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DELINEATION OF ASPECTS OF KANOSAMINE BIOSYNTHESIS

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John W. Frost

Major Professor's Signature

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DELINEATION OF ASPECTS OF KANOSAMINE BIOSYNTHESIS

By

Xiaofei Jia

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DELINEATION OF ASPECTS OF KANOSAMINE BIOSYNTHESIS

By

Xiaofei Jia

The syntheses of kanosamine 1-phosphate and uridine 5'-diphospho-3-amino-3-deoxy-α-D-glucose (UDP-kanosamine), possible intermediates in the biosynthesis of kanosamine, are detailed. UDP-kanosamine is established to be the biosynthetic precursor to kanosamine in both *Amycolatopsis mediterranei* and *Bacillus pumilus*. RifM from *Amycolatopsis mediterranei* is demonstrated to be the phosphatase responsible for the conversion of UDP-kanosamine into kanosamine. Kanosamine is the source of nitrogen for the biosynthesis of the ansamycins, which is a structurally diverse family of bioactive natural products.

To my family and friends

For their love and support

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LIST OF ABBREVIATIONS

Ac	acetyl
AHBA	3-amino-5-hydroxybenzoic acid
aminoDAHP	4-amino-3,4-dideoxy-D-*arabino*-heptulosonic acid 7-phosphate
aminoDHQ	5-amino-5-deoxy-3-dehydroquinate
aminoDHS	5-amino-5-deoxy-3-dehydroshikimate
aminoF6P	3-amino-3-deoxy-D-fructose 6-phosphate
aminoS7P	4-amino-4-deoxy-sedoheptulose 7-phosphate
aminoSA	5-amino-5-deoxyshikimate
Amp	ampicillin
bp	base pair
Ca	carbenicillin
Cm	chloramphenicol
DAHP	3-deoxy-D-*arabino*-heptulosonic acid 7- phosphate
DCIP	dichloroindophenol
DEAE	diethylaminoethyl
DHQ	3-dehydroquinate
DHS	3-dehydroshikimate
DMAP	4-(dimethylamino)pyridine
DMF	dimethylformamide
DNA	3-deoxyribonucleic acid
DO	dissolved oxygen
DTT	dithiothreitol

E4P	D-erythrose 4-phosphate
Ery	erythromycin
F6P	D-fructose 6-phophate
FAB	fast atom bombardment
FBR	feed back resistant
G6P	D-glucose 6-phosphate
Gln	L-glutamine
h	hour
His	L-histidine
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
iminoE4P	1-deoxy-1-imino-D-erythrose 4-phosphate
IPTG	isopropyl β-D-thiogalactopyranoside
K1P	kanosamine 1-phosphate
K6P	kanosamine 6-phosphate
Kan	kanamycin
kg	kilogram
LB	Luria Broth
M	molar
mg	milligram
mL	milliliter
μL	microliter
mM	millimolar

MS	mass spectrometry
min	minute
NAD	nicotinamide adenine dinucleotide, oxidized form
NADH	nicotinamide adenine dinucleotide, reduced form
NADP	nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NMR	nuclear magnetic resonance
OD	optical density
PEP	phosphoenolpyruvate
PCR	polymerase chain reaction
P_i	inorganic phosphate
PMS	phenazine methosulfate
PP_i	inorganic pyrophosphate
ppm	parts per million
psi	pounds per square inch
R5P	D-ribose 5-phosphate
RNA	ribonucleic acid
rpm	revolutions per minute
rt	room temperature
S7P	D-sedoheptulose 7-phosphate
SA	shikimate
SDS	sodium dodecyl sulfate
Tc	tetracycline

THF	tetrahydrofuran
TLC	thin-layer chromatography
TMS	trimethylsilyl
TSP	sodium 3-(trimethylsilyl)propionic acid-2,2,3,3-d_4
UDP	uridine 5'-diphosphate
UDPK	uridine 5'-diphospho-3-amino-3-deoxy-α-D-glucose (UDP-kanosamine)
UMP	uridine 5'-monophosphate
UTP	uridine 5'-triphosphate
UV	ultraviol

CHAPTER ONE

INTRODUCTION

Natural products represent a rich source of chemical diversity and potential drug leads. Many important medicines, particularly in the anti-infective, anticancer and hypertension areas, have come from natural product sources. The ansamycins[1] are a remarkable group of natural products, derived from microorganisms. A large variety of biological effects have shown to be caused by ansamycins. Given their important medicinal applications, understanding the way ansamycins are put together by living organisms has been vigorously pursued by scientists.[2]

The work carried out in completion of this thesis focused on elaborating the precursors to the aminoshikimate pathway, which results in the formation of 3-amino-5-hydroxybenzoate, a biosynthetic precursor to the ansamycins.[3] Chapter One will present an overview of ansamycin biosynthesis and the origin of the aromatic starter unit of ansamycin antibiotics, which is derived from the aminoshikimate pathway. Chapter Two details research that elaborates the biosynthetic precursors to kanosamine biosynthesis. Two classical methods used in the study of natural product biosynthesis are applied in this chapter. The first method comprises synthesizing the putative biosynthetic precursor and incubating it in *Amycolatopsis mediterranei* and *Bacillus pumilus* cell-free lysates, which is then analyzed for the formation of kanosamine biosynthetic intermediates. The second method involves isolating and characterizing enzymes associated with this pathway. By combining the results obtained in this research, UDP-kanosamine (uridine

5'-diphospho-3-amino-3-deoxy-α-D-glucose) is confirmed as the biosynthetic precursor to kanosamine. RifM is also identified as a specific UDP-kanosamine phosphatase.

Biosynthetic studies on natural products

Natural products have served as major sources of medicinal agents for centuries. The first records, written on hundreds of clay tablets in cuneiform, are from Mesopotamia and date from about 2600 BC. However, it was not until the early 1800's that the active principles from plants were isolated. Among the first active principles to be isolated were strychnine, morphine, atropine, and colchicine. This resulted in E. Merck producing the first commercially pure natural product, morphine, in 1826.[4] Following that, the first natural product-based semi-synthetic pure drug, aspirin, was introduced by Bayer in 1899.[5]

Natural or semi-synthetic products derived from microorganisms and used in the pharmaceutical industry have a shorter history. Their impact on medicine has only dated back about 60 years ago to the introduction of penicillin.[6] Since its discovery, it was suddenly possible to treat diseases that before had been untreatable and sometimes even deadly. After the discovery of penicillin, numerous commercial antibiotics and anticancer drugs have been developed, bringing a "golden age of antibiotics". To name a few: streptomycin, chlorotetracycline, erythromycin A (antibacterial), chloroquine, artemisinin (antimalarial), spongouridine, spongothymidine (precursors for antiviral drug AZT), podophyllotoxin, etoposide, teniposide, vinblasine, vincristine, taxol, (+)-discodermolide, and epothilone A (anticancer).

Natural products continue to play a dominant role in the drug discovery and development process. Between 1983 and 1994, 41% of newly approved drugs have natural products as their source. This was particularly evident in the areas of cancer and infectious diseases, where over 60% and 75% of these drugs, respectively, were shown to be of natural origin.[7]

Biosynthetic studies on natural products are therefore of essential importance. By understanding the biosynthetic pathways, scientists are able to gain useful knowledge on the chemistry performed by the cell machinery to produce these compounds. Beyond suggesting new approaches to the chemical total synthesis of complex organic molecules (biomimetic chemistry), the insights gained from such biosynthetic studies may lead to improved methods for the production of medicinally important natural products. Amplified expression of biosynthetic gene clusters and genetic modification of biosynthetic pathways have become widely employed alternatives for improving microbial synthesis of natural products.[8] For example, an increase of 176% in the penicillin productivity (g/L) was achieved when the whole penicillin gene cluster was amplified in *Penicillium chrysogenum* Wis 54-1255.[9] This increase in productivity of penicillin corresponds to about 5 years (1972–1977) of strain improvement using random mutagenesis.[10]

Biosynthetic studies on natural products may also enable scientists to manipulate the pathway to produce analogues of known natural products, which may possess unique biological activities. This has become important in terms of overcoming the increasing resistance to existing antibiotics developed by microbes. Together with semi-synthetic modifications of existing natural products, total syntheses and combinatorial syntheses,

another way of exploring new drugs is manipulation of biosynthetic pathways to produce analogues of natural products. The most notable class under study is the manipulation of polyketide biosynthetic gene clusters.[11] By replacement of acyltransferases or replacement of the starter units, the structure of complex polyketides can be changed in a predictable manner.[12] Diversity could also be achieved through this so-called "combinatorial biosynthesis". More importantly, the analogues produced in this manner structurally resemble original natural products, which have been selected as biologically active compounds through long lasting evolution. Manipulation of biosynthetic pathways, however, is still in its infancy and no drugs have been discovered through this approach.

In addition, biocatalytic synthesis of intermediates in biosynthetic pathways can provide new starting materials for chemical synthesis. For example, shikimate is a seven-carbon carbocyclic intermediate in the common pathway of aromatic amino acid biosynthesis.[13] The stereogenic centers and functional groups in shikimate make it an attractive chiral synthon for use in various synthetic schemes.[14] It is originally isolated from the fruits of the *Illicium* plants,[15] which complicated its commercial use as a starting material for the synthesis of the neuraminidase inhibitor Tamiflu.[16] Based on the biosynthetic database available for the shikimate pathway, recombinant *E. coli* biocatalysts have been designed, which are capable of producing high concentrations of shikimate from glucose in good yields.[17] This microbial synthesis enables shikimate to be a viable starting material in the commercial synthesis of Tamiflu.

Ansamycin Biosynthesis

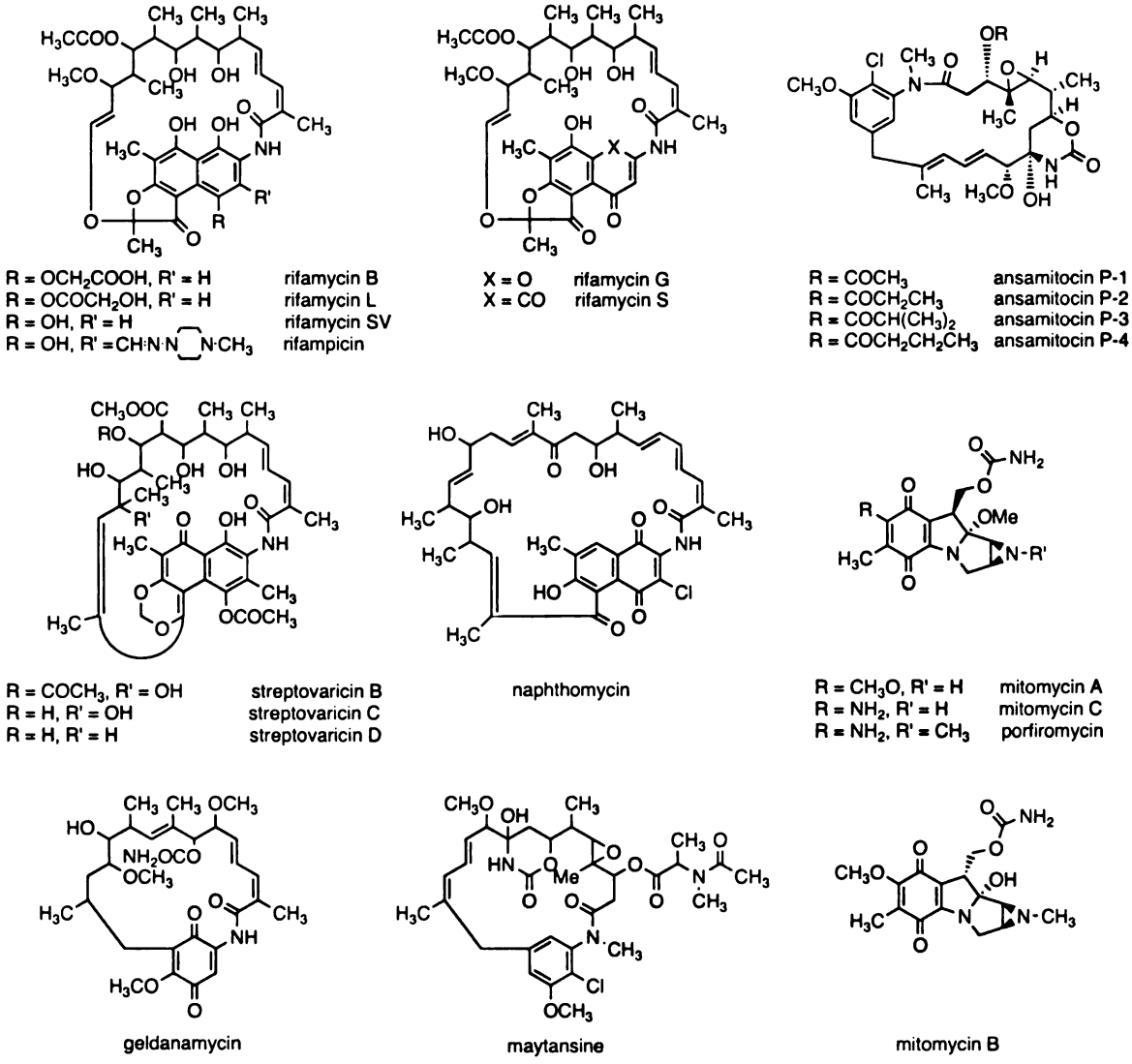

Figure 2. Structures of several ansamycins.

The ansamycins are a remarkable group of natural compounds varying widely in both their chemical structures and their biological activities. They have mostly been isolated from prokaryotic microorganisms, but one group, the maytansines, occurs in plants.[18] The ansamycin antibiotics (Figure 1) derived their name from their characteristic molecular structures, which consist of an aromatic nucleus and a long

aliphatic *ansa* bridge joining two opposite positions of the nucleus (chromophore). Two types of aromatic units can be distinguished based on the structure of the chromophore: a naphthalenic ring system (as in rifamycins, streptovaricins, tolypomycins, halomicins, naphthomycin, actamycin, and rubransarols) or a benzenic ring system (as in geldanamycin, herbimycins, macbecins, ansatrienins, mycotrienins, maytansinoids, and ansamitocins).

Ansamycins have been shown to cause a large variety of biological effects on bacteria, eukaryotes and viruses.[1c] The most powerful and selective mode of action is the specific inhibition of bacterial RNA synthesis by rifamycins, streptovaricins, tolypomycins, etc. Detailed investigations have shown that DNA-dependent RNA polymerase, the enzyme responsible for DNA transcription, forms a very stable 1:1 complex with these ansamycins and as a consequence, is inactivated.[19] Eukaryotic and viral enzymes do not interact with the drug in the same manner. The maytansines have been found to exert a very potent antimitotic action on eukaryotic cells and to show interesting antitumor activity.[18a] Besides these two, many other biological effects caused by ansamycins have been reported. The origin and biological activities of several ansamycins are listed in Table 1.

Rifamycins were first isolated[20] from *Amycolatopsis mediterranei* (*Nocardia mediterranei*) as a complex mixture (rifamycins A-E) in the Lepetit Research Laboratories in 1957. Addition of diethylbarbiturate to the fermentation medium led to the sole production of rifamycin B,[21] which was obtained in crystalline form. Its structure has been determined by chemical[22] and X-ray analysis[23]. They are the first isolated compounds of the ansamycins family. The rifamycins, as well as streptovaricins and

tolypomycins, specifically inhibit DNA-dependent RNA polymerases at the initiation step at very low concentrations (10^{-8} M). These antibiotics also have antiviral and antitumor activities, but only at very high concentrations.[1c] Although rifamycin B, the first isolated rifamycin, has very low antibacterial activities, the clinical application of the semisynthetic rifamycin, rifampicin (Figure 1), has been proven to be an excellent, orally active antibiotic and is now in widespread clinical use, especially in the treatment of tuberculosis in combination with other drugs.[24]

Table 1. Origin and biological activities of ansamycins.

Compound	Origin	Biological activities
Rifamycins	*Amycolatopsis mediterranei* *Micromonospora halophytica* *Streptomyces tolypophorus*	Antibacterial (antifungal, antiviral, antitumor)
Streptovaricins	*Streptomyces spectabilis*	Antibacterial (antifungal, antiviral, antitumor)
Tolypomycin	*Streptomyces tolypophorus*	Antibacterial
Naphthomycin	*Streptomyces collinus*	Antibacterial, antifungal
Geldanamycin	*Streptomyces hygroscopicus*	Antibacterial, antiprotozoal
Maytansines	*Maytenus serrata* *Maytenus buchananii* *Putterlickia verrucosa* *Colubrina texensis*	Antimitotic, antileukaemic, antitumor

The structure of the *ansa* chain in ansamycins suggests a polyketide synthesis origin. By incorporation of ^{14}C- and ^{3}H-labeled precursors followed by chemical degradation[25] and by ^{13}C-labeled precursors combined with ^{13}C-NMR spectroscopy,[26] it has been shown that the *ansa* chain of rifamycin S is derived from propionate, acetate and methionine, as depicted in Figure 2. Eight propionate units are incorporated into the *ansa*

chain (one of which has lost its methyl from C28 during biosynthesis) via methylmalonyl coenzyme A. C12, C13 and C29 originate from the same propionate unit, which is later split off by the introduction of oxygen between C12 and C29. Two acetate groups are incorporated through malonyl coenzyme A and another acetate group through acetyl coenzyme A (C35, C36). The methyl group, C37, comes from methionine. Interestingly, a seven-carbon amino unit including C1-C4 and C8-C10 of the naphthoquinone part of rifamycin S is not derived from acetate/propionate units.

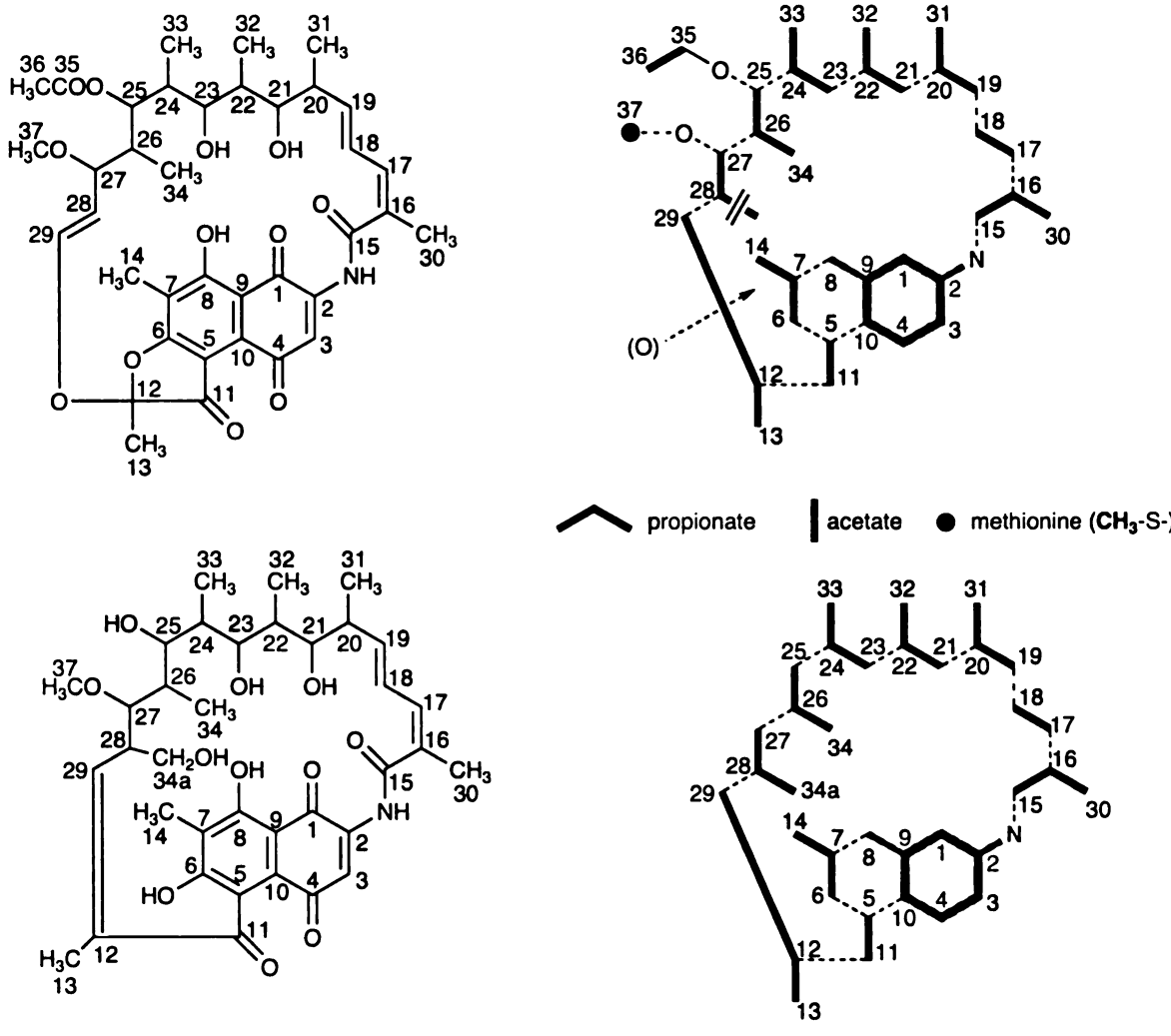

Figure 3. Incorporation of acetate/propionate/methionine into rifamycin S and rifamycin W.

From a mutant strain of *A. mediterranei*, a biogenetic precursor of rifamycin S was isolated and designated rifamycin W.[27] In transformation experiments it was demonstrated that radiolabeled rifamycin W was transformed into labeled rifamycin B via rifamycin S. The structure of rifamycin W is in accordance with the biogenetic model derived from the incorporation pattern of acetate/propionate into rifamycin S. C34a is still present in rifamycin W and the carbons C12, C13 and C29 are not yet separated by the introduction of oxygen. Rifamycin W showed an incorporation pattern identical to the pattern found earlier for rifamycin S.[28]

Biosynthetic studies on streptovaricins,[29] antamycin,[30] geldanamycin,[31] herbimycin A[32] and micotrienins[33] also indicated that the *ansa* chain is derived from acetate (or in geldanamycin glycerate/glycolate) and propionate units, whereas the remaining seven-carbon amino (mC_7N) unit must be synthesized from a different biosynthetic pathway.

Even though propionate and acetate are not incorporated into the mC_7N unit, radiolabeled [3,4-$^{14}C_2$]glucose and [1-^{14}C]glycerate did show good incorporation into this unit.[34] Later the incorporation patterns of [1-^{13}C]glucose and [1-^{13}C]glycerate were studied using ^{13}C-NMR.[35] The results showed that among the seven carbons in the mC_7N unit only C1 and C10 were enriched by [1-^{13}C]glucose, while C3 and C8 were enriched by [1-^{13}C]glycerate (Figure 4). This labeling pattern on [1-^{13}C]glucose resembles that obtained for [^{14}C]shikimate using [1-^{14}C]glucose as a precursor,[36] suggesting a shikimate pathway (Figure 3) origin of the chromophore. However neither [U-^{14}C]shikimate nor the ^{14}C-labeled aromatic amino acids were shown to label this part of the molecule. Based

on the information obtained, White suggested that the mC_7N was derived from shikimate pathway at a level earlier than shikimate (Figure 4). The results of the corresponding incorporation experiments with geldanamycin[31c] and mitomycin C[37] are also in agreement with a shikimate origin of the mC_7N unit.

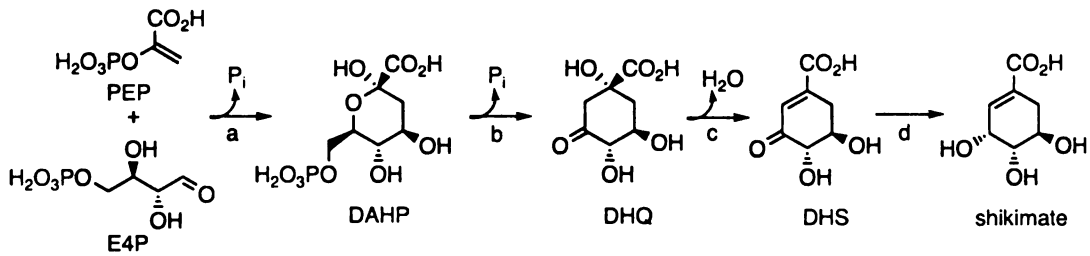

Figure 4. The shikimate pathway.

