THE BIOSYNTHESIS OF RAVIDOMYCIN

by

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I. Introduction

Natural product chemistry has been studied for many years, but the study of the biosynthetic pathways leading to the natural product is a much newer research endeavor. Previously, biosynthetic studies involved long, tedious, and complex chemistry which was focused mainly toward structure elucidation. In the last twenty years, however, the advent of modern spectroscopic techniques and isotopically labeled compounds has greatly simplified structure determination. This has led to surges in hypothesizing and testing biosynthetic pathways with attempts to determine the origins of these natural products.

Secondary metabolites, the compounds arising from biosynthetic pathways, are the compounds of interest to a biosynthetic chemist. They have a restricted distribution, being found mostly in plants and microorganisms, and are formed from primary metabolites along specialized pathways.[1] Primary metabolites, on the other hand, have a broad distribution and are essential to life. Many compounds such as terpenes, steroids, alkaloids, and polyketides are the products of secondary metabolism. Of these compounds, many display a bactericidal property. These compounds are termed antibiotics and are potentially useful to man.

An antibiotic, by definition, is an organic compound produced by one organism that, at great dilution, inhibits the growth of or kills another microorganism or microorganisms.[2] It is for this reason that much time and effort have been expended in determining the biosynthetic pathways which produce them.

Streptomyces ravidus, a recently discovered microorganism, was first isolated from a soil sample obtained in Guatemala.[3] It was found to be a producer of a new and potentially useful antibiotic. This antibiotic, called ravidomycin, is a bright yellow crystalline compound with a melting point of 248-250°C, a molecular weight of 563 g/mol, and a molecular formula of $C_{31}H_{33}NO_9$. The structure of ravidomycin was first reported by Findlay et al. and was based on chemical and spectroscopic data.[4] The reported structure was determined to be a polycyclic aromatic hydrocarbon with a C-glycosyl group. The amino sugar is 3,6-dideoxy-3-N,N-dimethylamino pseudo altropyranose (ravidosamine).[3] The aglycone moiety is identical with those of the antibiotics toromycin and gilvocarcin, and several other antibiotics. Ravidomycin is also produced by some closely related organisms, notably Streptomyces grieso-olivaceus.

Ravidomycin, Figure 1, has received much interest since its discovery. It displays a great deal of biological activity which, perhaps, may prove useful in the treatment of disease. Ravidomycin is mainly active against Gram-positive bacteria including mycobacteria. It also shows weak activity against Gram-negative bacteria and no activity against fungi (Table 1).[3] The minimum inhibitory concentration (MIC) of ravidomycin can be seen to be quite low for Gram-positive organisms ranging from 3.2 μg/ml for Staphylococcus pyrogenes (penicillin R and S) to less than 0.2 μg/ml for Streptococcus faecalis. The MIC is also quite low for mycobacteria, ranging from 0.5 μg/ml for Mycobacteria fortuctum to 25 μg/ml for Photochromogenic bacteria (Group I). Finally, ravidomycin shows very limited activity for Gram-positive organisms with

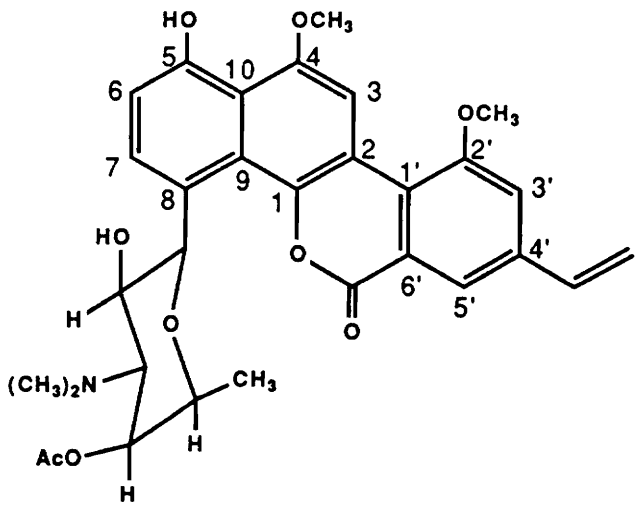


Figure 1. Ravidomycin.

Table 1. Minimum inhibitory concentration (MIC) of ravidomycin.

Bacteria	MIC (μg/ml)	Bacteria	MIC (μg/ml)
Staphylococcus pyogenes (penicillin^S)	3.2	*Klebsiella pneumoniae*	25
Staphylococcus pyogenes (penicillin^R)	3.2	*Serratia marcescens*	50
Streptococcus faecalis	<0.2	*Mycobacterium tuberculosis* var. *hominis*	1~5
Escherichia coli	100	Photochromogenic mycobacteria	25
Enterobacter aerogenes	50	(Group I)	
Salmonella pullorum	50	Scotochromogenic mycobacteria	5
Pseudomonas aeruginosa	50	(Group II)	
Proteus mirabilis	25	*Mycobacterium fortuitum* (Group IV)	0.5

MICs on the order of 25 µg/ml for Klebsiella pneumonia to 100 µg/ml for Escherichia coli.

The most important biological activity associated with ravidomycin is its potent antitumor activity. It is for this reason that much time and effort have been given to the study of ravidomycin. Sehgal et al.[3] have submitted ravidomycin to the National Cancer Institute for antitumor screening. Table 2 shows the activity of ravidomycin in mice implanted with P388 lymphocytic leukemia. Mice were treated with ravidomycin (in hydroxypropylcelluose) on days 1, 5, and 9 via single intraperitoneal injections. As can be seen from Table 2, the median survival time (MST) expressed as a percentage of test mice to control mice for mice treated with ravidomycin ranged from 86% to 238%. Any valve of 125% or greater is considered significant prolongation of host survival. Ravidomycin was also tested for antitumor activity in mice implanted with Colon 38 tumor and in rats implanted with CD8F1 mammary tumor. As in the test for lymphocytic leukemia, all mice were treated with a single intraperitoneal injection of ravidomycin in hydroxypropyl cellulose. Mice containing Colon 38 tumor were treated on days 2, 9, and 16 while rats with CD8F1 mammary tumor were treated on days 1, 8, 15, 22 and 29. Table 3 shows the results of these experiments.[3] The median tumor weight (MTW) has been reduced for all test animals treated with ravidomycin. When the MTW is expressed as a percent for test animals versus control animals, it can be seen that the sizes of the tumors are reduced significantly. Any value less than 42 is regarded as significant tumor growth inhibition. The most effective dosages for mice with Colon 38 tumor are 100 and 200 mg/kg while that for rats with

Table 2. Effect of ravidomycin on the survival time of mice implanted with lymphocytic leukemia P388.

Dose/injection (mg/kg)	Average weight difference of	Survivors on day 5	MST(days)		T/C(%) (MST)
			T	C	
400	-3.4	6/6	7.9	12.7	-----
200	-4.6	5/6	11.0	12.7	86
100	-1.0	6/6	19.0	12.7	149
100	-1.2	6/6	25.0	10.5	238
50	-1.4	6/6	14.3	10.5	136
25	-0.3	5/6	12.0	10.5	114

Table 3. Effect of ravidomycin on Colon 38 tumor mice and CD8F1 mammary tumors in rats.

Tumor	Dose/ injection (mg/kg)	Average weight difference of animals (T-C,g)	Survivors on day 5	MTW(mg) T	MTW(mg) C	T/C(%) (MTW)
Colon 38	400	3.4	10/10	0	1,273	0
	200	0.4	10/10	175	1,273	13
	100	3.2	10/10	384	1,273	30
	50	3.8	10/10	1,080	1,273	84
	25	7.2	10/10	661	1,273	51
	12.5	6.2	10/10	668	1,273	52
CD8F1	400	-4.8	0/10	0	1,116	-----
	200	-7.6	5/10	0	1,116	-----
	100	-7.4	10/10	1	1,116	0
	50	-3.8	10/10	1	1,116	0
	25	-2.4	9/10	576	1,116	51
	12.5	-0.5	9/10	864	1,116	77

CD8F1 mammary tumor is 50 and 100 mg/kg. All evaluations of ravidomycin antitumor activity were done thirty days after the implanted animals were given their first injection. The intense antitumor activity displayed in Tables 1-3 is further enhanced by the low toxicity of ravidomycin. The acute intraperitoneal LD_{50} in mice is 400mg/kg of body weight.

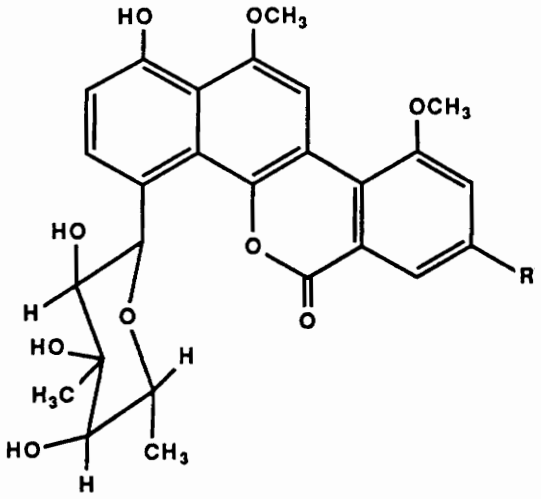
Chemically, there is much interest in ravidomycin. The biological activities associated with ravidomycin make the molecule an attractive target for synthesis. To date there have been no complete syntheses of ravidomycin i.e. aglycone along with the sugar moiety, but there have been several attempts to synthesize the ravidomycin aglycone. The first synthesis by Findlay et al.[5] uses a convergent strategy. Vanillin is converted to a protected substituted benzoic acid derivative through a series of synthetic manipulations. It is then coupled with a functionalized Grignard reagent of napthalene in an 81% yield using conditions pioneered by Meyers et al.[6] This gives a phenylnapthalene which is then deprotected, demethylated, and lactonized resulting in the desired product which was identical in all physical and spectroscopic properties with an authentic sample.

A second synthesis is by McKenzie et al.[7] The synthesis employs a sequential Meerwein and Diels-Alder route with the regiochemistry of the Diels-Alder reaction being directed by a chlorine atom. 3,5-Dimethyl anisole is oxidized to the diacid and eventually converted to a substituted aniline. The aniline is diazotized and then coupled to 2,6-dichloroquinone. The coupled product is then reacted with 1-trimethylsiloxy-1,3-butadiene using a chlorine atom on the quinone to

control the regiochemistry. The resulting Diels-Alder adduct is then rearomatized followed by reductive lactonization giving the final product. There are several drawbacks to this synthesis. First, all attempts to remove the residual chlorine used to control the regiochemistry of the Diels-Alder reaction were unsuccessful. Second, the vinyl side chain, crucial to antitumor activity, was not attached to the aglycone. These problems were corrected in a later publication by McKenzie et al.[8] The residual chlorine was removed by, first, conversion to the phenyl selenide followed by reductive lactonization and concomitant loss of phenyl selenide. Finally, the vinyl side chain was added by coupling vinyltributyltin with a tetracyclic bromide using a palladium catalyst.

A final synthesis by Danishefsky et al.[9] also uses a convergent strategy. This synthesis employs a unique Meyers coupling reaction to link a bromojuglone derivative with a vanillin derivative. 2-bromojuglone was converted via several synthetic steps to the bromonaphthohydroquinone and subsequently to a Grignard reagent. This was then coupled to an oxazoline derivative of vanillin by a Meyers coupling reaction. This gave a biaryl ring system which was lactonized by treatment of the ring system with dilute acid giving rise to the ravidomycin aglycon. All spectral data was in full agreement with that of an authentic sample.

The polycyclic aromatic aglycone of ravidomycin is not unique to this antibiotic. There are several other antibiotics which possess the same aglycone. They are chrysomycin V and M, toromycin, gilvocarcin V, M, and E, albacarcin V and M, and virenomycin V and M (V, M and E are



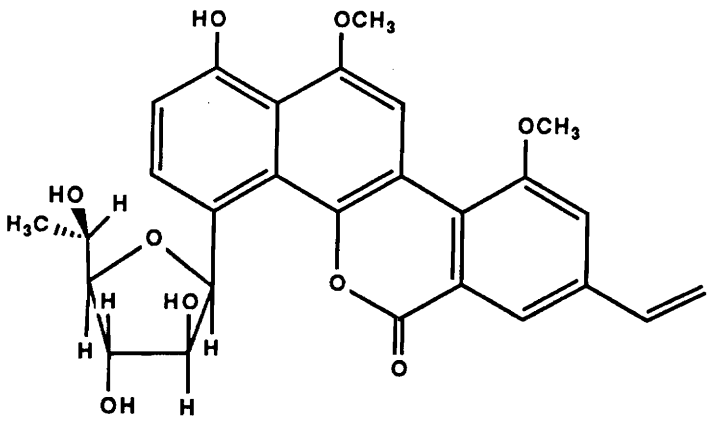
R = -CH=CH$_2$ Chrysomycin V
R = -CH$_3$ Chrysomycin M

Figure 2. Chrysomycin V and M.

vinyl, methyl, and ethyl side chains respectively).

