BIOSYNTHESIS OF RAPAMYCIN

BY STREPTOMYCES HYGROSCOPICUS

by

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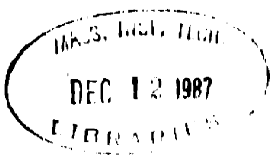

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ABSTRACT

Conditions for the small-scale production and purification of rapamycin were developed. Based on the literature, a biosynthetic scheme was proposed for rapamycin, and the corresponding labelled precursors were tested.

1- and 2-^{13}C-acetate enriched four acetate units as predicted (showing approximately 100% enrichment). Each labelled two carbons in the triene region, but not in the order predicted, suggesting that either the acetate units had been cleaved or an error was present in the published ^{13}C-NMR spectral assignments. Incorporation of 1,2-$^{13}C_2$-acetate indicated the presence of six acetate units, in agreement with the proposed scheme, thus proving that there were errors in the published assignments. Carbons 10 and 11 were not labelled significantly by any form of acetate, revealing that they were not derived from acetate as had been initially predicted.

1-^{13}C-Propionate administered in two doses provided very significant enrichment of six of the seven predicted propionate units. The peak assigned to the carbon corresponding to the carboxyl group of the seventh propionate (C32) was not enriched, but a peak assigned to a similar methylene group (C39) adjacent to the cyclohexane ring showed a small amount of enrichment. The observed incorporation pattern could possibly be explained by scrambling, but there was also the possibility that the enriched peak should be reassigned to correspond to the seventh propionate unit. 2-^{13}C-Propionate enriched seven carbons corresponding to the seven propionate groups predicted initially and none of the other carbons of rapamycin. Therefore it was concluded that there was an error in the published ^{13}C-NMR assignments, and that the peak assigned to C39 should be reassigned to C32. The incorporation patterns of 1- and 2-^{13}C-propionate indirectly eliminated certain possibilities for the origin of carbons 10 and 11.

^{14}C-(Methyl)-methionine showed a high degree of incorporation into rapamycin. In cultures receiving 0.3 g/l L-^{13}C-(methyl)-methionine, only the three methoxy carbons of rapamycin were enriched (15 to 20 fold), as was predicted by the proposed scheme for biosynthesis.

The addition of L-lysine and D,L-pipecolate to the medium did not affect the specific production of rapamycin. In a new medium containing reduced amounts of L-lysine, L-^{14}C-lysine and D,L-^{3}H-pipecolate were incorporated to a high degree, D,L-^{3}H-pipecolate being incorporated at a somewhat higher efficiency. Unlabelled pipecolate reduced the incorporation of radioactive lysine much more than unlabelled lysine could decrease the incorporation of radioactive pipecolate. These results imply that pipecolate is the more direct precursor of rapamycin, lysine being converted to pipecolate and free pipecolate being incorporated directly into rapamycin. Rapamycin samples enriched with ^{14}C-lysine and ^{3}H-pipecolate were hydrolyzed and analyzed to provide evidence of specific incorporation of these precursors into the pipecolate moiety,

especially in comparison with ^{14}C-acetate enriched samples.

Carbons 10 and 11 of rapamycin had not been labelled in any of the previous experiments. $^{13}C_2$-Glycine was added to cultures in an attempt to label these carbons but no direct incorporation into these two carbons was observed. The three methoxy carbons were highly enriched, apparently due to conversion of the labelled glycine to labelled methionine. Unlabelled glycine (up to 2.0 g/l) had no effect on the production of rapamycin.

The addition of shikimic acid and aromatic amino acids to the medium had no effect on the production of rapamycin. ^{14}C-Shikimic acid was incorporated into rapamycin to a very high degree. ^{13}C-Shikimic acid was successfully prepared from 1-^{13}C-glucose using a mutant of Klebsiella pneumoniae, and then used in an attempt to label rapamycin. After allowing for possible errors in the spectral assignments, it was concluded that ^{13}C-shikimic acid had been successfully incorporated into the cyclohexane moiety of rapamycin, thereby establishing the shikimic acid pathway origin of the seven carbon starter unit.

Using ^{1}H-^{1}H and ^{1}H-^{13}C decoupling techniques, the ^{13}C-NMR spectral assignments in the literature were checked, and found to be in error as had been shown by the labelling studies.

The origins of all but two of the 51 carbons of rapamycin have been identified. Several possible means for identifying the origins of the unlabelled two-carbon unit are discussed.

ACKNOWLEDGEMENTS

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I would like to thank Ayerst Laboratories for their generous donations of the rapamycin-producing strain of Streptomyces hygroscopicus, information on the culture conditions of the organism, samples of pure rapamycin, and 1-^{13}C-acetate, 2-^{13}C-acetate, and ^{13}C-(methyl)-methionine. I would especially like to thank Dr. S.N. Sehgal (Ayerst Laboratories) for his many helpful suggestions. I would like to thank Dr. D.I.C. Wang for the donation of 1-^{13}C-glucose and Drs. D.I.C. Wang and M.F. Roberts for the donation of 2-^{13}C-propionate.

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I cannot possibly name all the people who have helped me get through M.I.T. These include the post-docs that have shown me that there is life after graduate school, my labmates with whom I commiserated for hours, and various members of the BEAM (Biochemical Engineering and Applied Microbiology) group outside the lab. I must give special thanks to "my kids", the gentlemen of C-Entry in MacGregor House, who allowed me to live with them for the past five years and whose friendship helped keep me going.

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I. INTRODUCTION

Rapamycin is potentially a valuable antibiotic, produced during fermentation by a strain of Streptomyces hygroscopicus. While the mode of action remains unknown, rapamycin has very strong antifungal activity but little antibacterial activity and surprisingly low toxicity in animals. It also shows good antitumor activity against certain types of tumors and some immunosuppresant activity. Some structural features of rapamycin appear similar to those of some well-studied antibiotics, but others are novel. Despite these interesting characteristics, little was known about the biosynthesis of rapamycin when my thesis work was begun.

While much could be done empirically to improve rapamycin production, biosynthetic information could be useful in this respect and for many other reasons. Knowledge about the biosynthetic pathway could lead to improvements in the production of rapamycin via (1) improved medium design to increase the supply of precursors, (2)rational mutant screening to eliminate competing pathways, and (3)rational genetic manipulation, possibly including increasing the flow through rate determining steps of the pathway. Once the pathway and its mechanisms are understood, new rapamycins could be obtained by specifically blocking steps in the pathway or by the incorporation of compounds very similar to the natural precursors. These rapamycin derivatives could have improved pharmacological properties, and also give clues as to what structural features are important for activity.

Also biosynthetic information which is learned about rapamycin may be applicable to antibiotics with similar structural features, such as those antibiotics recently described as containing cyclohexane moieties.

The overall goal of this project is to elucidate the biosynthetic pathway of rapamycin. The main focus is on determining the precursors and their orientations in the molecule, primarily by the incorporation of ^{13}C-labelled precursors and identification of the sites of incorporation by ^{13}C-NMR. Additional information is gained by the use of radioactive precursors and by observing the effects of the addition of possible limiting precursors and inhibitory precursor analogs.

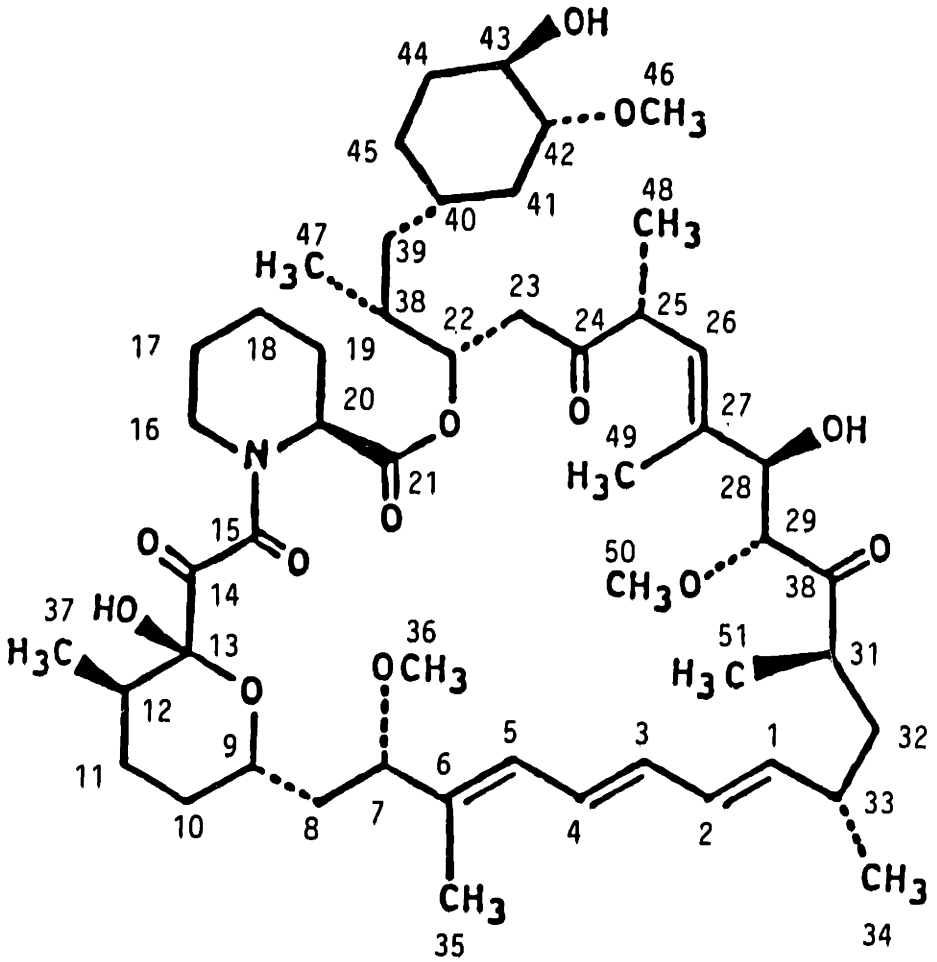
II. LITERATURE SURVEY

II. A. Rapamycin

Rapamycin (see Figure 1) is an antibiotic which was first reported as an antifungal agent in 1975 by Ayerst Research Laboratories (Montreal). It is produced by a strain of Streptomyces hygroscopicus isolated from an Easter Island soil sample (Vezina et al., 1975). It is very active against Candida species (common human pathogenic yeasts) and moderately active against dermatophytes, while having no activity against bacteria (Sehgal et al., 1975). Its specificity, relatively low toxicity, and good oral absorption in mice and dogs (Baker et al., 1978) make rapamycin a potentially valuable antifungal agent. Later, rapamycin was shown to have good activity against mammary, colon, and brain tumor model systems (Douros and Suffness, 1981) and was selected for further testing by the National Cancer Institute. Ayerst also conducted a study on its activity against transplanted tumors and toxicity (Eng et al., 1984). In one test, rapamycin had activity similar to adriamycin, a dangerously toxic but currently used anticancer drug. Rapamycin significantly inhibited tumor growth at any stage of development and had maximal activity similar to that of 5-fluorouracil and cyclophosphamide while appearing to be less toxic than these drugs in the active dose range.

The mode of action of rapamycin is still unclear, although it appears to differ from many known antifungal agents (Singh et al., 1979; Vining, 1979). It does not seem to affect membrane permeability or inhibit protein synthesis; its largest effect in yeasts seems to be on

Figure 1: The Structure of Rapamycin. (adapted from Findlay et al., 1982)



RAPAMYCIN

$C_{51}H_{79}O_{13}N$

phosphate incorporation into nucleic acids. There is no pH dependence of activity between pH 6 and 8, but all activity is lost at pH 4. Rapamycin has also been shown to inhibit the immune response in rats, apparently by inhibiting the lymphatic system (Martel et al., 1977).

Rapamycin is fairly stable as a white crystalline solid. It is insoluble in water but soluble in many organic solvents. Published methods for the recovery from fermentation broths include organic extraction of the mycelium, followed by a series of organic extraction and concentration steps, and finally silica gel column chromatography. In a complex medium, the titer was approximately 30 mg/l of fermentation broth using initial soil isolates of the producing organism (Sehgal et al., 1975).

The structure of rapamycin was determined by a combination of X-ray crystallography and ^{13}C- and ^{1}H-NMR (White et al., 1978; Findlay and Radics, 1980). It shares structural features with several classes of known antibiotics, but cannot easily be placed in any of these established categories (see Literature Survey, Section II. B.).

A major portion of the molecule is a very large (31-membered) "macrolide ring" (carbons 20 to 33 and 1 to 15, plus a nitrogen and an oxygen). Typical macrolides are much smaller (12- to 16-membered ring), having unusual amino- and deoxysugars attached to the ring. Along rapamycin's ring are various methyl, keto, hydroxyl, and methoxyl functionalities. Three conjugated double bonds (carbons 1 to 6) allow rapamycin to be classified as a "polyene", although most known polyenes contain 4 to 7 conjugated double bonds; trienes are rare. The

6-membered "hemiketal" ring (including oxygen 13 and carbons 9 to 13) could exist in solution in equilibrium with the keto-alcohol equivalent, but this isomerization does not appear to take place, based on NMR evidence (Findlay and Radics, 1980). A rare "diketo" functionality (including carbons 14 and 15) is located just outside the hemiketal ring. There does appear to be hindered rotation about the amide bond between C15 and the nitrogen atom, giving rise to 2 isomeric forms in approximately a 1 to 4 ratio in solution. The heterocyclic ring including this nitrogen and carbons 16 to 20 plus the C21 carboxyl can be excised by acid- or base- catalyzed hydrolysis as L-pipecolic acid. This imino acid occurs naturally in plants, animals, and bacteria, but is not as common as L-proline, its C_5N analog. Outside the macrolide ring is a trisubstituted cyclohexane ring (carbons 40 to 45). Many antibiotics contain C_6 rings, but they are usually aromatic. Recently some antibiotics have been isolated which contain cyclohexane rings, but little has published about their biosynthesis (see Literature Survey, Section II. B.).

Another antibiotic was isolated from the same rapamycin-producing culture. Now referred to as demethoxyrapamycin, its structure is identical to that of rapamycin, except that the methoxy group at C29 is replaced by a hydrogen (Findlay *et al.*, 1982). It is only one-fourth as active against *C. albicans*, and shows little or no activity against tumors (Sehgal *et al.*, 1983).

Little was known about the biosynthesis of rapamycin when this project was begun. The complete structure, stereochemistry, and ^{13}C-NMR

spectral assignments had been published. For such a complex molecule, the method of choice for the determination of the biosynthetic precursors is the incorporation of ^{13}C-labelled precursors and subsequent analysis by ^{13}C-NMR, supplemented by other biochemical experiments. The following sections contain a brief review of what was known about the biosynthesis of structurally similar natural products at the time this study was begun. This information provides clues as to the probable precursors, reaction mechanisms, and other features of rapamycin biosynthesis.

II. B.) Biosynthesis of Compounds Structurally Similar to Rapamycin

Rapamycin has structural features similar to those found in the ("polyoxo") macrolides, polyenes, peptidolactones, and ansamycins. These structures are shown in Figure 2 and can be described as follows:

1) "Typical" macrolides (Figure 2a) ("polyoxo macrolides") contain a 12-, 14-, or 16-membered lactone ring (the aglycone), heavily oxygenated and often methyl substituted, to which one or more aminosugars or deoxysugars are attached (Woodward, 1957). "Atypical" macrolides (Figure 2b) are often larger and contain odd bicyclic structures, but are apparently synthesized by routes initially similar to those of the typical macrolides (Omura and Tanaka, 1983).

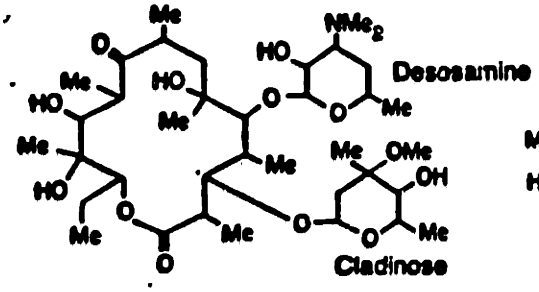
2) Polyenes (polyene macrolides) (Figure 2c) often contain larger lactone rings (26-38 atoms) than the macrolides, one portion containing a series of 4 to 7 conjugated double bonds, while the rest is highly oxygenated. An aminosugar is often attached to the lactone, and aromatic moieties are not uncommon (Martin, 1983).

3) Ansamycins (Figure 2d) contain a nitrogen-substituted chromophore (of either naphthalene or benzene type), to which both ends of an aliphatic chain are attached. One end is attached via an amide bond at the nitrogen, and the other end is attached at a non-adjacent site via a carbon-carbon bond. No sugars are attached, but methoxy and O-acetyl groups are common (Lancini, 1983).

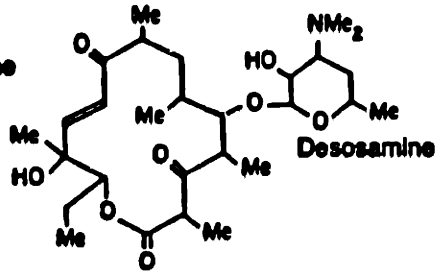
4) Peptidolactones of the virginiamycin type (Figure 2e) consist of a peptide chain linked end to end to an unsaturated and/or oxygenated acyl chain, to form a lactone ring, which also contains more than one

Figure 2: The structures of representative antibiotics.

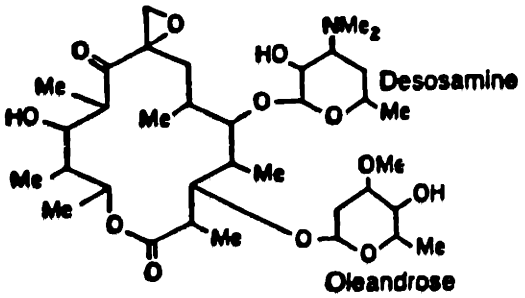
a.) "Typical" Macrolides:



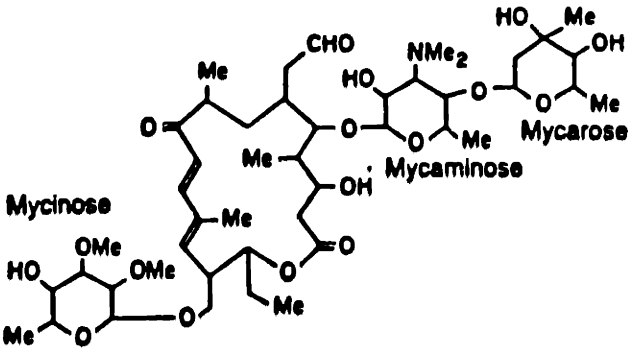
Erythromycin A



Pikromycin

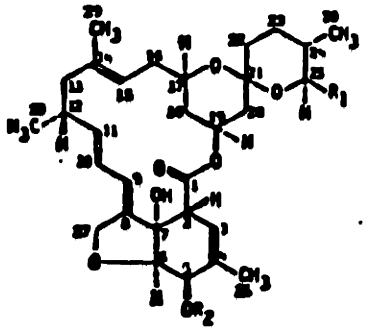


Oleandomycin

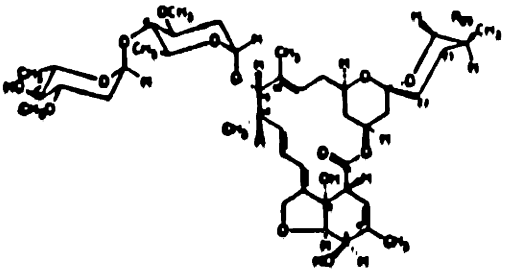


Tylosin

b.) "Atypical" Macrolides:



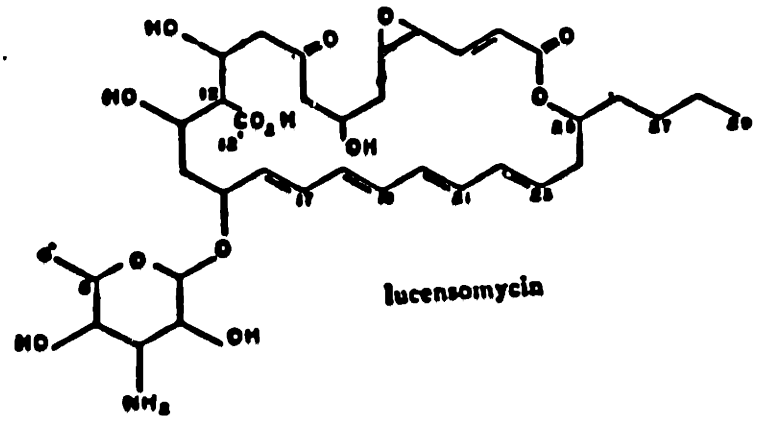
milbemycins



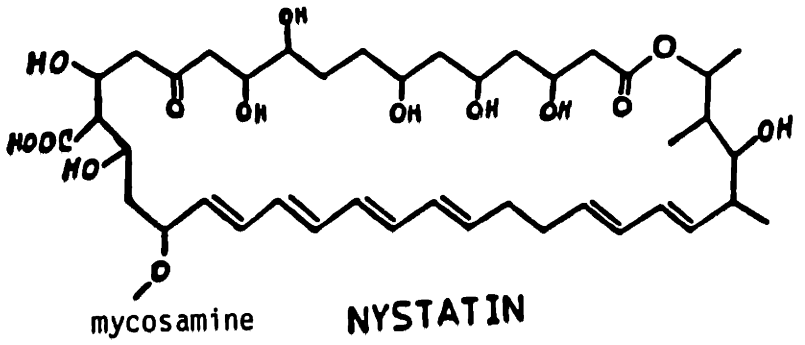
ivermectin

Figure 2 (continued):

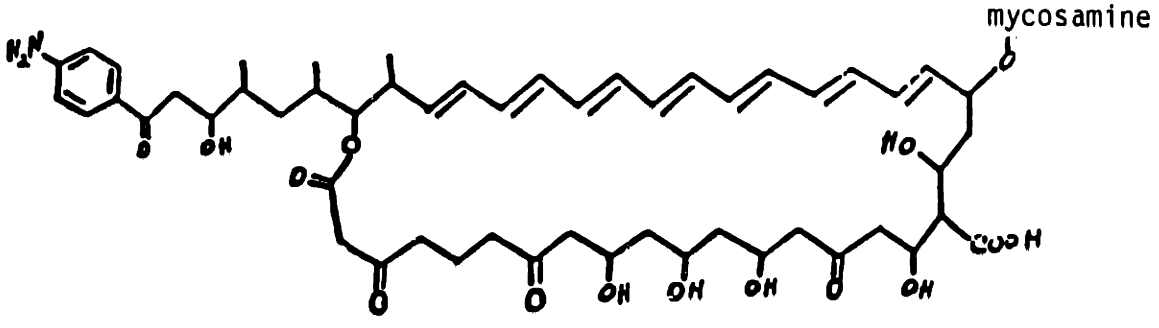
c.) Polyenes:



lucensomycin



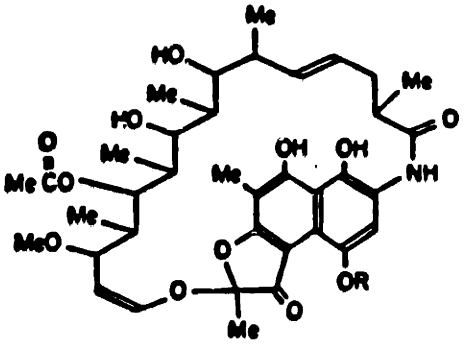
mycosamine NYSTATIN



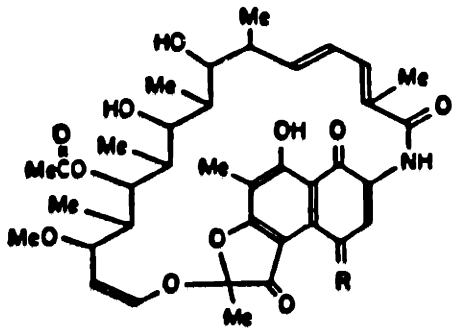
CANDICIDIN

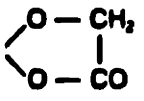
Figure 2 (continued):

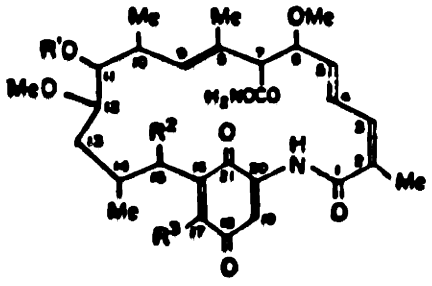
d.) Ansamycins:
(naphthalene and benzene types)



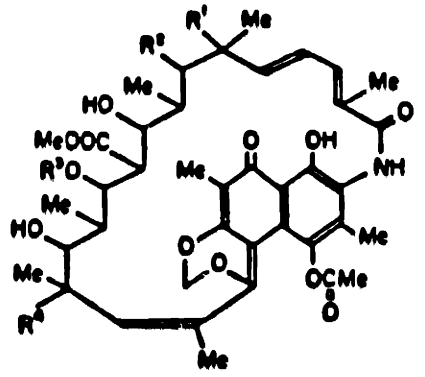
	R
Rifamycin B	$-CH_2COOH$
Rifamycin SV	-H



	R
Rifamycin S	=O
Rifamycin O	



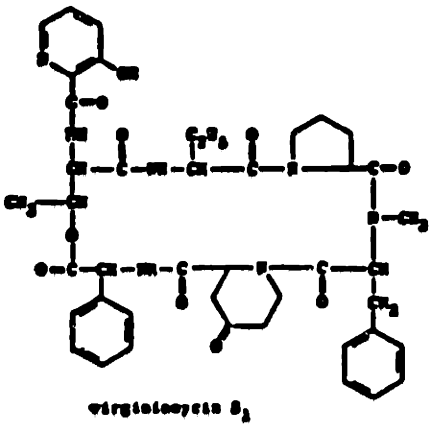
	R¹	R²	R³
Geldanamycin	-Me	-H	-OMe
Herbimycin A	-Me	-OMe	-H
Herbimycin B	-H	-H	-H

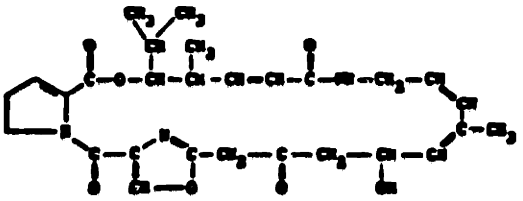


	R¹	R²	R³	R⁴
Streptovaricin A	OH	OH	$COCH_3$	OH
B	H	OH	$COCH_3$	OH
C	H	OH	H	OH
D	H	OH	H	H
E	H	=O	H	OH
G	OH	OH	H	OH
J	H	$OCOCH_3$	H	OH

Figure 2 (continued):

e.) Peptidolactones:





amide (peptide) bond. (Actinomycin-type peptidolactones contain a phenoxazinone chromophore and two peptide chains, and are less related to rapamycin.) Peptidolactones often contain unusual (non-protein) amino acids (Okumura, 1983).

