THE BIOSYNTHESIS OF VIRGINIAMYCIN S_1

by

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Introduction

Virginiamycin S_1 is a cyclic hexadepsipeptide antibiotic produced by Streptomyces virginiae and a few other closely related Streptomyces species. Its importance as an antibiotic is demonstrated by its use commercially as a feed additive to help promote growth in poultry, swine, and cattle. Radiolabeled precursors are used to study the biosynthetic pathways by which the Streptomyces microorganism produces the antibiotic. The results of these radiotracer experiments provide information to help substantiate the proposed biogenesis from particular precursors.

The term "antibiotic" was first proposed by Selman Waksman in 1942. He defined it as an organic compound produced by a microorganism, which at high dilution has the capacity to inhibit growth of or completely destroy another microorganism or microorganisms.[1] This definition obviously does not include materials extracted from green plants, purely synthetic chemotherapeutic agents, or other substances from non-microbial sources. It also excludes organic acids or amines that may inhibit microbial growth, but which are not active in low concentrations. Despite the exclusion of many organic compounds by the limitation of the definition, over 2,000 antibiotics have been reported, though most

are of no economic value. The bewildering array of organic compounds which comprise antibiotics have furnished a stimulating challenge to the organic chemist.

Bacteria, Streptomyces, Norcardia, and fungi are the primary producers of antibiotics. Although several other classes of microorganisms have this capability, it is to a much more limited extent.[2] The metabolic pathways that lead to the formation of antibiotics are usually not a part of the normal metabolic pathways responsible for the growth and reproduction of microorganisms, hence antibiotics are termed secondary metabolites. In most cases these secondary metabolites are not only non-essential, but appear to have no useful purpose to the organism that produces them. Because of this, antibiotic biosynthesis has even been regarded as a "series of inborn errors of metabolism" superimposed on the normal metabolism of the producing organism.[3] In general, antibiotics are selective in their action, each one acting on a specific group of microorganisms, called its "inhibition spectrum". Although not universally true, microorganisms are not sensitive to the antibiotic they produce.

Interest in the study of the origin of antibiotics or any chemical compound produced by a living organism, henceforth called biosynthesis, has only arisen over the last two decades. The increase in the time devoted to biosynthetic studies has been prompted to a large extent

by the increased ease of structure elucidation with the advent of nuclear magnetic resonance spectroscopy (n.m.r.), mass spectroscopy, and routine X-ray crystallography. The increase in availability of isotopically labeled precursor molecules (^{14}C, ^{3}H, and most recently ^{13}C), materials on which many of these studies rely, has helped advance the ease by which these studies can be accomplished.

A variety of reasons exist for undertaking biosynthetic studies on an antibiotic or any other natural product. Originally, these studies were approached with the hope of gaining insights into the biological processes that existed in the world around us. Hypothesized biosynthetic pathways often guided the chemist's approach to the structure elucidation, total synthesis, or classification of these compounds.[4] Today, with many of the pathways better understood, the biological processes becoming of interest are enzyme mechanisms. Currently, very little is known about the enzymes' involvement in the biosynthetic pathways, but with use of previously gained information, new biosynthetic studies can be designed to give insights and information regarding these mechanisms.

Biosynthetic pathways can be used as models by the synthetic chemist in designing syntheses. At one time the idea existed that the biosynthesis of natural products proceeded via biological trickery, i.e. a "vital force", but this concept has long since perished. The success

of biomimetic syntheses demonstrated that nature is bound by the same laws, and uses the same chemical reactions, though enzyme catalyzed, as the synthetic chemist. Considering this, plus the fact that nature's methods are highly efficient, makes it profitable for the synthetic chemist to at least examine, if not duplicate, many of the steps nature uses in the biosynthesis of natural products.

A knowledge of chemical precursors involved in the biosynthesis of an antibiotic offers the potential of modifying compounds.[5] By substituting chemical analogs it may be possible to alter the composition of an antibiotic, resulting in a new one which might have similar or completely different characteristics. Some may even prove to be effective where the original has little or no effect.

Finally, the future holds the possibility of increased yields of antibiotics, or other natural products, by using recombinant DNA techniques. This technique would generate increased yields by producing either larger amounts of the enzymes controlling the biosynthesis or perhaps altered regulatory enzymes, which are insensitive to the control mechanisms of the system. This reason may be of particular value when dealing with virginiamycin S_1, since it is always produced as a minor component by the microorganism. Being a marketable product, it

would be a great advantage to increase the yields of virginiamycin S_1, especially since most companies operate on the principle of producing the highest feasible quantities of these agents with minimum cost in time, labor and materials.

The biosynthesis of peptidolactones such as virginiamycin S_1 lends itself to a unique approach. In past years, the biosynthesis of peptidolactones was studied by incorporating possible precursors, isolating the pure antibiotic, degrading it, and then using adsorption or ion exchange chromatography to isolate enough of each component present to determine the location of the radioactivity. These studies proved to be difficult and time consuming, especially when the microorganisms produced the antibiotic in limited quantities. With the advent of high pressure liquid chromatography (HPLC) in the past decade, the approach can be modified to degradation, derivatization, and HPLC purification. Despite an additional step, using this technique biosynthetic studies can be accomplished much easier, faster, and with very small amounts of antibiotic.

The derivative used is of key importance. It provides not only the means for detection in the HPLC system, but will also effect the separation of the various components in the degraded antibiotic. The literature contains a large host of amino acid derivatives to select

from. When choosing, certain criteria must be kept in mind. First and of foremost importance is the presence of a UV adsorption chromophore. Most amino acids lack this feature, and it is essential if the component is to be detected. Secondly, the derivative must have good chromatographic properties. In general, the smaller the additional chromophore, the better the properties, since the amino acid portion, the only different part of each derivative, contributes more to the structure. Thirdly, the derivatization reagent must be reactive with secondary as well as primary amino groups. Secondary amino acids such as proline or N-methyl amino acids commonly occur in peptide antibiotics and must be dealt with if the modified technique is going to be of significant advantage. Fourth, the derivative should be produced by an easy one step reaction and, if possible, react with both the amino and carboxyl groups. This last criterion, though not essential, would provide an additional benefit if realized.

A close examination of the structure of virginiamycin S_1 (Figure 1) shows that it is comprised of seven components. Of the seven present only two, L-proline and L-threonine, are common protein amino acids. L-4-oxopipecolic acid and D-α-aminobutyric acid, two other components of the antibiotic are of particular interest. L-4-Oxopipecolic acid is of interest not only because its biogenesis is

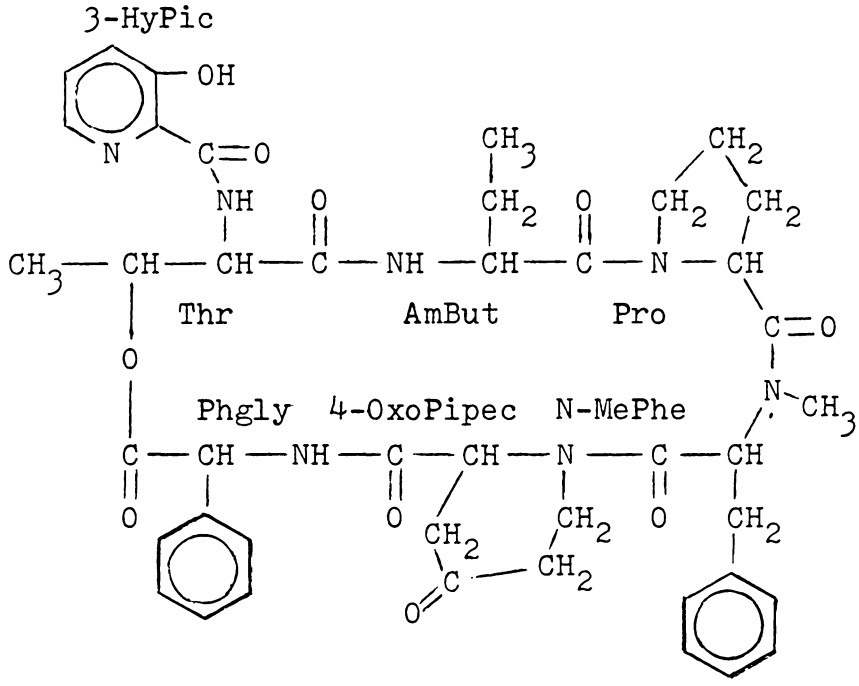


FIGURE 1. Structure of Virginiamycin S_1

currently unknown, but also because of the rarity of keto amino acids in nature. The origin of D-α-aminobutyric acid is of interest because most naturally occuring amino acids exist in the L isomer.

The research at hand was an investigation of the biosynthesis of virginiamycin S_1 by short term incubations of Streptomyces virginiae strain 1830 in broth cultures with various carbon-14 labeled precursors. A review of the literature and an examination of the structure indicated that L-threonine, L-proline, L-aspartic acid, L-lysine, L-phenylalanine, L-alanine, L-methionine, and L-tryptophan might be possible precursors. The probable location of incorporation of each precursor is shown in Figure 2. Each sample of purified virginiamycin S_1 was hydrolyzed and then derivatized with benzoyl chloride to give six N-benzoyl amino acid derivatives and 3-hydroxypicolinic acid which remained underivatized. Using analytical HPLC, a small quantity of each derivatized amino acid was collected to determine the distribution of the radioactivity. The results from these experiments provided information to confirm the proposed precursors in the biogenesis of virginiamycin S_1.

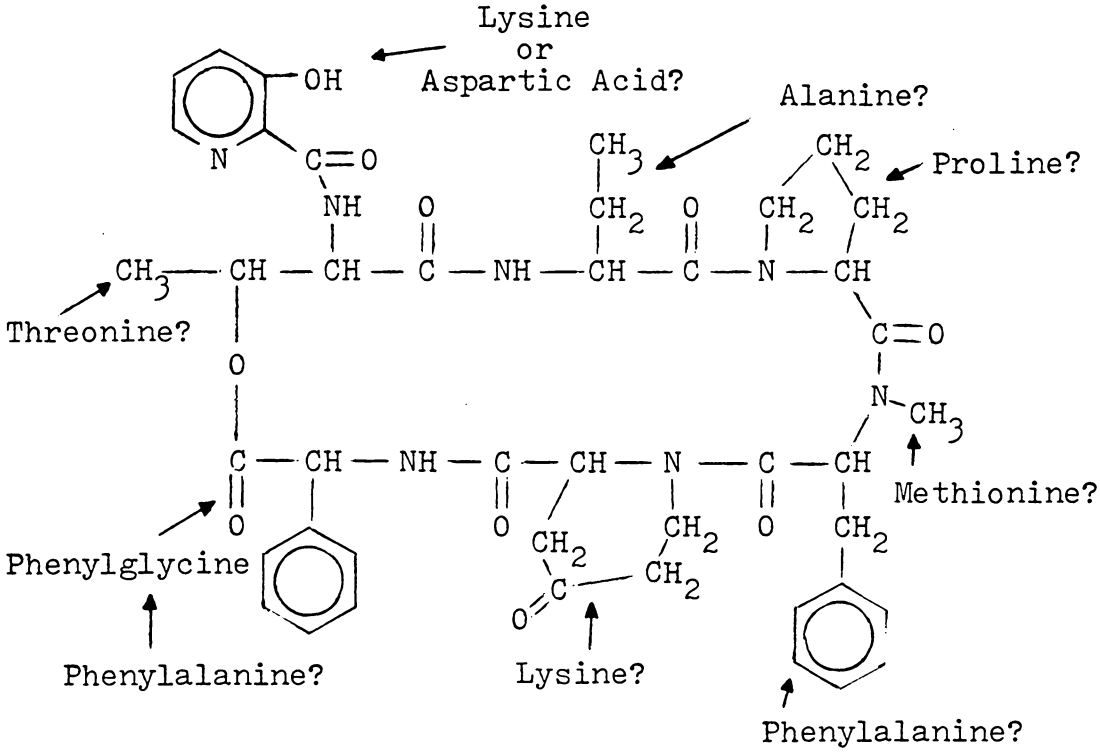


FIGURE 2. Location of Possible Precursors

Literature Review

Virginiamycin S_1 (Figure 1) is a macrocyclic peptidolactone antibiotic composed of six amino acids joined by peptide bonds and linked through the hydroxyl group of threonine to form a macrocyclic lactone ring. In addition it contains 3-hydroxypicolinic acid linked to the cyclic peptide through the amino function of threonine. Its structure places it in the group of heteromeric heterodetic cyclic peptides[7] along with etamycin[8], pyridomycin[9], and the streptogramin group[10-12].

Virginiamycin S_1 was first isolated by P. Van Dijck in 1955 from the culture broth of an unidentified Streptomyces species obtained from a Belgian soil sample.[13] It was isolated as part of a four component mixture, designated number 899, that exhibited properties very similar to streptogramin. It was not until 1957 that it was isolated in a pure form and its chemical properties determined.[14] At the same time it was also renamed staphlomycin factor S_1, the name used until 1972[15], at which time it became known as virginiamycin S_1.

The molecular formula of virginiamycin S_1 is $C_{43}H_{49}N_7O_{10}$ and its molecular weight is 823.91. It is considered a weak acid (pKa 9.0 in ethanol, pKa 7.7 in 1:2 dimethylformamide-water).[14,16] It shows almost no solubility in water or petroleum ether, but is soluble in most organic solvents.

It has UV maxima at 305 mμ in neutral or acid solution and 333 mμ in alkaline solution.

Vanderhaeghe and Parmentier reported its structure in 1959.[16,17] The structure was established by chemical identification of the total hydrolysis products combined with partial hydrolyses and the Edman degradation. The total hydrolysis products were identified as 3-hydroxy-picolinic acid, L-threonine, D-α-aminobutyric acid, L-proline, N-methyl-L-phenylalanine, 4-oxo-L-pipecolic acid and L-phenylglycine. The Edman degradation, a reaction used to sequence amino acids in peptides, and partial hydrolyses provided the information to determine the proper sequence of components.