(a) DAHP synthase; (b) 3-dehydroquinate synthase; (c) 3-dehydroquinate dehydratase; (d) shikimate dehydrogenase. Abbreviations: PEP, phosphoenolpyruvate; E4P, D-erythrose 4-phosphate; DAHP, 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate; DHQ, 3-dehydroquinate; DHS, 3-dehydroshikimate.

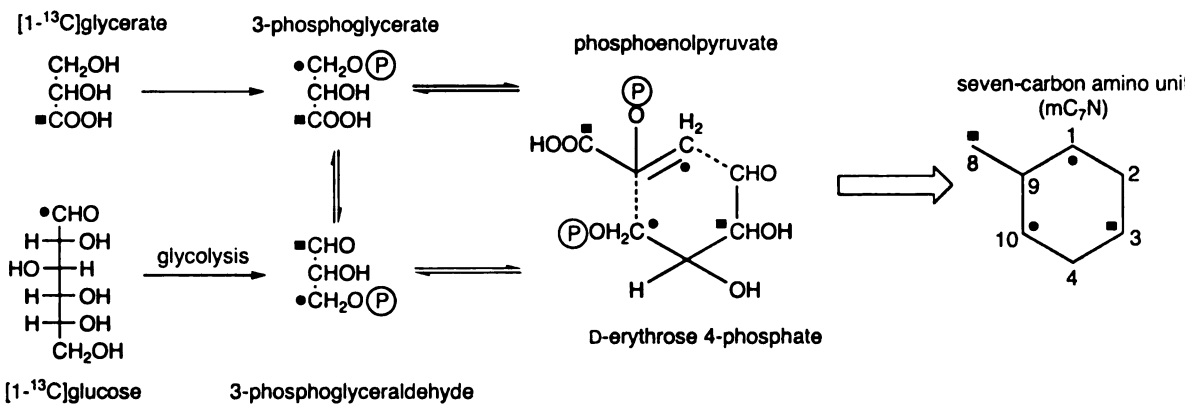

Figure 5. Suggested metabolic scheme explaining the pattern of labeling obtained in the seven-carbon amino unit with [1-^{13}C]glucose and [1-^{13}C]glycerate as precursors.

Genetic approaches on the biosynthesis of the mC_7N unit conducted by Ghisalba and colleagues also indicated a shikimate pathway origin. A mutant under study, designated A8, is derived from an *Amycolatopsis mediterranei* strain, N813, which is a

rifamycin B producer. A8[38] is auxotropic for aromatic amino acids and produces much less rifamycin B than the parent strain N813. No inactivation of the shikimate pathway enzymes was found and only a block in the transketolase activity was detected. A mixture of pentoses with D-ribose as the major product was found to accumulate in the culture broth of mutant A8. It was shown that A8 was affected in its transketolase activity since no D-sedoheptulose 7-phosphate (S7P) from pentose-phosphates could be detected *in vitro* using crude extracts. No other pathway for synthesizing D-sedoheptulose 7-phosphate (S7P) except the transketolase reaction is known. Likewise D-erythrose 4-phosphate (E4P) is only known to be synthesized from D-sedoheptulose 7-phosphate by means of the transaldolase reaction or from fructose 6-phosphate and glyceraldehydes 3-phosphate by the transketolase reaction. Thus a mutant lacking transketolase activity is not able to synthesize both D-erythrose 4-phosphate and D-sedoheptulose 7-phosphate. This is a strong indication that the mC_7N unit is derived from shikimate pathway because no other pathway except the shikimate pathway is known which starts from D-sedoheptulose 7-phosphate or D-erythrose 4-phosphate and leads to aromatic compounds.

Another mutant, A10,[39] is also auxotrophic for aromatic amino acids but unlike A8 produces the same amount of rifamycin as the parent. It was shown that the mutant was blocked in one of the enzymes leading from shikimate to chorismate. As mutant A10 is only defective in the biosynthesis of aromatic amino acids and not in the biosynthesis of rifamycins, it suggests that the mC_7N unit of the rifamycin chromophore must be derived from an intermediate of the shikimate pathway prior to shikimic acid.

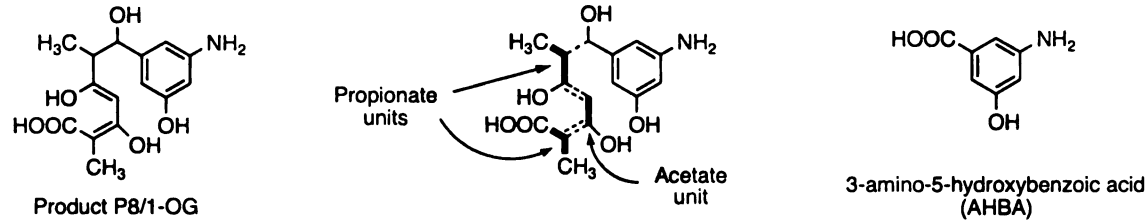

Figure 6. Structure of product P8/1-OG and 3-amino-5-hydroxybenzoic acid (AHBA).

Further genetic studies on several UV-mutants of *Amycolatopsis mediterranei* strain N813, including A10, revealed that an identical aromatic component, P8/1-OG, instead of rifamycin B, was accumulated and was identified spectroscopically as 2,6-dimethyl-3,5,7-trihydroxy-7-(3'-amino-5'-hydroxyphenyl)-2,4-heptadienoic acid (Figure 5).[40] Based on the structure of this intermediate, Ghisalba proposed that 3-amino-5-hydroxybenzoic acid (Figure 5) might serve as the starting unit for the biosynthesis of product of P8/1-OG and of the rifamycins. Later the supplementation studies with 3-amino-5-hydroxybenzoic acid and *Amycolatopsis mediterranei* A8 demonstrated that this compound could indeed substitute for the seven carbon amino unit.[41] The original rifamycin production capacity of *Amycolatopsis mediterranei* N813 can be restored in strain A8 by supplementation with 3-amino-5-hydroxybenzoic acid.

Rickards also predicted the direct precursor to the mC_7N unit as 3-amino-5-hydroxybenzoic acid based on analysis of the structures of known ansamycins and maytansinoids.[42] Since the unit is required to initiate a polyketide chain, the one-carbon substituent is presumably at the oxidation level of the carboxy-group. Also the mC_7N unit must carry an oxygen function at the 5-position of the ring. This oxygen function may subsequently be methylated or otherwise etherified as in the maytansinoids and some rifamycins, respectively, acetylated as in the streptovaricins, or commonly

oxidized, with involvement of the 2-position, to the *p*-quinone (or *p*-quinol) level. By incubation of [carboxy-^{14}C]-3-amino-5-hydroxybenzoic acid in the culture of the actamycin producer, *Streptomyces* sp. E/784, they established 3-amino-5-hydroxybenzoic acid to be the key mC_7N nuclear precursor.

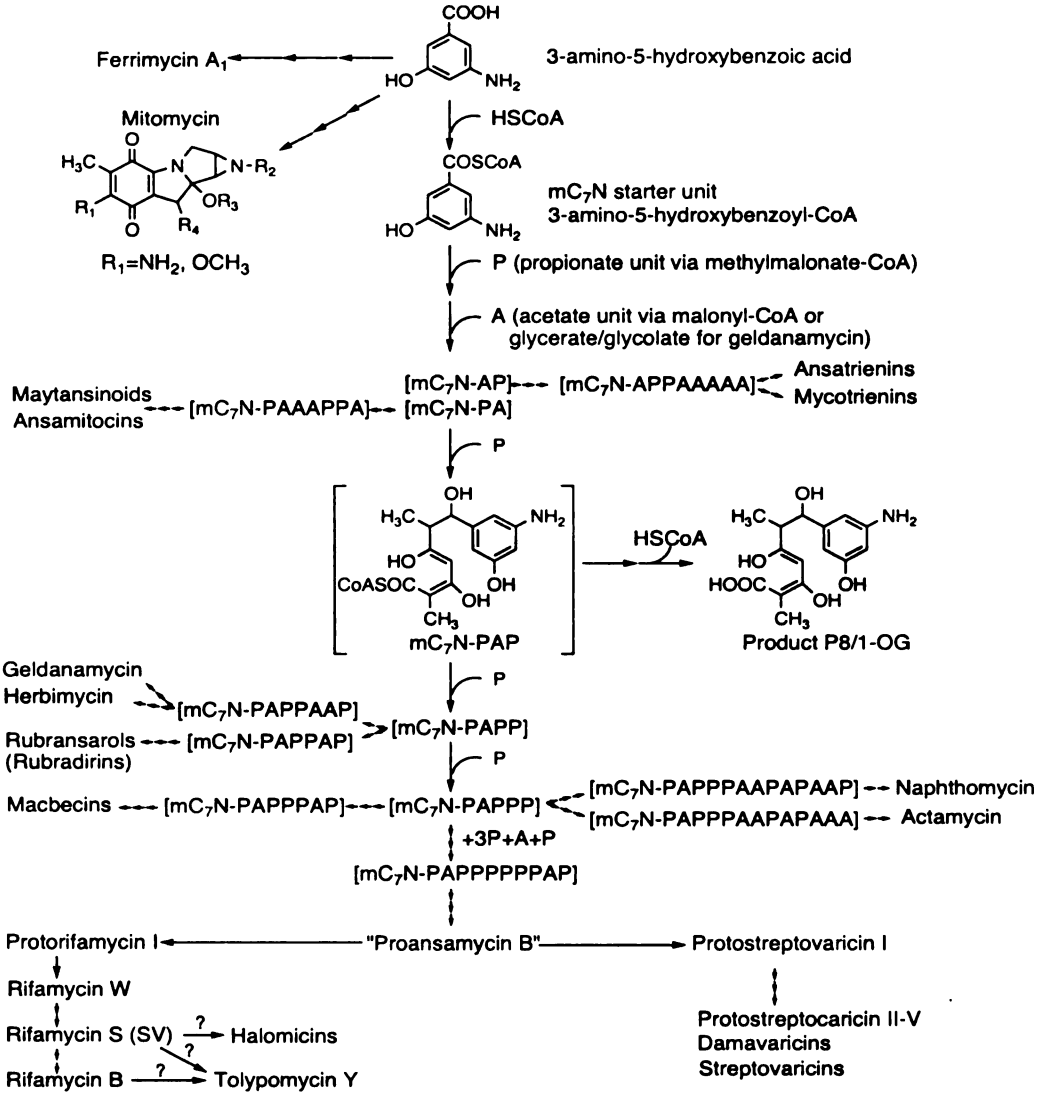

Figure 7. Possible pathways for the biosynthesis of ansamycins starting from shikimate pathway intermediates via 3-amino-5-hydroxybenzoic acid as the direct precursor of the mC_7N starter unit (proposed by Ghisalba).

Following Ghisalba and Rickards' efforts, [carboxy-^{13}C]-3-amino-5-hydroxybenzoic acid was also found to label the mC_7N units of the mitomycin-type

antibiotics porfiromycin[43] and ansamitocin P-3.[44] Furthermore, 3-amino-5-hydroxybenzoic acid (abbreviated as AHBA) was demonstrated to re-establish the biosynthesis of rifamycin B in an inactive mutant *Amycolatopsis mediterranei* rif 2.[45]

With the available data and by analyzing structure analogies, Ghisalba proposed a general biogenetic model for the known ansamycins (Figure 6).[2a] Starting with 3-amino-5-hydroxybenzoyl-CoA, a polyketide chain (*ansa* chain) is built up by subsequent condensation with propionate and acetate, via methylmalonyl-CoA and malonyl-CoA, respectively. Different antibiotics branch points in the polyketide synthesis were proposed by analyzing structural analogies of the ansamycin-types or by the known incorporation pattern for [^{13}C]acetate and [^{13}C]propionate.

With 3-amino-5-hydroxybenzoic acid (AHBA) being established as the direct mC_7N precursor, the biosynthetic origin of the chromophore of ansamycins then traced to the biosynthesis of AHBA. Even though an agreement had been reached that the ansamycin chromophore had a shikimate pathway origin, the exact branch point was not clear. As described earlier, the mC_7N unit must be derived from the shikimate pathway prior to shikimic acid.[39] White then suggested 3-dehydroquinate and 3-dehydroshikimate as possible branch points since the carbonyl functions of both molecules are in the correct position to give rise to an amino group *meta* to the carboxyl function on transamination.[34] However no incorporation was observed when labeled 3-dehydroquinate was tested as a precursor. Hornemann also demonstrated that [1-^{14}C]pyruvate, [3-^{14}C]pyruvate and D-[4-^{14}C]erythrose all labeled mitomycin antibiotics.[37c] The labeling pattern of D-[4-^{14}C]erythrose suggested that nitrogen was attached to the six-membered ring at a position corresponding to C5 of 3-dehydroquinate (Figure 7). This result further contradicted the

previous proposal by White due to the fact that transamination of the carbonyl groups of either 3-dehydroquinate or 3-dehydroshikimate would result in nitrogen being attached to a position corresponding to C3 of 3-dehydroquinate (Figure 7). Genetic studies by Ghisalba further excluded 3-dehydroquinate or 3-dehydroshikimate as precursors.[46] An *A. mediterranei* mutant, B9, with 3-dehydroquinate synthase being inactive was found to still be able to produce rifamycin B with slightly reduced titer compared to the parent strain, *A. mediterranei* N813. Since 3-dehydroquinate synthase catalyzes the reaction converting DAHP to 3-dehydroquinate, 3-dehydroquinate as well as intermediates behind it on the shikimate pathway (Figure 3) are then excluded as possible precursors for AHBA. Combining the information, for the first time, Hornemann proposed the yet unknown compound 4-amino-3,4-dideoxy-D-*arabino*-heptulosonic acid 7-phosphate (aminoDAHP), or a close relative, as an early precursor in formation of AHBA (mC_7N unit).[37c]

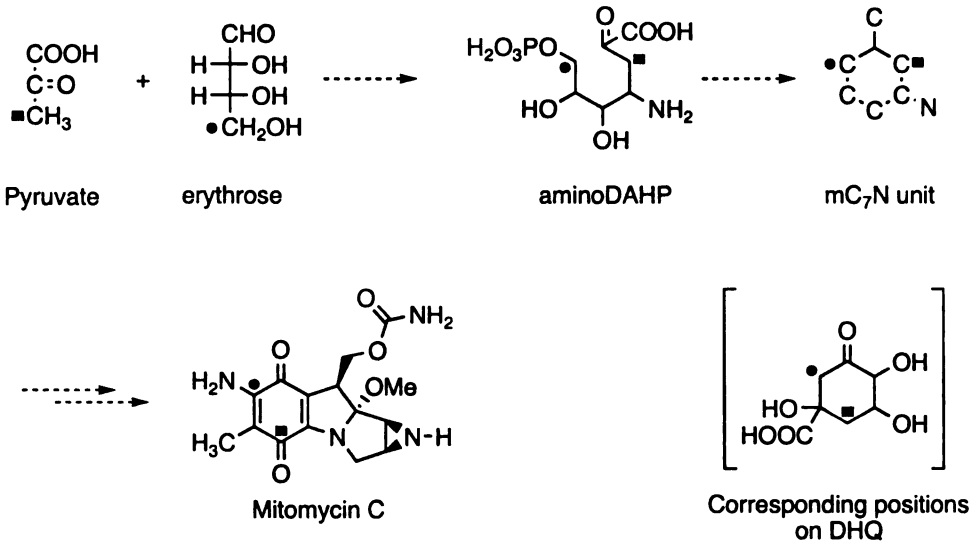

Figure 8. Labeling patterns of mitomycin C by [3-^{14}C]pyruvate and D-[4-^{14}C]erythrose.

A major step forward in the delineation of the pathway came with the proposal by Floss[47] that aminoDAHP was formed by condensation of phosphoenolpyruvate (PEP) with 1-deoxy-1-imino-D-erythrose 4-phosphate (iminoE4P), which could be formed by the reaction between E4P and a molecule of ammonia released from glutamine in the active site of a modified DAHP synthase. Cyclization and dehydration of aminoDAHP, either by the normal shikimate pathway enzymes or by a separate set of enzymes, then produce 5-amino-5-deoxy-3-dehydroshikimate (aminoDHS), which aromatizes to form 3-amino-5-hydroxybenzoic acid (AHBA) (Figure 8).

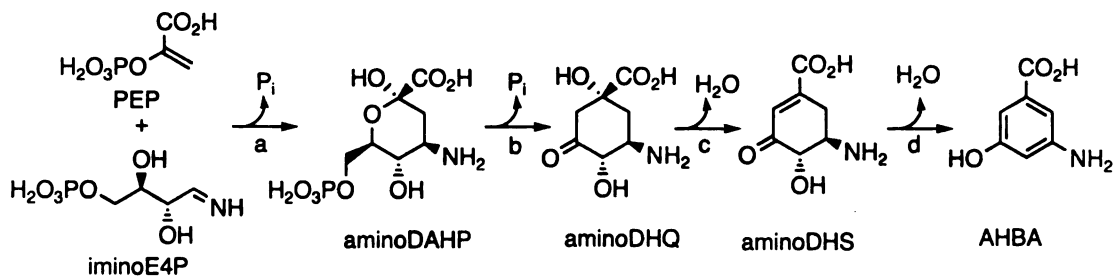

Figure 9. The proposed aminoshikimate pathway.
Enzymes (encoding genes): (a) aminoDAHP synthase (*rifH*); (b) 5-amino-5-deoxy-3-dehydroquinate synthase (*rifG*); (c) 5-amino-5-deoxy-3-dehydroquinate dehydratase (*rifJ*); (d) 3-amino-5-hydroxybenzoic acid synthase (*rifK*). Abbreviations: iminoE4P, 1-imino-1-deoxy-D-erythrose 4-phosphate; PEP, phosphoenolpyruvate; aminoDAHP, 4-amino-3,4-dideoxy-D-*arabino*-heptulosonic acid 7-phosphate; aminoDHQ, 5-amino-5-deoxy-3-dehydroquinate; aminoDHS, 5-amino-5-deoxy-3-dehydroshikimate; AHBA, 3-amino-5-hydroxybenzoic acid.

Floss synthesized aminoDAHP,[47,48] 5-amino-5-deoxy-3-dehydroquinate (aminoDHQ),[49] and 5-amino-5-deoxy-3-dehydroshikimate (aminoDHS)[49] and demonstrated that each of these substrates could be converted to 3-amino-5-hydroxybenzoic acid (AHBA) in cell-free lysate of the rifamycin B producer *A.*

mediterranei. They subsequently purified 3-amino-5-hydroxybenzoic acid synthase and cloned the encoding gene *rifK*.[50] Based on the observation that the genes encoding for a given antibiotic biosynthesis are clustered together, the *rifK* gene was subsequently used to identify the *rif* biosynthetic gene cluster required for biosynthesis of 3-amino-5-hydroxybenzoic acid in *A. mediterranei* S699.[51] A region of 95-kb of DNA surrounding the *rifK* gene was isolated and sequenced (Figure 9). This revealed five large open reading frames (ORFs) coding for a modular type I polyketide synthase, various putative modifying and regulatory genes, and a subcluster of ORFs, the *rifG--N* genes, some of which are homologous to genes involved in the shikimate biosynthetic and quinate utilization pathways of plants, bacteria and fungi. The genes that are related to AHBA biosynthesis in this gene cluster are shown in Table 2.

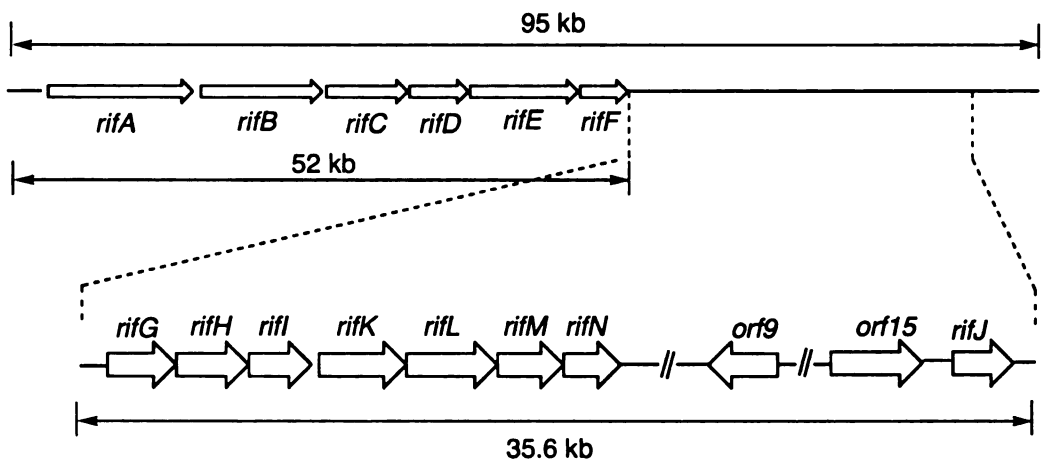

Figure 10. Rifamycin biosynthetic gene cluster of *A. mediterranei* S699 and proposed enzyme functions. Proposed functions (encoding gene): modular type I polyketide synthase (*rifA* to *rifE*); amide synthase (*rifF*); 5-amino-5-deoxy-3-dehydroquinate synthase (*rifG*); aminoDAHP synthase (*rifH*); aminoshikimate dehydrogenase (*rifI*); 3-amino-5-hydroxybenzoate synthase (*rifK*); oxidoreductase (*rifL*); phosphatase (*rifM*); glucokinase (*rifN*); transaminase (*orf9*), transketolase (*orf15*); 5-amino-5-deoxy-3-dehydroquinate dehydratase (*rifJ*).

Table 2. Proposed functions of the *rif* biosynthetic gene products.[a]

entry	*rif* biosynthetic gene products	sequence homology (species, homology%/identity%)	proposed function
1	RifG	AroB (*E. coli*, 49/33)	aminoDHQ synthase
2	RifH	AroG (*L. esculentum*, 54/34)	aminoDAHP synthase
3	RifI	AroE (*Synechocystis* sp. 56/29)	aminoshikimate dehydrogenase
4	RifJ	AroD (*A. pleuropneumoniae*, 63/41)	aminoDHQ dehydratase
5	RifK	AHBAS (*S. collinus*, 86/70)	AHBA synthase
6	RifL	Pur10 (*S. alboniger*, 55/29)	oxidoreductase
7	RifM	CbbzP (*R. eutropha*, 55/32)	phosphatase
8	RifN	XylR (*Synechocystis* sp. 52/29)	kinase
9	Orf9	YokM (*B. subtilis*, 58/30)	transaminase
10	Orf15	TktA (*E. coli*, 58/32)	transketolase

[a] Abbreviations: aminoshikimate, 5-amino-5-deoxyshikimate; aminoDHQ, 5-amino-5-deoxy-3-dehydroquinate; aminoDAHP, 4-amino-3,4-dideoxy-D-*arabino*-heptulosonic acid 7-phosphate; AHBA, 3-amino-5-hydroxybenzoic acid.

