. Typically two 250 mL fermentations were worked up together. The supernatant was adjusted to pH 3.0 with 6N HCl to remove some colloidal impurities and was centrifuged again (10,000 x g, 20 minutes). After centrifugation, the solution was immediately adjusted to pH 6.0. Bioassay and radioactivity measurements were done at this point. The solution was loaded onto a column of AG 2-X8(OH^-) resin (5 cm x 13 cm, bed volume 249 cm^3) which was pre-equilibrated with water. Elution was performed (rate=4.5 mL/min) with simultaneous titration of the eluant with conc HCl to maintain the pH at 6-7.

The anion exchange column was continuously washed with 3 bed volumes of distilled water. The radioactivity and bioassay were checked for the combined eluant and this mixture was then loaded onto a column of AG 50W-X2 (H+) resin (3.3 cm x 23.3 cm, bed volume 199 cm^3) which was pre-equilibrated with water. The elution rate was set to 2.5 mL/min.

After the sample was completely loaded on the cation exchange column, the column was washed with 2-3 bed volumes of distilled water. This wash effluent was discarded. Next the column was eluted with 3 bed volumes of 5% pyridine. Bioassay and radioactivity measurements of this eluant were also performed. The cation exchange column was finally eluted with 1.2% NH_4OH and blasticidin S typically began to elute after approximately 600 mL. The fractions (6 mL each) were spotted on a silica gel TLC plate, checked under a UV light, and then sprayed with ninhydrin. Fractions that were positive in both tests were analyzed by TLC (silica gel $60F_{254}$, solvent: n-BuOH:MeOH:Conc NH_4OH:H_2O = 5:2:2:5) before combining appropriate fractions. Radioactivity and bioassay were checked for each combined fraction. The fractions containing blasticidin S were rotovaped ca. 30 minutes to expel residual ammonia and then lyophilized. The fractions were then recrystallized with CO_2 free water - methanol to give pure blasticidin S.

Administration of Precursors. For feeding experiments, precursors were first fed at the time corresponding to, or shortly following, the appearance of blasticidin S in the broth, typically 48 hours after the production medium was inoculated. Precursors were introduced as aqueous solutions and were sterilized by passage through a Gelman membrane filter (product No. 4192, pore size 0.2 μm). A second

portion of the precursor was fed sterilely after an additional 12 hours.

First DL-[2,3,3-$^{2}H_3$]-Arginine, 8, Feeding. A 2% inoculum (5 mL) of S. griseochromogenes ATCC 21024 was used to start each of two 250 mL production cultures. Deuterio-arginine 8. HCl (200 mg, 0.94 mM) and DL-(1-^{14}C)-arginine (10.6 μCi) were mixed, divided equally and added aseptically to these production broths at 48 hours and 60 hours after inoculation with the seed culture. The fermentation was stopped after 60 additional hours. Work-up was done as described in Section III. As a result of improper fractionation after the second chromatography column, pure blasticidin S was not obtained. However, according to the bioassay, total production of antibiotic activity had been 159 mg/500 mL, with a 12.9% ^{14}C incorporation (assuming only the L-isomer was used).

Second DL-[2,3,3-$^{2}H_3$]-Arginine, 8, Feeding. Using the same parameters described for the previous arginine feeding, a mixture of DL-(1-^{14}C)arginine (23.4 x 10^6 dpm) and 8·HCl (200 mg, 0.94 mM) was added aseptically to two 250 mL production flasks and fermentation continued for an additional 60 hours. Bioassay indicated 159 mg of 1 had been produced. Standard work-up and purification yielded 1a (16.2% ^{14}C incorporation, assuming only L-isomer was used); ^{2}H NMR (deuterium depleted water, 61.43 MHz, 283°K)

δ 1.28 (t-butanol reference), 2.63, 3.74 and 4.9 (residual HOD). Based on 0.4 μmole natural abundance deuterium for the t-butanol reference, the observed deuterium content was 16 μmole for H-8 and 13 μmole for H-9.