Chrysomycin V and M (Figure 2) produced by Streptomyces A-419 was first isolated in 1954 from a soil sample obtained from the grounds of the New York Botanical Garden.[10] The structure of chrysomycin remained unknown until Weiss et al.[11] deduced it through the application of ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy. The structure was shown to have the same aglycone as ravidomycin, but the sugar moiety is a methyl glycoside of the sugar viranose. Biological studies were undertaken with emphasis on bacteriophages with the hopes that any antiphage activity could serve as a guide for action against the poliomyelitis virus. From these studies it was found that chrysomycin shows activity against bacteriophages, bacteria, and fungi. It was later tested for antitumor activity against P388 lymphocytic leukemia and found to be very active with low toxicity.[11] At 400 mg/kg chrysomycin increased the life span of 54% of the mice while showing no lethal toxicity.

A second antibiotic, which resembles ravidomycin, is toromycin (Figure 3). Toromycin was isolated from the culture broth of Streptomyces collinus subspecies albesceas which was isolated from a soil sample collected at the Toro remains in Japan.[12] Toromycin exhibits biological activity against pleuropneumonia-like organisms, DNA-type viruses, trichomonases, and gram-positive bacteria including mycobacteria.[12] The structure, first elucidated by Horii et al.[13], was determined by chemical degration and spectral studies. It has the same aglycone as ravidomycin with a C-glycosyl sugar which is either a substituted furanose or pyranose.



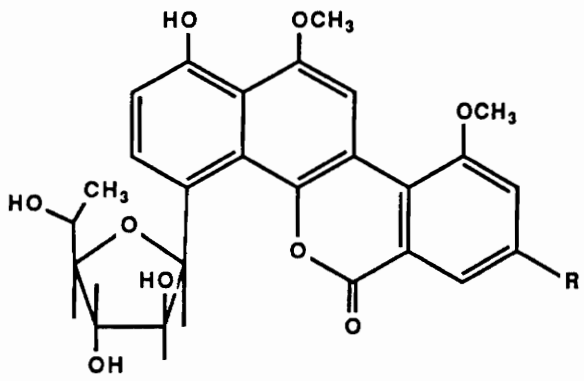
Toromycin (GilvocarcinV)

Figure 3. Toromycin.

Gilvocarcins V, M, and E are other antibiotics similar to ravidomycin. The producing organism, Streptomyces gilvotonareus, was isolated from a soil sample collected in Kochi-shi, Kochi, Japan.[14] The structure of gilvocarcin was first determined by Takahushi et al.[15] (Figure 4) using chemical degradation, nuclear magnetic resonance, and mass spectrometry. The aglycone is identical with ravidomycin, but the sugar is a furanose derivative which is connected to the aglycone via a C-glycosyl linkage. Gilvocarcin shows strong activity against Gram-positive bacteria and weak activity against Gram-negative bacteria. Gilvocarcin also shows strong antitumor activity with significant tumor regression being observed with P-388 lymphocytic leukemia, sarcoma 180, ascites, and Ehrlich carcinoma.[16]

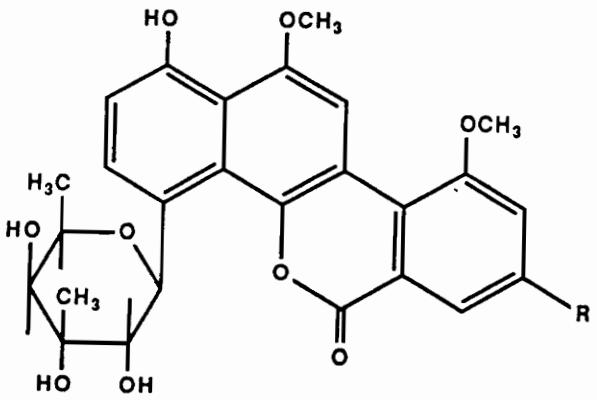
Streptomyces albaduncas produces the antibiotics albacarcin V and M (Figure 5)[17], which have been shown to be effective against bacteria and mammalian tumors. The aglycone is identical to that of ravidomycin. The sugar moiety is the distinguishing characteristic between albacarcin and ravidomycin.

A final antibiotic similar to that of ravidomycin is virenomycin V and M (Figure 6).[18] Once again the antibiotic is identical to ravidomycin except for the sugar moiety.



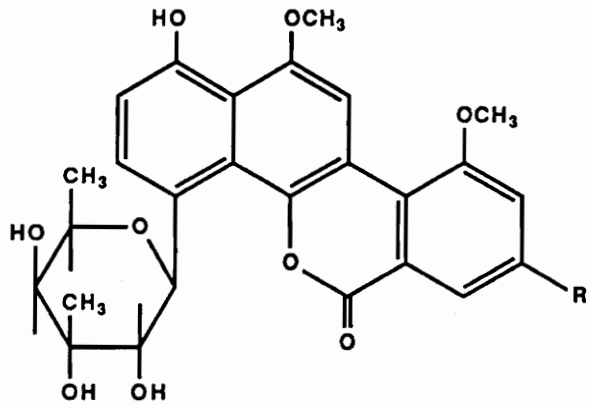
R = -CH=CH$_2$ Gilvocarcin V (Toromycin)
R = -CH$_2$-CH$_3$ Gilvocarcin E
R = -CH$_3$ Gilvocarcin M

Figure 4. Gilvocarcin V, M, and E.



R = -CH=CH$_2$ Albacarcin V
R = -CH$_3$ Albacarcin M

Figure 5. Albacarcin V and M.



R = -CH=CH$_2$ Virenomycin V
R = -CH$_3$ Virenomycin M

Figure 6. Vivenomycin V and M.

II. BIOSYNTHESIS OF POLYKETIDES

Since prehistoric times man has used crude plant extracts to help him survive. Many accounts dating to earliest recorded time describe the use of plant extracts to heal and to kill. Primitive man used these crude extracts to relieve pain and alleviate symptoms of disease. He also used the extracts as poisons for hunting and as agents of warfare. With the coming of modern science, the prehistoric uses of crude plant extracts have yielded way to the isolation and purification of natural products from plants and bacterial cultures with aspirations of benefiting man.

Since many natural products isolated from common sources can be used to combat disease, early studies in biosynthesis were focused on revealing many of the biological processes in biosynthetic pathways. Many of the proposed biosynthetic pathways enabled the early scientist to draw inferences about chemical make-up and structure. This in turn gave insights towards the total synthesis and classification of the natural products. Contemporary interest in biosynthesis has shifted away from the above and has focused instead on a host of other issues with biosynthetic relevance.

Today many of the pathways of secondary metabolism are understood with great certainty, but many of the enzymatic systems governing these pathways are not understood. Through biosynthetic studies the scientist is able to gain information and a thorough understanding of how enzymatic mechanisms operate. Having a clear understanding of the mechanisms of enzymes may lead chemists and biochemists to develop the

first artificial enzyme, thereby revolutionizing synthetic chemistry.

A thorough knowledge of biosynthetic pathways can be used by chemists as models when designing syntheses. In the past the biosynthetic transformations which produced the natural products were considered mysterious, but these mysteries are now better understood. Successful biomimetic syntheses populate the literature expounding the fact that biosynthetic pathways, although enzymatically controlled, are governed by the same chemical laws as those which govern the chemist. Reflecting upon this and the fact that nature is extremely efficient makes it worthwhile for the chemist to try to duplicate nature's steps in his own synthetic design.

Once a biosynthetic pathway is known it then becomes possible to modify existing compounds. Some element of control over the biosynthetic processes could be induced by administering certain analogs of known precursors. By varying the precursors, a potentially useful but toxic antibiotic could be chemically modified in vivo giving rise to a promising, less toxic antibiotic. For example, this controlled synthesis is routinely being done in the production of penicillin. Different species of penicillin can be synthesized by adding various precursors to the growth medium of Penicillium chrysogenum.[19]

Finally, a knowledge of biosynthetic pathways would theoretically enable scientists to produce antibiotics in higher yields, making them cheaper and readily available. This is possible because enzymes present in the biosynthetic pathways could be produced in larger amounts or altered in some way which would make nature's processes even more efficient, giving greater yields of antibiotics. This could have a

large impact on the health community by giving a more diverse distribution of medication such as to Third World or economically depressed countries whose people and government cannot afford the benefits of modern medicine. Since most antibiotics are marketable, it would also be a great economic advantage to be able to produce large quantities of antibiotics with minimal costs in labor, time, and materials.

Ravidomycin is not only an interesting antibiotic biologically, but it is also interesting biosynthetically, especially the formation of the propionate derived vinyl side chain on the aglycone moiety. Before this can be properly addressed, it is necessary to discuss the biosynthesis of polyketides.

Acetate is one of the most common and useful subunits used by organisms to produce larger and more complex molecules. Acetate is not only used in fatty acid biosynthesis, it is also used in the formation of two other classes of natural products, namely terpenes and polyketides. Polyketides are produced by a host of organisms such as bacteria, fungi, and lichens. In some cases plants also produce polyketides, but the pathway is not as well developed as for the aforementioned compounds.[20] Polyketides are responsible for a wide spectrum of substances such as several mycotoxins, flavonoids, xanthones, quinones, and phenols. This illustrates the fact that there appears to be an endless number of molecules which can be produced from one polyketide chain. The key polyketide chain is built up and then undergoes any of a variety of chemical changes which leads to products which have subtle differences from the rest. Although many of these

pathways may be occurring in an organism at one time, a particular pathway may be much more pronounced than the others giving rise to a particular antibiotic or other natural product.

As previously mentioned, acetate is responsible for the formation of polyketides. The C_2 units of acetate are assembled to form polyketomethylene chains of the formula $L\text{-}(CH_2\text{-}CO)_n\text{-}J$, formally ketene polymers. Fatty acids are also an assemblage of acetate units, but unlike polyketides, the carbonyl unit of the growing chain is reduced to a methylene group. The growing polyketide chain does not undergo such reductions; poly-β-ketoacids are formed instead. These ketoacids not only have active methylene groups which act as nucleophiles, but they also have carbonyl groups which can be potential electrophiles. It is this high reactivity of the growing chain which enables the formation of a wide variety of substances.

The formation of polyketide chains by the addition of acetate groups was first envisioned by Collie in 1907[21], but his hypothesis was largely ignored until Birch rediscovered it in 1953 and proposed a general hypothesis for the formation of polyketide chains.[22] His hypothesis states that the formation of polyketide chains occurs in four sequential steps:[23]

A. Acetic acid units are joined with each other or with other naturally occurring carboxylic acids to form β-polyketomethylene chains;

B. The β-polyketomethylene chains can undergo secondary changes such as cyclizations by Aldol or Claisen condensations to form aromatic rings;

C. The carbon skeleton formed may be further modified by the addition of alkyl groups;

D. Reduction (with or without dehydration) and oxidation may occur before or after cyclization.

Several experimental observations have been made in extension of the above rules. Steps B and D do not always occur; steps A and C can mix with each other; after step B and D an additional step A is very uncommon or virtually impossible; finally, step B occurring via intermolecular cyclizations is also very unusual if not impossible.[20]

The formation of polyketide chains probably takes place by way of Claisen-type condensations. The process can best be shown schematically in Figure 7.[20] An acetyl-CoA or propionyl-CoA starter unit bound to a thiol group of a multienzyme complex is condensed with a malonyl-CoA unit also bound to the same complex. Carbon dioxide is lost and the process is free to occur again in successive steps giving rise to polyketides of varying lengths. During the formation of the polyketide chain, alkylations, reductions, and oxidations may occur. While the polyketide chain is forming it is protected by the multienzyme complex from intermolecular reactions. This probably takes place by hydrogen bonding or metal chelation. Once the polyketide chain reaches a certain length, the folding pattern of the multienzyme complex allows for intramolecular cyclizations to take place. Finally, the newly formed polyketide is released from the complex and the process can start again. Since the whole process of polyketide formation is enzymatically bound there are no intermediates which are accepted or released outside the complex.