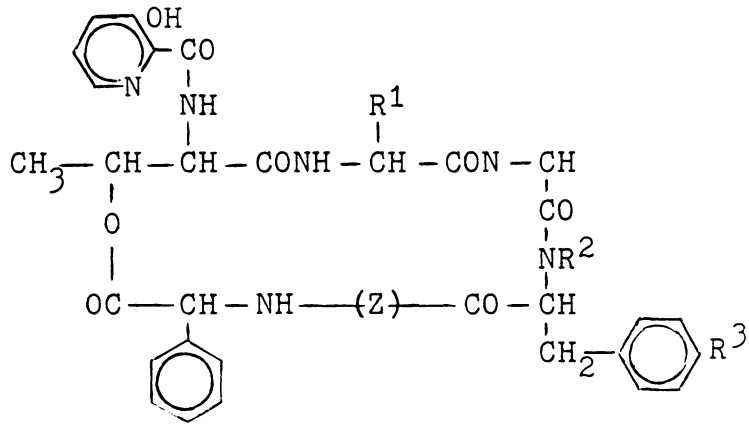
Several species of Streptomyces (e.g. mitakensis, ostreogriseus, virginiae) produce virginiamycin S_1 as one of several minor components, the others being virginiamycin M_2, S_2, S_3 and S_4. The major component produced by these species is virginiamycin M_1. These antibiotics belong to the streptogramin family; M_1 and M_2 to the A group and virginiamycin S_1, along with the other S components, to the B group. A characteristic of this family is the marked synergism of the A group antibiotics with those in group B.[18]

The chemical structure of group B antibiotics, similar to virginiamycin S_1, consists of a polypeptide containing unusual amino acids linked through the hydroxyl

group of threonine to form a macrocyclic lactone ring. In addition, all the antibiotics in the B group contain 3-hydroxypicolinic acid linked to the cyclic peptide. The primary structure which was largely established by identification of the hydrolysis products, is shown in Figure 3.

These streptogramin group B antibiotics are active mainly against Gram-positive bacteria and show little or no activity against Gram-negative bacteria, yeasts, fungi or protozoa. They are also known to be bactereostatic for Staphylococcus aureus, Bacterium agri[19], and Bacillus subtilis, although Bacillus subtilis mutants resistant to streptogramin B antibiotics have been reported[20]. In vivo studies have shown that few of the group B antibiotics have any effect in protecting mice infected with Staphylococcus aureus or Diplococcus pneumoniae. They are toxic to mice only in high doses (2-3g/kg) and they do not appear to accumulate in specific tissues or organs. Most of the antibiotic seems to be rapidly excreted in the urine and feces.[21]

At growth inhibitory concentrations, group B antibiotics appear to initially block protein synthesis.[22] This process being somewhat sensitive can be detected within 30 seconds of addition of the antibiotic. With longer exposure times, group B antibiotics also reduce the synthesis of RNA, DNA and cell walls, but to a lesser extent. The accumulation of low molecular weight



Names	R^1	R^2	R^3	⟨ Z ⟩
Virginiamycin S_1	C_2H_5	CH_3	H	4-Oxopipecolic acid
Virginiamycin S_4	CH_3	CH_3	H	4-Oxopipecolic acid
Virginiamycin S_2	C_2H_5	H	H	4-Hydroxypipecolic acid
Virginiamycin S_3	C_2H_5	CH_3	H	3-Hydroxy-4-oxopipecolic acid
Streptogramin B Mikamycin IA Pa 114 B 1 Pristinamycin IA Vernamycin Bα Ostreogrycin B	C_2H_5	CH_3	$N(CH_3)_2$	4-Oxopipecolic acid
Pristinamycin IC Vernamycin Bγ Ostreogrycin B_1	CH_3	CH_3	$N(CH_3)_2$	4-Oxopipecolic acid
Pristinamycin IB Vernamycin Bβ Ostreogrycin B_2	C_2H_5	CH_3	$NHCH_3$	4-Oxopipecolic acid
Vernamycin Bδ	CH_3	CH_3	$NHCH_3$	4-Oxopipecolic acid
Ostreogrycin B_3	C_2H_5	CH_3	$N(CH_3)_2$	3-Hydroxy-4-oxopipecolic acid
Vernamycin C Doricin	C_2H_5	CH_3	$N(CH_3)_2$	Aspartic acid
Patricin A	C_2H_5	CH_3	H	Proline
Patricin B	C_2H_5	CH_3	H	Pipecolic acid

FIGURE 3. Streptogramin Group B Antibiotics[15]

constituents in the pool is also affected. Accompanying the inhibition of protein synthesis is halt of the assembly of ribosomal particles. Studies with Bacillus subtilis treated with streptogramin group B antibiotics shows that a transient block of ribosomal assemblies is induced, but the normal ribosomal pattern is resumed when the antibiotic is removed.[23] These antibiotics have no effect on endogenous respiration, oxidation of ethanol or glucose, or anaerobic fermentation of glucose by Staphylococcus aureus.[22]

This inhibitory effect on protein synthesis by group B antibiotics has been confirmed in cell free systems. However, the polynucleotide used as the mRNA in the test system appears to have an effect on the extent of inhibition.[22] The incorporation of proline and lysine directed by polycytidylic acid (poly C) and polyadenylic acid (poly A), respectively, was observed, but little or no inhibition was seen in studying the polyuridylic acid-directed (poly U) synthesis of polyphenylalanine.

Present experimental evidence supports the idea that the site of action of streptogramin group B antibiotics is the 50S ribosomal subunit. Initially they were observed to inhibit uptake of ^{14}C-chloramphenicol by Bacillus megaterium and Staphylococcus aureus.[19] Then the inhibition of binding of ^{14}C-spiramycin I[24] and ^{14}C-Chloramphenicol[25,26] to bacterial ribosomes in cell free systems was

noted. Spiramycin and chloramphenicol in both cases are known to interact with the 50S ribosomal subunit. In addition, it has been reported that H^3-dihydrovirginiamycin S was incorporated into bacterial polyribosomes.[27] The fact that group B antibiotics enhance the interaction of streptogramin group A antibiotics with ribosomes in cell free systems also supports the ribosome as the site of action. An attempt to identify the virginiamycin S-binding protein was made, but unfortunately the experiments failed to do so. They did however demonstrate the stringent requirements for the drug attachment.[28]

The elongation step in protein synthesis is currently considered to be the most probable target of the group B antibiotics. Evidence to substantiate this hypothesis is extremely limited, and much work needs to be done. However, it has been shown that in the process of elongation the step of peptide bond formation catalyzed by the peptidyl transference activity of the 50S ribosomal subunit is not affected.[24] By the process of elimination, the specific step which might be blocked by the group B antibiotics is the translocation from the aminoacyl site (A-site) to the peptidyl site (P-site) of the growing polypeptide chain.[30]

All the previous information is characteristic of streptogramin group B antibiotics, a group to which virginiamycin S belongs. However, much of the information

was obtained from tests with group B antibiotics other than virginiamycin S (e.g. mikamycin B, vernamycins B). Regardless, the literature is not lacking studies done with the virginiamycins. Perhaps the most interesting characteristic of the streptogramin group of antibiotics is the synergistic effects they exhibit when an A group and a B group antibiotic are mixed. It is under these conditions that much of the research on the virginiamycins has been done. The original work was done by Van Dijck, Vanderhaeghe and DeSomer in the late 1950's.[13,31] Most recently Cocito and coworkers have been the principal contributors.

The virginiamycins (VM + VS mixture), like the streptogramin B group, show greater activity against Gram-positive bacteria than Gram-negative. The difference in sensitivity to the various bacteria is probably due to permeability, since the ribosomes, which are the site of action of these antibiotics, are just as sensitive in Gram-negative bacteria as those from Gram-positive organisms.[18] The significant difference of a VM + VS mixture is the enhanced inhibition of bacterial growth compared to the activity of the individual components. For example, high levels (~ 50 μg/ml) of either individual component is required to completely block the turbidity of growing cultures, whereas a much lower concentration (~ 0.5 μg/ml) of the mixture is all that is necessary to produce the

same effect. The mixture increases the antibiotic activity by 100-fold in this case.[32]

Studies dealing with the metabolism of macromolecules in bacteria treated with virginiamycin have produced conflicting results. It has been determined that the discrepancy in results can be explained by differences in length of culture incubations or evaluation techniques used.[18] Despite this it has generally been accepted that peptide chain formation is the primary target of the virginiamycins and that translation, not transcription, is prevented by these antibiotics. Formation of ribosomal subunits also is blocked in treated bacteria, an effect shared by other protein inhibitor antibiotics.[27] The individual components were observed to produce a reversible inhibition of protein and ribosomal subunit synthesis, whereas a combination irreversibly blocks the synthesis of both. Upon removal of the single virginiamycins the synthesis of protein and the formation of ribosomal subunits resume without delay. The recovery process for the formation of ribosomal subunits is rather puzzling, for the rRNA which accumulates under the condition of halted protein synthesis is undermethylated and has a lower molecular weight than normal.[33]

Studies in cell-free systems have also been controversial. This is largely due to the fact that the synergistic inhibitory effect of the two components on protein synthesis in cell-free systems has not been

observed in the systems used.[18] This is not surprising since virginiamycin S, a group B component, shows a range of activities depending on the test system used. Virginiamycin M is known to block protein synthesis by interacting with the 50S ribosomal subunit, whereas no biochemical reaction in protein biosynthesis has been conclusively shown to be blocked by virginiamycin S. Current evidence favors the idea of virginiamycin interaction with the A site of 50S ribosomal subunits, with the target of interference being elongation.[18]

The analysis of protein synthesis in cell free systems has indicated that the 50S ribosomal subunit is the target of virginiamycin M.[34] The target of virginiamycin S and its mechanism of action are still unknown. In order to help elucidate the mechanism a study of bacterial mutants, sensitive and resistant to each virginiamycin component, was made. It was found that a mixture of M and S inhibits growth of double sensitive organisms and for S-sensitive M-resistant mutants. It partially inhibits growth of M-sensitive S-resistant organisms and is without effect on double resistant cells.[20] The single components are bacteriostatic, whereas the mixture is bactericidal for double sensitive organisms and S-sensitive M-resistant mutants. No lethal effect can be seen in organisms that are S-resistant. The conclusion of this work is that, in Bacillus subtilis, the bactericidal effect directly linked to the gene controlling S-sensitivity in these

organisms.[20,35]

Another good example of the synergistic activity of virginiamycin M and S is the research done with Bacillus subtilis after infection with virulent phage 2C. Results from this work showed that virginiamycin blocks the preferential translation of the viral message, the mechanism by which the virus inhibits host-macro molecule formation, and the synthesis of viral DNA.[34] A mixture of M and S showed a 1,000-fold increase in activity over the single components. A suggested explanation of these results was the inhibition of the synthesis and function of virus dictated proteins.[34]

A few studies of the virginiamycins on organisms other than bacteria have been done. The virginiamycins were tested on Euglena gracilis, an algae, but no growth alteration was observed. However, virginiamycin M was found to reversibly block chlorophyll formation, causing the algae to appear bleached, while virginiamycin S had no apparent action. As in bacteria, a mixture exhibited synergistic effects.[18] Further research indicated that in addition to inhibition of chlorophyll production, chloroplast ribosomes and rRNA production was also blocked. In an effort to explain these effects it has been postulated that the biosynthetic pathways for chlorophyll, RNA, and protein formation are integrated within the chloroplasts.[18] Work has also been done with isolated spinach chloroplasts.[18]

In test systems used virginiamycin S showed no activity while virginiamycin M exhibited a strong effect. No synergistic effect was observed with a mixture of M and S.

Since its discovery, virginiamycin has been largely used as a feed additive to improve growth of poultry, swine and cattle. The reason behind the growth promotion by the virginiamycins has not been determined, but most likely the effect is due to the inhibition of intestinal flora, particularly of Gram-positive organisms.[18] Evidence in support of this is the fact that no growth enhancement is observed in germ-free animals, unless they are contaminated by conventional animals. The virginiamycins have been extensively investigated in chickens[36-38], turkeys[39], pigs[40-47], lambs[48], and calves[49]. In addition, the virginiamycins have been successfully used as therapeutic agents in veterinary medicine. The main use has been for the treatment and prevention of swine dysentery[50,51], but it is likely the future will see increased use of these antibiotics for other diseases.

The use of virginiamycin as an antibiotic for humans is limited at the present time. Virginiamycin has mainly seen topical applications in clinical use. This is largely due to the poor resorption of the antibiotic. The virginiamycins have been used in the fields of pediatrics, surgery, stomatology and dermatology.[18] One of the most successful applications has been in the treatment

of whooping cough in infants. One third of those treated showed remarkable recovery and the other two thirds had moderate improvements.[52-54] In addition it has been reported to have been successfully used in protection of the stumps of amputated limbs, and of surgical wounds from superinfection.[55] A variety of other examples also exist.[56-59]

The biosynthetic origins of virginiamycin S_1 or any part therein has not received any previous attention. However, the biogenesis of several of the components have been studied in other antibiotics. The 3-hydroxypicolinic acid residue has received attention in both the biosynthesis of etamycin[60,61] and of pyridomycin[62]. In etamycin evidence suggested its origin was from lysine whereas in pyridomycin it was shown to have arisen from L-aspartic acid, glycerol, and sodium pyruvate. The L-threonine residue is likewise present in both etamycin and pyridomycin. In both cases, it was found to have arisen from L-threonine, as would be expected. In addition, etamycin contains several N-methyl amino acids, though none are the same as the one that is present in virginiamycin S_1. In each case the N-methyl group was found to arise from L-methionine. Phenylsarcosine (N-methylphenylglycine) in etamycin resembles the phenylglycine residue in virginiamycin S_1. Its origin was determined to be from L-phenylalanine in etamycin. A streptogramin

group B antibiotic like virginiamycin S_1, etamycin provides interesting information to help speculate on the possible biogenesis of many of the virginiamycin S_1 components.