DL-[3-^{13}C,2-^{15}N]Arginine, 9, Feeding. The inoculation of two 250 mL production flasks with a 2% seed inoculum was followed by aseptic addition of 9·HCl (55 mg, 0.26 mM) and DL-(1-^{14}C)arginine (53.0 x 10^6 dpm) in equal portions to each flask at 48 and 60 hours after inoculation. The fermentation was stopped after 60 additional hours of fermentation and the blasticidin S was isolated in the usual manner. Production was low in this experiment and bioassay indicated only 55 mg equivalents of blasticidin S.

After the second ion exchange chromatography, the fractions showing ninhydrin positive and UV active material were examined by TLC (silica gel 60 F_{254}, n-BuOH:CH_3OH:NH_4OH:H_2O = 5:2:2:5). The fractions showing the same Rf (0.25) as authentic blasticidin S were combined and to expel all residual ammonia were rotovaped until the pH dropped below 9.0. The combined fractions were adjusted to pH 6.5 with 1N HCl and were lyophilized. In this manner two relatively pure fractions (A and B) were obtained. The dry weight/bioassay weight of these fractions were 9 mg/9mg and 6.5/4.7, respectively, and fraction A had a melting point of 219-224°C (literature 226°C).

In the ^{13}C NMR spectrum of fraction A (100 MHz, D_2O, Figure 16) all but two of the expected resonances (those due to the guanido and pyrimidone carbons) of blasticidin S were present. Additional weak resonances were observed at 150.2 and at δ 47.7. The natural abundance resonances of C-13 and C-15 (δ 48) were obscured by an intense doublet (J_{CN} = 5.1 Hz) reflecting an apparent 7.0% enrichment in ^{13}C from 9. To obtain a better spectrum, this sample was diluted with 21 mg of authentic antibiotic and recrystallized from CO_2-free water/methanol. The ^{13}C NMR spectrum (Figure 17) now contained all the expected resonances. Those due to C-15 and C-12 were each shifted slightly. The natural abundance C-13 resonance appeared as a singlet at δ 47.9 and the low field satellite of the ^{13}C-^{15}N spin-coupled doublet (J_{CN} = 5.1 Hz) appeared at δ 47.95.

Synthesis of Labelled Precursors

Synthesis of DL-[2,3,3-$^2H_3$]Arginine, 8.[23] In a 500 mL round bottom flask with a neck approximately 15 cm long and with a 1 cm diameter was placed L-arginine (5.10 g, 30 mM), pyridoxal:HCl (0.61 g, 30 mM) and ^2H_2O (30 mL). The mixture was wrapped with aluminum foil, frozen, and lyophilized. After lyophilization, pyridine (1.26 mL, 15 mM) and ^2H_2O (30 mL) were added. The solution was frozen again, and the flask was sealed under vacuum and heated at

125°C for two days. After cooling to room temperature the flask was opened and the contents poured into a 500 mL Erlenmeyer flask containing H_2O (200 mL), sodium oxalate (0.80 g, 6 mM), collidine (1.98 mL, 15 mM) and conc HCl (1.25mL). Using 3N HCl, the solution was adjusted to pH 4.0 and loaded onto a column of Dowex 50Wx8 100 (H+) resin (1 cm x 25.5 cm). The ion exchange resin was washed with distilled water (2 L) and then eluted with 0.25 N NH_4OH. Fractions giving a positive reaction with ninhydrin were pooled, rotovaped, and lyophilized to yield 2.0 g of an off-white solid. Decolorization using active carbon, and recrystallization from water-ethyl alcohol (pH 6-7) afforded 1.6 g (31%) of pure white crystals: mp. > 230° (lit.[64] 235°C decomp.); IR (1%KBr) 3150-3400 (broad), 1670, 1620, 1490 cm^{-1}; ^1H NMR (80 MHz, ^2H_2O) δ 1.68 (t, 2H, J = 8.0 Hz) 3.25 (t, 2H, J = 8.0 Hz); ^{13}C NMR (20 MHz, ^2H_2O) δ 24.1 (C-4), 39.5 (C-5), 155.8 (C-6), 173.3 (C-1).