Among these genes, *rifK* encodes 3-amino-5-hydroxybenzoic acid synthase. It was proven to be a PLP-dependent enzyme and catalyze the aromatization of aminoDHS stereospecifically to form AHBA.[50a] Inactivation of *rifK* in the *A. mediterranei* genome resulted in loss of rifamycin formation. Production of antibiotic could be restored when the mutant was supplemented with AHBA.[50a]

The *rifG*, *-H*, and *-I* genes encoding homologues of a DHQ synthase, a plant-type DAHP synthase, and a shikimate or quinate dehydrogenase, respectively, are located

immediately upstream of the *rifK* gene.[51] The *rifJ* gene, which appears to encode a type II DHQ dehydratase homologue, is located outside this subcluster about 26 kb downstream from the *rifK* gene. Presumably, the *rifH* product has a similar enzymatic activity with DAHP synthase and condenses phosphoenolpyruvate and 1-deoxy-1-imino-D-erythrose 4-phosphate to form aminoDAHP. The formation of aminoDHQ and aminoDHS would be expected to involve *rifG* and *rifJ* products to catalyze cyclization and dehydration, respectively. The *rifK* product, AHBA synthase, then aromatizes aminoDHS to AHBA. The *rifI* gene product is presumably responsible for the interconversion between aminoDHQ and 3-deoxy-3-aminoquinate or that between aminoDHS and 3-deoxy-3-aminoshikimate. The presence of *rifI* gene is surprising since 3-deoxy-3-aminoquinate or 3-deoxy-3-aminoshikimate are not expected to form in the pathway leading to the formation of AHBA. Inactivation of *rifH* and *rifJ* resulted in inhibition of rifamycin production by 99% and 90%, respectively, in *A. mediterranei.* Full rifamycin B production can be restored by supplementation of the culture with AHBA. Inactivation of *rifG* and *rifI* had no impact on biosynthesis of rifamycin B.[52]

Located immediately downstream of *rifK* are three genes, *rifL*, *rifM*, and *rifN*.[51] The gene product of *rifL* is similar to a class of oxidoreductases that have been implicated in interconversion between hydroxyl and carbonyl groups. The *rifM* gene product has considerable sequence similarity with the CBBY family of phosphoglycolate phosphatases. The deduced gene product of *rifN* shows a significant similarity with the glucose kinase from *Streptomyces coelicolor* and *Bacillus megaterium* involved in glucose repression. Unlike *rifG*, *-H*, *-I*, and *-J*, none of the three genes, *rifL*, *-M*, and *-N* has counterpart in the shikimate pathway. Inactivation of each of *rifL*, *-M*, and *-N* all

resulted in total loss of rifamycin production. Production can be restored to wild-type level by supplementation with AHBA.[52] In complementary experiments, introduction of a shuttle vector carrying *rifG,-H,-I,-J,-K,-L,-M,* and *-N* under the control of an appropriate promoter into *Streptomyces coelicolor* YU105 resulted in the biosynthesis of AHBA.[52] *S. coelicolor* YU105 does not synthesize AHBA in the absence of these genes. Deletion of individual genes in the cassette heterologously expressed in *S. coelicolor* YU105 almost all resulted in great or total loss of AHBA production except for *rifI*, deletion of which had no impact on biosynthesis of AHBA. It indicates that the products of the seven genes (*rifG,-H,-J,-K,-L,-M,* and *–N*) are necessary and sufficient for AHBA biosynthesis.

Combining all the information, including the fact that the amide nitrogen of glutamine is arguably the best source of nitrogen in the biosynthesis of AHBA,[45] the AHBA biosynthetic pathway has been proposed as follows. Condensation of iminoE4P with phosphoenolpyruvate gives aminoDAHP and is catalyzed by the *rifH* gene product. AminoDAHP is then cyclized to aminoDHQ, which is catalyzed by *rifG*-encoded aminoDHQ synthase. A *rifJ*-encoded aminoDHQ dehydratase-catalyzed dehydration reaction gives aminoDHS, which is converted into 3-amino-5-hydroxybenzoic acid by dehydration and enolization. Since the pathway diverges from the shikimate pathway at early steps and also involves several amino-counterparts of the shikimate pathway products, Floss has named it as the aminoshikimate pathway (Figure 8).

The four genes, *rifH*, *rifG*, *rifJ*, and *rifK*, are clearly involved in the AHBA biosynthesis (Figure 8). The enzymatic activities of *rifH*,[53] *rifG*,[54] *rifJ*,[55] and *rifK*[50a] have also been demonstrated. However, the functions of the gene products of *rifL*, *rifM*, and

rifN are still not clear, even though these three genes have been shown to be absolutely necessary for AHBA biosynthesis. Based on the fact that they are not required in the AHBA biosynthesis after the formation of aminoDAHP, Floss suggested that the enzymes encoded by these genes must be associated with the formation of aminoDAHP.[52]

The next breakthrough in elaborating the formation of aminoDAHP was provided by the Frost group. Instead of E4P going through a transamination reaction to form iminoE4P, Frost proposed that iminoE4P was formed from 3-amino-3-deoxy-D-fructose 6-phosphate (aminoF6P) through a transketolase-catalyzed reaction.[56] In order to test this hypothesis, aminoF6P was synthesized. Through an in situ generation/trapping strategy for iminoE4P, aminoDAHP was demonstrated to form when aminoF6P was incubated together with D-ribose 5-phosphate (R5P) and phosphoenolpyruvate (PEP) in both purified *rifH*-encoded aminoDAHP synthase/*E. coli tktA*-encoded transketolase solution and *A. mediterranei* cell-free extract. 3-[^{15}N]-Amino-3-deoxy-D-6,6-[$^{2}H_2$]-fructose 6-phosphate was also demonstrated to be converted to aminoDAHP with retention of all three heavy atom labels in *A. mediterranei* cell-free extract, indicating that aminoF6P is the precursor of iminoE4P and not simply a transaminase source of nitrogen.[56]

The Frost group further identified 3-amino-3-deoxy-D-glucose (kanosamine) as an early precursor to aminoDAHP.[57] Kanosamine was suggested to undergo a phosphorylation reaction to form kanosamine 6-phosphate, which then isomerizes to form aminoF6P and subsequently aminoDAHP. Kanosamine and kanosamine 6-phosphate were both synthesized. Incubation of kanosamine 6-phosphate together with D-ribose 5-phosphate (R5P) and phosphoenolpyruvate (PEP) both in yeast phosphoglucose isomerase/*rifH* encoded aminoDAHP synthase/*E. coli* TktA

transketolase solution and in *A. mediterranei* cell-free extract resulted in formation of aminoDAHP together with DAHP. Similar incubation reactions for kanosamine and ATP also produced very small amount (~1%) of aminoDAHP. Furthermore, kanosamine was demonstrated to form in *A. mediterranei* cell-free lysate from UDP-glucose (UDPG).

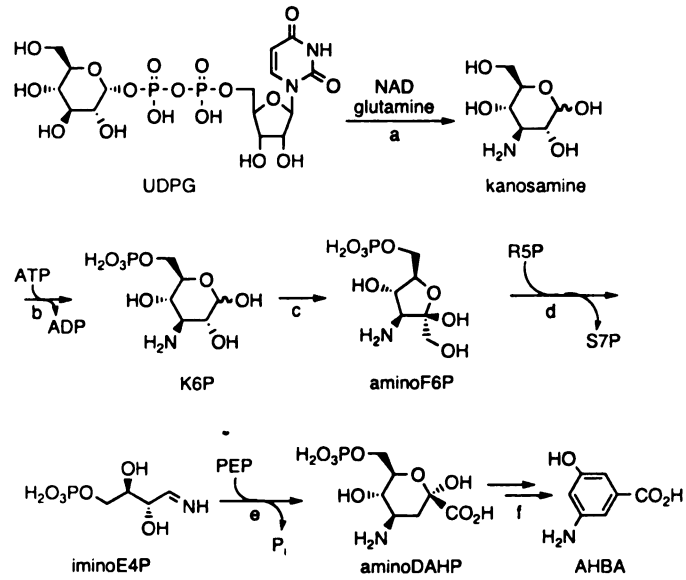

Figure 11. Modified biosynthetic pathway for AHBA.

Conversions (genes): (a) *A. mediterranei* cell-free extract; (b) kanosamine 6-kinase (*rifN*); (c) glucoisomerase; (d) transketolase (*tktA, orf15*); (e) aminoDAHP synthase (*rifH*); (f) aminoshikimate pathway; K6P, kanosamine 6-phosphate; aminoF6P, 3-amino-3-deoxy-D-fructose 6-phosphate; R5P, D-ribose 5-phosphate; S7P, D-sedoheptulose 7-phosphate; iminoE4P, 1-deoxy-1-imino-D-erythrose 4-phosphate; P_i, inorganic phosphate; aminoDAHP, 4-amino-3,4-dideoxy-D-*arabino*-heptulosonic acid 7-phosphate; AHBA, 3-amino-5-hydroxybenzoic acid.

Floss then verified the *rifN* gene product, which is essential for AHBA biosynthesis, to be a specific kanosamine 6-kinase.[58] Combining these facts, a clearer picture of the aminoshikimate pathway could be drawn (Figure 10). As a consequence, elaboration of the source of the aminoshikimate pathway's nitrogen atom turned to include the elaboration of the biosynthesis of kanosamine. Attention now turns to tracing

the mechanism and nitrogen source of the very first steps in which UDP-glucose is converted to kanosamine.

CHAPTER TWO

IN VITRO ELABORATION OF URIDINE 5'-DIPHOSPHOKANOSAMINE AS AN INTERMEDIATE IN KANOSAMINE BIOSYNTHESIS

Introduction

The aminoshikimate pathway was first discovered in a rifamycin B producer, *Amycolatopsis mediterranei*. 3-Amino-5-hydroxybenzoic acid (AHBA), one of the most important products of this pathway, was demonstrated to be a common aromatic starting unit for the biosynthesis of ansamycin antibiotics.[59] Floss and colleagues identified the *rif* gene cluster responsible for rifamycin B biosynthesis in *Amycolatopsis mediterranei* and assigned possible functions to these genes by comparing nucleotide sequences with identified genes in a gene database.[51] Seven genes, *rifH*, *rifG*, *rifJ*, *rifK*, *rifL*, *rifM*, and *rifN*, are found to be necessary and sufficient for AHBA biosynthesis.[52] They also established aminoDAHP,[47] aminoDHQ,[49] and aminoDHS[49] as precursors for AHBA biosynthesis (Figure 8, Chapter One) and confirmed the functions of the gene products of *rifG* (aminoDHQ synthase),[52] *rifJ* (aminoDHQ dehydratase),[52] and *rifK* (AHBA synthase)[50a]. The *rifH* gene product was also proposed to be an aminoDAHP synthase. But for the three genes, *rifK* (the suggested aminotransferase activity besides its function as AHBA synthase), *rifL*, and *rifM*, no experimental evidence has been obtained to support the proposed functions of the proteins encoded by them.

Recent research in the Frost group successfully identified 3-amino-3-deoxy-α-D-glucose 6-phosphate (kanosamine 6-phosphate, K6P)[57] and 3-amino-3-deoxy-D-fructose

6-phosphate (aminoF6P)[56] as precursors in the biosynthesis of aminoDAHP and therefore that of AHBA and confirmed the function of the *rifH* gene product. Floss further established the *rifN*-encoded protein as a specific kanosamine 6-kinase.[58] Furthermore, 3-amino-3-deoxy-α-D-glucose (kanosamine) was also demonstrated to form when uridine 5'-diphosphoglucose (UDP-glucose) was incubated together with β-NAD and L-glutamine in the cell-free extract of *Amycolatopsis mediterranei* (Figure 10, Chapter One).[57] These results confirmed the hypothesis that an aminosugar is the molecular source from which the aminoshikimate pathway derives its nitrogen atom. Thus delineation of the source of nitrogen for the aminoshikimate pathway must include investigation of the biosynthesis of kanosamine.

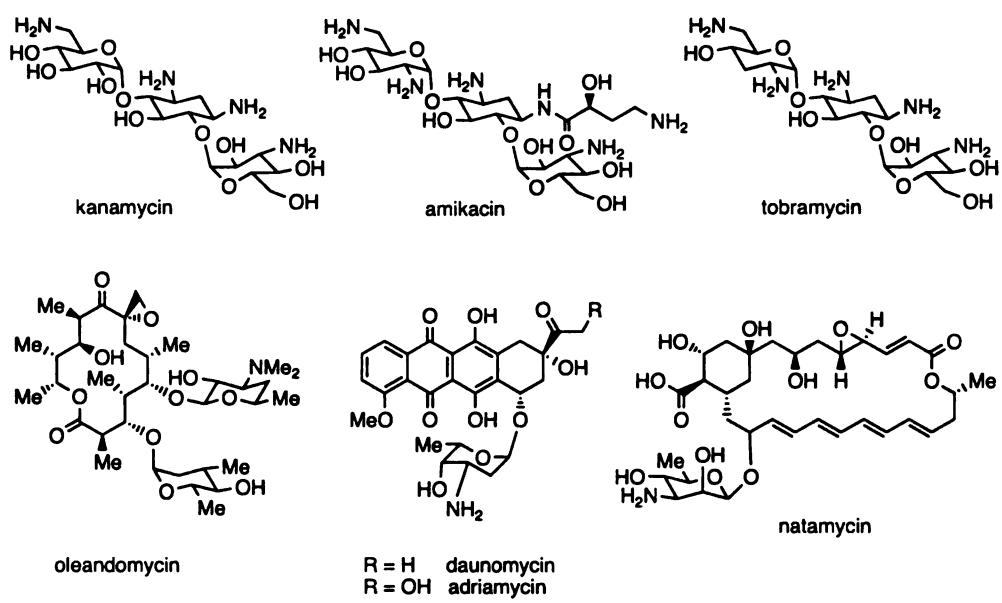

Figure 12. Antibiotics containing kanosamine or kanosamine-like structure.

Kanosamine is a natural product first found in a culture of *Bacillus pumilus* (formerly *Bacillus aminoglucosidicus*), which was isolated from a soil sample collected

at the shore of Lake Haruna, Gunma Prefecture.[60] Many naturally occurring antibiotics contain kanosamine or kanosamine-like moiety (Figure 11). Therefore, the importance of elaboration of kanosamine biosynthesis is not limited to the study of the biosynthetic precursors to iminoE4P biosynthesis. Incubation of [U-^{14}C]-glucose, ATP, UTP, NAD, glutamine, and Mg^{+2} in dialyzed cell-free lysate of *B. pumilus* led to the formation of [^{14}C]-kanosamine.[60b] Likewise, incubation of UDP-[U-^{14}C]-glucose in *B. pumilus* cell lysate with NAD and glutamine led to the formation of [U-^{14}C]-kanosamine.[60b] The distribution of radioactivity in kanosamine produced from [1-^{14}C]-glucose and [6-^{14}C]-glucose was measured. Kanosamine synthesized from [1-^{14}C]-glucose contained 73% of the total radioactivity at C-1 and kanosamine synthesized from [6-^{14}C]-glucose contained 58% of the radioactivity at C-6. These results suggest that kanosamine is biosynthesized from glucose via the intermediacy of UDP-glucose, and that the whole carbon skeleton of glucose is incorporated into kanosamine. This led to a proposed route[60b] for kanosamine biosynthesis from glucose as summarized in Figure 12. D-Glucose is first converted into UDP-glucose by way of D-glucose 1-phosphate. The requirement of NAD suggests that UDP-glucose is oxidized to UDP-3-keto-glucose, which undergoes transamination with glutamine to afford UDP-kanosamine. Subsequent hydrolysis of UDP-kanosamine generates kanosamine.

The fact that L-glutamine is the source of nitrogen of kanosamine biosynthesis coincides with the results obtained by the Jiao group[45] that the amide nitrogen of L-glutamine is putatively the best source of nitrogen for AHBA biosynthesis. This also suggests that kanosamine biosynthesis is possibly related to the aminoshikimate pathway. The Floss group also did research in identifying the source of nitrogen for the

aminoshikimate pathway. Starting from Floss' initial proposal that iminoE4P is obtained from E4P through a transamination, they attempted to identify a transaminase that catalyzes this reaction. Following inspection of the *rif* biosynthetic gene cluster, the *orf9* gene was discovered to have a high sequence homology to genes that encode dNTP-hexose aminotransferases.[51] Floss therefore suggested that *orf9* possessed the necessary enzymatic activity for introducing the nitrogen atom into the aminoshikimate pathway. Unfortunately, inactivation of *orf9* showed no impact on rifamycin B production.[52]

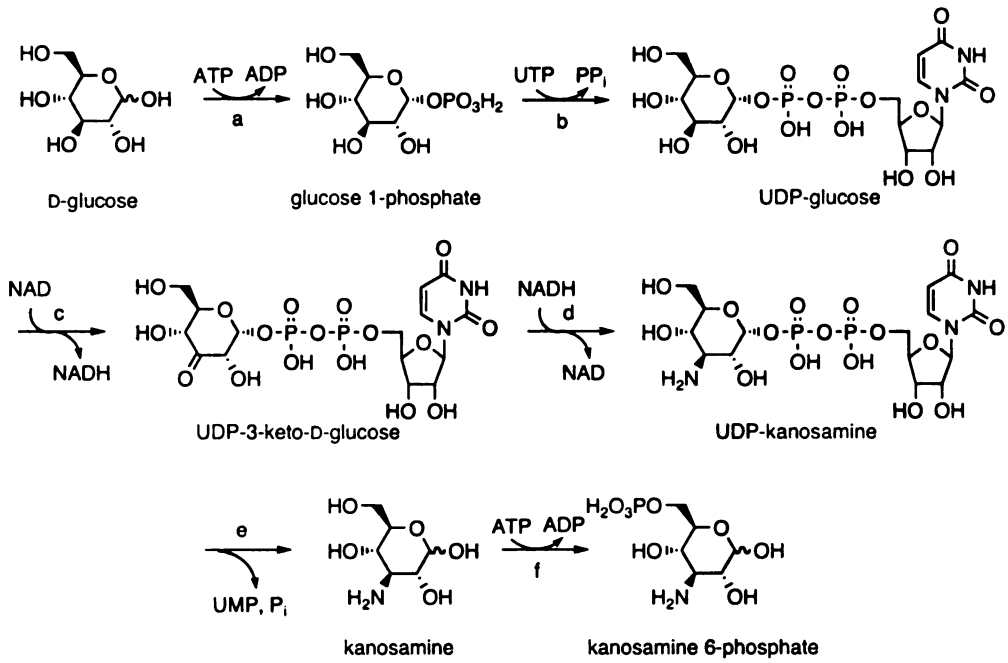

Figure 13. Hypothetical kanosamine and kanosamine 6-phosphate biosynthesis. (a) (i) glucokinase; (ii) phosphoglucomutase; (b) UDP-glucose pyrophosphorylase; (c) UDP-3-keto-D-glucose dehydrogenase; (d) UDP-3-keto-D-glucose transaminase; (e) UDP-kanosamine phosphatase; (f) kanosamine kinase. Abbreviations: AT(D)P, adenosine 5'-tri(di)phosphate; UD(M)P, uridine 5'-tri(mono)phosphate; PP_i, inorganic pyrophosphate; P_i, inorganic phosphate.

With *orf9* removed from consideration, the search for a dedicated transaminase continued. Previous experiments showed that heterologous coexpression of *rifG*, *rifH*,

rifK, *rifL*, *rifM*, *rifN*, and *rifJ* resulted in production of 3-amino-5-hydroxybenzoic acid in *Streptomyces coelicolor* YU105.[52] Based on this observation, Floss and coworkers proposed that the transaminase activity must reside on one of these seven genes. The *rifK* gene product, which had been identified as 3-amino-5-hydroxybenzoate synthase, also showed high sequence homology to transaminases in deoxysugar biosynthesis. More importantly, the *rifK* gene product binds pyridoxamine 5-phosphate (PMP) as well as pyridoxal 5-phosphate (PLP), which is a widely used cofactor in transamination reactions.[50] Based on these observations, Floss proposed that the *rifK* gene product carries two functions: transaminase and 3-amino-5-hydroxybenzoate synthase. The transaminase activity of the *rifK* gene product was proposed to either catalyze transamination of E4P or to introduce a nitrogen atom into another biosynthetic precursor of the aminoshikimate pathway in conjugation with the *rifL*, *rifM*, or *rifN* gene products.[52] After E4P was excluded as a precursor to aminoDAHP, it became more convincing that the enzyme encoded by *rifK* might catalyze a transamination reaction in conjunction with reactions involving the *rifL*, *rifM*, or *rifN* gene products.

The *rifL* gene is located directly downstream to *rifK*. The enzyme encoded by *rifL* shows a high degree of similarity at the amino terminus to the product of *pur10*, which is involved in the biosynthesis of the aminoglycoside antibiotic puromycin.[61] The gene product of *pur10*, which has been implicated in the oxidation of a hydroxyl to a carbonyl group, in turn is similar to the glucose-fructose oxidoreductase of *Zymomonas mobilis*. This enzyme oxidizes glucose to gluconolactone and reduces fructose to sorbitol.[62] The enzyme encoded by *rifL* also shows a high degree amino acid sequence identity to the gene product of *orf10* of the rifamycin biosynthetic gene cluster, which is

adjacent to the *orf9* gene that codes for a putative pyridoxal 5-phosphate (PLP)-dependent transaminase. Significantly, *rifL* homologs are located immediately downstream of all the AHBA synthase genes so far isolated: two AHBA synthases genes from *Streptomyces collinus*, presumably involved in naphthomycin and ansatrienin biosynthesis, one from *Actinosynnema pretiosum* and one from the mitomycin biosynthetic gene cluster of *Streptomyces lavendulae*. The juxtaposition of these oxidoreductases genes with genes coding for PLP-dependent proteins may be an indication of coordinated catalytic activity of the products of these genes. The *rifM* gene encodes for a protein, which is similar to a class of phosphatases in the CBBY family that appear to be involved in converting 2-phosphoglycolate to glycolate in the Calvin carbon reduction cycle.[63]

This information together with the facts that *rifK*, *rifL*, *rifM*, and *rifN* are located adjacently in the *rif* biosynthetic gene cluster (Figure 8) and that the *rifN* gene product has been proven to be a specific kanosamine 6-kinase, a kanosamine biosynthetic pathway in *A. mediterranei* involving all three genes, *rifK*, *rifL*, and *rifM*, can be proposed (Figure 13).

UDP-glucose is first oxidized by the *rifL*-encoded oxidoreductase, followed by transamination catalyzed by a *rifK*-encoded transaminase. Cleavage of UDP-kanosamine by *rifM*-encoded phosphatase affords kanosamine. Kanosamine is then phosphorylated by *rifN*-encoded kanosamine 6-kinase to give kanosamine 6-phosphate, which then enters the aminoshikimate pathway leading to formation of AHBA.

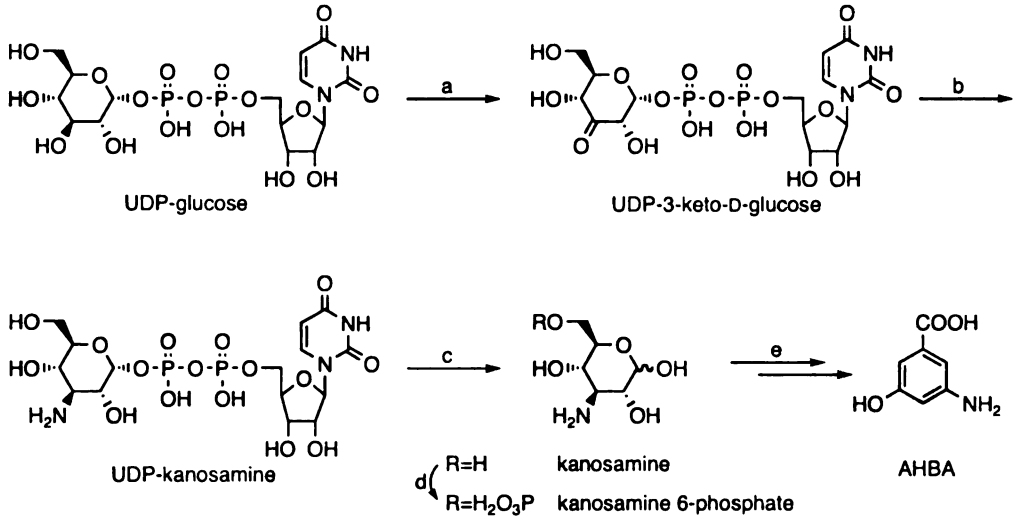

Figure 14. Enzymes and encoding genes associated with the hypothetical kanosamine biosynthesis in *A. mediterranei.* (a) *rifL*-encoded oxidoreductase; (b) *rifK*-encoded transaminase; (c) *rifM*-encoded phosphatase; (d) *rifN*-encoded kanosamine 6-kinase; (e) the aminoshikimate pathway.

In proving this hypothesis, we decided to tackle kanosamine biosynthesis by going after both possible intermediates, UDP-3-keto-D-glucose and UDP-kanosamine, respectively. This chapter will focus on the UDP-kanosamine part of the research. UDP-kanosamine was synthesized and incubated in either *Amycolatopsis mediterranei* or *Bacillus pumilus* cell-free extract, followed by analysis for the formation of kanosamine and kanosamine 1-phosphate. The role of UDP-kanosamine as an intermediate in kanosamine biosynthesis as well as in the aminoshikimate pathway was therefore established. The RifM enzyme involved in the conversion from UDP-kanosamine to kanosamine was isolated and characterized. The possibility of kanosamine 1-phosphate being an intermediate of the aminoshikimate pathway was also investigated. Research accomplished by other group members concerning the intermediacy of UDP-3-keto-D-glucose, the enzymatic functions of RifL and RifK, and L-glutamine being the direct nitrogen source of kanosamine biosynthesis will also be mentioned later in this chapter.