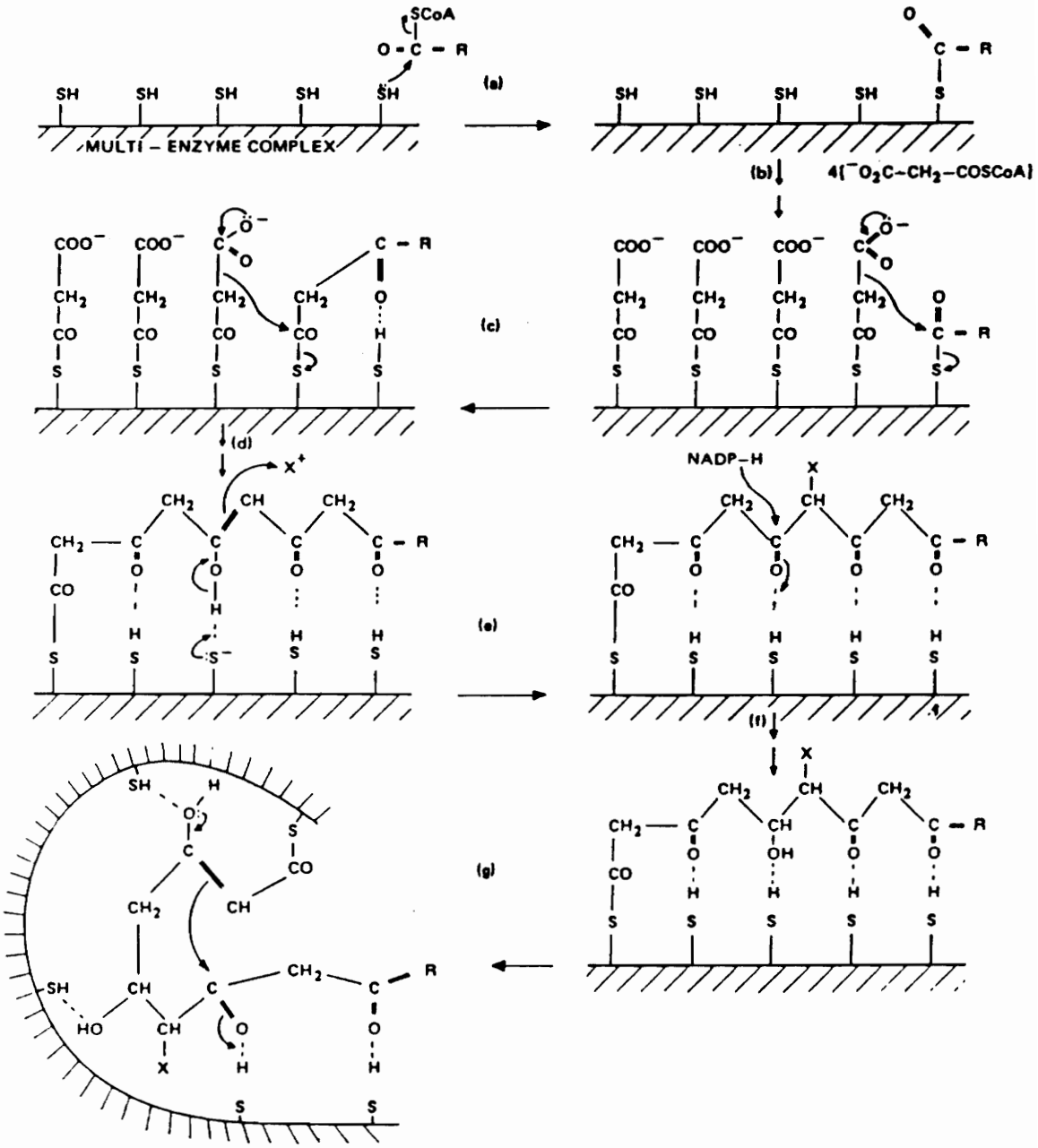


Figure 7. Model for Polyketide Formation.

As mentioned earlier, structural modifications of the polyketide may occur. These alterations may occur while the polyketide chain is forming or after it has formed. Modifications such as reductions, oxidations, and alkylations are difficult, if not impossible to determine. Fortunately, however, some have been identified by means of specific labeling experiments. Enough of these labeling experiments have been done that the frequency of several transformations occurring during or after the polyketide forms can be summarized in Table 4.[20]

Reductions of polyketide chains occur with NADH (nicotinamide adenine dinucleotide) and NADPH (nicotinamide adenine dinucleotide phosphate). Since both NADH and NADPH are in their reduced forms, both readily transfer a proton across a carbon-carbon double bond or a carbon-oxygen double bond. The double bonds then become reduced while the NADH or NADPH becomes oxidized to NAD^+ or $NADP^+$ respectively.

Most secondary modifications to polyketide chains are oxidations. Oxidations usually occur after the polyketide has undergone any cyclizations and occur by activated oxygen. One of the most common secondary biosynthetic processes is the oxidation of an aromatic methyl group into a carboxylic acid. This process occurs through successive oxidations, first to the alcohol, then to the aldehyde and finally to the acid. At this stage the carboxyl group can be replace by a hydroxyl group (oxidative decarboxylation) or with a hydrogen (non-oxidative decarboxylation).

Another common modification to a polyketide chain is alkylation. Alkylations occur via S-adenosylmethionine or dimethylallyl pyrophosphate. Alkylations may occur on a carbon or an oxygen.

Table 4. Frequency of Reactions Introducing Structural Variations into Polyketides.

	Reactions	On product polyketide	Before formation of polyketide product
1.	Reduction	common	rare
2.	Oxidation	rare	common
3.	C-methylation	very frequent	infrequent
4.	O-methylation	--	very frequent
5.	C-prenylation	rather frequent	common
6.	O-prenylation	--	common
7.	C-glycosylation	--	rare
8.	O-glycosylation	--	common

C-alkylations usually occur on a polyketide that already has formed. Sometimes it occurs on polyketides that are in the process of forming, but these occur rather infrequently.[20] O-alkylation, on the other hand, is very common on a forming polyketide.[20] C and O alkylations are important in polyketide biosynthesis because the alkyl groups not only play a role in the biological activity of the molecule, but they have a direct affect on the cyclization pattern of the resulting product.[1]

Polyketides are very reactive compounds. As stated above, they have active methylene groups which can act as nucleophiles as well as carbonyl groups which are potential electrophiles. Therefore, one fate of a polyketide chain is intramolecular cyclization, and many polyketides are cyclic products. The cyclizations that take place are of four types. The first two of the cyclizations form polyphenolic aromatic compounds and occur by crotonic condensations and Claisen condensations (Figure 8).[20] In crotonic condensations (pathway a) the active methylene group adjacent to the carboxyl group of the polyketide chain attacks a carbonyl at a suitable proximity in the chain. The cyclization occurs with loss of water to give a crotonic acid derivative which then aromatizes to give a resorcinol derivative characteristic of this type of cyclization.

Claisen-type condensations occur in the reverse manner as the crotonic condensations (Figure 8, pathway b).[20] Nucleophilic attack of an active methylene on the carboxylic acid forms a cyclic polyketone which, upon aromatization, forms an acylphloroglucinol which is common to this type of cyclization process.

The second two types of cyclizations form ethers and lactones.

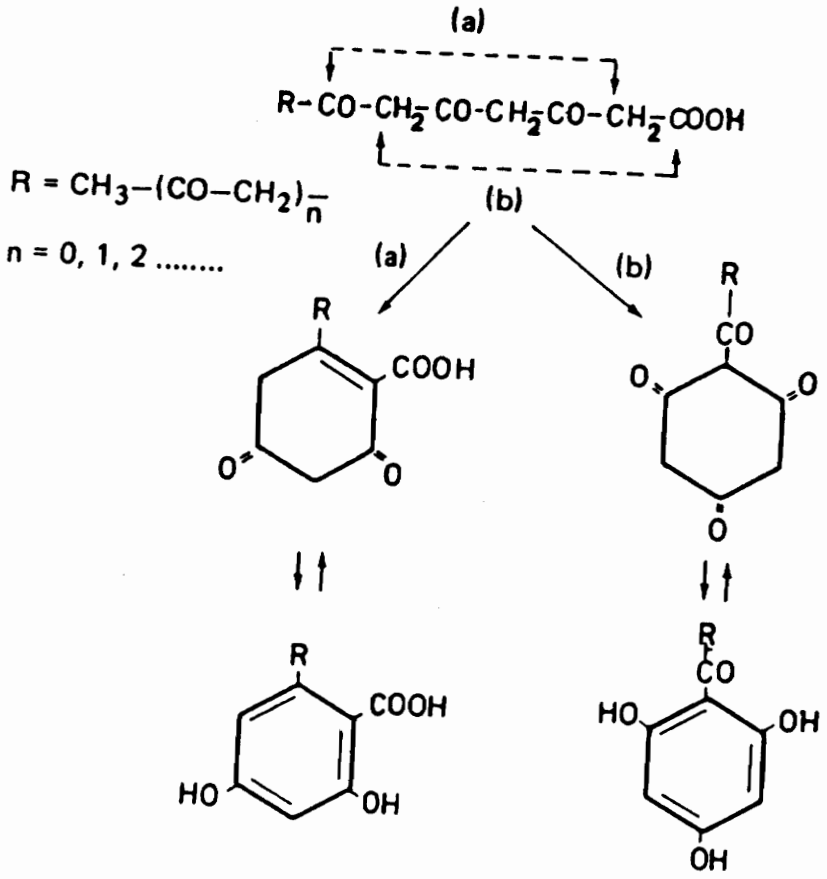


Figure 8. Intramolecular Cyclizations of Polyketides.

Etherification occurs by formation of oxygen bridges between two carbonyl functionalities. This process is not favored, but when it does happen, γ-pyrones such as those found in chromones are formed[20] (Figure 9, pathway d).[20] When the oxygen of a carbonyl group attacks a carboxyl group α-pyrones are formed (pathway c). This type of cyclization process is common to isocoumarins and sometimes predominates over the first two methods previously listed.

The biosynthesis of ravidomycin utilizes many of the previously discussed modifications and generalizations of polyketide formation. Its study involves the incorporation of ^{13}C labeled precursors, more specifically [1-^{13}C] propionate, [1-^{13}C] acetate, [2-^{13}C] acetate, and [1,2-^{13}C] acetate, and the analysis of the resulting antibiotic by NMR methods. When [1,2-^{13}C] acetate was fed and analyzed by 2-D INADEQUATE NMR the carbon connectivity patterns and enrichment factors enabled Carter, et al. to determine the incorporation pattern of intact acetate units.[24] The results of feeding [1,2-^{13}C] acetate show carbon-carbon connectivities between carbon pairs 1-2, 3-4, 5-10, 6-7, 1'-2', and 5'-6' (Figure 10). The lactone carbonyl and 3' carbon gave only singlet NMR signals. Since they are both acetate-derived, the carboxyl carbons must have been lost during the antibiotic's biosynthesis. The 4' carbon also gave no signal, which helps support the idea of the vinyl side chain being propionate derived.

When [1-^{13}C] propionate was fed ravidomycin was extensively labeled at the 4' position, which further supports the idea of a propionate-derived side chain. The carbons of the vinyl side chain were labeled by [2-^{13}C] acetate and not [1-^{13}C] acetate, which can be explained by

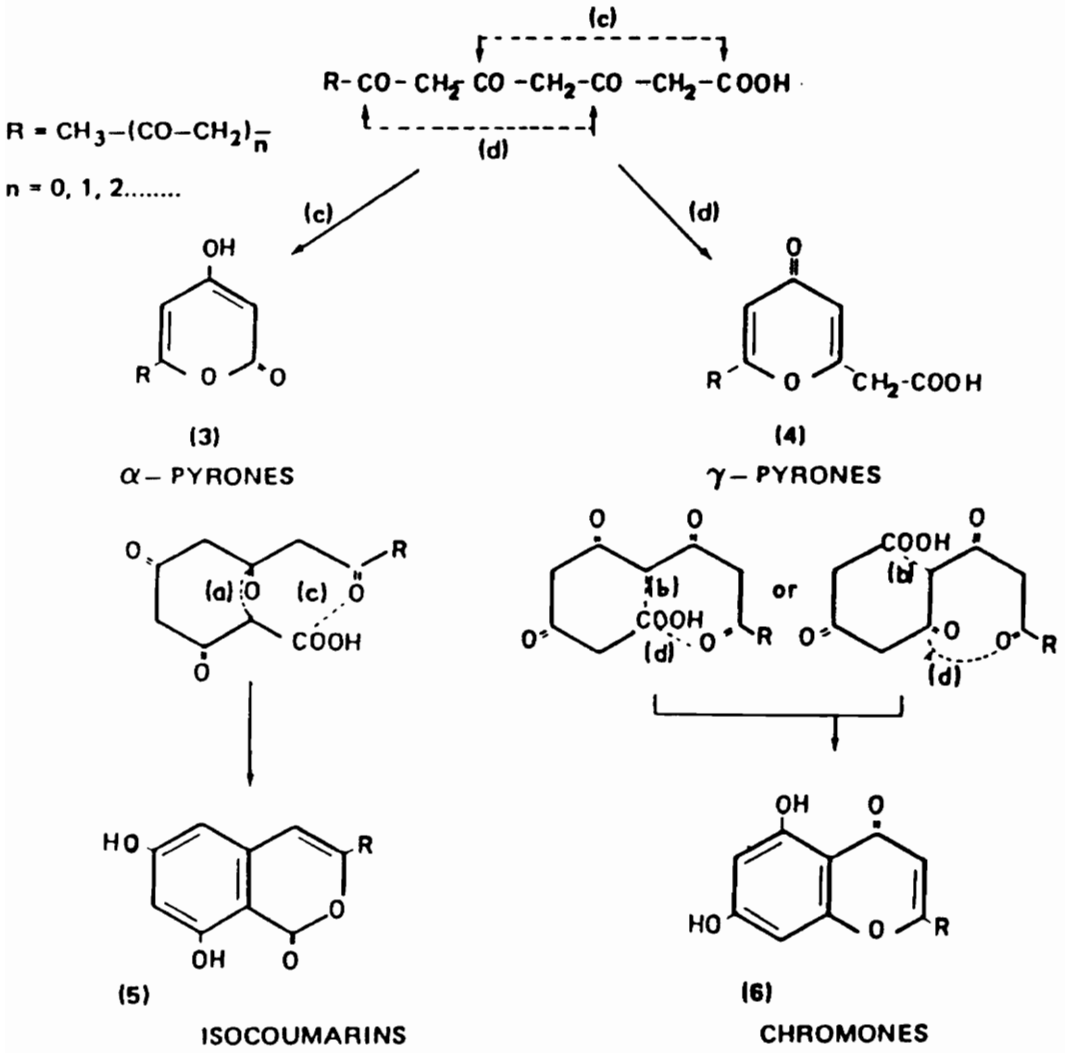


Figure 9. Intramolecular Cyclizations of Polyketides.

conversion of acetate to propionate via the tricarboxylic acid cycle similar to davinomycin biosyntheses.[24,25] Carbons 2,4,5,7,9,2' and 6' were labeled by [1-^{13}C] acetate and carbons 1,3,6,8,10,1',3',5' and the lactone carbonyl were labeled by [2-^{13}C] acetate. The results of the feeding study is summarized in Table 5.[24]

In light of the previous results Carter and coworkers proposed the following biosynthetic pathway (Figure 10).[24] The dekaketide chain **1**, which is propionate initiated, is condensed and cyclized in an intramolecular fashion. Oxygen is eliminated at C-7 and reintroduced at C-1 to give a tetracyclic intermediate **2**. The designated double bond is oxidatively cleared. This is then followed by loss of CO_2 at the terminal carboxylic acid to give a phenyl naphthalene intermediate **3**. Rotation about the single bond and subsequent lactone formation gives compound **4**. O-methylation of the C-4 and C-2' nydroxyls, dehydrogenation of the ethyl group, and C-glycosylation at C-8 give rise to the ravidomycin antibiotic.