Synthesis of DL-[3-^{13}C, 2-^{15}N]Arginine,9. N-[3-^{13}C]-2-Cyanoethylphthalimide,11.[53,56] To a 100 mL round bottom flask was added a mixture of N-2-bromo-ethylphthalimide (8.50 g, 33.69 mM), sodium [^{13}C] cyanide (1.69 g, 33.7 mM, 90 atom % enriched) and dimethyl sulfoxide (30 mL). The solution was heated at 65-67°C in a silicone oil bath for 6 hours, then cooled and poured onto crushed ice (150 mL). The mixture was stirred for 1 hour, filtered,

washed with cold water (20 mL) and dried under vacuum to give 6.80 g of crude product. Recrystallization from 95% ethanol-water yielded pure 9 (5.43 g, 80% yield): mp 153-155°C (lit.[65] 153-154°C); IR (nujol) 3010, 2220 cm^{-1}; ^{1}H NMR (80 MHz, $CDCl_3$) δ 7.85 (m, 4H), 3.96 (dt, 2H, J = 6.8 Hz, $^{3}J_{CH}$ = 3.3 Hz), 2.83 (dt, 2H, J = 6.8 Hz, $^{2}J_{CH}$ = 6.7 Hz).

N-[3-^{13}C]-p-Toluenesulfamidopropylphthalimide, 13.[53,57] In a 250 mL round bottom flask equipped with stir bar, were placed N-[3-^{13}C]-2-cyanoethylphthalimide (2 g, 0.01 mole), absolute ethanol (75 mL), conc HCl (1 mL) and PtO_2 (0.05 g). Hydrogenation at 1 atm was carried out until the theoretical amount of hydrogen gas was taken up (448 mL). The solution was filtered and after washing the catalyst with ethanol, the combined filtrates were evaporated to dryness. After drying under vacuum for 12 hours, methylene chloride (100 mL), triethylamine (3 mL), and p-toluenesulfonyl chloride (1.980 g, 1.04 mM) were added and the resulting solution was stirred at room temperature for 24 hours. After removal of the solvent with the rotary evaporator, crystallization was accomplished from an ethanol-water solution to yield 1.78 g of 13 (59% from 9) : mp 158-160°C (lit.[65] 154-156°C; ^{1}H NMR (80 MHz, $CDCl_3$) δ 7.70 (m, 6H), 7.24 (m, 2H), 5.25 (t, 1H, J = 7.5 Hz, NH),

3.70 (m, 2H of C-1 plus 0.9 H of C-3, J_{CH} = 150.4 Hz), 2.90 (m, 0.1H due to 10% unenriched sample of C-3, barely observable), 2.40 (s, 3H, CH_3), 1.82 (m, 2H of C-2 plus 0.9 H of C-3, J_{CH} = 150.4 Hz); ^{13}C NMR (20 MHz, $CDCl_3$) δ 21.5, 28.3, 34.7, 40.1 [C-3, observed as a 10 fold signal enhancement by diluting (3:37) the original 90% enriched sample with unlabeled sulfonamide], 123.4, 127.0, 127.1, 129.7, 131.1, 134.2, 137.2, 143.3, 168.6.

N-[3-^{13}C]-p-Toluenesulfamidonitrosopropylphthalimide, 14.[53,58,59] Finely powdered sodium nitrite (4.58 g, 67.1 mM) was added slowly with stirring over an 8 hour period to a cold (-5 to -7°C) solution containing N-[3-^{13}C]-p-toluenesulfamidopropylphthalimide 13 (1.1 g, 3.05 mM), concentrated acetic acid (10 mL), and acetic anhydride (20 mL) in a 250 mL round bottom flask. Following addition, the reaction was stirred an additional 10 hours at 0°C and then poured into an ice-water mixture and stirred for 30 minutes. After dissolving all the ice, the solution was vacuum filtered and dried under vacuum overnight. Recrystallization from CH_2Cl_2-petroleum ether yielded 0.94 g (79%) of 14: mp 106-110°; IR (1% KBr) 3490, 2940 cm^{-1}; ^1H NMR (80 MHz, $CDCl_3$) δ 7.72 (m, 6H), 7.20 (m, 2H), 4.65 (dd, 0.9 H of

C-3, J = 10,8 Hz, J_{CH} = 144 Hz), 3.65 (m, 2H, J = 6 Hz), 2.85 (dd, 0.9 H of C-3, J = 10.8 Hz), J_{CH} = 144 Hz), 2.41 (s, 3H), 1.81 (m, 2H).