Synthesis of uridine 5'-diphospho-3-amino-3-deoxy-α-D-glucose (UDP-kanosamine)

The synthesis of UDP-kanosamine started from the aminosugar, kanosamine (3-amino-3-deoxy-D-glucose). Kanosamine was synthesized from glucose using *Bacillus pumilus* followed by a one-step purification using Dowex 50 (H^+) cation exchange resin.

Enzymatic synthesis of UDP-kanosamine starting from kanosamine was first attempted by mimicking the bioconversion from D-glucose to UDP-D-glucose (Figure 12). Three enzymes, hexokinase, phosphoglucomutase, and UDP-glucose pyrophosphorylase were incubated together with kanosamine. However, no formation of UDP-kanosamine was detected probably due to the low activity of phosphoglucomutase toward the unnatural substrate, kanosamine 6-phosphate. Chemical synthesis of UDP-kanosamine was then pursued.

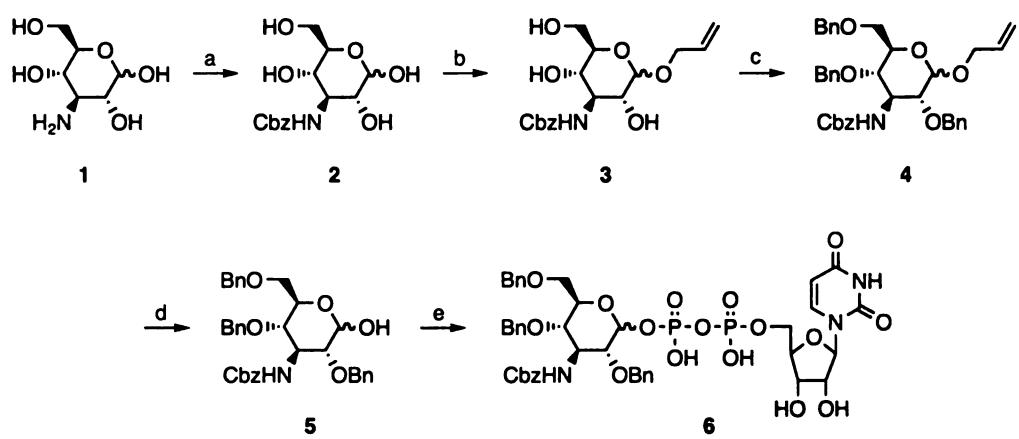

Figure 15. First attempted synthesis of UDP-kanosamine.

(a) $BnOC(O)Cl$, $NaHCO_3$, rt, 79%; (b) 4% HCl in allyl alcohol, reflux, 79%; (c) $Ba(OH)_2$, BnBr, DMF, ultrasound, rt, 60%; (d) (i) $RhCl(Ph_3P)_3$, ethanol:toluene:water=7:3:2, reflux, (ii) formic acid, rt, 80%; (e) (i) $(CO)_2Br_2$, DMF, CH_2Cl_2, rt, (ii) $UDP(NBu_4)$, CH_2Cl_2.

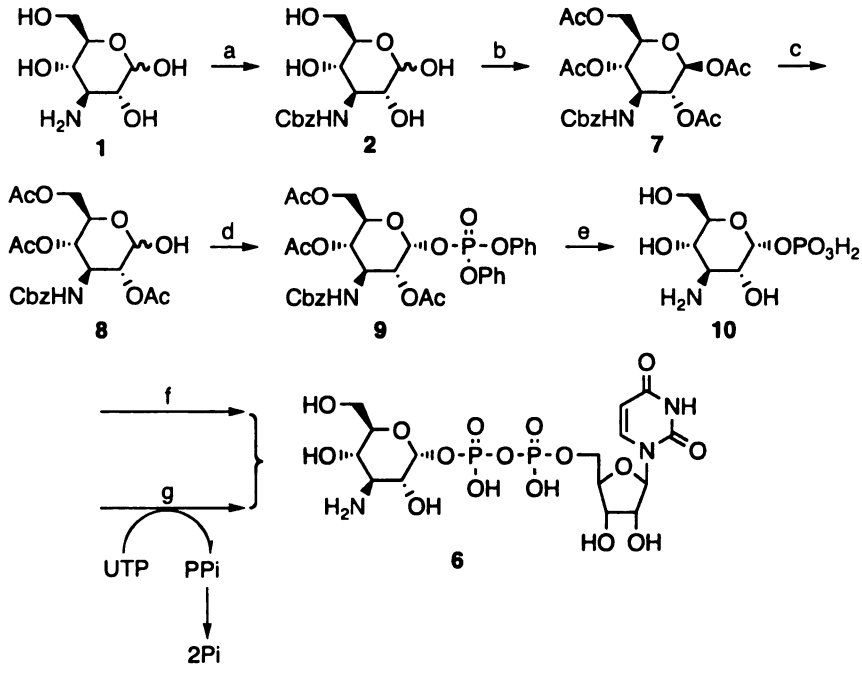

Figure 16. Second attempted synthesis of UDP-kanosamine.

(a) $BnOC(O)Cl$, $NaHCO_3$, rt, 79%; (b) NaOAc, Ac_2O, reflux, 68%; (c) $BnNH_2$, THF, rt, 88%; (d) diphenyl chlorophosphate, 4-(dimethylamino)pyridine (DMAP), CH_2Cl_2, -10°C-rt, 61%; (e) (i) H_2 (55 psi), PtO_2, CH_3OH, rt; (ii) NaOMe, CH_3OH, rt, 55%; (f) uridine 5'-monophosphomorpholidate, pyridine, 1*H*-tetrazole; (g) uridine 5'-diphosphoglucose pyrophosphorylase, inorganic pyrophosphatase, $MgCl_2$, 0.1 N triethanolamine buffer, pH 8.0.

The synthesis showed in Figure 14 was first performed. Kanosamine **1** was reacted with benzyloxy chloroformate in an aqueous suspension of sodium bicarbonate to produce Cbz-kanosamine **2** followed by protection of the anomeric hydroxy with an allyl group to give compound **3**. The remaining three free hydroxy groups were then protected as benzyl ethers by reacting **3** with benzyl bromide catalyzed by barium hydroxide in DMF under ultrasonic conditions. Deprotection of the anomeric allyl group using Wilkinson's catalyst afforded compound **5**. However, bromination of **5** with oxalyl bromide, followed by coupling reaction with the tetrabutyl ammonium salt of uridine 5'-diphosphate failed to give compound **6**. This route of synthesis was not further pursued

also because all the reaction intermediates and the final product are anomeric mixtures, which not only would make it difficult for the purification, but also complicated characterization of each individual compound.

The second attempted synthesis (Figure 15) again started with kanosamine. The amino group was first protected with a benzyloxycarbonyl group (Cbz). Slow addition of Cbz-kanosamine **2** into a refluxing suspension of anhydrous sodium acetate in acetic anhydride resulted in the formation of compound **7** in pure β form. Selective deprotection at the anomeric position by reaction of **2** with 1 eq of benzylamine in THF at room temperature gave **8**. Compound **9** was obtained in pure α form by reaction of the partially deprotected sugar **8** with diphenyl chlorophosphate and DMAP in dichloromethane under a steady temperature increase from –10°C to rt.[64] Hydrogenation at 55 psi catalyzed by a stoichiometric amount of PtO_2 followed by treatment in 50 mM NaOMe in CH_3OH for 2 days at rt afforded kanosamine 1-phosphate. However, kanosamine 1-phosphate produced this way was not pure. The deprotection of the phenyl groups was not complete and acetate groups were also not completely removed. The purity was not improved despite several purification attempts. In hope that partially protected kanosamine 1-phosphate would not undergo coupling or that a successful purification of the final product would eliminate the undesired byproducts, the conversion of impure kanosamine 1-phosphate to UDP-kanosamine was attempted both chemically and enzymatically. The chemical route involved reaction of kanosamine 1-phosphate triethylammonium salt with uridine 5'-monophosphomorpholidate, stirring in dry pyridine at room temperature for 5 days with 1*H*-tetrazole acting as a catalyst. The enzymatic approach involved reaction of kanosamine 1-phosphate with UTP in the

presence of uridine 5'-diphosphoglucose pyrophosphorylase (UGPase) and inorganic pyrophosphatase (PPase). However, in both cases, the UDP-kanosamine produced was contaminated due to the fact that impure kanosamine 1-phosphate was utilized as the starting material. No separation could be achieved between impurities and the desired UDP-kanosamine.

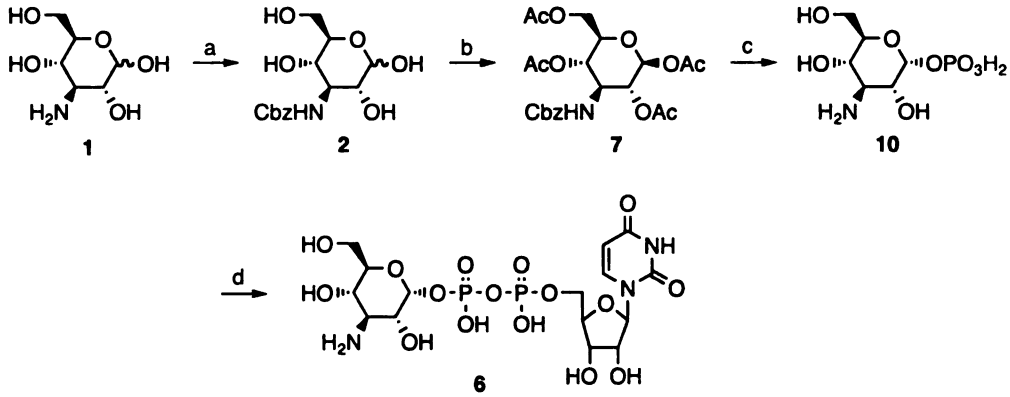

Figure 17. Final synthesis of UPD-kanosamine.
(a) $BnOC(O)Cl$, $NaHCO_3$, rt, 79%; (b) NaOAc, Ac_2O, reflux, 68%; (c) (i) crystalline H_3PO_4, 65°C; (ii) 1 N LiOH, rt, 46%; (d) uridine 5'-diphosphoglucose pyrophosphorylase, inorganic pyrophosphatase, uridine 5'-triphosphate, $MgCl_2$, 0.1 N triethanolamine buffer, pH 8.0, 24%.

UDP-kanosamine was finally synthesized in pure α-form as shown in Figure 16. Compound **7** was prepared as previously described. It was then treated with melted crystalline phosphoric acid at 65°C under vacuum followed by stirring in 1 N aqueous lithium hydroxide for 4 days. One-step purification using Dowex 50 (H^+) cation exchange resin eluted with water gave kanosamine 1-phosphate **10** in pure α–form. The structure was confirmed by ^1H NMR, ^{13}C NMR, two-dimentional NMR, and high resolution mass spectrometry. The conversion to UDP-kanosamine **6** was achieved enzymatically in a one-pot reaction containing two enzymes, uridine 5'-

diphosphoglucose pyrophosphorylase and inorganic pyrophosphatase, both purchased from Sigma. Kanosamine 1-phosphate **10** was first coupled with uridine 5'-triphosphate (UTP) catalyzed by uridine 5'-diphosphoglucose pyrophosphorylase to give UDP-kanosamine and pyrophosphate (PP_i). The pyrophosphate was subsequently cleaved to inorganic phosphate by inorganic pyrophosphatase in order to drive the coupling reaction forward.

Purification of UDP-kanosamine was complicated by its labile nature as well as by contaminations with either UTP or kanosamine 1-phosphate (K1P). The successful purification involved use of an AG1-X8 anion exchange column eluted with 0~1 N triethylammonium bicarbonate (TEAB) followed by azeotropic evaporation with isopropanol. Dissolving the resulting white solid in a minimum amount of water, followed by addition of 100% ethanol finally precipitated out UPD-kanosamine as a white solid in its monotriethylammonium salt form. The final product was characterized by ^1H NMR, ^{13}C NMR, two-dimentional NMR, and high resolution electrospray mass spectrometry.

Synthesis of UDP-3-keto-D-glucose

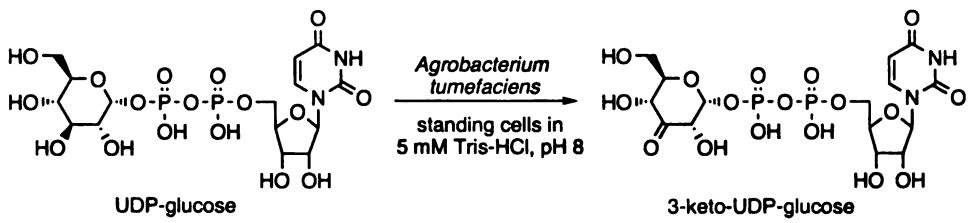

Figure 18. Synthesis of UDP-3-keto-D-glucose.

UDP-3-keto-D-glucose was synthesized from UDP-D-glucose by a whole cell-catalyzed bioconversion (Figure 17) following a procedure worked out by a coworker. *Agrobacterium tumefaciens* IAM-1525 (NCPPB 396) contains 3-keto-D-glucose dehydrogenase activity. It was originally reported that this strain could oxidize sucrose to 3-keto-sucrose.[65] It was also suggested that this strain could oxidize UDP-D-glucose to UDP-3-keto-D-glucose.[66] *A. tumefaciens* was grown at 30°C with sucrose supplementation. Cells were then washed with 5 mM Tris-HCl buffer (pH 8.2) and resuspended in 5 mM Tris-HCl (pH 8.2). UDP-glucose was added and the reaction mixture was shaken at 30°C until no UDP-D-glucose was detected by ^{1}H NMR. After removal of cells by centrifugation, the crude UDP-3-keto-D-glucose was passed through a Dowex 50 (H^+) column. Concentration to a small volume followed by lyophilization afforded UDP-3-keto-D-glucose as a white powder in a yield of 73% from UDP-D-glucose.

Experimental design for examination of UDP-kanosamine as the biosynthetic precursor to kanosamine

Direct isolation of kanosamine from the reactions of UDP-kanosamine being incubated in the cell-free extracts of either *B. pumilus* or *A. mediterranei* would verify that UDP-kanosamine is an intermediate in kanosamine biosynthesis in both organisms. Kanosamine 1-phosphate also needed to be investigated as a possible precursor to kanosamine. The possibility of kanosamine 1-phosphate being an intermediate in iminoE4P biosynthesis came from an observation that kanosamine 1-phosphate was isolated, along with the desired kanosamine, from the reaction mixture when UDP-D-

glucose was incubated together with β-NAD and L-glutamine in *A. mediterranei* cell-free extract.[67] This led to another possible route connecting kanosamine biosynthesis with iminoE4P biosynthesis. UDP-kanosamine might be first hydrolyzed to kanosamine 1-phosphate instead of kanosamine. Isomerization of Kanosamine 1-phosphate to kanosamine 6-phsopahte might then be catalyzed by a phosphoglucomutase-like enzyme (Figure 18). The possible involvement of kanosamine 1-phosphate in iminoE4P biosynthesis might be implicated if kanosamine 6-phosphate was isolated in the cell-free reaction of kanosamine 1-phosphate in *A. mediterranei.*

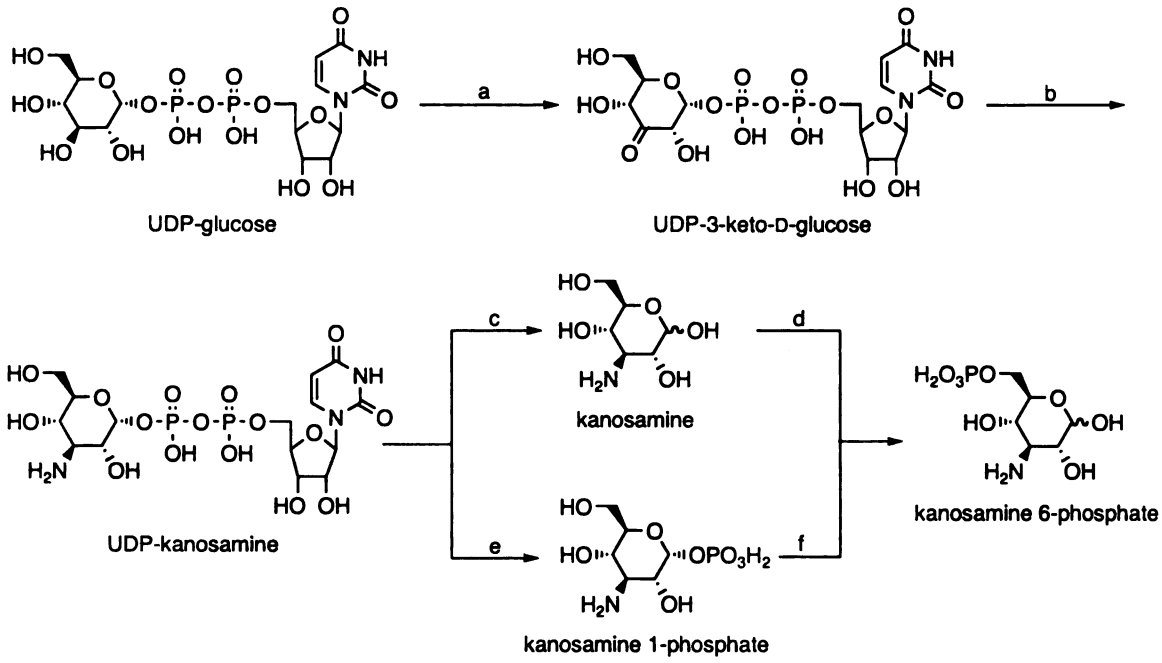

Figure 19. Kanosamine 6-phosphate biosynthesis.

(a) (i) UDP-D-glucose dehydrogenase; (ii) UDP-3-keto-D-glucose transaminase; (b) phosphatase; (c) UDP-sugar diphosphatase or pyrophosphorylase; (d) kanosamine kinase (*rifN*); (e) phosphomutase.

Enzyme purification and preparation of cell-free extracts

The *rifM*-encoded phosphatase from *A. mediterranei* S699 was purified as a recombinant 6-His tagged protein. Cell-free lysate was prepared from *Amycolatopsis mediterranei* (ATCC 21789), which contains all the enzymes involved in biosynthesis of rifamycin B (kanosamine biosynthetic enzymes, iminoE4P biosynthetic enzymes and the aminoshikimate pathway enzymes), and also from *Bacillus pumilus* (ATCC 21143), which contains kanosamine biosynthetic enzymes but no iminoE4P biosynthetic enzymes or aminoshikimate pathway enzymes.

The *A. mediterranei rifM* gene product was suggested to be a phosphatase. It was purified as a 6-His tagged protein from *E. coli* BL21(C$^+$RP)/pJG8.115 (*T5*, *lacO*, *His$_6$*, *rifM*, *lacI^Q*, Ap^R) by chromatography using a Ni-NTA Agarose column following the manufacture protocol.

A. mediterranei (ATCC 21789) cell-free lysate was prepared following literature procedures with slight modifications.[49] The harvested mycelia were resuspended in Tris-HCl buffer (pH 6.8) and disrupted by two passes through a French press at 16000 psi. The cell debris was removed by centrifugation. Diafiltration was then carried out using a Millipore PM-10 membrane and an Amicon stirred cell (300 mL). Dilution to 250 mL followed by concentration to approximately 25 mL was repeated 2 to 3 times.

B. pumilus (ATCC 21143) cell-free extract was prepared similarly as that of *A. mediterranei*. The harvested mycelia were resuspended in Tris-HCl buffer and disrupted by two passes through a French press. The cell debris was removed by centrifugation and the supernatant was directly used in the cell-free reactions.

Table 3. Reactions of UDP-kanosamine.

entry	conditions	kanosamine (%)	K1P (%)	UDPK (%)
1	*B. pumilus* (ATCC 21143)	20	22	28
2	*A. mediterranei* (ATCC 21789)	10	23	65
3	*A. mediterranei* S699 (*rifM*)	trace	55	36
4	RifM	8	4	66

^{1}H NMR yields were calculated using response factors based on integration relative to 3-(trimethylsilyl)propionate-2,2,3,3-d_4; Entry 1 was run in crude extract, while entry 2, 3 and 4 were run in dialyzed solution; Abbreviations: K1P, kanosamine 1-phosphate (3-amino-3-deoxy-α-D-glucose 1-phosphate); UDPK, UDP-kanosamine (uridine 5'-diphospho-3-amino-3-deoxy-α-D-glucose).

Reaction of UDP-kanosamine in *B. pumilus* cell-free extract

To test whether UDP-kanosamine is the precursor for kanosamine biosynthesis in *Bacillus pumilus*, UDP-kanosamine was incubated in the cell-free extract of *B. pumilus*. After 6 h incubation, both kanosamine and kanosamine 1-phosphate were formed in 20% and 22% yields, respectively, based on ^{1}H NMR analysis (entry 1, Table 3). The formation of kanosamine and kanosamine 1-phosphate was confirmed by partial purification on Dowex 50 (H^+) eluted with 1 N HCl followed by ^{1}H NMR and MS analysis. Kanosamine formation indicated that UDP-kanosamine is a precursor to kanosamine. The formation of kanosamine 1-phosphate, however, was unexpected. Since *B. pumilus* is a kanosamine producer, but not a rifamycin producer, kanosamine should be the end product and there is no aminoshikimate pathway following the kanosamine biosynthesis. The formation of kanosamine 1-phosphate thus might be better explained as a side reaction of certain phosphatase(s) or pyrophosphorylase(s) on UDP-

kanosamine. Another possibility is that UDP-kanosamine was first cleaved to give kanosamine 1-phosphate, which then is further hydrolyzed to produce kanosamine.

Reaction of UDP-kanosamine in *A. mediterranei* cell-free extract

To test whether UDP-kanosamine is a precursor in the kanosamine biosynthesis in *A. mediterranei*, UDP-kanosamine was incubated in the cell-free extract of *A. mediterranei*. After 6 h incubation, kanosamine and kanosamine 1-phosphate were formed in 10% and 23% yields, respectively, according to ^{1}H NMR (entry 2, Table 3). The formation of kanosamine and kanosamine 1-phosphate was confirmed by partial purification on Dowex 50 (H^+) eluted by 1 N HCl followed by ^{1}H NMR and MS analysis. No kanosamine 6-phosphate was observed to form. Kanosamine formation indicated that UDP-kanosamine was a precursor to kanosamine. The formation of kanosamine 1-phosphate again might lead to several possible explanations. Kanosamine 1-phosphate might be produced by the action of certain phosphatases using UDP-kanosamine as an unnatural substrate. It is also possible that kanosamine 1-phosphate is the intermediate between UDP-kanosamine and kanosamine. The possible role of kanosamine 1-phosphate as a precursor in iminoE4P biosynthesis will be further discussed later in this chapter.

Reaction of UDP-kanosamine in the cell-free extract of *A. mediterranei* S699 (*rifM*)

The *Amycolatopsis mediterranei* S699 (*rifM*) strain was generously provided by Professor Heinz Floss. The gene product of *rifM* was proposed to be a phosphatase[51] and possibly involved in the conversion of UDP-kanosamine to kanosamine. After

establishing that UDP-kanosamine is a precursor for kanosamine, the question then turned to the verification that RifM (gene product of *rifM*) catalyzes this reaction. If kanosamine production from UDP-kanosamine disappears or is substantially reduced in the *A. mediterranei* S699 (*rifM*) strain, RifM is implicated to be a UDP-kanosamine phosphatase.

Incubation of UDP-kanosamine in the cell-free lysate of *A. mediterranei* S699 (*rifM*) at 30°C for 6 h only afforded a trace amount of kanosamine. Kanosamine 1-phosphate was produced in 55%, according to ^{1}H NMR analysis (entry 3, Table 3). The reduced production of kanosamine is an indication that RifM is the enzyme responsible for hydrolysis of UDP-kanosamine. The formation of kanosamine 1-phosphate indicated the involvement of other enzymes in the production of kanosamine 1-phosphate. This supports the previous proposal that kanosamine 1-phosphate formed in the cell-free extracts of both *B. pumilus* and *A. mediterranei* might be produced by certain phosphatase(s) or pyrophosphorylase(s) using UDP-kanosamine as an unnatural substrate.