A comparison of rapamycin with the general properties of the above four classes of antibiotics is summarized in Table 1.

There are many well-written reviews on the biosynthesis of antibiotics and other natural products. I summarize below only information which is relevant to possible biosynthetic schemes for rapamycin. For additional information, I recommend the book by Vining (1983) and the following reviews: macrolides, Omura and Nakagawa, 1981, and Masamune et al., 1977; polyenes, Martin, 1979, and Hamilton-Miller, 1973; rifamycins, Lancini and Parenti, 1978.

A.) Biosynthesis of Lactone Ring / Carbon Chain

More than half of the carbons in rapamycin form a long methylated and oxygenated carbon chain, which comprises most of the large lactone ring. While the above-mentioned antibiotics have a wide range of structures and origins, they all contain portions resembling this carbon chain in rapamycin. These portions are believed to be synthesized via the polyketide pathway. Similar to fatty acid biosynthesis, a long carbon chain is built from the "head-to-tail" condensation of "activated" acid units. Acetic acid is activated as the carboxylated thioester malonyl-CoA (CoA = coenzyme A). However, polyketide biosynthesis can also incorporate propionate and butyrate units; the activated forms are usually methyl- and ethylmalonyl-CoA, respectively .

Table 1:

Comparison of rapamycin to other related antibiotics.

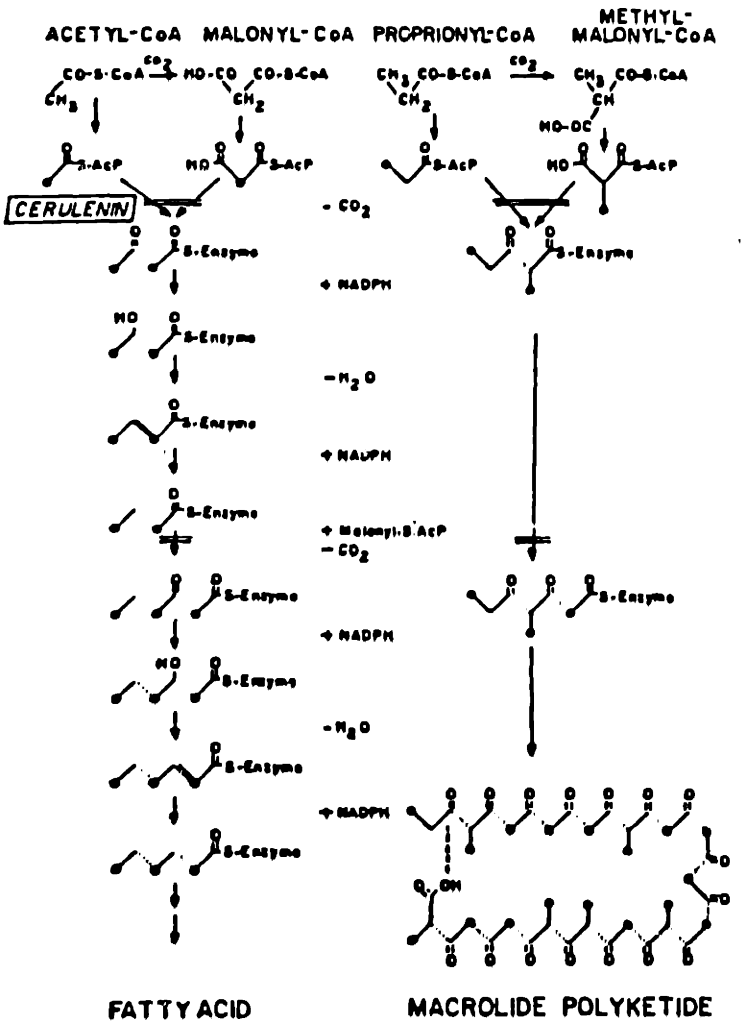
	Rapamycin	Macrolides (Typical)	Polyenes	Ansamycins	Peptidolactones (Virginiamycin type)
1) lactone ring present	yes	yes	yes	yes	yes
2) size of ring	31	12-16	26-38	9-23	18-24
3) peptide bonds	yes	no	no	yes	yes
4) aromatic moieties	no	no	often	yes	often aromatic amino acids
5) sugars	no	yes (1 to 3)	often 1	no	no
6) cyclohexane moiety	yes	no	no	no	no
7) pipecolic acid	yes	no	no	no	often a modified form
8) conjugated double bonds	3	usually ≤ 2	usually 4 to 7	often 2 to 3	usually ≤ 3

In fatty acid biosynthesis, the condensation of an incoming C_2 unit is followed by a reduction of the resulting keto group, a dehydration and another reduction, leaving a non-oxygenated alkyl thioester, to which the next C_2 unit is then added. In polyketide biosynthesis, the next carbon unit can be added before the keto group is modified. The keto group may be left as is or undergo some or all of the reactions occurring in fatty acid biosynthesis, resulting in either a hydroxyl, a double bond, or a fully saturated bond. The newly formed chain can also undergo intramolecular cyclization, yielding fused (e.g. tetracyclines) (Korte, 1977) or single (e.g. methylsalicylic acid) (Vogel and Lynen, 1975) aromatic rings.

While the polyketide condensing enzymes have different substrate specificities, the mechanism appears to be the same as that for fatty acid synthesis. Cerulenin ((2S) (3R)2,3-epoxy-4-oxo-7,10-dodecadienoyl amide) is a non-competitive inhibitor of the fatty acid synthetases of both yeasts and bacteria (Omura, 1976). Cerulenin also inhibits formation of the aglycones of leucomycin (Takeshima et al., 1977) candicidin (Martin and McDaniel, 1975), tylosin (Omura et al., 1978), tetracycline and other antibiotics (Omura 1976), often at subinhibitory levels for growth of the producing organism. A comparison of fatty acid and polyketide biosynthesis is shown in Figure 3 (Martin, 1975), and the proposed sites of cerulenin action are indicated.

In the case of fatty acid biosynthesis, the growing fatty acid chain remains attached to an acyl-carrier protein through a thioester bond. This is also believed to be true for polyketide enzymes.

Figure 3: Comparative pathways of biosynthesis of fatty acids and polyene macrolides and suggested site of action of cerulenin on polyene macrolide biosynthesis. Enzymatic steps inhibited by cerulenin (⊣). Malonyl-CoA in addition to propionyl-CoA and methyl-malonyl-CoA is used as a building unit for macrolide biosynthesis. The circles indicate the methyl group of either acetate or propionate units used. ACP, acyl carrier protein. (from Martin, 1975)



Excepting the cases of specifically blocked mutants, no intermediates before the completion of the lactone ring are accumulated. 6-Methylsalicylic acid synthetase has been purified from Penicillium species and shown to contain two types of SH groups (one identified as part of 4'-phosphopantetheine), through which a growing polyketide chain could be attached to the enzyme (Vogel and Lynen, 1975). The enzyme complex and cell extracts of S. griseus capable of synthesizing candicidin both require NADPH and malonyl-CoA, as was predicted (Martin, 1983).

For many antibiotics, this type of pathway has been established by the feeding of specifically labelled precursors and locating the label in the purified antibiotics. Birch (1964) was the first to apply this approach to the polyenes. He obtained significant incorporation of 1-^{14}C-acetate and 1-, 2-, or 3-^{14}C-propionates into nystatin, and was able to establish the location of the label by extensive chemical degradation. With improvements in instrumentation and the increasing availability of ^{13}C-labelled precursors, ^{13}C-NMR has become the method of choice for location of the sites of incorporation in complex molecules. Figure 4 shows some examples of the results of ^{14}C- and ^{13}C-incorporation studies. For the most part, methyl groups along the carbon chains are derived from the C3 of propionate, although sometimes they arise from methionine (e.g., geldanamycin). These methyl groups can also be removed by oxidation to CO_2 (e.g., rifamycin).

In the unsupplemented medium, the producing organism does not necessarily take up and activate free acids from the medium, although

Figure 4: The results of precursor incorporation studies on the polyketide portions of several antiobiotics.

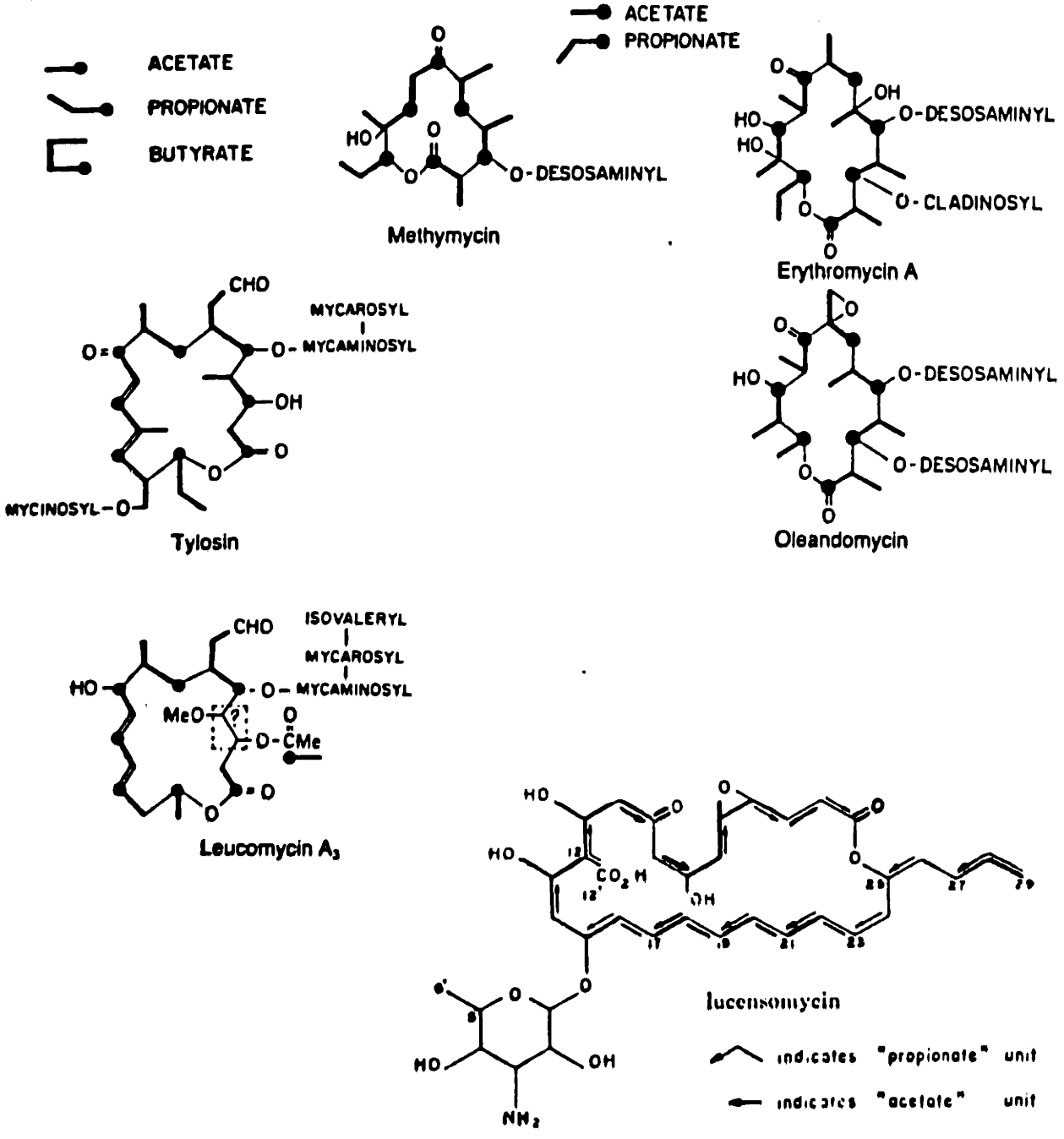
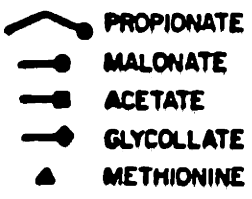
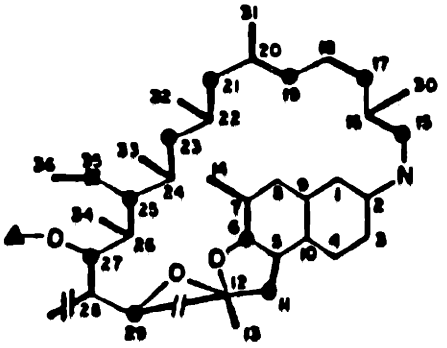


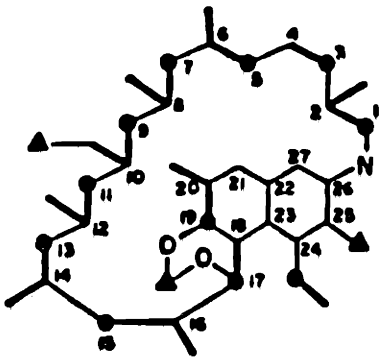
Figure 4 (continued):



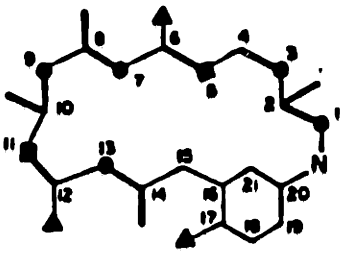


Rifamycin

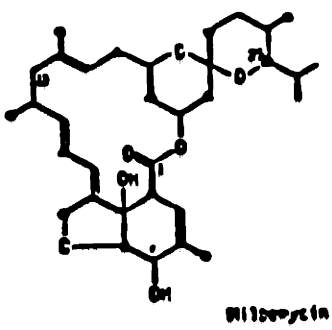
lost as CO_2



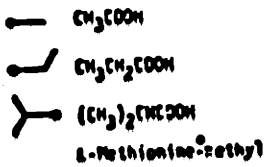
Streptovaricin

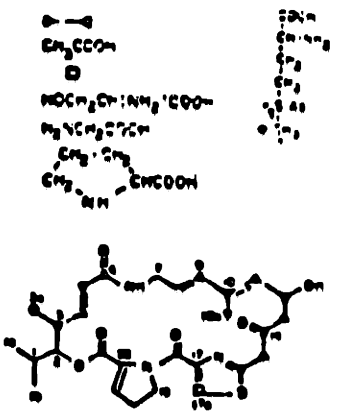


Geldanamycin



Milbemycin D





virginiamycin M_1

for nystatin it has been observed that acetic and other acids are excreted during growth and then utilized during antibiotic production (Torpova et al., 1972). Acetyl-CoA and propionyl-CoA can be generated directly from the metabolism of sugars, fatty acids, and amino acids. These can then be carboxylated (CO_2 or HCO_3^- as carboxyl donor) by a non-specific acetyl-CoA carboxylase. The dominant route to malonyl-CoA in Streptomyces aureofaciens (tetracycline producer) was shown to be the carboxylation of phophoenolpyruvate to oxaloacetate, followed by displacement of CO_2 by HSCoA (Behal et al., 1977). It has also been shown that transcarboxylation (oxaloacetate as CO_2 donor) may be significant for propionate incorporation (Rafalski, 1972). Methylmalonyl-CoA ("active propionate") can be obtained by a mutase reaction from succinyl-CoA, as in erythromycin formation (Hunaiti and Kolattukudy, 1984); similar incorporation patterns can be obtained by the feeding of appropriately labelled propionate, succinate, or methylmalonyl-CoA molecules. Butyrate and other acids are believed to be activated by similar mechanisms.

B.) Starter Units / Shikimic Acid Pathway

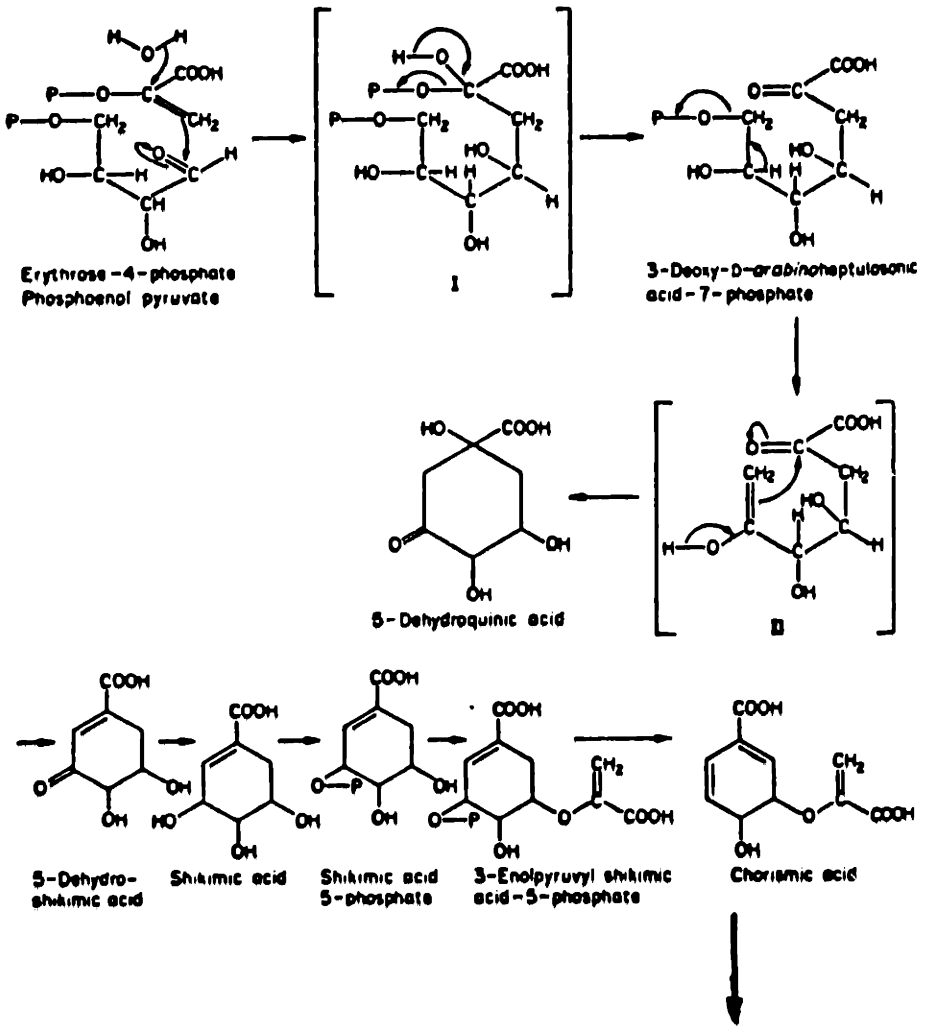
In the biosynthesis of fatty acids, most macrolides, and some polyenes, acetate or propionate activated as the CoA derivative (but not carboxylated to the malonyl-CoA form) serves as the "starter unit" in the initial condensation. The chain grows from the carboxyl group of the starter unit. Two recently discovered "fused" or "atypical" macrolides, milbemycin D (Ono et al., 1983) and avermectin (Cambell et al., 1983) appear to use isobutyrate to initiate the synthesis of their

carbon skeletons.

After assigning on paper acetate or propionate origins to most of the carbons in rapamycin (based on the polyketide pathway discussed above), a cyclic C_7 unit (C39 to C45) remains which should be the "starting" unit of the chain. The starter units of serveral polyenes and the ansamycins are cyclic C_7 compounds which apparently are derived from the shikimic acid pathway, which normally leads to aromatic amino acids (see Figure 5). Candicidin biosynthesis is initiated by p-aminobenzoic acid (PABA) (Liu et al., 1971), which is derived from chorismate via PABA synthetase. This has been demonstrated by specific incorporation of ^{14}C-labelled PABA into the aromatic moiety of candicidin and in experiments with PABA-requiring mutants (Gil et al., 1981). Aromatic amino acids, especially tryptophan, appear to feedback inhibit and/or repress the enzymes which synthesize 2-keto-3-deoxy-arabino-heptulosonate-7-P (DAHP) and PABA synthesis, and also decrease candicidin biosynthesis (Gil et al., 1980; Martin, 1983). The production of candicidin is well-correlated with the level of PABA synthetase activity under a variety of conditions, indicating that the production of PABA may be the rate-controlling step in the biosynthesis. Levorin (another heptaene) also appears to initiate with PABA, while perimycin uses N-methyl-PABA (Martin, 1983).