Though no work has been previously done on virginiamycin S_1, this is not true for its partner, virginiamycin M_1. Kingston and coworkers have elucidated much of the basic biosynthesis[63] and are currently working on further details.

Experimental

A. Culture Conditions

The microorganism used in this work, *Streptomyces virginiae* strain 1830, was obtained from SmithKline Animal Health Products, West Chester, Pennsylvania. The strain was maintained on soluble starch agar slants[64], the content of which is outlined in Table I. New slants were prepared every 3-4 months. The newly inoculated agar slants were incubated at 28°C for seven days and then stored at 4°C until needed.

For experimental work the microorganism was grown in broth cultures. It was initially grown in a medium[64] designed to promote only cell growth. The contents of the medium are listed in Table II. The vegatative inoculum was prepared by transferring the mycelium and spores from an agar slant to a 250 ml baffled flask containing 30 ml of medium. The broth culture was incubated at 25-26°C on a rotary shaker (Lab Line Orbit Environ-Shaker) set at 330 rpm for a 48 hour period. This medium accelerated the attainment of a stationary phase of cell growth, a state which generally is achieved before bacterial cultures commence antibiotic production, by being rich in a carbon source (dextrose) that promotes cell growth.

After cell growth stabilized (48 hours) 1 ml of this vegetative inoculum was transfered to each baffled flask

TABLE I. Agar Slant Medium

Distilled Water	1000 ml
Soluble Starch	10 g/L
$(NH_4)_2SO_4$	2 g/L
K_2HPO_4	1 g/L
$MgSO_4 \cdot 7H_2O$	1 g/L
NaCl	1 g/L
$CaCO_3$	3 g/L
Agar	20 g/L
pH 6.8	

TABLE II. Contents and Preparation of Vegetative Medium

Distilled Water	1000 ml
Corn Steep Solids	20 g/L
Raise pH to 7.5 with NaOH	
Peanut Oil Cake	8 g/L
Boil two minutes/Filter	
$CaCO_3$ (light, precipitated)	5 g/L
$MnSO_4$	0.01 g/L
Dextrose	50 g/L

containing 30 ml of a complex medium designed to promote antibiotic production. The contents of this fermentation medium are shown in Table III. These cultures were incubated under the same conditions as the vegetative culture except that the temperature was lowered to 22-23°C. These cultures were harvested after 48 hours growth.

All cultures were grown in baffled flasks. These flasks are known to increase the yields of the antibiotic produced.[65] The increased yields observed are thought to be due to the additional aeration the baffles create by forcing the liquid medium to roll and foam over them. In addition all media was autoclaved for 30 minutes at 120°C to achieve sterility and all transfers were made under sterile conditions. The incubator-shaker was not equipped to cool the cultures below the normal room temperature of 25°C. It has been reported[64] that the optimum temperature for antibiotic production is 20-22°C. The problem was circumvented by using the air conditioning in the laboratory to maintain a room temperature of 21-23°C. A reasonable amount of success was achieved using this technique.

B. Virginiamycin S_1 Growth Curve

In order to determine the time of optimum virginiamycin S_1 production, an investigation was undertaken with the only variable being the time of harvest. The

TABLE III.

Contents and Preparation of Fermentation Medium

Distilled Water	1000 ml
Corn Steep Solids	20 g/L
Yeast Autolyzate	5 g/L
Raise pH to 7.5 with NaOH	
Peanut Oil Cake	10 g/L
Boil two minutes/Filter	
Glycerol	25 g/L
Dextrose	5 g/L
Linseed Oil	10 g/L
$CaCO_3$ (light, precipitated)	5 g/L

study was initiated by growing a vegetative inoculum for the normal length of time (48 hours). Twenty-four flasks of the fermentation medium were then inoculated. Four flasks were harvested at twelve hour intervals, the first four being harvested twelve hours after inoculation of the media. The amount of virginiamycin S_1 production was measured and the results plotted. The results of this investigation can be seen in Figure 4.

The results indicate that the optimum time of production is 48 hours, after which the amount of virginiamycin S_1 actually appears to decrease. It is thought that this decrease is due to the fact that the nutrients in the medium are used up in the initial 48 hours and the microorganisms begin to use the antibiotics produced as a carbon source. It is also interesting to note that no antibiotic production is evident in the initial 24 hours, after which the antibiotic is produced rapidly.

C. Isolation of Virginiamycin S_1

The sides of the baffled flasks used to grow the cultures were washed down and then the culture was filtered to remove the mycelial cake. Hyflo Super Cel was used to aid filtering. The aqueous filtrate was extracted twice with a one third volume of hexane to remove unwanted oils. The hexane was discarded and the aqueous layer was extracted three times with a half volume of

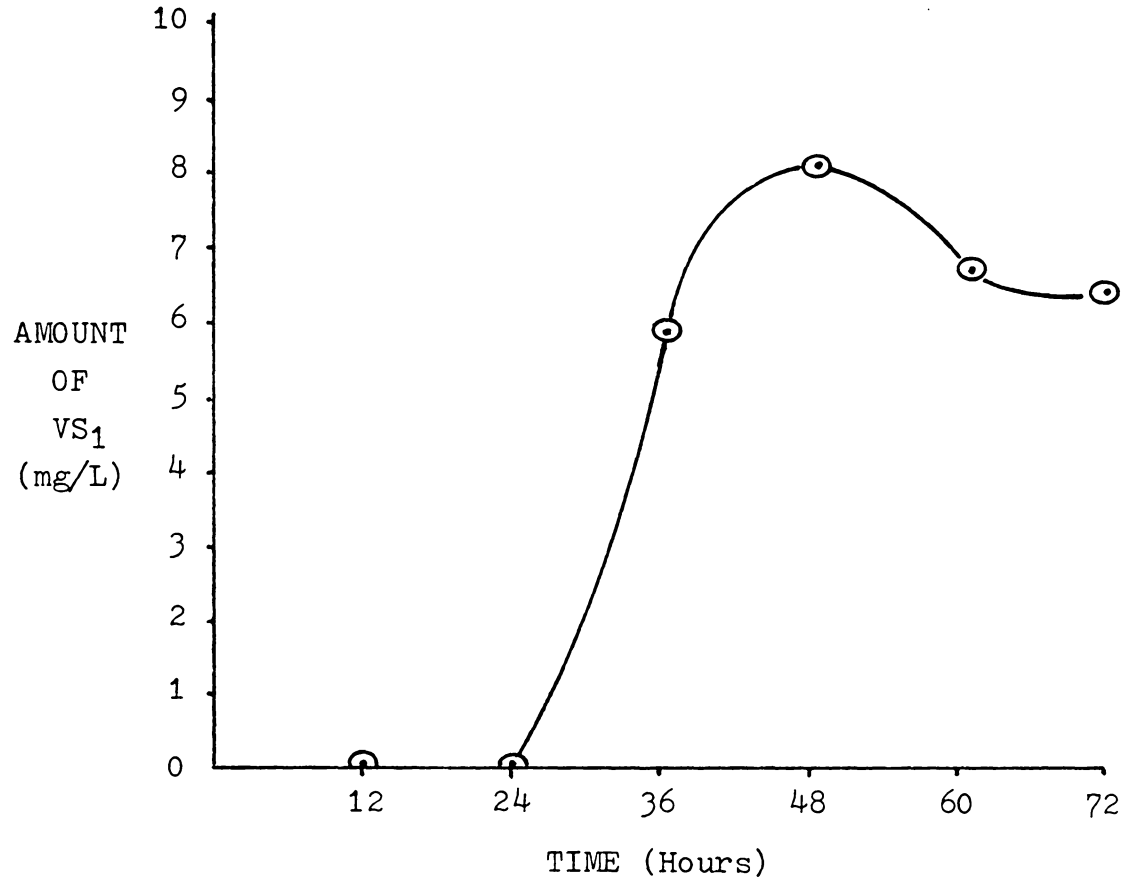


FIGURE 4. Virginiamycin S_1 Growth Curve

ethyl acetate. The aqueous layer was then discarded. The three ethyl acetate extractions were combined and washed once with an equal volume of distilled water. After drying with anhydrous sodium sulfate the ethyl acetate was evaporated to dryness on a rotary evaporator. The resulting residue was disolved in 4 ml (2 X 2 ml) of acetonitrile and the virginiamycin S_1 present was obtained by preparative high pressure liquid chromatography (HPLC). This procedure is depicted in Figure 5.

For the HPLC preparative scale separation of the crude mixture a LiChrosorb RP-8 10μm reverse phase column (300 mml X 10 mm ID) was used. An acetonitrile:water (46:54) solvent at a 6 ml/minute flow rate provided the necessary separation to obtain pure virginiamycin S_1. A representative chromatogram is shown in Figure 6.

D. Chromatographic Techniques

All chromatographic analyses were accomplished using a Waters Associates Chromatographic pump Model 6000A. In most cases a Waters Associates Model 440 UV Detector with 254 nm adsorption was used. Since virginiamycin S_1 is a fluorescent compound, a Waters Associates Model 420-C Fluorescence Detector was used in a few instances, which are noted as they arise.