Table 5. ^{13}C-NMR data for the aglycone carbons of ^{13}C labeled ravidomycin

Carbon	δ(Hz)[b]	Enrichment Factor[a] [1-^{13}C]propionate	[1-^{13}C]acetate	[2-^{13}C]acetate	Coupling Constant (Hz) [1,2-^{13}C]acetate
1	142.93	1.22	.665	1.49	69.7
2	113.92[c]	.967	2.09	.548	69.6
3	102.25	1.21	1.04	2.28	74.7
4	151.99	1.22	2.75	.885	74.6
5	154.64	.934	2.08	.723	63.1
6	112.41	1.57	1.14	3.28	56.5
7	129.51	1.19	2.77	1.09	56.5
8	124.82	.867	.595	1.08[d]	57.4
9	125.37	1.40	1.89	.599	57.4
10	116.31	1.04	1.28	5.66	63.1
1'	123.59	1.30	.652	1.62	69.6
2'	157.23	1.73	2.76	.949	69.8
3'	113.92[c]	.967	1.28	2.67	nc
4'	138.63	6.39	.802	.902	nc
5'	119.88	1.15	1.14	2.37	62.0
6'	122.37	1.79	3.26	1.08	61.9
$\underline{CH}=CH_2$	135.20	1.26	1.04	2.26	nc
$\underline{CH_2}=CH$	116.46	1.58	1.33	2.77	nc
CO lactone	160.66	1.08	.639	1.33	nc

[a]Ratio of normalized signals between labeled and natural abundance spectra; [b]ppm downfield from TMS in $CDCl_3$; [c]Unresolved in natural abundance spectrum; [d]Although enrichment is low labeling by [2-^{13}C]acetate was established by coupling with C-9 in double labeled material; nc=no coupling.

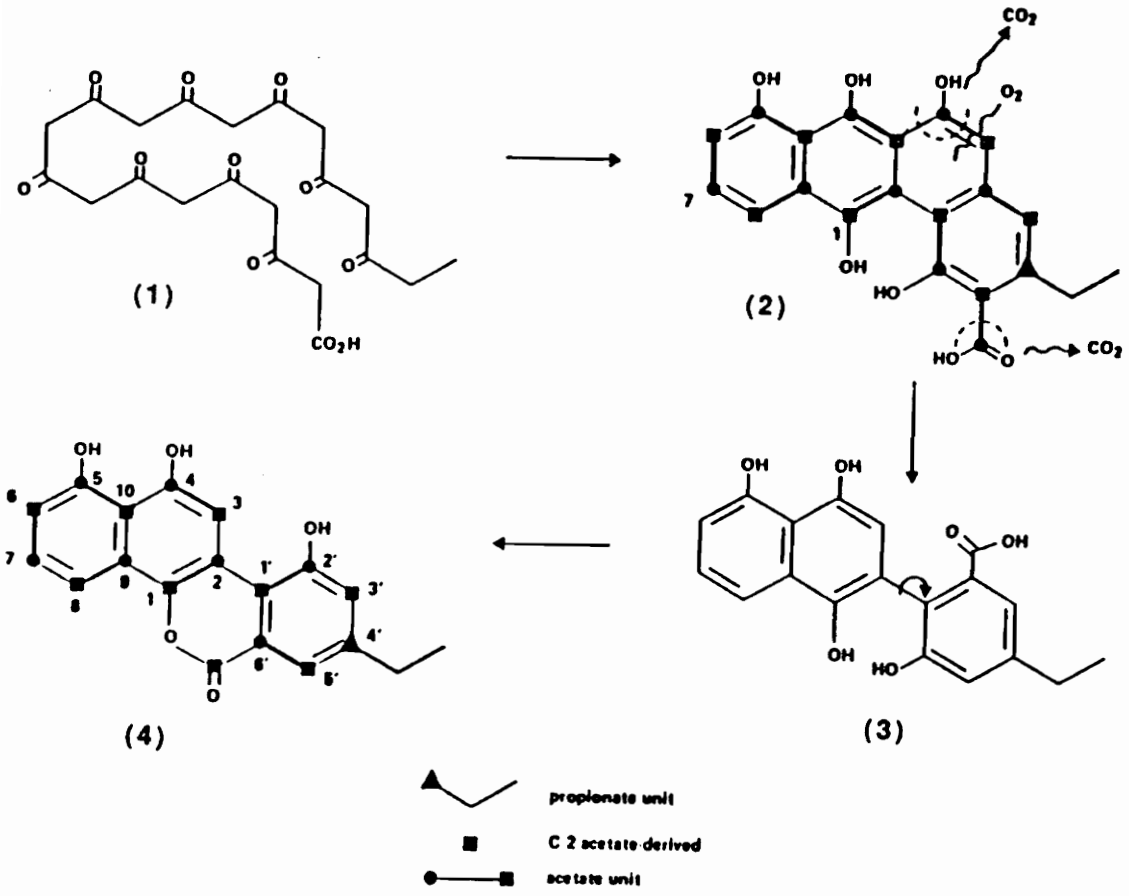


Figure 10. Biosynthesis of Ravidomycin.

III. Statement of Problem

An antibiotic that has been shown to possess biological activity could be potentially useful to the health community. However, it's biological properties, such as its antimicrobial or antitumor activity, while effective in the laboratory, may not be feasible for use in a clinical situation. The antibiotic may be toxic to the host organism, it may not be able to be administered in an efficient way, or it may not be readily taken up by the cell. If this is the case, it then becomes necessary to study the structure-activity relationships of the antibiotic to determine the active portions of the molecule. With this knowledge in hand, it becomes possible to structurally modify the antibiotic, thereby eliminating any undesirable characteristics while still retaining the desired biological activity. Structure-activity relationships have to be determined by derivatizing or cleaving off a portion of the molecule and studying, at a molecular level, how the molecule interacts with DNA, RNA, proteins, and the like, and determining which functionality or functionalities are necessary for biological activity.

Greenstein and coworkers have shown that ravidomycin is most effective when activated by light.[26] Further studies by Singh have shown that when ravidomycin is irradiated with light, DNA synthesis followed by RNA synthesis is inhibited.[27] The vinyl group of ravidomycin is essential to its full biological activity and mechanism of action as described above. This has been shown by several studies of structure-activity relationships of antibiotics of the ravidomycin type.

Elespuru and Gonda studied the activation of gilvocarcins by visible light.[28] Gilvocarcins M and V (Figure 4) were tested. The vinyl analog, gilvocarcin V, showed strong prophage-inducing activity, but gilvocarcin M, the methyl analog, showed no prophage induction. These results imply that the vinyl group is an important structural feature essential for biological activity.

Rakhit and coworkers have made derivatives of ravidomycin.[29] The derivatives were tested for antitumor activity against P388 leukemia in mice. Dihydro derivatives were found to be less active against tumors. Antimicrobial activity was diminished when the vinyl side chain was saturated.[30]

Rakhit also determined the effect of the derivatives on DNA synthesis in Bacillus subtilis by measuring the incorporation of labelled thymidine into acid-insoluble precipitates.[30] The dihydro derivative did not inhibit DNA synthesis, while ravidomycin did. This is indicative of the importance of the vinyl side chain in the molecule's mechanism of action. It is thus evident from these results, as well as the previous, that the antimicrobial and antitumor activities of ravidomycin derivatives parallel each other.

Based on the results described above, it can be seen that the vinyl side chain is a very important functional group on ravidomycin; without it, biological activity is greatly reduced. Because of the importance of the vinyl group to the activity of ravidomycin, its mechanism of formation is of great interest, particularly since related antibiotics may contain an ethyl group in place of the vinyl group. An understanding of the biosynthetic mechanism of formation of the vinyl

group might enable molecular biologists to isolate the gene(s) responsible for this step in the biosynthetic pathway and incorporate these into organisms lacking them, thus ensuring production of vinyl-containing rather than ethyl-containing products.

A first step in elucidating the biosynthetic mechanism of formation of the vinyl group is the determination of the stereochemistry of the dehydrogenation process. As discussed earlier, the vinyl side chain rises from a propionate starter unit.[24] It should, therefore, be possible through appropriately designed feeding experiments to determine whether or not there is any stereochemical selectivity. In other words, it should be experimentally determined if the pro-R or pro-S α proton on the propionic acid starter unit is lost or retained during elimination to form the vinyl side chain during the biosynthesis of ravidomycin (Figure 11). Propionic acid, doubly labeled at the α position could be fed to a growing culture of Streptomyces grieso-olivaceus to unequivocally determine if propionic acid is indeed incorporated. Although this will not tell which proton is being retained in the biosynthetic process, it will tell approximately how much incorporation can be expected. To determine whether the pro-R or pro-S α proton of propionic acid is being retained in the formation of the vinyl group a chiral propionic acid could be fed and analyzed for incorporation. Chiral R and S[2-^{2}H] propionic acid could be synthesized, and the labeled materials could be fed to the producing organism in successive runs. The resulting antibiotic from each run could then be analyzed for isotope retention. The retention or loss of the label will determine if the pro-R or pro-S is retained or lost in the actual biosynthesis of ravidomycin.

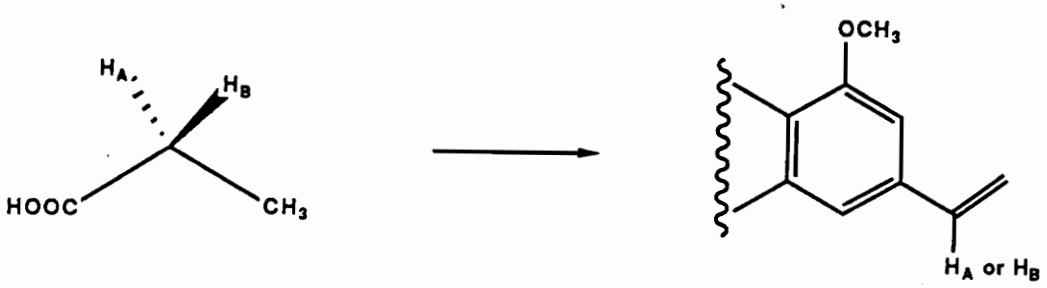


Figure 11. Elimination of Pro R and Pro S Protons of Propionic Acid to Form the Vinyl Side Chain.

IV. RESULTS AND DISCUSSION

When doing research on the biosynthesis of an antibiotic, many obstacles must be overcome. The three most important barriers are; first, synthesis of the labeled precursors which will be used for the incorporation studies. Second, a suitable method of analysis of the antibiotic must be chosen. Finally, in addition, the producing organism must not only be successfully grown, but it must produce a sufficient amount of the desired antibiotic. Each of these obstacles must be overcome in order to ensure accurate and reliable results.

Propionic acid has been shown by Carter et al. to be the starter unit in the formation of the aglycone of ravidomycin.[24] It was also shown to be the precursor for the origin of the vinyl side chain. Therefore, in order to determine if there is any stereochemical selectivity in the vinyl group formation, labeled propionic acid was synthesized. Deuterium labeling was chosen as the method for the synthesis. Before the synthesis of the labeled compounds can be addressed, the advantages and disadvantages of the use of stable isotopes must be discussed.