The starter unit in rifamycin (and presumable all ansamycins) is 3-amino-5-hydroxybenzoic acid. The origins of all but a "C_7N unit" of rifamycin had been determined to be acetate, propionate, and methionine (White et al., 1973). While shikimate itself did not label rifamycin

Figure 5: The Shikimic Acid Pathway.
(adapted from Luckner, 1972)



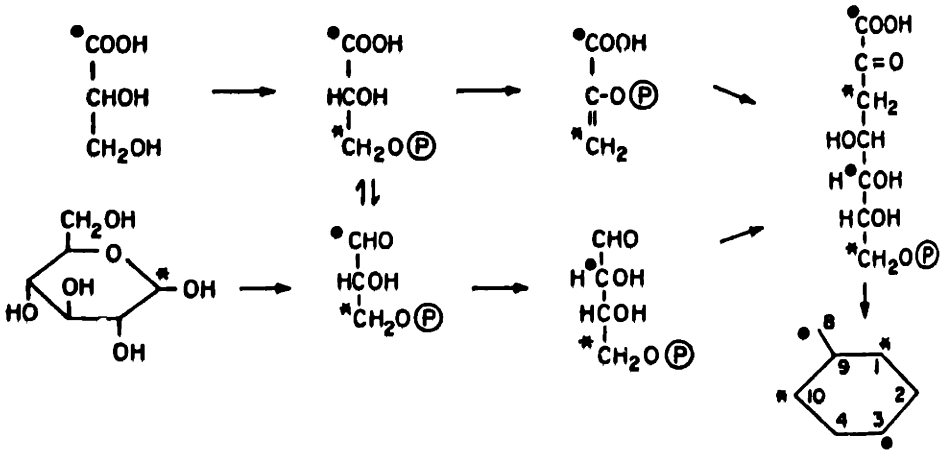
phenylalanine
tryptophan
tyrosine
p-amino-benzoic acid (PABA)
p-hydroxy-benzoic acid (PHBA)
Vitamin K

(Karlsson et al., 1974), White and Martinelli (1974) were able to show in an elegant experiment that the C_7 unit was from the shikimate pathway. 1-^{13}C-Glucose and 1-^{13}C-glycerate enriched only those carbons in the ring which would be expected to be labelled from this pathway (see Figure 6). Later, labelling studies showed that 3-amino-5-hydroxy-(7-^{14}C)-benzoic acid (Kibby et al., 1980) was readily incorporated unlike other benzoic acid derivatives, while genetic studies (Ghisalba and Nuesch, 1981) showed the C_7N unit could restore antibiotic production in mutants blocked at DAHP synthetase. It has not yet been determined which of three possible intermediates of the shikimate pathway (3-dehydroquinic acid, 3-dehydroshikimic acid, or DAHP) serves as the direct precursor of 3-amino-5-hydroxybenzoic acid.

In Nocardia mediteranei (Christ et al., 1981) (the rifamycin producer) as well as Micrococcus luteus and several Streptomyces species (Jensen and Stenmark, 1970; Lowe and Westlake, 1971), DAHP synthetase is under no apparent feedback control by aromatic amino acids or intermediates. This could explain various "abnormalities" in the control of rifamycin biosynthesis (Lancini, 1983), such as the lack of apparent carbon, nitrogen, or phosphate control and the growth-associated production in some media.

Based on ^{14}C-labelling experiments on geldanamycin biosynthesis, it was proposed that the amino group in 3-amino-5-hydroxybenzoic acid was attached to the carbon derived from C1 of erythrose-4-P (Hornemann, 1980). However, an alternative orientation is also possible, wherein the amino group would be attached to the carbon from C3 of phosphoenol

Figure 6: Incorporation of 1-^{13}C-glycerate and 1-^{13}C-glucose into rifamycin chromophore. (Vining, 1983, p.242)



pyruvate (PEP). Rinehart (1982) used $^{13}C_6$-glucose to distinguish between these two possibilities. $^{13}C_6$-Glucose generates in vivo $^{13}C_4$-erythrose and $^{13}C_3$-PEP, in the formation of DAHP (see Figure 7). By interpretation of the ^{13}C-^{13}C coupling patterns, the orientation suggested for geldanamycin was confirmed. Using the same approach on pactamycin, the second possible orientation was found. (Pactamycin contains an N-linked m-amino-acetophenone unit derived from m-aminobenzoic acid and methionine.) (See Figure 8.)

The above-mentioned "shikimate pathway derived" C_7 units all contain aromatic rings, whereas rapamycin contains a fully-saturated ring. Validamycin A contains two non-aromatic C_7 units, validamine and valienamine (Figure 9). Preliminary data showed that both are derived from the shikimate pathway, and that in validamine, PEP and erythrose are aligned in the same orientation with respect to the nitrogen as in m-aminobenzoic acid in pactamycin. While these rings are more highly substituted than that in rapamycin and are not attached to a carbon chain, these studies demonstrate that non-aromatic derivatives of shikimate pathway intermediates can occur in secondary metabolites (Rinehart, 1982).

Other direct precursors of 6-membered rings include acetate and glucose. Glucose-6-P is cyclized to form meso-inositol-1-P (Luckner, 1972) and deoxystreptamine (Kakinuma, 1982) (Figure 10). The resulting cyclohexane ring is highly oxygenated (5 oxygens compared to the 2 on the corresponding ring of rapamycin) and the seventh predicted carbon is missing. Methionine could donate a seventh carbon to the ring, as in

Figure 7: Two possible labelling patterns of m-amino benzoic acid based on the incorporation of $^{13}C_6$-glucose via the shikimic acid pathway. (from Rinehart, 1982)

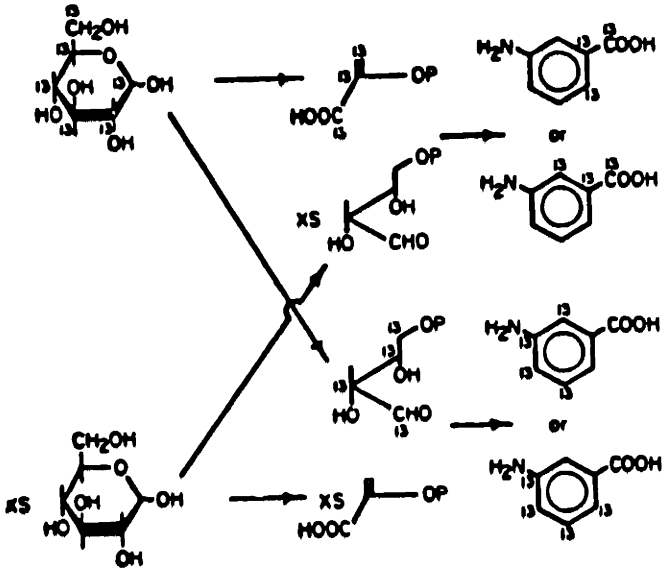


Figure 8: Two routes to 3-amino substituted aromatic rings from erythrose-4-P and phosphoenol pyruvate. In the route to pactamycin, the amino group is attached to the carbon derived from C3 of erythrose, while in geldanamycin biosynthesis, the carbon is derived from C1 of erythrose. (Rinehart, 1982)

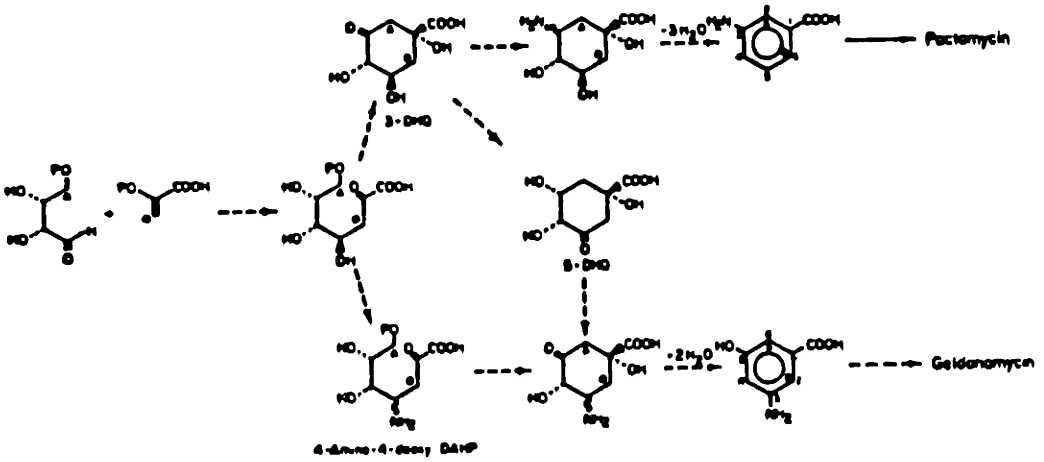


Figure 9: Validamycin A

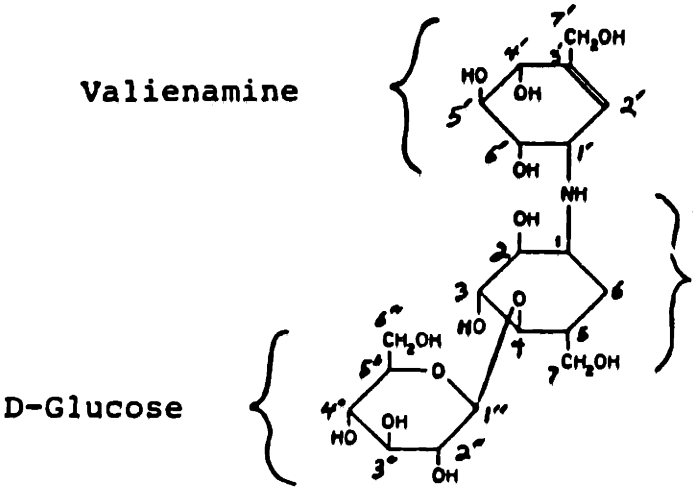
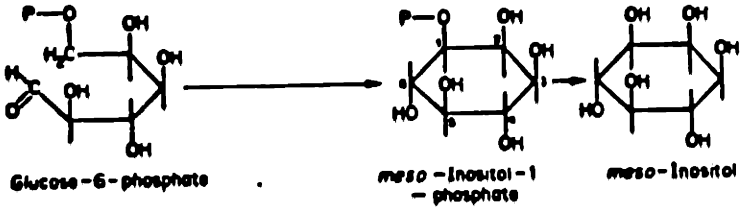
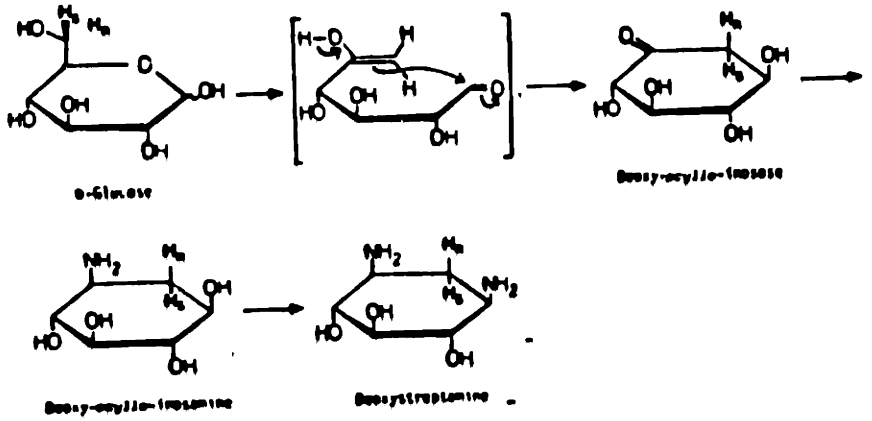


Figure 10 :

a.) Formation of meso-inositol-1-phosphate. (Luckner, 1972)



b.) Formation of deoxystreptamine. (Kakimura, 1982)



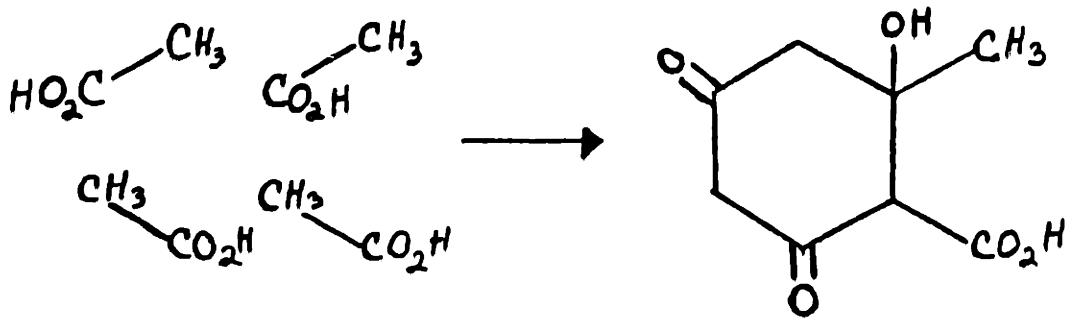
the case of pactamycin (Rinehart, 1982), and this carbon could be later oxidized and linked to the polyketide chain. A polyacetate chain could be folded in two possible conformations to form C_6 rings (see Figure 11). The first conformation occurs in the production of palitantin and orsellinic acid (Hutchinson, 1982) (Figure 12). The initial compounds formed by this route contain an additonal methyl group on the ring and only one O-functional group is in the correct position when compared to rapamycin (Figure 13). The second folding pattern also results in an "extra" carbon and an oxygen in the wrong location. Shikimate (Figure 14) (or a closely related compound) contains exactly seven carbons, has only one extra oxygen on the ring and contains oxygens at the two sites oxygenated in rapamycin in the correct stereochemical orientation. There remains the possibility that an aromatic amino acid is incorporated and then oxygenated and reduced, but examples of this type of pathway are unknown to me. A few natural products containing cyclohexane rings have recently been isolated (Figure 15) but little is known about their biosynthesis (Rinehart, 1982). In studies on bacilysin biosynthesis, ^{14}C-tyrosine was not incorporated into this dipeptide antibiotic, while ^{14}C-shikimate showed high incorporation (Roscoe and Abraham, 1966). Unfortunately, ^{14}C-phenylalanine was not tested, so the possibility remains that phenylalanine is incorporated, followed by oxidation and reduction of the aromatic ring.

C.) Pipecolic Acid Moiety

The unit comprised of carbons 16 to 21 and the nitrogen of rapamycin is identical to L-pipecolic acid. Pipecolic acid is a

Figure 11: Two possible folding patterns of a polyacetate chain to form 6-membered rings.

a.)



b.)

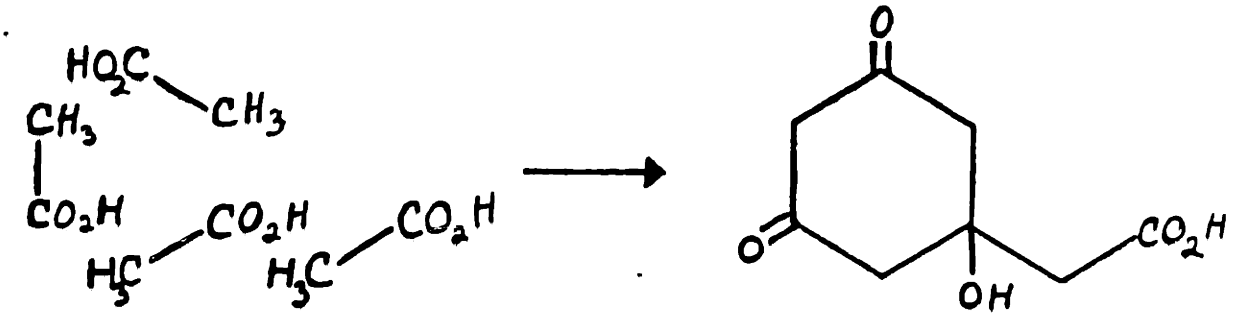
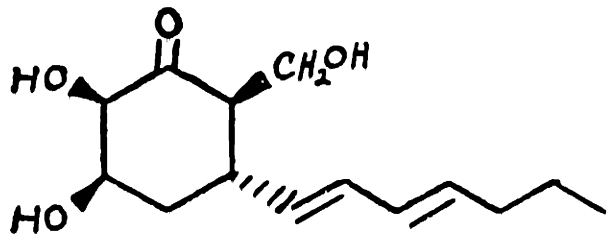
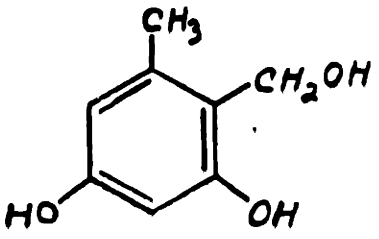


Figure 12: Two polyketide-derived six-membered rings. (Hutchinson, 1982)



palitantin



orsellinic acid

Figure 13: Six-membered ring of rapamycin.

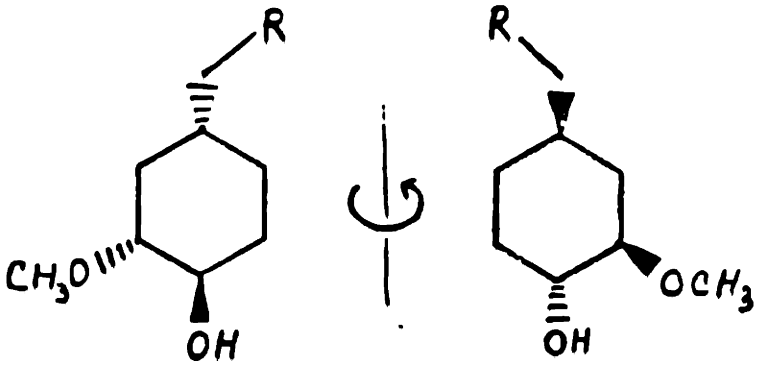


Figure 14: Shikimate.

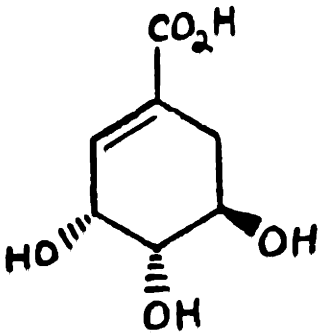
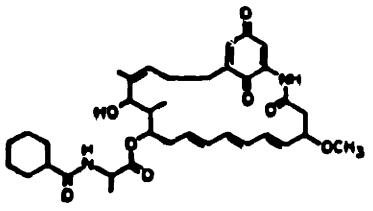
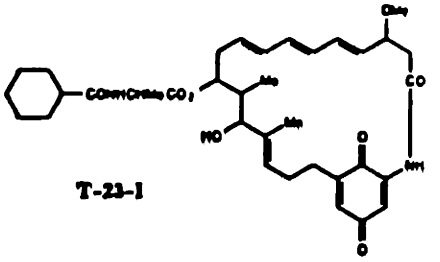


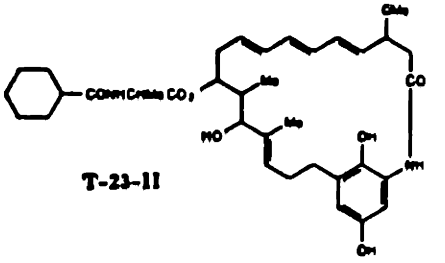
Figure 15: Natural products containing non-aromatic C_6 rings of undetermined origin. (Rinehart, 1982; Nisshin, 1983; Roscoe and Abraham, 1966)



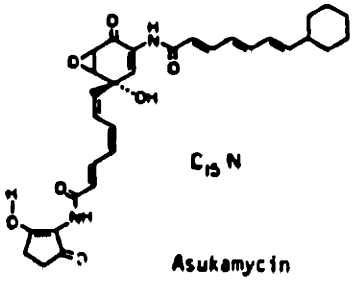
Ansatrienin A (Mycotrienin I)



T-23-I

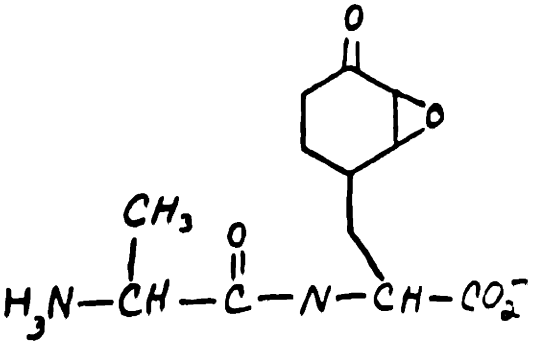


T-23-II

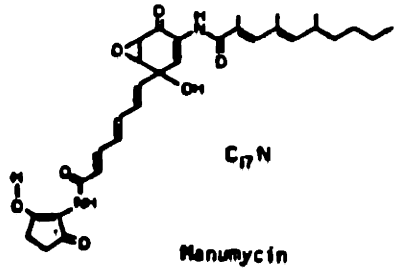


$C_{15}N$

Asukamycin



bacilysin*



$C_{17}N$

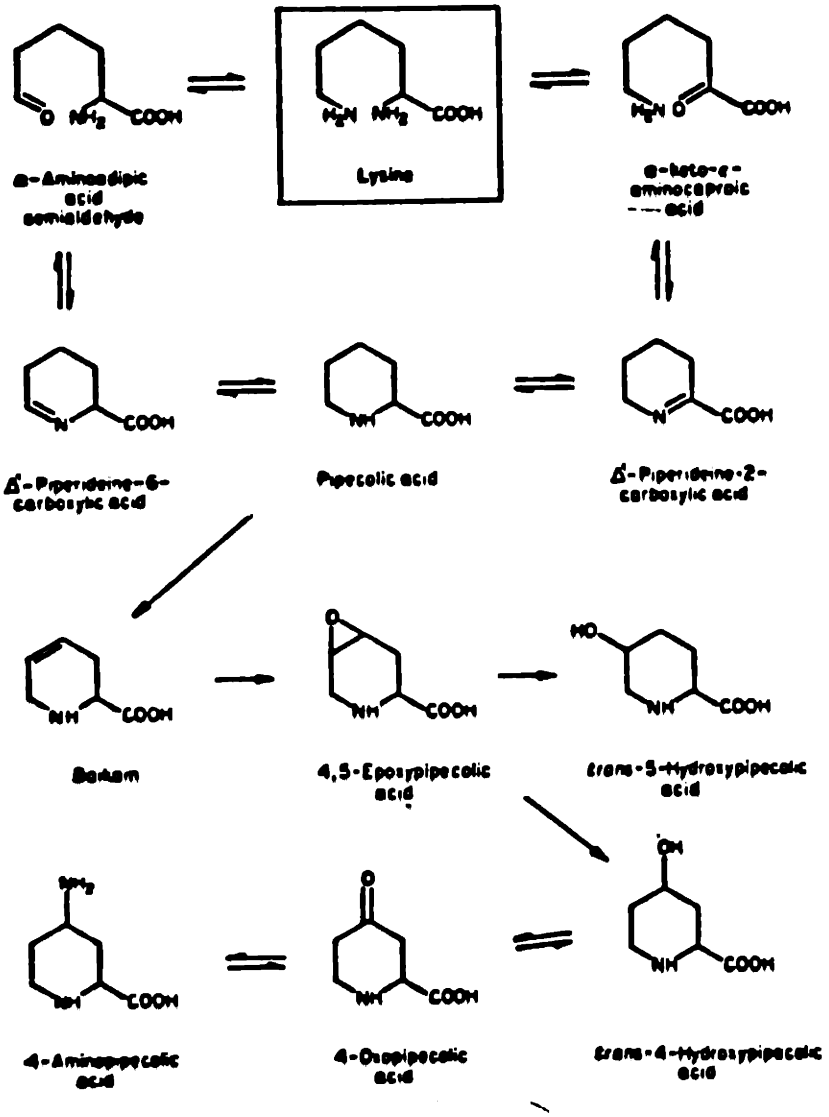
Manumycin

*: ring apparently derived from shikimate pathway, but branch-point is uncertain.

non-protein imino acid, with a 6-membered heterocyclic ring in contrast to the 5-membered ring of proline. Its presence has been detected in many higher plants, animals, and microorganisms (Luckner, 1972). While pipecolic acid itself does not usually appear in natural antibiotics, 4-oxo- and 3-hydroxypipecolic acids appear to be common components of the virginiamycin group B-1 antibiotics (Okumura, 1983). 3-Hydroxypicolinic acid (the aromatic analog of 3-hydroxypipecolic acid and possibly biosynthetically related) appears in virginiamycin groups B-I and B-II and other peptidolactones. Pipecolic acid has been incorporated into actinomycins in place of proline by directed biosynthesis (addition of pipecolic acid to the production medium) and into patricins A and B by chemical synthesis.