All analytical work was done with a LiChrosorb

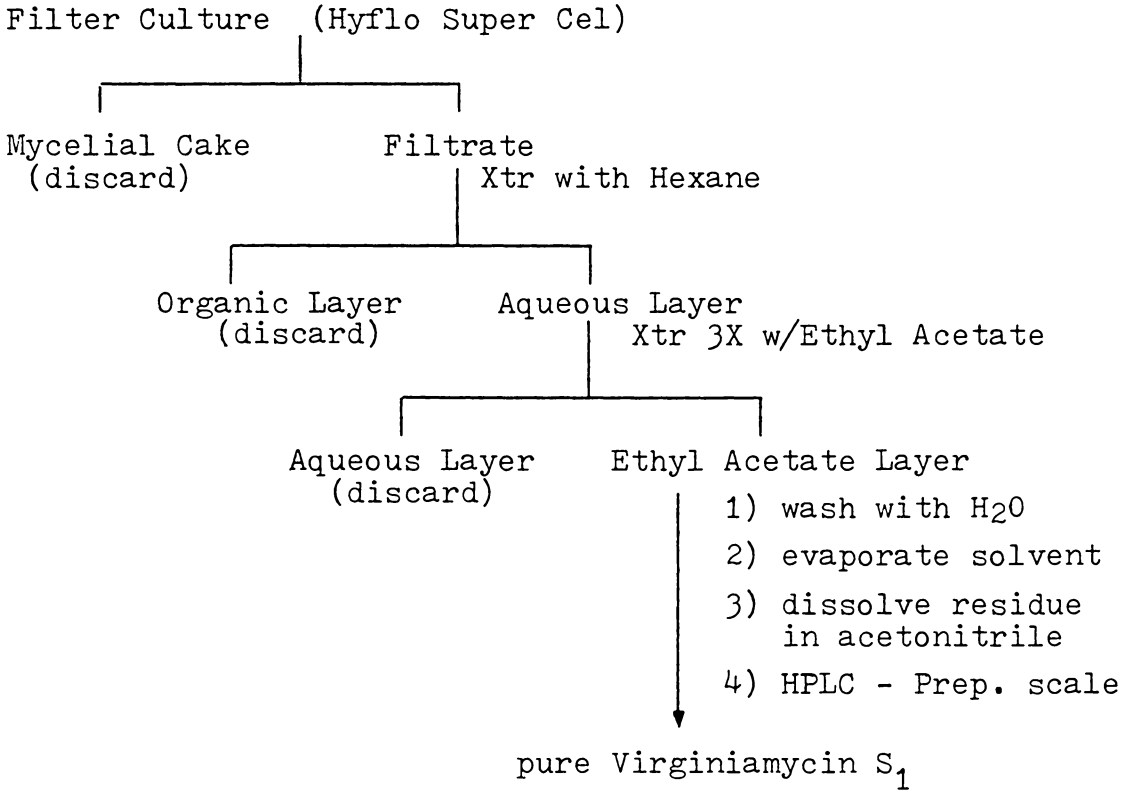


FIGURE 5. Virginiamycin S_1 Purification Procedure

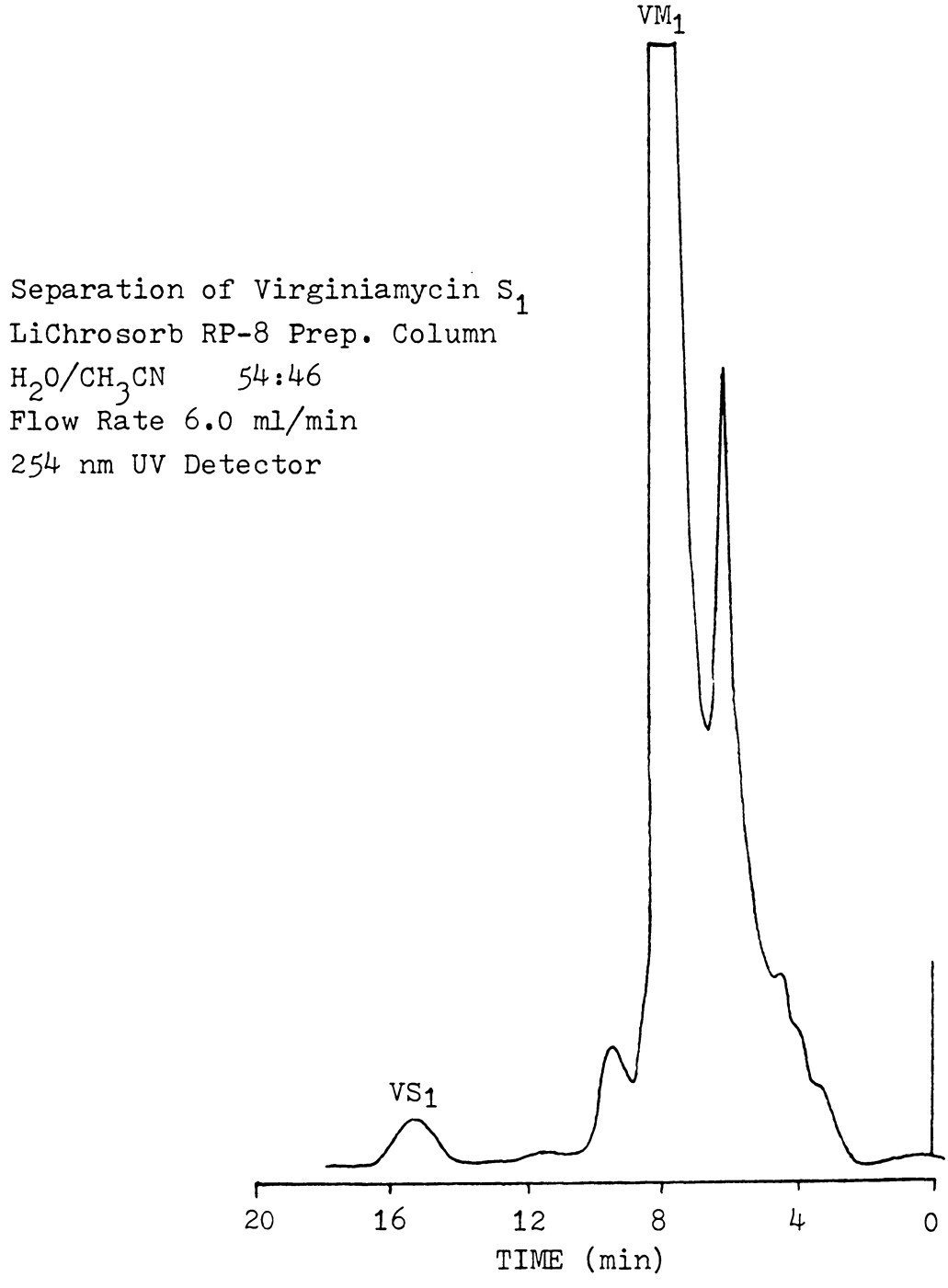


FIGURE 6. Prep HPLC Purification of Virginiamycin S_1

RP-8 10 m reverse phase column (250mm L X 4.6mm ID). A flow rate of 2 ml/minute was used in all separations. For all preparative scale separations, the column described under the isolation section was used, except in one instance which is discussed later.

A standard reference sample of virginiamycin S_1 was obtained by a preparatory scale separation of a mixture obtained from SmithKline Animal Health Products. The mixture was determined to be comprised of virginiamycin S_1 and S_4. The crude mixture (200 mg) was dissolved in acetonitrile (10 ml), and injected on the preparatory HPLC column in small increments (1 ml). The two major fractions present were collected. A sample chromatogram is shown in Figure 7. The two fractions collected were evaporated to dryness on a rotary evaporator. The resulting white residue in each flask was recrystallized in ethanol. The first fraction contained a white crystalline compound (19 mg) identified by melting point and n.m.r. to be virginiamycin S_4.[65] The second fraction also contained a white crystalline compound (35 mg) identified by melting point and n.m.r. to be virginiamycin S_1.[65]

Five milligrams of the purified virginiamycin S_1 were dissolved in five milliliters of acetonitrile. This solution was used to calibrate the HPLC in quantitative determinations. The quantity of a sample present was determined by comparing the peak height of the standard to that of the sample.

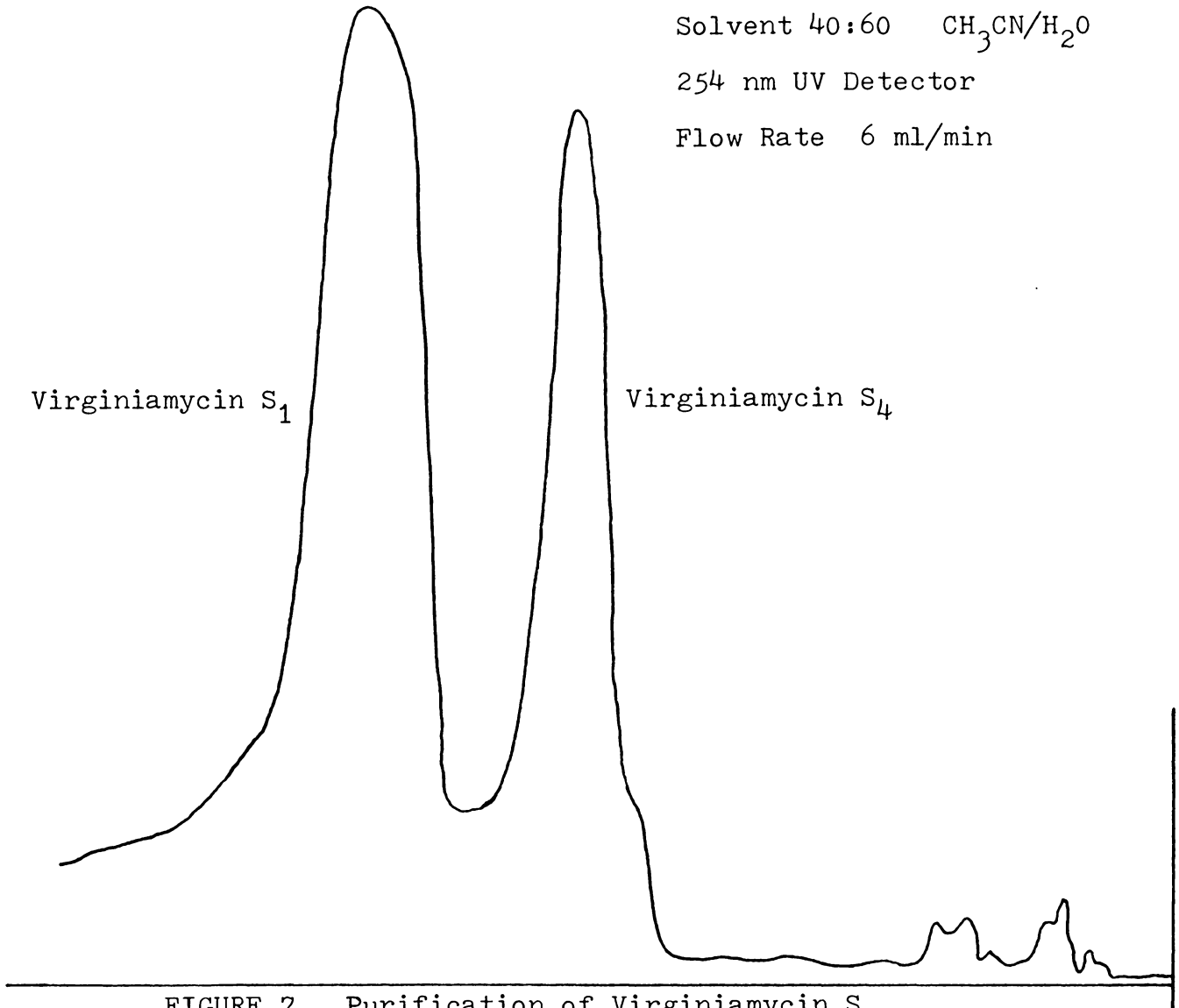


FIGURE 7. Purification of Virginiamycin S_1

E. N-Benzoyl Amino Acid Derivative Standards

The N-benzoyl amino acid derivatives were prepared by the Schotten-Baumann reaction. The general procedure is as follows: 3.8 millimoles of an amino acid were added to 10 ml of 1 N NaOH and stirred using magnetic stirring until dissolved. To this 10 ml of ether and 4.3 millimoles of benzoyl chloride were added. The heterogeneous solution was vigorously stirred at room temperature for a 24 hour period. After 24 hours the ether layer was removed and the aqueous layer acidified using 6 N HCl. Upon acidification an oil appeared. In some cases it solidified after a few minutes and in others it remained an oil. Regardless it was extracted with an equal volume of ethyl acetate. The ethyl acetate layer was dried with sodium sulfate and then evaporated to dryness. The resulting residue in most cases was a crystalline solid, but in a few an oil remained. Upon recrystallization in an ethyl acetate/hexane mixture all samples produced white crystalline solids. All melting points agreed with the literature values. The average yields for these derivatives was 40 percent from the starting amino acid.

F. N-Benzoyl-4-oxo-L-pipecolic Acid

A sample of virginiamycin S_1 and S_4 (2 grams) was dissolved in 6 N HCl (150 ml). This solution was heated in a sealed container to 105°C for a 24 hour period.

After the hydrolysis the solution was neutralized and then made alkaline (approximately 1 N in NaOH) by the addition of 6 N NaOH. Benzoyl chloride (2.9 ml) was dissolved in ether (100 ml) and added to the aqueous solution. This solution was vigorously stirred, using magnetic stirring, at room temperature for 36 hours. Removal of the ether layer and acidification of the aqueous layer with 6 N HCl resulted in a white precipitate. This was extracted using ethyl acetate (3 X 75 ml). The ethyl acetate layer was evaporated to dryness. Using a Partisil PAC preparatory column (500mm L X 10mm ID) with a chloroform/isopropanol/acetic acid (85:12:3) solvent system the N-benzoyl-4-oxo-L-pipecolic acid (24.2 mg) was collected. The N-benzoyl-4-oxo-L-pipecolic acid was obtained in four percent yield. Using a LiChrosorb RP-8 analytical column it was determined to be 97 percent pure. All attempts to recrystallize the yellowish-green gummy substance failed. The sample was identified by comparison to a pure standard prepared by using the general derivatization procedure on a very small quantity of 4-oxopipecolic acid hydrochloride generously provided by Dr. J. W. Clark-Lewis, The Flinders University of South Australia, Australia.

G. Synthesis of 3-Hydroxypicolinic Acid

Quinolinic imide was prepared in 46 percent yield from quinolinic acid by the method of Sucharda.[66] From

the imide 3-aminopicolinic acid was obtained using the method of Fibel and Spoerri[67] in an overall yield of 55 percent. 3-Hydroxypicolinic acid was prepared according to Kirpal[68] by the diazotization of the amino-picolinic acid. The crude material was recrystallized from methanol. The overall yield was 5.3 percent. The infrared spectrum was indistinguishable from an authentic sample purchased from the Rare Chemical Division of Aldrich Chemical Company.

H. Measurement of Radioactivity

Samples, dissolved in a suitable scintillation cocktail, were counted in a Beckman LS 100C liquid scintillation counter. The cocktail used was obtained from Beckman Technical Report 1042-NUC-76-55T.[69] The composition of the scintillation fluid used is shown in Table IV.

I. Radiochemicals

The radiolabeled compounds used as precursors were L-(U-14C)-threonine, L-(U-14C)-aspartic acid, L-(U-14C)-alanine, L-(methyl-14C)-methionine, L-(U-14C)-lysine, L-(U-14C)-proline, L-(U-14C)-phenylalanine, and DL-(methylene-14C)-tryptophan. All radiochemicals were purchased from either ICN Pharmaceuticals, Inc., New England Nuclear, or Amersham.

TABLE IV. Liquid Scintillation Cocktail Formula

2,5-Diphenyloxazole (PPO)	4 grams
p-bis (2-(4-Methyl-5-phenyloxazolyl))-benzene (dimethyl-POPOP)	0.2 grams
Triton X-100	333 ml
Toluene	Dilute to final volume of 1 liter

J. Radiotracer Experiments

The cultures were prepared and grown as described under culture conditions. Aliquots (0.5 ml) of a sterilized aqueous solution containing 2 μCi of the radioactive precursors were added to each culture eight hours after innoculation of the fermentation medium. The cultures were grown for an additional 40 hours before harvesting. The virginiamycin S_1 produced was isolated and purified as previously described. Ten percent of the antibiotic produced was used to determine the amount of radioactivity present. All samples were dissolved in acetonitrile before addition of the scintillation fluid used for counting.

K. Location of Radioactivity

The remaining sample (90%) was hydrolyzed with 6 N HCl (1 ml) in a sealed vial for a 24 hour period. After hydrolysis, the resulting solution was made 1 N in NaOH by the addition of solid NaOH (0.28 grams). To the alkaline solution ether (1 ml) containing benzoyl chloride (1.1 equivalents) was added. This heterogeneous solution was vigorously stirred, using magnetic stirring for a 24 hour period. The aqueous layer was pipeted off the bottom and transferred to a clean vial and the ether layer was discarded. The aqueous layer was acidified with 6 N HCl. The resulting precipitate was extracted with ethyl

acetate (2 X 1 ml). The ethyl acetate layer was evaporated to dryness. Both the aqueous layer and the ethyl acetate extract were stored at 4°C until analysis.

The N-benzoyl amino acid derivatives from the ethyl acetate extraction were separated by analytical HPLC, collected, and the amount of radioactivity in each sample determined. A LiChrosorb RP-8 10μm reverse phase column with a gradient solvent system was used to separate the components. The solvent compositions were (A) water: methanol:tetrahydrofuran:formic acid (85:11.5:2.5:1) and (B) methanol:water:tetrahydrofuran:formic acid (58:40:1:1). A Tracor 980A solvent programmer was programed for an initial delay (4 minutes) followed by a linear gradient (10% per minute) and then held at 100% solvent B. A flow rate of 2 ml/minute was sufficient for a good separation. From this separation six of the components in virginiamycin S_1 were collected and the amount of radioactivity in each determined. The six components, collected as N-benzoyl derivatives, were 4-oxo-L-pipecolic acid, L-threonine, D-α-aminobutyric acid, L-proline, L-phenylglycine, and N-methyl-L-phenylalanine. All samples collected were evaporated to dryness and redissolved in 1 ml methanol before the scintillation cocktail was added. In addition, any unusual peaks noted in the extract were also checked for radioactivity. A representative chromatogram can be seen in Figure 10.

