AN ABSTRACT OF THE THESIS OF

Michael D. Jackson for the degree of Doctor of Philosophy in Chemistry presented on August 4, 2000. Title: Advanced Studies on the Biosynthesis of the Streptolidine Moiety of Streptothricin F

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Streptothricin F (STF, **12a**) is a peptidyl nucleoside antibiotic produced by *Streptomyces lavendulae* var. L-1689-23. The biosynthesis of the streptolidine moiety in streptothricin F was investigated using a combined approach of whole-cell incorporation experiments and cell-free extract studies.

[*guanidino*-^{13}C]Streptolidine was synthesized and introduced to production flasks of *S.* L-1689-23. Analysis of the purified streptothricin F by ^{13}C NMR revealed a 1.9% enrichment of the guanidino carbon (C-6) of STF. Based upon these results, further support for a convergent biosynthesis of STF has been obtained.

When cell-free extracts of *S.* L-1689-23, were incubated with [U-^{14}C]arginine enzyme activity leading to the formation of three unidentified compounds was observed. While one of the compounds (t_R = 30 min) had an identical retention time to that of dansyl-capreomycidine, the identity of this compound could not be confirmed when it was isolated and analyzed by FABMS. The remaining two compounds (t_R = 18 min and t_R = 35 min) were identified as dansyl-citrulline and didansy-ornithine, respectively. The conversion of arginine to citrulline may be a result of arginine deiminase activity. This is the first time this enzyme activity has been observed in the species *Streptomyces*.

A new synthesis of L-capreomycidine (**17**) was developed in order to allow incorporation of a ^{13}C-label at the guanidino position. Beginning with D-serine methyl ester, the key intermediate 1,1-dimethylethyl (4*R*,1'*S*)-4-1',3'-diazidopropyl)-2,2-dimethyl-3-oxazolidinecarboxylate (**99**) was obtained after 11 synthetic steps. Reduction of **99** followed by reaction with $Br^{13}CN$ afforded the corresponding diprotected amino alcohol which was converted to L-[*guanidino*-^{13}C]capreomycidine. Incorporation studies using the labeled material with *S.* L-1689-23 resulted in no observable incorporation into STF. These results suggest that either the amino acid was not transported into the cell, is an enzyme-bound intermediate, or is not a free biosynthetic intermediate in STF formation.

Advanced Studies on the Biosynthesis of the Streptolidine Moiety of Streptothricin F

by

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Major Professor, representing Chemistry

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Chair of Department of Chemistry

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Dean of Graduate School

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Michael D. Jackson

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List of Abbreviations

ACV	L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine
Arg	arginine
AT	amidinotransferase
BOC	*t*-butoxycarbonyl
bs	broad singlet
BzCl	benzyl chloride
C	celcius
Cap	capreomycidine
CAS	clavaminate synthase
CbzCl	benzyloxycarbonyl chloride
Ci	curie
CI	chemical ionization
Cit	Citrulline
CFE	cell-free extract
d	doublet
DABS	(dimethylamino)azobenenesulfonyl
DAOCS	deacetoxycephalosporin C synthase
DCC	dicyclohexylcarbodiimide
DEAD	diethylazodicarboxylate
DIBALH	diisobutylaluminum hydride
DMF	*N*,*N*-dimethylformamide
DMP	2,2-dimethylpropane
Dns	Dansyl
DPPA	diphenylphosphoryl azide
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
FABMS	fast atom bombardment mass spectrometry
hr	hour
Harg	β-hydroxyarginine

List of Abbreviations (Continued)

HPHT	hydroxypyridylhomothreonine
HPLC	high performance liquid chromatography
HRCIMS	high resolution chemical ionization mass spectrometry
HRFABMS	high resolution fast atom bombardment mass spectrometry
IPNS	isopenicillin N synthase
α-KG	α-ketoglutarate
LAH	lithium aluminum hydride
Leu-BS	leucylblasticidin S
m	multiplet
min	minute
MTPA	α-methoxy-α-(trifluoromethyl)phenyl acetate
MOPS	(3-[N-morpholino]propanesulfonic acid
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NMR	nuclear magnetic resonance
Orn	ornithine
OPA	*ortho*-phthalaldehyde
PEP	phosphoenolpyruvate
PMA	phosphomolybdic acid
ppm	parts per million
Pyr	pyridine
R_f	retardation factor
s	singlet
sec	second
STF	streptothricin F
Str	streptolidine
t	triplet
TBAF	tetra-*n*-butylammonium fluoride
TBDMS	*tert*-butyldimethysilyl

List of Abbreviations (Continued)

TCA	tricarboxylic acid cycle
THF	tetrahydrofuran
TLC	thin-layer chromatography
TPAP	tetra-*n*-propylammonium perruthenate
TPP	triphenyl phosphine
t_R	retention time
p-TsCl	*p*-toluenesulfonyl chloride
p-TsOH	*p*-toluenesulfonic acid
UDP	uridine diphospate
YME	yeast malt extract

List of Schemes

List of Schemes (Continued)

List of Schemes (Continued)

Advanced Studies on the Biosynthesis of the Streptolidine Moiety of Streptothricin F

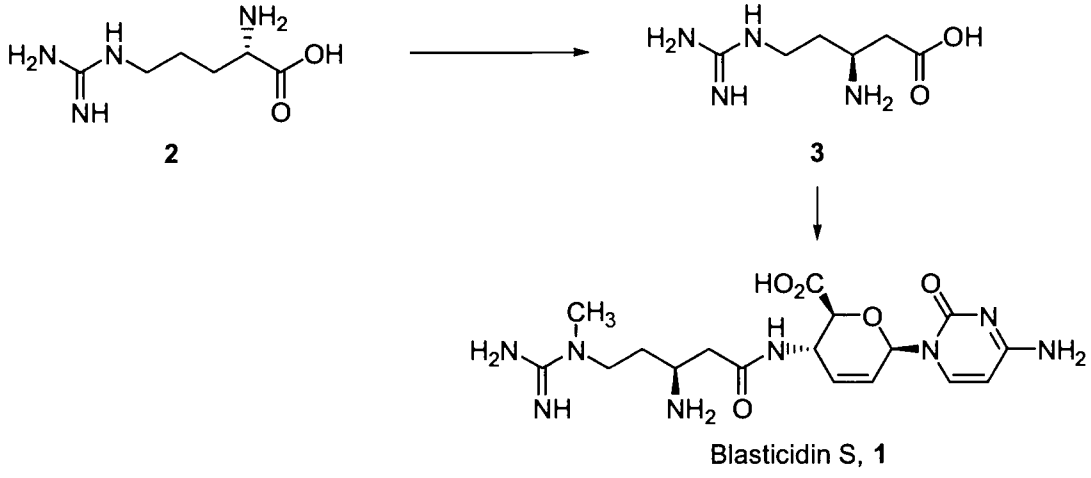
Chapter 1
Introduction to Arginine Secondary Metabolism in *Streptomyces lavendulae* var. L-1689-23

Examples of Arginine Metabolism in Various *Streptomyces* Species

The use of amino acids in the formation of secondary metabolites is quite abundant in nature. These metabolites can range from alkaloids, derived from tyrosine or tryptophan, to cyclic peptide antibiotics, containing both the twenty common amino acids and more unusual amino acids, to aminoglycoside antibiotics. Arginine is the most basic amino acid, and has been incorporated into a number of natural products. Examples of the incorporation of arginine by *Streptomyces* species are especially interesting. Some examples show only slight modification of the arginine molecule while others show such extensive modification that, at first hand, arginine would not be considered the most direct primary precursor.

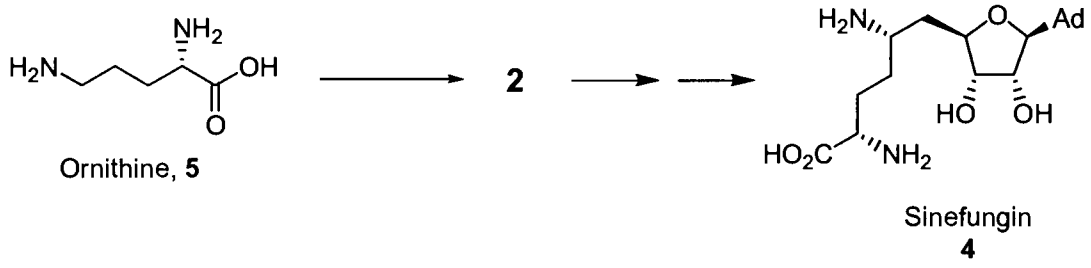
Blasticidin S (**1**), an antifungal antibiotic produced by *Streptomyces griseochromogenes*, contains a δ-*N*-methylated β-arginine residue in its structure. Work by the Gould group has shown direct incorporation of arginine[1] (**2**) and β-arginine[2] (**3**) into **1** as shown in Scheme 1.1. *N*-Methylation was originally proposed to occur as the last step in the biosynthesis of **1**.[3] But this hypothesis was recently modified by work from our laboratory. Leucylblasticidin S (Leu-BS), modified by the addition of leucine to the β-nitrogen of the β-arginine portion of **1**, is formed prior to *N*-methylation and acts as a self-resistance mechanism.[4] As the molecule is transported out of the cell, the leucine residue is most likely removed to liberate the active antibiotic.

Scheme 1.1. Incorporation of Arginine Into Blasticidin S



An example of near-intact incorporation of arginine into a secondary metabolite can be found in sinefungin (**4**), a nucleoside antibiotic produced by *S. griseolus* and *S. incarnates*. Both arginine (**2**)[5] and ornithine (**5**)[6] are incorporated into **4** using cell-free extracts of *S. incarnates* and *S. griseolus*, respectively (Scheme 1.2). Pyridoxal phosphate was required in the enzyme assays and was proposed to activate arginine for coupling with C-6 of ribose. A mechanism for the activation of arginine via formation of a Schiff-base intermediate with pyridoxal phosphate and its incorporation into **4** has been proposed by Malina and coworkers.[5]

Scheme 1.2. Incorporation of Ornithine and Arginine Into Sinefungin

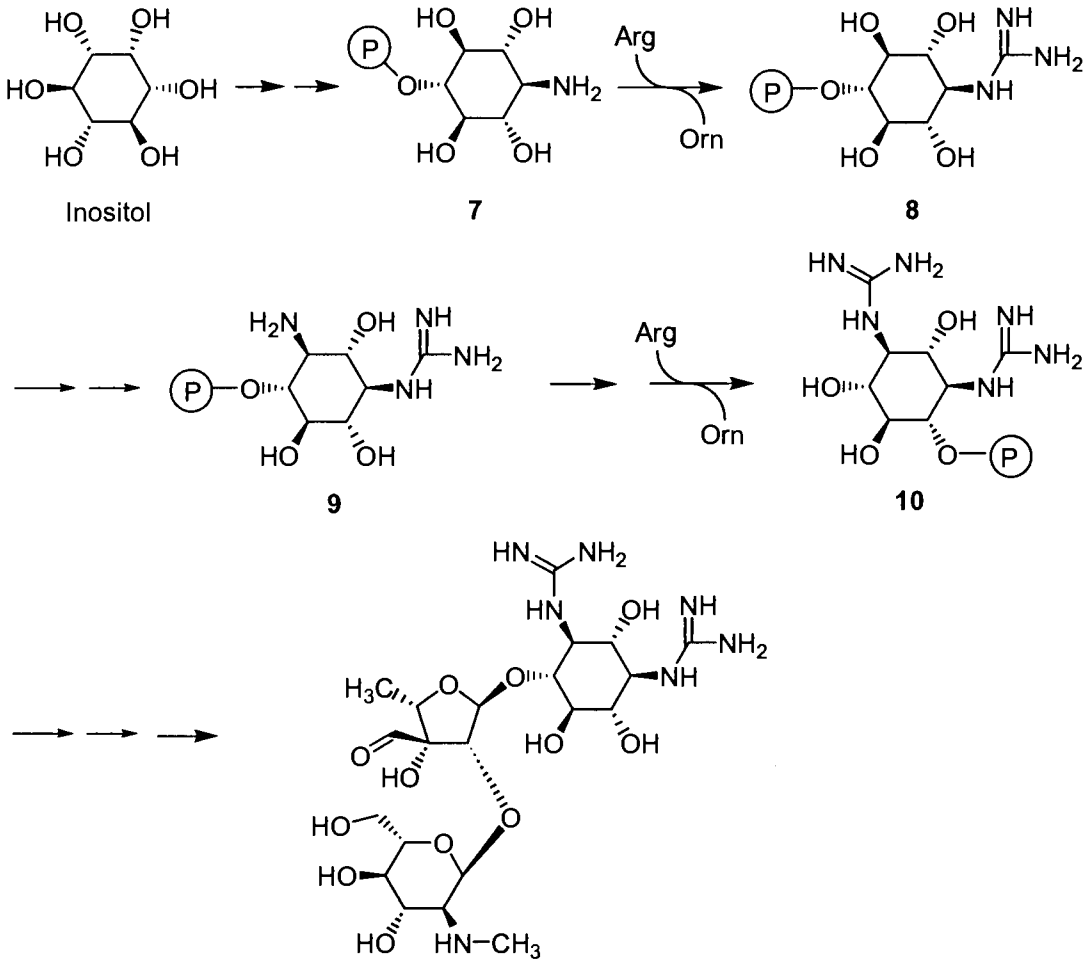


Ornithine, **5**

Sinefungin
4

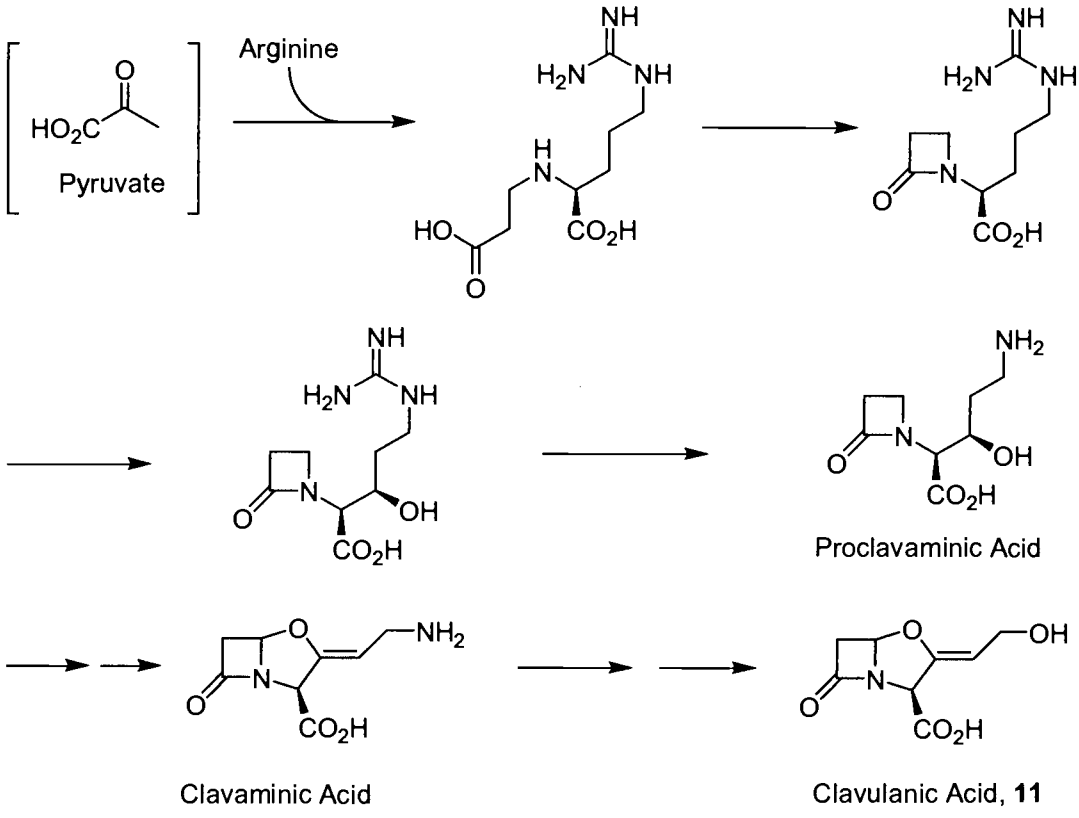
The aminoglycoside antibiotic, streptomycin (**6**, Scheme 1.3) contains two guanidino moieties that are derived from arginine. The biosynthesis of **6** has been extensively studied and most of the enzymes utilized in the formation of streptomycin have been identified by several different groups.[7-9,10] The gene that codes for the amidinotransferase StrB1 from *S. griseus* has been isolated and characterized.[11] The gene product catalyzes the amidination of *scyllo*-inosamine 4-phosphate (**7**) into *N*-amidino-*scyllo*-inosamine 4-phosphate (**8**) and N^1-amidinostreptoamine 6-phosphate (**9**) into streptidine 6-phosphate (**10**). The crystal structure of StrB1 was recently reported and indicated that conserved residues in the active site of the enzyme shared equivalent topological positions to that of other amidinotransferases (AT).[12] The authors therefore suggested a similar mechanism among AT.

Scheme 1.3. Partial Biosynthetic Pathway of Streptomycin



Probably the best example of the extensive metabolism of arginine into a secondary metabolite is the β-lactamase inhibitor, clavulanic acid (**11**), a β-lactam produced by *S. clavuligerus*. As depicted in Scheme 1.4, most of the biosynthetic intermediates in clavulanic acid biosynthesis have been identified[13-15] as well as several of the enzymes.[16] The initial C-3 unit that is condensed with arginine has yet to be identified, however, strong evidence has been reported[17] suggesting that pyruvate is the primary C-3 precursor.

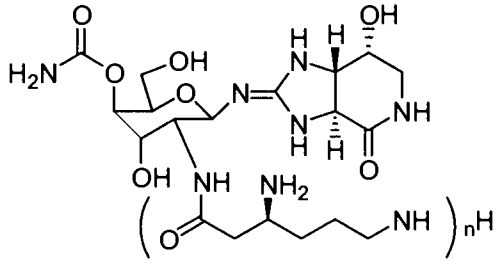
Scheme 1.4. Biosynthesis of Clavulanic Acid From Arginine



Our laboratory is actively involved in studying the biosynthesis of secondary metabolites containing basic amino acids. One of these compounds, blasticidin S, has already been discussed. Two other compounds, which will be discussed in greater detail, are steptothricin F and capreomycin, antibiotics produced by two different *Streptomyces* species; *S. lavendulae* and *S. capreolus*, respectively.

Isolation of Streptothricin F and Related Homologs

Streptothricin F (STF, **12a**) is a broad-spectrum peptidyl nucleoside antibiotic first isolated from *Streptomyces lavendulae* in 1943 by Waksman and Woodruff.[18] It is a member of the Streptothricin class of antibiotics; the only difference among the members is the number of β-lysine residues present (Figure 1.1).



12a: Streptothricin F; n = 1
12b: Streptothricin E; n = 2
12c: Streptothricin D; n = 3
12d: Streptothricin C; n = 4
12e: Streptothricin B; n = 5
12 f: Streptothricin A; n = 6
12g: Streptothricin X; n = 7

Figure 1.1. The Streptothricin Class of Antibiotics

Because of their wide-spread occurance and potent biological activity, it was thought that several different streptothricin analogues had been isolated. For most cases, however, it was determined that the compounds were actually a mixture of the streptothricin antibiotics. A list of the many *Streptomyces* strains as well as other variants of *S. lavendulae* that produce streptothricins have been summarized in the thesis of John Wityak.[19] While several streptothricin producers are variants of *S. lavendulae*, other strains such as *S. xanthophaeus,*[20,21,22] *S. purpeofuscus*[23-25] and *S. roseochromogenes*[26] are also representatives of the diverse species of *Streptomycetes* that produce these antibiotics.

Biological Activity of the Streptothricin Group of Antibiotics

Streptothricin F and its homologues exhibit a broad spectrum of biological activity. The streptothricins show antibacterial activity toward both Gram-positive and Gram-negative bacteria, mycobacteria, as well as some antifungal activity.[27,18] A cocktail of streptothricins (streptothricins A-E) has also been reported to show *in vitro* antiviral activity.[28] Besides microorganisms and lower eukaryotes, the streptothricins have demonstrated toxicity towards insects[29,30] and some species of fish.[29]

The streptothricin class of antibiotics, while exhibiting very broad activity, is not used clinically due to its high toxicity, which has been observed in mice and rats. The streptothricins were first shown to exhibit an increase in protein binding to serum proteins *in vitro* in relation to an increase in the number of β-lysine residues in the molecule.[31] It was later reported that kidney damage was found to result from the formation of the free acid of the streptothricins (Figure 1.2) which had been isolated from the urine of mice and rats.[32,33] The streptothricins were reported to have no hematotoxic potential.[34] The use of the streptothricins as taeniacidal agents in dogs and cats has been reported,[35,36] but they are not currently used in veterinary medicine.

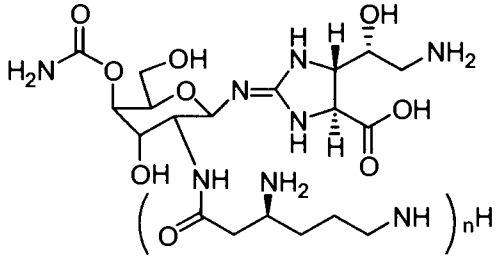


Figure 1.2. Proposed Toxic Metabolite of the Streptothricins

The mode of action of the streptothricins has been reported in some detail. The streptothricins were first reported to form weak DNA-antibiotic complexes in low salt concentrations.[37] It was later reported that antibiotics, streptothricin F (**12a**), effected protein synthesis by binding to the 30S and 50S subunit of the bacterial ribosomes,[38,39] thus preventing the amino acid-tRNA from binding to the mRNA-ribosome complex.

Chemical and Physical Properties of Streptothricin F (12a)

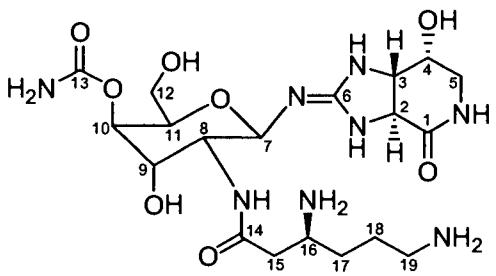
The streptothricins are solids that decompose upon melting above 250 °C. During the isolation and purification of the streptothricins, several salts have been reported such as the helianthate,[40,41] picrolonate,[40] reineckate,[40,41] picrate,[40] sulfate,[42] phosphotungstate,[40] and hydrochloride.[40,41]

Streptothricin F has three ionizable groups with pKa's of 7.1, 8.2, and 10.1.[43] The antibiotic is insoluble in organic solvents with the exception of slight solubility in methanol and dimethylsulfoxide. It is completely soluble in water at pH ranges of 1-9, but substantial decomposition occurs at higher or lower pH. The hydrochloride salt of **1a** can be stored for over a year at -10 °C in a dessicator without appreciable decomposition. For long-term storage of STF, formation of the helianthate salt, which can be stored in a dessicator at 25 °C, is preferred.

Over the years, several researchers have assigned partial ^{1}H NMR[44,45] and ^{13}C NMR[23,46,47] spectra of STF (**12a**) or its components. Martinkus[48] was the first to assign the complete ^{1}H and ^{13}C NMR spectra of **12a** as presented in Table 1.1.

Table 1.1. Proton and Carbon Chemical Shift of STF (**12a**) at pH 6.8[48]

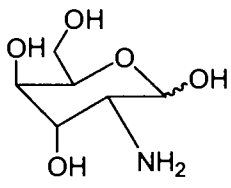
Proton	Mult.	δ(ppm)	*J* (Hz)	Int.	Carbon	Mult.	δ(ppm)
					1	s	167.29
2	d	4.66	14.2	1 H	2	d	51.80
3	dd	4.12	<1, 13.6	1 H	3	d	58.34
4	bs	4.76		1 H	4	d	58.17
5 (*pro S*)	dd	3.85	4.9, 14.6	1 H	5	t	46.56
5 (*pro R*)	dd	3.45	<1, 14.6	1 H			
					6	s	159.94
7	d	5.14	9.3	1 H	7	d	76.25
8	dd	4.29	2.9, 9.8	1 H	8	d	46.35
9	dd	4.21	2.9, 3.4	1 H	9	d	63.88
10	dd	4.83	<1, 3.4	1 H	10	d	67.38
11	dt	4.38	<1, 5.9	1 H	11	d	70.79
12	d	3.77	5.7	2 H	12	t	57.64
					13	s	155.08
					14	s	169.75
15 (*pro S*)	dd	2.66	8.3, 16.6	1 H	15	t	33.83
15 (*pro R*)	dd	2.78	4.4, 16.6	1 H			
16	m	3.65		1 H	16	d	45.67
17	bs	1.79		2 H	17	t	26.51



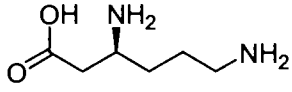
Streptothricin F
(**12a**)

Structural Elucidation of Streptothricin F (12a)

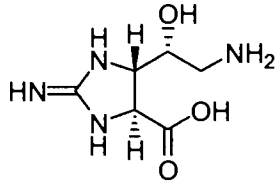
The structure of streptothricin F was initially elucidated from the hydrolysis products of the antibiotic. Three basic components were isolated after vigorous hydrolysis and purified by various chromatographic techniques. These components were identified as the amino sugar D-gulosamine (**13**), and the basic amino acids β-lysine (**14**) and streptolidine (**15**)[23,42,49-54] (Figure 1.3). The structures of **13**[55,56,57] **14**,[58] and **15**[59,60] were confirmed by total synthesis, and the absolute stereochemistry of streptolidine (**15**) was determined by x-ray crystallography.[61]



D-Gulosamine
13



β-Lysine
14



Streptolidine
15

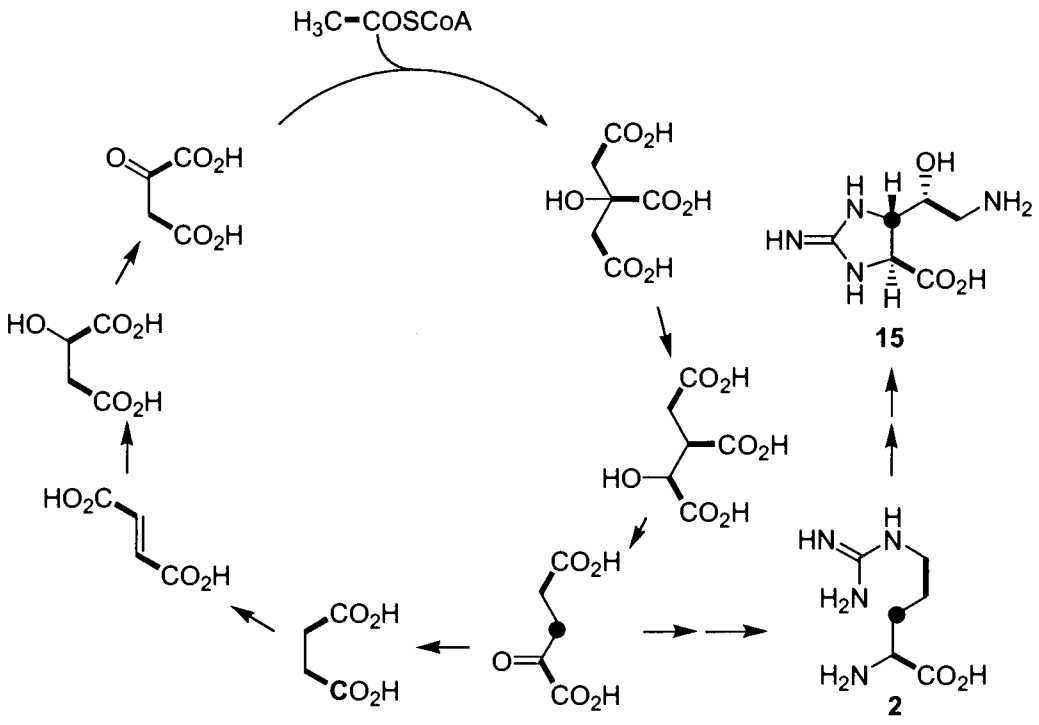
Figure 1.3. The Structural Components of the Streptothricin Class of Antibiotics

Under mild hydrolysis of STF (**12a**) it was found that carbon dioxide and ammonia were generated.[42] It was proposed that a urethane substituent was present on either the C-3 or C-4 hydroxy group of the gulosamine moiety in STF.[43] The complete structure of **12a** was finally elucidated by total synthesis[59,62] which confirmed the location of the carbamate substituent at C-4 of gulosamine. The Gould group also independently determined the location of the urethane as residing at C-4.[48]

Previous Biosynthetic Studies on the Streptolidine Moiety of STF

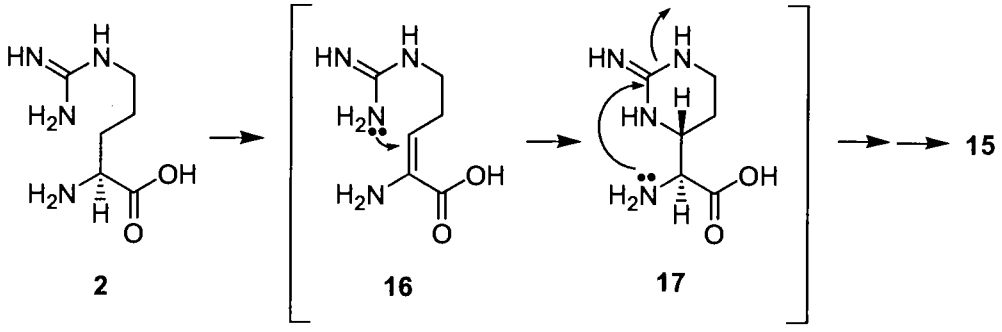
In the mid 1970s, two different groups[47,63,64] began biosynthetic studies on **12a** which produced conflicting results. Sawada and co-workers concluded that two different pathways leading to the formation of streptolidine (**15**) were present in *Streptomyces* species: one derived from arginine (**2**) and the other from acetate. The use of universally labeled materials made it difficult to determine the specificity of incorporation as well as which pathway was involved in the biosynthesis of **15**. Sodium [1-^{13}C]- and [2-^{13}C]acetates were specifically incorporated, however, Sawada and co-workers were unable to propose a pathway that explained the observed labeling pattern in **12a**.[47] Gould and co-workers disputed the proposal that two different pathways were used in the formation of **15**.[65] Using [1,2-^{13}C]acetate, they found that **12a** was specifically, albeit indirectly, labeled via arginine (**2**) and the Tricarboxylic Acid Cycle (TCA). This result strongly supported a single pathway for the formation of **15** as shown in Scheme 1.5.

Scheme 1.5. Labeling of Streptolidine by [1,2-^{13}C]Acetyl-CoA via α-Ketoglutarate and the TCA Cycle



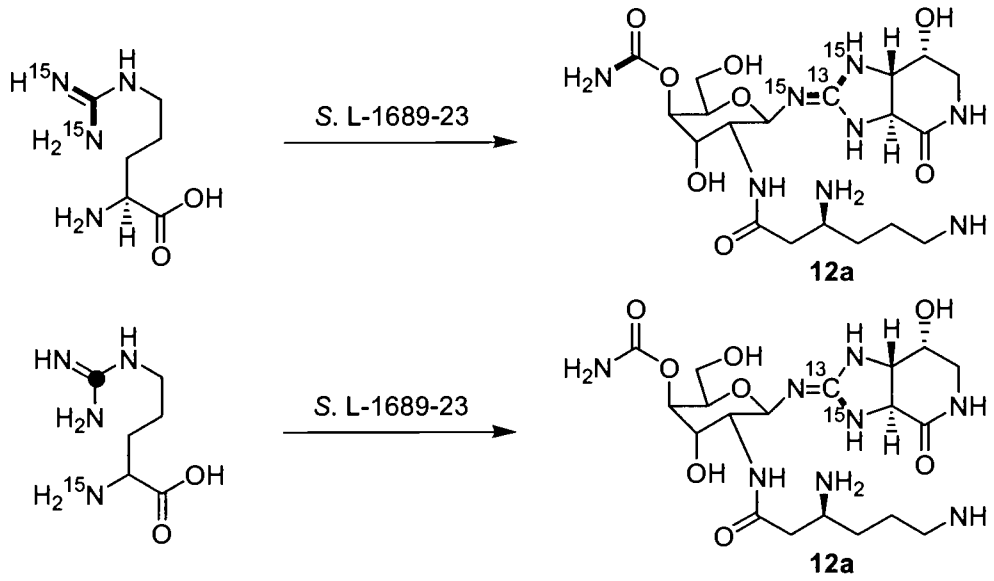
In 1972, Bycroft and King[61] proposed the intermediacy of a dehydroarginine derivative (**16**) and the amino acid, capreomycidine (**17**), in the biosynthesis of **15** as shown in Scheme 1.6. The [1,2-^{13}C]acetate incorporation study supported the proposed pathway in Scheme 1.6 as well as suggesting the direct biosynthesis of capreomycidine from arginine.

Scheme 1.6. Biogenesis of the Streptolidine Portion of Streptothricin F from Arginine (**2**)



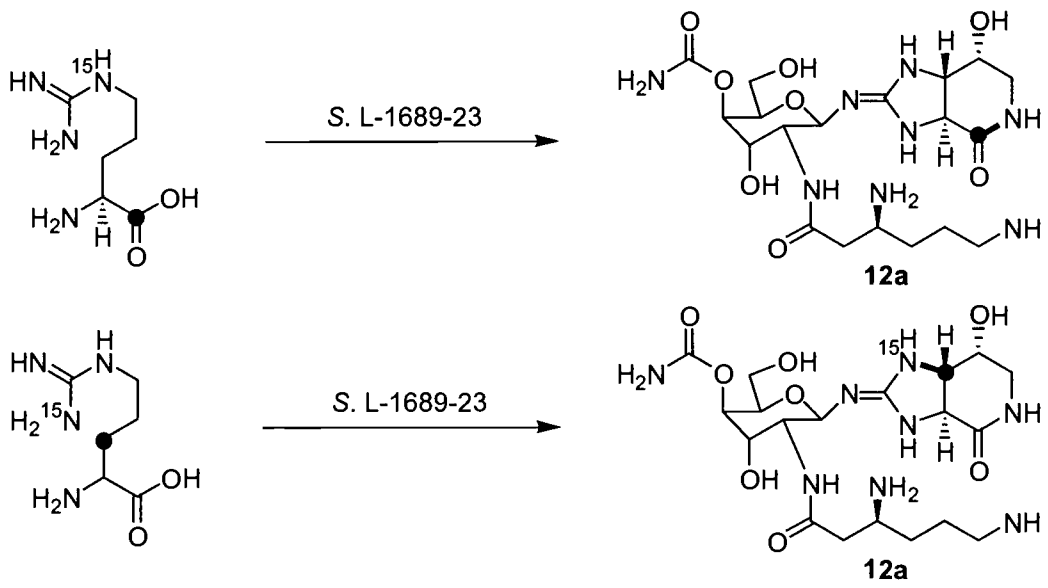
Additional support for Bycroft and King's proposal was obtained through dual-labeling studies using L-[*guanidino*-^{13}C,$^{15}N_2$]- and DL-[*guanidino*-^{13}C,2-^{15}N]arginine.[66] The incorporation of ^{13}C and ^{15}N into **12a** was observed as shown in Scheme 1.7. Spin coupling between ^{13}C and ^{15}N demonstrated the formation of two new C-N bonds and established that all three guanidino nitrogen atoms in streptolidine are derived from arginine. It was also determined that only L-arginine is incorporated into streptothricin F (**12a**) further supporting the direct incorporation of arginine into **15** and **12a**.