If pipecolic acid is a direct precursor of rapamycin, it could be derived from lysine or from an intermediate in lysine biosynthesis. The conversion of L-lysine to L-pipecolic acid has been demonstrated in intact plants and rats, as well as their extracts (Meister et al., 1957). This apparently takes place via deamination of lysine to the α-keto analog which spontaneously cyclizes, followed by selective reduction by NADH. (Formation via ϵ-deamination is also possible, but is less substantiated.) (See Figure 16.) L-Pipecolic acid has been suggested as an intermediate in the conversion of D-lysine to L-lysine by Neurospora crassa, as evidenced by the migration of ^{15}N-label from the ϵ-position of D-lysine to the α-position of L-lysine during the isomerization (Fangmeier and Leistner, 1980). D- and L-lysine appear to be catabolized by two separate pathways in Pseudomonas putida, L-lysine

Figure 16: Formation of pipecolic acid and pipecolic acid derivatives. (Luckner, 1972)



via δ-aminovalerate to glutarate and D-lysine via L-pipecolate to L-α-aminoadipate (Chang and Adams, 1974; Miller and Rodwell, 1971). Interconversion of L- and D-lysine by lysine racemase was very slow in this organism, and D-lysine selectively induced the pipecolate pathway (Chang and Adams, 1971). Degradation of lysine via pipecolate may be a common pathway in pseudomonads (Miller and Rodwell, 1971).

Pipecolic acid has been implicated as an intermediate in lysine synthesis in Euglena gracilis and Aspergillus nidulans, two eukaryotes (Luckner, 1972). L-Pipecolate itself is not an intermediate of either of the established biosynthetic pathways of L-lysine (Lehninger, 1975). It is interesting to note, however, that α-aminoadipic semialdehyde from the aminoadipic (fungal) pathway could spontaneously cyclize and be reduced to pipecolic acid, and Δ'-piperideine-2,6-dicarboxylate from the diaminopimelic (bacterial) pathway is only two steps (decarboxylation and reduction) away from L-pipecolic acid. While the diaminopimelic pathway is the one most likely to occur in Streptomyces hygroscopicus, a remote chance exists for the presence of the aminoadipic pathway.

D.) Peptide Bond Formation

Rapamycin contains a single amide (peptide) bond between C15 and the nitrogen of the pipecolic acid moiety. For peptidolactone and small peptide antibiotics, it has been demonstrated that the peptide bonds are formed via a "protein thiotemplate" mechanism (Lehninger, 1975; Stryer, 1981; Kleinkauf and von Dohren, 1983; Okumura, 1983). According to this model, the carboxyl groups of the amino acids are activated as enzyme-bound thioesters at specific enzyme sites. The peptide chain

grows from the N-terminal to the C-terminal, with the thioester linkage of the growing chain being attacked by the amine or imine nitrogen of the next amino acid. 4'-Phosphopantetheine is involved in carrying the growing peptide chain from one amino acid-activation site to the next and keeping the chain bound to the enzyme via a thioester linkage.

By analogy, in rapamycin biosynthesis, the peptide bond could be formed by an attack of the pipecolate or lysine nitrogen on the terminal thioester of the polyketide chain. The carboxyl of the pipecolate or lysine could be activated as the thioester to facilitate the closing of the lactone ring.

Thiotemplate peptide biosynthesis is not affected by antibiotics which inhibit ribosomal protein synthesis, such as chloramphenicol, puromycin, and tetracycline (Kamal and Katz, 1976). Furthermore, the enzymes tend to have broad substrate specificities, and supplementation of the medium with amino acids can cause the incorporation of these amino acids into peptidolactones (Okumura, 1983).

E.) Hydroxylation/Oxidation of the Carbon Skeleton

As mentioned above, the carbonyls formed on alternating carbons during the condensation of the acyl-CoA derivatives can be left as is, reduced to hydroxyls, dehydrated to double bonds, or further reduced to leave carbon-carbon single bonds. Additional oxygen functionalities can be introduced specifically at other carbons by more than one route. In erythromycin biosynthesis, direct hydroxylation of C6 of 6-deoxyerythronolide B is carried out by a soluble cytochrome P450 hydroxylase (Corcoran and Vygantas, 1982) while hydroxylation at C12 is

carried out by a membrane- associated P450-independent enzyme (Corcoran and Vygantas, 1977). An examination of the structures of tetrins A and B, pimaricin, and lucensomycin (which contain very similar carbon-skeletons), suggested that an epoxide might be an intermediate in the interconversion of these antibiotics in vivo (Pandey et al., 1971). (See Figure 17.)

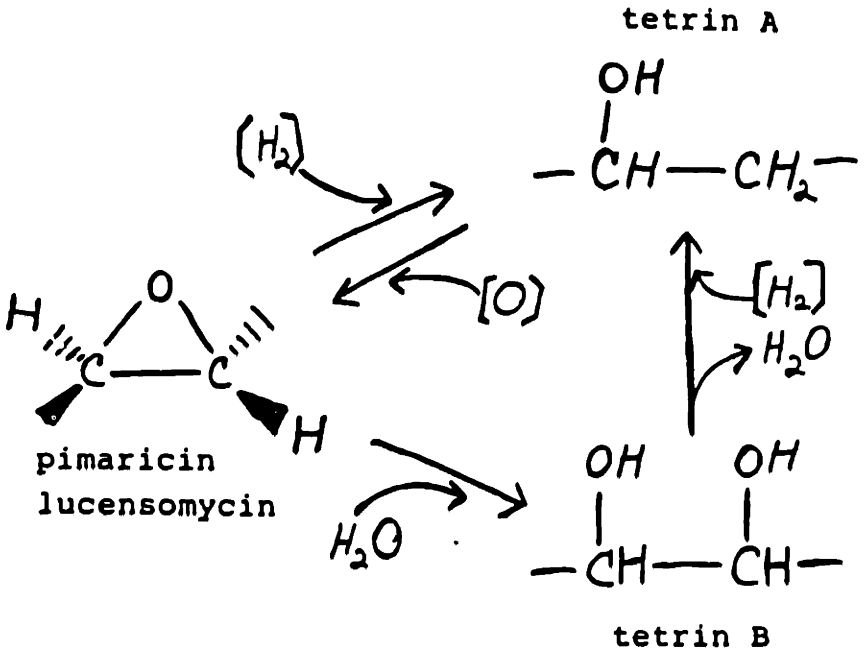
In tylosin biosynthesis, one methyl goup (C23) is oxidized to a primary alcohol, while a second methyl goup (C20) undergoes two oxidation steps to the aldehyde stage (Baltz and Seno, 1981). During the formation of rifamycin, one backbone methyl group (derived from a propionate) is successively oxidized to a carboxyl group and is eventually lost as CO_2, while seven other propionate-derived methyl groups remain unoxidized (White et al., 1973). Intermediates corresponding to the non-oxidized tylosin and rifamycin have been isolated mainly from blocked mutants and do not normally accumulate in the medium.

F.) O-Methylations

In general, the methyl donor in biological methylations is methionine, activated in the form of S-adenosylmethionine (SAM) (Greenberg, 1963). The resultant S-adenosylhomocysteine is hydrolyzed, and homocysteine is "re-methylated" by N-methyltetrahydrofolate to regenerate methionine.

The O-methyltransferases isolated from erythromycin and tylosin producers both require SAM and exhibit very high substrate specificity. SAM:Erythromycin C O-methyltransferase will O-methylate the L-cladinose

Figure 17: Proposed scheme for hydroxylation and interconversion of antibiotics via an epoxide intermediate. (adapted from Pandey et al., 1971)



of erythromycin C to form erythromycin A, but will not O-methylate erythromycin B; B and C only differ by a hydroxyl at C-12, a site far removed from the methylation site (Corcoran, 1975) (See Figure 18). The above methylation reaction has been shown to be irreversible. In the biosynthesis of tylosin, 2'''O-methylation must preceed 3'''O-methylation in the formation of the mycinose moiety, each methylation being carried out by a separate enzyme (Baltz and Seno, 1981). The 3'''O-methylation is the final step in tylosin biosynthesis and increased 3'''O-methytransferase activity was well correlated with increased tylosin production (Seno and Baltz, 1981 and 1982).

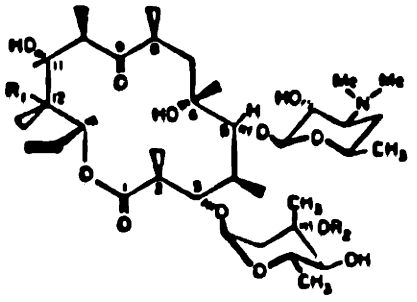
The effects on antibiotic production of several methylation inhibitors have been studied. C-, N-, S-, and O-Methylations have all been found to be inhibited by ethionine, and most by sulfonamides (Argoudelis et al., 1970 and 1973; Lancini and White, 1973). The presence of ethionine can also cause analogous "transethylations" to occur.

G.) Unusual Sugars

Peptidolactones and ansamycins do not contain sugars, but typical macrolides and many polyenes do have at least one sugar moiety attached to the aglycone ring. D-Glucose is incorporated without skeletal rearrangements into the amino- and deoxysugars found in macrolides (Omura, 1981). These sugars often contain O-, N-, or C-methyl groups arising from methionine. Less is understood about the biosynthesis of the aminosugars of the polyenes, but the carbon skeleton probably arises from glucose or mannose (Martin, 1979 and 1983).

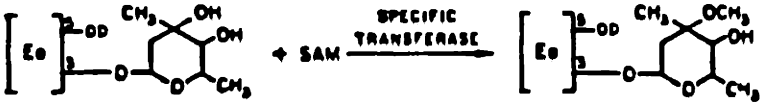
Figure 18: Conversion of erythromycin C into erythromycin A in the presence of S-adenosyl-L-methionine (SAM).

(Corcoran, 1975)



	R_1	R_2
ERYTHROMYCIN A	OH	CH_3
B	H	CH_3
C	OH	H

Structure of erythromycins A, B, and C.



Erythromycin C

Erythromycin A

From studies on candicidin and candihexin (Martin and Gil, 1979; Martin and McDaniel, 1975a), it has been suggested that attachment of the aminosugars takes place during excretion of these polyenes. Only the non-glycosylated forms are found intracellularly, while the glycosylated forms are mainly found extracellularly. Without the aminosugar attached, candihexins show no antifungal activity. The aglycones of several macrolide antibiotics have also been isolated. Some were accumulated by blocked mutants or after the addition of sodium arsenite (Maezawa et al., 1974c) or organic acids (Maezawa et al., 1974a and 1974b) to the medium, while others accumulate in small amounts in the normal production of sugars, while the mechanism by which the organic acids act is unclear.

H.) Co-Products

Many antibiotics are produced as members of families, i.e. mixtures of biosynthetically related antibiotics and intermediates. Given the number of steps and enzymes necessary for the complete production of representative antibiotics, it is not surprising that a certain percentage of the products might result from the bypassing of steps, especially when diffusible intermediates are involved. Also, after biosynthesis is complete, the antibiotic can be non-enzymatically oxidized or hydrolyzed. The candihexin (polyene) complex contains at least six different components, two of the major components containing aminosugars while two others correspond to their aglycones (Martin and McDaniel, 1975b). The remaining two are very unstable and their structures ar unknown. The relative amounts of the components change

during the course of the fermentation (Martin and McDaniel, 1975a). Organisms have been found which coproduce polyenes containing different lengths of the polyene chromophore. Amphotericin B (a heptaene) is co-produced with amphotericin A (a tetraene plus diene); mycoheptin is coproduced with mycopentin; the candihexin complex contains a small amount of a heptaene component (Martin, 1979). Two strains which are producers of the macrolides methymycin and neomethymycin were also found to produce picromycin, the three antibiotics being isomers in their sites of hydroxylations and attachment of the aminosugar (Devoe, 1963). The only structural difference between the macrolides tylosin and relomycin is that the aldehyde group of tylosin is reduced to the corresponding alcohol in relomycin. Early in the tylosin fermentation, enzymes quickly oxidize the alcohol so that mainly tylosin is accumulated, while late in the fermentation tylosin is slowly reduced to relomycin (Seno and Baltz, 1982). The original rifamycin-producing strain of Norcardia mediterranea produces at least seven rifamycin-type compounds, two of which are biologically inactive (Lancini, 1983). Peptidolactones are also produced as mixtures, the components differing mainly in the degree and location of methylation and oxidation (Okumura, 1983).

The presence of products very similar to the desired antibiotic is usually undesirable, as they are often difficult to remove during purification procedures and compete for precursors. In the case of rifamycin, however, intermediates B and S have been chemically modified to produce semisynthetic derivatives; one of these, rifampicin, is

commercially important (Lancini and White, 1973). Co-products are also useful in examining the structure activity relationships for differing types of antibiotics.

II. C.) ^{13}C-NMR: Applicability to Biosynthetic Studies

The number of references using ^{13}C-NMR for the determination of the precursors of natural products grows every year (Omura and Nakagawa, 1981; Scott and Baxter, 1981; McInnes et al., 1976). While radioactive labelling is still the method of choice for some experiments, advances in NMR instrumentation and interpretation (Neuss, 1975; Cooper, 1976) have made ^{13}C-NMR increasingly attractive. Pulsed Fourier-transform techniques (in which successive scans are added together to enhance the signal to noise ratio) and the use of larger sample tubes have resulted in greatly increased sensitivity. Small sample sizes can be compensated for by increasing the number of scans recorded. Once the assignments have been made to the ^{13}C-NMR natural abundance spectrum (sometimes a noteworthy achievement in itself), ^{13}C-NMR can give both the location and the degree of enrichment of a sample after incorporation of ^{13}C-labelled precursors. The sample is not destroyed during analysis and can be recovered, unlike ^{14}C experiments which involve tedious degradation of the molecule to locate the sites of incorporation.

An enrichment of approximately 1% is generally considered significant. (Given a natural abundance of 1.1%, an enrichment of 1% will approximately double the height of the spectral peak corresponding to the enriched carbon.) It is important to first check and optimize the level of incorporation using ^{14}C-labelled precursors. One must interpet the corresponding ^{13}C-NMR spectra carefully, always keeping in mind the following complications of labelling studies:

1.) Permeability : In working with any living organism, the

precursor must get through the cell membrane, either by way of diffusion or by way of a transport system, to be incorporated. (However, for a few reactions, i.e. attachment of aminosugars to aglycones, the enzymes may be located in or near the membrane.) If the proposed precursor is not taken up by the cells, various approaches can be used. Methyl and ethyl esters of precursors are sometimes taken up when the free form is not. The cells can be "permeabilized" with ether (Felix et al., 1980) or other chemicals (Ghisalba et al., 1982) or cell free systems can be used, although these methods tend to decrease the production of antibiotics.

2.) "Scrambling" : When a labelled compound enters a cell, it may be incorporated directly, or it may be metabolized to another compound and then incorporated. In the latter case, the label is "scrambled" into more than just the originally added precursor and is found in more sites in the product than would otherwise be predicted. In studies on rifamycin biosynthesis, 2-^{13}C-acetate enriched the three carbons predicted to be derived from the acetate methyl group, but also enriched sites labelled by 2-^{13}C- and 3-^{13}C-propionate in separate experiments (White et al., 1973). This is consistent with the expected conversion of acetate to methylmalonyl-CoA via citrate and succinate (i.e. via the tricarboxylic acid cycle and methylmalonylmutase) (Lehninger, 1975). In the case of tylosin, 2-^{13}C-acetate labelled propionate-derived carbons, again suggesting scrambling via the tricarboxylic acid cycle. Also, 1-^{13}C-butyrate labelled carbons derived from C1 of propionate, and 1'-^{13}C-ethylmalonate enriched carbons derived from C2 of propionate

(Omura, 1981). This implied the incorporation of butyrate following conversion to methylmalonyl-CoA, involving the migration of a carboxyl group. This was further substantiated by the appearance of ^{13}C-^{13}C coupling patterns between propionate-derived carbons when 1,3,1'-^{13}C-ethylmalonate was fed.

In the case of virginiamycin M1 (a peptidolactone), D,L-3-^{13}C-serine labelled the serine moiety to the largest extent, but also carbons believed to be derived from acetate, methionine, and valine (Kingston and Kolpak, 1980). This is consistent with the formation of acetyl-CoA from serine via pyruvate, transfer of C3 from serine to methionine via tetrahydrofolate, and the biosynthesis of valine from serine. 1-^{13}C-Glucose and 6-^{13}C-glucose were shown to be interconvertible via triosephosphate intermediates in the glycolytic and gluconeogenic pathways (Hollander and Shulman, 1983) in intact yeast cells.

Low levels of incorporation via scrambling are tolerable, while high levels make labelling studies difficult to interpret. To determine the direct precursors, several experiments can be done, and in each a singly labelled substrate is fed. The resulting spectra are compared, and interpreted in terms of known pathways. The scrambling is often "unidirectional", i.e. 2-^{13}C-acetate will label carbons derived from the 2- and 3-carbons of propionate, but 3-propionate will not label 2-acetate derived carbons, so assignments of precursors can be made by observing the differences between spectra. Addition of unlabelled "scrambling products" along with labelled precursors may suppress

scrambling, i.e. addition of valine along with 1-^{13}C-serine may prevent labelling of a valine moiety by serine. The use of doubly or multiply labelled precursors (^{13}C label on adjacent carbons) allows for the direct detection of intact carbon units, a feat not feasible with ^{14}C labelling. For example, 1,2-$^{13}C_2$-acetate has been fed (London et al., 1975) and the resulting spectra examined for ^{13}C-^{13}C coupling patterns (satellite peaks), which should only arise at carbons enriched directly by this precursor. Metabolic scrambling will still transfer some of the label to propionate carbons, but after this conversion the ^{13}C labelled carbons are no longer adjacent, and thus there would be no ^{13}C-^{13}C coupling patterns at carbons enriched by this generated propionate.

Indirect incorporation can be put to good use, if the major routes of metabolism are understood. The use of 1-^{13}C-glucose and 1-^{13}C-glycerate were used to indirectly establish the shikimate pathway origins of various structures (see Literature Survey, II. B. Starter Units/Shikimic Acid Pathway).

3.) Competing pathways : A potential precursor of the product under study may also be a precursor of several other products of the cell, or may be utilized for energy production. For example, acetate can be used to make fatty acids or converted to CO_2 and energy. If competing pathways are very active, large amounts of precursors will have to be added before significant incorporation into the desired product is observed. To reduce the amount of (usually expensive) precursor required, attempts are made to suppress the competing pathways. For studies involving secondary metabolism, precursors are

usually added when the growth is slowing down and product formation is just starting. In this way, primary metabolism will compete less for precursor. Also, inhibitors of competing pathways can be added, but these may also suppress production of the desired products as well by upsetting the balance of the cell's metabolism.

4.) Endogenous Precursor : Labelled precursor added to the medium will be isotopically diluted by any unlabelled precursor present in the medium or produced by metabolism within the cell. This isotopic dilution increases the need for labelled precursor, and thus medium ingredients which contain or readily generate unlabelled precursor are to be avoided. Cells could also be grown in one medium, then transferred to a fresh medium or buffer for addition of the label and production, thereby removing any organic acids and other products excreted during growth. While this technique has been successful in some cases, disturbing the cultures can have detrimental effects on the production of antibiotics.

5.) Toxicity : While increasing the concentration of labelled precursor in the medium should increase the degree of enrichment of products, above certain concentrations precursors can be toxic to the producing strain. For example, acetate and other organic acids are known to cause cell lysis (McInnes, 1976). The maximum concentration tolerable can be determined using unlabelled precursors. The problem of toxicity can sometimes be lessened by feeding the precursor in several small doses.

6.) Repression/Inhibition : Many readily utilized carbon- and

nitrogen-containing compounds are known to cause catabolite repression of secondary metabolism (Demain et al., 1983). Unlabelled forms of potential precursors should be used to determine whether the concentrations required for significant incorporation will cause a decrease in the total titer of product. Again, slow feeding of the precursor may improve production while still maintaining the necessary incorporation level.

7.) Commercial Availability : While the number of commercially available ^{13}C-labelled compounds is increasing, many useful compounds are only available through custom synthesis. The same routes for the synthesis of ^{14}C-labelled compounds can be applied to ^{13}C analogs, without the problem of radioactive contamination.

III. MATERIALS AND METHODS

A.) Strains:

1) Streptomyces hygroscopicus strain AY-B1206 was obtained from Ayerst Research Laboratories, Montreal, Canada. This strain was a direct soil isolate found during a re-examination of the soil sample which contained the original rapamycin-producing strain (AY B994, deposited as ATCC 29253 and NRRL 5491). It was reported (personal communication, Dr S.N. Sehgal) to produce higher levels of rapamycin and little or no demethoxyrapamycin when compared to the original isolate.

2) Klebsiella pneumoniae ATCC 25597 (formerly Aerobacter aerogenes, Tabor A170-40) was obtained from the American Type Culture Collection. This mutant is missing the enzyme which converts shikimic acid-5-phosphate to 3-enolpyruvyl shikimic acid-5-phosphate. It is therefore auxotrophic for all aromatic amino acids and vitamins and cannot use shikimic acid for growth.

B.) Media:

1) Sporulation agar (Ayerst M84 agar): One liter of agar contains 20 g glucose, 1 g KNO_3, 0.5 g K_2HPO_4, 0.1 g KH_2PO_4, 0.5 g NaCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, and 22 g agar (Difco). The pH was adjusted to 6.8. The agar surface was allowed to dry for 24 to 48 hours before being inoculated with Streptomyces hygroscopicus spores.

2) Fermentation medium for labelling studies:

a) Basal medium: The following were added to 900 ml distilled water: 2.0 g K_2HPO_4, 2.0 g KH_2PO_4, 5.0 g NaCl, 1.5 g $CaCO_3$ 20 ml

glycerol, 1.0 g L-leucine, 2.0 g L-glutamic acid, 4.0 g L-lysine·HCl, 5.0 g yeast extract (Difco), 0.06 g $ZnSO_4$, 0.256 g $MgSO_4.7H_2O$, 0.012 g $MnSO_4.H_2O$, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.018 g $(NH_4)_6Mo_7O_{24} \cdot H_2O$, 0.01 g $Na_2B_4O_7 \cdot 10H_2O$, 0.01 g $CoCl_2 \cdot 6H_2O$, 0.0013 $CuCl_2 \cdot 2H_2O$, 0.51 g $MgCl_2 \cdot 6H_2O$, and 0.36 g $NaSO_4$. (The latter 10 inorganic salts were added as 10 ml of a 100-fold concentrated solution.) The pH of the solution was adjusted to 6.0. Forty-five ml of this solution were measured into each 500 ml unbaffled Erlenmeyer flask. The flasks were autoclaved 20 min at 121 C. After cooling, 5.0 ml of a 20 g glucose/100 ml H_2O solution were added to each flask.

b) Ammonium phosphate-based medium (AmP medium): The composition of AmP medium is the same as the basal medium, except that it contains 5 g/l $NH_4H_2PO_4$ and lysine HCl is omitted.

3) Modified Davis A medium for the production of shikimic acid by Klebsiella pneumoniae: The original Davis A medium contained citrate, which was later found to inhibit the accumulation of shikimic 5-phosphate by Klebsiella pneumoniae ATCC 25597 (formerly Aerobacter aerogenes mutant A170-40) (Weiss and Mingioli, 1956). Citrate was also found to decrease the total production of shikimate and shikimate-5-phosphate and was therefore omitted from the medium. The following components were dissolved in 900 ml distilled water: K_2PO_4, 7.0g; KH_2PO_4, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $(NH_4)_2SO_4$, 1.0 g; L-tryptophan, 5 mg; L-tyrosine, 10 mg; D,L-phenylalanine, 20 mg; p-aminobenzoic acid, 10 ug; p-hydroxybenzoic acid, 10 ug. The medium was distributed into flasks (90 ml per 500 ml flask) and autoclaved for 15 min at 121 C. After

cooling, 10 ml of a sterile glucose solution was added, and the flasks were then inoculated with a colony of K. pneumoniae from an agar plate. Unlabelled glucose was autoclaved while ^{13}C-glucose was filter-sterilized.