N-[3-^{13}C]-Iodopropylphthalimide,16.[53,58,59] In a 250 mL round bottom flask were placed dried 14 (1.01 g, 2.60 mM), CCl_4 (100 mL), and anhydrous Na_2CO_3 (0.3 g, 2.7 mM), and the mixture was heated at reflux under an argon atmosphere for 24 hours. After cooling, the solvent was removed with a rotary evaporator. Chloroform was added and the insoluble residue removed by filtration. The solvent was again removed by evaporation, and drying the residue under vacuum overnight gave 1.0 g of crude tosylate. The tosylate, sodium iodide (4 g, 26.7 mM), and acetone (100 mL) were placed in a 250 mL round bottom flask and the mixture was heated at reflux for 24 hours. After cooling the solvent was removed by rotary evaporation and the residue was dried under vacuum for 24 hours. Distilled water (50 mL) was added and the solution was filtered. The solids were dried under vacuum. Purification was accomplished by flash chromatography on silica gel (4.4 cm x 22 cm) using $CHCl_3$ as solvent. The fractions were checked by TLC and after pooling the appropriate fractions, the solvent was removed. The solid remaining was dried under vacuum, yielding 0.88 g of 16 (27% overall yield starting from sulfonamide 13): mp 86-88°C (lit.[65] 88°C); ^{1}H NMR (80 MHz, $CDCl_3$)

δ 7.65 (m, 4H), 4.11 (t, 0.9 H of C-3, J_{HH} = 8 Hz, J_{CH} = 149.6 Hz), 3.78 (m, 2H, C-1), 3.15 (t, 0.1H due to 10 % unenriched sample of C-3), 2.24 (m, 2H of C-2 plus 0.9 H of C-3, J_{CH} = 149.6 Hz); ^{13}C NMR (20 MHz, $CDCl_3$, diluted with natural abundance iodide) δ 168.2, 134.0, 133.9, 132.0, 123.3, 38.6, 32.6, and 1.24, (C-3).

DL-[3-^{13}C,2-^{15}N]Ornithine, 18.[21,60] Under a nitrogen atmosphere, NaH (102 mg, 50% oil suspension) was placed in a flame dried 250 mL three neck round bottom flask equipped with a septum, stir bar, drying tube, and vacuum adaptor. Absolute ethanol (5 mL) was slowly added while under an N_2 flow and the flask was heated at 60°C for 30 minutes. Diethyl [^{15}N]phthalimidomalonate (648 mg, 2.12 mM) was then added and the reaction mixture was stirred at 120°C for 30 minutes. The color of the solution had turned to orange. After 30 minutes, the stopper was replaced with a vacuum distillation apparatus and the ethanol was removed, first with an aspirator then at high vacuum. During this time the temperature was maintained at 120°C while a very weak flow of N_2 was necessary to prevent bumping. The addition of N-[3-^{13}C]iodopropyl phthalimide, 16, (703 mg, 2.22 mM) in dry toluene (5 mL) followed and this solution was stirred at 120°C for 30 minutes. Under the N_2 flow, the toluene was removed first with an aspirator and then under high vacuum. The septum was replaced by a drying tube

and the mixture was heated at 160°C for 3 hours. Since the mixed solid did not melt completely, the temperature was raised to 175°C and the mixture was heated for an additional 3 hours. The TLC at this point showed no remaining starting materials.