Scheme 1.7. Incorporation of L-[*guanadino*-^{13}C,$^{15}N_2$]- and DL-[*guanidino*-^{13}C,2-^{15}N]Arginine Into **12a**.



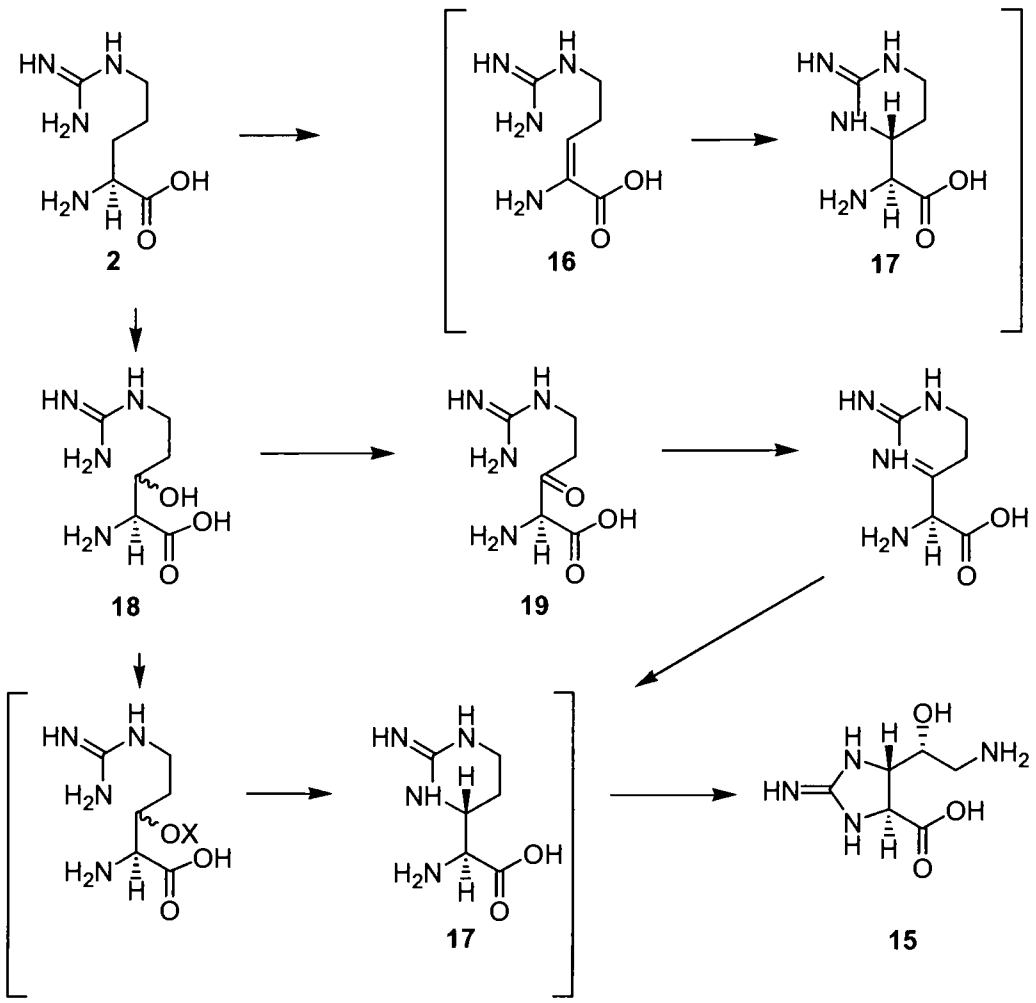
The remaining two new C-N bonds formed in the conversion of arginine to the streptolidine moiety in **12a** were identified through incorporation studies using DL-[1-^{13}C,5-^{15}N]- and DL-[3-^{13}C,*guanidino*-$^{15}N_2$]arginines (Scheme 1.8).[67]

Scheme 1.8. Incorporation of DL-[1-^{13}C,5-^{15}N]- and DL-[3-^{13}C,*guanidino*-^{15}N]Arginine Into **12a**.



While Bycroft and King's proposal of a Michael addition to dehydroarginine (**16**) was a plausible mechanism, other possibilities for the formation of streptolidine (**15**) were proposed.[67] As Scheme 1.5 illustrates, sequential oxidation of arginine to β-hydroxyarginine (**18**) then β-ketoarginine (**19**) then followed by formation of an imine intermediate would occur. Subsequent reduction of the imine could lead to capreomycidine (**17**). Alternatively, activation of **18** as a phosphate ester followed by nucleophilic displacement with the terminal guanidino nitrogen could also lead to **17**.

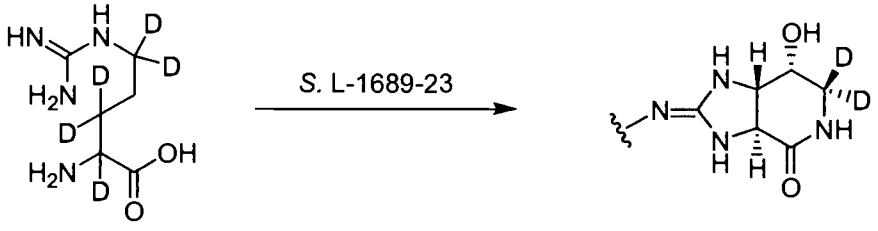
Scheme 1.9. Alternative Mechanisms for Streptolidine Formation in **12a**



To distinguish between the three different pathways, incorporation studies using DL-[2-^{2}H]-, DL-[3,3-$^2H_2$]-, and DL-[2,3,3-$^2H_3$]arginines were conducted.[67] In each example, the isolated streptothricin F showed no deuterium incorporation. These negative results were interpreted as support for the formation of β-hydroxy- and β-ketoarginine because the loss of deuterium at C-3 could be explained by the formation of a keto group, while loss of deuterium at C-2 could result from simple enolization of the β-dicarbonyl moiety.

Further exploration of the fate of the arginine hydrogens was conducted by performing an incorporation study using DL-[2,3,3,5,5-$^2H_5$]arginine (**20**, Scheme 1.10).[68] It was found that deuterium was completely lost from C-2 and C-3, but was retained at C-5. These findings substantiated the earlier results in which deuterium was lost from C-2 and C-3.

Scheme 1.10. Incorporation of DL-[2,3,3,5,5-$^2H_5$]Arginine (**20**) Into the Streptolidine Portion of STF (**12a**)

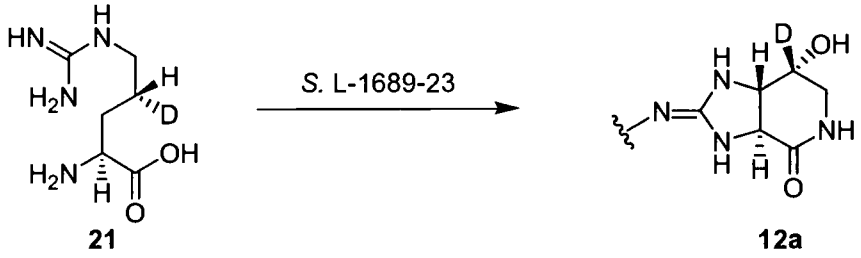


20 **12a**

When *erythro*- and *threo*-DL-[5,5-$^2H_2$]-β-hydroxyarginine were introduced to production cultures of *S.* L-1689-23, no deuterium was observed in the isolated streptothricin F, and isotope-trapping experiments failed to detect *de novo* biosynthesis of either β-hydroxy- or β-ketoarginine.

The stereospecific oxidation of arginine to form the hydroxyl group in streptolidine was examined next using DL-[4,4-$^2H_2$]-, L-[4,4-$^2H_2$]-, L-[4*S*-^2H]-, and L-[4*R*-^2H]arginines (**21**, Scheme 1.11). Results of these incorporation studies indicated retention of the *pro*-4*R* hydrogen at what had been C-4 of arginine.

Scheme 1.11. Incorporation of L-[4*R*-^{2}H]Arginine (**21**) Into the Streptolidine Portion of STF (**12a**)



21 **12a**

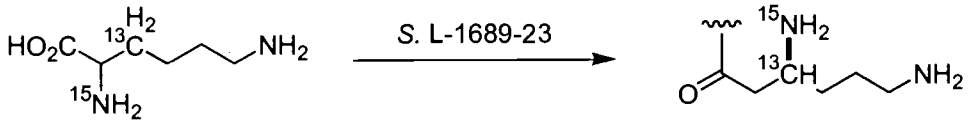
Previous Biosynthetic Studies on the β-Lysine Moiety of STF

Initial work by Sawada and coworkers first reported the incorporation of α-lysine (**22**) into the β-lysine (**14**) portion of streptothricin F.[63] This finding was later indirectly supported by Gould et al. in their incorporation studies using labeled acetate.[65] One question that remained was how β-lysine is formed from α-lysine, **22**. β-Lysine has been identified as a degradation product from various species of *Clostridium*.[69] The enzyme responsible for this conversion, *Clostridium* (*S*)-α-lysine 2,3-aminomutase, has been isolated and cofactor requirements were reported.[70] Recently, the gene for lysine 2,3-aminomutase (KAM, EC 5.4.3.2.) from *Clostridium subterminale* SB4 has been cloned and sequenced.[71] This enzyme catalyzes the stereospecific migration of the 3-*pro*-*R* hydrogen of **22** with inversion of configuration at both C-2 and C-3.[72] Recently, Chen and coworkers have reported on a lysine 2,3-aminomutase encoded by the yodO gene of *Bacillus subtilis*.[73]

To confirm the direct incorporation of **22** into the β-lysine portion of streptothricin F, Gould and Thiruvengadam synthesized DL-[3-^{13}C,2-^{15}N]lysine (**23**, Scheme 1.12) and introduced it to production flasks of *S.* L-1689-23.[74] Analysis of the ^{13}C NMR spectrum of isolated **12a** showed clear incorporation of

labeled material as well as the formation of a new ^{13}C-^{15}N bond. Because a ^{13}C-^{15}N spin coupling was observed in derived **12a**, the result clearly showed that the migration of nitrogen from C-2 to C-3 in **22** was intramolecular.

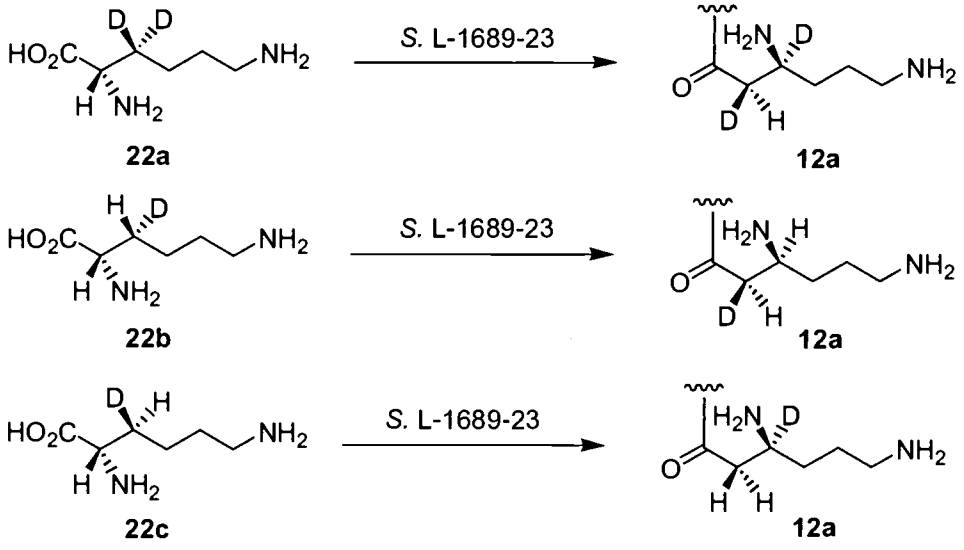
Scheme 1.12. Incorporation of DL-[3-^{13}C,2-^{15}N]Lysine (**23**) Into the β-Lysine Portion of STF (**12a**)



23 **12a**

To determine whether the 2,3-aminomutase used in the biosynthesis of streptothricin F utilized the same mechanism as the *Clostridium* enzyme, α-[(3*RS*)-$^2H_2$]-, α-[(3*R*)-^2H]- and α-[(3*S*)-^2H]lysine (**22a**, **22b** and **22c**, respectively, Scheme 1.13) were tested.[75] From the analysis of the ^2H NMR spectra of the derived antibiotic from the individual incorporation experiments, it was determined that the migration of the alpha nitrogen proceeded through inversion of configuration at C-3 via an intramolecular process. The migration of the 3-*pro-R* hydrogen to C-2 was also demonstrated to occur with an inversion of configuration. Lastly, the incorporation of β-[(2*S*)-^2H]lysine was observed in the β-lysine portion of **12a** suggesting that **14** is a true intermediate in the formation of **12a**.[75]

Scheme 1.13. Incorporation of Deuterated Lysine Into the β-Lysine Portion of **12a**



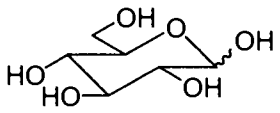
22a **12a**

22b **12a**

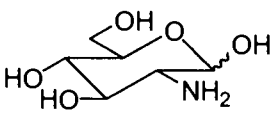
22c **12a**

Previous Biosynthetic Studies on the Gulosamine Moiety of STF

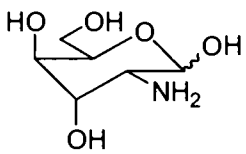
The origin of the gulosamine moiety (**13**) in **12a** was initially addressed by Sawada and coworkers[47,63] in the late 1970's. Glucose (**23**) and glucosamine (**24**) were identified as precursors by adding [U-^{14}C]-D-glucose and [1-^{14}C]-D-glucosamine to production cultures of *S. lavendulae*, and observing in 0.71% and 4.46% incorporation into **12a**, respectively. The higher level of incorporation of labeled glucosamine, with respect to labeled glucose, suggested that **24** was a more direct precursor to **13**. Radioactivity was also found in the β-lysine and streptolidine portions of **12a** as well, implying extensive degradation of glucose.



D-Glucose
(23)



D-Glucosamine
(24)

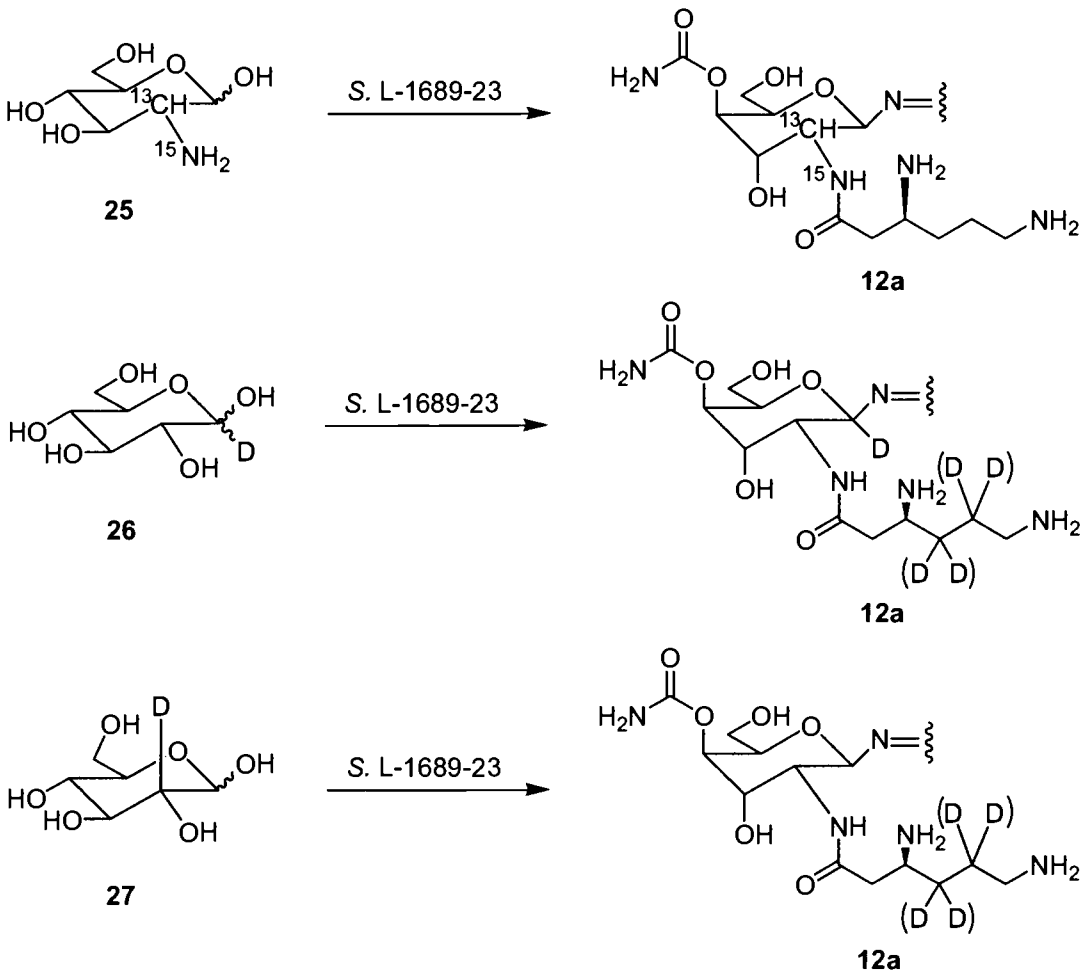


D-Gulosamine
(13)

Figure 1.4. The structure of D-Glucose (**23**), D-Glucosamine (**24**) and D-Gulosamine (**13**)

Palaniswamy and Gould[76] established D-glucosamine as a direct precursor of streptothricin F by observing the direct incorporation of [2-^{13}C,^{15}N]-D-glucosamine (**25**) into the gulosamine moiety of **12a** (Scheme 1.14). To probe the conversion of glucose to **13**, [1-^{2}H]- and [2-^{2}H]-D-glucose (**26** and **27**, respectively) were tested and the resulting STF was analyzed by ^{2}H NMR. While the incorporation study using [1-^{2}H]-D-glucose showed reasonable incorporation(1.8%) into H-8 of **12a**, and minor incorporation (0.4%) into the β-lysine residue at H-17/H-18, [2-^{2}H]-D-glucose resulted in only minor incorporation (0.09%) at H-17/H-18 further illustrating the extensive degradation of glucose and random redistribution of label into streptothricin F (**12a**). From these results, it was proposed that H-2 of glucose is lost in the phosphoglucose isomerase reaction.

Scheme 1.14. Incorporation of Labeled Glucose and Glucosamine Into STF (**12a**)

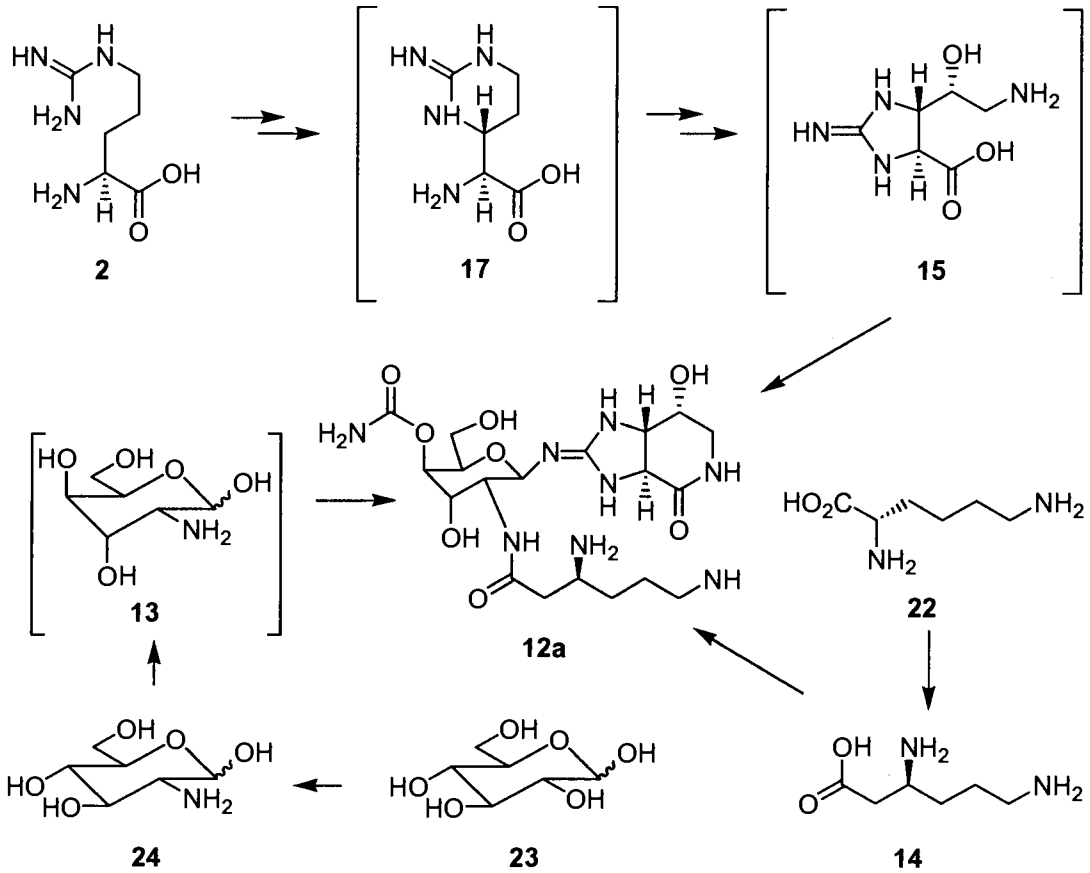


Objectives and Significance of the Present Studies

Through work that has been done as of 1994, our understanding of the biosynthesis of streptothricin F can be summarized as shown in Scheme 1.15. Gould has demonstrated that key components of streptothricin F, namely β-lysine[74] (**14**) and D-glucosamine[76] (**13**) are formed prior to assembly of the antibiotic suggesting a convergent biosynthesis of **12a**. Extensive work in the

area of arginine metabolism in STF biosynthesis[65-68] provided insight into the conversion of arginine into the streptolidine portion of STF. Streptolidine (**15**), however, has not been identified as a true intermediate in the biosynthesis of **12a**. Through the use of ^{13}C-^{15}N dual labeling studies, the amino acid, capreomycidine (**17**) has been proposed as an intermediate in the formation of streptolidine but has not been unequivocally shown to be a true intermediate.

Scheme 1.15. Proposed Biosynthetic Pathway to STF (**12a**) as of 1994



The first objective of this work was to determine whether streptolidine is an intermediate in the biosynthesis of STF. This would conclusively determine whether STF biosynthesis occurs through a convergent pathway. A primary goal was to synthesize ^{13}C-labeled **15** with the label located at the guanidino carbon and test whether the labeled material is incorporated into the streptolidine moiety of STF (**12a**).

While there is strong evidence for the involvement of capreomycidine (**17**) in the biosynthesis of the streptolidine moiety in STF, no intermediates have been identified. Therefore, a second objective was to investigate the intermediacy of **17** in the formation of the streptolidine portion of **12a**. Using cell-free extracts (CFE) from *S.* I-1689-23, [U-^{14}C]arginine would be added and radioactive compounds that are produced would be characterized.

An alternative method in determining the intermediacy of capreomycidine (**17**) in the biosynthesis of streptolidine (**15**) would be through incorporation studies using ^{13}C-labeled **17**. Based upon what has been published in the literature, a more efficient synthesis of capreomycidine would need to be designed and subsequently developed so that a ^{13}C label could be placed at the guanidino carbon of **17**. This would provide labeled capreomycidine for use in incorporation studies. Elucidation of this portion in the pathway could identify the order of oxidations that occur in the conversion of arginine to **15**.

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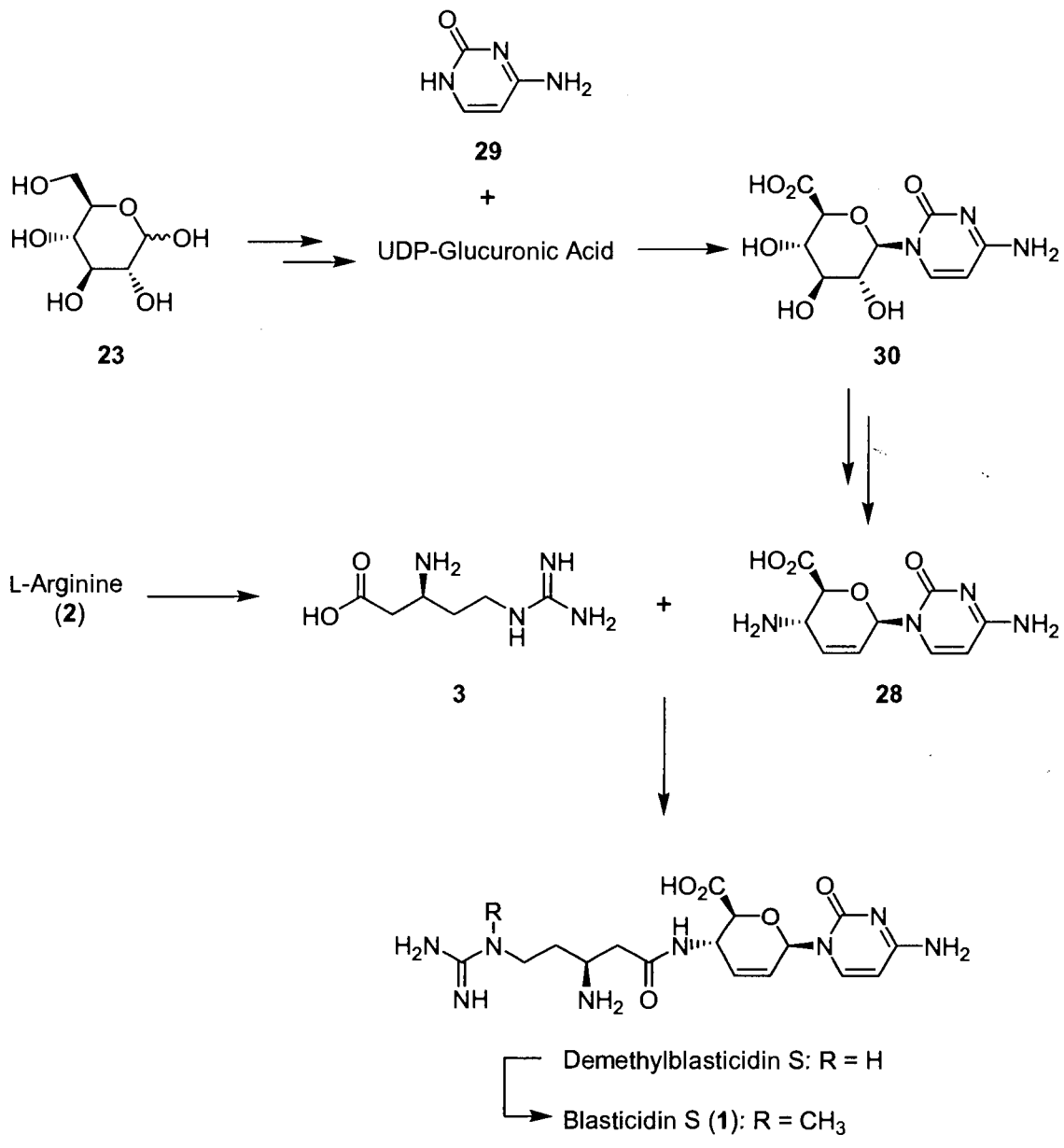
Chapter 2
The Intermediacy of Streptolidine in the Formation of Streptothricin F

Introduction

Several organisms have developed what can be identified as a convergent biosynthesis for the formation of particular natural products. Convergent biosynthesis is defined as a biosynthetic pathway in which the key structural components of the molecule are made independently of each other, and then coupled to form the core structure of the natural product. Post assembly modifications such as methylations, acetylations, and oxidations or reductions often occur before the molecule is presented in its biologically active form.

An example of a convergent pathway that has been extensively studied in our laboratory pertains to the biosynthesis of blasticidin S (**1**). The identity of free β-arginine (**3**, Scheme 2.1) as a direct precursor to the β-arginine moiety in **1** was first reported by Gould in 1997,[1] and later shown to be derived from α-arginine (**2**).[2] The nucleoside cytosinine (**28**) was identified as an intermediate in the biosynthesis of **1** using cell-free extracts (CFE) of *S. griseochromogenes*.[3] Cytosinine is derived from D-glucose (**23**) and the nucleoside cytosine (**29**).[4] Cytosyl glucuronic acid (CGA, **30**) is the only other compound identified as a true intermediate in the formation of **28**.[5]

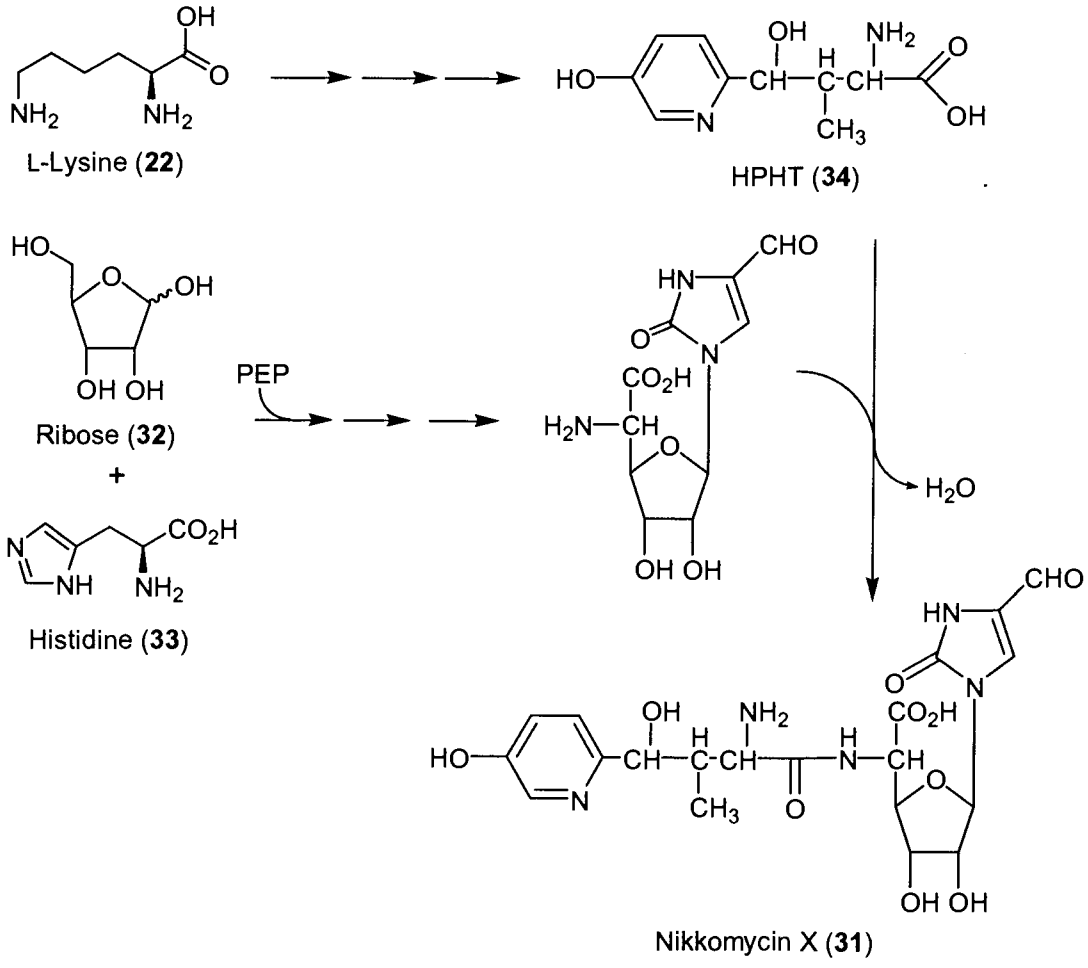
Scheme 2.1. Proposed Pathway for the Biosynthesis of Blasticidin S (**1**) by *S. griseochromogenes*



The nikkomycins, isolated from *Streptomyces tendae* Tü901, are also examples of peptidyl nucleoside antibiotics. Studies on the biosynthesis of the nikkomycins have indicated that separate pathways synthesize the peptidyl and

nucleoside moieties, which are then linked together by the formation of a peptide bond (Scheme 2.2).[6] The primary biosynthetic precursors to the nucleoside moiety in nikkomycin X (**31**) were identified as ribose (**32**), histidine (**33**) and phosphoenolpyruvate (PEP).[7,8] For the unusual amino acid hydroxypyridylhomothreonine (HPHT, **34**), lysine (**22**) has been identified as the primary precursor. All of the nikkomycin biosynthetic genes have been identified from *S. tendae* Tü901,[9] and the first gene involved in HPHT formation was shown to be a L-lysine 2-aminotransferase by Bruntner in 1998.[10] More recently, six additional genes were identified with HPHT biosynthesis, lending further insight into the biosynthesis of the nikkomycins.