4) Luria-Bertani (LB) Agar (for enteric bacteria): The following were dissolved in 1000 ml of distilled water: 10 g Bacto-tryptone, 5 g Bacto yeast extract, and 10 g NaCl. The pH was adjusted to 7.2 with NaOH. 20 g/l Bacto-agar was added and the solution autoclaved at 121 C for 15 min. Plates containing approximately 30 ml were poured, allowed to cool, and dried for one to two days before inoculation.

C.) Culture conditions:

1) Streptomyces hygroscopicus strain AY-B1206 (Ayerst Laboratories, Montreal, Canada), a direct soil isolate, was used for all fermentations. A stock of spores mixed with sterile soil was stored at room temperature under vacuum in a desiccator. To prepare the inoculum for fermentations, a small portion of spore/soil mixture was transferred to a bottle containing a layer of M84 agar medium. One ml of sterile water was added and the spores were distributed across the agar surface. Bottles were incubated for 14-21 days at 25 C. During this time, the culture changed from white to dark grey and sporulated. A spore suspension was prepared by adding sterile H_2O to a bottle and suspending the spores by scraping the agar surface with an inoculating loop or spatula. This dilute suspension was transferred to the next bottle, the spores suspended, and so on until spores from 200 to 250 cm^2 (5 to 6

Blake bottles) had been collected. The volume of the dark black suspension was adjusted to approximately 15 ml. For labelling work, one ml of the suspension was used to inoculate each 50 ml of fermentation medium in 500 ml unbaffled flasks. (For some experiments, especially where large numbers of flasks were required, 25 ml of fermentation medium was placed in 250 ml flasks.) Fermentation flasks were incubated at 25 C with constant shaking (2" stroke, 250 rpm) until the pH reached 7.0, usually 6 to 7 days.

During the course of the fermentation, sterile 1 ml samples of broth were removed. The pH of each sample was measured. These samples were also assayed for rapamycin as descibed below.

To determine the dry cell weight of the cultures, 2 ml 3 N HCl were added to each 50 ml flask to dissolve any remaining $CaCO_3$. The complete contents of a flask were then collected by filtration, dried at 90 C for 24 hours, and weighed.

A minimum of two flasks were sampled for each determination of pH, dry cell weight, and rapamycin content.

2) Klebsiella pneumoniae was stored on either LB plates or plates made from Davis A medium with 2% agar added. The culture was transferred to a fresh plate every 2-3 months to maintain a viable stock culture. To prepare the fermentation inoculum, an LB plate was inoculated and incubated for two days at 37 C to obtain large colonies. These plates were used to inoculate fermentations within a week.

D.) Procedures:

1) HPLC Assay for Rapamycin:

Nine ml of methanol were added to each 1 ml sample of fermentation broth, the tubes tightly sealed, and shaken several times during the next 20 min to extract the rapamycin. The tubes were then centrifuged and the supernatants filtered through glass fiber filters or solvent-resistant 0.45 um filters. These samples were either analyzed immediately or stored at -20 C until they could be analyzed. The rapamycin content was determined by reverse phase HPLC (Waters uBondapak C-18 column, 4 mm x 30 cm; mobile phase 70% methanol/30% water, 2 ml/min; detected at 254 nm and 280 nm, Waters Model 440 UV and Waters Lambda-Max Model 480 absorbance detectors at 0.01 aufs). 50 ul of extract was injected, rapamycin having a retention time of approximately 30 min. After the rapamycin peak had eluted the column was washed with 100% methanol for 5 min to elute the remaining components in the extract. (Other than the large peak which elutes near the void volume, the rapamycin peak is the largest peak in the first 30 minutes of the chromatogram. If the elution is continued with 70% methanol, several large 254 nm-absorbing peaks elute after rapamycin.) The column is then re-equilibrated with 70% methanol before the next injection.

Purified rapamycin (a gift of Ayerst Laboratories) was dissolved in methanol and used to prepare a series of standard solutions. A 10 ug/ml standard solution gave a peak of approximately 50% to 70% of the full scale. The concentration of rapamyin was found to be directly proportional to peak height.

2) Small-Scale Recovery and Purification of Rapamycin:

To prepare rapamycin samples for NMR and TLC analysis, the contents of several fermentation flasks were combined. The mycelium was collected by centrifugation for 10 min (4000x g, GSA rotor) at room temperature. The pellet was resuspended in distilled water and centrifuged again. The pellet was then resuspended in approximately 4 times its volume of methanol. This mixture was shaken for approximately 20 min to extract the rapamycin, and then centrifuged for 10 min. The supernatant (methanol) was removed, and the extraction repeated two more times with similar volumes of fresh methanol. The extracts were combined and the methanol removed by rotary evaporation. The resulting oil was dissolved in CH_2Cl_2 (150 ml per liter of broth being processed), to which an equal volume of water was added. A dark emulsion layer often appeared at the interface. The CH_2Cl_2 layer was removed, and the oil/emulsion was extracted 2 more times with similar volumes of CH_2Cl_2. The CH_2Cl_2 extracts were pooled and the solution evaporated to dryness. The resulting residue was then extracted with several portions of hexane. The hexane was removed by rotary evaporation, leaving a dark yellowish oil. At this stage, the sample could be redissolved in a known volume of hexane and a portion applied to TLC plates (as in the experiments for determining ^{14}C incorporation). Alternatively, the sample could be dissolved in approximately 2 ml methanol and further purified by semipreparative-scale HPLC.

A portion of the sample was diluted and used to optimize the

conditions for the semipreparative reverse phase column (Waters uBondapak C18, 8 mm x 30 cm; 254 and 280 nm UV detectors; typically 65% methanol/35% water, 5 to 7 ml/min). The sample was injected approximately 100 ul at a time. The center portion of the rapamycin peak was collected. (The beginning and end portions of the peak were discarded as they were relatively dilute and possibly contained contaminants from neighboring peaks.)

The collected fractions were pooled and concentrated by one of two procedures. For most of the samples, the fractions were chilled in an acetone/dry ice bath, and the methanol/water evaporated under vacuum in a lyophilizer. For the last three samples (2-^{13}C-propionate, $^{13}C_2$-glycine, and ^{13}C-shikimic acid enriched rapamycin), C-18 reverse-phase cartridges (Maxi-Clean Cartridges, Alltech, Deerfield, Illinois) were used to concentrate the purified rapamycin. Sufficient water was added to the pooled fractions to bring their concentration to approximately 40% methanol, and then the solution was passed through the cartridges to bind the rapamycin to the C-18 packing. Air was passed through the cartridges briefly to remove as much solvent as possible, and then the rapamycin was eluted with pure methanol. The methanol was then removed by rotary evaporation without heating, and any remaining traces of water were removed by lyophilization. After either concentration procedure, the rapamycin (white fluffy solid) was dissolved in CH_2Cl_2, the solution transferred to a small flask, and the CH_2Cl_2 removed by rotary evaporation. The sample was stored at -20 C until the analysis by ^{13}C-NMR.

3) ^{13}C-NMR Analysis Conditions:

For the early samples, a Bruker 270 NMR Spectrometer with proton noise decoupling was used for the observation of the ^{13}C-NMR spectra at 67.9 MHz. A sweep width of 16000 Hz was covered by 16K data points. A pulse width of 12 to 16 usec (equivalent to a 40 degree pulse) was used, followed by an accumulation time of 0.4 sec and a further delay of 1.5 sec. To enhance sensitivity, each free induction decay was exponentially multiplied by 4 Hz. Spectra were recorded at an ambient probe temperature of approximately 30 to 35 C. The number of transients accumulated ranged from 14000 to 16000 for the natural abundance sample (100 mg sample) and 8000 (for highly enriched samples) to 17000 (12 to 18 hours) for the enriched samples (approximately 10 mg sample).

For some of the later samples, a home-built 360 MHz wide-bore spectrometer was available, providing both increased resolution and sensitivity. The ^{13}C observation frequency was 90.4 MHz. A pulse width of 18.5 usec (equivalent to a 90 degree pulse) was used to accumulate data. Protons were decoupled with a WALTZ decoupling routine for 5 sec prior to acquisition. The 90 degree ^{1}H pulse in this mode was equal to 180 usec. The relaxation delay time after data acquisition was 4 sec. The sweep width of 20000 Hz was covered by 16K data points. To enhance sensitivity, each free induction decay was exponentially multiplied by 2 Hz. Spectra were recorded at ambient probe temperature, approximately 22 C. The number of transients accumulated ranged from 4000 to 5000 for the natural abundance sample (100 mg sample) and 5000 to 6000 (up to 12

hours total accumulation) for the enriched samples (approximately 10 mg sample).

All samples were dissolved in $CDCl_3$, except where noted.

4) Determination of Amino acids by OPA Technique:

The method of Hill et al. (1979) was used to determine the amino acid content of various samples. This method is most accurate for samples containing 20-500 pmol of each amino acid. The amino acids are first reacted with orthophthaldehyde (OPA) and ethanethiol to form fluorescent derivatives, and then these derivatives are separated and quantitated by reverse-phase HPLC. Only primary amino acids will react with OPA under these conditions, so proline is not detected. Cysteine also cannot be detected without first modifying the thiol group.

Before derivatization, the sample is centrifuged and diluted as necessary. To derivatize the sample, 100 ul sample is mixed with 100 ul saturated borate buffer, 50 ul OPA cocktail, and 250 ul methanol. (OPA cocktail contains 50 mg OPA, 4.5 ml methanol, 0.5 ml saturated borate buffer, pH 9.5, and 50 ul ethanethiol.) After 2 min, 20 ul were injected into the HPLC system (Waters C-18 uBondapak reverse phase column, Model 660 Solvent Programmer, Model 420 Fluorescence Detector with 395 nm band pass excitation filter and 455 nm long pass emission filter). At the time of injection the column is equilibrated with 15 mM sodium phosphate buffer, pH 7.2 (buffer A), 2.0 ml/min. Immediately after injection, a linear gradient was initiated, changing the solvent composition to 50% buffer A, 50% acetonitrile, after 45 minutes. After

the last amino acid (lysine) has eluted, the column was returned to the initial conditions using a 5 min gradient and re-equilibrated for 10 min before the next sample was injected. Amino acid concentration was found to be protional to the peak height.

5) HPLC Method for the Determination of Organic Acids and Sugar Content:

A Biorad HPX87H organic acids column with Aminex precolumn was used to determine acetate, glucose, and glycerol concentrations in the media. This column resolves many organic acids, sugars, and other fermentation products and substrates, including glucose, glycerol, and shikimic acid. After centrifugation, 50 ul of sample was injected into the HPLC system (0.5 ml/min flow rate, 5 mM H_2SO_4, pH 2.3, mobile phase; refractive index detector; Waters WISP Automatic Injector 710B; Hewlett Packard 3390A Integrator).

6) Hydrolysis of radioactive rapamycin samples:

Preparative experiments showed that high concentrations of NaCl (as would be generated by neutralization of an NaOH hydrolysis mixture with HCl) caused severe band spreading of pipecolate standards in the TLC systems tested. $Ba(OH)_2$ was therefore used, since it can be neutralized with H_2SO_4 and the resulting $BaSO_4$ is insoluble and can be removed by centrifugation. Heating ^{3}H-pipecolate in methanolic solutions which had been acidified with H_2SO_4 caused the counts to appear at the solvent front of the TLC chromatogram, implying that pipecolate had been converted to a less polar compound. Drying the ^{3}H-pipecolate hydrolysates in the presence of $Ba(OH)_2$ and $BaSO_4$ (basic solution)

hydrolysates in the presence of $Ba(OH)_2$ and $BaSO_4$ (basic solution) caused the counts to remain in the tube (bound to the glass or the $BaSO_4$ precipitate), but they could be removed by the addition of H_2SO_4. Therefore several pH changes are incorporated into the final hydrolysis procedure, such that pipecolate is always heated under basic conditions and transferred under acidic conditions.

Rapamycin samples were purified by semipreparative HPLC, concentrated, and dissolved in a small volume of methanol. An aliquot was removed and the specific activity of the solution was determined. An aliquot (depending on the specific activity of the solution, 50 to 300 ul, 2,000 to 20,000 cpm) was transferred to a 1 ml glass ampule. Additional methanol was added to bring the total volume to 300 ul. 100 ul of a pipecolate solution (2 mg/ml) and 100 ul 0.6 M $Ba(OH)_2$ solution was added to each ampule. An aliquot of a methanolic solution of ^3H-pipecolate, methanol, unlabelled pipecolate solution and $Ba(OH)_2$ was transferred to an additional ampule. Another ampule received only unlabelled pipecolate solution, methanol, and $Ba(OH)_2$. Ampules were sealed using a glass-blowing torch. All ampules were placed inside 13 mm test tubes and placed inside a heating block at 80 C. After 24 hours, the ampules were cooled and opened. The hydrolysate solution in each ampule was acidified with 1 M H_2SO_4. The resultant solution (and $BaSO_4$ precipitate) was transferred to a 1.5 ml snap-cap tube. The ampule was rinsed with additional methanol and these rinses were added to the snap-cap tube. The tubes were centrifuged at approximately 10,000 x g for 2-3 min. The supernatants were transferred to 13 mm

additional 1 ml aliquots of methanol, and these rinses were combined with the first supernatants. The solutions were made slightly alkaline with $Ba(OH)_2$. The tubes containing the supernatants were placed in a heating block and the evaporation of the solution was accelerated by a gentle stream of air blown into the tube. When the tubes were dry, they were immediately removed from the heating block. Approximately 50 to 100 ul 1 M H_2SO_4 and 100 ul methanol were added to each tube. After shaking, more acid was added if the solution was not yet acidic. These solutions were then applied as a streak to Silica-gel 60-F (Merck, 5 x 20 cm) glass TLC plates. The plates were developed simultaneously in a chamber containing methanol:$CHCl_3$:17% NH_4OH (2:2:1). After development, the plates were removed and allowed to dry in the hood for 20-30 min. The plate which had received the unlabelled pipecolate sample was sprayed with a ninhydrin solution (10 mg ninhydrin, 10 ml butanol, 1 ml acetic acid) and heated at approximately 90 C until the purple-blue pipecolate band was visible. The plates were then laid side-by-side and bands of equal width were drawn across the plates. The location of the pipecolate band was noted (relative to these markings). The bands were then removed from the glass plate by scraping with a razor blade and transferred to scintillation vials for counting.

7) Chemical assay for shikimic acid:

The method used was basically that of Yoshida and Hasegawa (1957), except that all reagent volumes were reduced by half. The periodate reagent and the aniline solution were prepared fresh daily. To 0.5 ml sample solution in a glass test tube was added 0.5 ml periodate reagent

(160.5 mg sodium periodate in 25 ml acetate buffer, pH 4.7, 0.5 M acetic acid and 0.5 M sodium acetate). The tube was mixed well and incubated at 30 C for 15 min or 25 min at room temperature. Next, 0.5 ml ethylene glycol solution (1.0 ml ethylene glycol in 100 ml distilled water) is added to the tube and the solutions mixed well and incubated for another 5 to 10 min at the same temperature. To the tube is then added 0.5 ml aniline solution (distilled water saturated with aniline at room temperature). The tube is then shaken and left at room temperature for 5 min. Absolute alcohol (2.5 ml) is then added to the tube with shaking and a clear red solution results if shikimate is present. The optical density at 510 nm is then recorded. All unknowns and standard curve samples must be treated in the same fashion, and standards must be run each time, since the results are sensitive to incubation time and temperature. If the samples have been acid hydrolyzed, the OD must be measured quickly and the standards must also be prepared in the same concentration of acid and heated for the same amount of time, since high concentrations of acid increase the intensity of the resulting red color but decrease its stability. This assay is specific for shikimic acid. Shikimic-5-phosphate must be hydrolyzed by heating in acid or by incubation with acid phophatase (Sigma). To hydrolyze the samples, they are mixed with an equal volume of 0.2 N HCl in glass tubes, then capped with plastic caps and autoclaved. Approximately 40 to 50% of the shikimate should be released after 1 hour of autoclaving, while autoclaving overnight releases approximately 70 to 80% (Weiss and Mingioli, 1956). Shikimic acid is very stable in acid, but unstable in

base.

8) Preparation of the trimethylsilyl derivative of shikimic acid:

Two groups had published procedures for the determination of shikimic acid and sugars in plant extracts using trimethylsilyl derivatization and gas chromatography. Ericsson et al. (1978) used DMF as the solvent and only a 30 min reaction time. Derivatives were prepared using this method but several peaks were observed, one of which had a mass spectrum resembling that of the desired derivative of shikimic acid. It was concluded that the derivatization was incomplete, and so the other procedure was tried. In this method pyridine is used as the solvent and the reaction is left overnight (Cranswick and Zabkiewicz, 1979).

Aliquots of solutions of either labelled or unlabelled shikimic acid were transferred to screw-cap vials. These were freeze-dried and then capped until derivatization. To each vial was added 200 ul pyridine, 100 ul hexamethyldisilazane, and 50 ul trimethylchlorosilane. The vials were then capped and left at 35 C overnight. The liquid was then evaporated by passing dry nitrogen through the vial. Hexane (200 ul) was added to the vial to dissolve the derivative. A white residue did not dissolve but did not interfere with the mass spectral analysis because it stayed on the walls of the vial. The addition of 1% trimethylsilylimidazole was recommended to improve the stability of the derivative. This was added to some duplicate samples but did not appear to affect the mass spectra. A Hewlett-Packard GC/MS system with

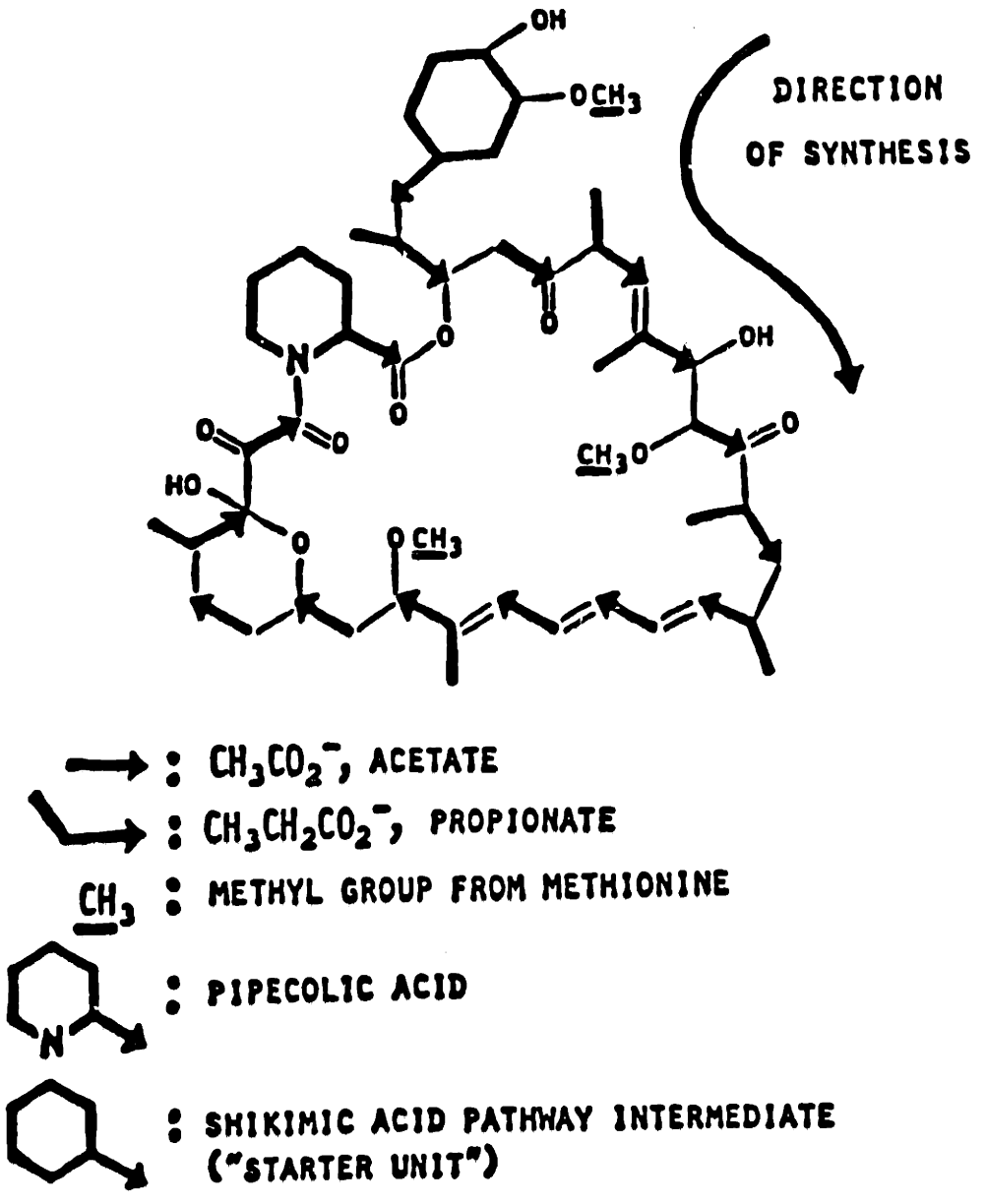
microbore column and electron-impact ionization was used to analyze the samples.

IV.) Proposed Scheme for Rapamycin Biosynthesis.

In order to design experiments to determine the biosynthetic precursors of rapamycin, a possible scheme for rapamycin biosynthesis was needed. The scheme I proposed is summarized in Figure 19. Based on previous findings for other systems, the proposed precursors of rapamycin, their orientations in the molecule, and the direction of synthesis are indicated. I proposed that the biosynthesis is initiated by a cyclic C_7 unit from the shikimic acid pathway ("starter unit"), activated as a thioester. A long polyketide chain would then be built up from the condensation of seven acetate and seven propionate units, participating in the reaction in the form of malonyl- and methylmalonyl-CoA. Chain growth would be terminated by the attachment of the pipecolate moiety, resulting in peptide bond formation between the last acetate carboxyl and the pipecolate nitrogen. The pipecolate carboxyl group and the C22 hydroxyl would then interact to close the ring by forming a lactone linkage. Either during the chain growth or after the molecule is released from the postulated thio-enzyme linkage, four oxygen functionalities or double bonds would be removed while two would be added, according to the simple polyketide model. Some of the keto groups would be reduced to hydroxyl groups and three hydroxyl groups would be methylated, presumably with S-adenosylmethionine acting as the methyl donor.

The above is perhaps the simplest scheme one can propose, but even it allows for a large number of possible pathways to rapamycin. These pathways would vary mainly in the order in which they modify the various

Figure 19: Proposed scheme for the biosynthesis of rapamycin.



sites along the carbon skeleton. Other simple variations include the following: (a) lysine could be incorporated directly and later cyclized to the pipecolic acid moiety, or (b) C_3 units thought to be derived from propionates could instead be formed from two carbons of acetate and one carbon from the methyl of methionine. Information gained from labelling studies will indicate if the proposed pathway is correct and if corrections are necessary. The above scheme clearly suggests certain precursors to be tested, along with a logical order for testing.

V. RESULTS

A) Development of Fermentation Medium

Table 2 shows the composition of the defined medium originally suggested by Ayerst (personal communication from Dr. S. Sehgal). It had been developed empirically but not optimized to any great extent, and was reported to support the production of 50 to 60 ug/ml rapamycin in 6 days. In my hands, however, cultures in this medium were severely pelletted and showed no production of rapamycin until 7 days; only 20 ug/ml was produced after 14 days, even when a very large spore inoculum was used. This medium appeared to be deficient in certain trace elements, and the concentrations of Co, Mo, Cu, B, Fe, and Zn were therefore increased. (Yields of 9 to 10 g/l dry cell weight had been observed in a complex medium, and the original concentrations of these elements were calculated to be insufficient to support 9 g/l cells.) Some improvements in rapamycin production and growth were observed. The medium was still apparently deficient in Mg, and a tripling of the Mg concentration improved the initial growth rate, the final dry cell weight (7 g/l) and rapamycin production (35 ug/ml after 9 days). A comparison of the fermentation profiles in the three media is shown in Figure 20.