There are two major reasons for choosing deuterium, namely cost and ease of handling. Aside from these obvious reasons there are several other factors which must be kept in mind when using stable isotopes. Stable isotopes can give more information more quickly than radioactive isotopes. The methods of analysis for stable isotopes such as NMR spectroscopy and mass spectrometry are less sensitive than those for radioactive isotopes, therefore more precursor must be administered to

the producing organism. This presents a risk in that the normal metabolism of the organism might be upset, leading to erroneous results.[1]

Another disadvantage of using stable isotopes is their natural abundance. Although not too critical for the deuterium isotope, stable isotopes which have a significant natural abundance such as ^{13}C, present difficulties in the amount of label which can be detected in a product. When using mass spectrometry for analysis of feeding experiments, the amount of incorporation is measured using the M+1 peak. Due to the natural abundance of stable isotopes, the M+1 peak can have a large intensity making it difficult to determine if there is any incorporation, especially if the incorporation is small.[1]

Finally, the use of stable isotopes is appealing because they are highly enriched. It therefore becomes an easy task to prepare a precursor with two or more labels. This becomes evident when attempting to synthesize presursors with two or more radioactive labels per molecule. Due to the low enrichment of a radioactive label in a compound it becomes statistically unlikely that a multiple labeled compound will contain more than one label.[1] Judging and weighing the advantages and disadvantages of using stable isotopes versus radioactive isotopes, it is apparent that the use of the stable deuterium isotope was the most feasible route to follow for the biosynthetic study.

The synthesis of the labeled precursors, sodium (R)-[2-$^{2}H_{1}$] propionate and sodium (S)-[2-$^{2}H_{1}$] propionate, followed a modification of the procedure of Brandange et al.[30] (Figure 12). (S)-(-)-Ethyl lactate **5** is tosylated according to the procedure of Kenyon et al.[31]

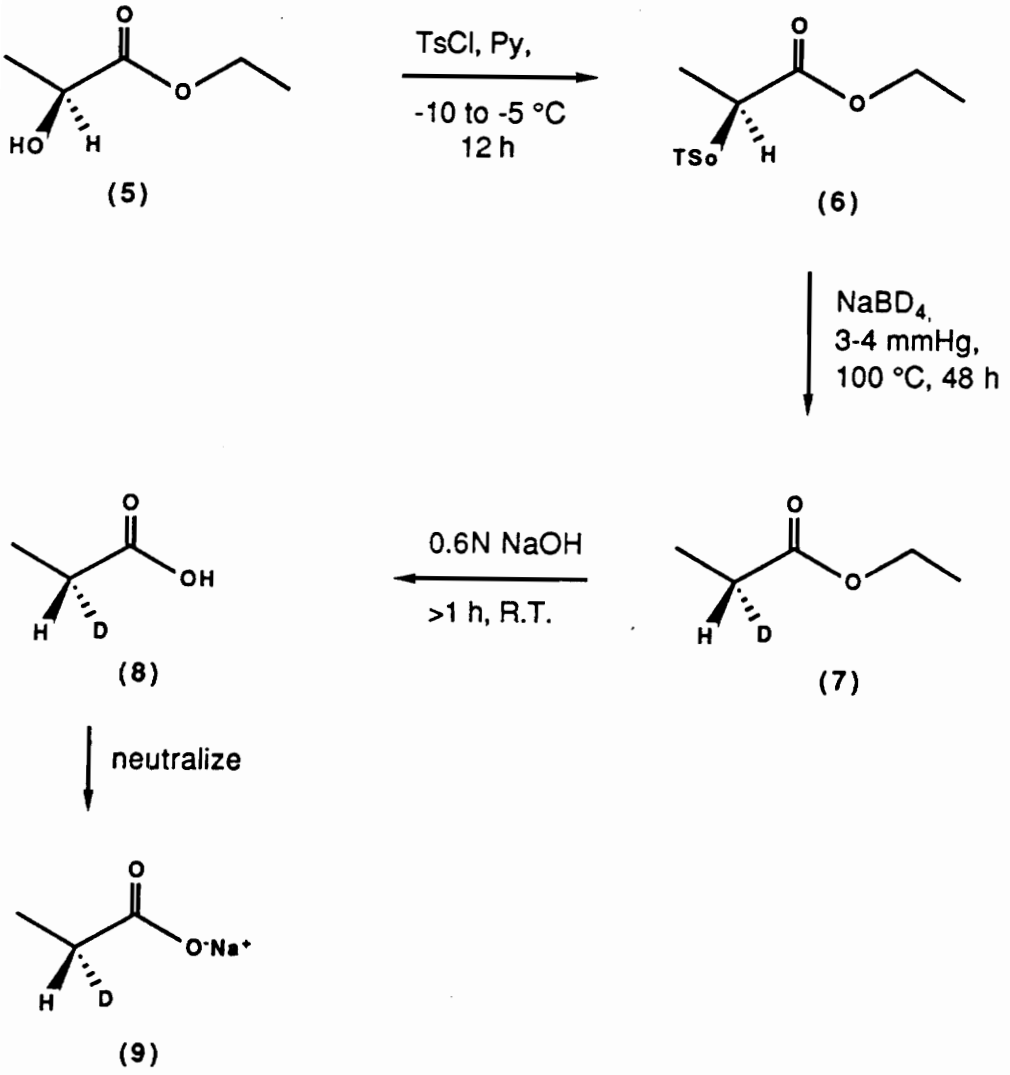


Figure 12. Synthesis of (R)-[2-$^{2}H_{1}$] Propionate.

Tosyl chloride was reacted with (S)-(-)-Ethyl lactate to convert the hydroxyl group of the ethyl lactate into a good leaving group. It was found that instead of reacting the mixture for twelve hours at 0°C as specified, the yield could be significantly increased by stirring the mixture on a stir plate in a freezer at approximately -10°C overnight.

Once the tosylate **6** was made, it was reduced with $NaBD_4$ to give the stereospecific deuterium labelling. $NaBD_4$ was stirred with the tosylate without the use of solvent to avoid the possibility of isotope exchange. The volatile products were removed by vacuum distillation and collected in a trap. Two major products, ethyl lactate and triethyl borate, were trapped in approximately a 2:1 ratio as determined by by GC analysis. Ethyl propionate **7**, the desired product, arises from the S_N2 attack of a deuteride ion on the α-carbon, with displacement of the tosylate as a toluene sulfonate ion. Since the attack is S_N2, there is inversion of configuration at the α-carbon, and the resulting etnyl propionate thus has the S configuration. The minor component trapped, triethyl borate, results from the reduction of the ester. The accompanying products of the reduction remain in the reaction flask and are not collected in the trap. This accounts for the typically low yield of this reaction, approximately 30% of the desired product.

At this point of the synthesis, Brandange et al. separated the compounds by spinning band distillation and then hydrolyzed the separated ethyl propionate to propionic acid.[30] It was found that the separation of triethyl borate and hydrolysis of ethyl propionate could be effectively accomplished in a simpler manner. The 2:1 mixture of

ethyl propionate and triethyl borate was hydrolyzed with two equivalents of sodium hydroxide, followed by neutralization with dilute hydrochloric acid. This converted the ethyl propionate to propionic acid and ethanol and the triethyl borate to boric acid and ethanol. The propionic acid could then be extracted into an organic solvent while the boric acid remained in the aqueous layer. The propionic acid was then neutralized to form sodium (R)-[2-$^{2}H_{1}$] propionate **9**, which had a specific rotation of -0.87°, in agreement with the literature value of -0.88°.[30]

Sodium (S)-[2-$^{2}H_{1}$] propionate was synthesized in exactly the same manner as previously discussed (Figure 13). The only modification is that (R)-(+)-methyl lactate **10** was used instead of the corresponding ethyl ester, since only the methyl ester was commercially available.

It was found to be necessary to dry the tosylate thoroughly before carrying out the reduction step to avoid hydrolysis to methyl lactate. When this was done, the yields and results were comparable to those described above.

Doubly deuterated propionic acid was purchased and converted to its sodium salt before use.

The next step was the development of a suitable analytical method for the deuterium labeled biosynthetic products. There are two methods of analysis of deuterium commonly used.[1] The first is nuclear magnetic resonance. Usually, deuterium is detected by the absence of signals in proton NMR. The development of deuterium NMR, however, now enables the direct observation of deuterium resonances.[32] The second most common method is mass spectrometry. Mass spectrometry offers a quick analysis by observation of the M+1 peak, after correction for the intensity of

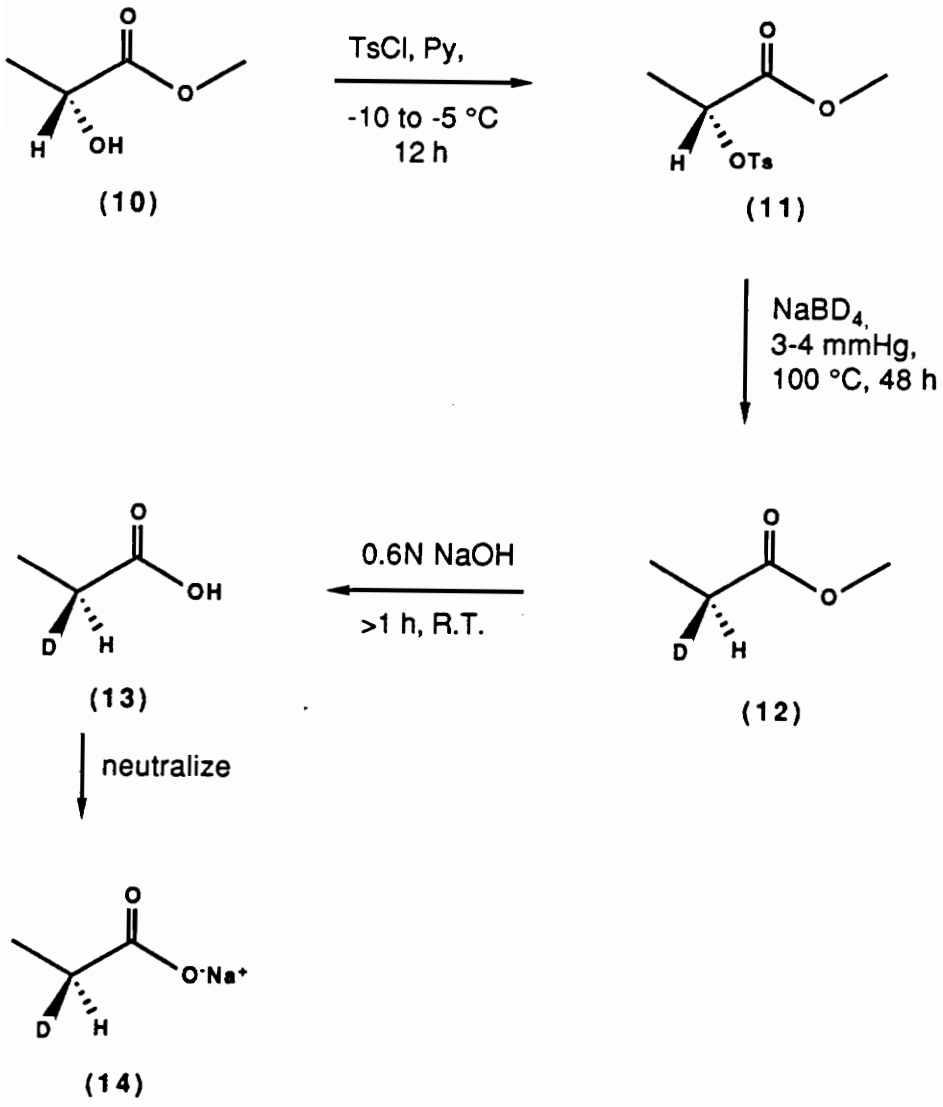


Figure 13. Synthesis of (S)-[2-$^{2}H_{1}$] Propionate.

this peak caused by the natural abundance of ^{13}C.

Mass spectrometry was the method of choice for our analysis, since it was anticipated that the isolated amounts of antibiotic would be inadequate for the NMR method. In addition, the NMR method lacks a convenient internal standard for determination of the extent of label incorporation. Usually for large natural products with relatively high molecular weights and low volatility fast atom bombardment (FAB) mass spectrometry is used. FAB, developed in 1981, is a technique in which samples, usually in a glycerol matrix, are ionized in the solid state by bombardment of the matrix with argon or xenon atoms with energies ranging from 5000 to 10,000 electron volts. Positive or negative ions are then desorbed from the surface where they can be detected.[33-36] A drawback of FAB is that the M+1 peaks can have variable intensities. This is because the ionization mechanism gives rise to molecular ions which contain $M+H^+$ and $M+Na^+$ ions, and the intensities of these ions depends on the exact ionization conditions.[33]

Because FAB has an unreliable M+1 peak, another mass spectrometry technique must be explored for the analysis. Other mass spectrometry methods such as chemical ionization and electron impact require molecules which are volatile and have a lower molecular weight. As a result, a degradation scheme for ravidomycin was developed which would produce fragments suitable for electron impact or chemical ionization mass spectrometry and that would also contain the vinyl side chain.