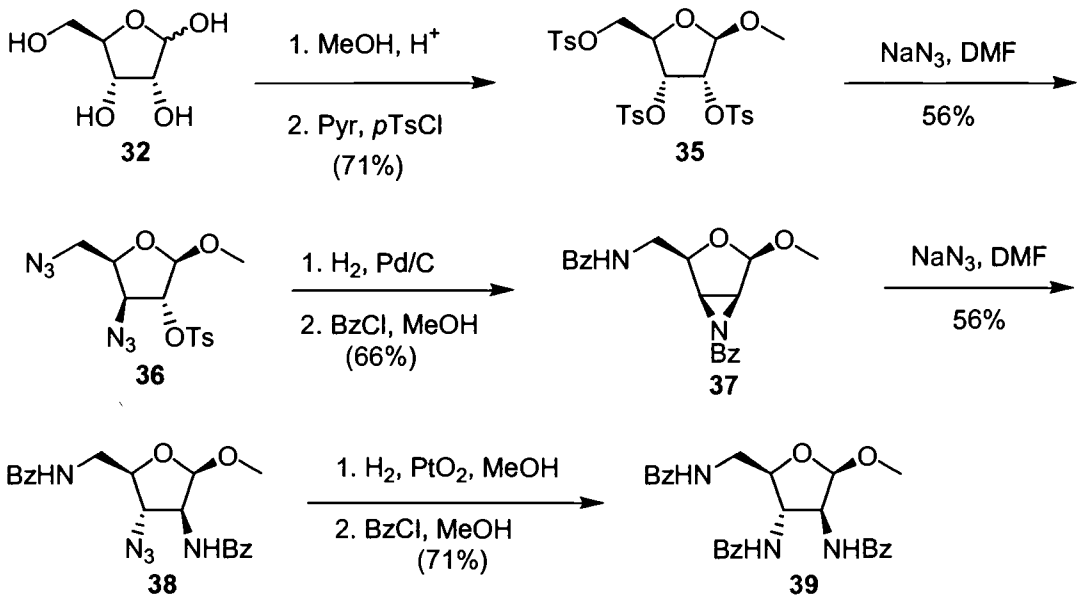
Scheme 2.2. Partial Biosynthetic Scheme for Nikkomycin X; Convergence of the Peptide and Nucleoside Pathways



To determine whether streptothricin F (**12a**, cf. Scheme 1.15) is also produced via a convergent biosynthesis, streptolidine (**15**) must first be identified as a true intermediate. The intact incorporation of both β-lysine (**14**)[11] and D-glucosamine (**24**)[12] into streptothricin F (STF, **12a**) has provided support for a convergent biosynthesis of **12a**. For additional support, labeled streptolidine (**15**) was needed in order to gain insight into the biosynthesis of the nucleoside portion of STF. It was desired that streptolidine have a ^{13}C label at the guanidino

carbon, to permit analysis of isolated STF by NMR spectroscopy. To obtain labeled **15**, a new synthesis could be developed, or a current synthesis be used to incorporate the ^{13}C label into the desired location. To date, two groups have synthesized streptolidine. Independently, Goto[13] and Kusumoto[14] have reported the synthesis of **15** using ribose derivatives that showed stereochemical resemblance to that of streptolidine (**32**, Scheme 2.3). The ribose derivatives used in Goto's and Kusumoto's synthesis were first reported by Hildesheim et al. in 1969.[15]

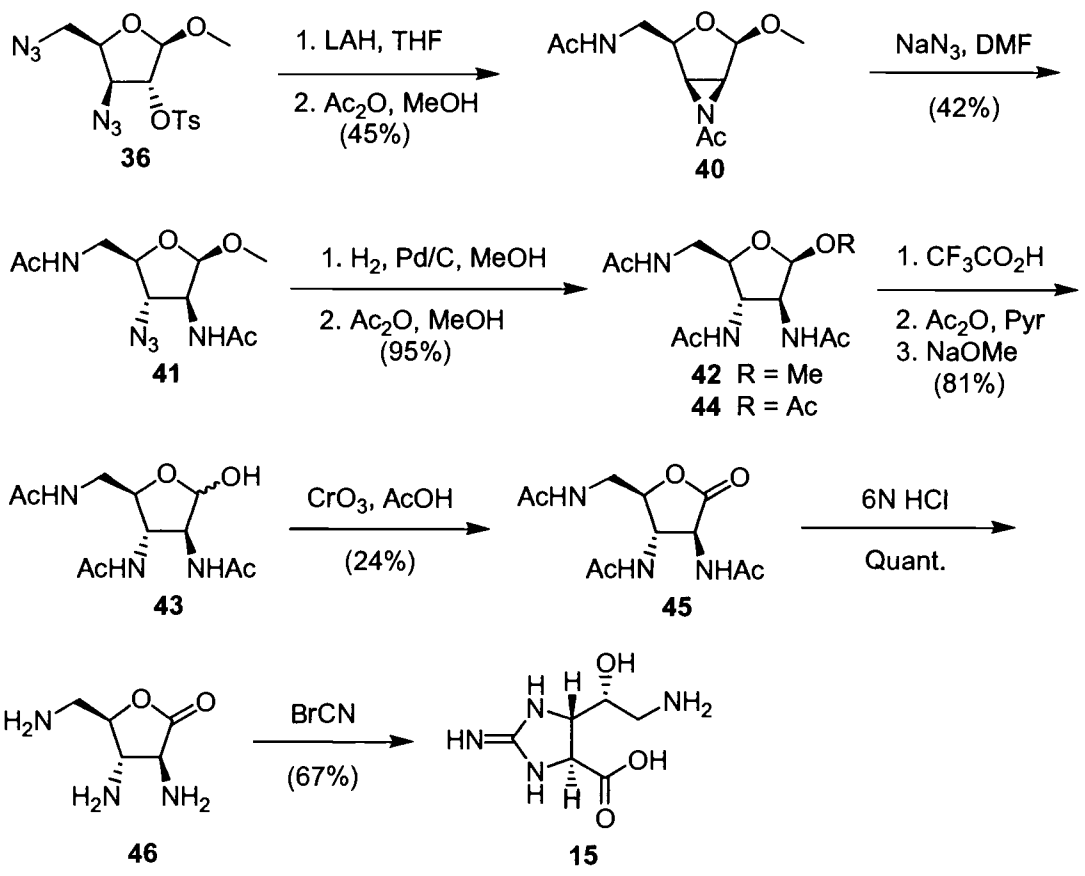
Scheme 2.3. Synthesis of Ribose Derivative **39** with Stereochemistry Related to Streptolidine (**15**)[19]



In Goto's synthesis of streptolidine,[13] diazide **36** (Scheme 2.4) was reduced and the resulting aziridine protected as the diacetate (**40**) in moderate yield. Nucleophilic opening of the aziridine using sodium azide afforded

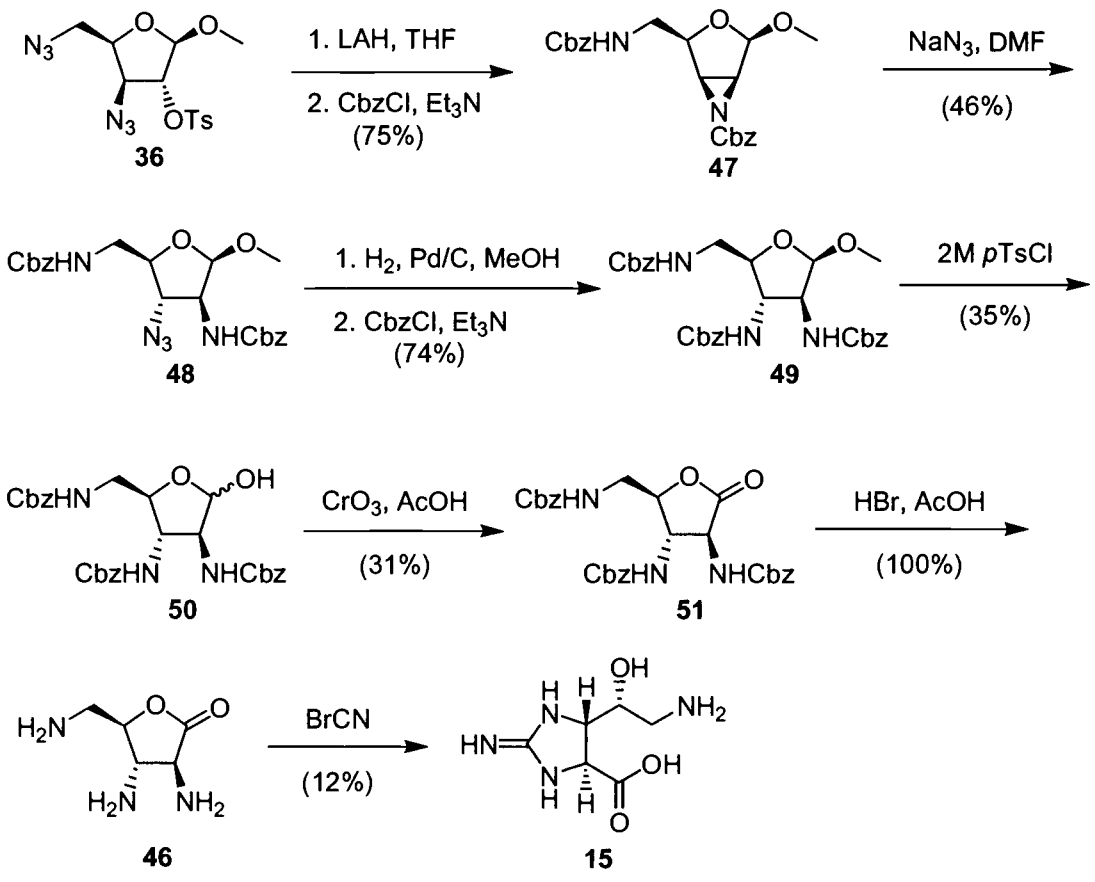
monoazide **41** which was subsequently reduced and protected as the triacetate, **42**. Because conversion of the acetal (**42**) to the corresponding hemiacetal (**43**) under acidic conditions resulted in partial cleavage of the amides in **42/43**, **42** was first converted to tetraacetate **44** followed by saponification to generate **43**. Oxidation of hemiacetal **43** to the lactone (**45**) was achieved in poor yield. Deprotection of the amines to generate triamine **46**, followed by reaction with cyanogen bromide to form the guanidino heterocycle, afforded **15** in moderate yield.

Scheme 2.4. Goto's Synthesis of Streptolidine (**15**) via Diazide **36**[17]



Kusumoto's synthesis of streptolidine (**15**) is nearly identical to Goto's, with the exception that the benzyloxycarbonyl (Cbz) protecting group was used (Scheme 2.5).[14] Beginning with diazide **36**, reduction using lithium aluminum hydride followed by protection of the resulting aziridine, provided intermediate **47**. Nucleophilic ring opening of the aziridine with sodium azide in dimethylformamide produced the desired monoazide (**48**) in reasonable yield. Conversion of **48** to the corresponding triamine via catalytic reduction and protection of the resulting triamine afforded the desired Cbz-protected triamine (**49**). Hydrolysis of **49** to hemiacetal **50**, followed by oxidation using chromium trioxide, yielded lactone **51** which was then deprotected to triamine **46**. Cyclization using cyanogen bromide afforded **15**, albeit in low yield.

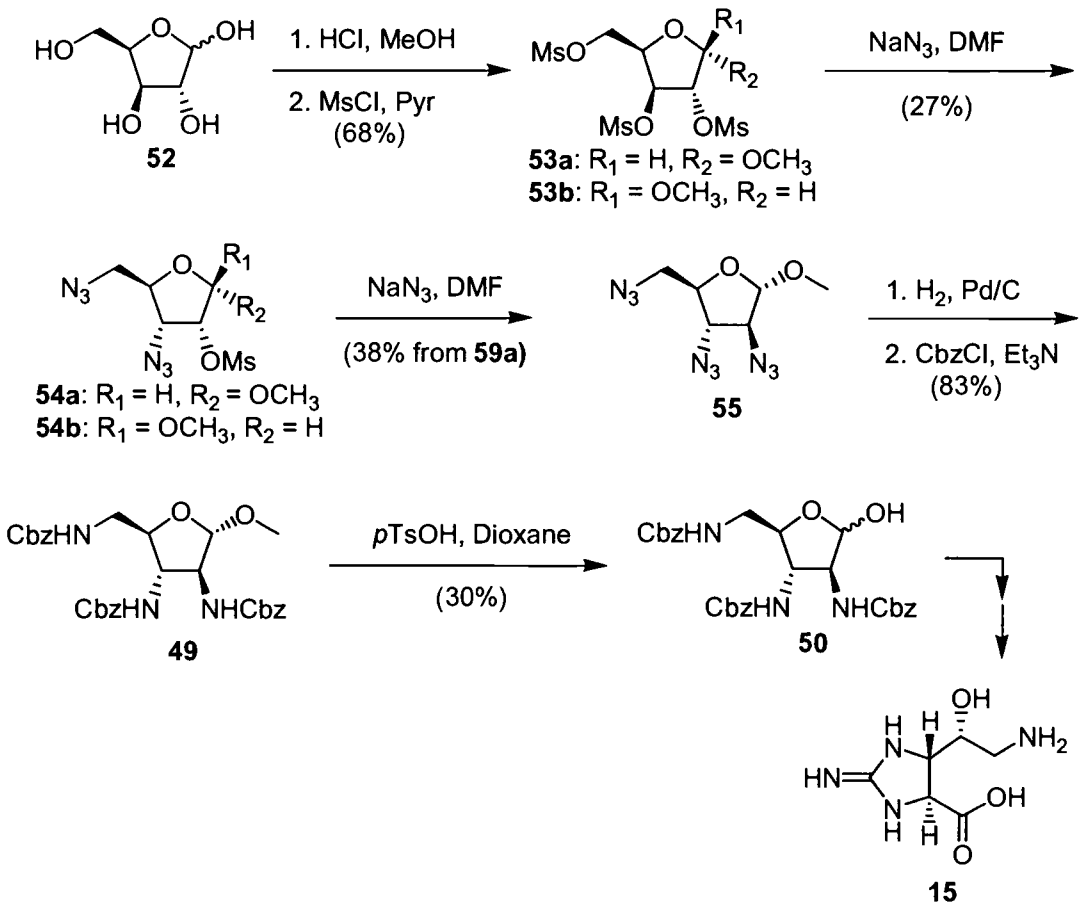
Scheme 2.5. Kusumoto's Synthesis of Streptolidine (**15**) via **36** Using Benzyloxycarbonyl Protecting Groups[18]



In an attempt to obtain **49** without going through an aziridine intermediate, Kusumoto later reported on the formation of **40** using D-xylose (**52**) as the starting sugar (Scheme 2.6).[16] In this synthesis, **52** was first treated with HCl in methanol followed by mesyl chloride in pyridine to afford acetals **53a** and **53b**, predominately as the β-anomer (**53**). Reaction of **53** with sodium azide produced the corresponding diazides **54a** and **54b** as well as a very small amount (0.75% yield) of triazide **55**. Because methyl β-furanosides do not readily undergo displacement reactions at C-2, **54b** was converted to the α-anomer (**54a**) by

bubbling dry hydrochloric acid into a solution of **54b** in methanol. Acetal **54a** was obtained in 16% yield with the recovery of 64% **54b**. The α-diazide was converted to the corresponding triazide **55**, which was subsequently reduced and protected to give acetal **49**. Hydrolysis of **49** afforded hemiacetal **50**, which could be converted to **15** in three steps.

Scheme 2.6. Kusumoto's Synthesis of Intermediate **50** via D-Xylose



Results and Discussion

Synthesis of [guanidine-^{13}C]Streptolidine

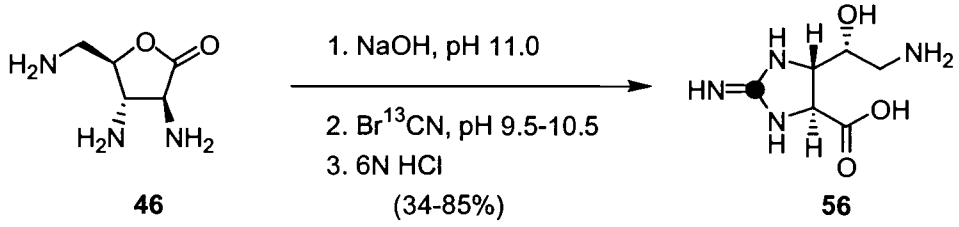
When synthesizing isotopically labeled molecules with either stable or radioisotopes, it is important to place the label in an appropriate intermediate in the synthetic scheme. Labeled compounds are very expensive. To minimize cost, the incorporation of the label into the target compound needs to be toward the end of the synthesis. Also of consideration when working with radiolabels is disposal cost of waste material from the individual reactions. For our synthesis of labeled streptolidine, it was decided to use Kusumoto's synthetic route. By using ^{13}C-labeled cyanogen bromide, the chemical label could be introduced at the end of the synthesis. While many of the synthetic steps in the Kusumoto synthesis were low in yield, unreacted starting material obtained in several of the reactions, could be recycled to increase the overall yield of certain synthetic steps. Goto's synthesis reported no isolation of unreacted starting material in any of the reaction sequences. Furthermore, the use of the Cbz-protecting group over the acetyl group had the additional advantage of being UV-active, making it easier to follow during the course of the synthesis. The most direct route to **15** appeared to be from the use of D-ribose (**32**) as the starting material, instead of D-xylose (**52**).

Following Kusumoto's synthesis of streptolidine (**15**) from D-ribose, hemiacetal **50** was synthesized without difficulty. At this stage, alternative oxidation chemistry was attempted in hopes of increasing the yield. Among the oxidative reagents tested were Dess-Martin's periodininane[17] and tetra-*n*-propylammonium perruthenate (TPAP).[18] These reagents, however, resulted in either little or no lactone **51**. It was discovered that hemiacetal **50** was insoluble or sparingly soluble in most organic solvents, including methylene chloride,

chloroform, acetonitrile, and tetrahydrofuran, which may explain the poor yield in the oxidation of **50** to **51**.

We also attempted to improve the addition of cyanogen bromide to triamine **46**. Initially, it was noted that the pH of the reaction solution gradually dropped from pH 11 to pH 6.5 and correlated with very poor yields of streptolidine. Because the pKa of a typical primary amine is between 8.5 and 9.0,[19] it was speculated that as the amines reacted with the cyanogen bromide, the resulting HBr generated would protonate the remaining amine, thus making it non-nucleophilic. Indeed, when the pH of the reaction mixture was maintained between 9.5 and 10.5, higher yields of **15** (up to 85%) were obtained (Scheme 2.7). This simple procedural modification was used in the synthesis of [*guanidino*-^{13}C]streptolidine (**56**) with greater than 98% ^{13}C enrichment in the guanidino carbon, based upon Fast Atom Bombardment Mass Spectrometry (FABMS). The ^{13}C NMR of labeled **56** clearly showed 1.9% enrichment at the guanidino carbon of streptolidine (Figure 2.1) when compared to the natural abundance spectrum of **12a**.

Scheme 2.7. Preparation of [*guanidino*-^{13}C]Streptolidine from Triamine **46**



46 **56**

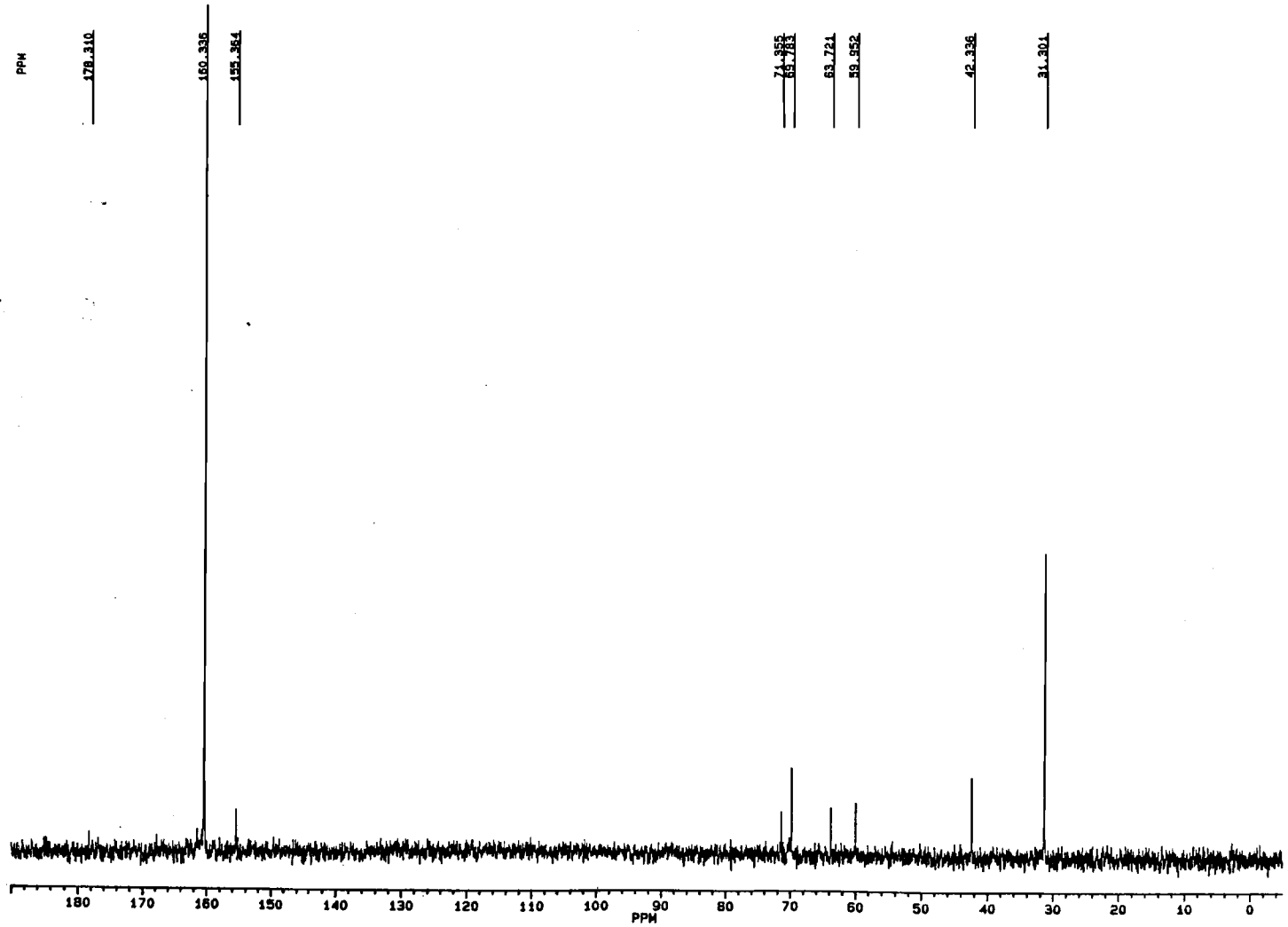


Figure 2.1. ^{13}C NMR Spectrum *of* [*guanidino*-^{13}C]Streptolidine

*Incorporation Studies Using [*guanidino-^{13}C*]Streptolidine*

Incorporation studies were performed in chemically defined O'Brein medium with slight modifications. The [*guanidino*-^{13}C]streptolidine (**61**) thus synthesized was introduced to production flasks of *S.* L-1689-23 in one pulse, twelve hours after inoculation.

The labeled streptothricin F that was produced was isolated (18 mg) using the reported procedure.[20] Based upon the intensity of the signal for C-6 (160 ppm) in the carbonyl region of the ^{13}C NMR spectra of both the control and labeled STF, a 1.9% enrichment of the guanidino resonance at 160 ppm was observed (Figure 2.2).

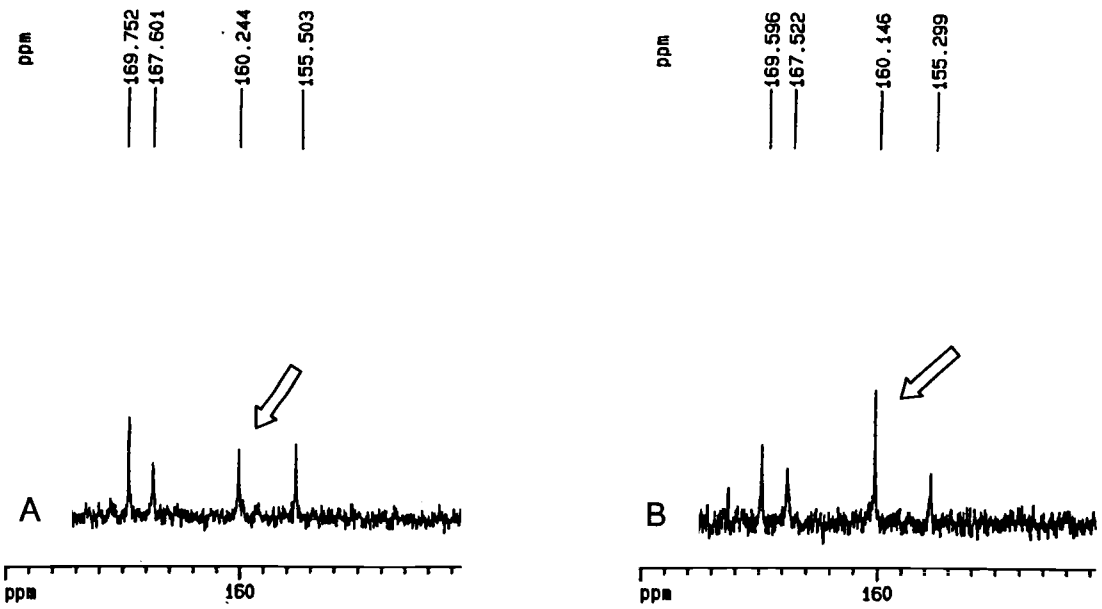
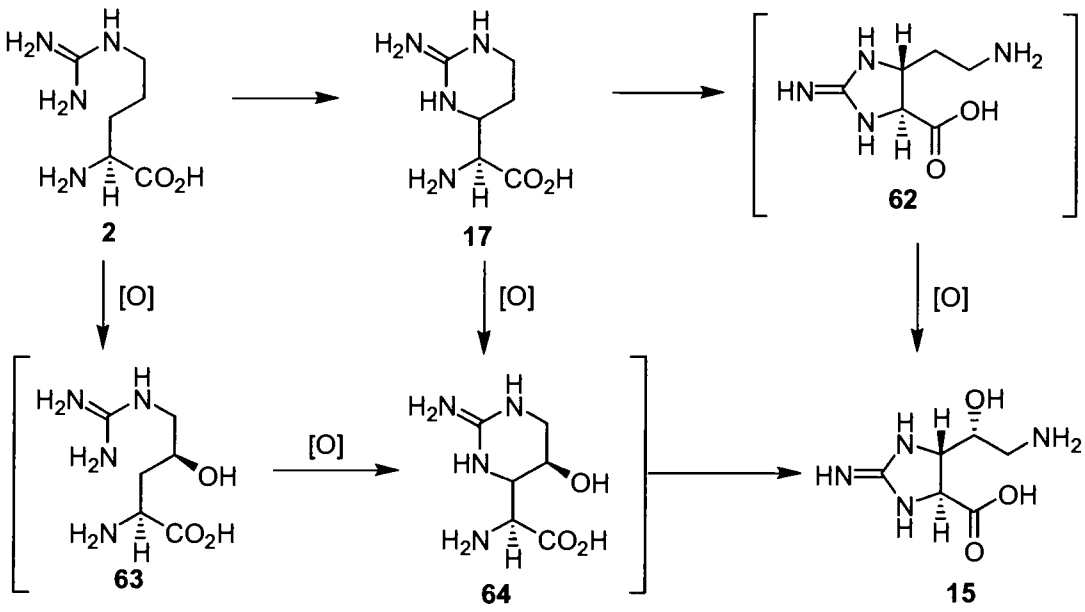


Figure 2.2. Partial ^{13}C NMR Spectra of A) STF Control and B) [*guanidino*-^{13}C]-STF From Incorporation Study

The clear enrichment of the guanidino carbon in the streptolidine moiety of streptothricin F (**12a**) confirms it as a free intermediate and provides further evidence for a convergent biosynthesis. It remains to be determined when, in the biosynthesis of **15**, formation of the secondary alcohol occurs. One possibility, as shown in Scheme 2.8, is after the initial formation of dehydroxystreptolidine (**57**). An alternative is the hydroxylation of arginine to form β-hydroxyarginine, **58**, which could then undergo the oxidative cyclization to form capreomycidine derivative **59**. The third possibility is the oxidation of capreomycidine (**17**) to form **59**, which then undergoes rearrangement to **15**. Also yet to be addressed is the timing of amide bond formation in the streptolidine moiety of STF.

Scheme 2.8. Alternate Biosynthetic Routes to Streptolidine (**15**)



Summary

[*guanidino*-^{13}C]Streptolidine was synthesized and added to production flasks of *Streptomyces lavendulae* L-1689-23. The resulting streptothricin F was purified and analyzed by both ^{13}C NMR and FABMS. From the ^{13}C NMR of the STF isolated, a 1.9% enhancement of the guanidino signal was calculated. This result clearly demonstrates that streptolidine is an intermediate in the biosynthesis of streptothricin F, and lends further support for a convergent biosynthesis.

Experimental

General

Streptomyces L-1689-23 used in the present studies was the same enhanced antibiotic producer used in prior biosynthetic studies by the Gould group. Bacillus subtilis ATCC 6633 spore suspension was obtained from Baltimore Biological Laboratories (Cockeysville, MD). Bacto agar, brain-heart infusion agar, yeast extract, and tryptose were purchased from Difco Laboratories (Detroit, MI). Beef extract was purchased from Sigma Chemical Company (St. Louis, MO). Commerical molasses and cornstarch was used.

All fermentation media were prepared with double-deionized water (Milli-Q, Millipore). Sterilization of media for use in the biosynthetic studies was performed in an AMSCO general-purpose steam powered sterilizer operating at 121 °C. Fermentation studies were carried out in a Lab-Line model 3595 gyrotatory incubator shaker having a one-inch throw and operating at 29 °C and 225 rpm. Incubations for bioassay and in the preparation of slants for short-term maintenance were done in VWR model 1520 incubators. All sterile procedures

were performed in an EdgeGARD™ hood manufactured by The Baker Company, Inc. (Sanford, ME). The paper disks used for bioassay (#740-E) were purchased from Schleicher and Schuell (Keene, NH). Ion exchange resins and LH-20 resin were purchased from Sigma Chemical Company (St. Louis, MO).

Sodium cyanide (99.99% ^{13}C enriched) was purchased from Cambridge Isotope Laboratories (Cambridge, MA). All other chemicals were purchased from Aldrich or Sigma Chemical Company and used without further purification. Anhydrous THF was obtained by refluxing over sodium metal in the presence of benzophenone. Anhydrous dimethylformamide (DMF) was obtained by first letting 500 mL stand over 4 Å molecular sieves for 24 hours. The DMF was then distilled under argon into a receiving vessel containing additional 4 Å molecular sieves. The DMF was stored under argon and used without further purification.

NMR spectra were reported on either a Bruker AM 400 or AC 300 spectrometer, where noted. Pyridine or was added as an internal chemical shift reference (δ 135.5 for ^{13}C, middle resonance for pyridine) when spectra were recorded in D_2O. Mass spectra were obtained with a Kratos MS 50 TC spectrometer. Infrared spectra were recorded on a Nicolet 5DXB FT-IR spectrophotometer.

Maintenance of S. lavendulae

S. lavendulae was maintained on yeast-malt extract agar slants, stored at 4 °C. Original slants were prepared using *S. lavendulae* spores stored on agar plugs in liquid nitrogen (-196 °C). For the generation of new slants, one agar plug was transferred into 50 mL of YME medium (consisting of 0.4% yeast extract, 1.0% malt extract, and 0.4% glucose dissolved in Milli-Q water, adjusted to pH 7.3 with 1 M KOH prior to sterilization) and this was incubated in a shaker at 29 °C and 225 rpm for 48 h. A portion of the resulting growth (0.1 mL per slant) was used to inoculate YME agar slants (15 mL, consisting of 0.4% yeast

extract, 1.0% malt extract, 0.4% glucose, and 2% Bacto agar in Milli-Q water, adjusted to pH 7.3 with 1 M KOH prior to sterilization), which were then incubated at 29 °C for seven days. After this time period, the slants showed dense pink/brown growth and were stored at 4 °C. New slants were prepared every nine months.

Seed and Production Medium

Seed medium was composed of 0.3% beef extract, 0.5% tryptose, 0.5% yeast extract, 0.1% glucose and 2.4% cornstarch in Milli-Q water. A seed medium of 100 mL was inoculated with 1 cm^2 of sporulated growth from a recently prepared slant using a sterile inoculating loop. The inoculated seed medium was incubated for 64 hrs at 29 °C and 225 rpm in a rotary shaker.

Production broth was prepared by inoculating with 8.0% (v/v) of the above seed culture to a chemically defined medium prepared in the following manner: Glycine (2.6 g), potassium phosphate-dibasic (0.2 g), and sodium acetate (1.36 g) were dissolved in 1 L Milli-Q water and to this was added 2 mL of 250 mL stock solutions containing magnesium sulfate-heptahydrate (31.22 g) and manganese sulfate-monohydrate (0.557 g), copper sulfate-pentahydrate (0.998 g) and zinc sulfate-heptahydrate (1.87 g) calcium chloride-dihydrate (4.14 g), and ferrous sulfate-heptahydrate (1.56 g). The solutions were autoclaved, and after cooling to room temperature, sterilized calcium carbonate (10 g) was added. A solution of glycerol (20 g) and glucose (2.5 g) in 20 mL Milli-Q water was then introduced via a sterile filter. Upon inoculation of the production medium, the flasks were incubated at 29 °C and 225 rpm for 48 hrs.

Streptothricin F Bioassay

The concentration of streptothricin F was determined by correlating the diameter of the zone of inhibition of growth of *Bacillus subtilis* on brain-heart infusion agar plates. A *B. subtilis* spore suspension was diluted 50-fold with 0.3% saline in 0.01% Tween 80 solution for bioassay use. To a bioassay plate (20mL brain-heart infusion agar/plate) was added 100 μL of the diluted spore suspension and spread evenly over the surface of the plate. Paper bioassay disks were evenly spaced on the surface of the plate (up to 4 disks per plate), and 50 μL of the sample to be tested was applied to each disk. The plates were incubated for fifteen hours at 35 °C, and then the diameter of the zone of inhibition was measured and correlated to a standard curve. The standard curve was prepared using known concentrations of streptothricin F.

Radial TLC of Streptothricin F

The radial TLC system used for the detection of STF (**12a**) was developed by Martinkus.[21] The apparatus consists of the bottom of a glass petrie dish, which is used as the solvent reservoir, and a glass plate with a 1.5 mm hole drilled into the center. A small diameter tube is inserted into the hole in the plate. The solvent (Pyridine/water/I-PrOH/AcOH, 15:12:5:3) is delivered, via capillary action, to the center of a 10 x 10 cm TLC plate (Whatmann aluminum-backed silica gel 60). Streptothricin F (R_f 0.46) was detected using 0.2% ethanolic ninhydrin spray.

Synthesis of [guanidino-^{13}C]Streptolidine

Methyl 2,3,5-tritosyl-β-D-ribofuranoside (35)

To a solution of D-ribose (**32**, 10.0 g, 66.7 mmol) in MeOH (200 mL) at 0 °C was added 1 mL of concentrated H_2SO_4 and the reaction stirred at 4 °C overnight (approx. 16 hrs). The solution was neutralized with amberlite IR-45 ($^-$OH form, 300-400 mesh) and the solvent removed to afford crude methyl β-D-ribose as colorless syrup (11.2 g). The crude syrup was dissolved in pyridine (150 mL) and *p*-toluenesulfonyl chloride (43.8 g, 0.23 mol) added with stirring. Upon completion of the addition, the solution was stirred at room temperature for 40 hrs. The reaction was terminated by the addition of water (100 mL) followed by extraction with CH_2Cl_2 (3 x 200 mL). The combined organic layers were washed with aqueous HCl (10% conc. HCl, 3 x 200 mL), sat. $NaHCO_3$ (3 x 200 mL), brine (1 x 200 mL) and then dried over Na_2SO_4. Filtration and evaporation of the solvent afforded a dark yellow residue (22.9 g). This residue was recrystallized from $CHCl_3$/Hexane to provide the tritosyl derivative as a light yellow solid (20.4 g, 68%). A NMR quality sample was obtained by purifying 100 mg of the material by flash chromatography (hexanes/EtOAc, 3:1, 2 x 15 cm column, detection: UV). $[\alpha]_D^{25}$ 29.0° (*c* 0.87, $CHCl_3$); IR (KBr) 1599, 1379, 1189 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C) δ (ppm) 2.45 (s, 3H), 2.46 (s, 6H), 3.82 (dd, *J* = 4.5 and 11 Hz, 1H), 4.02 (dd, J = 2.9 and 11 Hz, 1H), 4.22 (m, 1H), 4.65 (d, *J* = 4.6 Hz, 1H), 4.83 (dd, *J* = 4.6 and 7.4 Hz, 1H), 4.90 (s, 1H), 7.35 (t, *J* = 8.3 Hz, 6H), 7.67 (d, *J* = 8.2 Hz, 2H), 7.73 (d, *J* = 8.2 Hz, 2H), 7.79 (d, *J* = 8.2 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C) δ (ppm) 21.85, 21.93, 21.96, 55.51, 67.85, 74.67, 77.42, 79.27, 105.4, 128.2, 128.3, 128.4, 130.1, 130.2, 130.4, 132.1, 132.9, 145.3, 145.7, 146.1.