While improvements had been made over the original medium, the cultures still contained very large pellets, grew slowly, and produced less rapamycin than previously reported. The growth kinetics were also not very reproducible. Dr. S. Sehgal (Ayerst; personal communication)

Table 2: Comparison of Three Defined Media with the Calculated Requirements of 9.0 g/l Cells

Element	Amount** Required by 9.0 g/l cells (calculated) (g/l)	Amount in Original Medium (g/l)	Amount in Original Medium plus Increased Trace Elements	Amount in Original Medium plus Increased Trace Elements and Mg	Provided as
N	0.90	0.91	0.91	0.91	glu, leu, lys
Mg	0.045	0.025	0.025	0.085	$MgSO_4$, $MgCl_2$
P	0.27	0.812	0.812	0.812	KH_2PO_4, K_2HPO_4
S	0.045-0.090	0.042	0.051	0.051	SO_4
Fe	0.0027	0.004	0.020	0.020	$FeSO_4$
Zn	0.013	0.011	0.014	0.014	$ZnSO_4$
Mn	0.00045	0.0033	0.0033	0.0033	$MnSO_4$
Cu	0.00027	0*	0.00031	0.00031	$CuSO_4$
Co	0.0003	0*	0.0024	0.0024	$CoCl_2$
Mo	0.0002	0*	0.0096	0.0096	$(NH_4)_6Mo_7O_{24}$
B	0.0005	0*	0.0011	0.0011	$Na_2B_4O_7$
K	0.126	1.47	1.47	1.47	KH_2PO_4, K_2HPO_4
C	4.5	18	18	18	glucose, glycerol

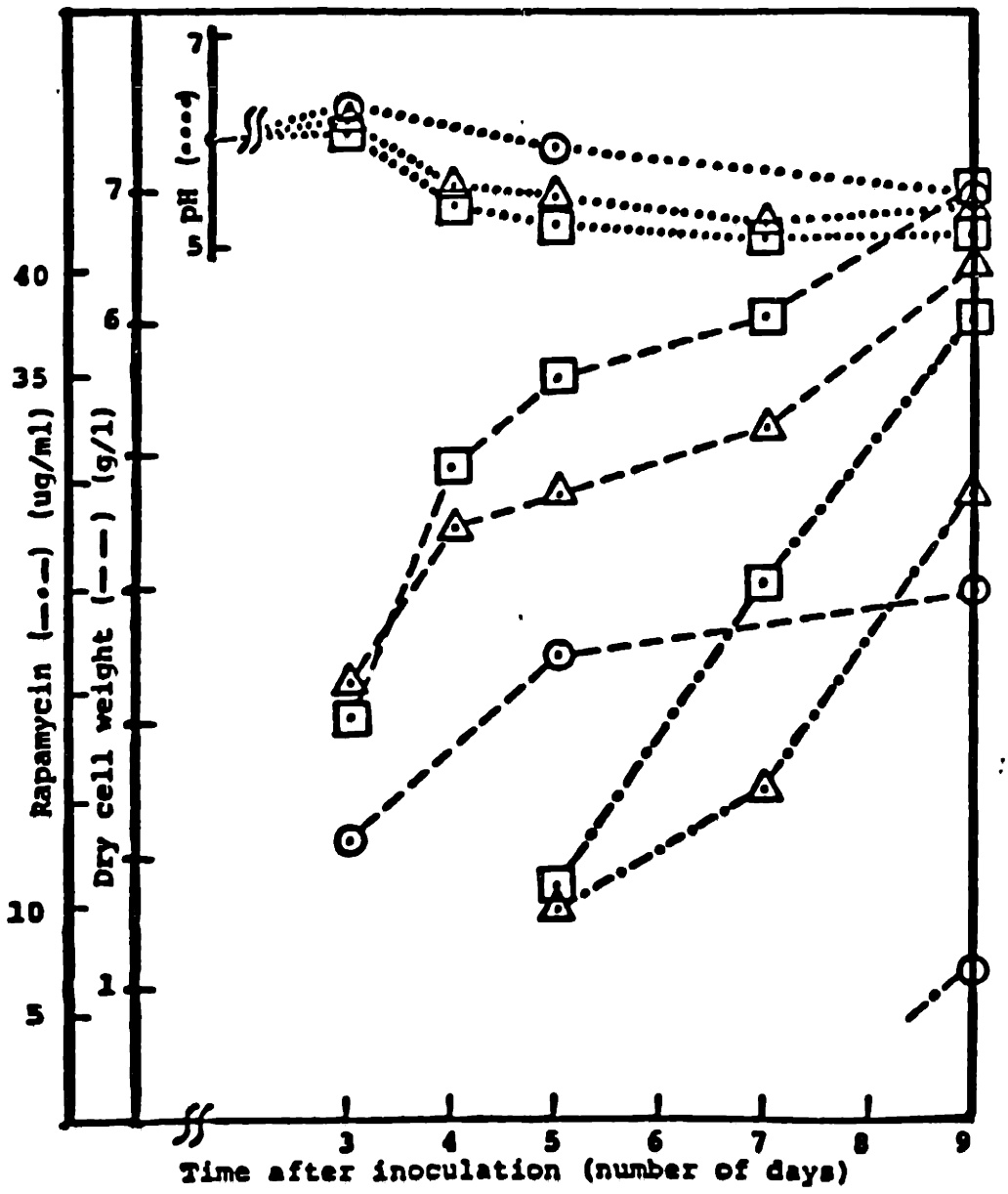
*= none added; Water and other media ingredients may provide trace amounts of these elements.
**= Haggstrom, 1973; Wang, 1979. Values estimated based on the reported average composition of various bacteria.

Figure 20: Comparison of Fermentation Profiles in Three Defined Media

⊙: original composition

△: concentrations of trace elements increased

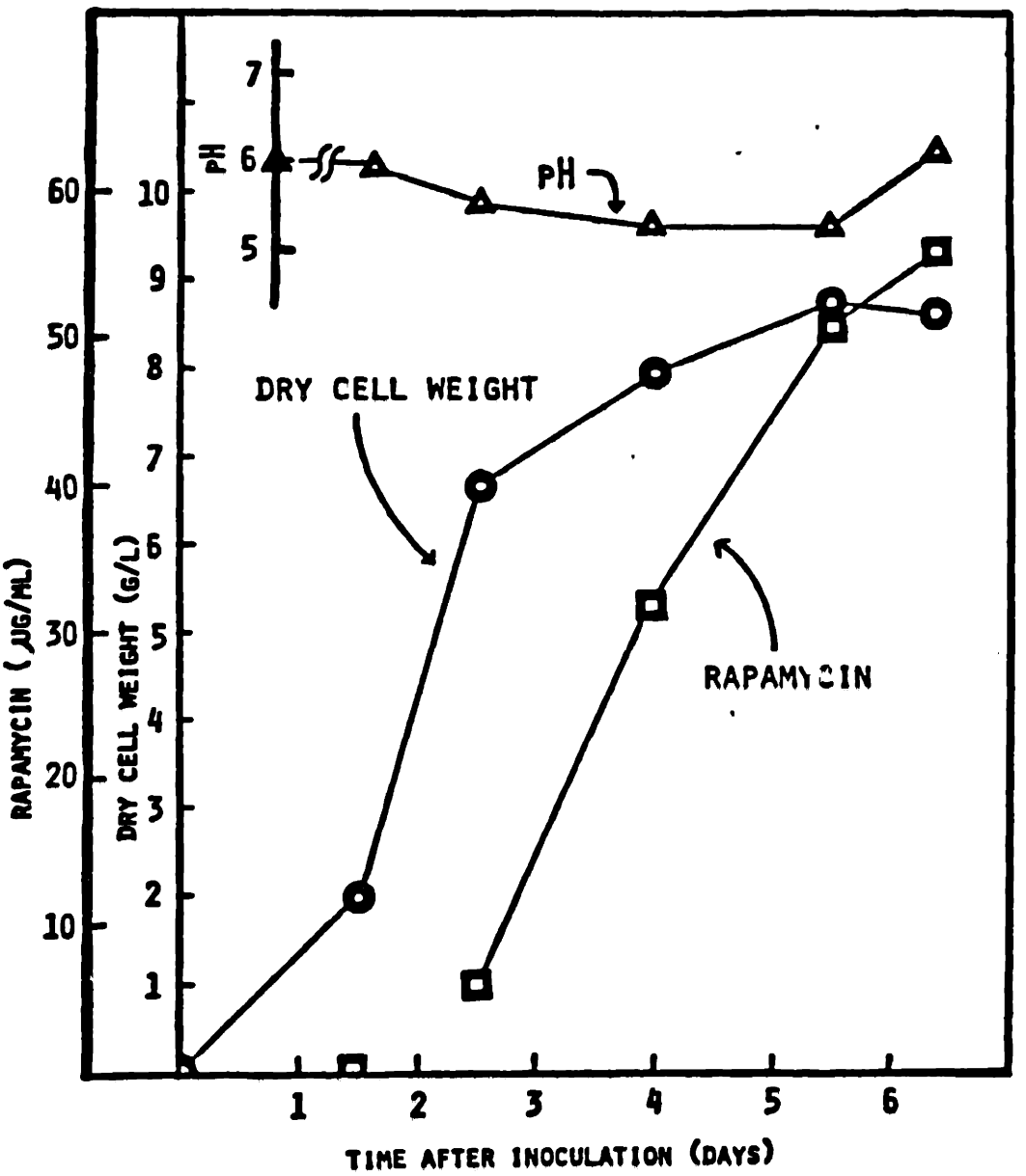
⊡: concentrations of trace elements and magnesium increased



had reported that when 0.5% yeast extract was added to their original defined medium, rapamycin production had doubled to 100 ug/ml. When I added yeast extract to the defined medium, now supplemented with the additional trace elements and Mg, rapamycin production improved to 50 to 60 ug/ml in only 6.5 to 7.0 days. The final dry cell weight was higher (9 to 10 g/l), the pellets much smaller, and the fermentations more reproducible. The addition of a complex ingredient such as yeast extract could be detrimental to labelling studies by contributing unlabelled precursors to the culture, resulting in isotopic dilution of added labelled precursor molecules. However, at this stage the beneficial effects on the fermentation seemed to merit its inclusion in the medium.

Calculations showed that sulfur could be growth- limiting, and the work of Karen Guerrero (UROP student) showed that more growth was obtained when Na_2SO_4 was included in the medium without yeast extract. Addition of Na_2SO_4 (0.36 g/l Na_2SO_4, providing 0.08 g/l S) to the medium containing yeast extract did not show a definite effect on dry cell weight or rapamycin, but the pellets appeared slightly smaller and less dense under microscopic examination. Based on this observation, Na_2SO_4 was included in the standard fermentation medium. The final composition of the medium (basal medium)is given in the Materials and Methods section. A typical fermentation profile in the basal medium is shown in Figure 21. (The development of a lysine-free medium and other observations on medium composition are discussed in a later section.)

Figure 21: Growth, rapamycin production, and pH during fermentation in basal medium.



B) Development of Small-Scale Recovery Method and Purification of Rapamycin

To obtain an NMR spectrum with a reasonable signal-to-noise ratio, approximately 10 mg of rapamycin are required. It is important that the sample be very pure so that contaminant peaks do not obscure or overlap with rapamycin NMR peaks.

The series of extractions using methanol, CH_2Cl_2, and hexane described in Materials and Methods were based on published procedures for rapamycin (Vezina et al., 1975; Sehgal et al., 1975). These procedures call for a silica gel column to be eluted with acetone/hexane followed by recrystallizations to purify rapamycin. For this was substituted a semi-preparative scale reverse phase HPLC step, using approximately 65% methanol and 35% H_2O. The conditions were easily reproduced for each injection and the resolution could be improved by increasing the water concentration. Rapamycin collected using this system appeared pure, as shown by analysis by analytical HPLC and by ^{1}H-NMR spectral comparisons. It was used without recrystallization in the ^{13}C-NMR analysis. The major drawback of this method is that the rapamycin is left in solution in a large volume of 65% MeOH/35% H_2O which is difficult to remove by rotary evaporation without heating, and heating causes the appearance of rapamycin breakdown products. Therefore the rapamycin solution was concentrated by one of two procedures. Most of the samples were concentrated by lyophilization, and the last three were concentrated by use of C-18 reverse-phase cartridges (Maxi-Clean Cartridges, Alltech, Deerfield, Illinios). In

the first procedure, the rapamycin solution was chilled in a dry ice/acetone bath or frozen in liquid nitrogen and the solvent removed in a lyophilizer. This step took 3 to 4 days due to the limited capacity of the solvent resistant lyophilizer available. In the procedure using the cartridges, the rapamycin was bound to the cartridges, then eluted into pure methanol, which could be removed by rotary evaporation without heating. After either procedure, the rapamycin sample was redissolved in CH_2Cl_2, the solution passed through a 0.45 um filter to remove particulates, and the solvent removed by rotary evaporation. The dry sample was stored at -20 C until the time of NMR analysis.

C) Toxicity of Sodium Acetate

To determine the maximum acetate concentration which could be used in the labelling experiments, the effect of sodium acetate on the cultures was investigated. The fermentation was started in 250 ml shake flasks in the basal fermentation medium described in the Materials and Methods section. After 2.5 days growth (when rapamycin production had just started), different volumes of a sterile sodium acetate solution were added to several pairs of flasks. At the same time, enough sterile water was added to make the combined volume of acetate solution and water equal to 2 ml, the control flasks receiving 2 ml H_2O. No significant difference in dry cell weight or rapamycin production was observed for flasks receiving up to 0.5 g/l (6.1 mM) sodium acetate (9 to 10 g/l dry cell weight and 50 to 60 ug/ml rapamycin after 6.5 days). In the flasks receiving 0.6 g/l (7.3 mM) sodium acetate, complete lysis of the cells occurred and no detectable rapamycin was produced.

D) Incorporation of Radioactive Acetate

To determine the level of incorporation of exogenously added acetate into rapamycin, tracer experiments were performed with 1,2-^{14}C-acetate (New England Nuclear; a mixture of 1-^{14}C- and 2-^{14}C-acetate). An aliquot of an ethanolic solution of ^{14}C-acetate (presumed to be sterile) was mixed with a sterile solution of unlabelled acetate to yield an acetate solution of the desired specific activity and concentration. Portions of this solution were added to three or four culture flasks at the desired time and the flasks incubated until the fermentation was complete. The contents of similar flasks were pooled, and the mycelium extracted as described above. The procedure for the recovery and purification of rapamycin was followed up to the point of obtaining a crude rapamycin solution in hexane. The concentration of rapamycin in this solution was determined by the standard HPLC procedure. A portion of the hexane solution (containing 100 to 200 ug of rapamycin) was applied as a streak on a silica gel TLC plate (Merck silica gel G with F-254 indicator, 20 x 20 cm). A streak of standard rapamycin solution was also applied to the plate. The plate was then developed in 50% acetone/50% hexane, and the rapamycin spot visualized with short wave UV and marked. The silica gel at the rapamycin spot and other sections of interest were removed from the plate by scraping and transferred directly to scintillation vials. Ten ml of Aquasol (New England Nuclear) were added, and the cpm ^{14}C recorded.

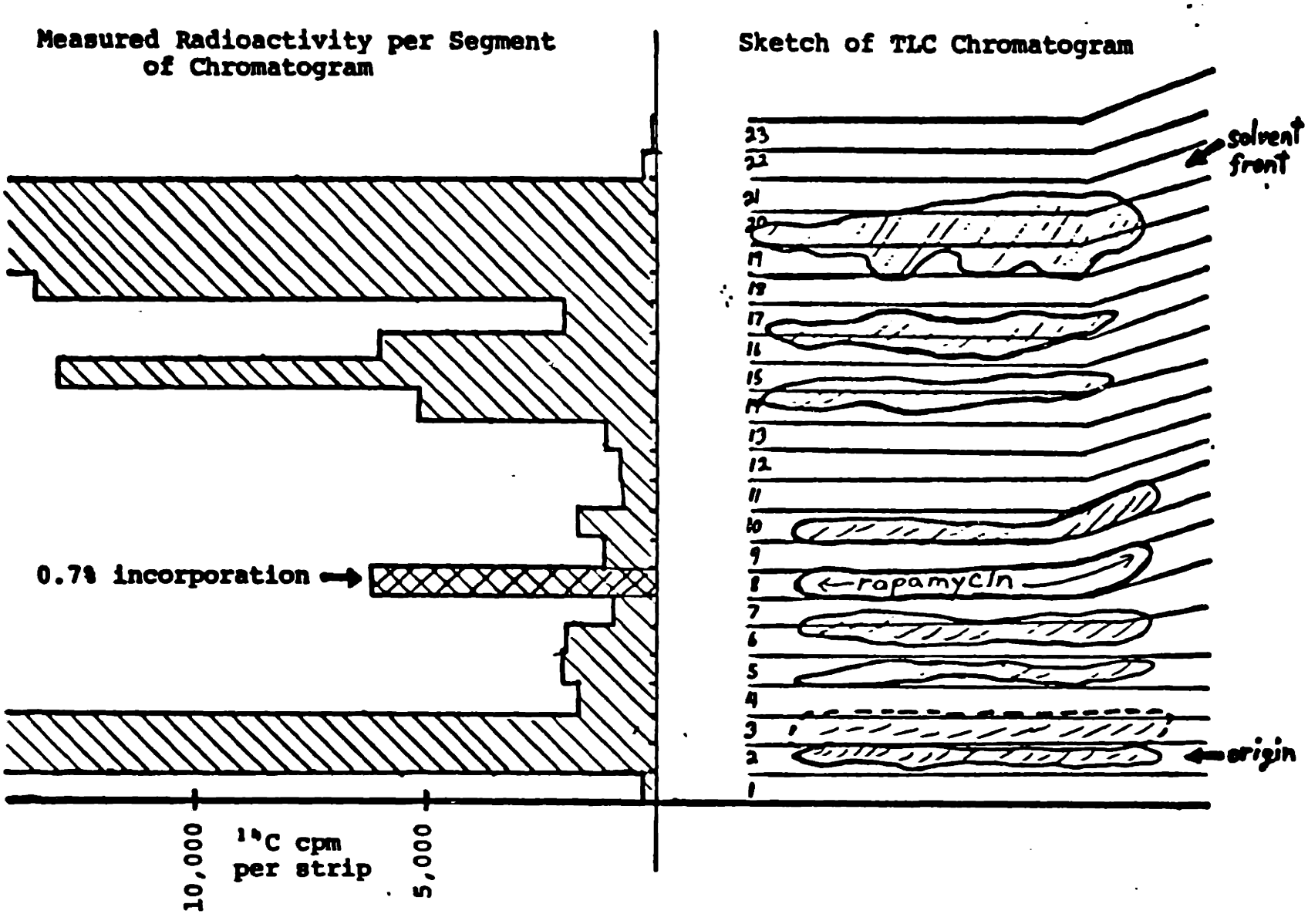
Given the calculated specific activity of the sodium acetate added

and the rapamycin recovered, the average number of acetate molecules incorporated per rapamycin molecule was calculated. Assuming an even distribution of label among the sites enriched by acetate and assuming (based on the proposed pathway of biosynthesis) that the number of acetate units incorporated is seven, I estimated the degree of incorporation of exogenous acetate at the sites.

To determine if the time of addition of the labelled acetate would have any effect on the level of incorporation, the following experiment was performed. Sodium ^{14}C-acetate (0.04 g/l) was added to one set of flasks at the time they were inoculated. The same amount of sodium ^{14}C-acetate was added to a second set of flasks after 2.5 days of incubation, about the time when rapamycin first became detectable in the cultures. The incorporation of acetate was analyzed as described above. The rapamycin isolated from the second set of flasks had approximately 3 times the incorporation (approximately 0.14% per site) observed in cultures which received the ^{14}C-acetate at the time of inoculation. The lower incorporation observed with early addition is probably due to the labelled acetate being used to synthesize fatty acids and other compounds during the first 2.5 days of growth, leaving less ^{14}C-acetate available for the synthesis of rapamycin. For all subsequent labelling experiments, the labelled compound was added at approximately 2.5 days.

Another fermentation was started, and 0.4 g/l sodium ^{14}C-acetate was added after 2.5 days. The final calculated incorporation was 0.7%. To determine where else the radioactivity was incorporated and how significant the rapamycin-associated radioactivity was relative to the

Figure 22:


Measured Radioactivity per Segment of Chromatogram
Sketch of TLC Chromatogram
0.7% incorporation
rapamycin
solvent front
origin
23
22
21
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
10,000
^{14}C cpm per strip
5,000

background, the entire TLC chromatogram was analyzed for radioactivity. The silica gel was removed from the plate in several strips, including one containing the complete rapamycin spot. The cpm of ^{14}C were measured, and the results are plotted in Figure 22, alongside a sketch of the chromatogram as visualized by UV. The peak of radioactivity associated with rapamycin is fairly distinct, although the radioactivity does not return to zero on either side of the peak. As much as a 15% overestimation of the rapamycin radioactivity may be introduced by this background. Large portions of the radioactivity were associated with the solvent front and the origin. These are the areas where fatty acids and phospholipids, respectively, would be expected to be located under the solvent conditions used. Whatever the nature of these radioactive substances, it is apparent that other compounds are competing with rapamycin for added acetate.

After it was determined that 0.5 g/l was the maximum dose of sodium acetate that could be added to the fermentations, this concentration of sodium ^{14}C-acetate was added after 2.5 days incubation. An incorporation of 1.0% was calculated, the minimum acceptable incorporation for the ^{13}C-labelling experiments.

It was suggested that the yeast extract in the medium might be contributing unlabelled acetate. This acetate could isotopically dilute the labelled acetate added, thus lowering the level of incorporation, or could add to the toxic effect of the added acetate, thus lowering the maximum tolerable dose of acetate. To check this possibility, the acetate concentration of the uninoculated medium was estimated by HPLC

(Biorad HPX87H organic acids column with Aminex precolumn, refractive index detector, 0.5 ml/min flow rate, 5 mM H_2SO_4, pH 2.3, mobile phase). Approximately 0.01 to 0.02 g/l sodium acetate (near lower limit of detection) was detected. It was therefore concluded that removing the yeast extract would not significantly raise the level of incorporation of acetate.

Samples of the culture supernatant after 2 and 6 days of growth were also assayed for acetate. After 2 days, approximately 0.2 g/l sodium acetate was detected, while after 6 days only 0.1 g/l was detected. From these results it appears that the concentration of the endogenous acetate present at the time of addition is insignificant compared to the amount of labelled acetate to be added (0.5 g/l). Reducing the amount of endogenous acetate by transferring the cells to fresh medium could improve the incorporation slightly, but disturbing the culture may lower rapamycin production.

E) Incorporation of Singly Labelled Acetate (1-^{13}C- and 2-^{13}C-Acetate)

A fermentation was started in twelve 500 ml flasks, using the procedures and basal medium described in Materials and Methods. After 2.5 days of incubation, rapamycin production had just begun, and 25 mg sodium 2-^{13}C-acetate (Merck Sharp and Dohme; 90% enriched) was added as a sterile aqueous solution to each flask. At the end of the fermentation, the contents were pooled and frozen at -20 C. Another 12 flasks were started and treated in an identical fashion. The broth from the two fermentations was combined, and the rapamycin extracted and purified by the procedures described in the Materials and Methods section. The final yield was approximately 8 to 9 mg rapamycin. This sample was then subjected to NMR analysis. (solvent: $CDCl_3$; approximately 12 hours accumulation; 12 usec pulse, 1.5 sec accumulation; the sample was kindly analyzed by Prof. M. F. Roberts at the Francis Bitter National Magnet Laboratory NMR facilities.)

Later, 1-^{13}C-acetate (sodium salt, 90% enriched; Merck Sharp and Dohme) was added to cultures, and the labelled rapamycin was recovered and purified in an identical fashion as the above sample. A total of 36 flasks were used, yielding 11 to 12 mg rapamycin, the larger sample size providing a better signal-to-noise ratio in the recorded spectrum under the same NMR conditions.