Reaction of UDP-kanosamine in the presence of purified heterologously expressed *rifM*-encoded phosphatase

UDP-kanosamine was incubated in the presence of purified RifM at 30°C for 18 h. Kanosamine was formed in 8%, together with kanosamine 1-phosphate, 4% (entry 4, Table 3). Formation of kanosamine and kanosamine 1-phosphate was again confirmed by partial purification using Dowex 50 (H^+) eluted with 1 N HCl followed by ^{1}H NMR and MS analysis. This result indicates that RifM functions as a phosphatase, which can take UDP-kanosamine as substrate.

It is also interesting that the heterologously expressed RifM can use UDP-kanosamine as substrate and produce kanosamine 1-phosphate, along with the production of kanosamine. It was originally thought that since RifM was heterologously expressed in *E. coli* BL21(C$^+$RP)/pJG8.115(*T5*, *lacO*, *His$_6$*, *rifM*, *lacI^Q*, Ap^R), it was possible that the purified RifM was still contaminated by certain protein(s) from the host cell. Therefore formation of kanosamine 1-phosphate might be a result from the action of the contamination protein(s) instead of RifM. However, by using the enzyme solution prepared the same way as that of RifM from culture of *E. coli* BL21(C$^+$RP), no reaction occurred and UDP-kanosamine was not consumed at all. Thus the activity of forming both kanosamine and kanosamine 1-phosphate was attributed to RifM only.

Two possible enzyme activities would account for the formation of kanosamine 1-phosphate from UDP-kanosamine. They are UDP-sugar diphosphatase activity (to produce kanosamine 1-phosphate and UMP) or pyrophosphorylase activity (to produce K1P and UTP, in this case pyrophosphate is required as another substrate besides UDP-kanosamine).

If pyrophosphorylase activity exists in RifM, the enzyme must be considered as a bifunctional enzyme. However, RifM does not have amino acid sequence homology to any identified pyrophosphorylases. Furthermore, formation of K1P did not require addition of any pyrophosphate. Thus, it seems unlikely that RifM may possess any pyrophosphorylase activity.

If UDP-sugar diphosphatase activity is the one responsible for producing K1P, RifM can still be considered as a bifunctional enzyme. The bifunctionality might be described as that the site for hydrolysis of the diphosphate group may not be restricted to

one phosphate, but may happen on both phosphate groups and therefore results in two different products.

Another possibility would be that kanosamine 1-phosphate is the intermediate between UDP-kanosamine and kanosamine. In other words, RifM first hydrolyzes UDP-kanosamine to form kanosamine 1-phosphate, and then further cleaves K1P to form kanosamine. If this is the case, incubation of kanosamine 1-phosphate with RifM solution would lead to formation of kanosamine.

Reaction of kanosamine 1-phosphate in the presence of purified heterologously expressed *rifM*-encoded phosphatase, UTP and *E. coli* inorganic pyrophosphatase

In order to investigate whether RifM also has pyrophosphorylase activity, K1P and UTP were incubated in the enzyme solution of RifM and inorganic pyrophosphatase. If RifM displays pyrophosphorylase activity, the coupling reaction between K1P and UTP should result in formation of UDP-kanosamine together with pyrophosphate which would be subsequently cleaved by inorganic pyrophosphatase to form phosphate and therefore drive the reaction forward.

Incubation of K1P and UTP in the presence of RifM and inorganic pyrophosphatase at 30°C for 6 h, however, did not result in formation of UDP-kanosamine (Figure 19). This observation, combined with the fact that RifM does not have amino acid sequence similar to any identified pyrophosphorylase enzymes, is strong evidence that RifM does not have any pyrophosphorylase activity.

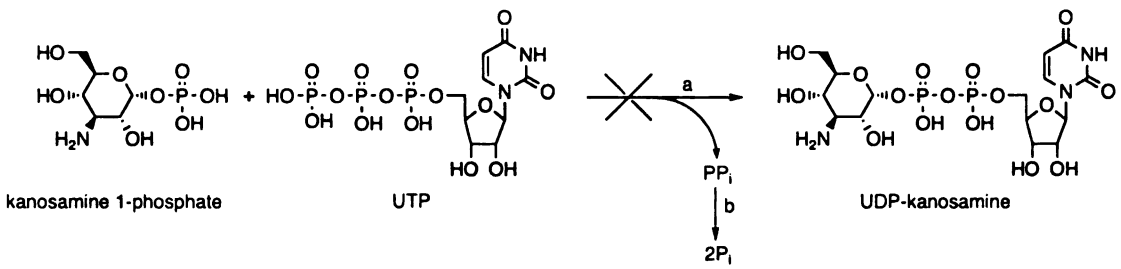

Figure 20. Absence of pyrophosphorylase activity of RifM.
(a) RifM; (b) inorganic pyrophosphatase. Abbreviations: UTP, uridine 5'-triphosphate; PP_i, pyrophosphate; P_i, phosphate.

Reaction of kanosamine 1-phosphate in the presence of purified heterologously expressed *rifM*-encoded phosphatase

As previously discussed, if kanosamine 1-phosphate is the intermediate between UDP-kanosamine and kanosamine, incubation of kanosamine 1-phosphate with RifM solution would result in production of kanosamine. To test whether kanosamine 1-phosphate (K1P) is a substrate for RifM and can be converted to kanosamine, K1P was incubated in the presence of purified RifM at 30°C for 18 h. A 4% yield of kanosamine was produced as analyzed by ^{1}H NMR (entry 1, Table 4), compared to 8% production when UDP-kanosamine was used as starting material. More importantly, when comparing the ratio of kanosamine and K1P present in both cases (UDP-kanosamine as substrate, kanosamine : K1P= 1:0.5; K1P as substrate, kanosamine : K1P= ~1:25), it seems unlikely that kanosamine is primarily produced directly from kanosamine 1-phosphate. However, the two-step hydrolysis (UDP-kanosamine to K1P, then to kanosamine) remains a viable option. One possibility is that UDP-kanosamine might bind to the active site of RifM better than kanosamine 1-phosphate. If this is the case, K1P produced from UDP-kanosamine would already reside in the active site of RifM and therefore might undergo subsequent hydrolysis more rapidly compared to incubating K1P

with RifM, which may suffer from low binding affinity of K1P to the RifM active site. Another possibility is that UMP, produced along with kanosamine 1-phosphate by the UDP-sugar diphosphatase activity of RifM, may activate the hydrolysis of kanosamine 1-phosphate. But no matter which one might be the case, UDP-kanosamine should still be considered as the natural substrate for RifM and the precursor to kanosamine in kanosamine biosynthesis. Kanosamine 1-phosphate might be described more appropriately as an intermediate of hydrolysis of UDP-kanosamine to kanosamine rather than an intermediate in kanosamine biosynthesis.

Table 4. Reactions of kanosamine 1-phosphate.

entry	conditions	kanosamine (%)	Kanosamine 1-phosphate (%)
1	RifM	4	97
2	*A. mediterranei* (ATCC 21789)	10	91
3	*A. mediterranei* S699 (*rifM*)	3	95
4	*B. pumilus* (ATCC 21143)	83	12

^{1}H NMR yields were calculated using response factors based on integration relative to 3-(trimethylsilyl)propionate-2,2,3,3-d_4; Entry 4 was run in crude extract, while entry 1, 2 and 3 were run in dialyzed extract.

Reaction of kanosamine 1-phosphate in *A. mediterranei* cell-free extract

As proposed in Figure 17, besides the earlier proposed pathway for kanosamine 6-phosphate formation resulting from hydrolysis of UDP-kanosamine to give kanosamine followed by phosphorylation of the C-6 hydroxy group of kanosamine, an alternative biosynthetic pathway was proposed. Hydrolysis of UDP-kanosamine might lead to

kanosamine 1-phosphate followed by an isomerase-catalyzed phosphate group transfer reaction to form kanosamine 6-phosphate. Kanosamine 1-phosphate was incubated in the cell-free extract of *A. mediterranei* to determine if kanosamine 6-phosphate formation could be observed.

Incubation of kanosamine 1-phosphate in *A. mediterranei* cell-free lysate at 30°C for 6 h resulted in formation of kanosamine in 10% and the rest of K1P remained unreacted (entry 2, Table 4). No kanosamine 6-phosphate was observed to form based on ^{1}H NMR and ^{31}P NMR analysis of reaction mixture partially purified using Dowex 50 (H^{+}). This observation, the absence of a gene in the *rif* biosynthetic gene cluster that encodes a phosphoglucomutase-like enzyme, and the kanosamine 6-kinase encoded by *rifN* are not consistant with kanosamine 1-phosphate being the precursor to kanosamine 6-phosphate.

Kanosamine 1-phosphate can either be cleaved by RifM or by some other phosphatases in *A. mediterranei* cell-free extract. The activity of heterologously expressed RifM on kanosamine 1-phosphate has already been demonstrated. However, the possibility of K1P being cleaved by enzymes other than RifM in *A. mediterranei* still exists and will be discussed in the following section.

Reaction of kanosamine 1-phosphate in *A. mediterranei* S699 (*rifM*) cell-free extract

To investigate whether there are enzymes other than RifM, which can hydrolyze kanosamine 1-phosphate to produce kanosamine, K1P was incubated in the cell-free extract of *A. mediterranei* S699 (*rifM*) at 30°C for 6 h to afford a 3% yield of kanosamine. The rest of K1P remained unreacted (entry 3, Table 4). This is a clear

indication that there are enzymes other than RifM in *A. mediterranei* that can hydrolyze kanosamine 1-phopshate to produce kanosamine albeit at activity levels apparently comparable to the hydrolysis of kanosamine 1-phosphate catalyzed by RifM. The presence of these enzymes may help convert more kanosamine 1-phosphate to kanosamine.

Reaction of kanosamine 1-phosphate in *B. pumilus* cell-free extract

Conversion of kanosamine 1-phosphate to kanosamine was also studied in *B. pumilus* cell-free extract. K1P was incubated in the cell-free extract of *B. pumilus* at 30°C for 6 h to afford an 83% yield of kanosamine (entry 4, Table 4). The surprisingly high productivity seems to indicate that kanosamine 1-phosphate is the intermediate between UDP-kanosamine and kanosamine in *B. pumilus*. However, since many genes and encoding enzymes of kanosamine biosynthesis in *B. pumilus* are currently unavailable, further research efforts are needed to answer this question.

Reactions of UDP-D-glucose and UDP-3-keto-D-glucose in the presence of RifM

RifM has been identified as the UDP-kanosamine phosphatase, which hydrolyzes UDP-kanosamine to produce kanosamine. However, one question still remained: is RifM specific for UDP-kansamine or also active toward other UDP-sugars present together with UDP-kanosamine? To answer this question, UDP-D-glucose and 3-keto-UDP-D-glucose were incubated in the presence of RifM, respectively, at 30°C for 18 h. In both cases, no reaction occurred and both starting UDP-sugars remained unconsumed. UDP-D-glucose is a common metabolite in organisms and has been proven to be a precursor to

kanosamine. Recently UDP-3-keto-D-glucose has also been demonstrated as a precursor to kanosamine.[68] The specificity of RifM toward UDP-kanosamine over UDP-D-glucose and UDP-3-keto-D-glucose would ensure that hydrolysis would happen only on UDP-kanosamine and not on its earlier UDP-sugar precursors so as to prevent disruption of the biosynthesis of kanosamine.

HPLC assay of RifM activity

The enzymatic activity of RifM was measured as 0.002 μmol/min·mg based on the consumption rate of UDP-kanosamine. The amount of UDP-kanosamine left has been followed by HPLC analysis of samples that were taken out at different time points, quenched with 10% TCA, centrifuged to remove proteins, and diluted 4 times before HPLC analysis. Paired-ion chromatography on the reverse-phase C18 HPLC column was employed since a better separation of nucleotide diphosphosugars could be achieved this way compared to normal reverse-phase HPLC methods.

Discussion

In this chapter, UDP-kanosamine has been established as a biosynthetic precursor to kanosamine. The *rifM* gene product, RifM, has been demonstrated to be a specific UDP-kanosamine phosphatase.

Formation of kanosamine by incubating UDP-kanosamine in both *B. pumilus* and *A. mediterranei* cell-free lysates established UDP-kanosamine as an intermediate in the kanosamine biosynthetic pathway in both organisms. Formation of kanosamine 1-

phosphate was also observed in both cell-free reactions. Only a trace amount of kanosamine was produced, along with formation of kanosamine 1-phosphate, when UDP-kanosamine was incubated in the cell-free extract of the mutant strain *A. mediterranei* S699 (*rifM*), suggesting that RifM is the enzyme responsible for the formation of kanosamine from UDP-kanosamine.

Formation of kanosamine in the reaction of treating UDP-kanosamine with purified, heterologously expressed RifM solution further confirmed RifM as a UDP-kanosamine phosphatase. The release of kanosamine could be either direct or through K1P as an intermediate. Even though the direct release of sugar from a sugar nucleotide catalyzed by a single enzyme seems to be not common, it does have been observed before.[69] It has been reported that guanosine diphospho-sugars, either guanosine diphospho-glucose or guanosine diphospho-mannose, can be hydrolyzed to yield GDP and corresponding sugars under catalysis of single enzymes respectively.

Kanosamine 1-phosphate was also observed to form in the RifM solution. Formation of kanosamine 1-phosphate from UDP-kanosamine suggests that RifM may have either pyrophosphorylase activity (converting UDP-kanosamine to K1P and UTP in the presence of pyrophosphate) or UDP-sugar diphosphatase activity (converting UDP-kanosmine to K1P and UMP). The possibility of RifM containing the pyrophosphorylase activity was then ruled out by the observation that no reaction occurred when kanosamine 1-phosphate was incubated together with UTP in the presence of RifM and inorganic pyrophosphatase. It thus left UDP-sugar diphosphatase activity as the remaining activity responsible for the formation of kanosamine 1-phosphate.

Since RifM is capable of producing both kanosamine and kanosamine 1-phosphate, two possibilities remain. One is that RifM is a bifunctional enzyme, which consists of both UDP-kanosamine glucosyl hydrolase activity (produce UDP and kanosamine directly from UDP-kanosamine) and UDP-kanosamine diphosphatase activity (produce UMP and K1P from UDP-kanosamine). Kanosamine 1-phosphate produced this way might be further cleaved to kanosamine by other enzymes or even recycled through pyrophosphorylation back to UDP-kanosamine and finally go to kanosamine. Bifunctionality has also been observed for another UDP-sugar hydrolase (or more oftenly called in this thesis as UDP-sugar diphosphatase).[70] This distinct UDP-glucose hydrolase (EC 3.6.1.45) hydrolyzes UDP-glucose to produce glucose 1-phosphate and UMP. The second activity of this enzyme is 5'-nucleotidase activity, which is capable of hydrolysis of 5'-ribonucleotide to produce ribonucleoside and phosphate.

Another possibility is that RifM is a processive enzyme and that kanosamine 1-phosphate is the intermediate between UDP-kanosamine and kanosamine. In other words, UDP-kanosamine is first hydrolyzed to kanosamine 1-phosphate, which is then further cleaved to form kanosamine. This is supported by the observation that kanosamine was formed when kanosamine 1-phosphate was incubated with purified RifM. However, the ratio of kanosamine/K1P present in solution when UDP-kanosamine was used as substrate (kanosamine : K1P= 1:0.5) was much higher than that when K1P was used as substrate (kanosamine : K1P= ~1:25). Based on this observation, it seems unlikely that kanosamine 1-phosphate is the direct precursor to kanosamine in the aminoshikimate pathway. However, the two-step hydrolysis (UDP-kanosamine to K1P,

then to kanosamine) remains a possibility since UDP-kanosamine might bind to the active site of RifM better than kanosamine 1-phosphate. Under this scenario, K1P produced from UDP-kanosamine would already reside in the active site of RifM and therefore undergo the subsequent hydrolysis much more easily, relative to incubating K1P with RifM, which may suffer from low binding affinity of K1P to the RifM active site. Another possible explanation could be that UMP, produced along with kanosamine 1-phosphate, may activate the hydrolysis of kanosamine 1-phosphate. But no matter which would be the case, UDP-kanosamine should still be considered as the natural substrate for RifM and the precursor to kanosamine in kanosamine biosynthesis. Kanosamine 1-phosphate might be described more appropriately as an intermediate of hydrolysis of UDP-kanosamine to kanosamine rather than an intermediate in kanosamine biosynthesis.

The specificity of RifM toward UDP-kanosamine has also been established with the observation that RifM showed no activity toward either UDP-D-glucose or 3-keto-UDP-D-glucose after incubation at 30°C for 18 h (the same condition used to measure the activity of RifM toward UDP-kanosamine).

Kanosamine 1-phosphate formation has been observed before when trying to study the kanosamine formation by incubating UDP-D-glucose together with β-NAD and L-glutamine in the cell-free extract of *A. mediterranei*. This led us to a different proposal of kanosamine 6-phosphate formation in the aminoshikimate pathway: UDP-kanosamine might be first hydrolyzed to kanosamine 1-phosphate, which then undergoes a phosphoglucomutase-like enzyme catalyzed phosphate group transfer reaction to form kanosamine 6-phosphate. To investigate this hypothesis, kanosamine 1-phosphate was

incubated in the cell-free extract of *A. mediterranei.* However, no kanosamine 6-phosphate formation was observed. Only kanosamine was produced in 4% yield and the rest of the kanosamine 1-phosphate remained unreacted. This observation, the absence of a gene in the *rif* biosynthetic gene cluster that encodes a phosphoglucomutase-like enzyme, and the kanosamine 6-kinase encoded by *rifN* are not consistant with kanosamine 1-phosphate being the precursor to kanosamine 6-phosphate in *A. mediterranei. B. pumilus* is a kanosamine producer, but does not contain the aminoshikimate pathway.[60] Therefore kanosamine 1-phosphate accumulation in *B. pumilus* is clearly also not involved in the formation of kanosamine 6-phosphate.

How then does kanosamine 1-phosphate accumulate in the cell-free reactions of *A. mediterranei* and *B. pumilus*? Based on the previous discussion, kanosamine 1-phosphate can be produced either by RifM (or RifM-like enzyme in *B. pumilus*) or by certain other phosphatase(s). No decisive role could be assigned to kanosamine 1-phosphate at current stage.

Research accomplished by other coworkers has established that UDP-3-keto-D-glucose is an intermediate in the kanosamine biosynthesis.[71] In *A. mediterranei*, RifL and RifK have been identified as UDP-D-glucose dehydrogenase and UDP-3-keto-D-glucosetransaminase, respectively. Incubation of UDP-D-glucose in the cell-free extract of *E. coli* BL21(C$^+$RP)/pJG7.275 (P_{tac}, *rifL*, Ap^R, *lacI*^{Q}), containing heterologously expressed RifL, in the presence of dichloroindolphenol (DCIP), phenazine methosulfate (PMS), and Mg^{2+} resulted in formation of UDP-3-keto-D-glucose in 38% yield.[68] Incubation of UDP-3-keto-D-glucose in the cell-free lysate of *E. coli* BL21(C$^+$RP)/pJG7.279a (T_5, *rifK*, *6xHis*, Ap^R, *lacI*^{Q}), containing heterologously

expressed RifK, L-glutamine, pyridoxal 5'-phosphate (PLP), and Mg^{2+} resulted in formation of UDP-kanosamine in 20% yield.[68] Results obtained from experiments dealing with [amine-^{15}N]-L-glutamine and [amide-^{15}N]-L-glutamine also suggested that the amide nitrogen of L-glutamine was the preferred source of nitrogen in kanosamine biosynthesis in *A. mediterranei* cell-free lysates.[72]

In conclusion, we have established the roles of two precursors (UDP-3-keto-D-glucose and UDP-kanosamine) and three pathway enzymes (*rifL*-encoded UDP-D-glucose dehydrogenase, *rifK*-encoded UDP-3-keto-D-glucose transaminase, and *rifM*-encoded UDP-kanosamine phosphatase) in kanosamine biosynthesis in *Amycolatopsis mediterranei*. This, in turn, provides the remaining details for how the nitrogen atom is incorporated into the aminoshikimate pathway (Figure 20).

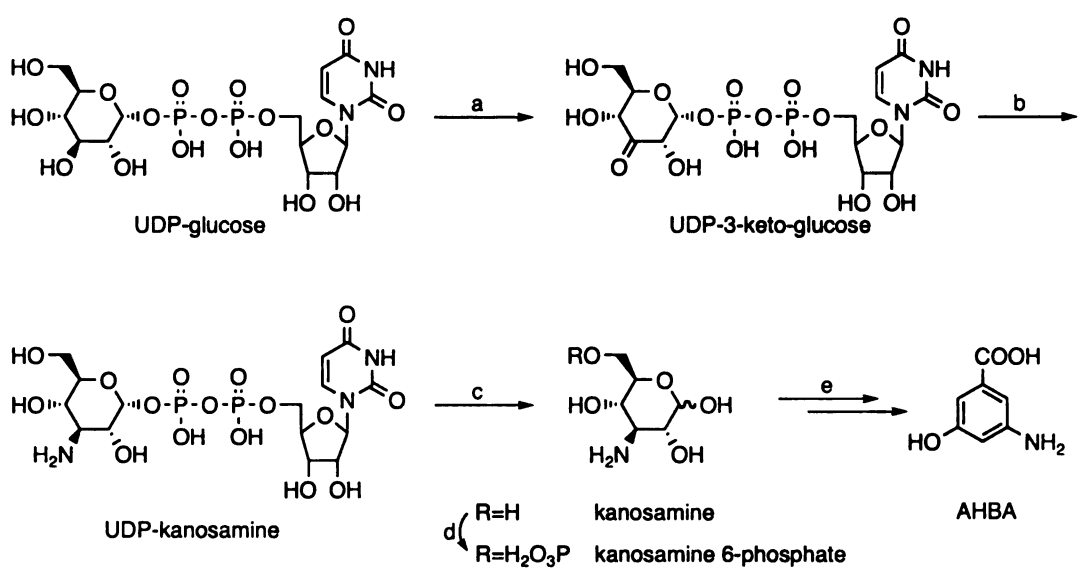

Figure 21. Verified kanosamine biosynthesis in *A. mediterranei*. (a) *rifL*-encoded UDP-D-glucose dehydrogenase; (b) *rifK*-encoded UDP-3-keto-D-glucose transaminase; (c) *rifM*-encoded UDP-kanosamine phosphatase; (d) *rifN*-encoded kanosamine 6-kinase; (e) the aminoshikimate pathway.

Kanosamine biosynthesis in *B. pumilus* was also verified to go through similar conversions. UDP-3-keto-D-glucose[73] and UDP-kanosamine (this thesis) were identified to be precursors to kanosamine. UDP-D-glucose dehydrogenase was isolated and its encoding gene was identified and characterized in *B. pumilus*.[74] The genes and their encoded UDP-3-keto-D-glucose transaminase and UDP-kanosamine phosphatase activities remain to be identified in *B. pumilus*.