Methyl 3,5-diazido-2-tosyl-β-D-xylofuranoside (36)

To the tritosyl derivative **35** (20.4 g, 0.033 mol) in DMF (250 mL) was added sodium azide (7.28 g, 0.11 mol) and the mixture heated at 145 °C for 2.5 hrs. After cooling to room temperature, the mixture was filtered through celite and the solvent removed in vacuo. The syrup that was obtained was dissolved in 150 mL of hot MeOH and allowed to cool to room temperature. The crystals (monoazide, 7.8 g, 45%) were filtered off and the mother liquor evaporated in vacuo. The resulting dark orange oil (8.2 g) was purified by flash chromatography (hexanes/EtOAc, 4:1, 6 x 20 cm column, detection: UV) affording the desired product (4.3 g, 35%) as oil. The monoazide can be converted to the diazide using the same conditions as above. Monoazide: $[\alpha]_D^{25}$ 79.3° (lit.[18] $[\alpha]_D^{25}$ 80.5°) (*c* 1.36, $CHCl_3$); IR (KBr) 2104, 1598, 1366 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C) δ (ppm) 2.46 (s, 3H), 2.47 (s, 3H), 3.05 (dd, *J* = 4.7 and 13 Hz, 1H), 3.35 (s, 3H), 3.41 (dd, *J* = 2.9 and 13 Hz, 1H), 4.22 (m, 1H), 4.69 (d, *J* = 4.6 Hz, 1H), 4.84 (dd, *J* = 4.7 and 7.8 Hz, 1H), 4.96 (s, 1H), 7.36 (t, *J* = 8.7 Hz, 4H), 7.70 (d, *J* = 8.3 Hz, 2H), 7.82 (d, *J* = 8.3 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C) δ 42.1, 42.2, 42.7, 57.0, 66.8, 68.6, 74.9, 102.6, 128.1, 128.2, 128.4, 128.5, 128.6, 135.4, 136.5, 156.5, 161.5. Diazide **36**: $[\alpha]_D^{25}$ -53.9° (*c* 2.86, Et_2O); IR (neat) 2108, 1597, 1379 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, 25 °C) δ 2.48 (s, 3H), 3.32 (s, 3H), 3.36 (dd, *J* = 5.3 and 13 Hz, 1H), 3.49 (dd, *J* = 7.5 and 12.8 Hz, 1H), 4.18 (dd, *J* = 2.6 and 6.3 Hz, 1H), 4.40 (m, 1H), 4.77 (m, 1H), 4.86 (s, 1H), 7.41 (d, *J* = 8.3 Hz, 2H), 7.84 (d, *J* = 8.5 Hz, 2H); ^{13}C NMR (75.5 MHz, $CDCl_3$, 25 °C) δ (ppm) 21.6, 51.7, 55.9, 64.9, 79.4, 86.2, 106.8, 128.1, 130.3, 132.4, 146.0; MS (CI): *m/z* 367.0 (M - 1); HRMS (CI): calcd for $C_{13}H_{15}N_6O_5S$ 367.0825, obsd 367.0822.

Methyl 5-benzyloxycarbamido-2,3-benzyloxycarbonyl-azido-2,3,5-trideoxy-β-D-lyxofuranoside (47)

To a suspension of lithium aluminum hydride (2.12 g, 54 mmol) in dry THF (20 mL) at 0 °C was slowly added a solution of the diazide **36** (4.3g, 11.2 mmol) in dry THF (25 mL) via cannula. The reaction mixture was stirred at 0 °C for 1.5 hrs and then refluxed for an additional 2 hrs. After cooling to room temperature, the excess lithium aluminum hydride was destroyed by the slow addition of saturated Na_2SO_4 at 0 °C. The aluminum salts were filtered off and washed with EtOH (3 x 100 mL) and Et_2O (3 x 100 mL). The washings were combined and the solvents removed in vacuo. The resulting light yellow residue was dissolved in 100 mL of EtOH and cooled to 0 °C. To the ice-cold solution was added Et_3N (3.9 mL, 28 mmol) followed by the slow addition of benzyl chloroformate (4.0 mL, 28 mmol) over 30 min. The mixture was stirred at 0 °C for 0.5 hours followed by 2.5 hrs at room temperature. Water (150 mL) was then added and the resulting white crystals were filtered off and dried to afford 3.1 g (69%) of the desired product. $[\alpha]_D^{25}$ -93.1° (*c* 0.8, $CHCl_3$); IR (KBr) 3311, 1717, 1694, 1552 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, 25 °C) δ (ppm) 3.24 (dd, *J* = 1.8 and 4.9 Hz, 1H), 3.35 (dd, *J* = 1.0 and 5.0 Hz) 3.46 (m, 1H), 3.49 (s, 3H), 3.61 (m, 1H), 4.05 (t, *J* = 4.6 Hz, 1H), 4.96 (d, *J* = 1.0 Hz, 1H), 5.10 (m, 4H), 5.34 (t, *J* = 5.0 Hz, 1H) 7.32 (s, 5H), 7.33 (s, 5H); ^{13}C NMR (75.5 MHz, $CDCl_3$, 25 °C) δ (ppm) 42.0, 42.2, 42.7, 57.1, 66.8, 68.6, 75.0, 102.6, 128.1, 128.2, 128.4, 128.5, 128.6, 135.4, 136.5, 156.5, 161.5; MS (CI): *m/z* 412.2 (M); HRMS (CI): calcd for $C_{22}H_{24}N_2O_6$ 412.1634, obsd 412.1632.

Methyl 3-azido-2,5-dibenzyloxycarbamido-2,3,5-trideoxy-β-D-arabionfuranoside (48)

To a solution of the Cbz-protected aziridine **47** (3.1 g, 7.5 mmol) in 50 mL anhydrous DMF was added sodium azide (2.4 g, 37.5 mmol) and the mixture stirred at 90 °C for 1 hour under argon atmosphere. After cooling, the excess sodium azide was filtered off and the solvent removed in vacuo. The resulting residue was purified by flash chromatography (hexanes/EtOAc, 2:1, 4 x 18 cm column, detection: UV) to afford 1.6 g (47%) of the desired product as a white solid. A total of 1.1 g of the undesired 2-azido isomer (**48b**) was also obtained. (**48a**) $[\alpha]_D^{25}$ –26.8° (*c* 2.20, EtOAc); IR (KBr) 3326 (br), 2110, 1693 (br) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C) δ (ppm) 3.39 (s, 3H), 3.45 (m, 2H), 3.69 (t, *J* = 8.8 Hz, 1H), 3.90 (dd, J = 4.6 and 12.4 Hz), 4.35 (ddd, *J* = 4.8 and 10.0 Hz, 1H), 4.78 (d, *J* = 4.6 Hz, 1H), 5.00 (bs, 1H), 5.16 (m, 5H), 7.34 (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C) δ (ppm) 43.8, 55.5, 58.9, 65.5, 67.1, 67.4, 80.2, 101.6, 128.4, 128.5, 128.6, 128.7, 136.2, 136.5, 155.9, 156.8; MS (FAB): *m/z* 456.2 (M + 1); HRMS (FAB): calcd for $C_{22}H_{26}N_5O_6$ 456.1883, obsd 456.1891. (**48b**) $[\alpha]_D^{25}$ –25.2° (*c* 1.85, EtOAc); IR (KBr) 3328 (br), 2109, 1696 (br) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C) δ (ppm) 7.36 (m, 10H), 5.66 (bd, *J* = 9.5 Hz, 1H), 5.25 (bs, 1H), 5.12 (m, 4H), 4.83 (s, 1H), 4.40 (m, 1H), 4.33 (dd, *J* = 6.0 and 11.5 Hz, 1H), 3.86 (s, 1H), 3.56 (m, 1H), 3.37 (s, 3H), 3.26 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C) δ (ppm) 156.6, 156.0, 136.0, 128.8, 128.6, 127.7, 106.7, 80.6, 69.7, 67.6, 67.0, 55.7, 41.5; MS (FAB): *m/z* 456.2 (M + 1); HRMS (FAB): calcd for $C_{22}H_{26}N_5O_6$ 456.1883, obsd 456.1891.

Methyl 2,3,5-tribenzyloxycarbamido-2,3,5-trideoxy-β-D-arabinofuranoside (49)

To a solution of the monoazide **48** (1.6 g, 3.5 mmol) and conc. HCl (1.05 mL) in 40 mL MeOH was added Pd/C (10%, 168 mg) and the resulting mixture stirred at room temperature under hydrogen atmosphere overnight. The catalyst was then filtered off and an additional 50 mL MeOH was added. To the solution was added triethylamine (2.4 mL, 17.5 mmol), the mixture was cooled to 0 °C, and then benzyl chloroformate (2.5 mL 17.5 mmol) added slowly over 30 minutes followed by stirring at room temperature for an additional 1 hour. Water (50 mL) was then added and the white precipitate was filtered off to afford 1.15 g (58%) of the desired product as a white solid. $[\alpha]_D^{25}$ –17.9° (*c* 2.01, $CHCl_3$); IR (KBr) 3409 (br), 1699 (br) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, 25 °C) δ (ppm) 7.32 (m, 15H, 5.50 (bd, 1H), 5.32 (bd, 1H), 5.07 (m, 6H), 4.76 (d, *J* = 4.1 Hz, 1H), 4.23 (m, 1H), 3.91 (m, 2H), 3.53 (m, 1H), 3.39 (m, 1H), 3.31 (s, 3H), 3.08 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C) δ (ppm) 157.2, 156.7, 136.9, 136.2, 128.8, 128.7, 128.6, 128.5, 128.4, 128.3, 128.2, 100.8, 81.6, 77.4, 67.5, 67.4, 66.8, 58.0, 55.3, 46.0, 44.5; MS (FAB): *m/z* 550.2 (M + 1); HRMS (FAB): calcd for $C_{29}H_{32}N_3O_8$ 549.2111, obsd 549.2118.

2,3,5-Tribenzyloxycarbamido-2,3,5-trideoxy-D-arabinofuranoside (50)

To a solution of the acetal **49** (1.15 g, 2.1 mmol) in 50 mL dioxane was added 50 mL of 2 M *p*-toluenesulfonic acid and the mixture heated under reflux for 2 hrs. The mixture was concentrated in vacuo to one-half the original volume and extracted with CH_2Cl_2 (3 x 100 mL). The combined organic layers were washed with sat. $NaHCO_3$ (3 x 100 mL), brine (1 x 100 mL), and dried over sodium sulfate. Filtration and removal of the solvent afforded a yellow residue which was purified by flash chromatography (CH_2Cl_2/EtOAc, 2:1, 4 x 18 cm

column, detection: UV) to obtain the desired hemiacetal (425 mg, 39%) as well as 400 mg of recovered starting material. $[\alpha]_D^{25}$ –19.2° (*c* 1.98, 1,4-dioxane); IR (neat) 3504 (br), 1694 (br) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, 25 °C) δ (ppm) 7.34 (m, 15H), 5.60 (m, 2H), 5.34 (d, *J* = 8.0 Hz, 1H), 5.09 (m, 6H), 4.78 (d, *J* = 4.5 Hz, 1H), 4.24 (ddd, *J* = 4.5, 4.5 and 4.7 Hz, 1H), 3.90 (m, 2H), 3.58 (ddd, J = 3.4, 4.8 and 5.8 Hz, 1H), 3.39 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C) δ (ppm) 157.2, 156.8, 136.9, 136.2, 128.8, 128.7, 128.5, 128.4, 128.2, 100.9, 81.6, 77.4, 67.5, 67.4, 66.8, 58.0, 55.3, 44.5; MS (FAB): *m/z* 536.2 (M + 1); HRMS (FAB): calcd for $C_{28}H_{30}N_3O_8$ 536.1955, obsd 536.1950.

2,3,5-Tribenzyloxycarbamido-2,3,5-trideoxy-D-arabino-1,4-lactone (51)

To a solution of the hemiacetal **50** (425 mg, 0.8 mmol) in acetic acid (35 mL) was added a drop of concd H_2SO_4 and CrO_3 (80 mg, 0.8 mmol) in $AcOH/H_2O$ (4:1, 2 mL). The mixture was stirred at room temperature for 1.5 hrs, then water (25 mL) was added and the mixture extracted with CH_2Cl_2 (3 x 50 mL). The combined organic layers were washed with sat. $NaHCO_3$ (3 x 50 mL), brine (1 x 50 mL) and dried over sodium sulfate. Filtration and removal of the solvent afforded a residue that was purified by flash chromatography (CH_2Cl_2/EtOAc, 2:1, 3 x 18 cm column, detection: UV) to give 210 mg (48%) of the corresponding lactone. An additional 200 mg of recovered starting material was also obtained. $[\alpha]_D^{25}$ -18.5° (*c* 1.88, 1,4-dioxane); IR (neat) 3321 (br), 1768 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C) δ (ppm) 7.36 (m, 15H, 5.51 (bd, 1H), 5.01 (m, 6H), 4.73 (d, *J* = 4.1 Hz, 1H), 4.19 (m, 1H), 4.01 (m, 2H), , 3.35 (m, 1H), 3.12 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$, 25°C) δ 7.11 (m, 15H), 7.01 (d, *J* = 8.7 Hz, 1 H), 5.41 (m, 1H), 5.34 (m, 6H), 5.11 (d, *J* = 6.2 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C) δ (ppm) 172.0, 157.5, 157.4, 157.2, 127.6, 127.5, 127.4, 127.3,

86.1, 70.2, 70.5, 69.9, 63.0, 49.5, ; MS (FAB): *m/z* 534.2 (M + 1); HRMS (FAB): calcd for $C_{28}H_{28}N_3O_8$ 534.1798, obsd 534.1796.

2,3,5-Triamino-2,3,5-trideoxy-D-arabino-1,4-lactone (46)

To a solution of the protected triamine **51** (210 mg, 0.4 mmol) in 5 mL AcOH/HBr (sat. solution) was added anisole (0.15 mL) and the solution allowed to stand at room temperature for 1.5 hours. Anhydrous ether (25 mL) was then added and crystals of the deprotected triamine were filtered off to afford 120 mg (77%) of the desired product as a white hygroscopic powder. $[\alpha]_D^{25}$ -18.5° (*c* 1.87, H_2O); IR (neat) 3415 (br), 1789 cm^{-1}; ^1H NMR (300 MHz, MeOH-d4, 25 °C) δ (ppm) 5.23 (m, 1H), 5.09 (d, J = 10 Hz, 1 H), 4.61 (m, 1H), 3.81 (m, 1H), 3.53 (m, 1H); ^{13}C NMR (75 MHz, MeOH-d4, 25 °C) δ (ppm) 167.6, 68.1, 53.3, 52.7, 44.2; MS (FAB): *m/z* 146.1 (M + 1); HRMS (FAB): calcd for $C_5H_{12}N_3O_2$ 146.0930, obsd 146.0936.

[guanidino-^{13}C]Streptolidine (56)

The hydrobromide salt of the unprotected triamine **46**(60 mg, 0.15 mmol) was dissolved in 6 mL water and the pH was adjusted to 11.0 by the addition of 1M NaOH and then left to stand at room temperature for 2 hours. A solution of $Br^{13}CN$ (85 mg, 0.8 mmol) in 3 mL water was added dropwise over 2.5 hours while maintaining a pH of 10.5 – 11.0. The mixture was stirred for an additional 30 minutes and then evaporated to dryness. The residue was dissolved in 15 mL of 6 M HCl and refluxed for 2 hours. Removal of the solvent afforded a dark brown residue that was dissolved in a small amount of water and loaded onto an Ag 50-X8 (NH_4^+ form, 100-200 mesh, 1.5 x 20 cm) cation exchange column. The

column was washed with water (400 mL) and then eluted with 0.2 M NH_4OH. Fractions of 10 mL were collected and ninhydrin positive fractions that contained only streptolidine (as compared to standard) were pooled and the solvent removed to afford 6 mg of the desired compound. ^1H NMR (400 MHz, D_2O, 25 °C) δ (ppm) 4.45 (t, J = 4.3 Hz, 1H), 4.23 (m, 2H), 3.38 (dd, J = 3.8 Hz and 9.1 Hz, 1 H), 3.26 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C) δ (ppm) 178.3, 160.3, 69.8, 63.7, 60.0, 42.3, ; MS (FAB): *m/z* 190.1 (M + 1); HRMS (FAB): calcd for $C_5{}^{13}CH_{13}N_4O_3$ 190.1022, obsd 190.1011.

Incorporation Study Using [guanidino-^{13}C]Streptolidine (56), First Experiment

To each of two flasks containing 100 mL of chemically defined fermentation medium were added 7 mg of [*guanidino*-^{13}C]streptolidine in 2 mL Milli-Q water via passage through a sterile filter. The addition of material was performed twelve hours after inoculation. The production flasks were incubated for an additional 32 hours.

After 48 hours from initial inoculation, the production broth was filtered over celite. The production flasks and filter cake were washed with additional 50 mL Milli-Q water and the total volume recorded. A small sample was saved for bioassay studies. The filtrate was passed through a column of Amberlite IRC-50 cation exchange resin (K^+ form, 16-50 mesh, 1.5 x 19 cm column) at a flow rate of 1.0 mL/min. Once all of the filtrate had been loaded onto the column, the column was washed with Milli-Q water (100 mL). After collection of the effluent and water wash as one large fraction, the volume was measured and a sample saved for bioassay. The column was eluted with 0.3 M HCl, taking 10 mL fractions at a flow rate of 1.0 mL/min. Ninhydrin-positive fractions were pooled, the pH adjusted to 6.8 using 2% KOH, the volume measured, and an aliquot saved for bioassay prior to lyophilization.

The white powder resulting from freeze-drying (approx. 0.6 g) was transferred to a 40 mL centrifuge tube and extracted with 10 mL portions of methanol until the extracts were ninhydrin negative (typically 8 extractions were necessary). After evaporation of the methanol, the material was dissolved in a small amount of water and loaded onto a column of LH-20 gel filtration resin (1 x 30 cm column) with a flow rate of 1.0 mL/min. The column was washed with water, collecting 3 mL fractions. Those fractions that were ninhydrin-positive were analyzed by TLC (pyridine/H_2O/*n*-PrOH/AcOH, 15:12:5:3; detection: 0.2% ninhydrin). Fractions containing only STF (R_f 0.46) were pooled and the pH adjusted to 6.8 using 2% KOH prior to lyophilization. From this procedure, 18 mg of STF was obtained, which was analyzed by ^{13}C NMR.

Incorporation Study Using [guanidino-^{13}C]Streptolidine (56), Second Experiment

Using an additional 22 mg of [*guanidino*-^{13}C]streptolidine, a second incorporation study was performed as described previously. From a total of 200 mL of production medium was obtained 55 mg of antibiotic. The antibiotic was analyzed by ^{13}C NMR spectroscopy and a 2.2% enrichment of the guanidino carbon of **12a** was observed.

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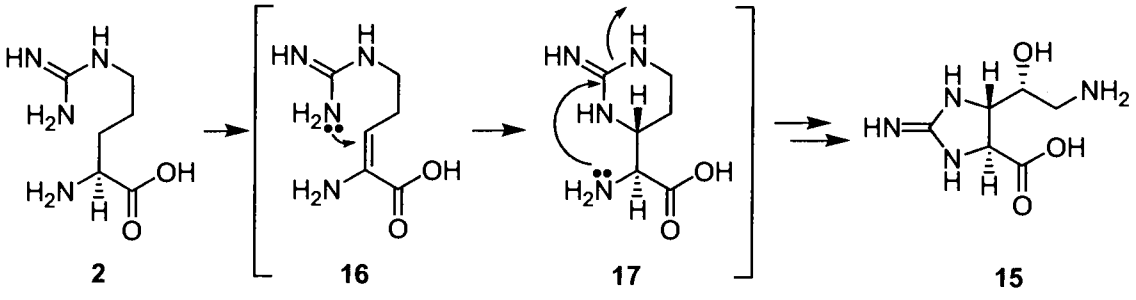
Chapter 3
Testing the Intermediacy of Capreomycidine in the Biosynthesis of Streptothricin F

Introduction

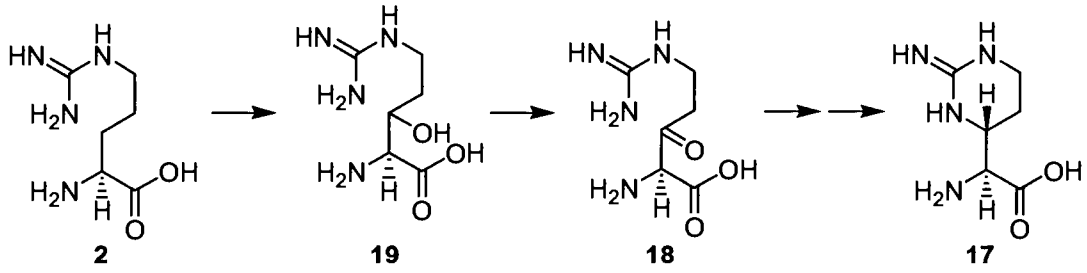
During the conversion of arginine (**2**) to the streptolidine moiety of streptothricin F, at least two separate oxidations are required. In early speculation on the biogenesis of the streptolidine moiety in streptothricin F, Bycroft and King proposed the intermediacy of capreomycidine (**17**) and arginine (**2**, Scheme 3.1).[1] In their studies on streptolidine formation, Gould and co-workers used ^{13}C- and ^{15}N-labeled arginine to support this hypothesis.[2,3] Although capreomycidine had not been determined as an intermediate, several experiments were designed to address this issue. One such experiment was an incorporation study using labeled β-hydoxy-L-arginine.[4] While there was no observable incorporation of β-hydroxy-L-arginine into streptothricin F (**12a**), the result does not exclude the intermediacy of such a compound.

Scheme 3.1. Biogenesis of the Streptolidine (**15**) Portion of Streptothricin F From Arginine (**2**)



Based upon the observed loss of deuterium labels at C-2 and C-3 of arginine (cf. Scheme 1.10),[4] the most likely explanation is in the intermediacy of 2-keto-L-arginine (**19**) which would have originated from the oxidation of 2-hydroxy-L-arginine (**18**, Scheme 3.2). β-Hydroxyarginine (**19**), however, was not incorporated into the streptolidine moiety of STF and isotope trapping experiments failed to confirm the intermediacy of **18**. If these intermediates were enzyme-bound, it could explain why no incorporation of labeled **19** was observed in STF (**12a**) and why **18** was not isolated. If capreomycidine can be identified as an intermediate, further experiments can be designed to address the conversion of arginine into **17**. To determine if **17** is synthesized *in vitro* from arginine, cell-free extracts (CFE) of *S.* L-1689-23 would be generated and an assay developed to test for enzyme activity related to the formation of **17**.

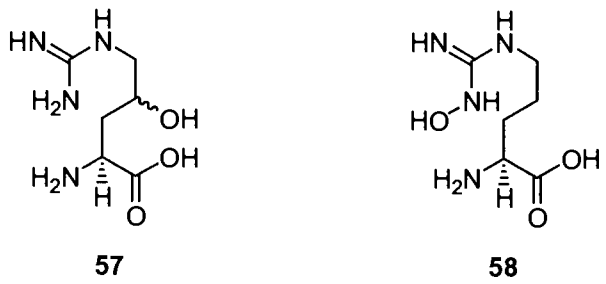
Scheme 3.2. Proposed Oxidation Pathway to Capreomycidine (**17**)



Examples of Hydroxylated Amino Acids

β-Hydroxyamino acids are very prevalent in nature and have been isolated from a variety of sources. In the thesis of John Wityak, an extensive table of naturally occurring β-hydroxyamino acids has been compiled,[5] and therefore, will not be discussed in detail here. Surprisingly, there are no reports on the isolation of β-hydroxyarginine from any source. There are a few examples, however, of

the isolation of both γ-hydroxyarginine (**57**, Figure 3.1) and N^{ω}-hydroxyarginine (**58**). With respect to γ-hydroxyarginine, it has been identified as a constituent in the antibiotic K-582, which is produced by the fungus *Metarrhizium anisopliae*.[6] Amino acid **57** is also a free amino acid found in lentil seed (*Lens culinaris Med.*),[7] and a constituent of polyphenolic proteins isolated from the adhesive plaques of the marine mussel *Mytilus edulis*.[8] N^{ω}-Hydroxyarginine (**58**) is an intermediate in the formation of nitric oxide in mammalian tissues[9] and is synthesized from arginine via the enzyme nitric oxide synthase.[10] Neither **58** nor nitric oxide synthase have ever been isolated from non-mammalian sources.



57 **58**

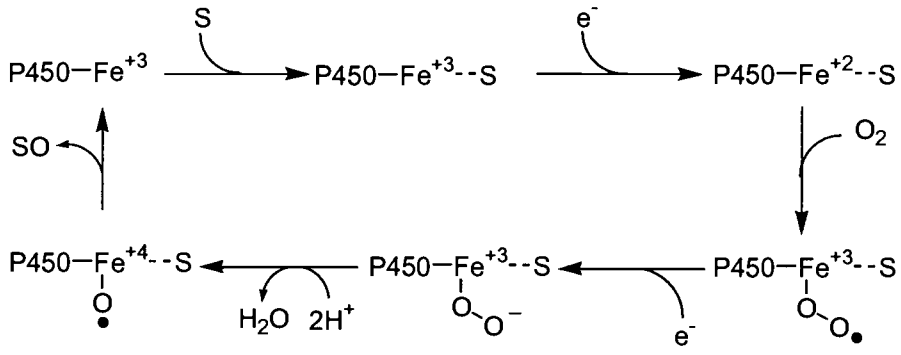
Figure 3.1. Hydroxylated Arginine Derivatives Found in Nature

Examples of Biological Oxidations

Many enzymes catalyze oxidations that are required in the formation of primary and secondary natural products. One of the most abundant classes of oxidases is the cytochrome P-450s and several cytochrome P-450-mediated reactions have been reported from *Streptomyces* species and other Actinomycetes.[11] Most bacterial cytochrome P-450s contain a heme-iron complex and require NAD(P)H for activity. Cytochrome P-450 catalyzed oxidations are initiated by binding of substrate (S), which occurs prior to binding of oxygen (Scheme 3.3). Two reducing equivalents are sequentially transferred

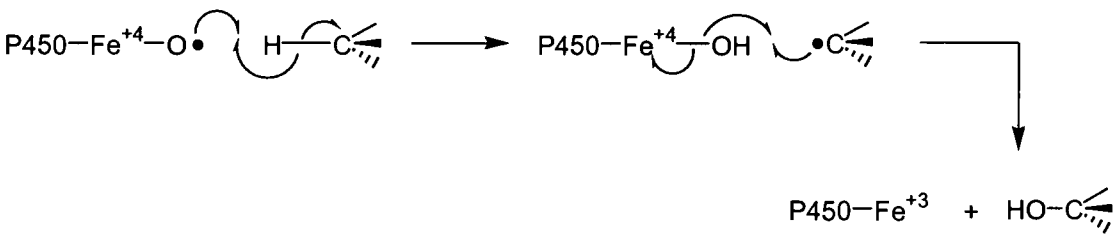
to the heme iron via a flavin nucleotide, ferredoxins, and/or cytochrome b_5. These reducing equivalents reduce one atom from molecular oxygen to water, while the second oxygen atom is incorporated into the substrate.

Scheme 3.3. Proposed Catalytic Cycle of Cytochrome P-450 Monooxygenases



The currently accepted mechanism by which cytochromes P-450 oxidize a C-H bond is represented in Scheme 3.4.[12] Upon formation of the iron(IV) O-porphyrin radical complex, abstraction of a hydrogen atom occurs to form the corresponding carbon radical. Transfer of hydroxy radical back to the substrate completes the reaction.

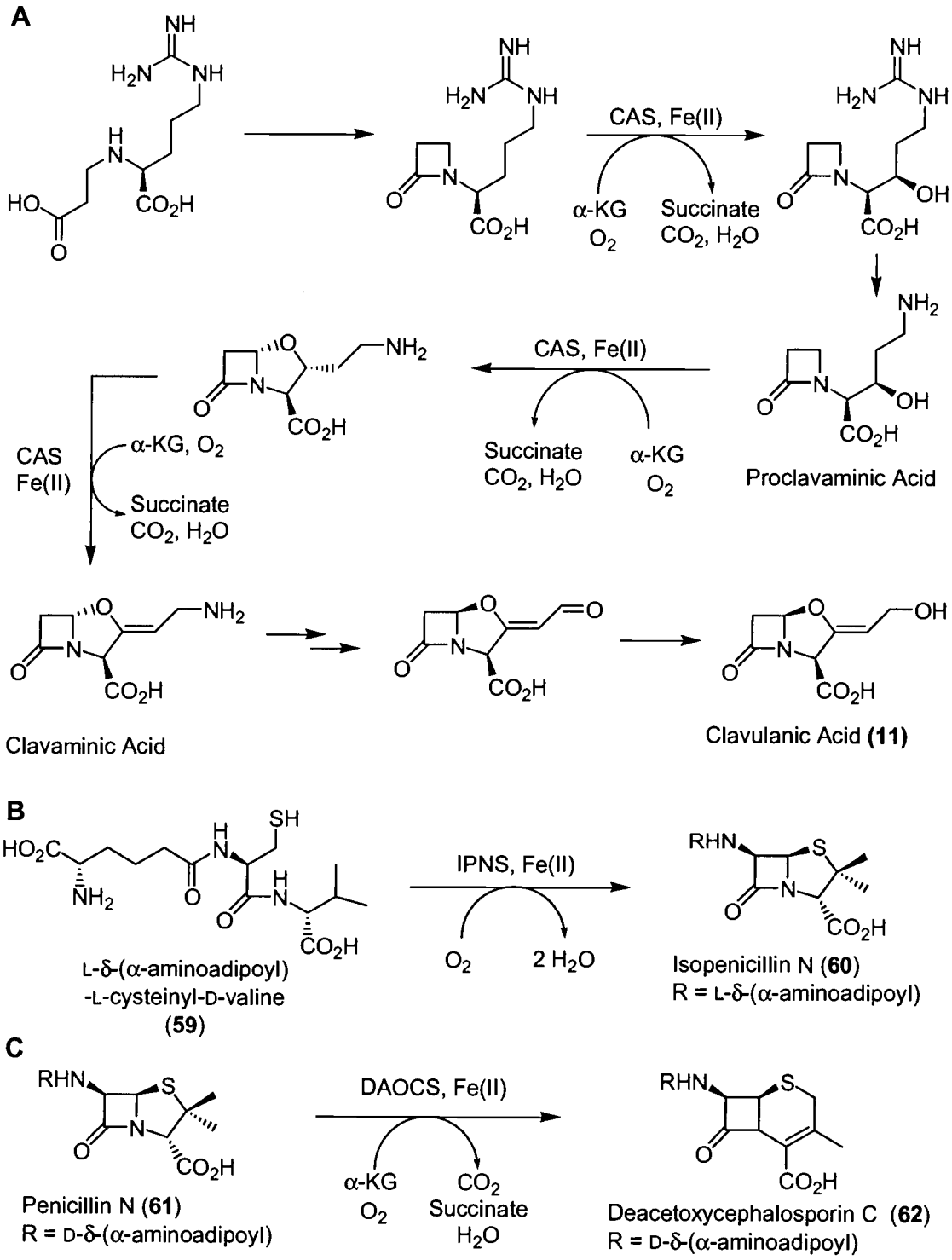
Scheme 3.4. Proposed Mechanism for Cytochrome P-450 Mediated Hydroxylation at a Saturated Carbon



Cytochromes P-450 catalyze the oxidation of activated and unactivated carbon centers and are capable of catalyzing more than one reaction in a biosynthetic sequence. The recombinant cytochrome P-450 DoxA, isolated from *Streptomyces* sp. strain C5 has recently been shown to catalyze three distinct reactions in the biosynthesis of doxorubicin; two separate hydroxylations, and further oxidation of one of the hydroxy groups to a ketone.[13]

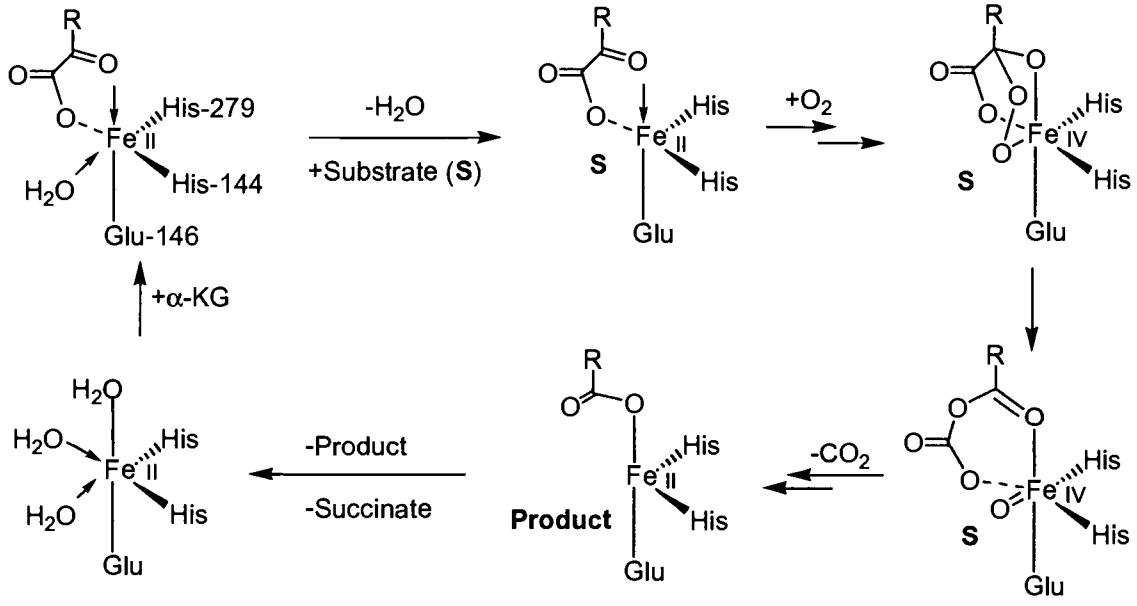
A special class of monooxygenases has been characterized over the last several years that contain an iron center, but are not heme proteins. These enzymes typically require α-ketoglutarate and molecular oxygen for activity, generating the oxidized substrate (product), carbon dioxide and succinate. One example is clavaminate synthase (CAS). Clavaminate synthase catalyzes three distinct reactions in the biosynthesis of the β-lactamase inhibitor, clavulanic acid (**11**, Scheme 3.5).[14] Other examples of this class of monooxygenases include IsoPenicillin N Synthase (IPNS), which catalyzes the oxidative cyclization of L-δ-(α-Aminoadipoyl)-L-Cysteinyl-D-Valine (ACV, **59**) to isopenicillin N (**60**),[15-17] and deacetoxycephalosporin C synthase (DAOCS), which catalyzes the ring expansion of penicillin N (**61**) to deacetoxycephalosporin C (**62**).[18-20]

Scheme 3.5. Examples of Non-Heme Monooxygenases Utilized in the Biosynthesis of A) Clavulanic Acid, B) Isopenicillin N, and C) Deacetoxycephalopsorin C



Clavaminate synthase has been purified and characterized from *S. clavuligerus* and shown to exist as two functional isozymes with 87% sequence identity.[19] Clavaminate synthase has also been isolated from *S. antibioticus*.[21] The genes that code for the two isozymes from *S. clavuligerus* have been cloned and over-expressed in *Escherichia coli*.[22] Recently, the crystal structure of CAS in complex with Fe(II), α-ketoglutarate, and the substrates N-α-acetyl-L-arginine or proclavaminic acid has been reported and provided further insight into this versatile monooxygenase.[23] A generalized mechanism for the oxidation of a substrate by CAS is shown in Scheme 3.6.