The flask was cooled, $CHCl_3$ (50 mL) was added and the mixture was stirred for 3 hours. Insoluble material was removed by filtration and the solid was washed with $CHCl_3$ (3 x 25 mL). The pooled filtrates were filtered through a silica gel column (4 cm x 4 cm) and eluted with more $CHCl_3$. Fractions that showed a single spot on TLC (silica plate, solvent 100% $CHCl_3$) were pooled and concentrated to dryness to give a solid (691 mg). This was hydrolyzed without further purification in a mixture of concentrated HCl (10 mL), glacial acetic acid (10 mL), and H_2O (10 mL), heating at reflux for 12 hours. After cooling, the pH was adjusted to 6.0 with pyridine. Distillation of ethyl acetate that had been generated was followed by lyophilization of the solution to give moderately pure ornithine·HCl 18 (119 mg, 33% from 16) : mp 225-230°C decomp. (lit.[66] 232-233°C); ^1H NMR (80 MHz, D_2O) δ 3.75 (m, 1H), 3.00 (m, 2H), 2.74 (m, 0.9 H of C-3, J_{CH} = 119.2 Hz), 1.76 (m, 2H), 1.25 (m, 0.9 H of C-3, J_{CH} = 119.1 Hz).

DL-[3-^{13}C,2-^{15}N]-Arginine, 9·HCl.[61,62] In a 100 mL Erlenmeyer flask was placed $CuCO_3$ $Cu(OH))_2$ (652.3 mg, 2.95 mM), and boiling water (20 mL) was added to form a green suspension which, upon addition of 18·HCl (330 mg, 1.9 mM) became blue in color. After brief boiling (10 sec), the green undissolved solid was collected and washed with water (20 mL). The combined deep blue filtrates were then concentrated in vacuo at 50°C to a volume of 5 mL. After the addition of O-methyl isourea tosylate 19 (511.4 mg, 2.08 mM), the pH was adjusted to 11.2 with 10% NaOH and stirred at room temperature for 16 hours. A supplemental addition of 19 (444 mg, 1.81 mM) was made after 16 hours and stirring was continued for a total of 43 hours. The pH had changed to 10 and was now adusted to 1.2 by adding conc HCl, resulting in a change of color to light blue. H_2S gas was bubbled through the solution for 15 minutes, immediately forming a black precipitate of CuS. The precipitate was filtered, washed with H_2O, and the combined filtrates boiled for 15 minutes to remove residual H_2S. Lyophilization afforded 1.5 g of an off white residue. A column of Dowex 50 W x 8-100 (H+) resin (1.5 cm x 9.5 cm) was prepared and equilibrated with H_2O. The lyophilisate was dissolved in H_2O, adjusted pH 4 with conc NH_4OH, and loaded onto the column. Water washing (1L) was followed by elution with 0.1 N NH_4OH. The fractions were checked by ninhydrin and

TLC (n-BuOH:AcOH:H_2O = 4:1:1 on cellulose plate), and the arginine containing fractions were combined and lyophilized yield 180 mg of solid. Recrystallization from water-acetone to yielded 112 mg (27.2%) of pure arginine•HCl,9: mp 231-235°C (decomp. lit.[64] 235°C decomp.); ^1H NMR (400 MHz, D_2O) δ 3.78 (t, 1H, J = 7.5 Hz), 3.24 (t, 2H, J = 7.5 Hz), 2.07 (m, 0.9 H of C-3, J_{CH} = 156 Hz), 1.91 (m, 2H of 10% unenriched sample C-3, barely observable), 1.75 (m, 2H), 1.68 (m, 1H of C-3, J_{CH} = 156 Hz); ^{13}C NMR (100 MHz, D_2O, diluted with natural abundance arginine) δ 175.2 (C-1), 157.7 (C-6), 55.3 (d, J_{CC} = 29 Hz, C-2), 41.4 (C-5), 28.4 (C-3, highly enriched), 24.8 (d, J_{CC} = 34 Hz, C-4).