The last component, 3-hydroxypicolinic acid remained underivatized and in the aqueous layer after the ethyl acetate extraction. A small portion was purified and counted for radioactivity. This was accomplished using a LiChrosorb RP-18 10μm reverse phase column and a solvent system of 100% water, at a flow rate of 2 ml/minute. A fluorescence detector was used to detect 3-hydroxypicolinic acid in the HPLC system. All samples were dissolved in water (1 ml) before the scintillation cocktail was added. A representative chromatogram can be seen in Figure 8.

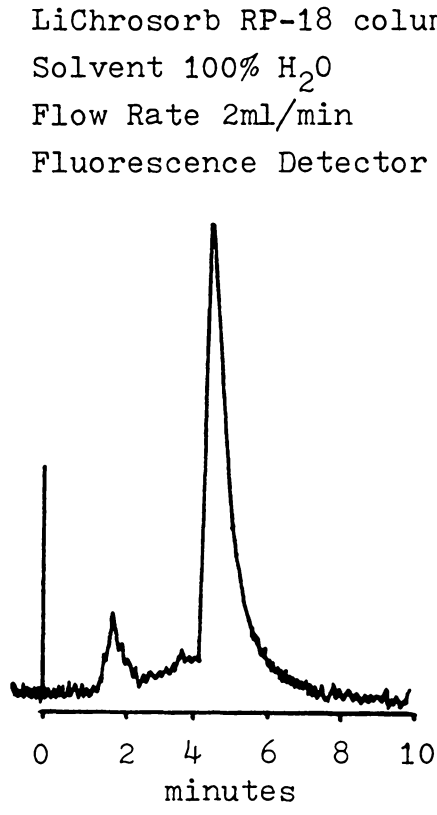


FIGURE 8. HPLC Separation of 3-Hydroxypicolinic Acid

Results and Discussion

When facing the challenge of a biosynthetic study of an antibiotic such as virginiamycin S_1, three problems must be dealt with before the study can be accomplished. These three problems are: 1) getting the microorganism to grow and produce the antibiotic, 2) getting the microorganism to incorporate the radiolabeled precursor to a reasonable extent, and 3) developing a technique that will be sufficient for determining the location of the radioactivity. Any or all of these problems may be accomplished with ease; on the other hand, any one of them could become a major source of research in itself.

The microorganism selected to study the biosynthesis of virginiamycin S_1 was Streptomyces virginiae strain 1830. This strain was selected since it was reported[64] to produce factor S_1 in yields almost equal to factor M_1 which is the major component produced by the microorganism. Initial studies on microorganism growth and antibiotic production showed production levels to be only 1-2 μg/ml, a figure which is too low to effectively work with. Further, research indicated that the microorganism was very sensitive to minor modifications in growth conditions. An example of this is shown by the improved yields obtained with the only changes being distilled water to tap water

and a switch in brands of corn steep solids used in the medium. The antibiotic production levels were effectively increased with these minor changes to consistently produce 8-10 μg/ml. An increase in temperature by a few degrees (from 21° to 23°C) also seemed to improve levels of production. Though these antibiotic production levels were still smaller than hoped for, they were sufficient to obtain the desired results.

A study of the time course of virginiamycin S_1 synthesis was undertaken with hopes of gaining additional growth information. The results showed that the maximum production of virginiamycin S_1 was reached after 48 hours growth in the fermentation medium. In addition, it was discovered that the antibiotic was not produced to any measurable extent until after 24 hours growth. This information is significant in determining not only the best time to harvest, but also the best time to add the radiolabeled precursors.

When adding labeled precursors certain criteria must be kept in mind. First, it is necessary to add the precursor early enough for the microorganism to use it in the antibiotic production. Secondly, it is important to add it late enough that it will not be taken into the major biosynthetic pathways and metabolized to produce incorporations without specificity. This criteria suggests that the optimum time of addition would be 1-2

hours before antibiotic production begins. In the case of virginiamycin S_1 this would be after 21-22 hours of growth by the microorganism.

Initial experimental additions of precursors were performed at the optimum time indicated. The result was that very low incorporations were obtained indicating that the precursors were added too late for effective use by the microorganism. In biosynthetic studies of pyridomycin, similar results were obtained upon precursor addition at 24 hours.[62] Increased incorporations were obtained in pyridomycin by adding the precursors at a much earlier time (8 hours). Since Streptomyces pyridomyceticus, the organism used in pyridomycin biosynthetic studies, exhibits a growth curve very similar to that of Streptomyces virginiae strain 1830, early addition seemed to provide the solution, and was used in the remaining experiments. The evaluation of this early addition procedure was complicated by the fact that increased production was always noted whenever good incorporations were observed. It was not clear whether the improvements of incorporations were due to the early addition of the precursor or increased production of the antibiotic. After the final results were examined the answer to this became evident and will be discussed along with their presentation.

The final problem to be overcome, and perhaps the

most critical is the development of the analytical technique to determine the location of the radioactivity. Because of the speed and accuracy of HPLC it was determined to be the best technique to use. Its use, however, requires that the various amino acid components in virginiamycin S_1 be derivatized. The reason for derivatization and the derivative criteria have previously been discussed, but the criteria may be reviewed on Table V.

An examination of the literature revealed that the phenylthiohydantoin (PTH) amino acids fit the criteria the best. PTH-amino acids have received a lot of attention because of the prime position they hold in the sequence determination of peptides and proteins (Edman degradation procedure). Several general synthetic procedures[70,71], as well as mechanistic studies[73], spectral analyses[74-77], and chromatographic studies[77-80] have appeared in the literature. A considerable amount of time and effort was spent on trying to prepare a standard of each derivative to be used. However, all efforts failed to produce PTH-N-methylphenylalanine. In addition, PTH-phenylglycine was produced only under vigorous conditions.

After the PTH derivatives were unsuccessful in producing the desired results several other derivatives were attempted. Perhaps as equally well known as the PTH derivatives are the 2,4-dinitrophenyl derivatives (DNP).

TABLE V. Amino Acid Derivative Criterion

1) UV Absorption chromophore

2) Good chromatographic properties

3) Reacts with secondary as well as primary amino groups

4) Easy one step reaction

5) Reacts with both the amino and carboxyl groups

All the hydrolysis products of virginiamycin S_1 except 3-hydroxypicolinic acid formed nice brightly colored crystalline derivatives. Unfortunately highly colored substances are not desirable when using liquid scintillation counting since they exhibit a quenching effect and produce inaccurate results. The N-benzoyl methyl ester amino acids and the N-p-nitrobenzoyl amino acid derivatives were also tried. The N-benzoyl methyl ester derivatives failed to give crystalline solids and the N-p-nitrobenzoyl derivatives were produced only in extremely low yields (5-10%), if at all. Although several systems have been developed for the derivatization of the common amino acids it is readily apparent that the less common ones have not received much attention. The basic structure of the derivatives attempted can be seen on Table VI.

The most successful derivative and the one finally chosen for use was the N-benzoyl derivative. It satisfies all the criteria, at least to some extent, except for reaction with both the amino and carboxyl groups, a criterion which is not absolutely essential. The N-benzoyl derivatives were produced by a variation of the Schotten-Bauman procedure. Each of the seven components which comprise virginiamycin S_1, except 3-hydroxypicolinic acid and 4-oxo-L-pipecolic acid, produced nice white crystalline derivatives (Table VII). 3-Hydroxypicolinic

TABLE VI. Derivatives Tried

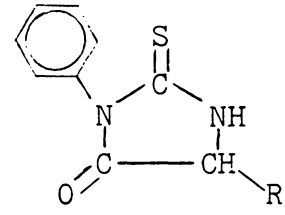
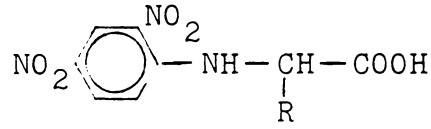
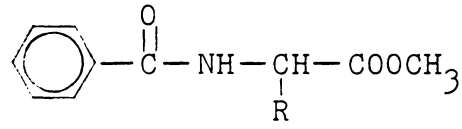
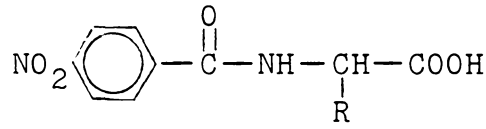
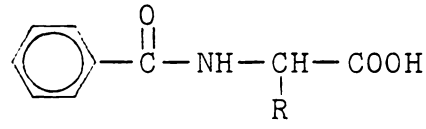
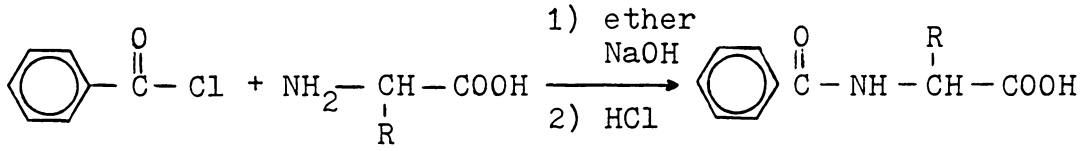
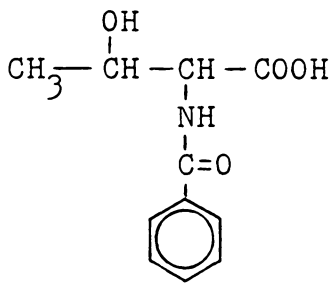
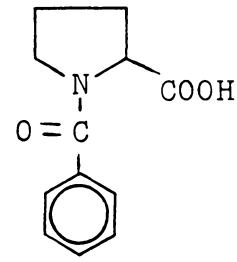
PHENYLTHIOHYDANTOIN (PTH)	
2,4-DINITROPHENYL (DNP)	
N-BENZOYL METHYL ESTERS	
N-p-NITROBENZOYL	
N-BENZOYL	

TABLE VII. Schotten-Baumann Reaction and N-Benzoyl Derivatives

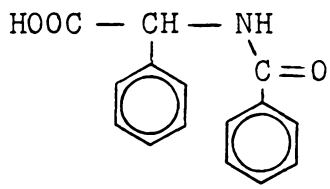




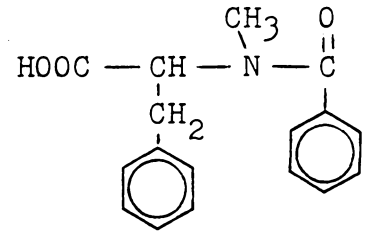
N-Benzoyl-L-Threonine



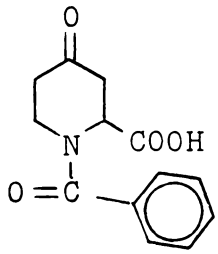
N-Benzoyl-L-Proline



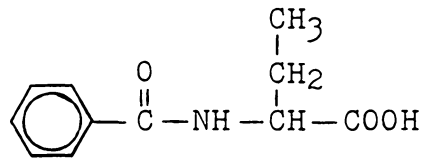
N-Benzoyl-L-Phenylglycine



N-Benzoyl-N-Methyl-L-Phenylalanine



N-Benzoyl-4-Oxo-L-Pipecolic Acid



N-Benzoyl-D-∝-Aminobutyric Acid

acid failed to produce a derivative, however it could be purified by HPLC without derivatization because of its fluorescence ability.

4-Oxo-L-pipecolic acid proved to be a special case. Preparation of the derivative for a standard requires the availability of the pure amino acid. Currently, 4-oxo-L-pipecolic acid is not available commercially nor is a synthetic route known. In order to obtain a pure standard it was necessary to hydrolyze and derivatize a large quantity of virginiamycin S_1. The N-benzoyl-4-oxo-L-pipecolic acid residue was obtained by preparatory HPLC purification. Despite all efforts, the purified component failed to crystallize. The yellowish, gummy residue which resulted was determined to be 97% pure by analytical HPLC. Though the sample is certainly not ideal, it was satisfactory for the work presented here.

Although the chromatographic properties of each individual N-benzoyl derivative appeared to be reasonably good, the resulting mixture of the six components provided a challenging problem. All the common systems attempted failed to produce a clear separation. Reverse phase RP-2, RP-8, RP-18, normal phase partisil and chemically bonded partisil PAC columns were tested using a variety of common solvent systems. In addition, reverse phase ion pairing chromatography was tried, using tetraalkylammonium[81-83] ions as well as inorganic salts[84]. Many of the systems were

examined using gradients as well. All the systems attempted, however, failed to provide the necessary chromatographic separation.

The solution to the problem was a four solvent HPLC system recently developed by DuPont.[85] Resolution, which is a measure of the quality of separation in HPLC, is affected by three factors. The first factor, column efficiency (theoretical plates), is essentially fixed for a given system, and of course, should be maximized. The second factor, the capacity factor, is a measure of relative retention and can be varied by changing solvent strength. This also should be optimized. The third factor, the selectivity factor, is a measure of the chemical difference of the components and can be changed by changing solvent types. It is on the third factor that the DuPont system is based.