Scheme 3.6. Proposed Mechanism for the CAS Catalytic Cycle[23]



Because clavaminate synthase (CAS) oxidizes an arginine derivative at an unactivated carbon center, the idea that an enzyme like CAS may be involved in the biosynthesis of streptolidine (**15**) was very appealing. Reported enzyme assay conditions using purified CAS can be modified to generate enzyme assays

from cell-free extracts of *S.* L-1689-23. Using [U-^{14}C]arginine, the cell-free extracts (CFE) can be probed for enzyme activity involved in the biosynthesis of **12a**. This would be a simple method for assessing the intermediacy of capreomycidine (**17**), as well as isolating potential biosynthetic intermediates, in the formation of streptothricin F.

Results and Discussion

Generation of Cell-Free Extracts of S. L-1689-23 and Analysis of Enzyme Activity Using a TLC Linear Analyzer

If a non-heme monooxygenase, similar to that of clavaminate synthase (CAS) is involved in the oxidation of arginine (**2**) to streptolidine (**15**), an established assay for CAS activity would hopefully permit detection of intermediates in the biosynthesis of streptolidine/streptothricin F. The assay conditions employed by Salowe et al.[19] and Lawlor et al.[24] are very straightforward, and were employed in the generation of CFEs from *S.* L-1689-23 with only slight modifications.

Based upon STF production profiles, the onset of antibiotic biosynthesis occurred at approximately twelve hours after initial inoculation of the production medium. The mycelia from 12 hour-old production flasks were harvested via centrifugation, re-suspended in buffer, and sonicated over ice. Removal of the cellular debris by centrifugation afforded a crude extract. An aliquot of this extract was incubated with iron (II) sulfate, dithiothreitol (DTT), sodium ascorbate, α-ketoglutarate (α-KG), and arginine (containing 0.016 μCi [U-^{14}C]arginine). After termination of the assays, a sample (approximately 60 μL) was initially developed by radial thin-layer chromatography (TLC) as described in Chapter 2. For the detection of radioactivity, the TLC plate was analyzed using a TLC-linear analyzer. The initial results showed two separate radioactive compounds, one

having an R_f of 0.75 and the other with an R_f of 0.50. Using [U-^{14}C]arginine as a standard, the high R_f component was identified as arginine. The other compound had an R_f close to that of capreomycidine, which had also been used as a standard. The low-R_f compound was absent from those assays that had been boiled prior to the addition of substrate, cofactors, and incubation. This demonstrated that the low-R_f compound was dependant upon active enzyme for synthesis and suggested it was capreomycidine.

The quantitation of radioactivity in a given spot could not be determined using the TLC-linear analyzer. Therefore, to determine the amount of radioactivity present in each compound, the compound and silica support was removed from the aluminum-backed TLC plate and analyzed by liquid scintillation counting (LSC). Approximately 3000 dpm was found in the high R_f spot (arginine, Table 3.1) and 1200 dpm in the low Rf spot (unknown).

Table 3.1. Conditions Used in Determining Initial Enzyme Activity

	Assay Number			
Component	2	3	4	5
DTT (1mM)	✓	✓	✓	✓
Na Ascorbate (1.5 mM)	✓	✓	✓	✓
$FeSO_4$ (45 μM)	✓	✓	✓	✓
α-KG (1 mM)	✓	✓	✓	✓
α-KG (2 mM)		✓	✓	
[U-^{14}C]Arginine (1.0 mM)	✓	✓	✓	✓
Incubation Time	24 h	24 h	24 h	24 h
Boiled Control	✓	✓		
High R_f Compound (dpm x 100)	40	40	30	29
Low R_f Compound (dpm x 100)	0	0	12	12

In the next series of assays, the effect of increased α-KG upon the formation of the low-R_f compound as well as incubation time was examined. Table 3.2 lists the assay numbers and components/conditions used for each assay.

Table 3.2. Conditions Used in Determining Dependency of α-Ketoglutarate and Incubation Time on the Formation of the Low-R_f Compound

	Assay Number							
Component	1	2	3	4	5	6	7	8
DTT (1mM)	✓	✓	✓	✓	✓	✓	✓	✓
Na Ascorbate (1.5 mM)	✓	✓	✓	✓	✓	✓	✓	✓
$FeSO_4$ (45 μM)	✓	✓	✓	✓	✓	✓	✓	✓
α-KG (1 mM)	✓	✓			✓		✓	✓
α-KG (2 mM)			✓	✓		✓		
[U-^{14}C]Arginine (1.0 mM)	✓	✓	✓	✓	✓	✓	✓	✓
Incubation Time	12 h	24 h	12 h	24 h	12 h	24 h	12 h	24 h
Boiled Control					✓	✓		
Low R_f Compound	✓	✓	✓	✓			✓	✓

Based upon the results from the TLC analysis, the low-R_f radioactive compound was present in all of the assays. These results indicated that the duration of the incubation did not affect the presence of the low-R_f compound in the assays. Assays #5 and #6 (boiled controls) showed only the presence of arginine.

Because the procedure used to grow *S.* L-1689-23 and generate the CFE was time-consuming, the next set of experiments examined ways of cutting the time needed to obtain a CFE while maintaining enzyme activity. To determine whether CFE could be used from harvested cells that had been stored at –80 °C, assays were conducted as represented in Table 3.2. CFE stored at either 4 °C or –25 °C (Table 3.3, Assays 3-6) was also examined for enzyme activity.

Table 3.3. Conditions Used in Determining Viability of CFE or Harvested Cells at Various Temperatures

	Assay Number					
Component	1	2	3	4	5	6
DTT (1mM)	✓	✓	✓	✓	✓	✓
Na Ascorbate (1.5 mM)	✓	✓	✓	✓	✓	✓
$FeSO_4$ (45 μM)	✓	✓	✓	✓	✓	✓
α-KG (1 mM)	✓	✓	✓	✓	✓	✓
CFE from Cells (-80 °C)	✓	✓				
CFE stored at 4 °C			✓	✓		
CFE stored at -25 °C					✓	✓
[U-^{14}C]Arginine (1.0 mM)	✓	✓	✓	✓	✓	✓
Incubation Time	12h	12h	12h	12h	12h	12h
Boiled Control	✓		✓		✓	
Low R_f Compound		✓				

The results from the analysis of assays #4 and #6 showed no formation of the low-R_f compound, while assay #2 contained the low-R_f compound, albeit in smaller amounts than what had been observed using freshly harvested cells. These results suggested that newly harvested mycelia should be used as the source of the CFE

In the last series of assays, the effect of the iron chelator ethylenediaminetetraacetic acid (EDTA) upon addition to the assay, or the absence of cofactors on the formation of the low R_f compound was examined. EDTA was added to determine if the enzyme might contain a non-heme iron in the active site. Table 3.4 lists the components and/or conditions used for each assay.

Table 3.4. Conditions Used in Determining Dependency of α-Ketoglutarate or Addition of EDTA on the Formation of the Low-R_f Compound

	Assay Number							
Component	1	2	3	4	5	6	7	8
DTT (1mM)	✓	✓		✓	✓	✓	✓	✓
Na Ascorbate (1.5 mM)	✓	✓	✓		✓	✓	✓	✓
$FeSO_4$ (45 μM)	✓	✓	✓	✓		✓		
α-KG (1 mM)	✓	✓	✓	✓	✓		✓	✓
EDTA (4 mM)							✓	
EDTA (8 mM)								✓
[U-^{14}C]Arginine (1.0 mM)	✓	✓	✓	✓	✓	✓	✓	✓
Incubation Time	12 h	12 h	12 h	12 h	12 h	12 h	12 h	12 h
Boiled Control	✓							
Low R_f Compound		✓	✓	✓	✓		✓	✓

When the assays were developed by radial TLC and analyzed, it was observed that all of the assays, with the exception of assays #1 and #6, contained the low R_f compound. In assays #7 and #8, where ferrous sulfate was omitted and EDTA added, the amount of the low R_f compound appeared to be much smaller (data not shown) than that present in the other assays. These findings strongly suggested that the formation of the low R_f compound was dependant upon the addition of α-KG to the assay. The observed decline in the formation of the R_f compound upon addition of EDTA also suggested the requirement of iron.

During the course of these experiments, it became apparent that the TLC Linear Analyzer would be of limited use. The greatest problem being that the amount of radioactivity that was contained within a spot on the TLC was difficult to quantitate, let alone duplicate. Standards containing known amounts of [U-^{14}C]arginine were used in order to obtain a rough estimation as to the amount of radioactivity that was in each radioactive compound present in the CFE. The

standards themselves, however, were not consistent. Another problem was due tto dynamic range. On several occasions, the amount of non-reacted arginine was so large that it prevented any detection of the low R_f compound. Also of concern was the degree of reproducibility with respect to the quantity of the sample that was being applied to the TLC plate. While graduated micro-capillary tubes were used to load samples onto the plates, the addition of sample was not consistent. From these concerns, it was obvious that a better method of detection and quantitation was required before the low R_f compound could be identified.

Analysis of Enzyme Assays using High Performance Liquid Chromatography

Because the low R_f compound in the enzyme assays was radioactive, it must be derived from [U-^{14}C]arginine. Initially, this unknown compound was to be isolated and identified through isotope trapping experiments. This approach however would not be the most efficient, in terms of time. An alternative method would be to derivatize the components of the enzyme assay followed by analysis using High Performance Liquid Chromatography (HPLC). This method had several advantages over the use of the TLC Linear Analyzer. First of all, dynamic range issues would be decreased. Secondly, the reproducibility between samples would be better, and quantitation of compounds would be easier.

There are several examples of the use of HPLC to isolate and qualitate amino acids.[25-28,29] The types of amino acid derivatives used have included (dimethylamino)azobenzenesulfonyl- (DABS),[30] *ortho*-phthalaldehyde (OPA) derivatives,[31] and 1-dimethylaminonaphthalene-5-sulfonyl- (Dns, Dansyl) derivatives,[32] with Dns-amino acids being the most popular.

It was decided to use the protocol reported by Tapuhi *et al.* for the formation of Dns-amino acids and analysis by HPLC.[32]. The use of [U-

^{14}C]arginine was continued because, with the use of a radiochemical detector, it would help in identifying compounds derived from arginine.

Conditions for the dansylation of amino acid standards and enzyme assays were first developed. Over the course of establishing a procedure for the derivitization of the enzyme assays, it was required to switch the initial sonication buffer used in generating the CFE from Tris to potassium phosphate. Also, the derivatization produced better results when 11 mM Dns-Cl was used. Once all of the conditions were established, an enzyme assay containing all of the cofactors was generated using CFE created from recently harvested mycelia. The assays were terminated by the addition of cold methanol and then derivatized using dansyl chloride. An aliquot of this mixture was analyzed by HPLC. Chromatograms from both the HPLC and radiochemical detector are presented in Figure 3.2 and Figure 3.3. Based upon Dns-[U-^{14}C]arginine used as a standard, as well as a co-injection of Dns-arginine (Dns-Arg), the compound having a retention time (t_R) of 23.9 minutes was identified as Dns-Arg (data not shown). Surprisingly, the assay contained three additional radioactive compounds having retention times of 19.0, 22.9, and 34.2 minutes. These compounds were not present in the boiled control, and were shown to be produced in a time-dependent manner.

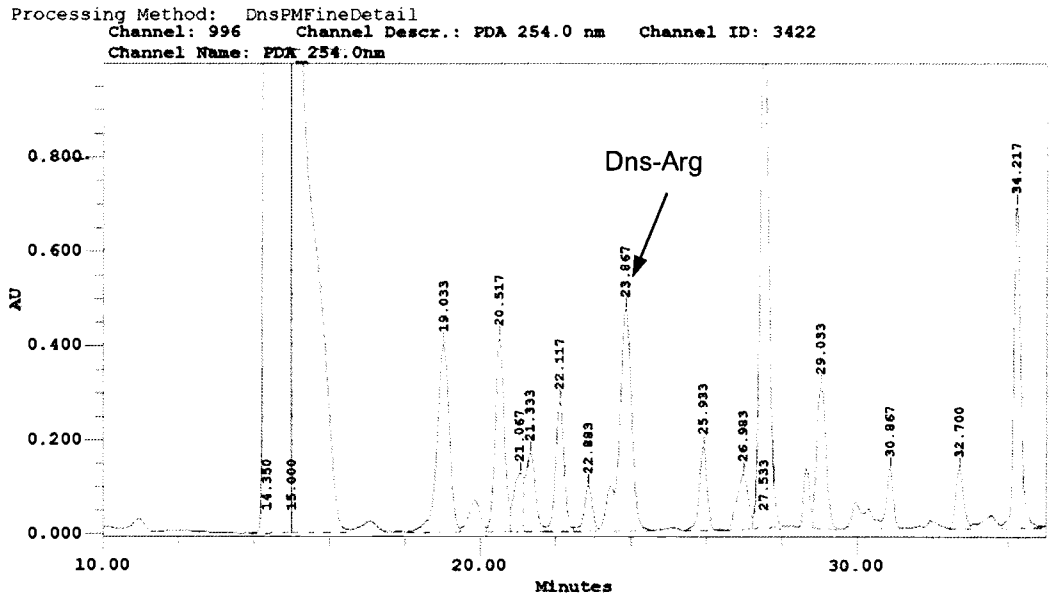


Figure 3.2. HPLC Chromatogram of a Typical Enzyme Assay Containing all Cofactors

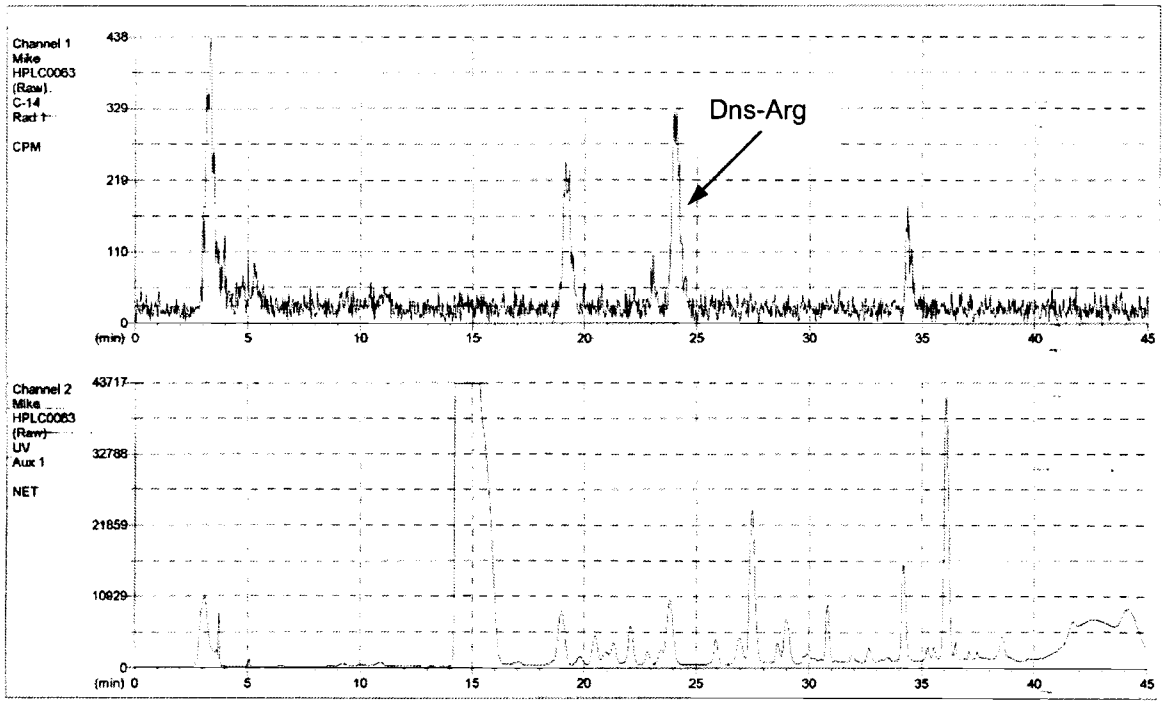


Figure 3.3. Radiochemical Chromatogram of a Typical Enzyme Assay Containing all Cofactors

It was observed that the compound having the retention time of 22.9 minutes had approximately the same t_R as a standard of Dns-capreomycidine (Dns-Cap). Using a new reverse-phase C-18 column from the same manufacturer (Altech), a standard of Dns-Cap and the enzyme assay was re-analyzed to determine the extent that retention times had changed. For some compounds, the retention times were dramatically different, especially for Dns-Cap, which had changed from 22.9 to 30.0 minutes (Figure 3.4). When a sample of Dns-Cap was co-injected with the assay mixture, a clear increase in the area of the peak at 30 minutes was observed. However, very little radioactivity could be detected for the compound at 30.0 minutes. To more accurately determine if this compound contained any radioactivity, several injections of the assay were performed, manually collecting the compound that eluted from the column at t_R = 30 minutes. Analysis of these combined fractions using a liquid scintillation counter showed no radioactivity. Using unlabeled Dns-Arg, the compound at 30 minutes was collected in the same manner and submitted for analysis by FAB-MS. No molecular ion with the same expected molecular mass as Dns-Cap were observed.

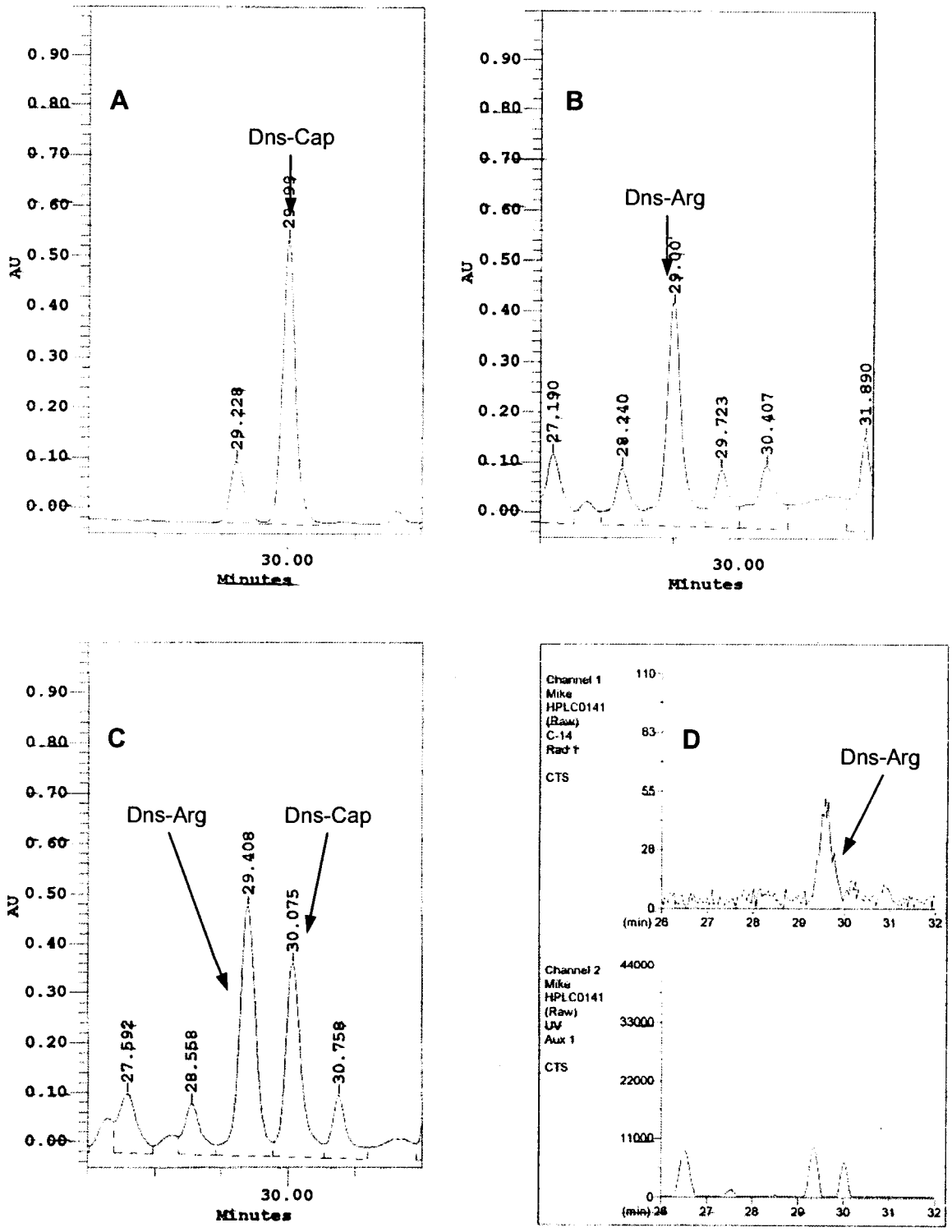


Figure 3.4. HPLC Chromatograms of A) Dns-Capreomycidine Standard, B) Dansylated Enzyme Assay Containing all Cofactors, C) Co-Injection of Enzyme Assay with Dns-Capreomycidine, and D) the Radiochemical Chromatogram of the Enzyme Assay

A set of enzyme assays was generated to determine if any of the radioactive components in the assay were dependent upon the cofactors added, or upon the addition of EDTA (Table 3.5). Earlier results had suggested that the low R_f compound was dependant upon α-KG for its formation, while the addition of EDTA also inhibited its formation.

Table 3.5. Conditions Used in Determining Dependency of Cofactors or Addition of EDTA on the Formation of the Low-R_f Compound by HPLC

	Assay Number									
Component	1	2	3	4	5	6	7	8	9	10
DTT (1mM)	✓	✓	✓	✓		✓	✓	✓	✓	✓
Na Ascorbate (1.5 mM)	✓	✓	✓	✓	✓		✓	✓	✓	✓
$FeSO_4$ (45 μM)	✓	✓	✓	✓	✓	✓		✓		
α-KG (1 mM)	✓	✓	✓	✓	✓	✓	✓		✓	✓
EDTA (4 mM)									✓	
EDTA (8 mM)										✓
[U-^{14}C]Arginine (1.0 mM)	✓	✓			✓	✓	✓	✓	✓	✓
Arginine (1.0 mM)			✓	✓						
Incubation Time	12 h	12 h	12h	12h	12 h	12 h	12 h	12 h	12 h	12 h
Boiled Control	✓		✓							
t_R = 19 min cmpd (dpm x 100)		29		31	20	26	29	22	24	32
t_R = 30 min cmpd (dpm x 100)		6		8	7	9	6	8	6	5
t_R = 34 min cmpd (dpm x 100)		13		17	16	21	15	18	18	12

Analysis of the derivatized assays showed no effect from the absence of any cofactors or upon the addition of EDTA. Only the boiled controls (assays #1 and #3) lacked the three unidentified compounds.

Lastly, the presence of cytochrome P-450 activity was examined in the CFE. NADH or NADPH was added to CFE containing [U-^{14}C]arginine (Table 3.6) and the assays analyzed by HPLC. All of the assays tested contained only the

three unidentified compounds, with the exception of the boiled controls (data not shown). This suggested that the radioactive compounds derived from [U-^{14}C]arginine were not produced from cytochrome P-450 activity.

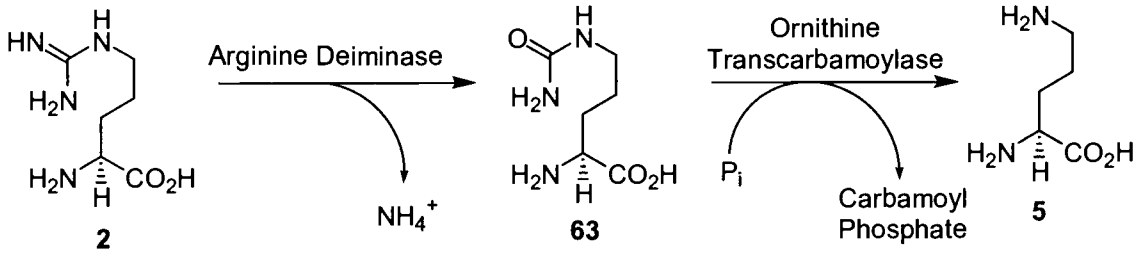
Table 3.6. Conditions Used in Determining Dependency of NADH or NADPH on the Formation of the Unknown Radioactive Compounds

	Assay Number							
Component	1	2	3	4	5	6	7	8
NADH (2.0 mM)	✓	✓	✓	✓				
NADPH (2.0 mM)					✓	✓	✓	✓
Arginine (1.0 mM)	✓	✓			✓	✓		
[U-^{14}C]Arginine (1.0 mM)			✓				✓	✓
Incubation Time	12 h	12 h	12 h	12 h	12 h	12 h	12 h	12 h
Boiled Control	✓		✓		✓		✓	
t_R = 19 min cmpd (dpm x 100)		15		12		18		16
t_R = 30 min cmpd (dpm x 100)		5		8		6		6
t_R = 34 min cmpd (dpm x 100)		10		8		15		14

Because it appeared that capreomycidine was not produced from cell-free extracts of *S.* L-1689-23, it remained to be determined whether the two remaining radioactive compounds at t_R = 19 and 34 minutes were intermediates in the biosynthesis of streptolidine. To identify these unknown compounds, Dns-streptolidine (Dns-Str) and Dns-β-hydroxyarginine (Dns-Harg) were first analyzed by HPLC to determine if these compounds had similar retention times to the radioactive compounds of interest. Neither standard had similar retention times (Dns-Str: t_R = 26.6; Dns-Harg: t_R = 21.2) and therefore suggested that the unidentified compounds may be derived from arginine catabolism.

In the catabolism of arginine, several species of bacteria, such as *Lactobacillus sake*,[33] *Synergistes jonessi*,[34] *Halobacterium salinarium*,[35] as well as protazoa such as *Trichomonas vaginalis*[36] have been shown to contain arginine deiminase, an enzyme that catalyzes the conversion of arginine (**2**) to citrulline (**63**, Scheme 3.7). There are no reports of arginine deiminase activity that has been described in *Streptomyces*.

Scheme 3.7. Arginine Catabolism to form Citrulline (**63**) and Ornithine (**5**)



When Dns-Citrulline (Dns-Cit) was used as a standard, it was found to have a nearly identical retention time to the unidentified compound at t_R = 19 minutes (Figure 3.5). Co-injection of Dns-Cit with the enzyme assay mixture resulted in an increase in the area of the peak corresponding to t_R = 19 minutes. Using unlabeled arginine as substrate, the desired compound was collected from several injections of the assay and analyzed by positive FAB-MS, revealing a molecular ion that matched the molecular ion of Dns-Cit. The same procedure was used in identifying the compound having a retention time of 34 minutes using DiDns-ornithine (DiDns-Orn) as the standard. As expected, both co-injections (data not shown) and mass spectral data confirmed this compound to be DiDns-Orn. Interestingly, when ornithine was added to enzyme assays prior to the addition of substrate, the amount of citrulline produced by the CFE was greatly decreased, suggesting an inhibitory role of ornithine for the enzyme that

catalyzes the conversion of **2** to **63**. Because ornithine is a known inhibitor of arginine deiminase, this may be the first example of arginine deiminase activity observed in the species *Streptomyces*.

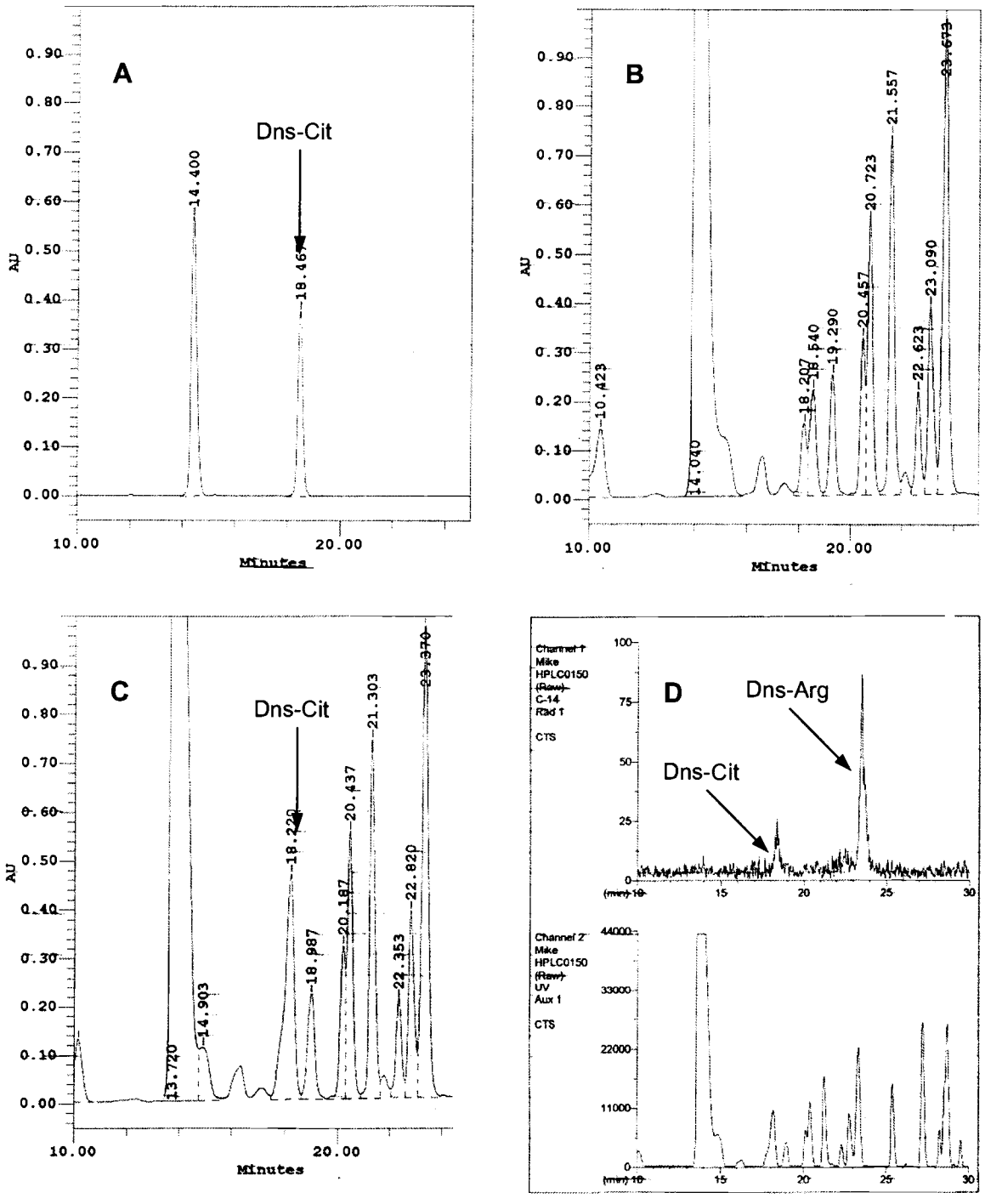


Figure 3.5. HPLC Chromatograms of A) Dns-Citrulline Standard, B) Dansylated Enzyme Assay Containing all Cofactors, C) Co-injection of Enzyme Assay with Dns-Citrulline, and D) the Radiochemical Chromatogram of the Enzyme Assay

Summary

Cell-free Extracts (CFE) of *S.* L-1689-23 were generated to determine if capreomycidine is synthesized *in vitro*. Development of the assays by radial TLC and analysis using a TLC-Linear Analyzer, revealed the presence of two radioactive compounds. Based upon the use of [U-^{14}C]arginine as a standard, one of the compounds was identified as arginine. The other compound, which contained a lower R_f than arginine, had a similar R_f to that of capreomycidine. The presence of this compound within the enzyme assay was protein dependent and appeared to be dependent upon the addition of α-ketoglutarate to the assay.

A High Performance Liquid Chromatography (HPLC) protocol was developed for the isolation and identification of compounds derived from arginine. A total of three unidentified compounds were present in initial assays. The omission of cofactors or the addition of EDTA did not affect the production of these compounds in the CFE. While one compound had an identical retention time (t_R) as that of capreomycidine, results obtained from this study suggested that capreomycidine is not produced by CFEs of *S.* L-1689-23. The two remaining compounds were identified as citrulline and ornithine. Using ornithine in the enzyme assays, the production of citrulline by the CFE was greatly decreased, suggesting that the enzyme, a putative arginine deiminase, is responsible for the conversion of arginine to citrulline. If arginine deiminase were the active enzyme, this would be the first report of this activity in *Streptomyces*.

Experimental

General

Fermentation conditions and media preparation used in the following experiments were described in Chapter 2. Analyses of radial TLC plates were performed on a Tracemaster 20 Automatic TLC-Linear Analyzer (Berthold Instruments, Berlin Germany). All enzyme assays were centrifuged using a Brinkmann Eppendorf centrifuge model 5415C. Assays were concentrated using a Savant Speedvac concentrator model SVC100H. All dansylated standards and enzyme assays were analyzed using a Waters 600 multisolvent delivery system, Hewlett Packard series 1050 autoinjector with a 200 μL injection loop, and a Waters 996 photodiode array detector (256 nm detection). The entire system was controlled using Millennium Chromatography Manager software (Waters v.2.1). A Packard Radiomatic Flo-One Series A-500 radiochemical detector was connected in series with the HPLC for the analysis of radioactive samples.

Culture Maintenance and Fermentation Conditions

Standard culture maintenance and seed growth have been described in Chapter 2. Chemically defined medium was used for the generation of all cell-free studies. Fermentations were harvested at 12 or 24 hours after inoculation, as noted.