IV. REFERENCES

1. H. Yonehara, S. Takeuchi, K. Hirayama, K. Ueda and H. Sakai, J Antibiot., 11A, 1 (1958).

2. T. Misato, I. Ishii, M. Asakawa and K. Fukunaga. Ann. Phytopathol. Soc. Japan, 24, 302 (1959).

3. N. Tanaka, Y. Sakagami, T. Nishimura, H.Yamaki and H. Umezawa, J. Antibiot., 14A, 123 (1961).

4. T. Hirai and T.Shimomura, Phytopath., 55, 291 (1965).

5. T. Misato, I. Ishii, M. Asakawa and K. Fukunaga, Ann. Phytopathol Soc. Japan, 26, 19 (1961).

6. K.T. Huang, T. Misato and M. Asuyama, J. Antibiot., 17A, 65 (1964).

7. Y. Sakagami, J. Antibiot, 14A, 247 (1961).

8. C.C. Cheng, Progress in Medicinal Chemistry, 7, 69 (1970) and references cited therein.

9. N. Otake, S. Takeuchi, T. Endo and H. Yonehara, Tet. Lett., 1405 (1965); idem., ibid., 1411 (1965).

10. S. Onuma, Y. Nawata and Y. Saito, Bull. Chem. Soc. Japan, 39, 1091 (1965).

11. V. Swaminathan, J.L. Smith, M. Sundaralingam, C. Coutsogeorgopoulos and G. Kartha, Biochim. Biophys. Acta, 655, 335 (1981).

12. H. Seto, I. Yamaguchi, N. Otake and H. Yonehara, Agr. Biol. Chem., 32, 1292 (1968).

13. H. Seto, I. Yamaguchi, N. Otake and H. Yonehara, Tet. Lett., 3793 (1966).

14. H. Seto and H. Yonehara, J. Antibiot., 29, 595 (1976).

15. S.J. Gould and T.K. Thiruvengadam, J. Am. Chem. Soc., 103, 6752 (1981).

16. T. Misato, Shokubutsu Boeki Sosho, 13, 51 (1961).

17. T. Shomura, M. Inoue, T. Niida and T. Hara, J. Antibiot., 17A, 253 (1964).

18. N. Otake, S. Takeuchi, T. Endo and H. Yonehara, Agr. Biol. Chem., 30, 132 (1966).

19. H. Seto and H. Yonehara, J. Antibiot., 30, 1019 (1977); idem., ibid., 1023 (1977).

20. D.J. Aberhart, S.J. Gould, H.J. Lin, T.K. Thiruvengadam and B.H. Weiler. J. Am. Chem. Soc., 105, 5461 (1983).

21. T.K. Thiruvengadam, S.J,. Gould, D.J. Aberhart and H.J. Lin, J. Am. Chem. Soc., 105, 5470 (1983).

22. D.J. Aberhart, H.J. Lin and B.H. Weiler, J. Am. Chem. Soc., 103, 6750 (1981).

23. D.M. LeMaster and F.M. Richards, J. Labelled Comp. and Radio-pharm., 19, 639 (1982).

24. K.J. Martinkus' Ph.D. Thesis, University of Connecticut (1982), Storrs Connecticut, p. 108.

25. R.F. Borch, M.D. Bernstein and H.D. Durst, J. Am. Chem. Soc., 93, 2897 (1971).

26. G.W. Gribble, J. Org. Chem., 37, 1833 (1972).

27. "Principles of Biochemistry;" 5th Ed., White A., Handler, P., Smith, E.L., Eds.; McGraw-Hill: New York, 1973; pp. 723-724.

28. "Enzymatic Reaction Mechanisms;" Walsh, C.; Ed.; W.H. Freeman: San Francisco, 1979; p. 801.

29. "Chemistry of the Amino Acids"; Greenstein, J.P. Winitz, M.,Eds.; John Wiley and Sons, Inc.: New York, 1961; p. 601.