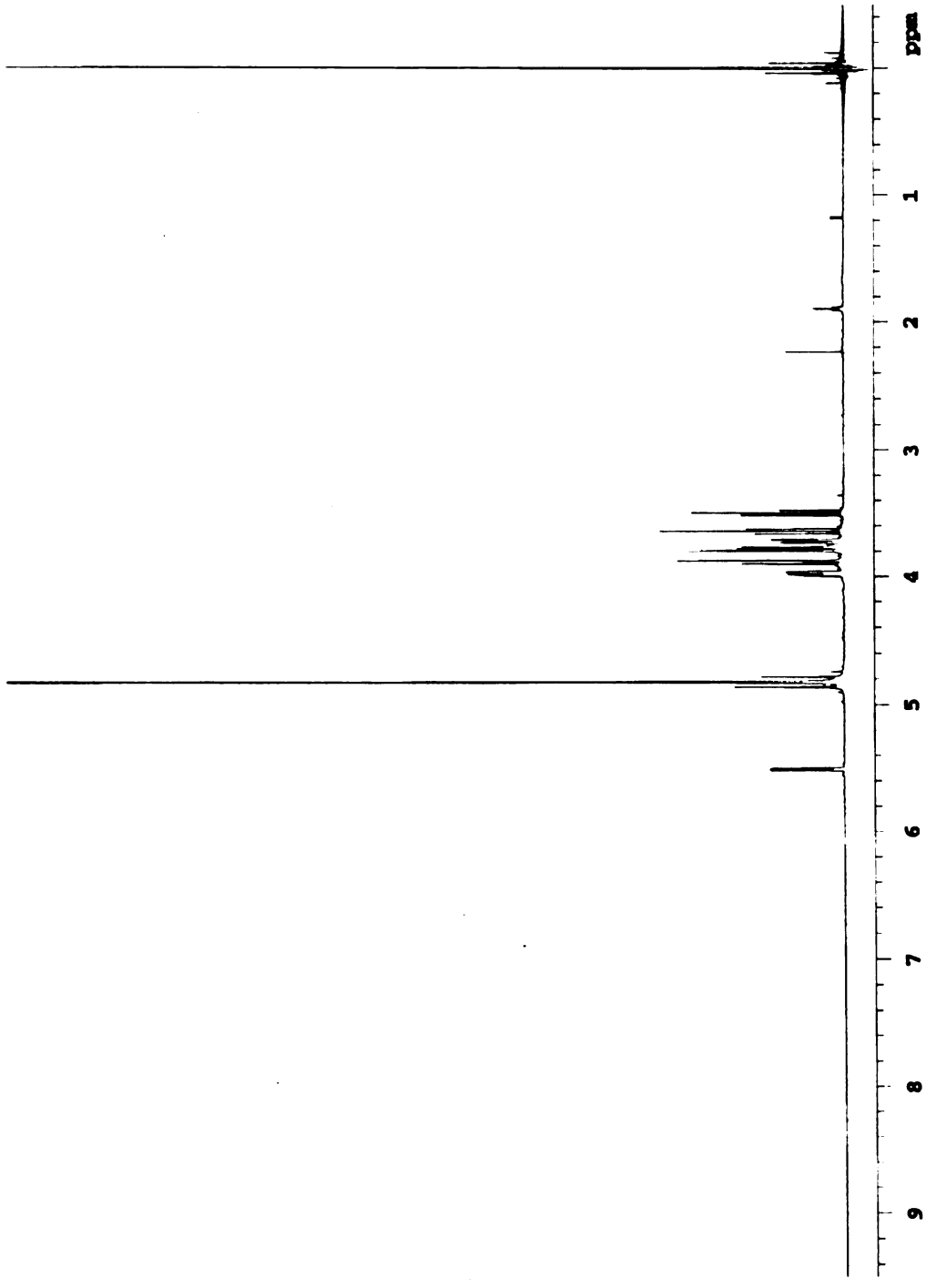

Figure 21. ^{1}H NMR of chemically synthesized kanosamine 1-phosphate.

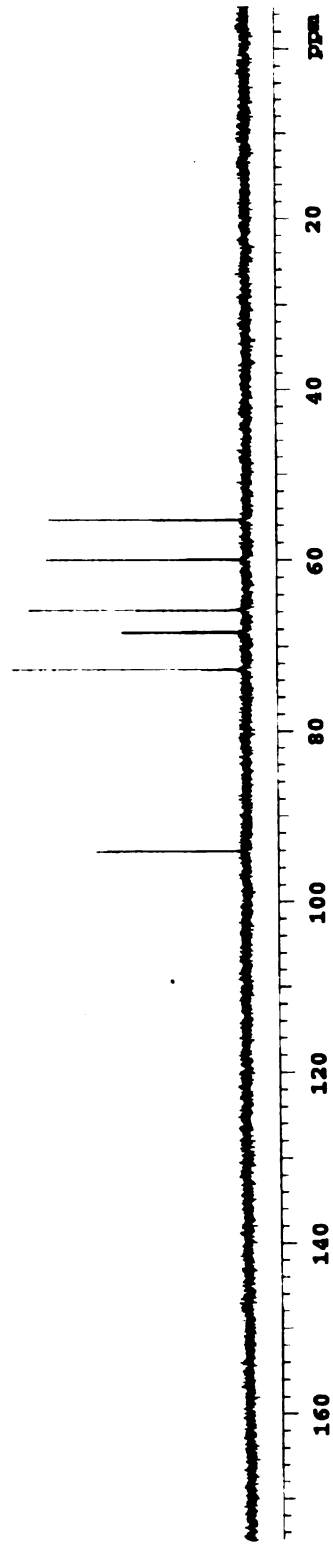

Figure 22. ^{13}C NMR of chemically synthesized kanosamine 1-phosphate.

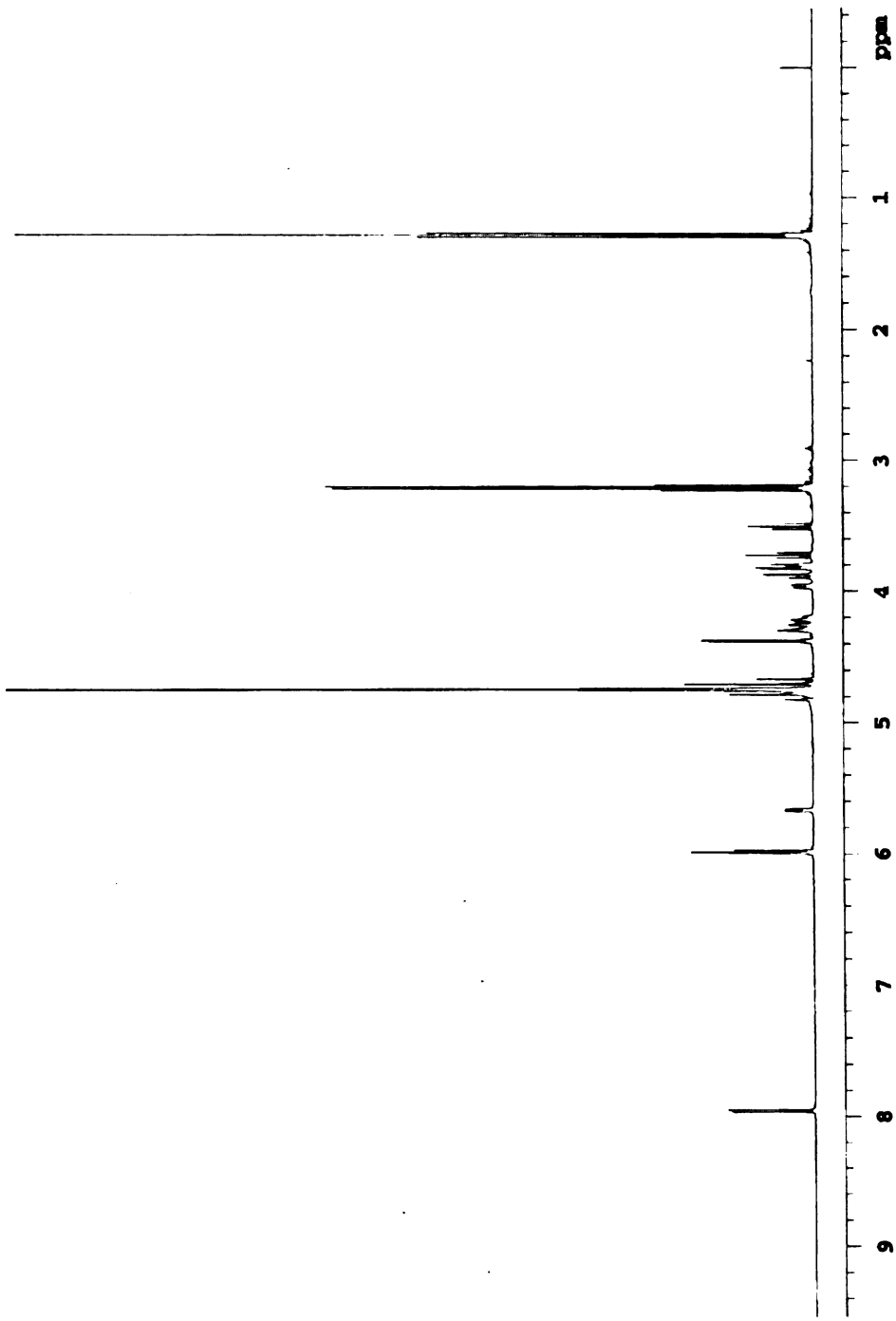

Figure 23. ^{1}H NMR of chemically synthesized UDP-kanosamine.

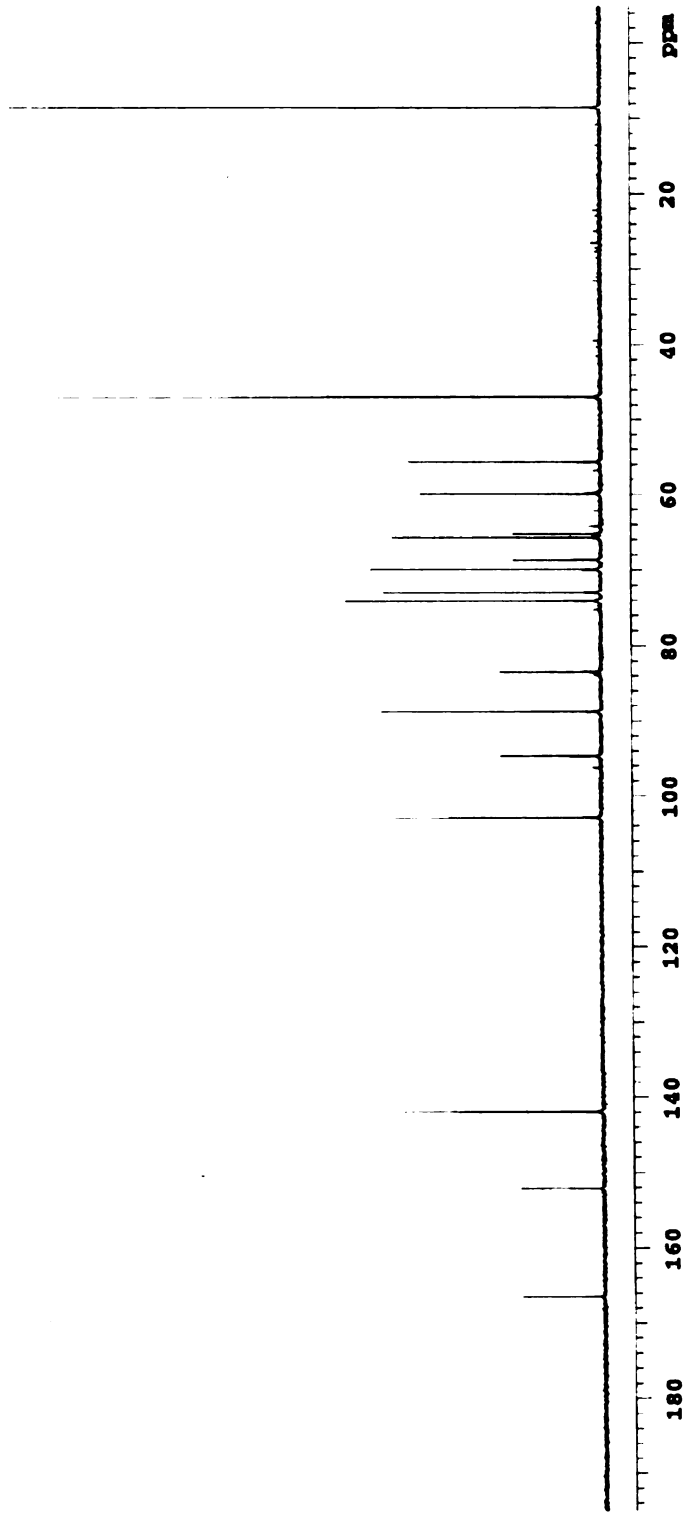

Figure 24. ^{13}C NMR of chemically synthesized UDP-kanosamine.

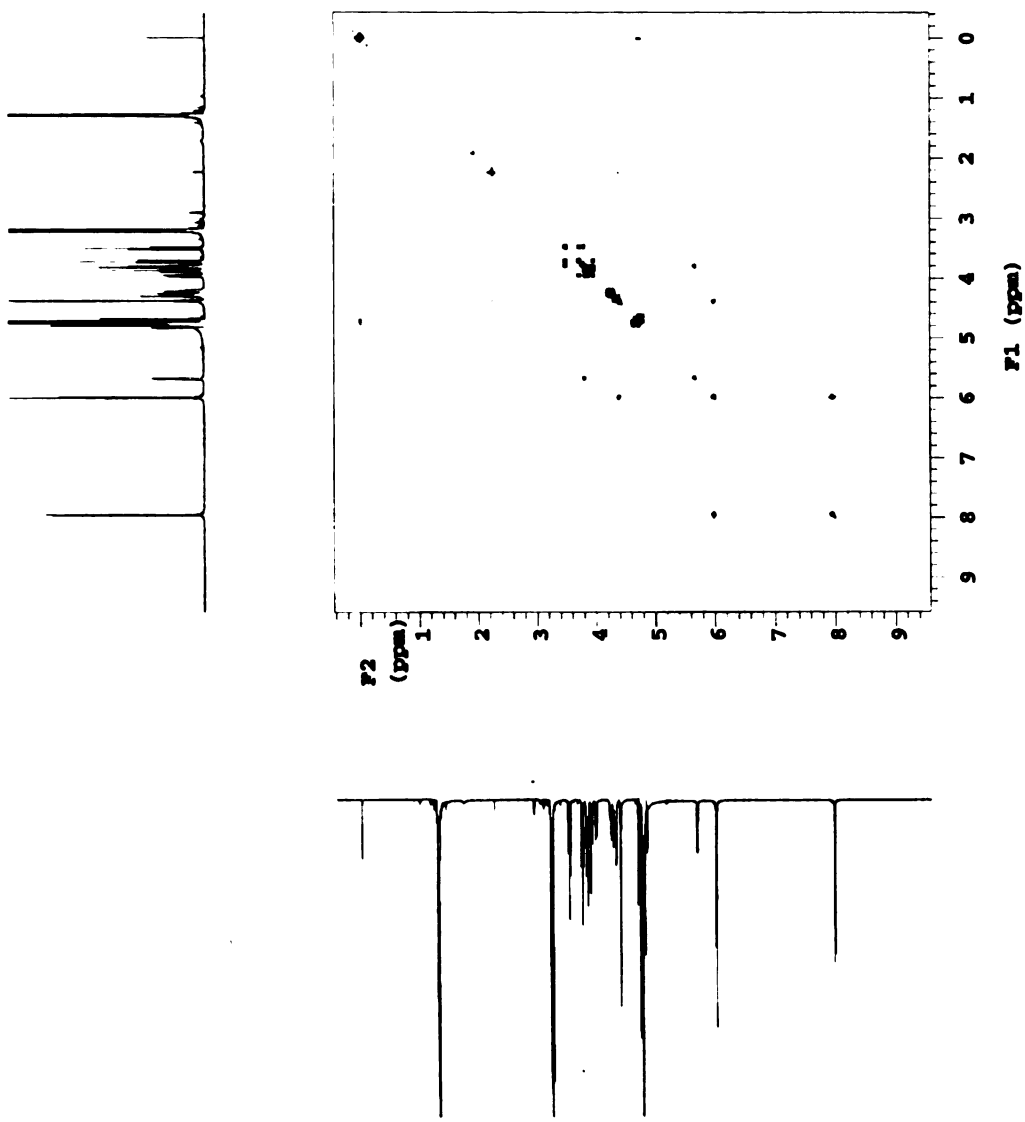

Figure 25. COSY of UDP-kanosamine.

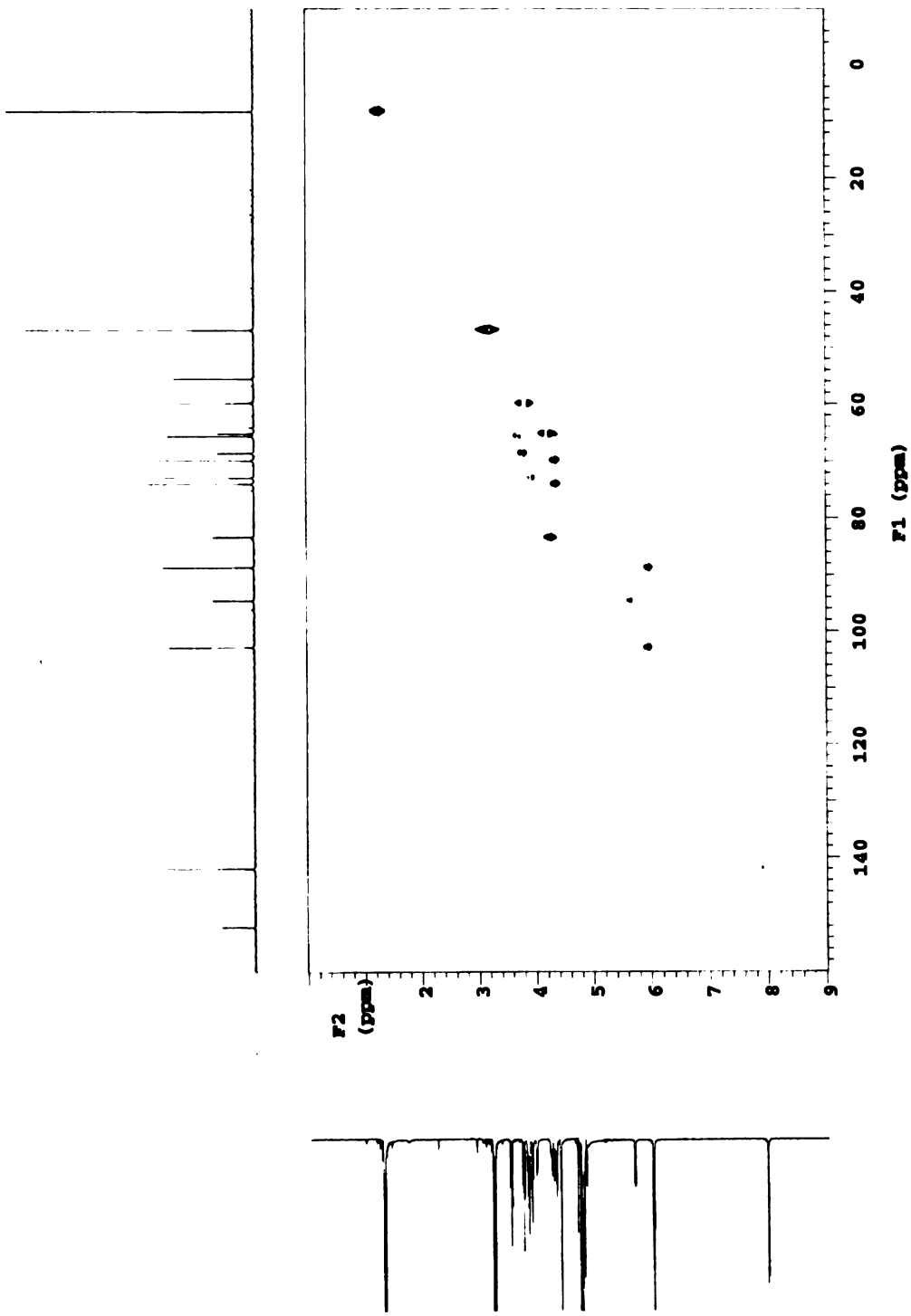

Figure 26. HMQC of UDP-kanosamine.

CHAPTER THREE

EXPERIMENTAL

General Methods

General Chemistry

All reactions sensitive to air and moisture were carried out in flame or oven-dried glassware under a positive atmosphere of argon. Air or moisture sensitive reagents and solvents were transferred to reaction flasks fitted with rubber septa via oven-dried syringes or cannula. Unless otherwise specified, all reactions were carried out at room temperature. Solvents were removed using either a Büchi rotary evaporator at water aspirator pressure or under high vacuum (0.5 mm Hg).

CH_2Cl_2, Et_3N, and pyridine were distilled from calcium hydride under nitrogen. Tetrahydrofuran and diethyl ether were distilled under nitrogen from sodium/benzophenone. Water used in synthesis was glass-distilled and deionized. All other reagents and solvents were used as available from commercial sources. Organic solutions of products were dried over anhydrous Na_2SO_4 or $MgSO_4$. The sodium salt of 3-(trimethylsilyl)propionic-*2,2,3,3-d*$_4$ acid (TSP) was purchased from Lancaster Synthesis Inc.

Chromatography

HPLC analysis was preformed on an Agilent 1100 series HPLC with ChemStation acquisition software (Rev. A.08.03). Columns used include Agilent ZORBAX C-18 reverse phase analytical column (4.6 mm x 150mm), Alltech C-18

reverse phase semi-prep column (22 mm x 250 mm), and sugar KS-801 strong cation exchange column (Showa Denko, 8 mm x 300 mm). Solvents were routinely filtered through 0.45-μm membranes (Gelman Science). Analytes were detected at 254 nm or 260 nm as specified.

AG1-X8 (acetate form and chloride form) was purchased from Bio-Rad. Ni-NTA resin was purchased from Qiagen. Dowex 1 (200-400 mesh, chloride form) and Dowex 50 (200-400 mesh, H^+) were purchased from Sigma-Aldrich.

Radial chromatography was performed on a Harrison Associates Chromatotron (model 7924), using 1, 2 or 4 mm layers of silica gel 60 PF_{254} containing gypsum (E. Merck). Silica gel 60 (40-63 μm, E. Merck) was used for flash chromatography. Analytical thin-layer chromatography (TLC) utilized precoated glass plates of silica gel 60 F-254 (0.25 mm, Whatman). TLC plates were visualized by UV or by immersion in anisaldehyde stain (by volume: 93% ethanol, 3.5% sulfuric acid, 1% acetic acid, and 2.5% anisaldehyde), or phosphomolybdic acid stain (7% phosphomolybdic acid in ethanol, w/v) followed by heating.

Spectroscopic Measurements

^1H NMR and ^{13}C NMR spectra were recorded on a Varian VX-300 FT-NMR or a Varian VX-500 FT-NMR spectrometer. Chemical shifts for ^1H NMR spectra are reported (in parts per million) relative to internal tetramethylsilane (Me_4Si, δ = 0.0 ppm) with $CDCl_3$ as solvent and to sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$ (TSP, δ = 0.0 ppm) when D_2O was the solvent. The following abbreviations are used to describe spin multiplicity: s (singlet), d (doublet), t (triplet), q (quartet), m (unresolved multiplet), dd (doublet of doublets), b (broad). ^{13}C NMR spectra were recorded at 125 MHz.

Chemical shifts for ^{13}C NMR spectra are reported (in parts per million) relative to internal tetramethylsilane (Me_4Si, δ = 0.0 ppm) with $CDCl_3$ as solvent and to sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$ (TSP, δ = 0.0 ppm) when D_2O was the solvent. ^{31}P NMR spectra were recorded on a 121 MHz Varian spectrometer and chemical shifts are reported (in parts per million) relative to external 85% phosphoric acid (0.0 ppm). UV and visible measurements were recorded on a Perkin-Elmer Lambda 3b UV-Vis spectrophotometer or on a Hewlett Packard 8452A Diode Array Spectrophotometer equipped with HP 89532A UV-Visible Operating Software. Fast atom bombardment (FAB) mass spectra were obtained on a double focusing Kratos MS50 mass spectrometer at Michigan State University and electrospray ionization (ES) mass spectra were obtained on a direct infusion electrospray mass spectrometer at Department of Chemistry and Biochemistry at University of South Carolina.

Concentrations of fermentation and cell-free reaction products were determined by comparison of the integrals corresponding to each compound with the integral corresponding to TSP (δ=0.00 ppm) in the ^1H NMR. Compounds were quantified using the following resonances: kanosamine (δ 5.27, d, 0.45 H), kanosamine 1-phosphate (δ 5.53, dd, 1 H), and UDP-kanosamine (δ 5.67, dd, 1 H). Concentrations of above compounds derived from their respective ^1H NMR integral values tended to be overestimated and their precise concentrations were calculated by application of the following formulas: $[\text{kanosamine (mM)}]_{actual} = 0.78 \times [\text{kanosamine (mM)}]_{NMR} + 0.15$; and $[\text{kanosamine 1-phosphate (mM)}]_{actual} = 0.81 \times [\text{kanosamine 1-phosphate (mM)}]_{NMR}$; $[\text{UDP-kanosamine (mM)}]_{actual} = 0.92 \times [\text{UDP-kanosamine (mM)}]_{NMR}$. These equations were obtained as follows: A known quantity of each compound was dissolved in 10 mL

of D_2O to obtain a stock solution. Various known volumes of the stock solution of each compound were concentrated under reduced pressure and redissolved in 1 mL of D_2O containing 10 mM TSP and their ^1H NMR spectra were recorded. The solute concentration in each sample that was estimated for ^1H NMR was plotted against the calculated concentration for that sample resulting in the calibration curve.