Preparation of Cell-free Extracts

Streptomyces lavendulae L-1689-23 cells were grown in a chemically defined medium and harvested at 12 hours after inoculation. The production broth (typically 1000 mL) was transferred to 250 mL centrifuge bottles and the mycelium was collected by centrifugation at 8,000 x g for 15 minutes at 4 °C. The pellets were washed (1 L total volume) with sonication buffer (0.05 M MOPS or Tris buffer, pH 7.0 at 4 °C) containing 0.1 mM DTT and then centrifuged at 8,000 x g for 15 minutes. The pellet was washed with 0.8 M sodium chloride followed by 1.0 M potassium chloride and this procedure repeated twice. The pellet obtained from the last potassium chloride washing was rinsed again with the original sonication buffer and centrifuged at 8,000 x g for 15 minutes. The pellets were combined and suspended in 50 mL of sonication buffer, cooled to 4 °C in an ice-water bath, and disrupted by sonication (Heat Systems Ultrasonic, Inc. model W-225, large sonication tip, power level 8, 90% duty cycle). Sonication consisted of three pulses twenty seconds long (ten bursts per pulse) with one minute cooling intervals.

Enzyme Assay Conditions

For a typical assay, 1.0 mM arginine (100 µL), 1 mM DTT (25 µL), 1.5 mM sodium ascorbate (25 µL), 1 mM α-ketoglutarate (25 µL) and 45 µM ferrous sulfate (25 µL) in 50 mM assay buffer (MOPS, Tris, or Phosphate, pH 7.0) was incubated with 300 µL CFE for a total volume of 500 µL in 1.5 mL amber centrifuge tubes. Incubations were performed for 12 hours in the dark at room temperature (23-25 °C). The assays were terminated by the addition of cold (4 °C) ethanol and then centrifuged. For those analyses done using radial TLC, the supernatant was used directly. For those analyses done using reverse phase

chromatography, the supernatant was evaporated and the residue redissolved in 500 μL Milli-Q water. For assays requiring [U-^{14}C]arginine, 1 mM arginine stock solution containing 3.49 x 104 dpm [U-^{14}C]arginine per 100 μL was added. Boiled controls were prepared by first placing 300 μL of CFE into 1.5 mL amber centrifuge tubes and submerging the tubes in boiling water for ten minutes. Once cooled to room temperature, all appropriate cofactors and substrate was added.

Analysis of Enzyme Assays Using a TLC Linear Analyzer

The radial TLC plates were prepared and developed as described in Chapter 2. The TLC plates were analyzed for radioactivity using a TLC Linear Analyzer (total measuring time: 360 minutes; Gain = 4; Y Resolution = 256; Start scan (cm) = 10, Stop scan (cm) = 30; Step size (cm) = 0.4; Slit Width (cm) = 0.4; Rf-Start-X (cm) = 0; Front-X (cm) = 20; Rf-Start-Y (cm) = 10; Front-Y (cm) = 30).

Synthesis of Dansyl-Amino Acids

To 2 mL of a 6 mM amino acid solution (buffered with 40 mM Li_2CO_3 buffer [adjusted to pH 9.5 using HCl]) was added a 11 mM solution of dansyl chloride in acetonitrile and the resulting mixture was shaken lightly for one minute. The reaction vial was then covered with aluminum foil and allowed to stand at room temperature for one hour. The reaction was terminated by the addition of 2% ethylamine hydrochloride solution and used without further purification.

Dansylation of Enzyme Assays

A total of 40 µL of the enzyme assay was transferred to a 1.5 mL amber colored centrifuge tube (VWR Scientific) and to this was added 40 µL of a 80 mM Li_2CO_3 buffer (pH 11.0) followed by the quick addition of 40 µL of 11 mM Dansyl chloride in acetonitrile. The reaction mixture was vortexed quickly followed by incubation in the dark at 35 °C for one hour. Reactions were terminated by the addition of 2% ethylamine hydrochloride solution (4 µL).

Effect of α-Ketoglutarate Concentration on Enzyme Activity

Cell-free extracts of *S.* L-1689-23 were prepared as described previously. Enzyme assays containing either 1 µM or 2 µM α-ketoglutarate were incubated at room temperature in the dark for 12 hours. Control experiments were prepared in a similar manner using boiled CFE. The assays were terminated by the addition of cold ethanol (500 µL) and then centrifuged. A total of 60 µL of the assay was spotted onto a 20 x 20 cm TLC plate and developed as previously described. The TLC plates were then analyzed for radioactivity using a TLC Linear Analyzer.

Assay for Enzyme Activity Using Stored Mycelia or Cell-Free Extracts

Enzyme assays containing the required cofactors and substrate were incubated with 300 µL of CFE generated from mycelia stored at –80 °C for two weeks, CFE stored at 4 °C for one week, or CFE stored at –25 °C for one week and incubated at room temperature in the dark for 12 hours. Control experiments were prepared in a similar manner using boiled CFE. Assays were terminated by

the addition of cold ethanol (500 µL) and then centrifuged. A total of 60 µL of the assay was spotted onto a 20 x 20 TLC plate and developed as previously described. The TLC plates were then analyzed for radioactivity using a TLC Linear Analyzer.

Effects of α-Ketoglutarate Omission or Addition of EDTA to Enzyme Activity

Cell-free extracts of *S.* L-1689-23 were prepared as described previously. Sonication buffer (25 µL) was added to enzyme assays lacking sodium ascorbate, ferrous sulfate, dithiothreitol, or α-ketoglutarate so as to maintain a final volume of 500 µL. Ferrous Sulfate was omitted from those assays in which either 4 mM or 8 mM EDTA (50 µL) was added. All assays were incubated at room temperature in the dark for 12 hours. Control experiments were prepared in a similar manner using boiled CFE. The assays were terminated by the addition of cold ethanol (500 µL) and then centrifuged. A total of 60 µL of the assay was spotted onto a 20 x 20 TLC plate and developed as previously described. The TLC plates were then analyzed for radioactivity using a TLC Linear Analyzer. For the analysis of enzyme assays by HPLC, the supernatant was evaporated to dryness and the residue dissolved in 500 mL Milli-Q water. Samples were filtered though a 0.45 µm membrane filter before analysis.

Addition of NADH or NADPH to Cell-Free Extracts of S. L-1689-23

Cell-free extracts of *S.* L-1689-23 were prepared as described previously. To freshly prepared cell-free extracts (300 µL), 2.0 mM NADH or 2.0 mM NADPH was added followed by the addition of substrate (100 µL) for a final volume of

500 µL. All assays were incubated at room temperature in the dark for 12 hours. Control experiments were prepared in a similar manner using boiled CFE. The assays were terminated by the addition of cold ethanol (500 µL) and then centrifuged. The supernatant was evaporated to dryness and the residue dissolved in 500 mL Milli-Q water. Samples were filtered though a 0.45 µm membrane filter before analysis.

Analysis of Enzyme Assays by High Performance Liquid Chromatography

Dansylated standards or enzyme assays (75 µL, filtered before analysis) were separated by gradient elution chromatography (0 to 100% solvent B in 30 minutes, curve 6), at a flow rate of 1.0 mL/min at 25 °C, using a 5 µm Econosphere C_{18} column (Alltech, 3.9 mm x 150 mm). An All-guard 5 µm Econosphere C_{18} guard column (Alltech) was placed between the injector and column. At the end of the analysis, initial column conditions were restored using a reverse gradient in ten minutes at a flow rate of 1.0 mL/min. A total of fifteen minutes was then required for column equilibration. Solvent A consisted of 30 mM sodium phosphate buffer (pH 7.4) containing 5% methanol and 6.5% tetrahydrofuran. Solvent B consisted of methanol and water (70:30 v/v). Both mobile phases were filtered using Whatman 0.45 µm membrane filters and degassed prior to use. For compound identification via co-injection, 75 µL of the appropriate standard was mixed with 75 µL of the assay and injected into the HPLC (total volume = 150 µL).

Identification of Dansyl-Citrulline in Cell-free Extracts of S. L-1689-23

Enzyme assays containing all required cofactors and substrate were prepared and derivatized as described previously. For co-injections, 100 µL of Dns-Citrulline was added to 100 µL of dansylated enzyme assay containing [U-^{14}C]arginine and 150 µL was injected into the HPLC. For analysis by positive FAB-MS, a total of 10 x 100 µL of dansylated assay, containing non-radioactive substrate, was injected and the desired compound collected manually. The solvent was removed *in vacuo* and submitted for mass-spectral analysis without further purification.

Identification of Dansyl-Ornithine in Cell-free Extracts of S. L-1689-23

Enzyme assays containing all required cofactors and substrate were prepared and derivatized as described previously. For co-injections, 100 µL of Dns-Ornithine was added to 100 µL of dansylated enzyme assay containing [U-^{14}C]arginine and 150 µL injected into the HPLC. For analysis by positive FAB-MS, a total of 10 x 100 µL of dansylated assay, containing non-radioactive substrate, was injected and the desired compound collected manually. The solvent was removed *in vacuo* and submitted for mass-spectral analysis without further purification.

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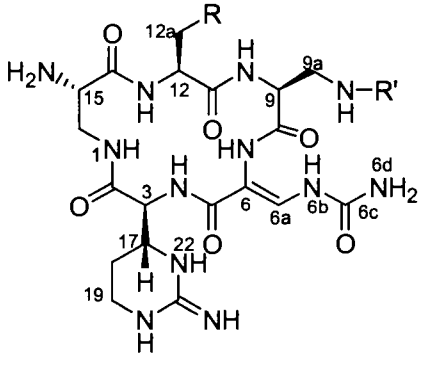
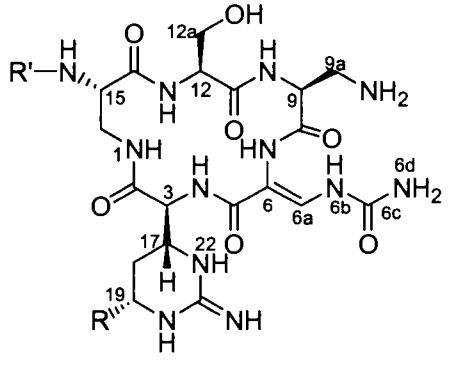
Chapter 4
Synthesis of L-[*guanidino*-^{13}C]Capreomycidine and Incorporation Studies using S. L-1689-23

Introduction

Capreomycidine (**17**) is an arginine-derived amino acid constituent found in many members of the tuberactinomycin class of peptide antibiotics, including capreomycins IA (**64**, Figure 4.1), IB (**65**), IIA (**66**) and IIB (**67**), and tuberactinomycins N (**70**) and tuberactinomycin O (**71**). Capreomycidine is also a proposed intermediate in the biosynthesis of the streptolidine (**15**) moiety in streptothricin F (**12a**).[1] While the chemical structure of capreomycidine was proposed by Herr,[2] Bycroft and coworkers proposed the stereochemistry of the amino acid.[3] The absolute configuration of capreomycidine was ultimately determined through the x-ray analysis of tuberactinomycin O.[4,5,6]



Capreomycins
IA (**64**): R = OH, R' = β-Lysine
IB (**65**): R = H, R' = β-Lysine
IIA (**66**): R = OH, R' = H
IIB (**67**): R = H, R = H

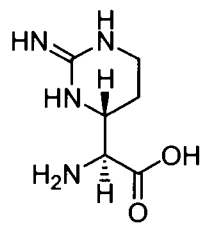


Tuberactinomycins
A (**68**): R = OH, R' = γ-hydroxy-β-Lysine
B (**69**): R = OH, R' = β-Lysine
N (**70**); R = H, R' = γ-hydroxy-β-Lysine
O (**71**): R = H, R' = β-Lysine

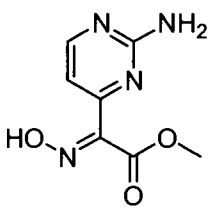
Figure 4.1. The Tuberactinomycin Class of Antibiotics

The first synthesis of DL-capreomycidine (**17**, Figure 4.2) was reported by Bycroft and coworkers and involved the hydrogenation and hydrolysis of 2-amino-4-(α-methoxycarbonyl-α-hydroxyimino)methylpyrimidine (**72**).[7] In an attempt to synthesize optically pure capreomycidine, Wakamiya and coworkers first developed a synthesis of DL-capreomycidine starting from the diethyl acetal of β-aminopropionaldehyde, **73**, to form the key intermediate β-hydroxyornithine (**74**) as a mixture of diastereomers.[8] This procedure was later applied to the synthesis of enantiomerically pure L-capreomycidine.[9] It was reported that the racemic *threo* diastereomer (**74a**) could be separated from the racemic *erythro* diastereomer (**74b**) via fractional crystallization. Enantiomerically pure Cbz-protected β-hydroxyornithine (**75**) was prepared by chloroacetylation of racemic **74a** followed by enzymatic resolution using acylase (Scheme 4.1). β-Hydroxyornithine (**75**) was then converted to (2*S*,3*R*)-capreomycidine in nine successive steps with a 0.1 percent overall yield starting from the protected aldehyde, **73**.

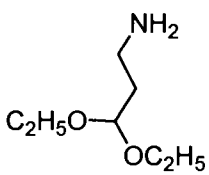
Figure 4.2. The Structure of L-Capreomycidine (**17**) and Other Key Intermediates Used in Prior Syntheses of **17**



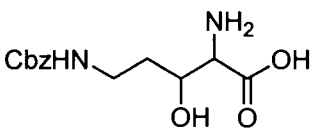
(2*S*,3*R*)-Capreomycidine
(**17**)



72

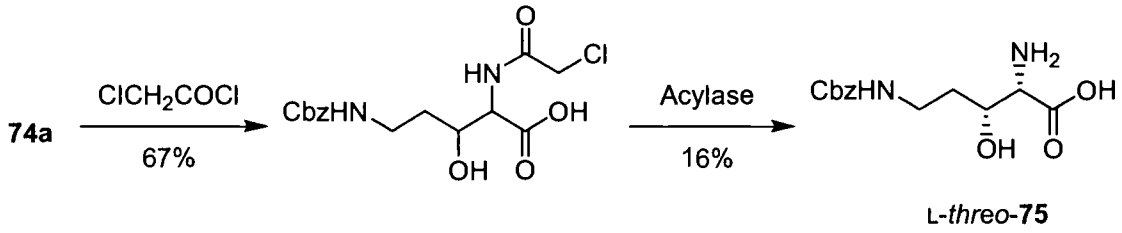


73



74a *threo*
74b *erythro*

Scheme 4.1. Synthesis of L-*threo*-**75**



L-*threo*-**75**

In order to study biosynthetic pathways in which capreomycidine may be involved, labeled forms of the amino acid are required. Stable isotopes, either deuterium or ^{13}C, should be located within the molecule at sites that would not be lost due to racemization or other biochemical events. When introducing the chemical label into a synthetic sequence, the label should be introduced as late in the sequence as possible. Based on these criteria, a study wherein L-[*guanidino*-^{13}C]capreomycidine is prepared and used in incorporation experiments was designed to determine whether capreomycidine is an intermediate in the biosynthesis of streptothricin F (**12a**).

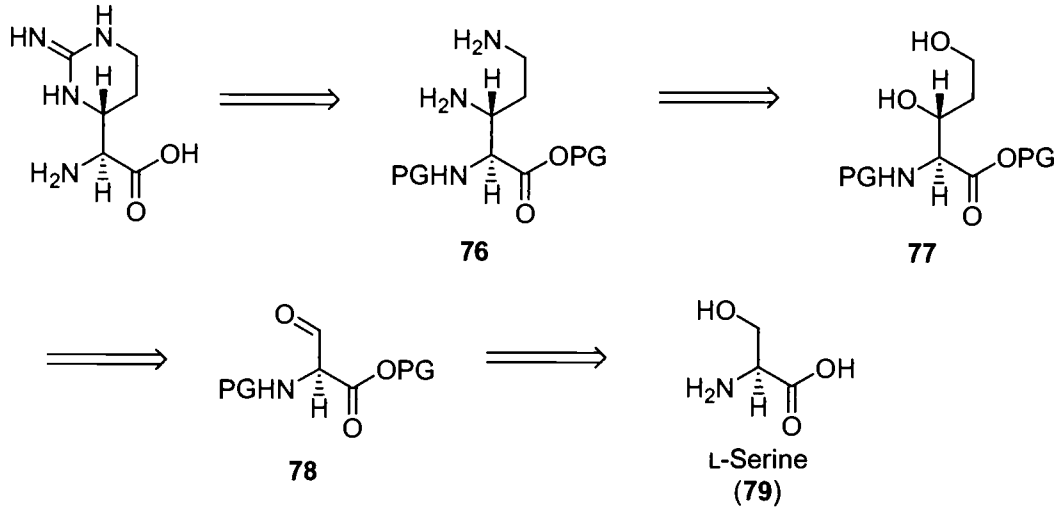
Results and Discussion

Synthesis of L-[guanidino-^{13}C]Capreomycidine (17)

Due to the enzymatic resolution step required in Wakamiya's synthesis[9] to produce the key intermediate **74a**, a more efficient synthesis of L-capreomycidine was sought for our incorporation studies. Scheme 4.2 illustrates the retrosynthetic analysis used in designing a synthesis for L-capreomycidine. It was envisioned that the guanidino moiety in capreomycidine results from the

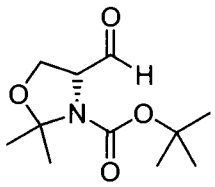
condensation of diamine **76** with cyanogen bromide. It was also recognized that this would be the best step to introduce a ^{13}C label into the amino acid. Diamine **76** could be obtained from diol **77** through functional group interconversion. Diol **77** can be derived from aldehyde **78** using a funtionalized Grignard reagent. The corresponding aldehyde (**78**) can be derived by oxidation of di-protected L-serine (**79**)

Scheme 4.2. Retrosynthetic analysis of L-Capreomycidine

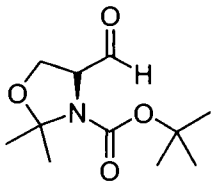


Instead of beginning directly with L-serine, use of the serine-derived oxazolidine aldehydes **80** (Figure 4.3) or **81** as potential starting materials for the synthesis of L-capreomycidine was examined. Oxazolidine aldehydes **80** and **81** are commonly referred to as Garner's aldehyde's and were first reported by Garner and Park in 1987[10] with various modifications published over the years.[11-13] The synthesis of a Cbz-protected oxazolidine aldehyde has also been reported,[14] but has attracted much less attention as synthetic starting material. Both **80** and **81** have been used extensively in the synthesis of enantiomerically

pure amino acids and derivatives,[15-25] as well as natural products and their derivatives.[26-34] Currently, both isomers of Garner's aldehyde are commercially available from Aldrich Chemical Company, but are too expensive to be used as starting material on a large scale. Initially, the desired oxazolidine, **80**, was obtained from D-serine (**82**) using Garner's procedure[10] as outlined in scheme 4.3. By using D-serine methyl ester (**83**), however, oxazolidine aldehyde **80** was prepared more efficiently.



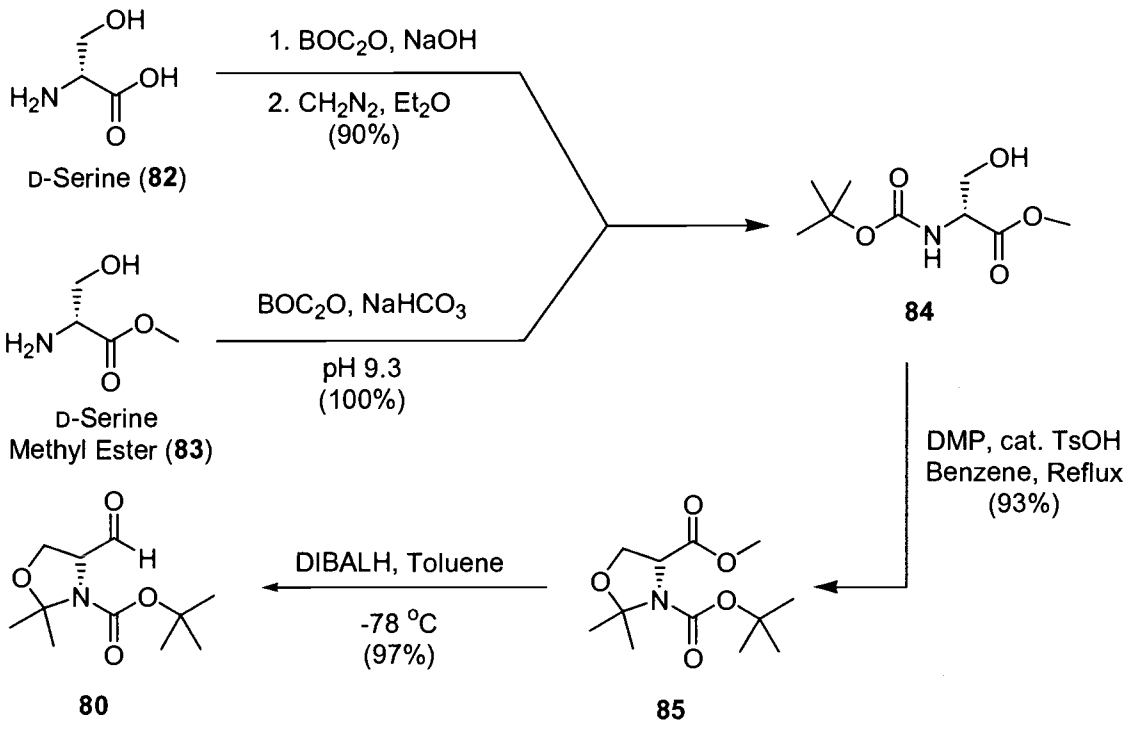
80



81

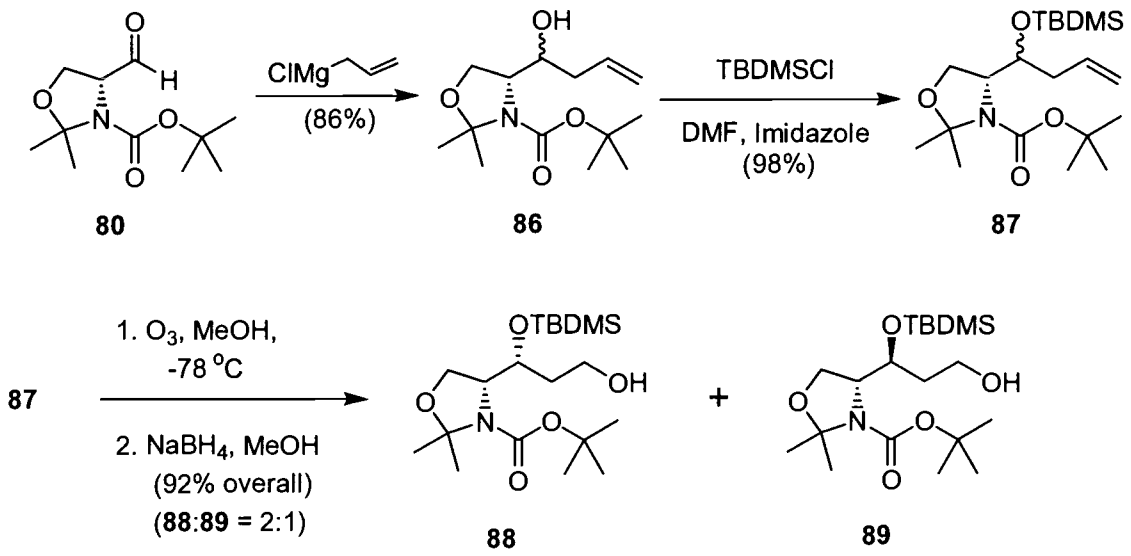
Figure 4.3. (4*R*)- and (4*S*)-Oxazolidine Aldehydes (Garner's Aldehydes)

Scheme 4.3. Synthesis of (4*R*)-Garner's Aldehyde



The synthesis of L-capreomycidine was initiated as outlined in Scheme 4.4. Reaction of aldehyde **80** with allylmagnesium chloride[35] cleanly produced a diastereomeric mixture of the corresponding homoallylic alcohols, **86**. Protection of the secondary alcohols with *tert*-butyldimethylsilyl (TBDMS) chloride[36] afforded the TBDMS-protected homoallylic alcohol (**87**) in near quantitative yield. Oxidative cleavage of the terminal double bond in **87** with ozone and *in situ* reduction with sodium borohydride,[37] afforded the desired primary alcohols, **88** and **89** in a 2:1 ratio, respectively. The diastereomers of **86** can be separated after protection with *tert*-butyldimethylsilyl chloride using flash chromatography, but the separation was tedious and difficult. Separation of the secondary alcohols **88** and **89** was achieved with greater efficiency.

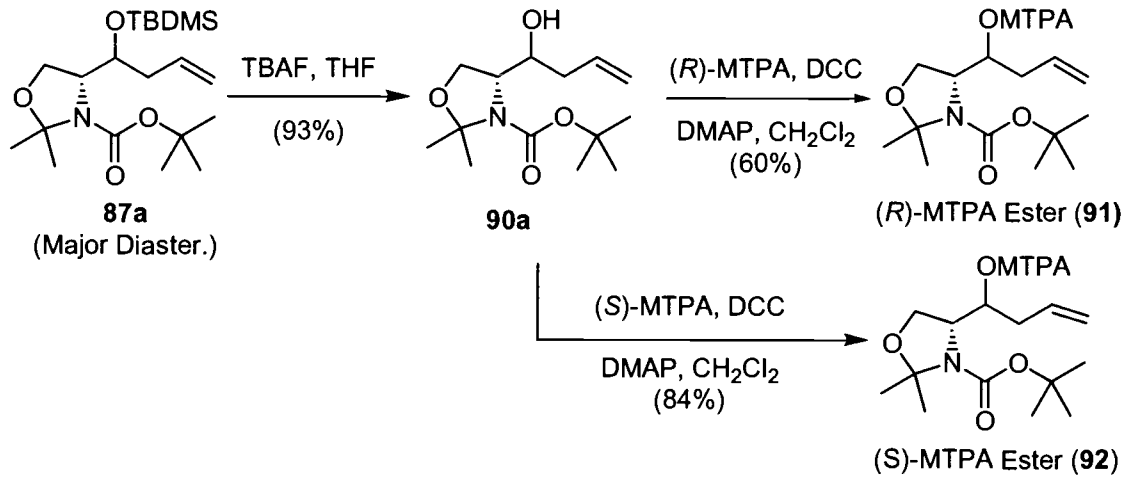
Scheme 4.4 Synthesis of Primary Alcohols **88** and **89**



To determine the relative stereochemical outcome of the Grignard reaction, the TBDMS-protected homoallylic alcohols were carefully purified by flash chromatography to afford pure samples of each diastereomer. The major diastereomer (**87a**) from the Grignard reaction was deprotected[36] to form **90a** (Scheme 4.5). Homoallylic alcohol **90a** was then converted to the α-methoxy-α-(trifluoromethyl)phenylacetate esters (MTPA)[38,39] **91** and **92**, respectively. Analysis of the proton NMR spectra of both diastereomers by the advanced Mosher's method[40] (Table 4.1) resulted in the tentative assignment of the stereochemistry of the major diastereomer **87a** as presented in Figure 4.4. Hafner and coworkers, in their synthesis of homoallylic alcohols using cyclopentadienyldialkoxyallyltitanium complexes, have synthesized the enantiomer to **87a**.[41] Further support for the stereochemistry in **87a** was therefore obtained through a comparison of the spectral data of **87a** to that of its the enantiomer, which revealed near identical chemical shifts for both the proton and carbon NMR spectral data. A further attempt to elucidate the absolute

stereochemistry of the major diastereomer included conversion of primary alcohol **88** to the corresponding *p*-nitrobenzoate derivative (**93**, Scheme 4.6), but crystals of **93** could not be obtained.

Scheme 4.5 Synthesis of the (*R*)- and (*S*)-MTPA Esters of **87a**



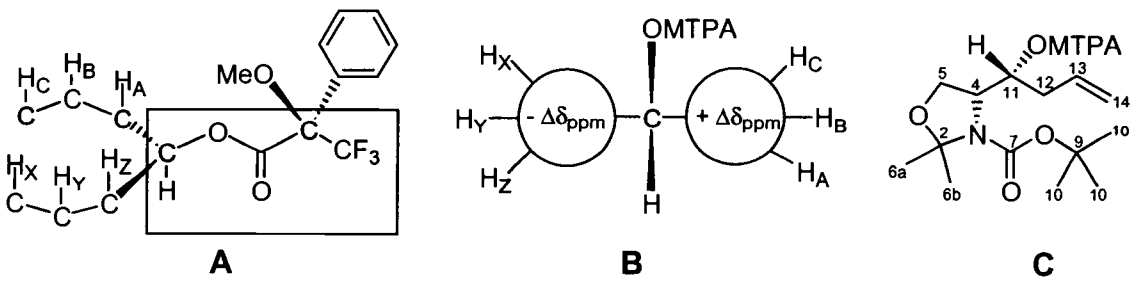
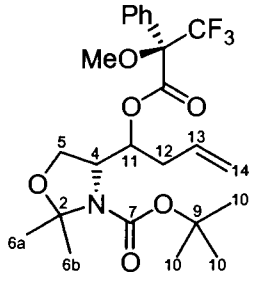


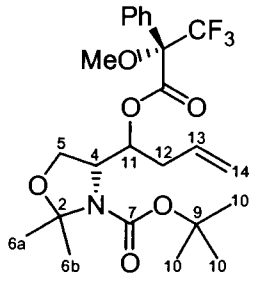
Figure 4.4. A) The MTPA plane of the (*R*)-MTPA ester of a secondary alcohol. B) The rule for determining the absolute configuration of secondary alcohols($\Delta\delta = \delta_S - \delta_R$). C) The proposed stereochemistry for the major diastereomer from the Grignard reaction.

Table 4.1. ^{1}H NMR analysis of the Mosher Esters **96** and **97**.

Proton	δppm (*S*)-MTPA	δppm (*R*)-MTPA	Δppm (δ*S* - δ*R*)
10	1.4757	1.4640	0.012
6a,b	1.6353	1.6604	-0.025
	1.3909	1.4283	-0.037
12a,b	2.5772	2.5428	0.034
	2.3538	2.3136	0.040
5a,b	3.8006	3.9157	-0.115
	3.5259	3.6344	-0.109
4	4.0846	4.1178	-0.033
14a,b	5.0244	4.9508	0.074
	4.9697	4.8870	0.083
13	5.7397	5.6606	0.079
11	5.8439	5.8002	0.044

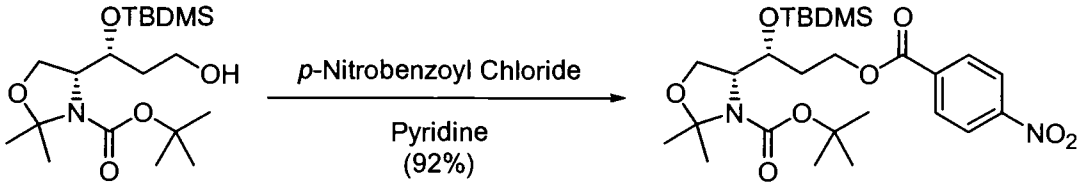


91



92

Scheme 4.6. Synthesis of *p*-Nitrobenzoate Derivative **93** from **88**



88 **93**

The stereochemical outcome of the Grignard reaction can be rationalized as depicted in Figure 4.5. The Felkin-Anh model for the addition of an organometallic compound to a chiral aldehyde predicts that the major product would result from attack of the nucleophile on the *si*-face of the aldehyde. This direction of attack would lead to *anti* product. Conversely, if the metal were chelated to the compound, as depicted in the Cram chelation model, nucleophilic attack by the Grignard reagent at the *re*-face of the aldehyde would occur. Based upon the analysis of the MTPA-esters of **90a**, and a comparison with the ^{13}C NMR spectral data of its enantiomer, the *anti* addition product is the major diastereomer produced in the reaction. This suggests that chelation dictates the predominant adduct.

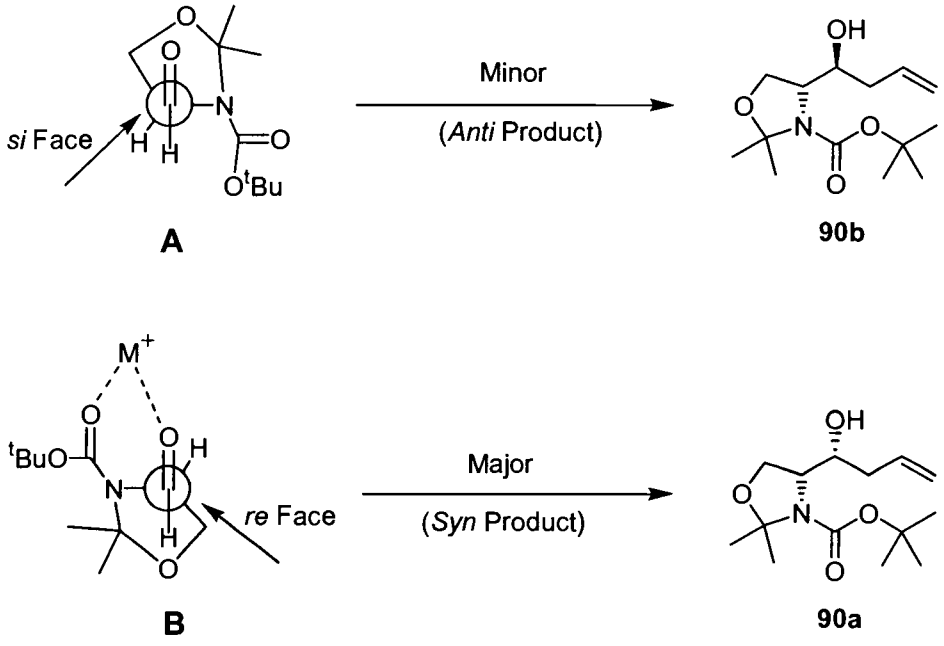
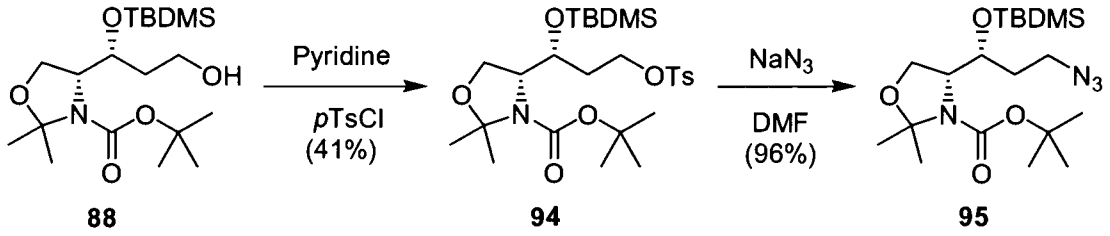


Figure 4.5. A) Felkin-Anh and B) Cram Chelation Model Illustrating Nucleophilic Attack from the Least Encumbered Face.