30. R.J. Parry and Z. Kurylo-Borowska, J. Am. Chem. Soc., 102, 836 (1980).

31. D.J. Picken and K.H. Overton, J. Chem. Soc. Chem. Comm., 80 (1981).

32. H. Seto, N. Otake and H. Yonehara, Arg. Biol. Chem., 37, 2421, (1973).

33. H. Seto, Agr. Biol. Chem., 37, 2415 (1973).

34. H. Seto and H. Yonehara, Abstract 2G-3. Annual Meet. Agr. Chem. Soc. Japan. Tokyo, 1973.

35. Z. Kurylo-Borowska, T. Abramsky, Biochem. Biophys. Acta, 264, 1 (1972).

36. J.M. Poston, Phytochemistry, 17, 401 (1978), and references cited therein.

37. E. Winterstein, A. Iatrides, Hoppe-Seyler's Z. Physiol. Chem. 117, 140 (1921); E. Winterstein, A. Guyer, ibid. 128, 175 (1923); E. Graf, H. Boedekker, Arch. Pharm., 289, 364 (1956).

38. R.E. Summons, Phytochemistry, 20, 1125 (1981).

39. "The Enzymes"; Havir, E., Hanson, K. and Boyer, P., Eds.; Academic Press, New York, 1973, p. 75.

40. "Microbial Assay - An Introduction to Quantitative Principles and Evaluation"; Hewitt, W., Ed.; Academic Press, New York, 1977, p. 2.

41. "The Aldrich Library of NMR Spectra"; Pouchert, C.J., Campbell, J.R., Eds.; Aldrich Chemical Company: 1974; Vol. IX, 77 D.

42. H. Kalinowski and H. Kessler, Org. Magn. Res., 6, 305 (1974).

43. R. Benn and H. Gunther, Angew. Chem. Int. Ed. Engl., 22, 350 (1983).

44. D.M. Doddrell, D.T. Pegg, M.R. Bendall, J. Magn. Reson., 48, 3232 (1982); D.T. Pegg, D.M. Doddrell and M.R. Bendall, J. Chem. Phys., 77, 2745 (1982).

45. "Carbon-13 NMR Spectra. A Collection of Assigned, Coded, and Indexed Spectra"; Johnson, L.F., Jankowski, W.C., Eds.; Wiley-Interscience, (1972), p. 213.

46. A.S. Kende, T.J. Bentley, R.A. Mader and D. Ridge, J. Amer. Chem. Soc., 96, 4332 (1974).

47. R.A. Olofson and D.E. Abbott, J. Org. Chem., 49, 2795 (1984).

48. H. Finkelstein, Ber., 43, 1528 (1910).

49. "Enzymatic Reaction Mechanisms"; Walsh, C.; Ed.; W.H. Freeman: San Francisco, 1979: p. 659.

50. T.C. Stadtman, Enzymes 3rd Ed., 6, 539 (1972).

51. M.R. Stetten, J. Biol. Chem., 189, 499 (1951).

52. K.J. Martinkus, C.H. Tann and S.J. Gould, Tetrahedron, 39, 3493 (1983).

53. Reference 24, p. 178-184.

54. Reference 24, p. 125.

55. "Reagents for Organic Synthesis"; Fieser, L.F., Fieser, M., Eds.; John Wiley and Sons, Inc.: New York, 1967; Vol. I, p. 333 and references cited therein.

56. S. Chodroff, R. Kapp and C.O. Beckman, J. Am. Chem. Soc., 69, 256 (1947).

57. P.J. Dechristopher, J.P. Adamek, G.D. Lyon and J.J. Galante, J. Am. Chem. Soc., 91, 2384 (1969).

58. E.H. White, J. Am. Chem. Soc., 77, 6008 (1955).

59. E.H. White, J. Am. Chem. Soc., 77, 6011 (1955).

60. Reference 24, p. 170.

61. "Organic Synthesis"; John Wiley and Sons, Inc.: New York, 1952; Collective Vol. 2, p. 49.

62. F. Turba, K. Schuster, Ber., 283, 27 (1968).

63. P. Yorgey, Note Book III, Oregon State University, p. 31-32.

64. "Dictionary of Organic Compounds"; Oxford University Press: New york, 1965; vol. 1, p. 276.

65. Reference 64, vol. 5, p. 2742.

66. Reference 64, vol. 4, p. 2574.