The spectra from both samples are shown in reduced form in Figures 23 a, b, and c, alongside the natural abundance spectrum. (The horizontal scales are identical but the vertical scales vary among the spectra.) Based on the assignments published by Findlay and Radics

Figure 23: Spectra from 2-^{13}C-actate and 1-^{13}C-acetate enriched rapamycin compared with the natural abundance ^{13}C-NMR spectrum.

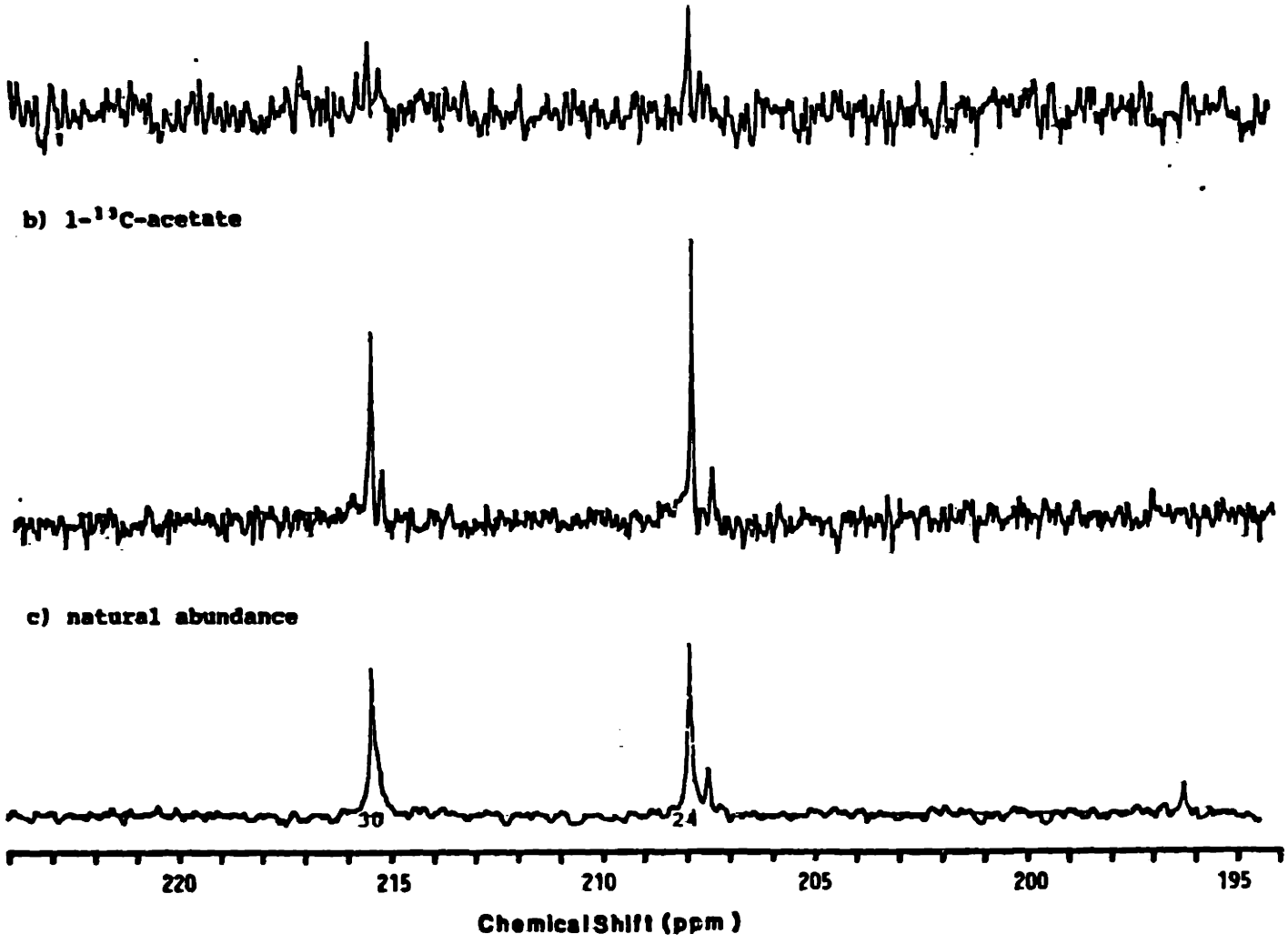


Figure 23 (continued):

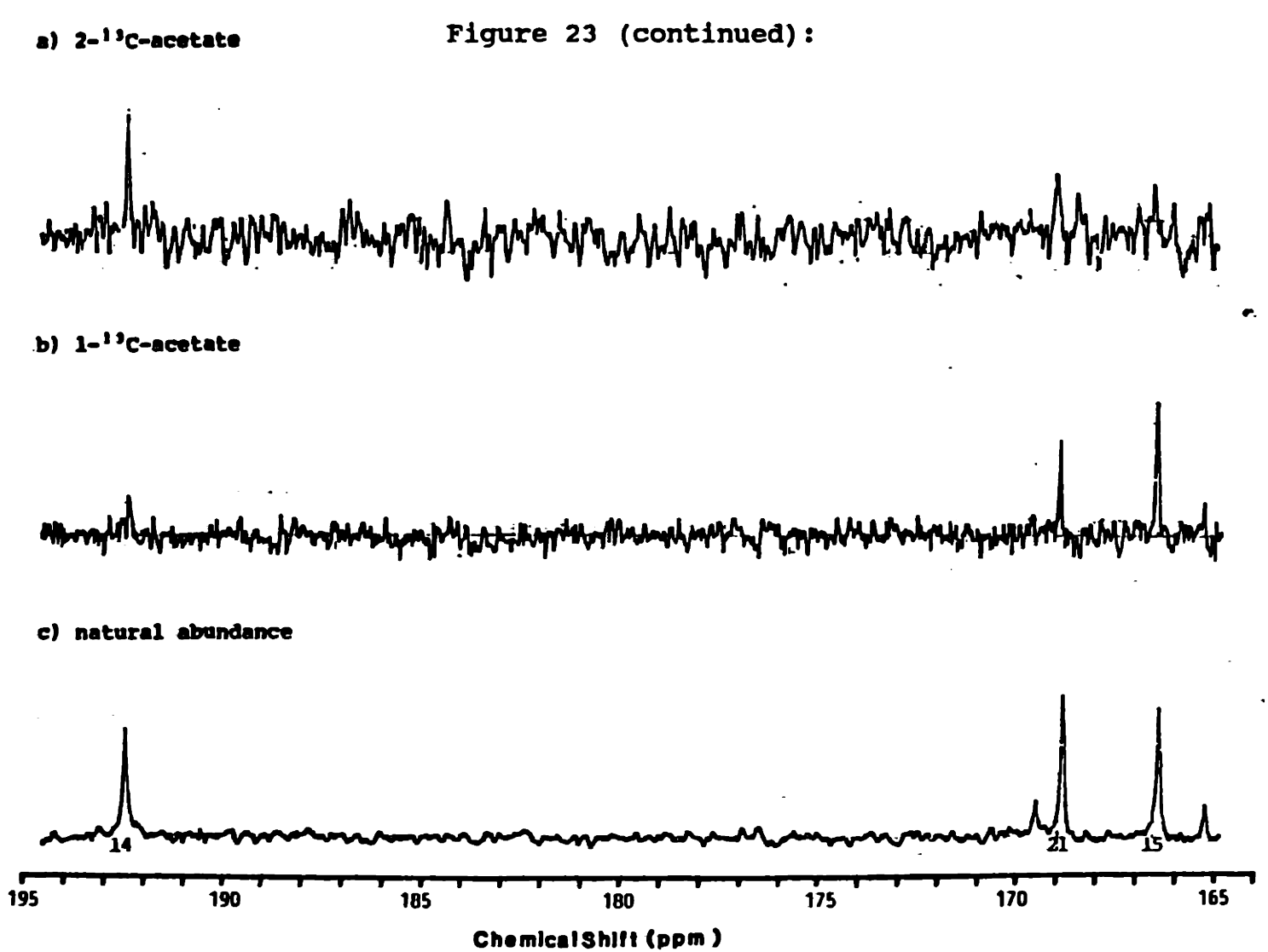


Figure 23 (continued):

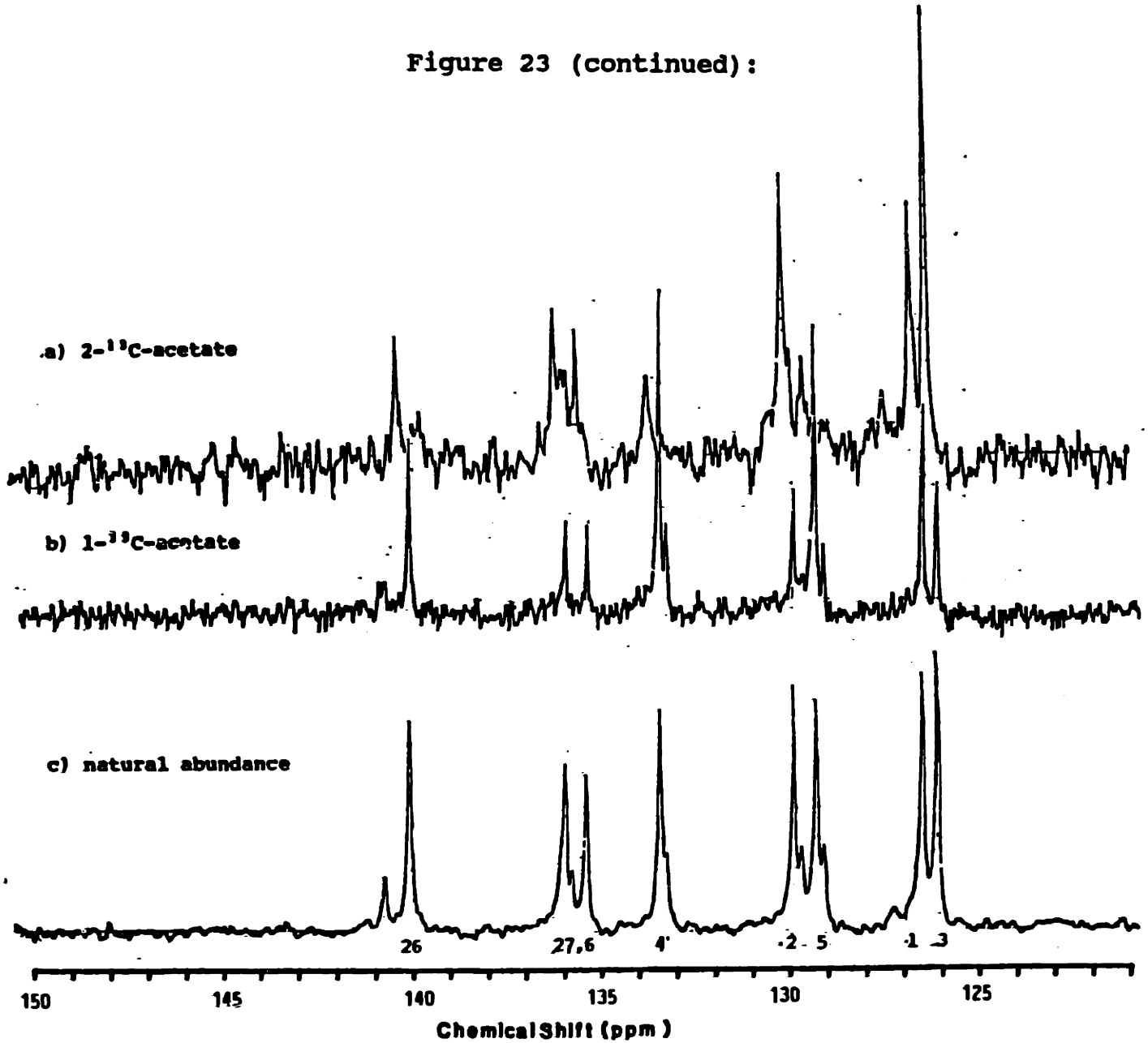
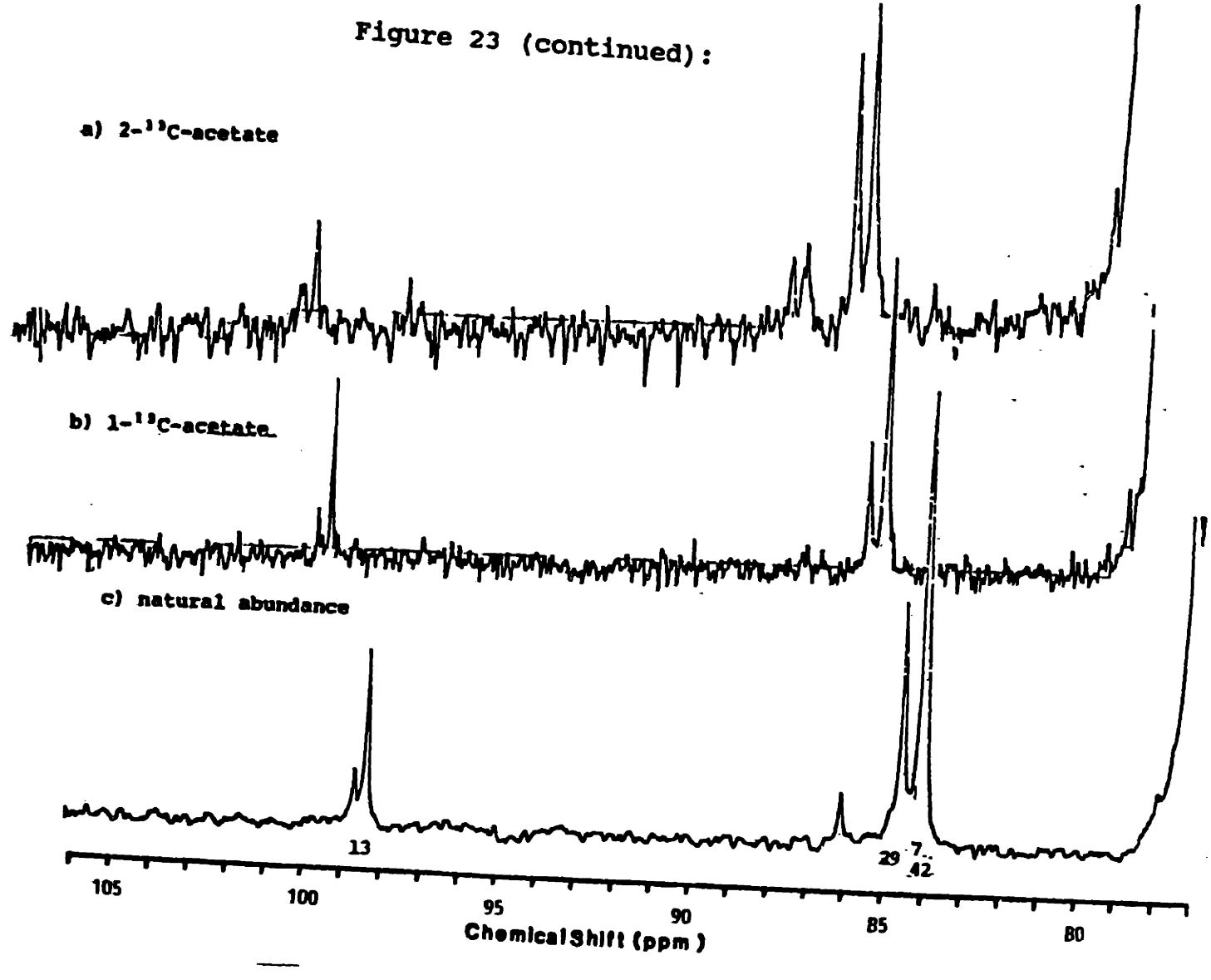


Figure 23 (continued):



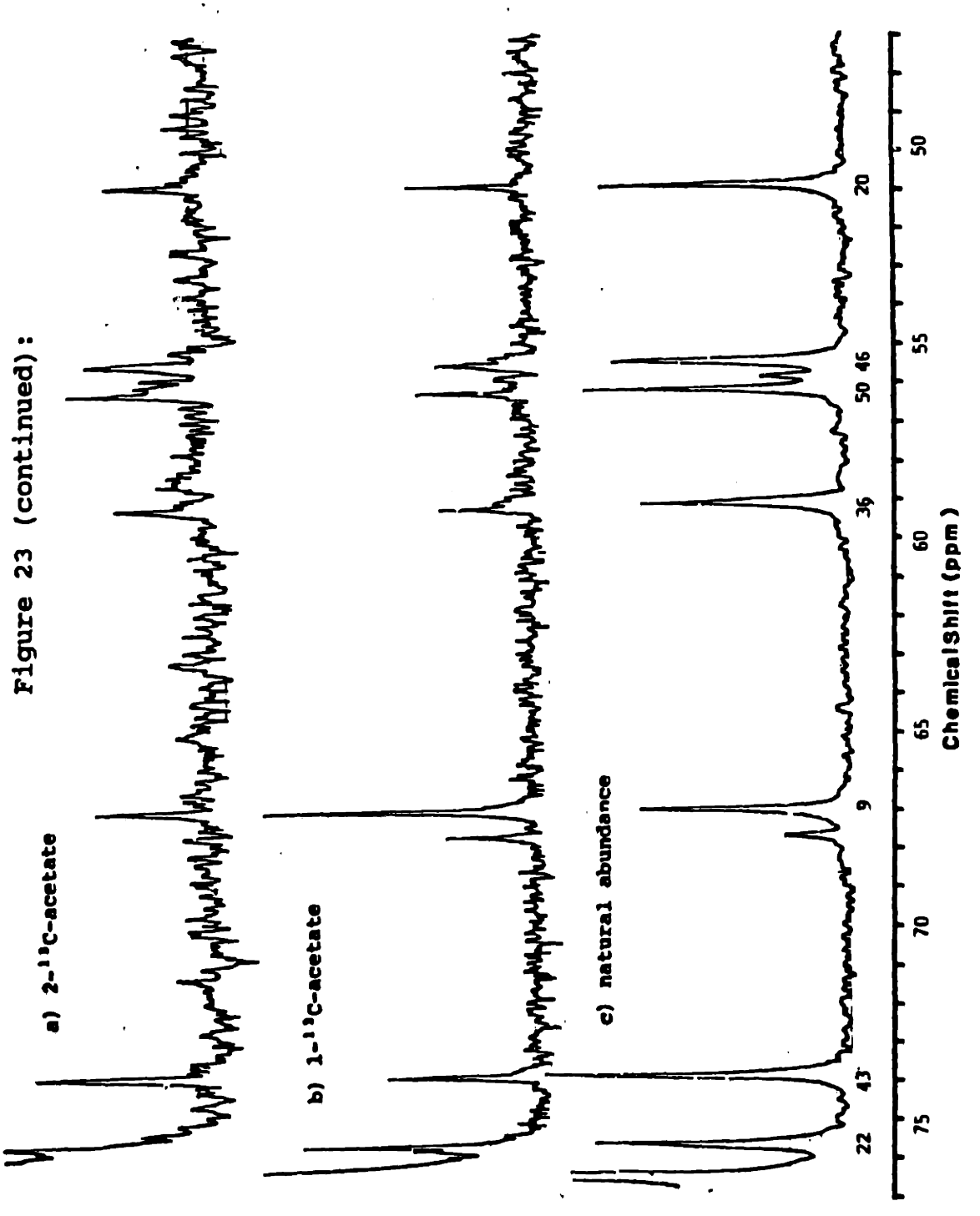


Figure 23 (continued):

Figure 23 (continued):

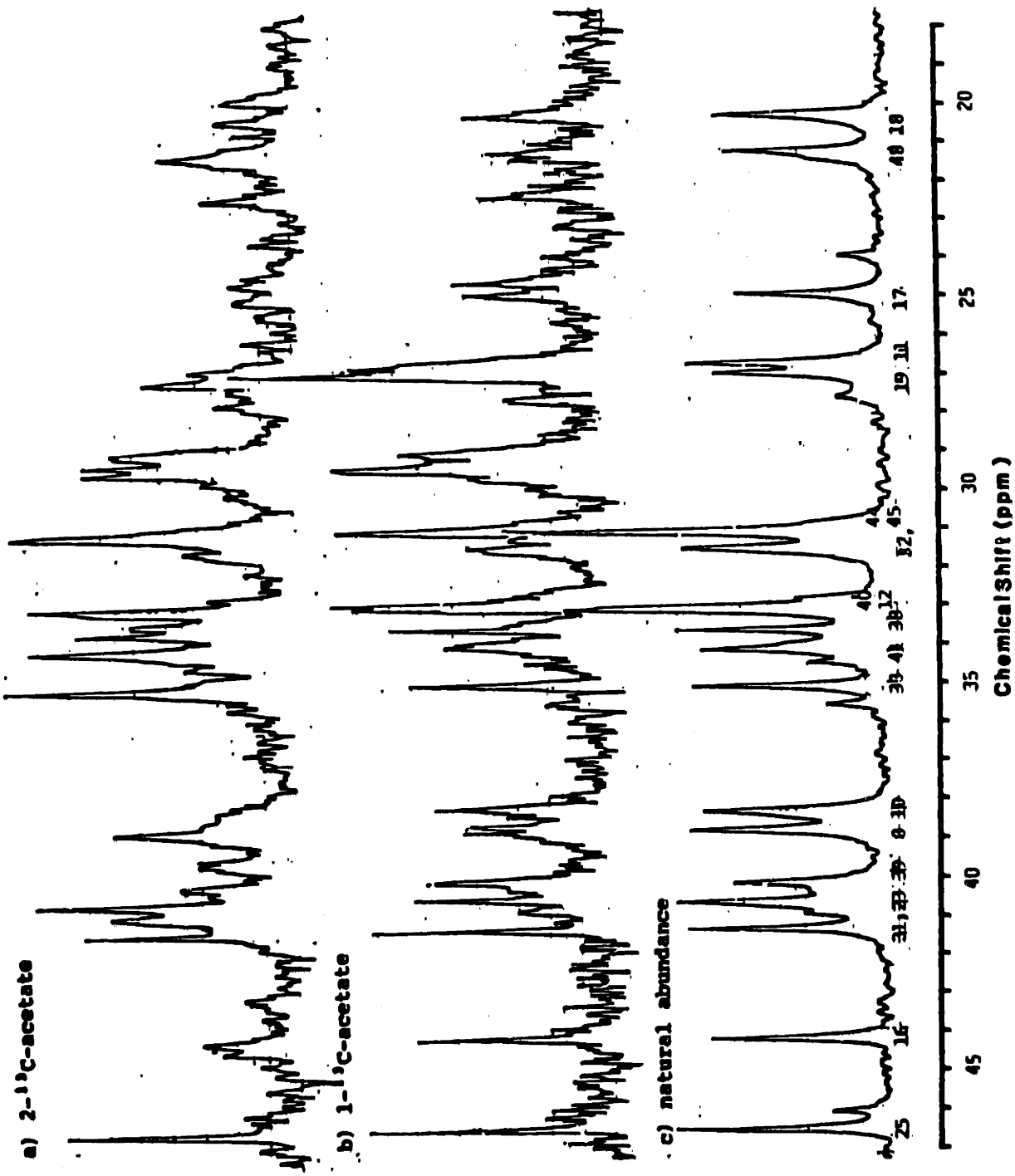
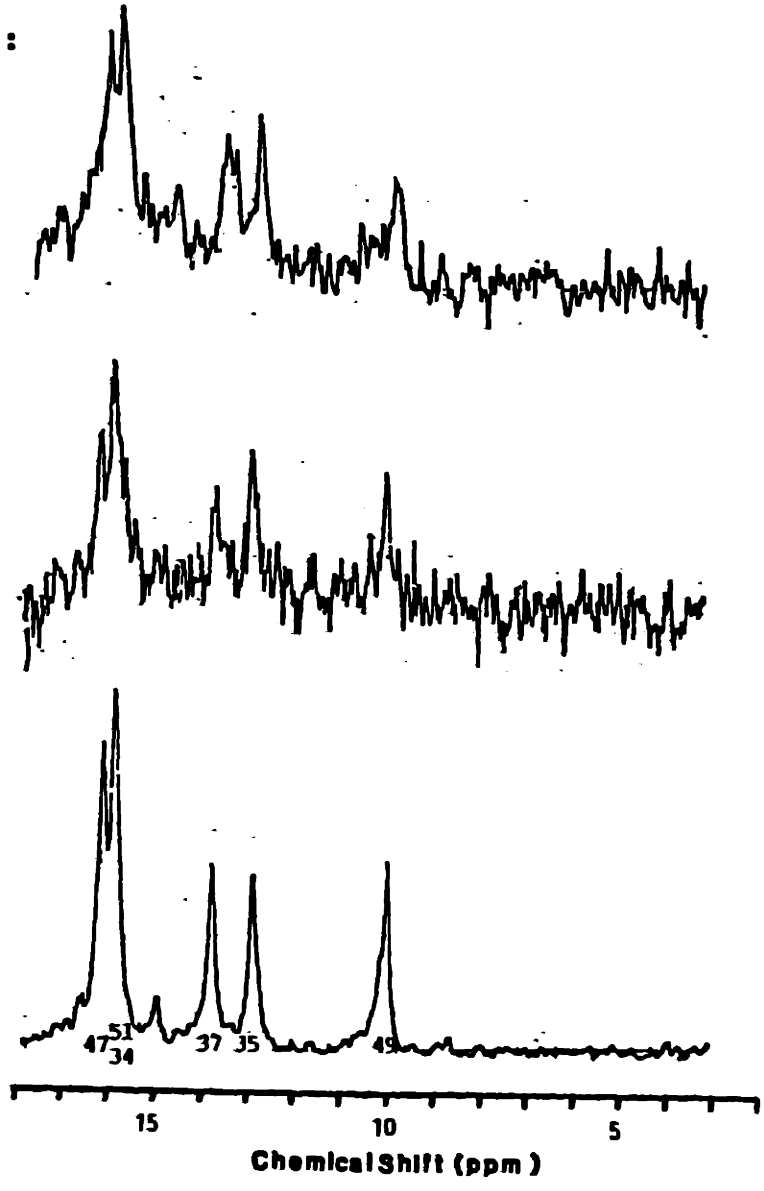


Figure 23 (continued):

a) 2-^{13}C-acetate

b) 1-^{13}C-acetate

c) natural abundance



(1980) (see Table 3), for each peak the number of the carbon assigned and its chemical shift are indicated. After careful examination and comparison of the three spectra it is apparent that several carbons are labelled.