Initially, primary alcohol **88** was to be converted to the corresponding primary azide via tosylation and nucleophilic displacement with sodium azide (Scheme 4.7). Unfortunately, the tosylation proceeded very slowly with an increase in the formation of side products over time. As a result, poor yields of tosylate **94** were obtained. Displacement of the tosylate with sodium azide, however, occurred cleanly and in high yield to generate the primary azide (**95**). Attempts were made to optimize the reaction by testing different bases and solvents (Table 4.2) but none of the conditions provided adequate yields for the synthesis of the desired primary azide.

Scheme 4.7. Synthesis of Primary Azide **95** via Tosylation



88 **94** **95**

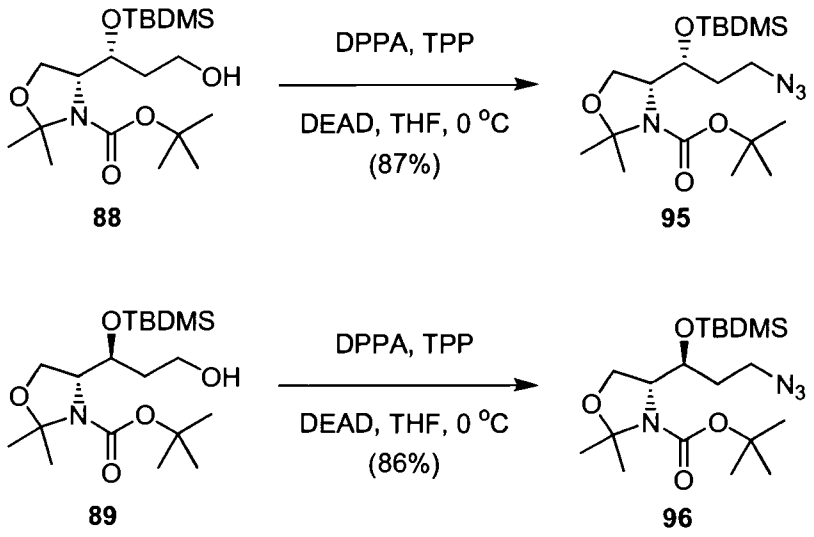
Table 4.2. Tosylation Conditions Used in the Formation of **94**

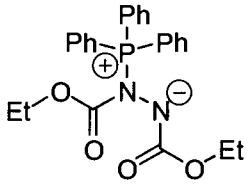
Entry No.	Mol. eq. *p*TsCl	Solvent	Base	Catalyst	t (hr)	% Yeild
1	1.6	$CHCl_3$	Pyridine	DMAP	64	19
2	1.6	Pyridine	Pyridine		48	41
3	1.6	Pyridine	Pyridine	DMAP	48	31
4	1.6	$CHCl_3$	Et_3N		64	29
5	1.6	$CHCl_3$	Et_3N	DMAP	64	33

Although alkyl azide formation via a sulfonate ester is a common practice, it was evident that an alternative route to azide **95** would need to be used. One

potential method was to use diphenylphosphoryl azide (DPPA) in the presence of triphenyl phosphine (TPP) and diethylazodicarboxylate (DEAD).[42] This modified Mitsunobu reaction has been applied towards the synthesis of a variety of secondary azides[43-45],[46] and therefore few problems were anticipated in the conversion of alcohol **88** to azide **95**. Also, the reaction was reported to proceed with inversion of configuration,[42] a feature that would be useful when converting the secondary alcohols to the corresponding azides. When alcohol **88** was exposed to the modified Mitsunobu conditions,[47] the corresponding primary azide was obtained in high yield (Scheme 4.8). When these same conditions were applied to diastereomer **89**, the reaction again proceeded smoothly to form primary azide **96**.

Scheme 4.8. Synthesis of Primary Azides **95** and **96**

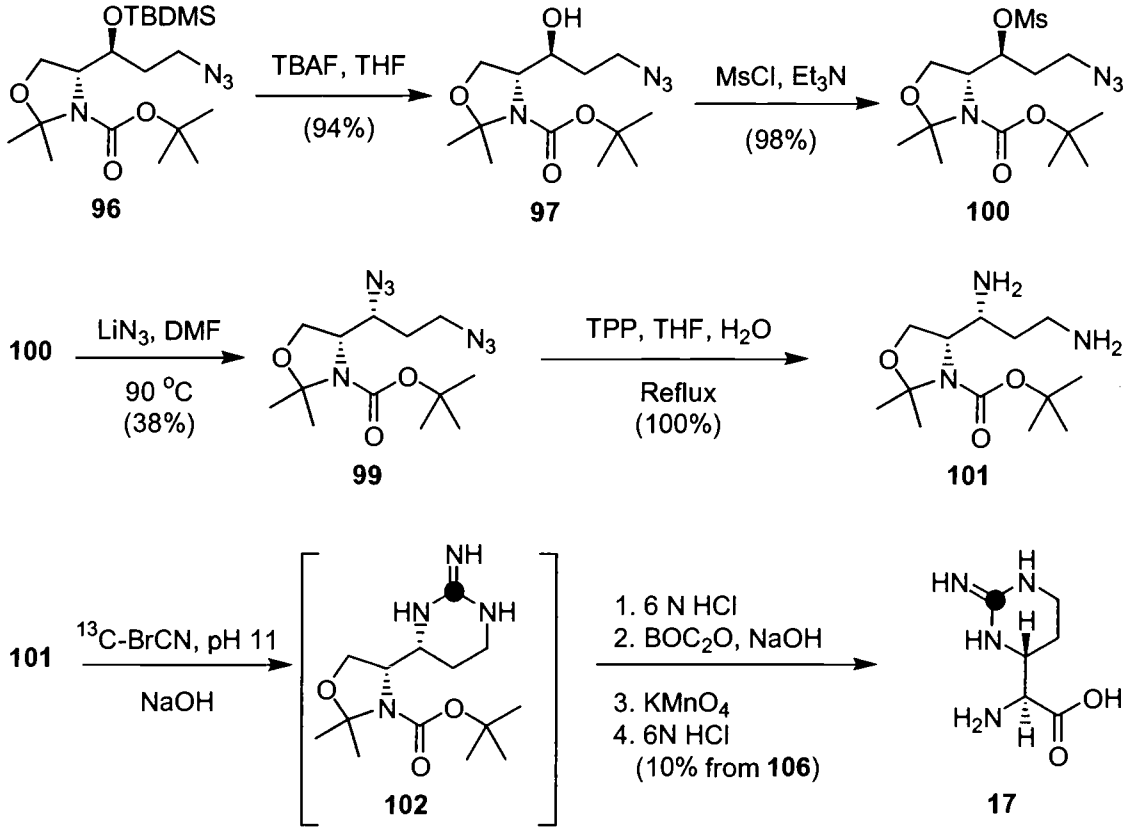




Betaine **98**

Primary azide **96** was deprotected (Scheme 4.9) using tetra-*n*-butylammonium fluoride[36] (TBAF) to generate the secondary alcohol **97** (Scheme 4.9). Compound **97** was subjected to the same Mitsunobu conditions used in the conversion of the primary alcohols to the primary azides, changing only the order that reagents were added to the reaction. Unfortunately, no diazide was detected and only starting material was recovered. There are reported cases in which the Mitsunobu reaction was unsuccessful in inverting the stereochemistry of a secondary alcohol[48],[49] as well as converting a secondary alcohol to the corresponding azide.[50] It was suspected that alcohol **97** was unreactive under the modified Mitsunobu conditions due to the presence of the large BOC group. The theory was that the BOC group may be positioned so that the nucleophilic hydroxyl group was unable to react with betaine **98**.[51-55] Diazide **99** was eventually obtained by converting **97** to the corresponding methanesulfonate ester **100**,[56] followed by displacement with lithium azide. Diazide **99** was reduced using TPP in wet tetrahydrofuran[57] (THF) to cleanly afford diamine **101**. Condensation of **101** with [^{13}C]BrCN[58] produced the guanidino derivative **102** in moderate yield. Deprotection of the oxazolidine using 6 N HCl followed by reprotection with di-*tert*-butyldicarbonate (BOC) and oxidation using potassium permanganate afforded L-capreomycidine (**17**) after acidic workup.

Scheme 4.9. Synthesis of L-Capreomycidine (**17**) from **96**.



Of the different types of sulfonates, methanesulfonates are the least reactive towards nucleophilic displacement.[59] This is due, in part, to the lack of stabilization of the resulting sulfonate anion that is generated as a by-product of the S_N2 reaction. Several attempts were made, therefore, to improve the conversion of secondary alcohol **97** to diazide **99** by exploring various sulfonate esters. As presented in Table 4.3 (entry 1), only the methanesulfonate was obtained in adequate yields. A recent report on the use of chloromethanesulfonyl chloride (McCl) as a much more efficient leaving group for displacement with azide ion was examined.[60] Unfortunately, the secondary

alcohol (**102**) did not react with McCl (entry 3). Attemps to convert the secondary alcohol to the corresponding triflate (entry 4) was also unsuccessful.[61]

Table 4.3. Attempted Synthesis of Various Sulfonate Esters from Alcohol **97**

Entry no.	Sulfonyl Chloride[a]	% Yield	% S.M.
1	MsCl	93	0
2	*p*TsCl	0	97
3	McCl	0	85
4	Tf_2O	0	0

[a]Abbreviations : MsCl = Methanesulfonyl Chloride; *p*TsCl = *p*-Toluenesulfonyl Chloride; McCl = Monochloromethanesulfonyl Chloride; Tf_2O =Triflic Anhydride

Incorporation Studies Using [guanidino-^{13}C]Capreomycidine

Enough [*guanidino*-^{13}C]capreomycidine (36 mg) was produced to allow two incorporation studies. After generating production flasks containing *S.* L-1689-23, 18 mg of labeled **17** was introduced into each of two 100 mL production flasks and the STF produced was isolated 24 hours after initial innoculation. Analysis of the purified antibiotic by ^{13}C NMR spectroscopy showed no appreciable incorporation into the guanidino carbon of STF. This experiment was repeated using the remaining labeled material with similar results. Based upon these findings, capreomycidine is not a true intermediate in the biosynthesis of streptolidine, or it is unable to enter the cell. It is also conceivable that a capreomycidine intermediate is enzyme-bound.

Summary

Beginning with D-serine or D-serine methyl ester, a new synthesis of L-capreomycidine has been established. The key intermediate in the synthesis was the oxazolidine diazide, which was converted to capreomycidine in five steps. While most of the steps in the synthesis produced high yields, the conversion of the mesylate to the diazide was troublesome and proceeded in low yield as did the final oxidation in the synthesis to produce capreomycidine. Nonetheless, an adequate amount of capreomycidine containing a ^{13}C label at the guanidino position was synthesized and added to production flasks of *S.* L-1689-23. Upon isolation of streptothricin F, no incorporation was detected. This may indicate that synthetic capreomycidine did not enter the cells, is not a true biosynthetic intermediate in the biosynthesis of streptolidine, or only occurs as an enzyme bound intermediate.

Experimental

General

Fermentation conditions and media preparation used in the following experiments were described in Chapter 2. All chemicals were purchased through Aldrich Chemical Company or Arcos Chemical Company and used without further purification. Sodium cyanide (99.99% ^{13}C enriched) was purchased from Cambridge Isotope Laboratories (Cambridge, MA). Anhydrous tetrahydrofuran, benzene, and toluene were obtained by refluxing over sodium in the presence of benzophenone. Anhydrous methanol was obtained by refluxing over magnesium methoxide. Anhydrous dimethylformamide (DMF) was obtained by first letting 500 mL stand over 4 Å molecular sieves for 24 hours. The DMF was then

distilled under argon into a receiving vessel containing additional 4 Å molecular sieves. The DMF was stored under argon and used without further purification. NMR spectra were obtained on either a BRUKER AC 300 or AM 400 spectrometer. *t*-BuOH or pyridine was added as an internal reference of chemical shift when spectra were recorded in D_2O. Mass Spectra were obtained on a Kratos MS 50 TL spectrometer. Optical rotations were obtained using a Perkin-Elmer model 141 Polarimeter. IR spectra were obtained using a Nicolet Model 510 FT-IR spectrometer. Ozone used for ozonolysis reactions was generated using an OREC Ozone Generator (Ozone Research & Equipment Corporation).

Synthesis of L-[guanidino-^{13}C]Capreomycidine

N-[(1,1-Dimethylethoxy)carbonyl]-D-Serine Methyl Ester (84)

To a solution of D-serine **82** (10.0 g, 9.52 mmol) in 1.0 N NaOH (195 mL) at 0 °C was added a solution of 1.20 eq Boc-anhydride (24.9 g, 1.14 mmol) in 1,4-dioxane (89 mL) with stirring. After 30 minutes at 0 °C, the mixture was warmed to room temperature over the course of four hours. Analysis by TLC (hexanes/EtOAc, 1:1, detection: 0.2% ethanolic ninhydrin) showed complete conversion of starting material (R_f 0.0) to product (R_f 0.34). The reaction mixture was then concentrated to one-half the original volume and cooled back down to 0 °C. To the resulting solution was slowly added 1.0 N $KHSO_4$ (240 mL) until the pH was 2-3. The aqueous mixture was extracted with EtOAc (3 x 200 mL) and the combined organic layers washed with brine (1 x 200 mL) and dried over anhydrous $MgSO_4$. Filtration and removal of the solvent afforded 19.5 g (100%) of the desired compound as a sticky, colorless foam. The carbamate was used without further purification.

To a solution of the Boc-protected amino acid (19.5 g, 0.952 mol) in 200 mL Et_2O at 0 °C was bubbled gaseous diazomethane in nitrogen until the color of the solution was yellow. Acetic acid was then slowly added (2.5 mL, N_2 evolution) until the solution was colorless. The reaction mixture was washed with half-saturated $NaHCO_3$ (500 mL), brine (1 x 200 mL) and dried over $MgSO_4$. Filtration and removal of the solvent afforded a light yellow oil which was purified by flash chromatography (hexanes/EtOAc, 1:1, 7 x 15 cm, detection: 0.2% ethanolic ninhydrin) to afford 14.5 g (70%) of the desired product. $[\alpha]_D^{25}$ -7.52° (*c* 6.08, $CHCl_3$); IR (neat) 3400, 1720 (br) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C) δ (ppm) 1.44 (s, 9H), 2.67 (bs, 1H), 3.77 (s, 3H), 3.87 (dd, *J* = 4.0 and 11 Hz, 1H), 3.94 (dd, *J* = 4.0 and 11 Hz, 1H) 4.37 (bs, 1H), 5.48 (bs, 1H); ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C) δ (ppm) 28.2, 52.4, 55.7, 62.9, 80.1, 155.9, 171.6; MS (FAB): *m/z* 220.2 (M + 1); HRMS (FAB): calcd for $C_9H_{18}NO_5$ 220.1185, obsd 220.1185.

Alternate route to N-[(1,1-Dimethylethoxy)carbonyl]-D-Serine Methyl Ester (84)

To a stirred solution of D-serine methyl ester (**83**), 15.0 g, 9.60 mol) in 1.0 M $NaHCO_3$ (280 mL, pH 9.3) at 0 °C was slowly added a solution of Boc-anhydride (25.2 g, 0.116 mol) in dioxane (90 mL) over one-half hour. The reaction mixture was removed from the ice-water bath and stirring continued for an additional five hours. Analysis by TLC (hexanes/EtOAc, 1:1, detection: 0.2% ethanolic ninhydrin) showed complete conversion of starting material (R_f 0.0) to product (R_f 0.39). The reaction was concentrated to one-half the original volume and cooled back down to 0 °C. Cold 1 M $KHSO_4$ was slowly added until no more carbon dioxide evolved and the pH of the solution was within the range of pH 1-2. The mixture was washed with EtOAc (3 x 200 mL) and the combined organic layers washed with saturated $NaHCO_3$ (3 x 200 mL), brine (1 x 200 mL) and

dried over Na_2SO_4. Filtration and removal of the solvent afforded **84**, as a light yellow oil (19.8 g, 94%), which was used without further purification.

3-(1,1-Dimethylethyl) (4R)-4-methyl-2,2-dimethyl-3,4-oxazolidinecarboxylate (85)

To a solution of **84** (19.8 g, 9.03 mmol) in dry benzene (314 mL) was added 2,2-dimethoxypropane (22.2 mL, 0.188 mol) followed by *p*-toluenesulfonic acid (0.242 g, 1.27 mmol) and the solution refluxed at 90 °C for one-half hour. A total of 269 mL of solvent was then slowly distilled off. Fresh benzene (125 mL) and 2,2-dimethoxypropane (5.8 mL, 47.2 mmol) was added to the reaction mixture and the procedure was repeated, this time collecting 100 mL of distilled solvent. Analysis by TLC (hexanes/EtOAc, 1:1, detection: 0.2% ethanolic ninhydrin) showed the complete conversion of starting material to product. The reaction mixture was partitioned between saturated $NaHCO_3$ (100 mL) and Et_2O (600 mL). The organic layer was washed with saturated $NaHCO_3$ (3 x 100 mL), brine (1 x 100 mL), and dried over Na_2SO_4. Filtration and removal of the solvent afforded crude product that was purified by flash chromatography (hexanes/EtOAc, 1:1, 7 x 15 cm column, detection: 0.2% ethanolic ninhydrin) to afford 20.1 g (86%) of the desired product. $[\alpha]_D^{25}$ +56.2° (*c* 3.84, $CHCl_3$); IR (neat) 2980, 1760, 1710, 1380 cm^{-1}; ^1H NMR (300 MHz, C_6D_6, 70 °C) δ (ppm) 1.40 (s, 9H), 1.56 (s, 3H), 1.84 (s, 3H), 3.33 (s, 3H), 3.73 (t, *J* = 8 Hz, 1H), 3.81 (dd, *J* = 3.2 and 9.0 Hz, 1H), 4.33 (m, 1H); ^{13}C NMR (75 MHz, C_6D_6, 70 °C) δ (ppm)24.8, 25.7, 28.2, 51.5, 59.6, 66.2, 95.2, 151.3, 171.3; MS (FAB): *m/z* 260.2 (M + 1); HRMS (FAB): calcd for $C_{12}H_{22}NO_5$ 260.1498, obsd 260.1499.

1,1-Dimethylethyl (4R)-4-formyl-2,2-dimethyl-3-oxazolidinecarboxylate (80)

To a stirred solution of the methyl ester **85** in dry toluene (150 mL) at -78 °C under argon atmosphere was added diisobutylaluminum hydride (1.50 M solution in toluene, 88 mL, 0.132 mol) dropwise with stirring. After the addition was complete, stirring was continued for another four hours at -78 °C. Analysis of the reaction mixture by TLC (hexanes/EtOAc, 4:1, detection: 0.2% ethanolic ninhydrin) showed nearly complete conversion of starting material (R_f 0.48) to product (R_f 0.36). At this point, dry methanol (164 mL) was slowly added at -78 °C (H_2 evolution). The milky solution was slowly poured into 190 mL of ice cold 1 N HCl with stirring. The mixture was extracted with Et_2O (3 x 500 mL), the combined organic layers washed with brine (1 x 500 mL) and dried over Na_2SO_4. Filtration and removal of the solvent produced crude product that was purified by flash chromatography (hexanes/EtOAc, 4:1, 7 x 15 cm column, detection: 2% Phosphomolybdic Acid (PMA)) to afford 15.9 g of the desired aldehyde (**80**). $[\alpha]_D^{25}$ +93.0° (*c* 1.13, $CHCl_3$); IR (neat) 2973, 1738, 1697, 1369 cm^{-1}; ^1H NMR (300 MHz, C_6D_6, 70 °C) δ (ppm) 1.38 (s, 9H), 1.44 (s, 3H), 1.62 (s, 3H), 2.10 (m, 1H), 2.19 (t, *J* = 6.2 Hz, 1H), 2.30 (m, 1H), 3.62 (dd, J = 6.2 and 9.2 Hz, 1H), 3.69 (m, 1H), 3.88 (m, 2H), 4.99 (t, *J* = 1.3 Hz, 1H), 5.05 (m, 1H), 5.92 (m, 1H); ^{13}C NMR (75 MHz, C_6D_6, 70 °C) δ (ppm) 24.5, 27.5, 28.8, 38.9, 39.2, 62.4, 64.9, 65.2, 72.4, 73.0, 80.5, 80.8, 94.8, 106.8, 117.3, 117.4, 136.0, 136.2, 153.7, 154.4; MS (FAB): *m/z* 272.35 (M + 1); HRMS (FAB): calcd for $C_{14}H_{26}NO_4$ 272.1862, obsd 272.1860.

1,1-Dimethylethyl (4R)-(1'hydroxy-3-butenyl)-2,2-dimethyl-3-oxazolidinecarboxylate (86)

To a solution of the aldehyde **80** (14.8 g, 6.45 mmol) in dry THF (350 mL) at –78 °C was slowly added 3.3 eq allylmagnesium chloride (0.213 mol, 107 mL of a 2 M solution in THF) with stirring and then slowly warmed to room temperature. Analysis by TLC (Hexanes/EtOAc; 3:1; detection: PMA) showed complete conversion of starting material to product at R_f 0.38. Saturated ammonium chloride (100 mL) was then added, and the mixture extracted with ether (3 x 300 mL). The combined organic layers were washed with sat. $NaHCO_4$ (2 x 300 mL), brine (1 x 300 mL) and dried over $MgSO_4$. Filtration and removal of the solvent afforded the desired product, which was purified by flash chromatography (hexanes/EtOAc; 3:1; 7 x 20 cm column; detection: PMA) to afford 15.0 g of the desired homoallylic alcohol (**86**) as a mixture of diastereomers. $[\alpha]_D^{25}$ +28.8° (*c* 3.90, $CHCl_3$); IR (neat) 3465 (br), 1696 cm^{-1}; ^1H NMR (300 MHz, C_6D_6, 70 °C) δ (ppm) 1.35 (s, 9H), 1.44 (s, 3H), 1.61 (s, 3H), 3.52 (t, *J* = 8.2 Hz, 1H), 3.65 (d, *J* = 6.9 Hz, 1H), 3.78 (m, 1H), 9.33 (s, 1H); ^{13}C NMR (75 MHz, C_6D_6, 70 °C) δ (ppm) 24.5, 26.5, 28.6, 64.0, 65.5, 80.9, 95.5, 152.0, 198.5; MS (FAB): *m/z* 230.131 (M + 1); HRMS (FAB): calcd for $C_{11}H_{20}NO_4$ 230.1392, obsd 230.1393.

1,1-Dimethylethyl (4R,1'R)-(1'-(tert-butyldimethysilyloxy)-3'-butenyl)-2,2-dimethyl-3-oxazolidinecarboxylate (88) and 1,1-Dimethylethyl (4R,1'S)-(1'-(tert-butyldimethysilyloxy)-3'-butenyl)-2,2-dimethyl-3-oxazolidinecarboxylate (89)

To 11.6 g (4.26 mmol) of the starting homoallylic alcohol in dry DMF (23.1 mL) was added imidazole (8.69 g, 0.128 mol) followed by *tert*-butyldimethysilyl chloride (8.98 g, 0.06 mol) and the mixture was stirred at room temperature for 24 hours. Analysis by TLC (hexanes/EtOAc, 4:1, detection: 2% PMA) showed

complete conversion of starting material (R_f 0.28) to product (R_f 0.81). Water (100 mL) was added and the solution extracted with Et_2O (3 x 500 mL). The combined organic layers were washed with saturated $NaHCO_3$ (3 x 500 mL), brine (1 x 500 mL), and dried over Na_2SO_4. Filtration and removal of the solvent afforded the diastereomers as a yellow oil (16.4 g, 100%). Both diastereomers were separated by flash chromatography (pentane/Et_2O, 9:1, 7 x 21 cm column, detection: 2% PMA) to afford 60% of the major diastereomer and 35% of the minor diastereomer. (**88**, major diastereomer) $[\alpha]_D^{25}$ +27.1° (*c* 3.84, $CHCl_3$); IR (neat) 1702, 1384 cm^{-1}; ^1H NMR (300 MHz, C_6D_6, 70 °C) δ (ppm) 0.11 (s, 3H), 0.12 (s, 3H), 0.84 (s, 9H), 1.42 (s, 9H), 1.46 (s, 3H), 1.70 (s, 3H), 2.31 (m, 1H), 2.49 (m, 1H), 3.80 (dd, *J* = 7.2 and 9.3 Hz, 1H), 4.01 (bs, 1H), 4.22 (dd, *J* = 2.2 and 9.4 Hz, 1H) 4.01 (bs, 1H), 5.07 (m, 2H), 5.91 (m, 1H); ^{13}C NMR (75 MHz, C_6D_6, 70 °C) δ (ppm) 18.6, 23.8, 26.5, 27.0, 29.0, 36.6, 61.7, 63.8, 72.2, 80.1, 95.3, 117.0, 137.3, 153.0; MS (FAB): *m/z* 386.2 (M + 1); HRMS (FAB): calcd for $C_{20}H_{40}NO_4Si$ 386.2727, obsd 386.2721. (**89**, minor diastereomer) $[\alpha]_D^{25}$ +50.7° (*c* 3.10, $CHCl_3$); IR (neat) 1695, 1363, 1254 cm^{-1}; ^1H NMR (300 MHz, C_6D_6, 70 °C) δ (ppm) 0.10 (s, 3H), 0.14 (s, 3H), 0.97 (s, 9H), 1.41 (s, 9H), 1.65 (s, 3H), 1.66 (m, 3H), 3.62 (bs, 2H), 3.70 (dd, *J* = 7.1 and 8.1 Hz, 1H), 3.98 (bs, 1H), 4.13 (dd, *J* = 3.3 and 8.4 Hz, 1H), 4.36 (bd, *J* = 6.2 Hz, 1H); ^{13}C NMR (75 MHz, C_6D_6, 70 °C) δ (ppm) -3.7, -3.7, 18.7, 25.2, 26.7, 27.5, 28.9, 38.9, 59.4, 62.0, 64.0, 70.0, 80.4, 94.9, 153.7; MS (FAB): *m/z* 386.2 (M + 1); HRMS (FAB): calcd for $C_{20}H_{40}NO_4Si$ 386.2727, obsd 386.2728.

1,1-Dimethylethyl (4R,1'R)-(1'-hydroxy-3'-butenyl)-2,2-dimethyl-3-oxazolidinecarboxylate (90)

To a solution of the TBDMS-protected homoallylic alcohol **87a** (0.519 g, 1.34 mmol) in dry THF (16 mL) at room temperature was added a solution of

tetra *n*-butylammonium fluoride in THF (3.36 mmol, 3.40 mL) with stirring. Upon completion of the addition, stirring was continued for two hours at room temperature. Analysis by TLC (hexanes/EtOAc, 3:1, detection: I_2) showed the desired product (R_f 0.19) at the expense of starting material. The reaction mixture was partitioned between water (100 mL) and Et_2O (150 mL). The organic layer was washed with saturated $NaHCO_3$ (3 x 100 mL), brine (1 x 100 mL), and dried over $MgSO_4$. Filtration and removal of the solvent afforded crude product (372 mg). Purification by flash chromatography (hexanes/EtOAc, 2:1, detection: I_2) afforded 363 mg (99%) of the desired product as an oil. $[\alpha]_D^{25}$ +19.2° (*c* 0.66, $CHCl_3$); IR (neat) 3472 (br), 3081, 2978, 2931, 1690 cm^{-1}; ^1H NMR (400 MHz, C_6D_6, 67 °C) δ (ppm) 1.38 (s, 9H), 1.44 (s, 3H), 1.62 (s, 3H), 2.09 (m, 1H), 2.30 (m, 1H), 3.62 (dd, *J* = 1.5 and 8.9 Hz, 1H), 3.70 (bd, *J* = 9.2 Hz, 1H), 3.91 (m, 1H), 5.00 (t, *J* = 1.3 Hz, 1H), 5.04 (m, 1H), 5.92 (m, 1H); ^{13}C NMR (100 MHz, C_6D_6, 67 °C) δ (ppm) 24.6, 27.5, 28.8, 39.1, 62.4, 65.2, 73.2, 80.8, 94.8, 117.5, 135.8, 154.5; MS (CI): *m/z* 272.1 (M + 1); HRMS (CI): calcd for $C_{14}H_{26}NO_4$ 272.1862, obsd 272.1863.

(R)-(+)-MTPA Ester of 1,1-Dimethylethyl (4R,1'R)-(1'-hydroxy-3'-butenyl)-2,2-dimethyl-3-oxazolidinecarboxylate (91)

To a solution of the secondary alcohol **90** (0.093 g, 0.34 mmol) in dry CH_2Cl_2 (2 mL) was added a catalytic amount of 4-dimethylamminopyridine (0.040 mmol, 5.0 mg), (*R*)-(+)-MTPA (0.391 mmol, 91 mg) and dicyclohexylcarbodiimide (0.37 mmol, 0.37 mL of a 1 M solution in CH_2Cl_2) and the mixture was stirred under argon for two hours. Analysis of the reaction mixture by TLC (hexanes/EtOAc, 3:1, detection: UV, I_2) showed conversion of the starting material to a UV-active compound (R_f 0.56). The organic layer was washed with saturated NH_4OH (1 x 2 mL), sat. $NaHCO_3$ (2 x 2 mL), brine (1 x 2 mL) and dried over sodium sulfate. Filtration and removal of the solvent first afforded a dark

yellow oil (143 mg), which was further purified by flash chromatography (hexanes/EtOAc, 3:1, 1 x 15 cm column, detection: UV) to afford 100 mg (60%) of the desired MTPA ester (**91**). $[\alpha]_D^{25}$ +29.2° (*c* 0.25, $CHCl_3$); IR (neat) cm^{-1}; ^1H NMR (300 MHz, C_6D_6, 70 °C) δ (ppm) 1.43 (s, 3H), 1.46 (s, 9H), 1.66 (s, 3H), 2.31 (m, 1H), 2.54 (m, 1H), 3.37 (s, 3H), 3.63 (dd, *J* = 6.8 and 9.7 Hz, 1H), 3.92 (dd, *J* = 1.7 and 9.9 Hz, 1H), 4.12 (bs, 1H), 4.89 (d, *J* = 10 Hz, 1H), 4.95 (dd, *J* = 1.3 and 17 Hz, 1H), 5.66 (m, 1H), 5.80 (m, 1H), 7.10 (m, 3H), 7.64 (d, *J* = 7.2 Hz, 2H); ^{13}C NMR (75 MHz, C_6D_6, 70 °C) δ (ppm) 23.5, 26.9, 28.8, 28.9, 33.7, 55.7, 58.8, 62.4, 64.1, 75.8, 81.0, 85.9, 95.4, 118.4, 122.8, 126.6, 130.2, 133.4, 133.8, 134.3, 152.8, 166.6; MS (FAB): *m/z* 488.2 (M + 1); HRMS (FAB): calcd for $C_{24}H_{33}F_3NO_6$ 488.2260, obsd 488.2268.

(S)-(-)-MTPA Ester of 1,1-Dimethylethyl (4R,1'R)-(1'-hydroxy-3'-butenyl)-2,2-dimethyl-3-oxazolidinecarboxylate (92)

Mosher ester **92** was prepared from the corresponding homoallylic alcohol (**90**) and (S)-(-)-MTPA using the same procedure outlined for the synthesis of **91**. Using 99 mg (0.36 mmol) of **90**, 210 mg of **92** (84%) was obtained. $[\alpha]_D^{25}$ -28.9° (*c* 0.55, $CHCl_3$); IR (neat) 3074, 2984, 2939, 1752, 1711, 1376 cm^{-1}; ^1H NMR (300 MHz, C_6D_6, 70 °C) δ (ppm) 1.39 (s, 3H), 1.48 (s, 9H), 1.64 (s, 3H), 2.35 (m, 1H), 2.58 (m, 1H), 3.44 (s, 3H), 3.53 (dd, *J* = 6.8 and 9.8 Hz), 3.80 (dd, *J* = 2.1 and 9.9 Hz), 4.08 (bs, 1H), 4.97 (d, *J* = 11 Hz), 5.02 (m, 2H), 5.74 (m, 1H), 5.84 (m, 1H), 7.08 (m, 3H), 7.64 (d, *J* = 7.9 Hz); ^{13}C NMR (75 MHz, C_6D_6, 70 °C) δ (ppm) 23.4, 26.8, 28.8, 33.3, 55.9, 58.4, 63.9, 75.6, 80.9, 85.8, 95.4, 118.3, 122.7, 126.6, 130.1, 133.5, 134.8, 152.7, 166.4; MS (FAB): *m/z* 488.2 (M + 1); HRMS (FAB): calcd for $C_{24}H_{33}F_3NO_6$ 488.2260, obsd 488.2269.

1,1-Dimethylethyl (4R,1'R)-4-(1'-(tert-butyldimethylsilyloxy)-3'-hydorxypropyl)-2,2-dimethyl-3-oxazolidinecarboxylate (88)

To a solution of **87a** (3.05 g, 0.0079 mol) in dry MeOH (106 mL) at -78 °C was bubbled ozone until the solution turned blue/gray in color. The excess ozone was removed by bubbling nitrogen through the solution at which point sodium borohydride (0.30 g, 0.0079 mol) was added. The reaction was stirred at -78 °C for one-half hour. This procedure was repeated twice more and then the mixture was allowed to slowly reach room temperature. Stirring was then continued at room temperature for twelve hours. The reaction was analyzed by TLC (hexanes/EtOAc, 3:1, detection: 2% PMA), which showed the desired product (R_f 0.55) at the expense of starting material. The excess sodium borohydride was destroyed by slowly adding saturated NH_4Cl and then the reaction mixture was transferred to a separatory funnel. The organic layer was washed with saturated $NaHCO_3$ (3 x 100 mL), brine (1 x 100 mL), and dried over Na_2SO_4. Filtration and removal of the solvent generated a light yellow oil which was purified by flash chromatography (hexanes/EtOAc, 3:1, 7 x 15 cm column, detection: 2% PMA) to obtain 2.81 g (91%) of the desired primary alcohol (**88**). $[\alpha]_D^{25}$ +17.9° (*c* 2.04, $CHCl_3$); IR (neat) 3493 (br), 1691, 1391 cm^{-1}; ^1H NMR (300 MHz, C_6D_6, 70 °C) δ (ppm) 0.14 (s, 6H), 0.93 (s, 9H), 1.44 (s, 9H), 1.46 (s, 3H), 1.71 (bs, 4H), 1.85 (m, 1H), 3.61 (t, *J* = 5.8 Hz, 2H), 3.81 (t, *J* = 7.8 Hz, 1H), 3.93 (q, *J* = 7.3 Hz, 1H), 4.02 (bs, 1H) 4.19 (bd, *J* = 9.4 Hz, 1H), 4.55 (bs, 1H); ^{13}C NMR (75 MHz, C_6D_6, 70 °C) δ (ppm) -4.24, -3.71, 18.5, 23.9, 26.5, 26.9, 29.0, 34.7, 60.2, 61.4, 63.9, 69.5, 80.3, 95.3, 153.1; MS (FAB): *m/z* 390.3 (M + 1); HRMS (FAB): calcd for $C_{19}H_{40}NO_5Si$ 390.2676, obsd 390.2675.