Chemical Assays

Organic and inorganic phosphate assay

Reagents used to quantify both organic phosphate and inorganic phosphate[75] include 10 % $Mg(NO_3)_2$ (w/v, dissolved in ethanol), 0.5 M HCl, 10% ascorbic acid (w/v, dissolved in H_2O), and 0.42% $(NH_4)_2MoO_4$ (w/v, dissolved in 1 M H_2SO_4). The assay solution (freshly mixed) consists of one volume of 10% ascorbic acid and six volumes of 0.42% $(NH_4)_2MoO_4$.

To assay for organic phosphate, 100 μL of 10% $Mg(NO_3)_2$ was added to a test tube (13 mm x 100 mm) containing 100 μL of sample. The resulting mixture was then evaporated to dryness over a flame, leaving a white solid. To this test tube was added 600 μL of 0.5 M HCl. After the white solid was dissolved, the solution was heated at 100 °C for 15 min in a boiling water bath. Assay solution (1400 μL, described above) was added to the cooled sample and the resulting mixture was kept at 45 °C for 20 min. If the original sample contains either inorganic phosphate or any organic phosphate, a blue color will develop.

To assay for inorganic phosphate, 600 μL of 0.5 M HCl and 1400 μL of assay solution were directly added to a test tube containing 100 μL of sample. The resulting

reaction mixture was then heated at 45 °C for 20 min. Blue color is developed if sample contains inorganic phosphate.

The phosphate concentration of a sample was quantified by comparing the absorbance at 820 nm of the sample to a standard curve that is prepared using a phosphorous standard solution (0.65 mM in 0.05 M HCl, Sigma 661-9).

Ninhydrin assay

The ninhydrin reagent[76] contains NaOAc (15%, w/v), sulfolane (40%, v/v), ninhydrin (2%, w/v), and hydrindantin (0.36%, w/v) in H_2O. The pH of the reagent was adjusted to 2.5 with glacial acetic acid and the reagent was then filtered through filter paper.

An aliquot (50 μL) of the sample was added to a test tube (13 mm x 100 mm) containing 500 μL of ninhydrin reagent. The resulting mixture was heated at 100 °C for 5 min. A purple color develops if the sample contains free amino group. The concentration of amino group containing compound in a sample was quantified by comparing the absorbance at 570 nm of the sample to a standard curve that is prepared using glycine as the standard.

Bacterial Strains and Plasmids

Amycolatopsis mediterranei (ATCC 21789) and *B. pumilus* (ATCC 21143) were purchased from the American Type Culture Collection (ATCC).

Storage of Bacterial Strains and Plasmids

All bacterial strains were stored at -78 °C in glycerol. Glycerol samples were prepared by adding 0.75 mL of an overnight culture to a sterile vial containing 0.25 mL of 80% (v/v) glycerol. The solution was mixed, left at room temperature for 2 h, and then stored at -78 °C.

Culture Medium

Bacto tryptone, Bacto tryptone peptone, Bacto yeast extract, Bacto malt extract, agar, and soytone were purchased from Difco. Soy flour and peanut meal were purchased from ICN Bioscience. Nutrient agar was purchased from Oxoid.

All solutions were prepared in distilled, deionized water. LB medium[77] (1 L) contained Bacto tryptone (10 g), Bacto yeast extract (5 g), and NaCl (10 g). Antibiotics were added where required to the following final concentrations: chloramphenicol (Cm), 34 μg/mL; ampicillin (Ap), 50 μg/mL; carbenicillin (Ca), 200 μg/mL; tetracycline (Tc), 12.5 μg/mL; kanamycin (Kan), 50 μg/mL; and erythromycin (Ery), 200 or 500 μg/mL, as specified. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to the culture medium of strains containing inducible promoters including P_{tac}, P_{T7}, or P_{T5}. Inorganic salts, D-glucose, and $MgSO_4$ solutions were autoclaved separately while antibiotics and IPTG were sterilized by passage through a 0.22-μm membrane. Solid medium was prepared by the addition of 1.5% (w/v) agar to the liquid medium. Soft agar (100 mL) contained Bacto tryptone (1 g), Bacto yeast extract (0.5 g), and agar (0.55 g).

A. mediterranei was grown in either YMG medium. YMG medium[78] (1 L) contained Bacto yeast extract (4 g), Bacto malt extract (10 g), and glucose (4 g). D-Glucose (60%, w/v) was autoclaved separately while trace element solution was sterilized

by passage through a 0.22-μm membrane. Solid YMG medium was prepared by the addition of 1.5% (w/v) agar to the liquid medium. YMG soft agar was prepared by the addition of 0.55% (w/v) agar to the liquid medium.

B. pumilus was grown either on solid nutrient agar or in liquid SSNG or SNG medium. Nutrient agar plates were prepared by the addition of 2.8% (w/v) nutrient agar to H_2O. SSNG medium[79] (1 L) contained soy flour (15 g), soytone (1 g), NaCl (6 g), and glucose (10 g). SNG medium[60b] contained (1 L) soytone (15 g), NaCl (3 g), and glucose (10 g). D-Glucose (20%, w/v) was autoclaved separately.

The standard *B. pumilus* fermentation medium[73] (1 L) contained soy flour (30 g), soytone (1 g), and NaCl (9 g). The pH of the medium was adjusted to 7.0 with 1 N NaOH prior to autoclaving. D-Glucose (30 g, 50% w/v) solution, which was autoclaved separately, was added to the medium immediately prior to initiation of the fermentation. One more batch of soy flour (10 g, autoclaved in 50 mL H_2O) was added at 48 h. Additional D-glucose (65%, w/v) was added to the fermentor vessel during a fermentation run to maintain a glucose concentration of 15-50 g/L. Glucose concentration was determined using the Glucose Diagnostic Kit from Sigma.

Agrobacterium tumefaciens was grown in AB/sucrose medium. AB/sucrose medium is a combination of four different solutions: solution 1 (90 mL), 2 (10 mL), 3 (1 mL), and 4 (1 mL). Solution 1 contains KH_2PO_4 (0.54 g), yeast extract (0.05 g), and Na_2HPO_4•2H2O (1.08 g) in 90 mL H_2O; solution 2 is 20% sucrose in water (20 g sucrose + 80 mL H_2O); solution 3 contains $MgSO_4$•$7H_2O$ (0.15 g), $CaCl_2$•$2H_2O$ (0.025 g), $FeSO_4$•$7H_2O$ (0.01 g), and citric acid (0.16 g) in 10 mL H_2O (pH 7.0); solution 4 contains

urea (0.9 g) in 10 mL H_2O (pH 7.0). Solution 1 and 2 were autoclaved separately, while solution 3 and 4 were filter sterilized, respectively.

Fermentation Conditions

General

Fermentations were conducted in a B. Braun M2 culture vessel with a 2 L working capacity. Environmental conditions were supplied by a B. Braun Biostat MD controlled by a DCU. Data was acquired on a Dell Optiplex GX200 personal computer utilizing B. Braun MFCS/Win software. PID control loops were used to control temperature, pH, and glucose addition. The temperature was maintained at 30 °C, and the pH was maintained at 7.0 by addition of concentrated NH_4OH or 2 N H_2SO_4. Glucose was added as a 65% (w/v) solution if required. Dissolved oxygen (D.O.) was monitored using a Mettler-Toledo 12 mm sterilizable O_2 sensor fitted with an Ingold A-type O_2 permeable membrane. D.O. was maintained at 10% air saturation throughout the course of the fermentations. Antifoam (Sigma 204) was added manually as needed.

Fed-batch fermentations of *B. pumilus*

Growth of an inoculant was initiated by introduction of a single colony from a nutrient agar plate into 100 mL of SSNG medium in a 500 mL flask with baffles and grown at 30 °C with agitation. Since SSNG medium was a heterogeneous medium (a suspension of soy flour), cell growth could not be monitored by following OD_{600}. The pH of the culture supernatant was used to monitor the stage of the cell growth. The pH of the culture supernatant decreased from 7.0 to approximately 5.0 during the first 24 h of

growth and increased from 5.0 to 8.5[80] thereafter due to the formation of kanosamine. When the pH of the culture supernatant reached 7.5 (30-48 h), the 100 mL culture was then transferred to the fermentation vessel. Three staged methods were used to maintain D.O. concentrations at 10% air saturation during the fermentation run. With the airflow at an initial setting of 0.06 L/L/min, the D.O. concentration was maintained by increasing the impeller speed from its initial set point of 200 rpm to its preset maximum of 1100 rpm. With the impeller rate constant at 1100 rpm, the mass flow controller then maintained the D.O. concentration by increasing the airflow rate from 0.06 L/L/min until a maximum airflow rate was reached (usually less than 0.5 L/L/min). Afterwards, airflow was maintained at 0.5 L/L/min, and the impeller was allowed to vary in order to maintain the D.O. concentration at 10% air saturation. The impeller speed typically varied between 500 and 1100 rpm during the remainder of the run. Additional D-glucose (65% w/v) was added to the fermentor vessel during the fermentation run to maintain a glucose concentration of 15-50 g/L. Glucose concentration was determined using the Glucose Diagnostic Kit from Sigma.

Analysis of Culture Supernatant

For strains being evaluated in shake flasks, samples (4 mL) of the culture were taken at timed intervals and the cells were removed by microcentrifugation. For strains being evaluated in fermentors, samples (5 mL) of broth were taken at indicated intervals, and cell densities of *E. coli* and *A. tumefaciens* were determined by dilution of fermentation broth with water (1:100) followed by measurement of absorption at 600 nm (OD_{600}). Dry cell weight for *E. coli* (g/L) was obtained using a conversion coefficient of

0.43 g/L/OD_{600}. The remaining fermentation broth was microcentrifuged to obtain cell-free broth. Cell densities of *A. mediterranei* and *B. pumilus* were not measured if heterogeneous medium was used. Cell-free samples of *A. mediterranei* and *B. pumilus* were obtained by microcentrifugation of the corresponding broth.

Solute concentrations in the cell-free culture supernatant were determined by ^{1}H NMR. Solutions were concentrated to dryness under reduced pressure, concentrated to dryness on additional time from D_2O, and then redissolved in D_2O containing a known concentration of the sodium salt of 3-(trimethylsilyl)propionic-*2,2,3,3-d*$_4$ acid (TSP). Concentrations were determined by comparison of the integrals corresponding to each compound with the integral corresponding to TSP (δ=0.00 ppm) in the ^{1}H NMR. Compounds were quantified using the following resonances: kanosamine (δ 5.27, d, 0.45 H), kanosamine 1-phosphate (δ 5.53, dd, 1 H), and UDP-kanosamine (δ 5.67, dd, 1 H). The concentrations of kanosamine, kanosamine 1-phosphate, and UDP-kanosamine were calculated by application of the previously described calibration formulae.

Purification of kanosamine from fermentation broth of *B. pumilus*

After the completion of the fermentation, the fermentation broth was passed through four layers of cheesecloth and the cells in the filtrate were removed by centrifugation (6400*g*, 4°C, 10 min). Activated charcoal (10 g/L) was then added to the supernatant and the mixture was shaken at 37°C and 250 rpm for 1 h. The charcoal was removed by filtration through Celite and the protein in the filtrate was removed by ultrafiltration. The resulting protein free solution was concentrated to about 200 – 300 mL and applied to Dowex 50 (H^+, 2.5 cm x 51 cm, 250 mL) resin. The column was

washed with H_2O (600 mL) and eluted with a linear gradient (600 mL + 600 mL, 0 to 0.5 M) of HCl. Fractions testing positive by the ninhydrin assay were collected and evaporated under reduced pressure to give kanosamine (14%, 39.8 g) as a slightly brown solid (very hygroscopic). ^{1}H NMR (500 MHz, D_2O, TSP as reference): δ 5.27 (d, J = 3.5 Hz, 0.4 H), 4.72 (d, J = 8 Hz, 0.6 H), 4.39 (m, 1 H), 3.74-4.38 (m, 2 H), 3.67 (ddd, J = 10, 10, 4.5 Hz, 1 H), 3.57 (ddd, J = 7.5, 5.5, 2 Hz, 0.5 H), 3.45 (dd, J = 10, 8 Hz, 0.5 H), 3.41 (dd, J = 10, 10 Hz, 0.5 H), 3.25 (dd, J = 10, 10 Hz, 0.5 H). ^{13}C NMR (125 MHz, D_2O, TSP as reference): δ 99.0, 94.1, 79.7, 74.2, 73.4, 71.0, 68.9, 68.8, 63.2, 63.0, 60.8, 58.1.

Preparation of and transformation of *E. coli* competent cells

Competent cells were prepared using a procedure modified from Sambrook et al.[81] A single colony was inoculated into 5 mL of LB containing the necessary antibiotics. After overnight growth, an aliquot (1 mL) of the culture was transferred to a 500 mL Erlenmeyer flask containing 100 mL of LB containing the necessary antibiotics. The cells were cultured at 37 °C with shaking at 250 rpm until an OD_{600} of 0.4-0.6 was reached. The entire culture was transferred to a centrifuge bottle that was sterilized with bleach and rinsed exhaustively with sterile water. The cells were harvested by centrifugation (4000g, 4 °C, 5 min) and the culture medium was discarded. All manipulations were carried out on ice during the remainder of the procedure. Harvested cells were resuspended in 100 mL of ice cold 0.9% NaCl. After centrifugation at 4000g and 4 °C for 5 min, the cells were resuspended in ice cold 100 mM $CaCl_2$ (50 mL) and stored on ice for 30 min. The cells were then collected by centrifugation (4000g, 5 min, 4

°C) and resuspended in 4 mL of ice-cold 100 mM $CaCl_2$ containing 15% glycerol (v/v). Aliquots (0.25 mL) of competent cells were transferred to 1.5 mL microfuge tubes, frozen in liquid nitrogen, and stored at -78 °C.

In order to perform a transformation, frozen competent cells were thawed on ice for 5 min prior to use. A small aliquot (1 to 10 μL) of plasmid DNA or a ligation reaction was added to the thawed competent cells (0.1 mL). The solution was gently mixed and stored on ice for 30 min. The cells were then heat shocked at 42 °C for 2 min and then placed on ice for 2 min. LB (0.5 mL) was added to the cells, and the sample was incubated at 37 °C for 1 h (or 30 °C for 1.5 h with agitation). After incubation, cells were collected by microcentrifugation. If the transformation was to be plated onto LB plates, 0.5 mL of the culture supernatant was removed. The cells was then resuspended in the remaining 0.1 mL of LB and spread onto LB plates containing the appropriate antibiotics. If the transformation was to be plated onto minimal medium plates, all the culture supernatant was removed. The cells were washed with 0.5 mL of M9 salts and collected by microcentrifugation. After removal of all the supernatant, the cells were resuspended in 0.1 mL of M9 salts and spread onto the appropriate plates. A sample of competent cells with no DNA added was also carried through the transformation procedure as a control. These cells were used to check the viability of the competent cells and to verify the absence of growth on selective medium.

General Enzymology

General information

E. coli and *B. pumilus* cells were harvested at 4000*g* for 5 min at 4°C and *A. mediterranei* were harvested at 11000*g* for 5 min at 4°C. Cells lysis was achieved by two passages through a French pressure cell (SLM Aminco) at 16,000 psi. Cellular debris was removed from the lysate by centrifugation (30000*g*, 30 min, 4 °C). Protein solutions were concentrated by ultrafiltration using either Millipore PM-10 membranes (10,000 MWCO) or Centricon concentrators (Amicon). Protein concentrations were determined using the Bradford dye-binding procedure[82] using protein assay solution purchased from Bio-Rad. The assay solution was prepared by diluting 20 mL of the Bio-Rad concentrate to 100 mL with water followed by gravity filtration of the resulting solution. Assay solution (5 mL) was added to an aliquot of protein containing solution (diluted to 0.1 mL) and the sample was vortexed. After allowing the color to develop for 5 min, the absorbance at 595 nm of the solution was measured. Protein concentrations were determined by comparison to a standard curve prepared using bovine serum albumin.

Protein gel (SDS-PAGE)

SDS-PAGE analysis was followed the procedure described by Harris et al.[83] The separating gel was prepared by mixing 3.33 mL of 30% acrylamide stock solution (w/v in H_2O), 2.5 mL of 1.5 M Tris-HCl (pH 8.8), and 4 mL of distilled deionized water. After degassing the solution using a water aspirator for 20 min, 0.1 mL of 10% ammonium persulfate (w/v in H_2O), 0.1 mL 10% SDS (w/v in H_2O), and 0.005 mL of N, N, N', N'-tetramethylethylenediamine (TEMED) was added. After mixing gently, the separating

gel was poured into the gel cassette to about 1.5 cm below the top of the gel cassette. *t*-Amyl alcohol was overlaid on the top of the solution and the gel was allowed to polymerize for 1 h at rt. The stacking gel was prepared by mixing 1.7 mL of 30% acrylamide stock solution, 2.5 mL of 0.5 M Tris-HCl (pH 6.8), and 5.55 mL of distilled deionized water. After degassing for 20 min, 0.1 mL of 10% ammonium persulfate, 0.1 mL 10% SDS, and 0.01 mL of TEMED was added, and the solution was mixed gently. *t*-Amyl alcohol was removed from the top of the gel cassette, which was subsequently rinsed with water and wiped dry. After insertion of the comb, the gel cassette was filled with stacking gel solution, and the stacking gel was allowed to polymerize for 1 h at rt. After removal of the comb, the gel cassette was installed into the electrophoresis apparatus. The electrode chamber was then filled with electrophoresis buffer containing 192 mM glycine, 25 mM Tris base, and 0.1% SDS. Each protein sample (10 μL) was diluted with Laemmli sample buffer[84] (10 μL, Sigma S-3401) consisting of 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, and 125 mM Tris-HCl (pH 6.8). Samples and markers (MW-SDS-200, Sigma) were loaded into the sample well and the gel was run under constant current at 30 mA when the blue tracking dye (bromophenol blue) was within stacking gel. After the blue tracking dye reached the separating gel, a higher current (50 mA) was applied to the gel. At the completion of electrophoresis (blue tracking dye reaches the bottom of the gel), the gels were removed from the cassettes and fixed in a solution of 10% (w/v) aqueous trichloroacetic acid for 30 min. After staining in a solution containing 0.1% (w/v) Coomassie Brilliant Blue R, 45% (v/v) MeOH, 10% (v/v) HOAc in H_2O for 3 h, the protein gels were destained in a

solution of 45% (v/v) MeOH, 10% (v/v) HOAc in H_2O. The gels were then sealed in a sheet protector.

Synthetic Procedures

3-Amino-3-deoxy-N-benzyloxycarbonyl (Cbz)-α, β-D-glucose (2). Kanosamine 1 (4.2 g, 23 mmol, purified from *B. pumilus* fermentation broth) was dissolved in a small amount of water and neutralized to pH 7 by addition of 10 N NaOH. The solution was then diluted to a volume of 25 mL. $NaHCO_3$ (5.0 g, 60 mmol) was added, followed by addition of benzyloxy chloroformate (5.0 mL, 35 mmol) in five portions over 1 h under vigorous stirring. The reaction was followed by the consumption of kanosamine 1 using ninhydrin test. After 24 h of stirring, the suspension was continuously extracted with EtOAc for 24 h. The organic phase was cooled in an ice bath and the precipitate was collected by filtration. The white solid was washed with cold EtOAc and dried overnight over $Mg(ClO_4)_2$ under vacuum. Yield: 5.79 g (79%). ^1H NMR (α-form in D_2O): δ 7.41-7.46 (m, 5 H), 5.25 (d, *J*=4 Hz, 1 H), 5.16 (s, 2 H), 3.74-3.91 (m, 4 H), 3.58 (dd, *J*=4, 11 Hz, 1 H), 3.43 (t, *J*=10 Hz, 1 H). ^{13}C NMR (*α* & *β*-form in D_2O): δ 161.7, 161.6, 139.3 (2 C), 131.6 (4 C), 131.2 (4 C), 130.5 (2 C), 99.3, 94.5 (2 C), 79.9, 75.4, 74.6, 72.7, 71.0, 70.9 69.8, 63.6, 63.5, 61.9, 58.5. HRMS (FAB) calcd for $C_{14}H_{20}O_7N$ ($M+H^+$): 314.1240. Found: 314.1241.

3-Amino-3-deoxy-N-benzyloxycarbonyl (Cbz)-(2-propenyl)-α, β-D-glucopyranoside (3). Compound **2** (3.0 g, 9.6 mmol) was added to dry acidic allyl alcohol (15 mL, 4% HCl in allyl alcohol). The reaction mixture was stirred and heated to reflux in a heating

mantle. Reaction ran overnight until TLC (CH_2Cl_2:MeOH = 12:1) showed a complete reaction. Reaction was cooled, neutralized with Na_2CO_3, followed by addition of charcoal. It was then filtered through Celite. Allyl alcohol was removed under reduced pressure. The resulting solid was dissolved in chloroform and subsequent flash column chromatography, eluted with 12:1 CH_2Cl_2-MeOH, yielded compound **3** as a white solid (2.7 g, 79%). ^{1}H NMR (CD_3OD): δ 7.38-7.24 (m, 5 H), 5.98 (m, 1 H), 5.36 (d, *J*=17 Hz, 1 H), 5.17 (d, *J*=11 Hz, 1 H), 5.08 (s, 2 H), 4.84 (d, *J*=4 Hz, 1 H), 4.26 (dd, *J*=5, 13 Hz, 1 H), 4.06 (dd, *J*=6 Hz, 1 H), 3.84-3.77 (m, 2 H), 3.68-3.60 (m, 2 H), 3.47 (dd, *J*=4, 11 Hz, 1 H), 3.34 (t, *J*=10 Hz, 1 H). ^{13}C NMR (CD_3OD): δ 159.7, 138.4, 135.7, 135.6, 129.4, 128.9, 117.6, 117.5, 104.0, 98.8, 79.1, 74.3, 73.3, 71.7, 71.0, 70.0, 69.3, 67.5, 62.7, 62.6, 61.1, 57.9. HRMS (FAB) calcd for $C_{17}H_{23}O_7N$ ($M+H^+$): 354.1553. Found: 354.1552.

3-Amino-3-deoxy-2, 4, 6-tri-*O*-benzyl-*N*-Cbz-(2-propenyl)-α, β-D-glucopyranoside (4). To the solution of compound **3** (0.56 g, 1.6 mmol) in dry DMF (10 mL) was added anhydrous Barium hydroxide (6.7 g, 39 mmol), followed by addition of benzyl bromide (1.3 mL, 11 mmol). The reaction mixture was sonicated in a Branson Ultrasonic cleaner for 10 h at rt. After the reaction was finished, the suspension was filtered and solid was further rinsed with chloroform. The organic phase was washed with 10% acetic acid, saturated $NaHCO_3$, and water. It was then dried over Na_2SO_4, filtered and concentrated. White solid crystallized out during removal of solvent and was later proven to be the right product. Flash column chromatography of the mother liquid, eluted with 3:1 hexanes-EtOAc, gave another crop of product. Totally 0.59 g of compound **4** was produced (60%). ^{1}H NMR ($CDCl_3$): δ 7.23-6.97 (m, 20 H), 5.77 (m, 1 H), 5.17 (dd, *J*=1,

16 Hz, 1 H), 5.05 (dd, *J*=1, 10 Hz, 1 H), 4.99 (d, *J*=12 Hz, 1 H), 4.95 (d, *J*=12 Hz, 1 H), 4.74 (d, *J*=3 Hz, 1 H), 4.49 (d, *J*=11 Hz, 2 H), 4.42 (d, *J*=2 Hz, 2 H), 4.39 (d, *J*=11 Hz, 2 H), 4.36 (d, *J*=12 Hz, 2 H), 4.27 (d, *J*=11 Hz, 1 H), 4.02 (dd, *J*=5, 13 Hz, 1 H), 3.85 (dd, *J*=6, 13 Hz, 1 H), 3.72 (s, 1 H), 3.68 (s, 1 H), 3.57 (dd, *J*=3, 11 Hz, 1 H), 3.49 (d, *J*=11 Hz, 1 H). ^{13}C NMR ($CDCl_3$): δ 138.0, 137.8, 136.6, 133.7, 128.4, 128.35, 128.27, 128.03, 127.96, 127.81, 127.71, 127.67, 117.8, 95.2, 75.6, 74.9, 74.4, 73.5, 72.4, 70.6, 68.5, 68.1, 66.5, 55.4. HRMS (FAB) calcd for $C_{38}H_{41}O_7N$ ($M+H^+$): 624.2961. Found: 624.2963.