All solvents in this system can be defined in terms of three characteristics. These characteristics are shown at the apexes of the optimization triangle in Figure 9. No solvent is considered to be exclusively one type, but all solvents exist at some point within the framework of the triangle, exhibiting some particular mixture of each characteristic. The point at which each solvent exists in the triangle was determined by the weighted contribution to total solvent strength that each of the three basic characteristics contribute to the selectivity. The three

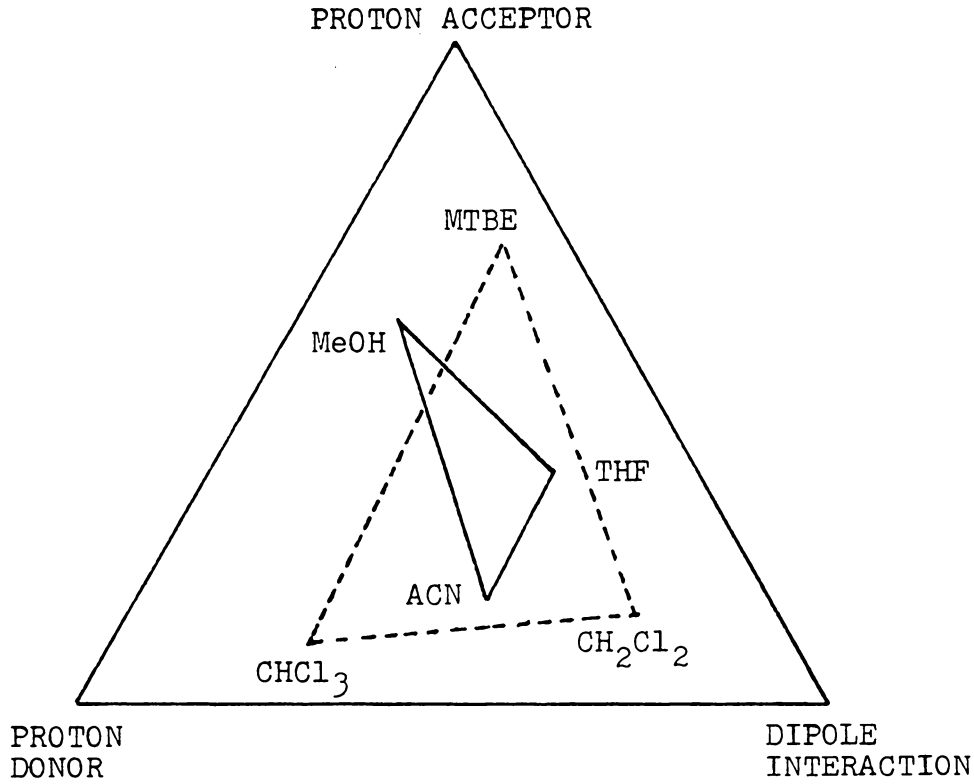


FIGURE 9. HPLC Optimization Triangle

solvents which are the most functionally different (greatest difference in characteristics) have been chosen (Figure 9), each for reverse phase and normal phase. These three solvents are considered the adjusting strong solvents and are mixed with a weak solvent. For reverse phase, acetonitrile, methanol and tetrahydrofuran are the strong solvents and water is the weak solvent. For normal phase methylene chloride, chloroform, and methyl-t-butyl ether are the strong solvents and hexane is the weak solvent. This means that four solvents are required to carry out the optimization routine.

In terms of an experimental approach this means that seven definitive isocratic experiments using combinations of the four solvents are necessary. The first of these experiments uses an appropriate binary composition (one strong solvent plus weak solvent) based on previous work. This choice defines one apex of an optimization triangle as well as the solvent strength to be used. Calculation of equivalent solvent strengths for the other two selectivity adjusting solvents, using the equation and weighting factors in Table VIII, produces the composition of the solvents to be used for definition of the remaining two apexes of the optimization triangle. Three more experiments can then be done by mixing equal volumes of the initial binary mixtures. The final experiment consists of mixing equal volumes of all three of the initial binary mixtures.

TABLE VIII. Solvent Strength Equation and Weighting Factors [85]

$$S_T = \sum_i s_i \psi_i$$

Solvent	Strength Weighting Factor (s_i)	
	(RP)*	(NP)**
Methanol	2.6	5.1
Acetonitrile	3.2	5.8
Tetrahydrofuran	4.5	4.0
Water	0	10.2
Chloroform		4.1
Methylene Chloride		3.1
Methyl-t-butyl ether		2.5
Ethyl ether		2.8
Hexane		0

*Reverse Phase

**Normal Phase

Statistically, this is sufficient to evaluate the entire selectivity space. A simple visual examination of the chromatograms from these seven experiments, plus a few additional chromatographic runs for refinement, will produce the optimum resolution.

Use of the equation in Table VIII is simply accomplished by using the appropriate volume fractions and weighting factors for the initially chosen binary solvent to calculate the solvent strength. Substituting this result plus the appropriate weighting factors back into the equation provides the solvent compositions for the remaining two binary systems with solvent strengths equal to the initial system.

Though a gradient was still required to separate the components of virginiamycin S_1, the use of this four solvent optimization technique provided a complete baseline separation which had previously proved impossible. A sample chromatogram from an actual incorporation run is shown in Figure 10. The six N-benzoyl amino acid derivatives are eluted in the following order: 1) N-benzoyl-4-oxo-L-pipecolic acid, 2) N-benzoyl-L-threonine, 3) N-benzoyl-L-proline, 4) N-benzoyl-D-α-aminobutyric acid, 5) N-benzoyl-L-phenylglycine, and 6) N-benzoyl-N-methyl-L-phenylalanine. (The numbering in the text corresponds to that shown in Figure 10.) The largest peak (labeled A) is benzoic acid, a side product of the Schotten-Bauman reaction.

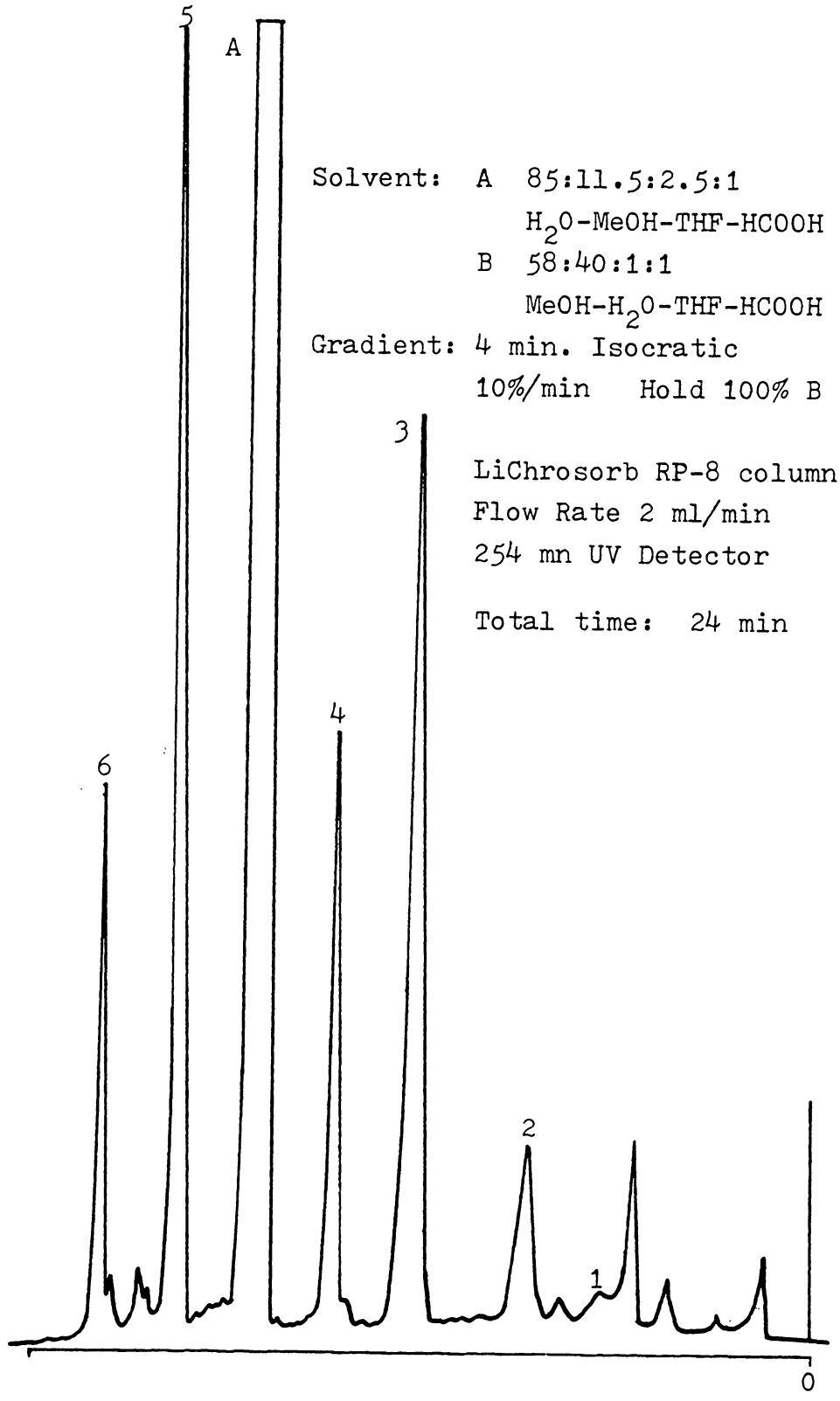


FIGURE 10. Separation of N-Benzoyl Derivatives

With the resolution of the three problems previously discussed the experimental incorporation of possible precursors was performed. An examination of the virginiamycin S_1 structure plus the previous work done on the biosynthesis of pyridomycin and etamycin suggested that L-phenylalanine, L-threonine, L-methionine, L-aspartic acid, L-lysine, L-proline, L-alanine and L-tryptophan might be precursors. The results of the radiolabeled incorporations are shown in Table IX and the distribution of the isotopic labels in the hydrolysis products of virginiamycin are in Table X. The incorporation of the possible precursor amino acids (Table IX) was calculated by two different methods, each significant in their own way. The percent incorporation is a measure of the total amount of radioactivity contained in the virginiamycin S_1 produced compared to the total amount added to the cultures. It does not take into account the amount of virginiamycin S_1 produced. This is significant, since normally the better the antibiotic production the better the incorporation. The specific incorporation is a direct measure of the amount of virginiamycin S_1 molecules labeled by the radiolabeled precursors. The amount of antibiotic produced shows little or no effect on this incorporation calculation. For example, it is interesting to note that the most radioactivity was incorporated from

TABLE IX. ^{14}C - Amino Acid Incorporations into Virginiamycin S_1†

Compound Administered	Time Added (hrs)	VS_1 Prod. (mg/L)	μCi Added	μCi in VS_1	% Inc.*	Spec. Act. Cmpd. (μCi/μm)	Spec. Act. VS_1 (μCi/μm) ($X10^{-2}$)	Spec. Inc.** ($X10^{-2}$)
L-(U-14C)-Phenylalanine	8	15.4	26	0.624	2.4	450	8.57	1.9
L-(U-14C)-Threonine	8	19.5	20	0.26	1.3	200	3.6	1.8
	22	1.5	24	0.026	0.11	200	4.02	2.0
L-(Me-14C)-Methionine	8	10.0	14	0.168	1.2	45.7	6.51	14.2
L-(U-14C)-Aspartic Acid	8	7.3	20	0.052	0.26	233	1.96	0.84
L-(U-14C)-Lysine	8	6.5	20	0.048	0.24	300	2.00	0.67
	8	1.7	14	0.012	0.08	300	1.78	0.59
L-(U-14C)-Proline	8	5.8	20	0.030	0.15	240	0.912	0.38
	18	5.8	20	0.024	0.12	240	1.41	0.59
L-(U-14C)-Alanine	22	13.3	18	0.011	0.06	174	0.284	0.16
DL-(3-14C)-Tryptophan	22	10.3	20	0.0016	0.008	56.5	0.043	0.08

†For method of calculation see Appendix.

*% Incorporation = (μCi in antibiotic produced/ μCi put in cultures) X 100.

**Specific Incorporation = (Specific activity of VS_1/Specific activity of precursor) X 100.

TABLE X. Distribution of Radioactivity in Virginiamycin S_1 Components†

Compound Administered	Sp. Inc.* (X 10^{-2})	Thr	AmBt	Pro	N-MePhe	4-Oxo	Phgly	3-HyPic	Error or RUF**
L-(U-14C)-Phenylalanine	1.9	0.2	0.1	0.0	44.9	0.5	39.7	0.3	14.3
L-(U-14C)-Threonine	1.8	96.2	14.5	0.2	0.2	1.4	0.7	1.1	-14.3
L-(Me-14C)-Methionine	14.2	2.3	0.6	0.2	88.4	15.2	0.2	1.9	- 8.8
L-(U-14C)-Aspartic Acid	0.84	25.2	3.1	16.4	0.4	9.1	0.4	43.7	1.7
L-(U-14C)-Lysine	0.67	2.1	1.0	2.1	2.8	11.9	1.4	***	78.6
Lysine (Repeat)	0.59	2.4	0.6	0.4	0.6	13.7	0.0	50.7	31.6
L-(U-14C)-Proline	0.38	1.8	1.0	69.1	0.6	1.4	0.4	5.2	20.5
L-(U-14C)-Alanine	0.16	14.3	0.0	5.8	8.1	0.0	7.9	***	63.9
DL-(3-14C)-Tryptophan	0.08	34.7	76.0	4.1	6.0	0.0	7.2	26.7	-54.7

† For method of calculation see Appendix.

* Specific Incorporation of precursor.

** Error or Radioactivity Unaccounted For (Amount of radioactivity needed to make 100%).

*** Activity of this component was not determined on this run.

NOTE: Thr = L-Threonine; AmBt = D-α-Aminobutyric Acid; Pro = L-Proline; N-MePhe = N-Methyl-L-phenylalanine; 4-Oxo = 4-Oxo-L-pipecolic Acid; Phgly = L-Phenylglycine; 3-HyPic = 3-Hydroxypicolinic Acid.