1,1-Dimethylethyl (4R,1'S)-4-(1'-(tert-butyldimethylsilyloxy)-3'-hydroxypropyl)-2,2-dimethyl-3-oxazolidinecarboxylate (89)

Compound **89** was prepared starting from diastereomer **87b** (1.99 g, 5.16 mmol), employing the procedure described for **93**. The primary alcohol **89** (R_f 0.46) was obtained in 94% yield (1.89 g): $[\alpha]_D^{25}$ +45.3° (*c* 0.96, $CHCl_3$); IR (neat) 3457 (br), 1695, 1370 cm^{-1}; ^1H NMR (300 MHz, C_6D_6, 70 °C) δ (ppm) 0.10 (s, 3H), 0.14 (s, 3H), 0.97 (s, 9H), 1.41 (s, 9H), 1.49 (s, 3H), 1.66 (s 3H), 1.67 (m, 3H), 3.62 (bs, 2H), 3.70 (dd, *J* = 6.9 and 8.2 Hz, 1H), 3.98 (bs, 1H), 4.13 (dd, J = 3.4 and 8.4 Hz, 1H), 4.36 (bd, *J* = 6.2 Hz, 1H); ^{13}C NMR (75 MHz, C_6D_6, 70 °C) δ (ppm) -3.73, 18.7, 25.2, 26.6, 27.5, 28.9, 38.9, 59.4, 62.0, 64.0, 70.0, 80.4, 94.9, 153.7; MS (FAB): *m/z* 390.3 (M + 1); HRMS (FAB): calcd for $C_{19}H_{40}NO_5Si$ 390.2676, obsd 390.2674.

1,1-Dimethylethyl (4R,1'R)-4-(1'-(tert-butyldimethylsilyloxy)-3'-(4-nitrobenzyloxy)-dihydroxypropyl)-2,2-dimethyl-3-oxazolidinecarboxylate (93)

To a solution of the primary alcohol **88** (60 mg, 0.15 mmol) in dry pyridine (10 mL) under an argon atmosphere at room temperature was added *p*-nitrobenzoyl chloride (40 mg, 0.22 mmol) and the reaction mixture was stirred at room temperature for one-half hour. Analysis of the reaction mixture by TLC (Hexanes/EtOAc, 4:1, detection: PMA, UV) showed complete conversion of starting material into a higher (R_f 0.67) UV-active component. The mixture was transferred to a separatory funnel and partitioned between water (10 mL) and CH_2Cl_2 (30 mL). The organic layer was washed with 1 M $CuSO_4$ (3 x 15 mL), water (3 x 15 mL), brine (1 x 15 mL) and then dried over anhydrous MgSO4. Filtration and removal of the solvent afforded a dark yellow oil (100 mg), which was purified by flash chromatography (Hexanes/EtOAc, 6:1, 15 x 1 cm column,

detection: PMA, UV) to afford 76 mg (92%) of the benzylated primary alcohol, **93**. $[\alpha]_D^{25}$ +15.7° (*c* 1.54, $CHCl_3$); IR (neat) 3111, 3078, 3054, 2957, 2859, 1729, 1697 cm^{-1}; ^1H NMR (300 MHz, C_6D_6, 70 °C) δ (ppm) 0.12 (s, 6H), 0.46 (bs, 1H), 0.93 (s, 9H), 1.37 (s, 9H), 1.43 (s, 3H), 1.70 (bs, 2H), 1.82 (m, 1H), 2.16 (m, 1H), 3.80 (dd, *J* = 7.1 and 9.4 Hz, 1H), 4.03 (m, 1H), 4.17 (dd, *J* = 2.0 and 9.5 Hz, 1H), 4.37 (m, 1H), 4.89 (m, 1H), 7.78 (d, *J* = 6.8 Hz, 2H), 7.88 (d, *J* = 9.0 Hz); ^{13}C NMR (75 MHz, C_6D_6, 70 °C) δ (ppm) -4.30, -3.67, 18.5, 24.0, 26.4, 27.1, 28.9, 31.0, 61.5, 63.6, 68.4, 80.4, 95.2, 123.8, 131.0, 136.3, 151.4, 153.0, 164.9; MS (CI): *m/z* 539 (M + 1); HRMS (CI): calcd for $C_{26}H_{43}N_2O_8Si$ 539.2789, obsd 539.2778.

1,1-Dimethylethyl (4R,1'R)-4-(1'-(tert-butyldimethylsilyloxy)-3'-(p-toluenesulfonyloxy)-dihydroxypropyl)-2,2-dimethyl-3-oxazolidinecarboxylate (94)

A total of 2.48 g (6.36 mmol) of the primary alcohol (**88**) were dissolved in dry pyridine (200 mL) followed by the addition of *p*-toluenesulfonyl chloride (1.94 g, 10.2 mmol) and the mixture stirred at room temperature under argon for twenty-four hours. Analysis of the reaction mixture by TLC (hexanes/EtOAc, 4:1, detection: PMA, UV) showed complete depletion of starting material so the reaction was transferred to a 1 L separatory funnel and $CHCl_3$ was added. The organic layer was quickly washed with cold 1 N HCl (3 x 200 mL) followed by water. The organic phase was then washed with saturated $NaHCO_3$ (3 x 200 mL) and brine (1 x 200 mL). The organic layer was dried over sodium sulfate, filtered, and the solvent removed to afford a dark brown syrup. The crude material was purified by flash chromatography (hexanes/EtOAc, 4:1, 6 x 16 cm column, detection: PMA, UV) to afford 1.61g (46%) of the desired primary tosylate (**94**). $[\alpha]_D^{25}$ +54.6° (*c* 2.82, $CHCl_3$); IR (neat) 2928, 1693 cm^{-1}; ^1H NMR (300 MHz, C_6D_6, 70 °C) δ (ppm) 0.09 (s, 6H), 0.92 (s, 9H), 1.41 (s, 3H), 1.45 (s,

9H), 1.60 (s, 3H), 1.92 (s, 3H), 2.05 (m, 1H), 3.73 (dd, *J* = 7.2 and 9.4 Hz), 3.95 (bs, 1H), 4.04 (dd, *J* = 1.7 and 9.4 Hz), 4.20 (q, *J* = 5.2 Hz, 1H), 4.48 (m, 1H), 6.81 (d, *J* = 8.0 Hz), 7.75 (d, *J* = 8.2 Hz); ^{13}C NMR (75 MHz, C_6D_6, 70 °C) δ (ppm) -4.3, -3.7, 18.5, 21.4, 23.6, 26.4, 26.9, 28.9, 31.7, 61.1, 63.5, 68.2, 80.6, 95.4, 130.1, 130.2, 135.5, 144.4, 153.0; MS (CI): *m/z* 415.1 (M + 1); HRMS (CI): calcd for $C_{19}H_{39}N_4O_4Si$ 415.2741, obsd 415.2734.

1,1-Dimethylethyl (4R,1'R)-4-(3'-azido-1'-(tert-butyldimethylsilyloxy)-propyl)-2,2-dimethyl-3-oxazolidinecarboxylate (95)

To a solution of the 94 **99** (1.61 g, 2.96 mmol) in dry DMF (40 mL) was added sodium azide (0.291 g, 4.44 mmol) and the mixture stirred at 90 °C for two hours. Analysis of the reaction mixture by TLC (hexanes/EtOAc, 4:1, detection: UV, PMA) showed complete conversion of starting material (R_f 0.42) to a high R_f compound (R_f 0.64). Upon cooling to room temperature, the mixture was partitioned between CH_2Cl_2 (75 mL) and water (75 mL). The organic layer was washed with saturated $NaHCO_3$ (2 x 100 mL), brine (1 x 100 mL), and dried over sodium sulfate. Filtration and removal of the solvent afforded a yellow oil, which was purified by flash chromatography (Hexanes/EtOAc, 4:1, 15 x 4 cm column, detection: PMA) to afford 1.18 g of the desired primary azide (**95**). $[\alpha]_D^{25}$ +47.9° (*c* 2.6, $CHCl_3$); IR (neat) 2097, 1691 cm^{-1}; ^{1}H NMR (300 MHz, C_6D_6, 70 °C) δ (ppm) 0.10 (s, 6H), 0.90 (s, 9H), 1.44 (s, 12H), 1.68 (s, 3H), 1.86 (m, 1H), 3.12 (m, 2H), 3.75 (t, *J* = 8.0 Hz, 1H), 3.98 (bs, 1H), 4.10 (d, *J* = 9.4 Hz, 1H), 4.44 (bs, 1H); ^{13}C NMR (75 MHz, C_6D_6, 70 °C) δ (ppm) -4.32, -3.72, 18.5, 23.7, 26.4, 26.9, 28.9, 30.8, 48.9, 61.3, 63.6, 68.8, 80.4, 95.3, 152.9; MS (FAB): *m/z* 415.1 (M + 1); HRMS (FAB): calcd for $C_{19}H_{39}N_4O_4Si$ 415.2741, obsd 415.2737.

1,1-Dimethylethyl (4R,1'R)-4-(3'-azido-(1'-tert-butyldimethylsilyloxy)-1'-hydroxypropyl)-2,2-dimethyl-3-oxazolidinecarboxylate (95) via Modified Mitsunobu Conditions

To a solution of the primary alcohol **88** (5.24 g, 0.0134 mol) in dry THF (200 mL) under argon atmosphere at 0 °C was added triphenylphosphine (4.23 g, 1.61 mmol) followed by diethylazodicarboxylate (2.5 mL, 1.61 mmol) and the mixture stirred at 0 °C in the dark for one-half hour. Diphenylphosphoryl azide (3.5 mL, 1.61 mmol) was then slowly added and the reaction mixture gradually warmed to room temperature, and then stirred in the dark overnight. Analysis of the reaction mixture by TLC (hexanes/EtOAc, 3:1, detection: 2% PMA) showed complete conversion of starting material to product (R_f 0.82). The solvent was removed and the resulting oil was purified by flash chromatography (hexanes/EtOAc, 4:1, 7 x 15 cm column, detection: 2% PMA) to afford 5.03 g (90%) of the desired azide (**95**). All physical data was identical to the primary azide prepared via nucleophilic displacement of the tosylate with sodium azide.

1,1-Dimethylethyl (4R,1'S)-4-(3'-azido-(1'-tert-butyldimethylsilyloxy)-1'-hydroxypropyl)-2,2-dimethyl-3-oxazolidinecarboxylate (96)

Diastereomer **96** was synthesized from compound **89** (1.89 g, 4.85 mmol), utilizing the Mitsunobu procedure outlined for the preparation of **95**. The desired azide **96** (R_f 0.75) was obtained in 90% yield (1.80 g). $[\alpha]_D^{25}$ +26.3° (*c* 2.48, $CHCl_3$); IR (neat) 2092, 1697 cm^{-1}; ^1H NMR (300 MHz, C_6D_6, 70 °C) δ (ppm) 0.06 (s, 3H), 0.10 (s, 3H), 0.93 (s, 9H), 1.43 (s, 9H), 1.49 (s, 3H), 1.68 (s, 2H), 3.11 (m, 1H), 3.26 (bs, 1H), 3.61 (dd, *J* = 6.7 and 8.2 Hz), 3.76 (bs, 1H), 4.00 (dd, *J* = 2.5 and 8.5 Hz), 4.17 (q, *J* = 4.7 Hz); ^{13}C NMR (75 MHz, C_6D_6, 70 °C) δ (ppm) -4.35, -4.25, 18.1, 24.4, 26.0, 27.3, 28.4, 34.2, 47.7, 61.2, 63.8, 69.8, 79.8, 94.4,

153.0; MS (CI): *m/z* 415.1 (M + 1); HRMS (CI): calcd for $C_{19}H_{39}N_4O_4Si$ 415.2736, obsd 415.2741.

1,1-Dimethylethyl (4R,1'S)-4-(3'-azido-1'-hydroxypropyl)-2,2-dimethyl-3-oxazolidinecarboxylate (97)

To a solution of the TBDMS-protected alcohol **96** (6.39 g, 1.54 mmol) in dry THF (129 mL) at room temperature was added tetra-*n*-butylammonium fluoride (1 M soln in THF, 38.5 mL, 3.85 mmol) and the mixture stirred at room temperature for 2.5 hours. Analysis by TLC (hexanes/EtOAc, 4:1, detection: 2% PMA) showed complete conversion of starting material to product (R_f 0.18). The mixture was partitioned between water (200 mL) and Et_2O (500 mL). The organic layer was washed with sat. $NaHCO_3$ (2 x 200 mL), brine (1 x 200 mL), and dried over $MgSO_4$. Filtration and removal of the solvent afforded a light yellow oil (7.63 g), which was purified by flash chromatography (Hexanes/EtOAc, 4:1, 7 x 15 cm column, detection: 2% PMA). A total of 4.33 g (94%) of the desired product (**97**) was obtained. $[\alpha]_D^{25}$ +24.3° (*c* 1.21, $CHCl_3$); IR (neat) 3451, 2099, 1695 cm^{-1}; ^1H NMR (400 MHz, C_6D_6, 70 °C) δ (ppm) 1.33 (s, 9H), 1.36 (s, 3H), 1.41 (m, 1H), 1.48 (m, 1H), 1.52 (s, 3H), 3.15 (m, 2H), 3.54 (bd, *J* = 5.6 Hz), 3.69 (m, 1H), 3.77 (bs, 1H); ^{13}C NMR (100 MHz, C_6D_6, 70 °C) δ (ppm) 24.4, 27.2, 28.7, 33.2, 49.2, 62.9, 65.2, 70.9, 81.0, 95.0, 154.3; MS (CI): *m/z* 301.1 (M + 1); HRMS (CI): calcd for $C_{13}H_{25}N_4O_4$ 301.1876, obsd 301.1878.

1,1-Dimethyl (4R,1'S)-4-(1'-(methanesulfonyloxy)-3'-azido-propyl)-2,2-dimethyl-3-oxazolidinecarboxylate (100).

The secondary alcohol **97** was dissolved in dry CH_2Cl_2 (6.0 mL) followed by the addition of dry triethylamine (1.22 mL, 8.74 mmol). The solution was

cooled to -48 °C and then methanesulfonyl chloride (0.260 mL, 3.30 mmol) was added dropwise with stirring over fifteen minutes. The reaction was slowly allowed to warm up to 0 °C over the course of two hours. Analysis of the reaction mixture by TLC (hexanes/EtOAc, 3:1, detection: 2% PMA) showed complete conversion of starting material (R_f 0.33) to a lower R_f compound (R_f 0.28). The reaction was partitioned between water (35 mL) and CH_2Cl_2 (75 mL) and then the organic layer was washed with water (1 x 35 mL), and dried over anhydrous magnesium sulfate. Filtration and removal of the solvent afforded a dark yellow oil (942 mg), which was purified by flash chromatography (hexanes/EtOAc, 2:1, 15 x 3 cm column, detection: PMA) to afford 615 mg of the desired mesylate (**100**). $[\alpha]_D^{25}$ +23.8° (*c* 1.34, $CHCl_3$); IR (neat) 2107, 1692 cm^{-1}; ^1H NMR (400 MHz, C_6D_6, 70 °C) δ (ppm) 1.41(S, 9 H), 1.43 (s, 3H), 1.62 (s, 3H), 2.41(s, 3H), 3.08 (m, 2H), 3.56 (dd, *J* = 6.8 and 9.0 Hz, 1H), 3.75 (bs, 1H), 3.79 (dd, *J* = 3.8 and 9.1 Hz, 1H), 5.17 (m, 1H); ^{13}C NMR (100 MHz, C_6D_6, 70 °C) δ (ppm) 24.6, 27.1, 28.7, 32.4, 38.6, 48.1, 60.2, 63.7, 78.2, 81.0, 95.3, 153.0; MS (CI): *m/z* 379 (M + 1); HRMS (CI): calcd for $C_{14}H_{27}N_4O_6S$ 379.1651, obsd 379.1656.

1,1-Dimethylethyl (4R,1'S)-4-1',3'-diazidopropyl)-2,2-dimethyl-3-oxazolidinecarboxylate (99)

The secondary mesylate **100** (3.04 g, 8.03 mmol) was dissolved in dry dimethylformamide (80 mL) followed by the addition of lithium azide (1.04 g, 21.3 mmol). The mixture was heated at 90 °C for twenty-four hours and then monitored by TLC (hexanes/EtOAc, 3:1, detection: 2% PMA), which showed the conversion of starting material to a major component at R_f 0.78. The reaction mixture was transferred to a separatory funnel and partitioned between water (200 mL) and Et_2O (500 mL). The organic layer was washed with water (2 x 200 mL), brine (1 x 200 mL) and dried over sodium sulfate. Filtration and removal of

the solvent afforded a dark yellow oil (1.41 g), which was purified by flash chromatography (hexanes/EtOAc, 7:1, 7 x 18 cm column, detection: 2% PMA) to afford 0.917 g (35%) of the desired product (**99**). $[\alpha]_D^{25}$ +37.2° (*c* 1.15, $CHCl_3$); IR (neat) 2146, 2098, 1697 cm^{-1}; ^1H NMR (400 MHz, C_6D_6, 70 °C) δ (ppm) 1.18 (m, 2H), 1.40 (s, 9H), 1.47 (s, 3H), 1.68 (s, 3H), 2.84 (m, 2H), 3.50 (m, 1H), 3.68 (dd, *J* = 3.2 and 9.4 Hz, 1H), 4.00 (bs, 1H); ^{13}C NMR (100 MHz, C_6D_6, 70 °C) δ (ppm) 23.3, 26.8, 28.4, 28.9, 48.7, 59.7, 61.2, 64.0, 80.5, 94.9, 152.4; MS (CI): *m/z* 326.2 (M + 1); HRMS (CI): calcd for $C_{13}H_{24}N_7O_3$ 326.1941, obsd 326.1940.

1,1-Dimethylethyl (4R,1'S)-4-(1',3'-diaminopropyl)-2,2-dimethyl-3-oxazolidinecarboxylate (101)

To a solution of the diazide **99** (0.917 g, 2.82 mmol) in THF (25 mL) was added triphenylphosphine (2.01 g, 6.18 mmol) and water (0.11 g, 6.18 mmol) and the mixture refluxed for sixteen hours. Analysis of the reaction mixture by TLC (CH_2Cl_2/MeOH/NH_4OH, 8:1:1, detection: 0.2% ethanolic ninhydrin) showed complete conversion of starting material to product at R_f 0.24. The solvent was removed, and the resulting yellow oil (3.15 g) was purified by flash chromatography (CH_2Cl_2/MeOH/NH_4OH, 8:1:1; column = 4 x 15 cm, detection: 0.2% ethanolic ninhydrin) to afford the desired diamine (**101**, 0.700 g, 100%) as yellow oil. $[\alpha]_D^{25}$ +26.2° (*c* 2.10, $CHCl_3$); IR (neat) 3405 (br), 1693 cm^{-1}; ^1H NMR (300 MHz, C_6D_6, 70 °C) δ 0.71 (bs, 5H), 1.14 (m, 1H), 1.29 (m, 1H), 1.42 (s, 9H), 1.52 (s, 3H), 1.67 (s, 3H), 2.64 (bs, 1H), 3.09 (bs, 1H), 3.28 (bs, 1H), 3.62 (t, *J* = 8.4 Hz, 1H), 3.69 (bs, 1H), 3.83 (dd, J = 1.9 and 8.5 Hz, 1H); ^{13}C NMR (75 MHz, C_6D_6, 70 °C) δ 24.4, 27.5, 28.9, 38.8, 40.5, 51.3, 63.2, 64.2, 80.0, 94.5, 153.3; MS (FAB): *m/z* 274.1 (M + 1); HRMS (FAB): calcd for $C_{13}H_{28}N_3O_3$ 274.2131, obsd 274.2133.

***L*-[guanidino-^{13}C]Capreomycidine**

A crude cyanogen bromide solution (2 mmol in 4 mL water) was slowly added to a solution of the diamine **101** (0.200 g, 0.730 mmol) in 21 mL water at pH 11.5 over 2.5 hours. During the addition, the reaction was continually adjusted to pH 11.5 using 1 N NaOH whenever the pH of the solution fell below 11.0. Upon completion of the addition, the mixture was stirred at room temperature for an additional hour and then transferred to a separatory funnel. The aqueous solution was extracted with Et_2O (3 x 100 mL), the combined organic layers were washed with water (1 x 100 mL), and then the aqueous washings were combined. The solvent from the aqueous washes was removed to afford a white, oily residue (1.05 g). The residue was washed with 100% ethanol (3 x 25 mL) and the solvent from the washings was removed in vacuo to afford a light tan residue (0.686 g). The residue was dissolved in water (8 mL) and 2 mL of the solution was loaded onto a Varian Mega Bond Elute C18 column (500 mg). The column was washed with water (3 x 3 mL) followed by elution with acetonitrile with 3 mL fractions being collected. The collected fractions were analyzed by TLC (*n*-BuOH/H_2O/AcOH, 4:2:1, detection: 0.2% ethanolic ninhydrin). Those fractions that contained the cyclized product at R_f 0.79 were pooled and the solvent removed to afford 150 mg of the desired product as the hydrobromide salt. Based upon ^{13}C NMR, the crude material was about 95% pure.

The crude residue was dissolved in 6 N HCl (10 mL) and allowed to stand for two hours at room temperature. Analysis of the solution by TLC (*n*-BuOH/H_2O/AcOH, 4:2:1, detection: 0.2% ethanolic ninhydrin) showed the conversion of starting material (R_f 0.82) to a ninhydrin-positive spot at the origin, so the solvent was removed and the resulting residue was dissolved in 1 M NaOH (5 mL) and cooled to 4 °C. To this was added BOC-anhydride (2.19 mmol, 480 mg) in dioxane (5 mL). The reaction was stirred at room temperature for four hours and then potassium permanganate (1.40 mmol, 64 mg) was added

and the mixture stirred for an additional six hours. The manganese dioxide was filtered off and then the filtrate was acidified with 6 N HCl to pH 1. After standing at room temperature for two hours, the solvent was removed to afford a white residue (140 mg). The residue was dissolved in water (25 mL) and then loaded onto an AG50W-X8 column (H^+ form, 13 x 2.5 cm) and then the column was washed with water (300 mL). The column was then eluted with a pH gradient (pH 7-9) of 0.14 NH_4OAc, taking 10 mL fractions. Those fractions that were ninhydrin-positive were pooled and the solvent removed to afford 15 mg (10% from diamine **106)** of the desired amino acid, L-capreomycidine. ^1H NMR (300 MHz, D_2O, 25 °C) δ (ppm) 2.05 (m, 1H), 2.21 (m, 1H), 3.49 (m, 2H), 3.90 (d, J = 5.3 Hz, 1H), 4.12 (m, 1H); ^{13}C NMR (75 MHz, D_2O, 25 °C) δ (ppm) 23.4, 37.9, 50.1, 58.2, 156.0, 172.4; MS (FAB): *m/z* 174.1 (M + 1); HRMS (FAB): calcd for $C_5{}^{13}CH_{13}N_4O_2$ 174.1072, obsd 174.1073.

Incorporation Study Using [guanidino-^{13}C]Capreomycidine (17), First Experiment

The generation of seed and production media for S. L-1689-23 has been described in Chapter 2. A total of 2 100 mL production flasks were prepared and inoculated with 8% seed medium, which had been prepared 64 hours earlier. The inoculated production flasks were incubated at 29 °C and 225 rpm for 12 hours, whereupon, 18 mg of [*guanidino*-^{13}C]capreomycidine in 2 mL Milli-Q water was divided between the two flasks and added aseptically via passage through a sterile filter. The production flasks were incubated at 29 °C and 225 rpm for an additional 36 hours and then worked up as previously described. After purification, a total of 13 mg of STF was obtained. The isolated antibiotic was analyzed by ^{13}C NMR spectroscopy.

Incorporation Study Using [guandino-^{13}C]Capreomycidine (17), Second Experiment

Using the remaining 18 mg of [*guanidino*-^{13}C]capreomycidine, a second incorporation study was performed as described previously. From a total of 200 mL of production medium was obtained only 8 mg of antibiotic. The antibiotic was analyzed by ^{13}C NMR spectroscopy to determine the extent of incorporation.

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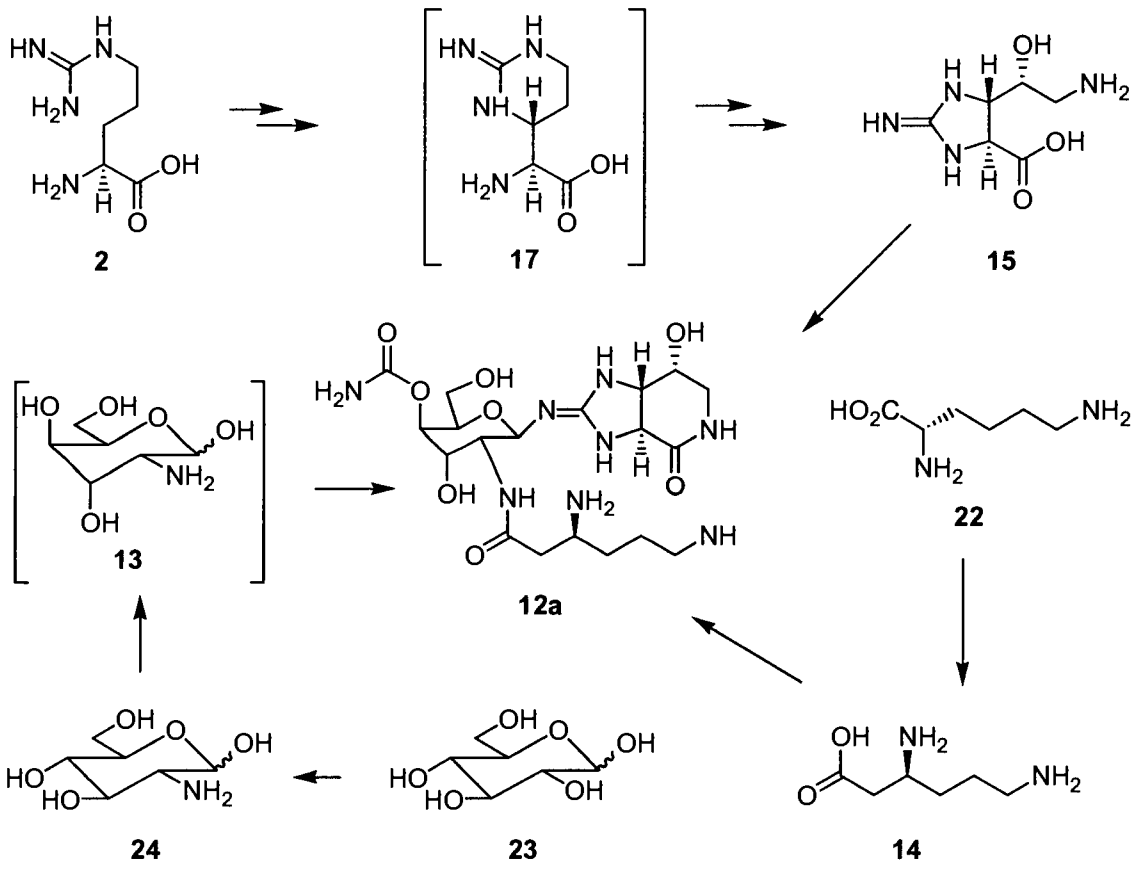
Chapter 5
Conclusions and Areas of Further Study

Through a combined approach using whole-cell incorporation studies and cell-free investigations, the intermediacy of streptolidine (**15**) and capreomycidine (**17**) in the biosynthesis of streptothricin F was investigated. From the results described in the previous chapters, the biosynthetic pathway leading to streptothricin F has been re-evaluated.

Scheme 5.1. Summary of the Biosynthetic Pathway to STF (**12a**)



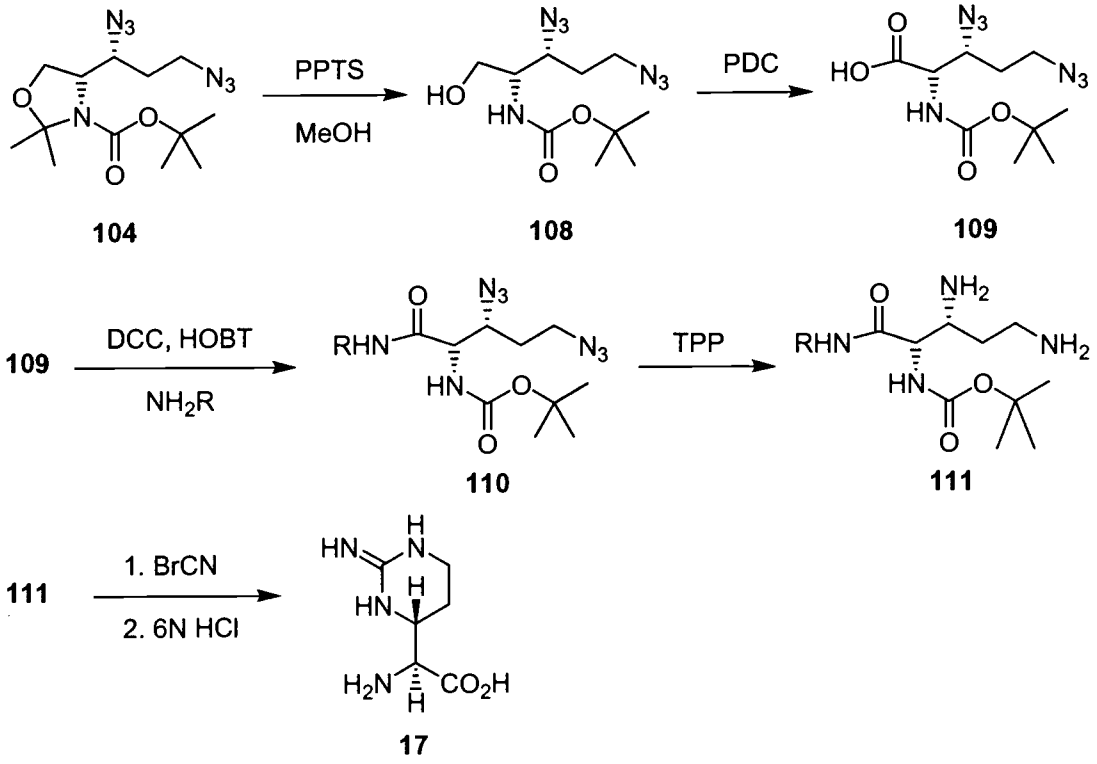
As shown in Scheme 5.1, the intermediacy of **15** in the biosynthesis of **12a** has been confirmed through studies using [*guanidino*-^{13}C]streptolidine. From the observed incorporation of labeled **15**, additional support for a convergent biosynthesis of **12a** has been obtained.

The intermediacy of capreomycidine (**17**) in the biosynthesis of streptolidine was also examined using cell-free extracts of *Streptomyces* L-1689-23. A HPLC protocol for the analysis of enzyme assays was developed and enzyme activity related to the formation of three compounds was observed. Two of the trhree compounds were identified as citrulline and ornithine, products formed from the degradation of arginine. One possible enzyme known to catalyze the conversion of arginine to citrulline is arginine deiminase, which has not been previously identified in *Streptomyces*.

To further probe whether capreomycidine is an intermediate to **15**, a new synthesis of **17** was developed. Using Garner's aldehyde (**85**), (2*S*,3*R*)-[*guanidino*-^{13}C]capreomycidine was synthesized and introduced to production cultures of *S.* L-1689-23. No incorporation of labeled **17** was observed. Because the incorporation study was performed with a single pulse of labeled compound, it is possible that the enzyme(s) responsible for the formation of streptolidine were not present at the time **17** was administered. Future work with capreomycidine may be successful if multiple pulses are administered prior to isolation of the antibiotic.

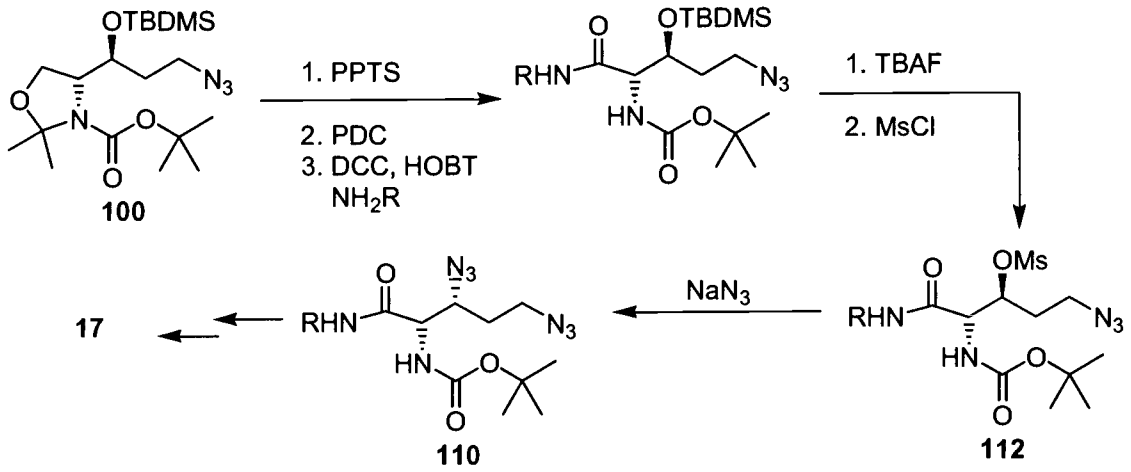
The current synthesis of capreomycidine, while efficient in the early steps, turned out to be inefficient at the end of the synthetic sequence. One possible way around this problem may be to change the order in which reaction with cyanogen bromide and final oxidation occur. Prior to reduction of the diazide (**104**), selective deprotection could afford primary alcohol **108**, which could then be oxidized to the carboxylic acid (**109**). After protection of the acid as an amide (**110**), the azides could be reduced to afford the desired diamine **111**. Addition of cyanogen bromide followed by acid hydrolysis should then generate capreomycidine.

Scheme 5.2. Alternate Route to Capreomycidine via Diazide **104**



If the oxazolidine was deprotected at the stage of the primary azide (**100**, Scheme 5.3), the formation of the diazide (**110**) from the corresponding mesylate (**112**) might also be improved.

Scheme 5.3. Alternate Route to Capreomycidine via Azide **100**



If adequate amounts of capreomycidine can be obtained, the intermediacy of capreomycidine in the biosynthesis of the tuberactinomycin class of antibiotics, capreomycidine 1A and 1B can also be examined.

To further identify potential biosynthetic intermediates in the formation of streptothricin F (**12a**), inhibitors of enzymes expected to be involved in the biosynthesis of **12a** could be added to production flasks of *S.* L-1689-23. This approach has been successful in identifying intermediates in blasticidin S biosynthesis.{ } Potential enzymes that could be targeted are those involved in lysine biosynthesis, lysine 2,3-aminomutase and carbamoylphosphate synthase. Aliquots of production medium obtained after introduction of the inhibitor could be derivatized as described in chapter two and analyzed using our HPLC protocol.

One additional experiment that can also be proposed is testing if (4*R*)-hydroxycapreomycidine is a reasonable intermediate to **15**. This compound could be used to determine when the oxidation that C-4 occurs in the conversion of arginine to streptolidine.

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