Two methods were attempted for the degradation of ravidomycin. The first method (Figure 14) involved reductive cleavage of the lactone moiety with sodium borohydride to give tetrahydroravidomycin **16.** This

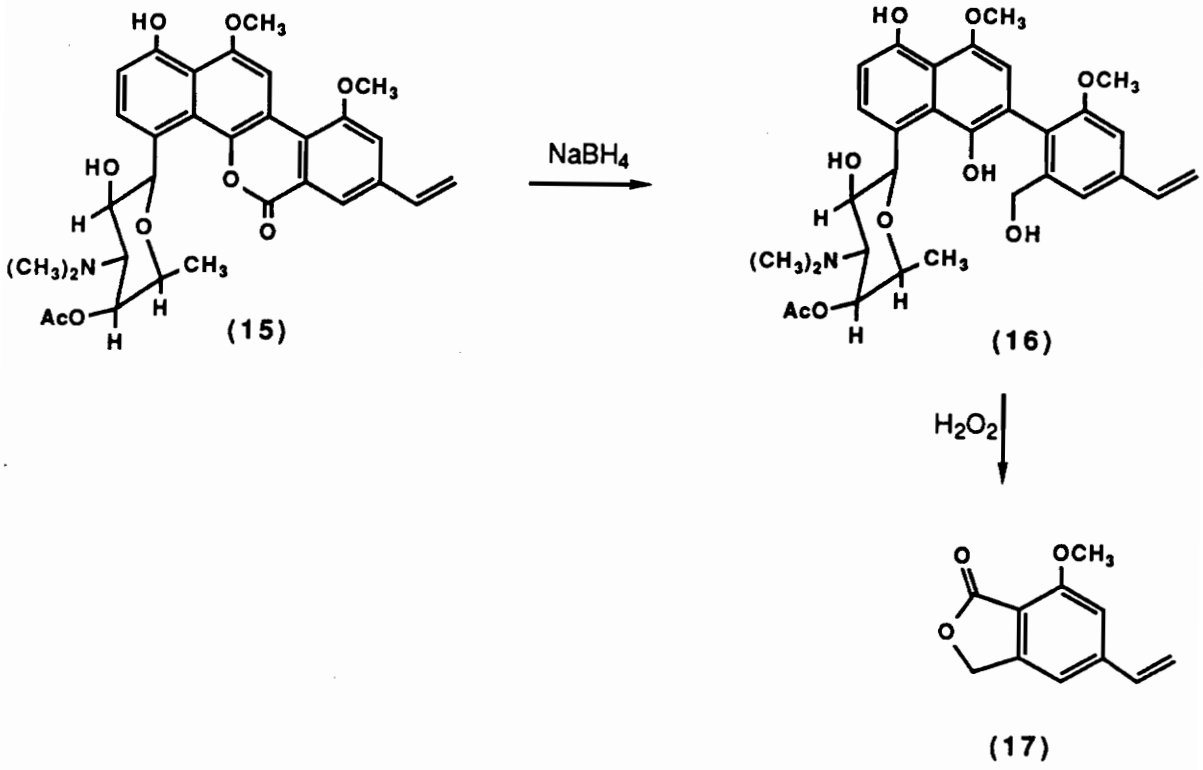


Figure 14. Degradation of Ravidomycin, Scheme 1.

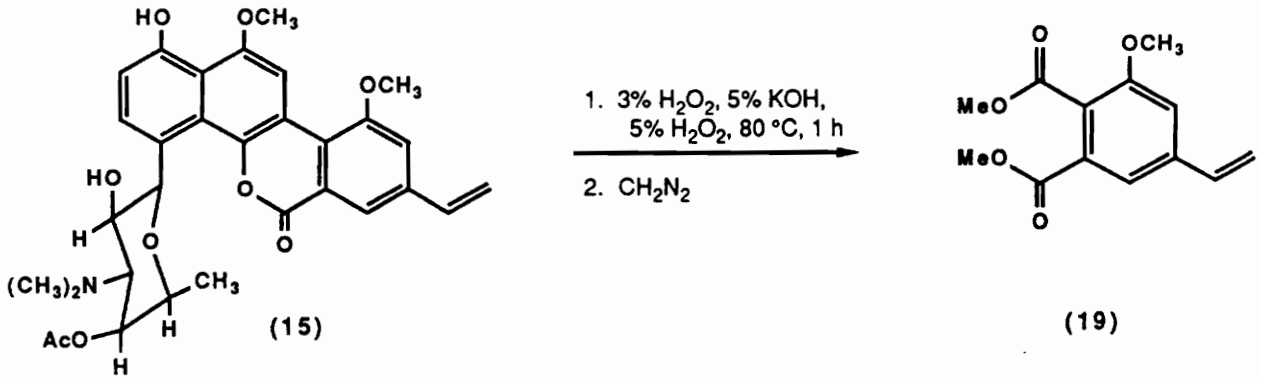


Figure 15. Degradation of Ravidomycin, Scheme 2.

method corresponds to that used by Horii et.al which was used to determine the structure of toromycin.[13] Once the lactone was successfully opened, alkaline hydrogen peroxide would yield a substituted phthalide **17**. Unfortunately, this method did not work for our compound. It was therefore decided to oxidize ravidomycin directly. Once again Horii and coworkers pioneered this method.[13] Ravidomycin was subjected to exhaustive alkaline oxidation using potassium hydroxide and hydrogen peroxide (Figure 15). The yield of this reaction was very low. It did, however, produce the desired product, 3-methoxy-5-vinyl-1,2-benzenedicarboxylic acid **18**. Since the reaction mixture was not purified and mass spectrometry was to be used for analysis, it was deemed necessary to use gas chromatography as the inlet to the mass spectrometer. As a consequence of using this technique, the diacid had to be made more volatile so it would pass through the GC. The diacid was, therefore, methylated with diazomethane prepared according to the method of de Boer[37] to give **19**. The diester **19** was then submitted for GC-MS analysis. The mass spectrum of the product had a base peak at m/z 219 and a molecular ion peak at m/z 250 (Figure 16); the mass spectrum was consistent with the structure.

Once the synthesis of labeled materials and the method of analysis established, perhaps the most difficult portion of a biosynthetic study remains, the biology. Conditions must be chosen that not only facilitate the growth of the producing organism, but the secondary metabolite in question must also be produced in sufficient quantity as to obtain the desired results. Many microorganisms are sensitive to growth conditions. Some are sensitive to amount of air, temperature,

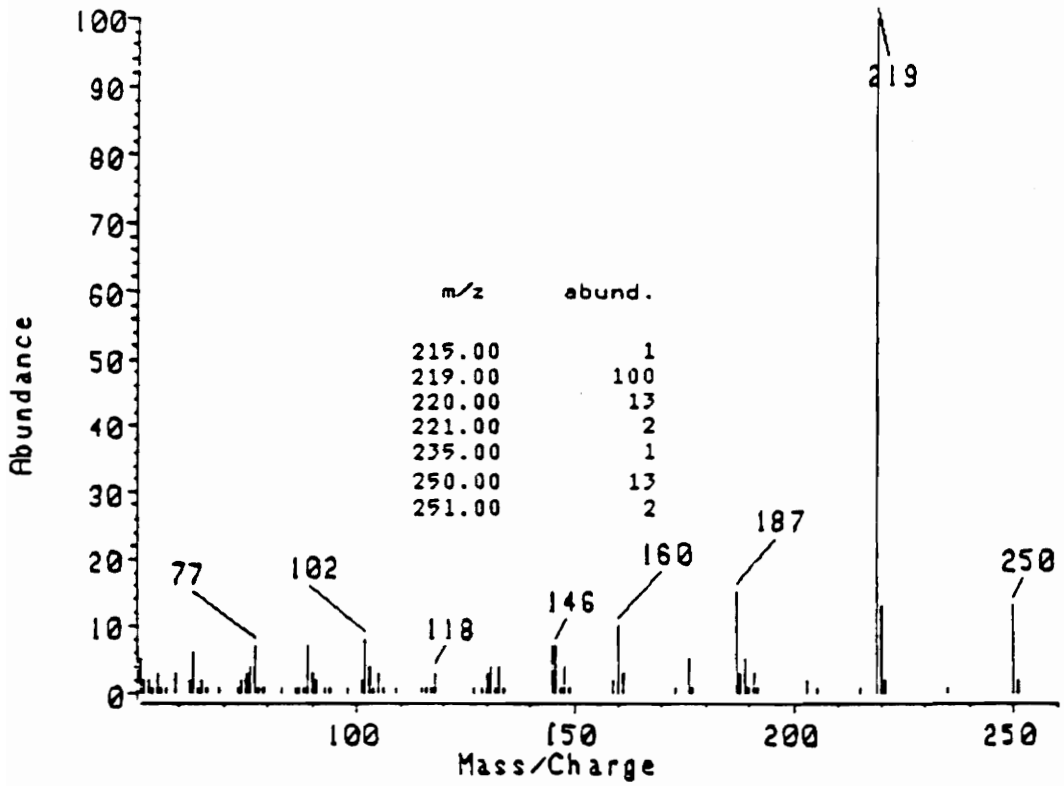


Figure 16. GC-MS of Ravidomycin.

and type of nutrients. If any of these are varied in any manner, then the amount of antibiotic produced may decrease or be entirely eliminated. Furthermore, the organism may not grow as hardily and ultimately result in diminished returns. When conditions are varied away from favorable, the microorganisms use alternate pathways in their metabolism. Therefore, an organism which normally gives high production may, under stressful conditions, switch to an alternate pathway in order to meet its demands. This alternate pathway may not produce the desired product or it may produce it by another route which does not utilize a particular precursor. This could then lead to false observations rendering invalid conclusions.

Fortunately, the producing organism used for these experiments, Streptomyces grieso-olivaceus, is a relatively hardy organism. It does not appear to be too sensitive to growth medium or conditions except temperature and amount of air the growing cultures receive.

Streptomyces grieso-olivaceus was grown in a two stage inoculum and a one stage fermentation.[24] It was necessary to get a good virulent growth of cells before they were inoculated into the fermentation medium. Although it was not specified, all the cultures were grown in baffled flasks, since these baffled flasks have been shown to increase the yield of antibiotics produced.[38] It is thought that the increased yields are due to the additional aeration caused by the baffles as they cause the liquid medium to roll and foam. No attempts were made to grow the culture in nonbaffled flasks, but production of ravidomycin in baffled flasks was generally high, on the order of 100-200mg per liter depending on the virility of the slant used. It was found that the

growth of the organism and the production of the antibiotic could be enhanced by growing the organism an extra day in each of the inoculum stages.

As mentioned above, Streptomyces grieso-olivaceus appeared to be slightly sensitive to temperature and aerobic conditions. The normal growth temperature used was 28°C. At times the temperature became difficult to regulate, sometimes fluctuating as many as 5°C in each extreme. It became evident that the organism grows best at 28°C±3°C. At temperatures below this, the rate of growth was depressed. As soon as the temperature was raised to its optimum, growth was stimulated. At temperatures above 28°C growth was poor, suggesting that the cells are somewhat heat labile and inhibited by the heat. The cultures were also sensitive to the air they received. Streptomyces grieso-olivaceus is an aerobic organism and therefore, to ensure virulent growth, they must be exposed to air. Due to the gyration of the shaker box, the cotton plugs which cover the baffled flasks got wet. When this happened, air could no longer reach the cells. As a result, the growth of the cells was stunted, but as soon as the plugs dried the cultures began growing as evident of the growth medium taking on a green color. The green color was typical of all growing cultures.

Once production of ravidomycin and growth of Streptomyces grieso-olivaceus was established (R)-[2-$^{2}H_{1}$] sodium propionate, (S)-[2-$^{2}H_{1}$] sodium propionate and sodium[2,2-$^{2}H_{2}$] propionate could be fed to the organism. Before labeled precursors can be added, certain criteria must be kept in mind. First, the labeled precursor must be administered early enough for the microorganism to metabolize it to form the

antibiotic. Second, the precursor must be added late enough that it is not incorporated into a primary metabolic pathway.

When doing feeding experiments several common problems may arise which will obscure the results. If a precursor used by a microorganism is incorporated at a low level or not at all may be explained either by difficulty in getting the precursor to the metabolic site or it may be more readily used in the production of a primary metabolite or another secondary metabolite. Finally, the precursor may be at a shunt and may or may not be incorporated.[1]

A time course study was undertaken in order to supplement previous knowledge about the production of ravidomycin. Two attempts were made. In the first attempt, [2,2-$^{2}H_{2}$] propionic acid was fed at 1g per liter of fermentation broth at time 20h, 28h, 36h, and 44h into the fermentation stage. The organism did not grow well and very small quantities of ravidomycin were isolated. It was theorized that, although the fermentation medium was buffered, the sudden addition of propionic acid killed a substantial number of cells, thereby inhibiting growth as well as production. A second attempt was thus made. At time 20h, 28h, and 36h, 1g of [2,2-$^{2}H_{2}$] sodium propionate was fed per liter of fermentation broth. By the end of the fermentation stage, ravidomycin was isolated in substantially larger quantities; in one instance there was almost a 17 fold increase. Therefore, growth and production can be enhanced by neutralizing the precursors and feeding them as a salt.