L-(U-14C)-phenylalanine, but by far the most efficient incorporation was L-(U-14C)-methionine. However, generally the specific incorporation tends to correspond with the percent incorporation. In the distribution of the radioactivity (Table X) several totals of the individual components of virginiamycin S_1 were significantly less than the total amount determined to be the whole antibiotic. The reason for this is unknown, but it is noted in the table as radioactivity unaccounted for. Most likely the difference is due to the small amount (20-30 μg in many cases) of each component measured when determining the distribution of the radioactivity.

In addition to the amino acid precursor incorporations gained from these results, the solution to the effect of time of addition on incorporations was discovered. Unlike pyridomycin, the result in this case was that the time of addition had very little or no effect on the amount of radioactivity incorporated. Three pieces of evidence support this conclusion. In the first experiment which was repeated twice, the L-(U-14C)-threonine incorporation, it can be noted that with the time variation a better than 10-fold percent incorporation increase was noted. But, there was also a better than 10-fold antibiotic production increase. From this it is difficult to tell what caused the difference. However, if the

specific incorporation, which takes into account the amount of antibiotic produced, is looked at, it can be seen that the difference in percent incorporations was due solely to increased antibiotic production. The second experiment which was performed twice, the L-(U-14C)-proline incorporation, supports the L-threonine evidence. Again, two different addition times were used, but this time the production was exactly the same. The result was that the percent incorporations are very close. In addition, the third experiment, the L-(U-14C)-lysine incorporation, also adds to the evidence. This time the same time of addition was used but a better than 3-fold increase in production was observed. The result was a 3-fold increase in percent incorporation. Further evidence was that, as was seen for the L-(U-14C)-threonine experiment, the specific incorporations for each trial of the last two experiments were very close for their respective experiments. From this evidence, little doubt is left that it was the production increases and not the time of precursor addition which effected the percent incorporations.

The results clearly show that L-(U-14C)-proline and L-(U-14C)-threonine were efficiently incorporated into the respective amino acid component in virginiamycin with a reasonably good amount of specificity. L-(U-14C)-Phenylalanine was also efficiently incorporated

and was located almost exclusively in the N-methylphenylalanine and phenylglycine residues. The distribution of radiolabel from L-(U-14C)-phenylalanine was split almost equally in these two components. In addition, very specific introduction of radioactivity from L-(methyl-14C)-methionine was obtained in the N-methyl phenylalanine residue.

L-(U-14C)-Aspartic acid and L-(U-14C)-lysine were both incorporated into virginiamycin S_1 with slightly less efficiency. The distribution of radioactivity in both cases is very similar. The only difference is that L-(U-14C)-aspartic acid was incorporated into the L-threonine residue whereas roughly the same amount is unaccounted for in the L-(U-14C)-lysine experiment.

Very little radioactivity entered into virginiamycin S_1 from DL-(methylene-14C)-tryptophan or L-(U-14C)-alanine. In addition, the little that was incorporated was not specific. This suggests that neither of these amino acids are precursor molecules.

The biosynthesis of virginiamycin S_1 in itself has not received any previous attention although some of the components have been studied in antibiotics produced by other microorganisms. The results presented here provide some definite answers as well as some interesting possibilities. In some cases they agree with previous biosynthetic origins whereas in other cases they are unclear or appear to conflict.

L-Threonine has been previously studied in both the biosynthesis of pyridomycin[62] and etamycin[60]. In both cases it was found to originate from L-threonine. Being a common protein amino acid this is what would be expected, since it would be readily present in the amino acid pools. In the case of virginiamycin S_1, the results concurred with the previous findings as expected. L-(U-14C)-Threonine was efficiently incorporated and with a high degree of specificity. It is likewise no surprise to find radioactivity from L-(U-14C)-aspartic acid. The biosynthetic pathway in Figure 11 has been reported[86,87] for bacteria as well as yeasts, Neurospora, E. coli, and higher plants. It is likely that this or a similar pathway is responsible for the radioactivity observed in L-threonine. These two incorporations provide substantial evidence for the precursor of the L-threonine portion of the molecule being L-threonine.

L-Proline, also being a common protein amino acid, would be expected to be the precursor of the L-proline fragment in virginiamycin S_1. Several previous studies have shown that proline derivatives arise from L-proline, so it would be natural to expect the L-proline fragment to arise from L-proline. The 4-hydroxyproline residue in etamycin[60] was seen to arise from proline. In addition, the dehydrated unit located in virginiamycin M_1, an antibiotic also produced by Streptomyces virginiae, is suspected to be from proline.[63]

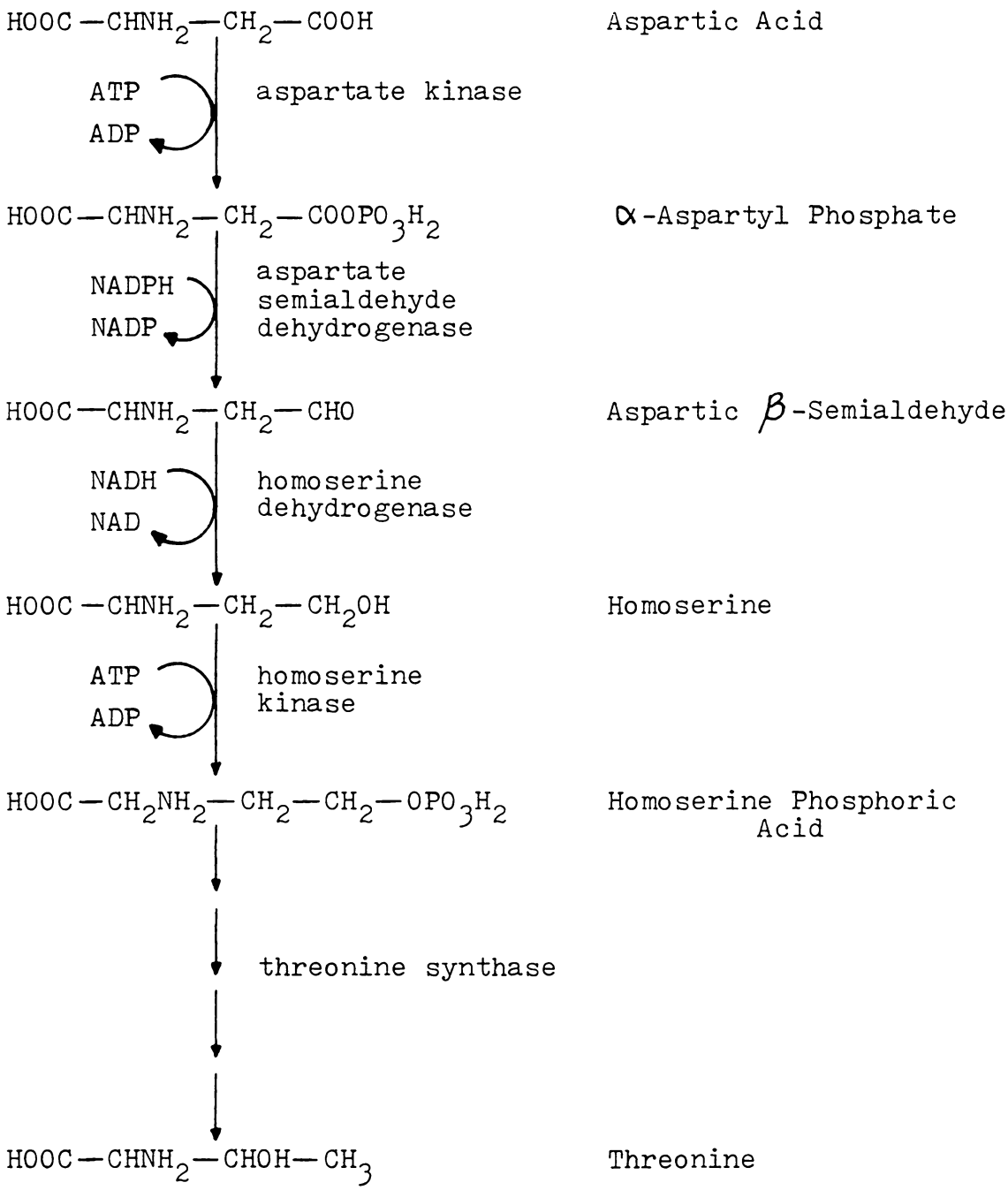


FIGURE 11. Threonine Biosynthesis From Aspartate[86]

These expectations were confirmed by the L-(U-14C)-proline incorporations into virginiamycin S_1 almost exclusively in the L-proline residue.

The biogenesis of several N-methyl amino acids has been previously studied, though N-methyl-L-phenylalanine has not. The three N-methyl amino acids in etamycin have been shown to have the N-methyl group arise from L-methionine.[60] This is certainly not unexpected since methionine is commonly known to be a methyl donor.[86] The parent compound of this N-methyl amino acid, L-phenylalanine is, like the previous two virginiamycin S_1 components, a common protein amino acid. The most likely possibility then, is the methylation of L-phenylalanine, and then incorporation. Both precursors, L-(methyl-14C)-methionine and L-(U-14C)-phenylalanine, were found to be very efficiently and specifically incorporated by Streptomyces virginiae strain 1830 into the N-methyl-L-phenylalanine residue of virginiamycin S_1. The location of the radiolabel from methionine has not yet been shown to be exclusively in the N-methyl group in N-methyl-L-phenylalanine, however this is the most likely location. It is also likely that very little radioactivity is located in the methyl group from the L-(U-14C)-phenylalanine incorporation. Experimental proof is necessary before this can be accurately stated.

The biosynthetic origin of L-phenylglycine has not

previously been elucidated, however, a closely related compound, phenylsarcosine (N-methyl-phenylglycine) was determined to be from L-phenylalanine.[60] It was also reported[60] that the phenylglycine portion was derived from the phenyl ring-, α-, and β-carbons of L-phenylalanine. The fact that L-phenylalanine is also the precursor of L-phenylglycine in virginiamycin S_1 is clearly evident, but more work needs to be done to determine if the same type of mechanism is present in the biogenesis. Precursors with labels specifically located on the α-, β-, and carboxyl carbons should provide the necessary evidence to elucidate the mechanism of rearrangement.

Up until this point the precursors from which the various virginiamycin S_1 components have arisen have been relatively obvious. The remaining three components, however, have not been so clearly labeled, but from the incorporations observed some interesting speculations can be made.

D-α-Aminobutyric acid showed very little labeling from any precursor. The expected precursor, L-(U-14C)-alanine, was incorporated into virginiamycin S_1 only to a small extent, and no radioactivity from this was found in D-α aminobutyric acid. There was, however, a small amount incorporated from L-(U-14C)-threonine, and an even smaller amount from L-(U-14C)-aspartic acid. The fact that these are the only two incorporations observed

at all support the idea that D-α-aminobutyric acid might be derived from L-threonine. The small incorporation of L-(U-14C)-threonine could easily be explained by the fact that L-threonine itself would absorb much of the labeled material in the biosynthetic pathways. Besides this, the L-(U-14C)-threonine incorporated would not only have to be converted to aminobutyric acid but also epimerized to the D isomer. Additional support is offered by the fact that a biosynthetic pathway between L-threonine and L-α-aminobutyric acid is known in higher animals and has also been demonstrated to occur in various microorganisms.[87] This pathway is shown in Figure 12. The last piece of evidence which supports this is the fact that L-(U-14C)-aspartic acid, a known precursor of threonine, also shows a very small incorporation into D-α-aminobutyric acid. This pathway is certainly not proven by the experimental evidence, and perhaps there is a better precursor, but it does provide a feasible explanation of the observed results, and a point from which to continue experimentation.

3-Hydroxypicolinic acid has received a substantial amount of attention in the past decade. Biosynthetic studies of pyridomycin[62] showed that it arose from L-aspartic acid, glycerol, and pyruvic acid. They found no incorporations from L-lysine. This evidence suggests that the mode of pyridine-ring biosynthesis is a route

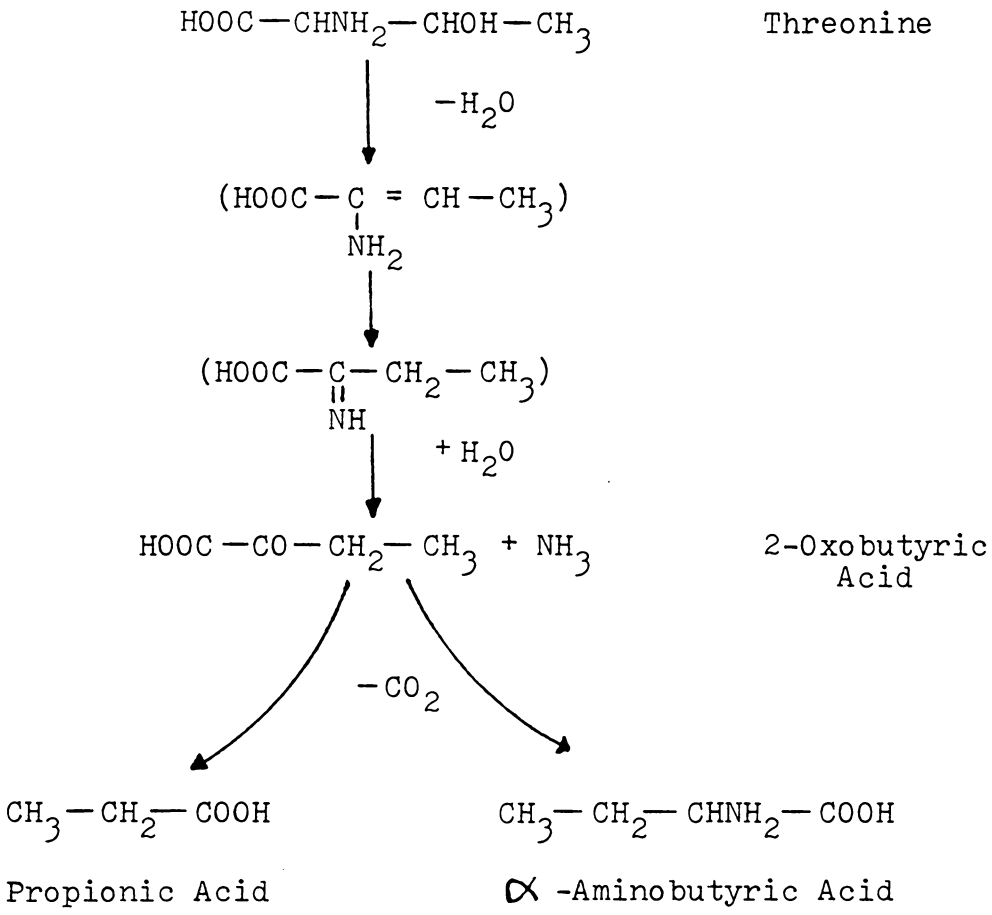


FIGURE 12. Catabolism of Threonine[87]

from 3- and 4-carbon precursors in Streptomyces. However, in the biosynthesis of etamycin it was discovered that 3-hydroxypicolinic acid was derived from L-lysine and not from L-aspartic acid. The existence of two pathways provided interesting speculations for the biogenesis in virginiamycin S_1. The results, however, showed that the L-(U-14C)-aspartic acid and L-(U-14C)-lysine were incorporated to nearly the same extent. An examination of the distribution did not provide any further helpful information since both precursors were again found to be incorporated in the 3-hydroxypicolinic acid residue to nearly the same extent. The only difference in specificity of incorporation was that L-(U-14C)-aspartic acid was incorporated into L-threonine and L-proline, and that L-(U-14C)-lysine had a significant amount of radioactivity unaccounted for.