3-Amino-3-deoxy-2, 4, 6-tri-*O*-benzyl-*N*-Cbz-α, β-D-glucopyranose (5). Compound **4** (2.2 g, 3.5 mmol) was dissolved in the solvent system, ethanol:toluene:water = 7:3:2 (80 mL). Wilkinson's catalyst, tris(triphenylphosphine)chlororhodium (0.5 g, 0.54 mmol), was then added. The reaction mixture was refluxed at ~90°C until TLC showed an almost complete isomerization (hexanes:EtOAc = 2:1). Formic acid (88%, 5 mL) was then injected into the reaction and refluxing was continued overnight. The reaction mixture was diluted with dichloromethane, washed with water, and dried over Na_2SO_4. Small amount of charcoal was added, followed by filtration after 1 h. The filtrate was concentrated to dryness. The resulting solid was dissolved in minimum amount of chloroform. Addition of 3:1 hexanes-EtOAc resulted in crystallization of **5** (1.63 g, 80%). HRMS (FAB) calcd for $C_{35}H_{37}O_7N$ ($M+H^+$): 584.2648. Found: 584.2653.

3-Amino-3-deoxy-*N*-Cbz-β-D-glucopyranose-1, 2, 4, 6-tetraacetate (7). A suspension of anhydrous NaOAc (1.43 g, 17.4 mmol) in 25 mL of Ac_2O was heated to reflux in a

heating mantle. Powdered **2** (5.0 g, 16.0 mmol) was added in portions. After the addition was complete, the suspension was continued to reflux for 1 h. The reaction mixture was then cooled and poured with stirring onto 80 mL of cracked ice. After standing for 3 h at rt with occasional stirring, the crystalline material was collected by filtration and then washed with cold water. The resulting brown solid was recrystallized from 95% EtOH. To avoid contamination with the α-anomer, the product (5.26 g, 68%) was collected by filtration as soon as the recrystallization solution cooled to rt. ^{1}H NMR ($CDCl_3$): δ 7.27-7.37 (m, 5 H), 5.72 (d, *J*=8.1 Hz, 1 H), 5.07 (s, 2 H), 4.31 (dd, *J*=12, 4.2 Hz, 1 H), 4.08 (m, 2 H), 3.86 (dd, *J*=2.7, 10.2 Hz, 1 H), 2.10 (s, 3 H), 2.09 (s, 3 H), 1.92 (s, 3 H), 1.90 (s, 3 H). ^{13}C NMR ($CDCl_3$): δ 170.6, 170.1 (2 C), 168.9, 156.1, 136.3, 128.5, 128.1, 128.0, 92.1, 73.7, 70.3, 67.7, 66.9, 61.5, 55.7, 20.8, 20.7, 20.4 (2 C). HRMS (FAB) calcd for $C_{22}H_{28}O_{11}N$ ($M+H^+$): 482.1663. Found: 482.1662.

3-Amino-3-deoxy-*N*-Cbz-α, β-D-glucopyranose-2, 4, 6-triacetate (8). To a solution of compound **7** (3.6 g, 7.5 mmol) in THF (18 mL) was added benzylamine (0.82 mL, 7.5 mmol) dropwise. The reaction was stirred overnight at rt. TLC showed a complete conversion (EtOAc:hexanes = 3:1). The mixture was diluted with water and extracted 3 times with chloroform. The combined organic phase was successively washed with ice-cold dilute HCl (0.05M), saturated NaCl, and water. It was then dried over Na_2SO_4 and concentrated to dryness under reduced pressure. The resulting syrup was applied to flash column chromatography, eluted with gradient 1:1~3:1 EtOAc-hexanes. Compound **8** was obtained as a white solid (2.9 g, 88%). ^{1}H NMR ($CDCl_3$): δ 7.32-7.28 (m, 5 H), 5,36 (s, 1 H), 5.07 (s, 2 H), 4.91 (t, *J*=10 Hz, 1 H), 4.83 (dd, *J*=3, 11 Hz, 1 H), 4.76 (d, *J*=8 Hz, 1

H), 4.40-4.08 (m, 4 H). ^{13}C NMR ($CDCl_3$): δ 171.1, 171.0, 170.9, 170.5, 170.3, 156.5, 156.4, 136.5, 128.5, 128.2, 128.03, 127.97, 95.8, 90.1, 73.3, 73.0, 71.1, 68.6, 67.8, 66.8, 62.4, 62.2, 55.3, 51.7, 20.7, 20.6, 20.5. HRMS (FAB) calcd for $C_{20}H_{25}O_{10}N$ ($M+H^+$): 440.1557. Found: 440.1556.

Diphenyl (2, 4, 6-tri-O-acetyl-3-amino-3-deoxy-*N*-Cbz-α-D-glucopyranosyl) phosphate (9). A solution of compound **8** (1.85 g, 4.2 mmol) in CH_2Cl_2 (30 mL) containing DMAP (1.20 g, 9.8 mmol) was stirred at rt for 15 min and then cooled to −10°C. Diphenylchlorophosphate (2.04 mL, 9.7 mmol) was added dropwise and the solution was stirred for 2 h between −10°C and 0°C and for 1 h at 4°C. The mixture was then diluted with CH_2Cl_2 and the organic phase was washed with ice-cold water, ice-cold 0.5M HCl and saturated $NaHCO_3$. Chromatographic purification using 2:3 EtOAc-hexanes afforded the title compound in pure α-form as a white solid (1.72 g, 61%). ^1H NMR ($CDCl_3$): δ 7.71-7.39 (m, 15 H), 5.99 (dd, *J*=3, 7 Hz, 1 H), 5.10 (s, 2 H), 4.89-4.98 (m, 2 H), 4.82 (d, *J*=10 Hz, 1 H), 4.37 (q, *J*=11 Hz, 1 H), 4.18 (dd, *J*=4, 12, 1 H), 4.05 (d, *J*=11 Hz, 1 H), 3.82 (d, *J*=12, 1 H), 1.99 (s, 3 H), 1.89 (s, 3 H), 1.74 (s, 3 H). ^{13}C NMR ($CDCl_3$): δ 170.5, 170.3, 170.0, 156.0, 150.3, 150.2, 150.1, 136.4, 129.9, 129.8, 128.5, 128.1, 128.0, 125.7, 120.5, 120.4, 120.1, 120.0, 95.1 (d, J_{POC}=6 Hz, 1 C), 70.3, 69.5 (d, J_{POCC}=7 Hz, 1 C), 67.3, 66.8, 61.1, 51.7, 20.5, 20.3, 20.1.

3-Amino-3-deoxy-α-D-glucose 1-phosphate (10). Crystalline phosphoric acid (4.0 g, 41 mmol) was dried over $Mg(ClO_4)_2$ *in vacuo* overnight. It was melted at 65°C in an oil

bath. Compound **7** (2.5 g, 5.2 mmol) was added and bubbling occurred at once. The mixture was stirred *in vacuo* at 65°C for 2 h when bubbling ceased and the solid disappeared. The sirup was dissolved, with gentle warming, in a small amount of THF, and the resulting solution was poured into 100 mL of ice-cold 1 N aq LiOH. The solution was stirred at rt for 4 days to saponify the acetyl groups. The reaction was followed by ^{1}H NMR. The solution was neutralized with 85% H_3PO_4 and the precipitated lithium phosphate was removed by filtration. The remaining solution was partially concentrated and passed through a column of Dowex 50 (H^+). The column was washed with water and fractions were checked both by ninhydrin test and by ^{1}H NMR. The fractions containing **10** were combined and concentrated to dryness to yield **10** as white solid (0.62 g, 46%), which is stable under rt. ^{1}H NMR (D_2O): δ 5.53 (dd, *J*=3, 7 Hz, 1 H), 3.90 (m, 1 H), 3.78-3.87 (m, 3 H), 3.72 (t, *J*=11 Hz, 1 H), 3.46 (t, *J*=11 Hz, 1 H). ^{13}C NMR (D_2O): δ 96.4 (d, J_{POC}=5.8 Hz, 1 C), 75.2, 71.2 (d, J_{POCC}=8.8 Hz, 1 C), 68.5, 62.7, 58.1. HRMS (FAB) calcd for $C_6H_{15}O_8NP$ ($M+H^+$): 260.0535. Found: 260.0533.

Uridine 5'-diphospho-3-amino-3-deoxy-α-D-glucose monotriethylammonium salt (6). Compound **10** (0.027 g, 0.10 mmol) and uridine 5'-triphosphate (UTP, 0.055 g, 0.10 mmol) were dissolved in 60 mL of TEA buffer (100 mM triethanolamine, 4 mM $MgCl_2$, and 10 mM mercaptoethanol, pH=8.0). The pH of the solution was adjusted to 8.0 with 1 N aq NaOH. The solution was degassed with Argon for 5 min. Uridine 5'-diphosphoglucose pyrophosphorylase and inorganic pyrophosphatase were then added. The solution was stirred gently at rt for 3 h when TLC (isopropyl alcohol/1 N NH_4OAc, 2:1, v/v) showed the disappearance of UTP. Compound **10** (0.027 g, 0.10 mmol) and

UTP (0.055 g, 0.10 mmol) was again added. After this process being repeated twice, ^{1}H NMR showed some unreacted **10**. More UTP (0.039 g, 0.071 mmol) was added. The solution was stirred for another 3 h and enzymes were removed by ultrafiltration. The resulting solution was then loaded on AG-1 X8 column (HCO_3^-) and eluted with 0~1 N triethylammonium bicarbonate (TEAB). Fractions were checked both by organic phosphate assay and by ^{1}H-NMR. Proper fractions containing the desired product were combined. TEAB was partially removed by azeotropic evaporation with isopropyl alcohol. The resulting solid was dissolved in 0.5 mL of water, followed by addition of 50 mL of EtOH. The suspension was centrifuged at 20000 *g* for 15 min. EtOH was poured out and the solid was washed twice with EtOH. The solid was then dissolved in water and lyophilized. Compound **6** was obtained as white solid (50.1 mg, 24%). ^{1}H NMR (D_2O): δ 7.95 (d, *J*=8 Hz, 1 H), 5.99 (d, *J*=4 Hz, 1 H), 5.98 (d, *J*=4 Hz, 1 H), 5.67 (dd, *J*=7, 3 Hz, 1 H), 4.39-4.36 (m, 2 H), 4.30-4.19 (m, 3 H), 3.98-3.94 (m, 1 H), 3.90-3.84 (m, 1 H), 3.83-3.79 (m, 2 H), 3.72 (t, *J*=10 Hz, 1 H), 3.51 (t, *J*=10 Hz, 1 H), 3.21 (q, *J*=7.5 Hz, 6 H), 1.29 (t, *J*=7.5 Hz). ^{13}C NMR (D_2O): δ 169.04, 154.64, 144.47, 105.48, 97.23 (d, J_{POC}=5.8 Hz, 1 C), 91.32, 86.01 (d, J_{POCC}=9.5 Hz, 1 C), 76.60, 75.53, 72.48, 71.21 (d, J_{POCC}=34.5 Hz, 1 C), 68.30, 67.83 (d, J_{POC}=4.8 Hz, 1 C), 62.52, 58.25, 49.53, 11.07. HRMS (ES) calcd for $C_{15}H_{25}O_{16}N_3P_2$($M+H^+$): 566.0784. Found: 566.078.

Uridine 5'-diphospho-3-keto-α-D-glucose. A single colony of *Agrobacterium tumefaciens* were inoculated into 5 mL AB/sucrose medium in a culture tube and incubated at 30°C for ~30 h. 50 λ of the culture were used to inoculate another 5 mL AB/sucrose medium and the new culture was incubated at 30°C for 24 h. The resulting

culture was then used to inoculate 3x100 mL of AB/sucrose medium (1 mL culture for each 100 mL medium). They were incubated at 30°C for ~12 h until OD_{600} reached 3. Cells were then harvested by centrifugation (5000 *g*, 4°C, 10 min) and subsequently resuspended in 20 mL of sterilized 5 mM Tris buffer (pH 8.2). UDP-α-D-glucose (1.00 g, 1.77 mmol) was added and the culture was incubated at 30°C, 250 rpm for ~10 h until ^{1}H NMR of the supernatant showed disappearance of starting UDP-α-D-glucose. Cells were then removed by centrifugation (5000 *g*, 4°C, 10 min), followed by removal of proteins by ultrafiltration. The filtrate was then applied onto Dowex 50 (H^+) column and eluted with water. The pH was adjusted to 7 and amount of product was determined through ^{1}H NMR. ^{1}H NMR (D_2O): δ 7.96 (d, *J*=8 Hz,1 H), 6.01 (d, *J*=4 Hz, 1 H), 5.98 (d, *J*=8 Hz, 1 H), 5.95 (dd, *J*=4, 7 Hz, 1 H), 4,62 (t, *J*=3 Hz, 1 H), 4.49 (d, *J*=10 Hz, 1 H), 4.44-4.36 (m, 2 H), 4.30-4.20 (m, 3 H), 4.08 (m, 1 H), 3.97 (dd, *J*=2, 13 Hz, 1 H), 3.88 (dd, *J*=4, 13 Hz, 1 H). HRMS (FAB) calcd for $C_{15}H_{22}O_{17}N_2P_2$ ($M-H^+$): 563.0316. Found: 563.0318.

Genetic Manipulations

Plasmid pJG8.115

The *rifM* gene was amplified by PCR from cosmid FKN108 using the following primers containing *Bam*HI terminal recognition sequences: 5'-ATGGATCCATGACATTCC CGATCGTCGAC and 5'-ATGGATCC ATACAGGCGGAGCCGGACTCG. The resulting 0.8-kb amplified fragment was digested with *Bam*HI and ligated into the *Bam*HI site of plasmid pJG7.246 to create

plasmid pJG8.115 (*T5*, *lacO*, His_6, *rifM*, $lacI^Q$, Ap^R) in which the *rifM* gene is oriented in the same orientation as *T5* promoter.

Enzyme Purifications

A. mediterranei rifM-encoded UDP-kanosamine phosphatase

UDP-kanosamine phosphatase was purified from *E. coli* BL21(C⁺RP)/pJG8.115 (*T5*, *lacO*, His_6, *rifM*, $lacI^Q$ Ap^R) by Ni-NTA Agarose column (Qiagen). Buffers used in the purification included buffer A: Tris-HCl (25 mM), imidazole (10 mM), NaCl (300 mM), pH 8.0; buffer B: Tris-HCl (25 mM), imidazole (20 mM), NaCl (300 mM), pH 8.0; buffer C: Tris-HCl (25 mM), imidazole (250 mM), NaCl (300 mM), pH 8.0; buffer D: Tris-HCl (25 mM), DTT (1 mM), glycerol (10%), pH 7.5.

A single colony of *E. coli* BL21(C⁺RP)/pJG8.115 (*T5*, *lacO*, His_6, *rifM*, $lacI^Q$ Ap^R) was used to inoculate 5 mL LB containing ampicillin and chloramphenicol. After 12 h of growth, the 5 mL culture was transferred to 1 L LB containing ampicillin and chloramphenicol and grown at 28°C until OD_{600} reached 0.5. IPTG was added to the culture to a final concentration of 0.1 mM. After an additional 9 h growth at 28°C, the cells were harvested by centrifugation (6000*g* for 10 min at 4 °C), resuspended in Buffer A (36 mL), and lysed by two passages through a French press at 16000 psi. Cellular debris was removed by centrifugation at 20000*g* and 4 °C for 30 min. To the supernatant, a 50% slurry (w/v) of Ni-NTA agarose resin was added (1 mL resin per 4 mL of crude lysate), and the mixture was stirred at 4 °C for an hour. The lysate resin slurry was transferred into a polypropylene column (Qiagen) and washed with Buffer B (2 x 4 mL per mL of Ni-NTA agarose resin). The 6-His tagged protein was eluted from

the column by washing with a solution of buffer C (2 x 1 mL per mL of Ni-NTA agarose resin). The eluted protein was concentrated, dialyzed against buffer D, quick frozen in liquid nitrogen, and stored at –80°C.

Cell-free Lysate Preparations

Cell-free lysate of *A. mediterranei*

A. mediterranei was first grown[49] on a YMG plate at 28°C for 4 days. This plate culture of *A. mediterranei* was then used to inoculate YMG medium. A 50 mL YMG culture was grown in a 250 mL flask with baffles at 28°C and 250 rpm. The synthesis of rifamycin B was checked by spectrophotometry[85] after 4 days of incubation at 28°C and 280 rpm. The culture that showed the highest synthesis of rifamycin B was used to inoculate 1000 mL YMG medium. After 3 days of incubation at 28°C and 300 rpm, the mycelia were harvested by centrifugation (8600*g*, 4°C, 5 min). After washing the mycelia with 50 mM Tris-HCl buffer (pH 6.8), the cells were harvested by centrifugation (11000*g*, 4°C, 5 min). The mycelia were resuspended in Tris-HCl buffer (1mM PMSF, 1mM DTT, 20% glycerol, pH 6.8) (5 mL/g of wet cells). Cells were then disrupted by two passages through a French press (16000 psi). The cell debris was removed by centrifugation (48000*g*, 4 °C, 25 min). Dialysis was performed using a Millipore PM-10 membrane and an Amicon stirred cell (300 mL). The cell lysate (about 30 mL) was first diluted to 300 mL followed by concentration (to about 25 mL). More buffer (225 mL) was added to the concentrator. The sample was concentrated to 25 mL again and the process was repeated until the concentration of the micro solute was sufficiently reduced.

Typically, 2 to 3 cycles were performed to remove most of initial salt content. Finally, the lysate was concentrated to about 30 mL.

Cell-free lysate of *B. pumilus*

B. pumilus was first grown on a nutrient agar plate at 37 °C for 18-24 h. A single colony from the plate was used to inoculate SSNG medium. A 100 mL culture was grown in a 500 mL flask with baffles for 1.5 to 2 days at 30°C and 250 rpm. The whole 100 mL culture was then transferred to a 4 L flask with baffles containing 1 L of the same medium. After 1 day of incubation at 30°C and 250 rpm, cells were collected by first passing through four layers of cheesecloth followed by centrifugation (6400*g*, 4°C, 10 min) of the filtrate. After washing the cells with 50 mM Tris-HCl buffer (pH 7.5), the cells were harvested by centrifugation (6400*g*, 4°C, 10 min). The cells were resuspended in 2 mL of 50 mM Tris-HCl buffer (1 mM DTT, 1 mM PMSF, 20% glycerol, pH 7.5) per 1 g of wet cells and were disrupted by two passages through a French press (16000 psi). The cell debris was removed by centrifugation (48000*g*, 4°C, 25 min).

In Vitro Enzymatic Reactions

Biosynthesis of kanosamine from UDP-kanosamine

UDP-kanosamine (0.020 g, 0.030 mmol) was incubated with *B. pumilus* cell-free lysate, or *A. mediterranei* cell-free lysate, or *A. mediterranei* S699 (*rifM*) cell-free lysate or partially purified RifM solution at 30°C for 6 h (18 h in case of RifM). The reaction was buffered in 10 mL of 50 mM Tris-HCl solution, which also contains 1 mM DTT, 1 mM PMSF (omitted in case of RifM) and 20% glycerol. Proteins were removed by

ultrafiltration. The filtrate was concentrated to dryness. To the resulting syrup was added 40 mL of 100% ethanol. The mixture was centrifuged (20000 *g*, 4°C, 30 min). Ethanol was removed and the solid pellet was rinsed twice with ethanol. It was then dissolved in water and prepared for NMR analysis. The final yields of kanosamine and kanosamine 1-phosphate were determined through ^{1}H NMR, respectively, based on previously obtained response factors. To further confirm the formation of kanosamine as well as kanosamine 1-phosphate, purification of the crude reaction mixture was performed. The mixture was applied to a Dowex 50 (H^{+}) cation exchange resin (2.5 cm x 6 cm, 30 mL). The column was first eluted with water (60 mL) and then with 1N HCl. Fractions that tested positive by organic phosphate assays were collected and neutralized with aqueous NaOH. Comparison of the NMR spectra between purified compounds with standard kanosamine and kanosamine 1-phosphate as well as HR-MS analysis confirmed the formation of kanosamine and kanosamine 1-phosphate.

Biosynthesis of kanosamine from kanosamine 1-phosphate

kanosamine 1-phosphate (0.010 g, 0.039 mmol) was incubated with *A. mediterranei* cell-free lysate, or *A. mediterranei* S699 (*rifM*) cell-free lysate, or partially purified RifM solution at 30°C for 6 h (18 h in case of RifM). The reaction was buffered in 10 mL of 50 mM Tris-HCl solution, which also contains 1 mM DTT, 1 mM PMSF (omitted in case of RifM) and 20% glycerol. Proteins were removed by ultrafiltration. The filtrate was concentrated to dryness. To the resulting syrup was added 40 mL of 100% ethanol. The mixture was centrifuged (20000 *g*, 4°C, 30 min). Ethanol was removed and the solid pellet was rinsed twice with ethanol. It was then dissolved in

water and prepared for NMR analysis. The final yields of kanosamine were determined through ^{1}H NMR based on the response factor. To further confirm that kanosamine was formed and more importantly that kanosamine 6-phosphate was not formed, purification of the crude reaction mixture was performed. The mixture was applied to a Dowex 50 (H^+) cation exchange resin (2.5 cm x 6 cm, 30 mL). The column was first eluted with water (60 mL) and then with 1N HCl. Fractions that tested positive by organic phosphate assays were collected and neutralized with aqueous NaOH. Comparison of the NMR spectra as well as HR-MS analysis confirmed the formation of kanosamine and no formation of kanosamine 6-phosphate.

Reaction of UDP-α-D-glucose or UDP-3-keto-α-D-glucose in the presence of RifM

UDP-α-D-glucose or UDP-3-keto-α-D-glucose (0.010 g) was incubated with partially purified RifM solution at 30°C. The total reaction volume was 5 mL and buffered in 50 mM Tris-HCl solution, which also contains 1 mM DTT and 20% glycerol. Proteins were removed by ultrafiltration. The filtrate was concentrated to dryness. To the resulting syrup was added 40 mL of 100% ethanol. The mixture was centrifuged (20000 *g*, 4°C, 30 min). Ethanol was removed and the solid pellet was rinsed twice with ethanol. It was then dissolved in water and prepared for NMR analysis. In both cases, no reaction occurred and starting materials remained unconsumed.

UDP-kanosamine phosphatase assay (RifM) by HPLC

UDP-kanosamine phosphatase activity was measured by HPLC according to the consumption rate of UDP-kanosamine. UDP-kanosamine (0.010 g, 0.015 mmol) was

incubated with partially purified RifM (0.54 mg) solution at 30°C. The reaction was buffered in 5 mL of 50 mM Tris-HCl solution, which also contains 1 mM DTT and 20% glycerol. At different time points (0h, 1h, 2h, 3h, 4h, 5h, 6h, 7h), 250 λ of reaction mixture was taken out. 50 λ of 10% TCA (trichloroacetic acid) was added to each sample and the proteins were removed by centrifugation (14000 *g*, 4 min). The supernatant was diluted 4 times (200 λ supernatant + 600 λ water). It was then filtered for HPLC analysis.

Paired-ion chromatography was utilized to analyze the amount of UDP-kanosamine left in each time point. Two mobile phases were prepared. The mobile phase A contains 50 mM potassium phosphate buffer (pH 6.9) and 2.5 mM tetrabutylammonium hydrogen sulfate (TBAHS). The mobile phase B was prepared in the same manner as A only different in that mobile phase B was diluted with 50% acetonitrile. The separations were accomplished on an Agilent ZORBAX C-18 reverse phase analytical column (4.6 mm x 150mm). Prior to analysis, the column was allowed to equilibrate for 30 min with a mixture of 97.5% eluent A and 2.5% eluent B, at a flow rate of 1 mL/min. At ambient temperature, 50 λ of sample was injected into the column. Separations were carried out with a 15 min linear gradient from 2.5% to 30% of eluent B, at a flow rate of 1 mL/min. The absorbance of the column effluent was monitored at 264 nm. UDP-kanosamine showed a retention time of 3.88 min under this condition. The RifM activity was calculated to be 0.002 units/mg, based on integration values obtained for UDP-kanosamine from each time point.

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