Degradation of the isolated ravidomycin was done as previously described. It was found that the ravidomycin had to be purified

Table 6. Production curve data

Time	m/z	%base	corrected labelled
20hr	219	100	4.1
	220	18.4	
	221	2.4	
	250	24.9	
	251	4.9	
	252	0.6	
28hr	219	100	8.1
	220	22.4	
	221	3.1	
	250	22.5	
	251	5.1	
	252	0.8	
36h	214	100	6.8
	220	21.1	
	221	3.1	
	250	23.3	
	251	5.1	
	252	0.8	

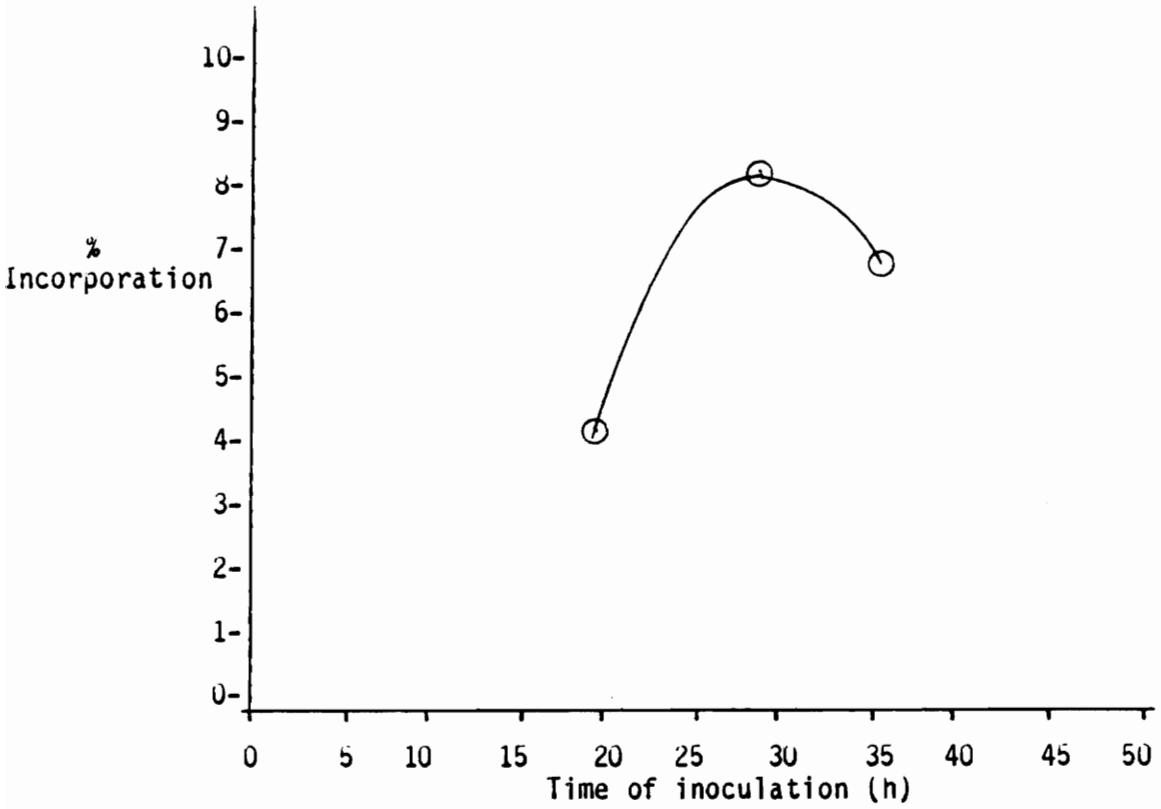


Figure 17. % Incorporation vs. time of inoculation of propionic-2,2-d_2-acid

carefully to avoid complications from contamination by co-produced lipids; this was best done by means of flash chromatography. GC-MS analysis of the degradation products showed that there was 4.1% incorporation at 20h, 8.1% at 28h, and 6.8% at 36h, as determined by MS analysis of the product as described earlier. (Table 6 and Figure 17). From the results of this feeding study it was evident that maxiumum incorporation occurred when the addition of precursors was at 28h post fermentation inoculation (Table 6). This coincides with the inoculation time used by Carter et al.[24]

Having established that 28h is the optimum time to add the precursors enabled the addition of (R)-[2-$^{2}H_{1}$] and (S)-[2-$^{2}H_{1}$] propionate to be made with the achievement of optimum incorporation. When (R)-[2-$^{2}H_{1}$] propionate was fed to ravidomycin, it was shown, after degradation, that this precursor had a 0% incorporation (Figure 18). When (S)-[2-$^{2}H_{1}$] propionate was fed, it showed a deuterium incorporation of 1.0% (Figure 19).

From these results it appears as if (S)-[2-$^{2}H_{1}$] propionate is being incorporated, although the amount of incorporation is rather low. Theoretically, if (S)-[2-$^{2}H_{1}$] propionate is indeed being incorporated, the percent incorporation should match that of the [2,2-$^{1}H_{2}$] propionate feeding, but this was not the case. It was reasoned that since the slants were getting old maybe the optimum time for feeding was not 28 hours but possibly earlier or later. It was decided to repeat the experiment feeding each fermentation flask at 20 hours, 28 hours, and 36 hours. This whould ensure that (S)-[2-$^{2}H_{1}$] propionate would have a maximum incorporation. It was hoped that the percent incorporation

would approach that of the [2,2-$^{1}H_{2}$] propionate feeding, but the percent incorporation was again 1.1%.

Table 7 summarizes the results of the feeding experiments. From these results, it is evident that (S)-[2-$^{2}H_{1}$] propionate is incorporated. Although the percent incorporation is lower than expected, the consistency between the two experiments supports the theory that (S)-[2-$^{2}H_{1}$] propionate is incorporated. This, therefore, suggests that there is some stereoselectivity in the formation of the propionate derived side chain namely the pro-S proton is being retained and the pro-R proton is being lost during elimination to form the vinyl side chain of ravidomycin.

The dehydrogenation of the side chain to form the vinyl group occurs late in the biosynthetic pathway according to Carter and coworkers.[24] Two possible mechanisms can be postulated, first, the benzylic position of the side chain could become hydroxylated, then lose water to form the vinyl side chain. This mechanism would be analogous to the conversion of hydroxylated precursors to dehydroamino acids in the antibiotics berninamycin and virginiamycin.[34,40]

The second possible mechanism involves the use of a complex enzyme system. This enzyme system has been shown by Strittmatter and colleagues to be composed of a hemoprotein cytochrome-b_5 component, a flavoprotein dehydrogenase (NADH-cytochrom-b_5 reductase) and coenzyme A desatorase.[41,42] This enzyme system has been extensively studied for the dehydrogenation of stearic acid to oleic acid. Dehydrogenation of the propionate derived side chain to the vinyl group may follow a very similar enzymatic pathway, which, when employed, will give rise to ravidomycin.

Mass	Abs.Ht	% Base	No.Smpls
203	419000	1.3	74
205	196000	0.6	62
207	371000	1.2	71
219	31180000	100.0	255 F
220	4153000	13.3	106 F
221	565000	1.8	88 F
235	249000	0.8	64
250	6686000	21.4	143 F
251	1005000	3.2	81 F
252	129000	0.4	57

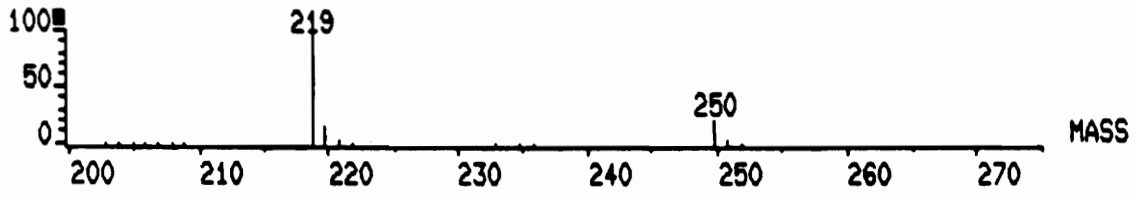


Figure 18. MS from (R)-[2-$^{2}H_{1}$] Propionate Feeding.

Mass	Abs.Ht	% Base	% Mass	No.Smpls
213	86000	0.6	2.2	6
218	167000	1.1	4.4	2
219	15154000	100.0	396.0	7
220	2146000	14.2	56.1	7
221	668000	4.4	17.5	6
227	631000	4.2	16.5	6
228	112000	0.7	2.9	3
235	93000	0.6	2.4	7
239	192000	1.3	5.0	4
241	127000	0.8	3.3	3
250	3827000	25.3	100.0	7
251	558000	3.7	14.6	5

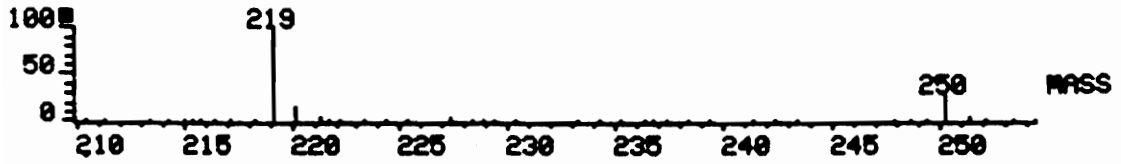


Figure 19. MS from (S)-[2-$^{2}H_{1}$] Propionate Feeding.

Table 7. Results of the feeding experiments.

expt. no.	precursor	Intensity of ions in range m/z 219-221[a] Uncorrected[b] 219	220	221	Corrected[b,c] 219	220	221	% labeled antibiotic
1	CH_3CH_2COONa	100	13.2	1.8	100	0	0	0
2	CH_3CD_2COONa	100	22.4	3.1	100	9.2	0	8.4
3	(R)-$CH_3CHDCOONa$	100	13.2	1.9	100	0	0.1	0
4	(R)-$CH_3CHDCOONa$	100	13.3	1.8	100	0.1	0	0.1
5	(S)-$CH_3CHDCOONa$	100	14.3	1.9	100	1.1	0	1.1
6	(S)-$CH_3CHDCOONa$	100	14.2	1.9	100	1.0	0	1.0

[a] Error of measurement is ± 0.1
[b] Intensities are the average of at least five scans.
[c] Ion intensities corrected for signal resulting from ions containing natural abundance ^{13}C and ^{18}O.

Summary

Ravidomycin is a polycyclic aromatic hydrocarbon with a C-glycosyl group. It has been shown to be active against Gram positive bacteria including mycobacteria. It is weakly active against Gram negative organisms and has no activity against fungi. It has been shown to possess strong antitumor activity against P388 lymphocytic leukemia, Colon 38 tumor, and CD8F1 mammary tumor. The mechanism of action of ravidomycin is by cleaving nucleic acids, but without the presence of the vinyl side chain the activity is greatly reduced. It is known that the vinyl side chain originates from propionic acid and is assembled during polyketide synthesis. It is therefore interesting to determine the stereochemical selectivity in the formation of the vinyl side chain.

(R)-[2-$^{2}H_{1}$] propionate and (S)-[2-$^{2}H_{1}$] propionate were fed to a growing culture of Streptomyces grieso-olivaceus. The antibiotic was isolated, purified, and degraded to a smaller unit which contained the vinyl side chain.

The analysis for deuterium incorporation was carried out by GC-MS. It was found that (S)-[2-$^{2}H_{1}$] propionate was incorporated in 1.0%. This suggests that the pro-S proton is retained during the biosynthesis of the vinyl group.

This opens up several other interesting biosynthetic studies for the future. Especially attractive, is the synthesis of and incorporation studies of (R) and (S)-[3-$^{3}H_{1}$, 3-$^{2}H_{1}$] propionic acid to further understand the formation of the vinyl side chain.

V. EXPERIMENTAL

General Procedures

Melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. Proton nuclear magnetic resonance (NMR) spectra were recorded at 270 MHz on a Bruker WP-270SY spectrometer. Infrared spectra were recorded on a Perkin-Elmer 710B spectrophotometer. GC-MS analysis was done on a Hewlett-Packard 5790 with a HP5 column (25m length, 0-32mm ID) with temperature programming from 75°C-200°C at 10°C/min and interfaced with VG Analytical 7070E-HF mass spectrometer operating at 70eV and a 200°C source temperature. All rotations were measured on a Perkin-Elmer 241 polarimeter. Growth of Streptomyces grieso-olivaceus was done in a Lab Line Orbit Environ-Shaker. All pH measurements were done on an Orion-Research model 701 digital pH meter. Thin layer chromatography was performed on E. Merck aluminum supported silica gel 60 (0.2mm, F_{254}) plates. Silica gel for flash chromatography was E. Merck 230-400 mesh. All solvents were distilled prior to use. Streptomyces grieso-olivaceus was obtained as a freeze dried pellet of culture C23201-NS7 from Lederle Laboratories of the American Cyanamid Company.

Synthesis of Labeled Propionic Acids

Ethyl-[S]-2-(p-toluenesulfonoxy)propionate 6.

To a stirred solution of 25ml(27.25g, 0.220mol) of (S)-(-)-Ethyl lactate and 13.2ml (13.86g, 0.330mol) of pyridine cooled to 0°C in an

ice bath was added 42g (0.220mol) of p-toluene sulfonyl chloride portion-wise. The mixture was then stirred for 12h in the freezer (-5°C to -10°C). The reaction was followed by TLC (50% ether, 50% hexanes). At the end of 12h, 100ml of water was added. The mixture was extracted with 3x100ml ether. The extracts were then washed with 3x50ml of 10% HCl, 3x50ml of water, and 1x150ml of brine. The extracts were then dried over sodium sulfate and evaporated in vacuo to yield a colorless oil. The oil was purified by recrystallization from an ether/petroleum ether mixture by slow cooling to -78°C to give **6** (40.42g, 70.7%) as a white crystalline solid. m.p. 32.5-33°C ^{1}H-NMR ($CDCl_3$ 270 MHz) δ1.21(t, 3H), 1.52(d, 3H), 2.43(s, 3H), 4.11(q, 2H), 4.92(q, 1H), 7.35(d, 2H), 7.81(d, 2H).