There are three possible explanations for the results obtained. The first is that 3-hydroxypicolinic acid may be derived from either precursor. Though possible, this is probably not likely. The second, and probably most likely explanation, is that the precursor is L-lysine and that L-aspartic acid is metabolized to L-lysine before incorporation. A known pathway, called the 2,6-diaminopimelate pathway, exists between aspartic acid and lysine. Though it has not been seen in Streptomyces before, it is known to occur in Actinomycetes as well as other

bacteria.[87] The basic intermediates of this pathway are shown in Figure 13. The third possibility is that the experimental results are erroneous and therefore the conclusions invalid. Though possible, this also is not likely since the technique clearly worked for the other cases. In order to determine if the diaminopimelate pathway or a similar biosynthetic route exists, further work must be done. Possible experiments could include pyruvic acid and glycerol as precursors.

The last component in virginiamycin S_1, 4-oxo-L-pipecolic acid, is perhaps the most interesting. Keto amino acids such as this component are extremely rare in nature. Besides the fact that L-pipecolic acid is known to arise from L-lysine in higher animals, little is known about the origin of this component. However, it is not difficult to envision a similar mechanism as that used by the higher animals.

As with 3-hydroxypicolinic acid, both L-(U-14C)-aspartic acid and L-(U-14C)-lysine were incorporated, and almost to the same extent. The overall incorporation was much lower than in the 3-hydroxypicolinic acid residue, nevertheless it is definitely present. From these experimental results it is not completely clear what the biosynthetic origin of this component is, but it is likely that one of the three possibilities listed for 3-hydroxypicolinic acid also holds true for

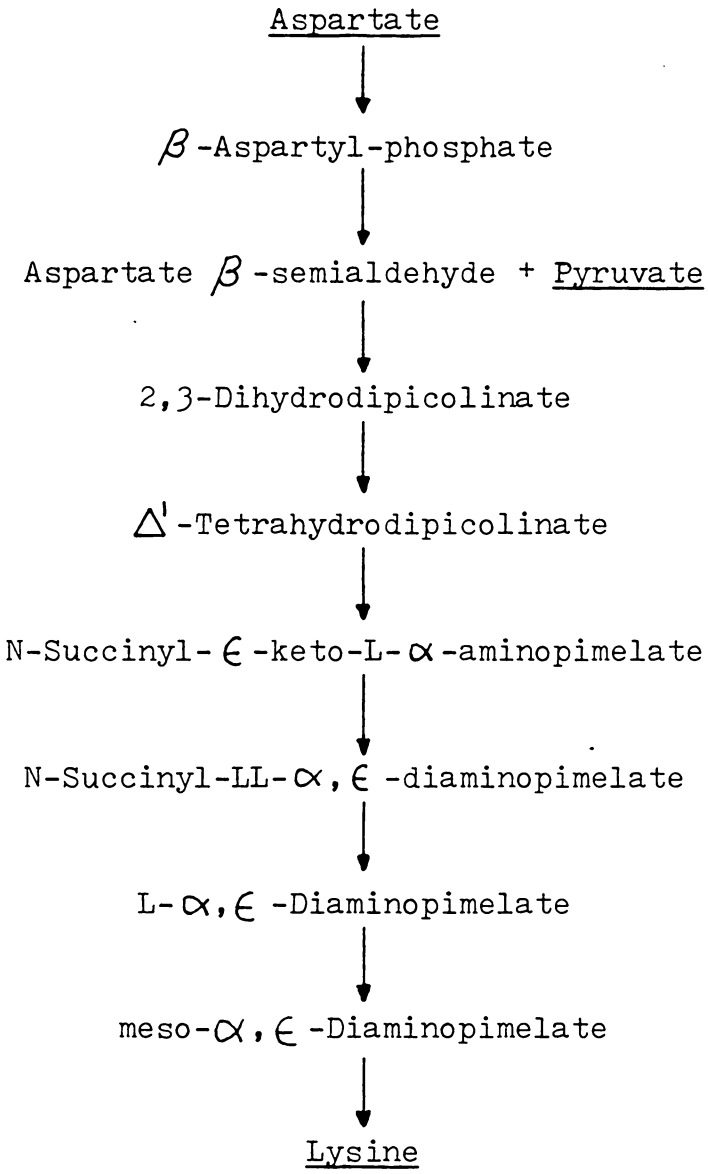


FIGURE 13.
Lysine Biosynthesis by Diaminopimelate Pathway[87]

4-oxo-pipecolic acid.

This last component also contained the most surprising of all the results. In addition to the L-(U-14C)-aspartic acid and L-(U-14C)-lysine incorporations a significant amount of L-(methyl-14C)-methionine was observed to be incorporated. L-methionine is known to be a methyl donor, but 4-oxo-L-pipecolic acid does not contain any methyl groups. At present the result is unexplainable, except for the possibility of the result simply being due to error.

The conclusions drawn from the radiolabeled precursor incorporations are depicted in Figure 14.

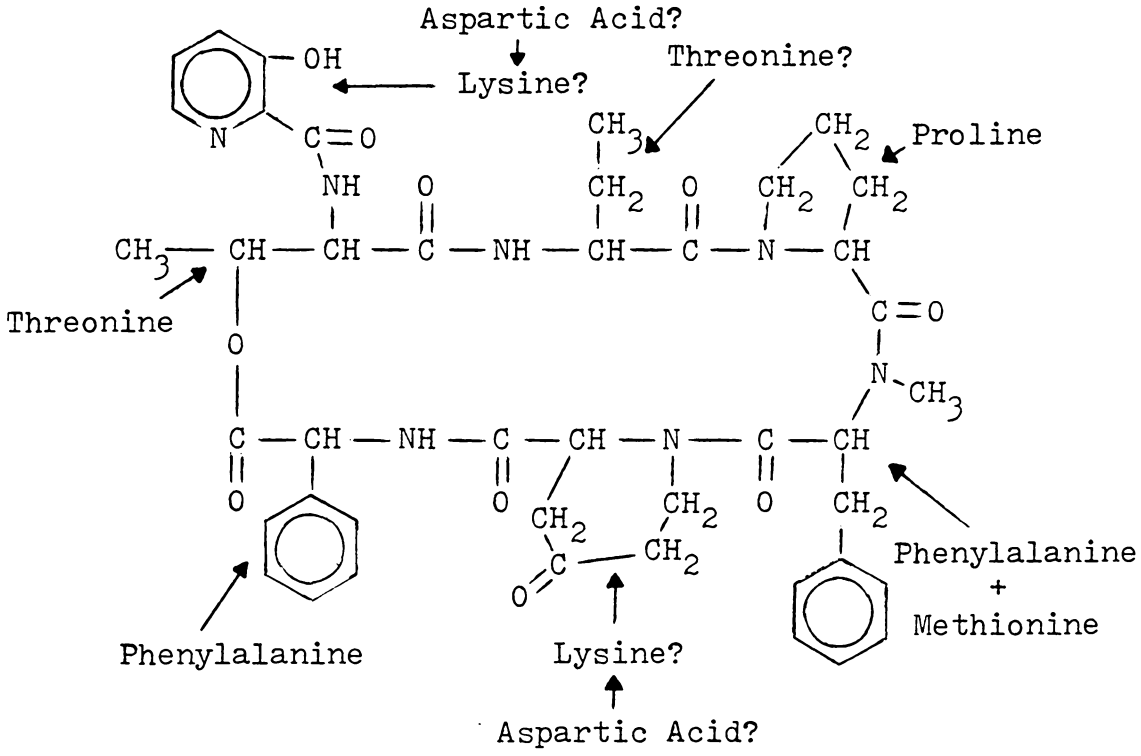


FIGURE 14. Radiolabeled Precursor Incorporations

Summary

Virginiamycin S_1 is a macrocyclic peptidolactone antibiotic produced by a Streptomyces species. It is currently used as a feed additive to promote growth in poultry, swine, and cattle. It is active mainly against Gram-positive bacteria. The biosynthesis of virginiamycin S_1 by Streptomyces virginiae strain 1830 was studied by growing the microorganism in a complex medium and observing the incorporation of a variety of labeled compounds. These radiotracer studies have established many of the biosynthetic precursors of virginiamycin S_1.

The location of the radioactivity in virginiamycin S_1 was determined by a modification of the old procedure. Where ion exchange or adsorption chromatography had been previously used to purify enough of the hydrolysis products to determine the location of the radioactivity, derivatization and HPLC were used. The amino acid derivative used was the N-benzoyl derivative. This modification provided a fast, easy method for compound analysis. In addition these studies can be accomplished with much smaller amounts of antibiotic.

Radiotracer studies indicated that L-threonine and L-proline were derived from their corresponding radiolabeled compounds. L-Phenylglycine was derived from

L-(U-14C)-phenylalanine and N-methyl-L-phenylalanine was from L-(U-14C)-L-phenylalanine and L-(methyl-14C)-methionine.

The biosynthetic precursors for the remaining three components, 4-oxo-L-pipecolic acid, 3-hydroxypicolinic acid and D-α-aminobutyric acid were less clear. Both L-(U-14C)-aspartic acid and L-(U-14C)-lysine were observed to be incorporated in virginiamycin S_1. The major precursor to D-α-aminobutyric was L-(U-14C)-threonine.

L-(U-14C)-Alanine and DL-(methylene-14C)-tryptophan were incorporated only in extremely small quantities and were not exclusively located. It is not likely that either of these two are precursors to the virginiamycin S_1 structure.

The interesting possibilities that were opened for the precursors of 3-hydroxypicolinic acid and 4-oxo-L-pipecolic acid suggest more work is needed in this area.

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Appendix

I. The method of calculation for all the percent incorporations is as follows:

$$\frac{\text{Total volume standard}}{\text{Volume of standard counted}} \times \text{cpm of counted volume} = \text{Total cpm in standard}$$

$$\text{Total cpm in standard} \times \frac{\text{Volume of radioactive materials added to cultures}}{\text{Volume of radioactive material in standard}} = \text{Total cpm added}$$

$$\frac{\text{cpm in sample counted} \times 10}{\text{Total cpm added}} \times 100 = \% \text{ Incorporation}$$

II. The method of calculation for all the specific incorporations is as follows:

$$\frac{\text{Ci in } VS_1}{\text{moles } VS_1} = \text{Specific activity of } VS_1$$

$$\frac{\text{Specific activity of } VS_1}{\text{Specific activity of precursor added}} \times 100 = \text{Specific Incorporation}$$

III. The method of calculation of the distribution of radioactivity is as follows:

$$\text{cpm of component counted} \times \frac{\text{Total amount of component}}{\text{Amount of component counted}} = \text{Total cpm of component}$$

$$\frac{\text{Total cpm of component}}{\text{Total cpm of sample}} \times 100 = \text{Percent in component}$$

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THE BIOSYNTHESIS OF VIRGINIAMYCIN S_1

by

Anthony A. Molinero

(ABSTRACT)

The biosynthesis of virginiamycin S_1, a macrocyclic peptidolactone antibiotic, was studied by growing a strain of Streptomyces virginiae in a complex medium and observing the incorporation of radiolabeled compounds into the antibiotic. These studies have established several of the biosynthetic precursors of virginiamycin S_1.

L-(U-14C)-Proline and L-(U-14C)-threonine were effectively incorporated into the respective amino acid components in the antibiotic. N-Methyl-L-phenylalanine was shown to arise from L-(U-14C)-phenylalanine and L-(methyl-14C)-methionine. L-(U-14C)-Phenylalanine was also efficiently incorporated into L-phenylglycine.

The origin of the remaining three components was less clear. A small amount of L-(U-14C)-threonine was observed in D-α-aminobutyric acid. A biosynthetic pathway is known between these two amino acids which suggests that L-threonine may be the biosynthetic precursor of D-α-aminobutyric acid. Both L-(U-14C)-aspartic acid and L-(U-14C)-lysine were incorporated into 4-oxo-L-pipecolic acid and 3-hydroxypicolinic acid. A biosynthetic pathway was hypothesized to explain these results.