R[2-$^{2}H_1$] ethyl propionate 7.

A stirred mixture of **6** (40.42g, 0.158mol) and $NaBD_4$ (3.3g, 0.079mol) was heated at 100°C for 48h under reduced pressure, 2-3mmHg. The volatile products were continously collected in a trap cooled by a dry ice/acetone mixture. The condensate contained about 6ml and GC analysis showed two major peaks in about a 2:1 ratio corresponding to **7** and triethyl borate. Peak identity was confirmed by comparison with authentic samples.

R[2-$^{2}H_1$] propionate 9.

Compound **9** was prepared by hydrolysis of **7** and $B(OC_2H_5)_3$ with 8 mol equivalents of sodium hydroxide (0.6M in H_2O,E+OH, 1:1) at room temperature for >1h. The reaction mixture was washed with ether until

the organics were removed. The aqueous layer was acidified with dilute HCl and the deuteriopropionic acid was then extracted with 3x200ml of ether. The extracts were washed with 2x50ml of water and 1x50ml brine and dried over $MgSO_4$. The solvent was removed in vacuo to give deuteriopropionic acid. The acid was neutralized to pH8 with 0.6M NaOH and the residual water was removed by lyophilization to give 1.30g of **9**. $[\alpha]_D^{28}$=-0.87°, literature $[\alpha]_D^{28}$=-0.88°; NMR(D_2O, dioxane as the internal standard, 270MHz) δ 1.01 (dt, 3H, J=0.9 and 7.7 Hz), 2.12(qt, 1H, J=2.3 and 7.7 Hz).

Methyl-[R]-2-p-toluenesulphonoxypropionate 11.

To a stirred solution of 23.85ml (26g, 0.25mol) of (R)-(+)-Methyl lactate and 30.33ml(29.7g, 0.375mol) of pyridine cooled to 0°C in an ice bath was added 47.66g(0.250mols) of p-toluenesulfonyl chloride portion-wise. The mixture was then stirred in the freezer for 12h (-5°C to -10°C). The reaction was followed by TLC (50% ether, 50% hexane). At the end of the 12h, 100ml of water was added. The mixture was extracted with 3x100ml of ether. The extracts were washed with 3x50ml of 10% HCl then 3x50ml of water and finally, 1x50ml of brine. The extracts were dried over sodium sulfate and evaporated in vacuo to yield a colorless oil. The oil was purified by recrystallization from an ether/petroleum ether mixture by slow cooling to -78°C to give **11** (43.43g, 76.4%) as a white crystalline solid. $[\alpha]_D^{28}$=+36.99°, NMR($CDCl_3$, 270MHz) δ 1.49(d, 3H, J=6.9 Hz), 2.44(s, 3H), 3.78(s, 3H), 4.94(q, 1H, J=6.9 Hz), 7.32(d, 2H, J-8.0 Hz), 7.80(d, 2H, J=8.3 Hz); IR (neat) 3040, 3000, 1770, 1370 cm^{-1}.

S[2-$^2H_1$] methyl propionate 12.

A stirred mixture of **11** (43.14g, 0.167mol) and $NaBD_4$ (3.50g, 0.084mol) was heated at 100°C for 48hr under reduced pressure, approximately 2-3mmHg. The volatile products were continously collected in a trap cooled by a dry ice/acetone mixture. The condenstate contained of a mixture of **11** and $B(OCH_3)_3$. NMR($CDCl_3$, 270 MHz) δ 1.14(dt, 3H, J=1.0 and 7.5 Hz), 2.32 (qt, 1H, J=2.5 and 7.5 Hz), 3.62(S), 3.71(S).

S[2-$^2H_1$] propionate 14 was prepared by hydrolysis of **12** and $B(OCH_3)_3$ with 8 mol equivalents of sodium hydroxide (0.6M in H_2O/MeOH, 1:1) at room temperature for >1hr. The reaction mixture was washed with ether until the organics were removed. The aqueous layer was acidified with dilute HCl. The deuteriopropionic acid was extracted with 3x100ml of ether. The extracts were washed with 2x50ml of water and 1x50ml of brine then dried over sodium sulfate. The solvent was removed in vacuo to give deuteriopropionic acid. The acid was neutralized to pH 8 with 0.6 N NaOH and the residual water was removed by lyophilization to give 1.90g of **14** $[\alpha]_D^{28}$=+0.81°; ^{1}H-NMR(D_2O, dioxane as the internal standard, 270MHz) δ 1.01(dt, 3H, J=0.9 and 7.7 Hz), 2.12(qt, 1H, J=2.3 and 7.7 Hz).

Degradation of ravidomycin

3-methoxy-5-vinyl-1,2-benzenedicarboxylic acid 18.

0.44ml of 3% H_2O_2 was added to a solution of ravidomycin in 1.76ml

of 5% KOH, and the solution stirred at 80°C for 1h. Three additional 0.44ml portions of 5% H_2O_2 were added at intervals of 1h at the same temperature. The resulting reaction mixture was cooled to room temperature and made slightly acidic with dilute HCl. The mixture was extracted with 3x15ml portions of EtOAc, the extracts washed with 2x15ml of water and 1x15ml of brine, dried over sodium sulfate and evaporated in vacuo to give a yellow solid containing **18** amoung other products.

Dimethyl 3-methoxy-5-vinyl-1,2-benzenedicarboxylate 19.

Crude 3-methoxy-5-vinyl-1,2-benzenedicarboxylic acid **18** was dissolved in 95% E+OH and diazomethane. CH_2N_2 was added until the yellow color persisted. The excess CH_2N_2 was destroyed by adding dilute HOAc. The resulting mixture was evaporated in vacuo. The crude material was subjected to GC-MS analysis. Four major peaks of retention times 6.85min, 7.97min, 9.12min, and 11.78min were observed. The peak eluted at 7.97min was **19**. m/z: 250(M+, 13), 219(100), 187(15), 160(10), 146(7), 118(3), 102(8), 77(7). MS results for the labeled compounds can be found in Table 9.

Culture conditions

Streptomyces grieso-olivaceus was maintained on agar slants, the content of which is outlined in Table 8. Newly inoculated slants were incubated at 28°C for 10-14 days and then stored at 4°C until needed.

For experimental work the microorganism was grown in broth cultures in three stages. The contents for the first inoculum state are listed in Table 9. This stage was prepared by transferring the mycelium from

Table 8. Contents of agar slant medium.

Yeast Extract	0.5%
Beef Extract	0.3%
Tryptose	0.5%
Dextrin	2.4%
Dextrose	0.5%
$CaCO_3$	0.4%
Agar	2.0%

Table 9. Contents of 1st and 2nd stage inoculum medium

Yeast Extract	0.5%
Beef Extract	0.3%
Tryptose	0.5%
Dextrin	2.4%
Dextrose	0.5%
$CaCO_3$	0.4%

an agar slant to two 250ml baffled flasks each containing 50ml of medium. The broth culture was incubated at 28°C on a rotary shaker at 200rpm for 4 days.

After the 4 day period the second stage inoculum was prepared by aseptically transferring 5% of the contents of the first stage inoculum to each of four 250ml baffled flasks containing 50ml of the same medium as above (Table 9). The cultures were grown for 3 days in a rotary shaker at 28°C and 200 rpm.

The second stage inoculum was aseptically transfered to the fermentation stage. 10ml of the second stage inoculum was inoculated into four 1000ml baffled flasks containing the fermentation medium, the contents of which are listed in Table 10. The cultures were grown for 6 days in a rotary shaker set at 200rpm and 28°C.

Isolation and Purification of Ravidomycin

Ethyl acetate (300ml) was added to each 1000ml baffled flask containing the culture. The contents were stirred overnight and then were filtered with the aid of Hyflo Super Cel to remove the mycelial cake. The aqueous and organic layers of the filtrate were separated and the aqueous layer was extracted with 2x200ml of EtOAc. The extracts were combined and washed with 2x200ml of water and 1x200ml of brine. The extracts were then dried over sodium sulfate and evaporated in vacuo to yield a dark residue containing ravidomycin.

Ravidomycin was purified by flash chromatography, eluting with 60% acetone 40% hexanes. The purity of the fractions were checked by thin layer chromatography using the above solvent system. The homogenous

Table 10. Contents of the fermentation medium.

Dextrose	1.5%
Glycerol	1.5%
Soybean Flour	1.5%
$CaCO_3$	0.1%
NaCl	0.3%

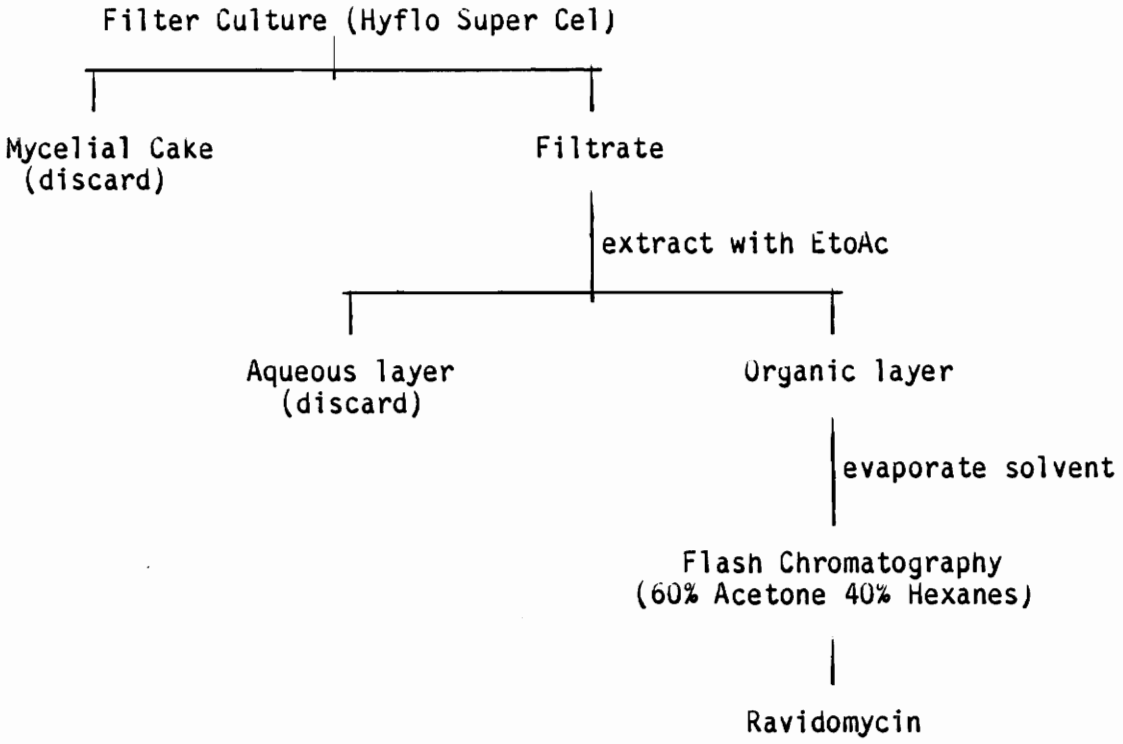


Figure 20. Isolation of ravidomycin from the fermentation mycelium.

fractions containing ravidomycin R_f=0.23, were collected and evaporated in vacuo to leave a bright yellow compound which was highly fluorescent under both short wave and long wave ultra violet light. Figure 20 depicts the isolation and purification scheme for ravidomycin.

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Vita

Robert Frederick Keyes was born on February 8, 1960 in Owosso, Michigan. In the summer of 1972, he moved with his parents and sister to Paris, Tennessee where he graduated from Henry County High School. Upon graduation he attended the United States Military Academy and later transferred to East Tennessee State University where he was awarded a Bachelor of Science degree in chemistry and microbiology.

In the fall of 1984 he enrolled at Virginia Polytechnic Institute and State University. During his tenure there he held a teaching assistantship in organic chemistry and also a research assistantship. In July 1988 he completed the requirements for the Master of Science degree.

Future plans include staying on at VPI & SU with Dr. David G. I. Kingston for further graduate studies.

Robert Frederick Keyes

Robert Frederick Keyes

THE BIOSYNTHESIS OF RAVIDOMYCIN

by

Robert Frederick Keyes

Committee Chairman: David G. I. Kingston
Chemistry

(ABSTRACT)

Ravidomycin is a yellow antitumor antibiotic produced by Streptomyces ravidus. Ravidomycin shows strong antitumor activity against P388 lymphocytic leukemia, the colon 38 tumor, and the CD8F1 mammary tumor. It is also very active against Gram positive bacteria. Biosynthetic studies have shown that the aglycone unit comes from the folding of a polyketide chain with the vinyl unit arising from propionic acid. Since this vinyl functionality is believed to play a role in the antitumor activity of the antibiotic, it is of interest to elucidate the stereochemical selectivity in its formation from propionic acid. The synthesis of (R) and (S)-[2-$^{2}H_{1}$] propionate, incorporation of the labelled material, and chemical analysis of the resulting antibiotic was be used to determine the stereochemistry of formation of the vinyl side chain. It was found that propionate was incorporated with ravidomycin with stereospecific loss of the 2-(pro-R)-proton.