Since the concentrations of the samples were not identical, the measured peak heights cannot be compared directly. Also, the heights for different types of carbons vary within a sample. However, the ratio of the heights of two peaks in a spectrum should be constant for all unlabelled samples. If one carbon site is later enriched with ^{13}C while another is not, the ratio of their peak heights should change. Based on this, the spectra were subjected to the following numerical analysis. The heights of all rapamycin peaks in the three spectra were recorded. The methoxy carbons (C36, C46, and C50) are believed to be derived from methionine and it is unlikely that they could be significantly enriched by labelled acetate. They also did not appear enriched upon examination of the spectra. The methoxy peaks were therefore chosen to serve as a type of "internal standard". The heights of all the peaks in the spectrum for a sample were divided by the average methoxy height for that sample, yielding a "normalized height". To determine the "enrichment factor", the normalized height calculated for a carbon in an enriched sample was divided by the normalized height calculated for the same carbon in the natural abundance spectrum. Thus, if a site had been enriched by 100% over the natural abundance of ^{13}C (approximately 1.1% incorporation), the final value of the "enrichment factor" for that site would be 2.0, indicating that the ^{13}C content had doubled.

Table 3:

Carbon-13 chemical shifts in rapamycin isomers.

Carbon	Major isomer	Minor isomer	Δδ[b]	Carbon	Major isomer	Minor isomer	Δδ
1	126.72	126.87	−0.15	27	136.09	136.19[c]	−0.10
2	130.16	129.93	0.23	28	77.23	77.13	0.10
3	126.38	126.34	0.04	29	84.87	84.46	0.41
4	133.60	133.42	0.18	30	215.48	215.35	0.13
5	129.55	129.35	0.20	31	41.39	40.81	0.58
6	135.54	135.93[c]	−0.39	32	31.66	31.76	−0.10
7	84.35	86.40	−2.05	33	35.18	35.64	−0.46
8	38.87	38.55[d]	0.32	34	15.94	16.17	−0.23
9	67.16	67.83	−0.67	35	13.01	12.85	0.16
10	38.37	38.46[d]	−0.09	36	59.40	59.29	0.11
11	27.00	26.93	0.07	37	13.81	14.97	−1.16
12	33.15	32.93	0.22	38	33.74	34.06	−0.32
13	98.49	98.78	−0.29	39	40.21	38.92	1.29
14	192.51	196.48	−3.97	40	33.22	33.29	−0.07
15	166.76	165.61	1.15	41	34.22	34.22	0
16	44.17	40.99	3.18	42	84.41	84.41	0
17	25.25	24.24	1.01	43	73.90	73.87	0.03
18	20.59	20.51	0.08	44	31.27	31.27[e]	0
19	27.26	27.85	−0.59	45	31.27	31.15[e]	0.12
20	51.37	56.19	−4.82	46	55.81	55.86	−0.05
21	169.22	169.86	−0.64	47	16.20	16.26	−0.06
22	75.66	75.77	−0.11	48	21.52	21.71	−0.19
23	40.68	40.44	0.24	49	10.13	10.28	−0.15
24	208.10	207.65	0.45	50	56.54	56.51	0.03
25	46.53	46.08	0.45	51	15.89	15.79	0.10
26	140.14	140.79	−0.65				

[a] In $CDCl_3$ solution, relative to internal TMS.
[b] $\Delta\delta = \delta_{major} - \delta_{minor}$.
[c, d, e] Assignments may be interchanged in this isomer.

(Findlay and Radics, 1980)

The results of such calculations for the samples enriched with 1-^{13}C- and 2-^{13}C-acetate are shown in Tables 4 and 5. The same results are also indicated on Figures 24 and 25 to show where in the molecule the carbons are located.

From these results it is apparent that carbons 2, 8 (weak), 14, 23, and 29 are significantly enriched by 2-^{13}C-acetate, exactly as predicted in the proposed scheme (see Figure 24 and Section IV). Surprisingly, C10 and C4 (predicted to be derived from the methyl group of acetate) were <u>not</u> enriched, while C3 (predicted to be derived from the carboxyl group of acetate) was very strongly enriched.

In addition, several carbons attributed to C2 and C3 of propionate were apparently enriched by 2-^{13}C-acetate (to varying degrees, carbons 35, 6, 37, 12, 47, 38, 48, 25, 27, 31, and 33). These could be enriched via "scrambling" of acetate carbons into propionate via the citric acid cycle. Acetate (as acetyl-CoA) could combine with oxaloacetate to form labelled citrate, which would then be converted to 3-^{13}C-succinyl-CoA and 2-^{13}C- and 3-^{13}C-succinate. These could be isomerized to 2-^{13}C- and 3-^{13}C-methylmalonyl-CoA ("active propionate") via the reverse of the methylmalonyl-CoA mutase reaction (see Figure 26). (This type of scrambling has been observed in studies on several polyketide antibiotics; see Literature Survey, Section III.2.) Carbon 41 also appears to be enriched. It is possible that the labelled acetate is incorporated into oxaloacetate via the citric acid cycle, and this labelled oxaloacetate could be used to generate labelled phosphoenol pyruvate, which could be combined with erythrose-4-phosphate to form the

Table 4 : Calculated Enrichment of Rapamycin Carbons by 2-^{13}C-Acetate

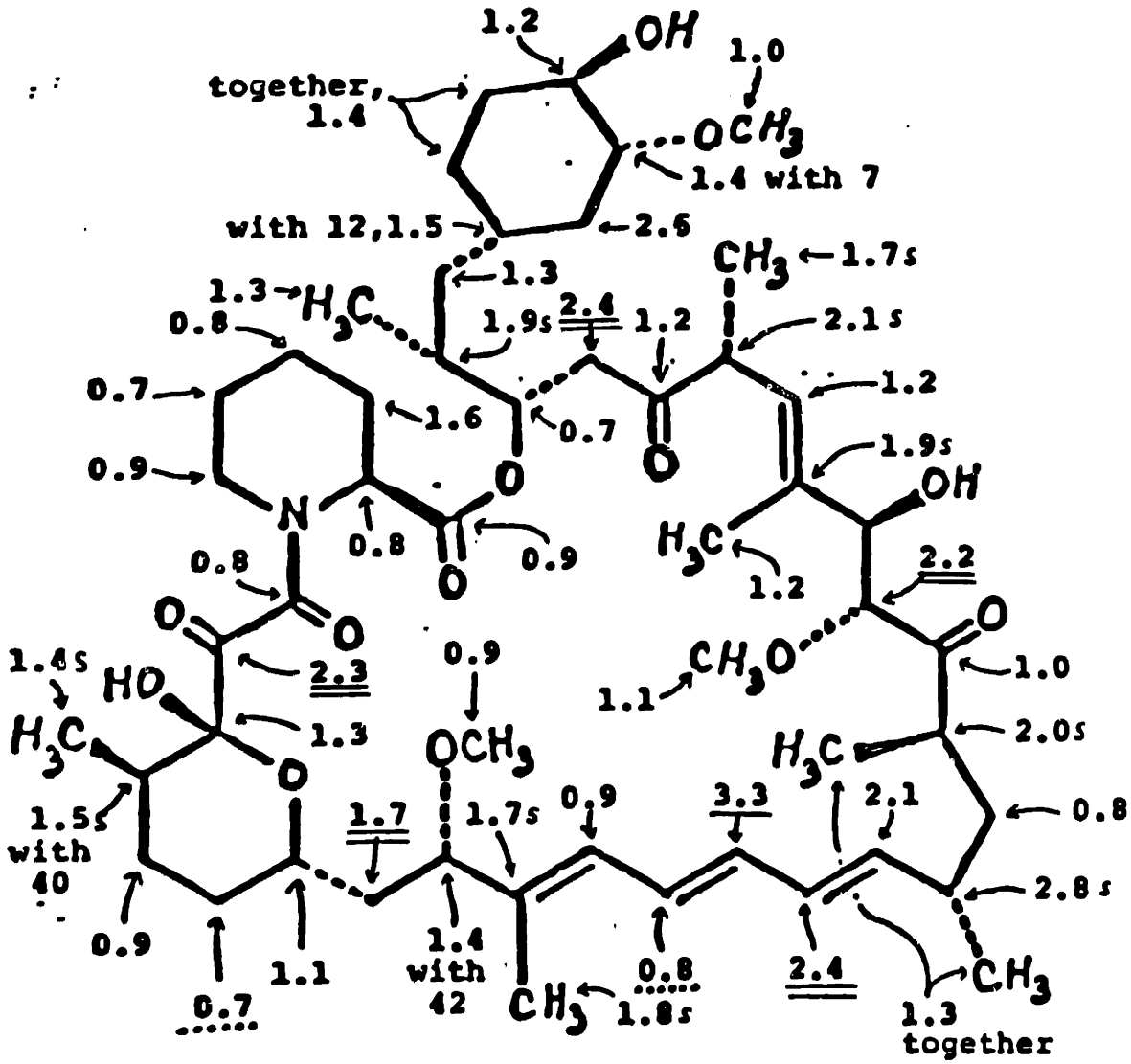
Carbon number assigned	Enrichment factor* calculated	Carbon number assigned	Enrichment factor* calculated
30	1.0	25	2.1
24	1.2	16	0.9
		31	2.0
14	2.3	23	2.4
21	0.9	39	1.3
15	0.8	8	1.7
		10	0.7
26	1.2	33	2.8
27	1.9	41	2.6
6	1.7	38	1.9
4	0.8	40 >	1.5
2	2.4	12 >	
5	0.9	32	0.8
1	2.1	45 >	1.4
3	3.3	44 >	
		19	1.6
13	1.3	11	0.9
29	2.2	17	0.7
7 >	1.4	48	1.7
42 >		18	0.8
(22)	(0.7)	47	1.3
43	1.2	51 >	1.3
9	1.1	34 >	
36	0.9	37	1.4
50	1.1	35	1.8
46	1.1	49	1.2
20	0.8		

*: Enrichment factor $\equiv$ $\dfrac{\left(\dfrac{\text{height of peak from enriched sample}}{\text{average height of methoxy peaks from enriched sample}}\right)}{\left(\dfrac{\text{height of peak from natural abundance sample}}{\text{average height of methoxy peaks from natural abundance sample}}\right)}$

Table 5: Calculated Enrichment of Rapamycin Carbons by 1-^{13}C-Acetate

Carbon number assigned	Enrichment factor calculated	Carbon number assigned	Enrichment factor calculated
30	3.0	25	1.3
24	3.9	16	1.3
		31	1.4
14	0.9	23	1.1
21	1.0	39	1.4
15	2.4	8	0.8
		10	1.1
26	2.0	33	1.1
27	1.3	41	0.9
6	1.4	38	1.1
4	3.5	40 >	0.9
2	1.3	12 >	
5	3.0	32	0.7
1	1.9	45 >	0.8
3	1.1	44 >	
		19	2.4
13	2.4	11	1.4
29	1.3	17	1.0
7 >	1.6	48	0.7
42 >		18	0.8
(22)	(1.9)	47	0.6
43	1.2	51 >	0.8
9	3.1	34 >	
36	1.0	37	0.6
50	1.0	35	0.9
46	0.9	49	0.8
20	1.1		

Figure 24 : Calculated Enrichment of Rapamycin Carbons by 2-^{13}C -acetate



═══ :significantly enriched as predicted in the proposed biosynthetic scheme

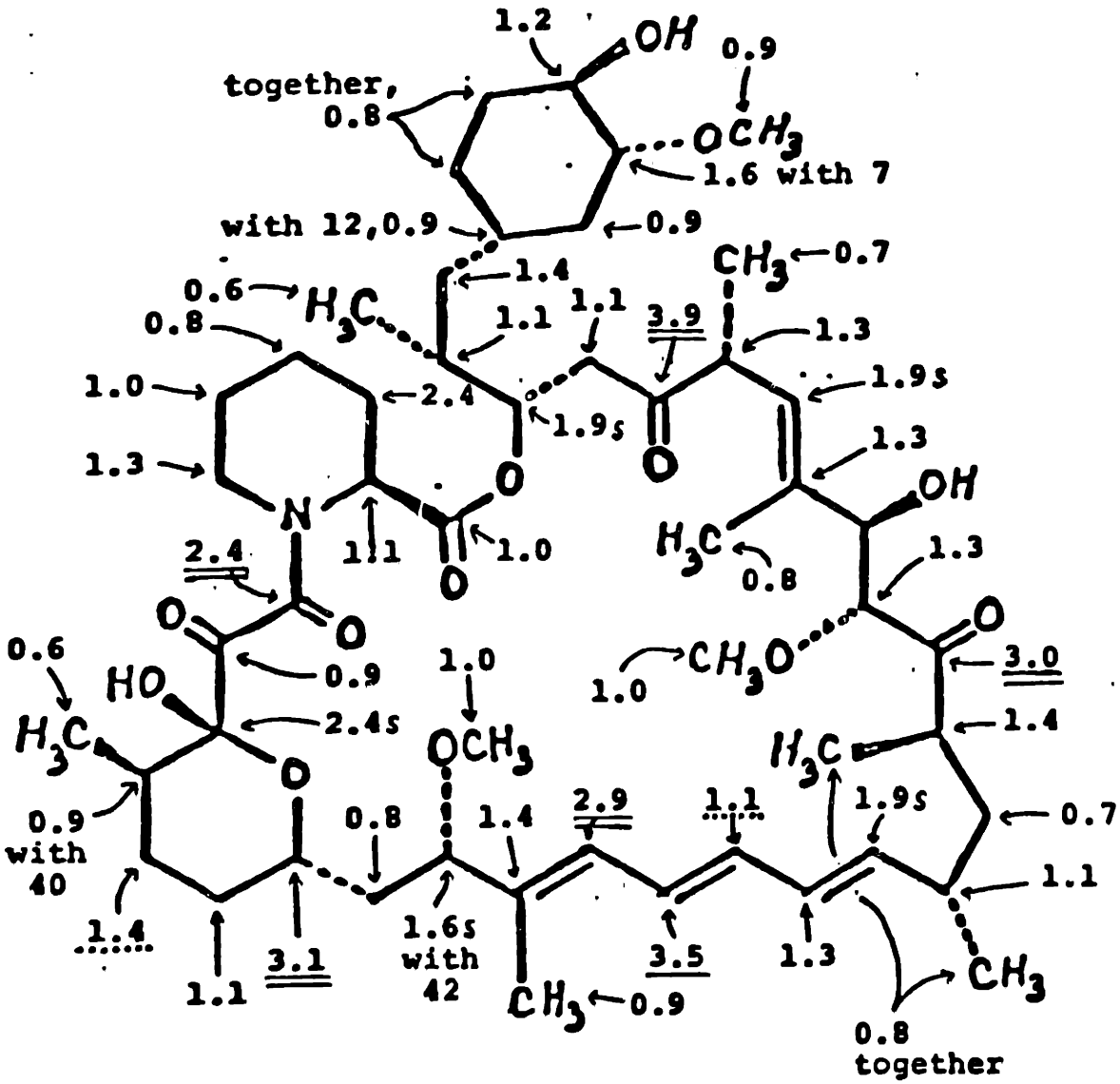
—— :enriched, but not predicted to be by the proposed scheme

s :enriched, but attributed to indirect incorporation (scrambling)

..... :predicted to be enriched by proposed scheme, but not significantly enriched

Carbons 40 & 12, 34 & 51, 44 &45, and 42 & 7 are not resolved under these conditions.

Figure 25: Calculated Enrichment of Rapamycin Carbons by 1-^{13}C -acetate



═══ :significantly enriched as predicted in the proposed biosynthetic scheme

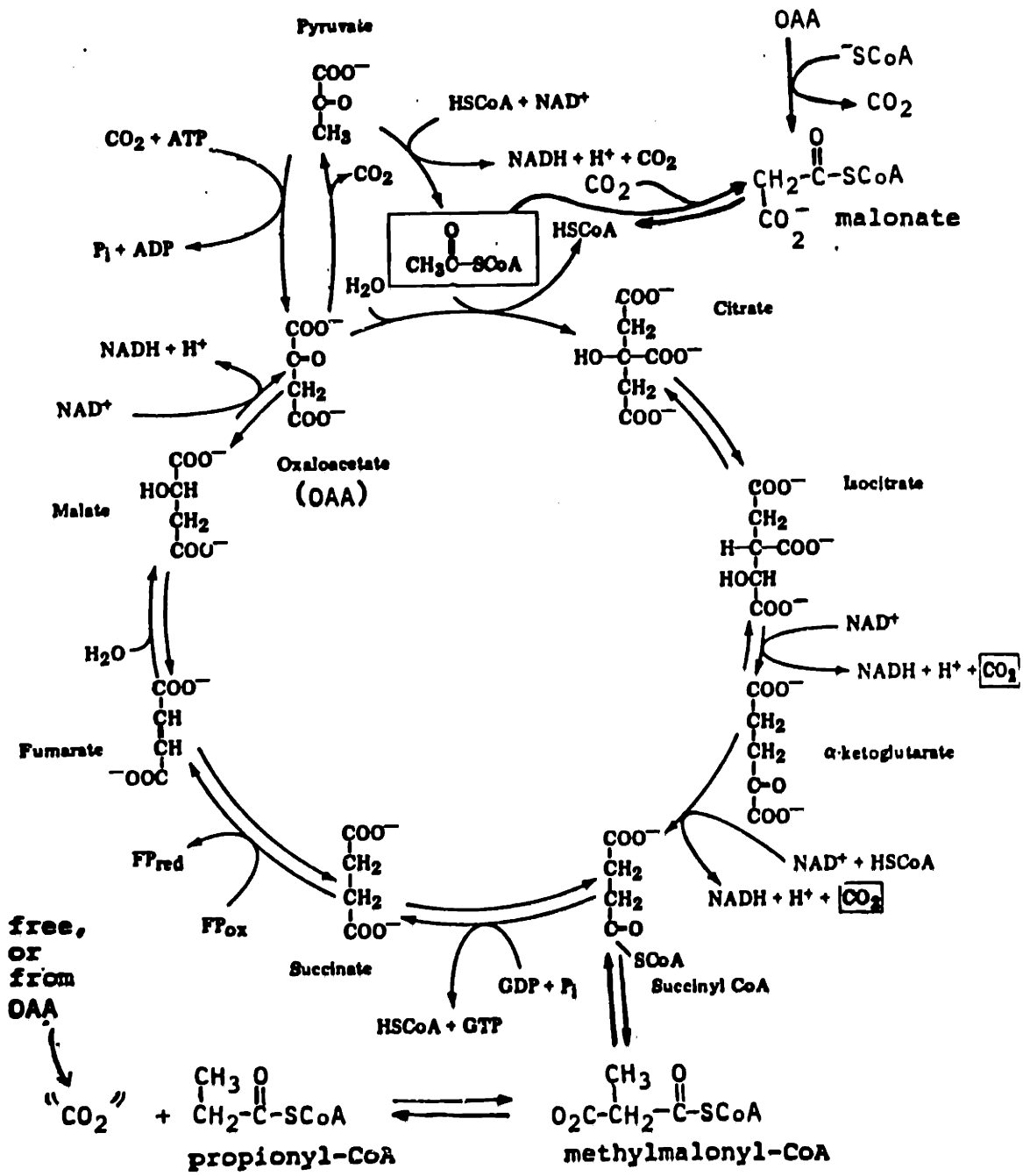
——— :enriched, but not predicted to be by the proposed scheme

5 :enriched, but attributed to indirect incorporation (scrambling)

..... :predicted to be enriched by proposed scheme, but not significantly enriched

Carbons 40 & 12, 34 & 51, 44 &45, and 42 & 7 are not resolved under these conditions.

Figure 26: Citric Acid Cycle and Scrambling



(adapted from W.B.Wood, J.H.Wilson, R.M.Benbow, and L.E.Hood. 1974. In Biochemistry: A Problems Approach. p. 242. W.A.Benjamin, Inc., Menlo Park, CA.

cyclic seven carbon starter unit. Carbons 1 and 7, corresponding to C1 of propionate in the proposed scheme are also apparently labelled, although it is not clear by what route.

For the sample enriched with 1-^{13}C-acetate, similar results were obtained. Carbons 5, 9, 15, 24, and 30 were enriched as predicted by the proposed scheme (see Figure 25). Carbons 3 and 11 (predicted to be derived from C1 of acetate) were not significantly enriched, while C4 (predicted to be derived from C2 of acetate) was.

Several carbons (1, 7, 13, 22, and 26) predicted to be derived from C1 of propionate were enriched. This could be explained by scrambling via a route similar to that outlined above for 2-^{13}C-acetate into propionate. Entry of 1-^{13}C- acetate into the citric acid cycle would result in the formation of 4-^{13}C-succinyl-CoA and 1-^{13}C- and 4-^{13}C-succinate. These could be converted to methyl- malonyl-CoA and the label either lost as $^{13}CO_2$ or incorporated as the C1 of propionate. If methylmalonyl-, succinyl-, and propionyl-CoA are being rapidly interconverted, any $^{13}CO_2$ (generated by the further metabolism of labelled acetate to $^{13}CO_2$) could be incorporated into the positions attributed to C1 of propionate. Also, 4-^{13}C-oxaloacetate could serve as the carboxyl donor in the carboxylation of propionyl-CoA, and the label could later be shifted to the C1 position via the above mentioned equilibrium. (This incorporation of $^{13}CO_2$ could also explain the enrichment of C7 and C1 by 2-^{13}C-acetate.)

One could also argue that carbons attributed to C1 and C2 of propionate are really derived from C1 and C2 of acetate and those

attributed to C3 of propionate were really from methionine, which is somehow enriched by C2 of acetate. An established route explaining the incorporation of the C2 of acetate into the methyl group of methionine is unknown to me, and further evidence against this alternate origin of the propionate carbons is presented in the next section.

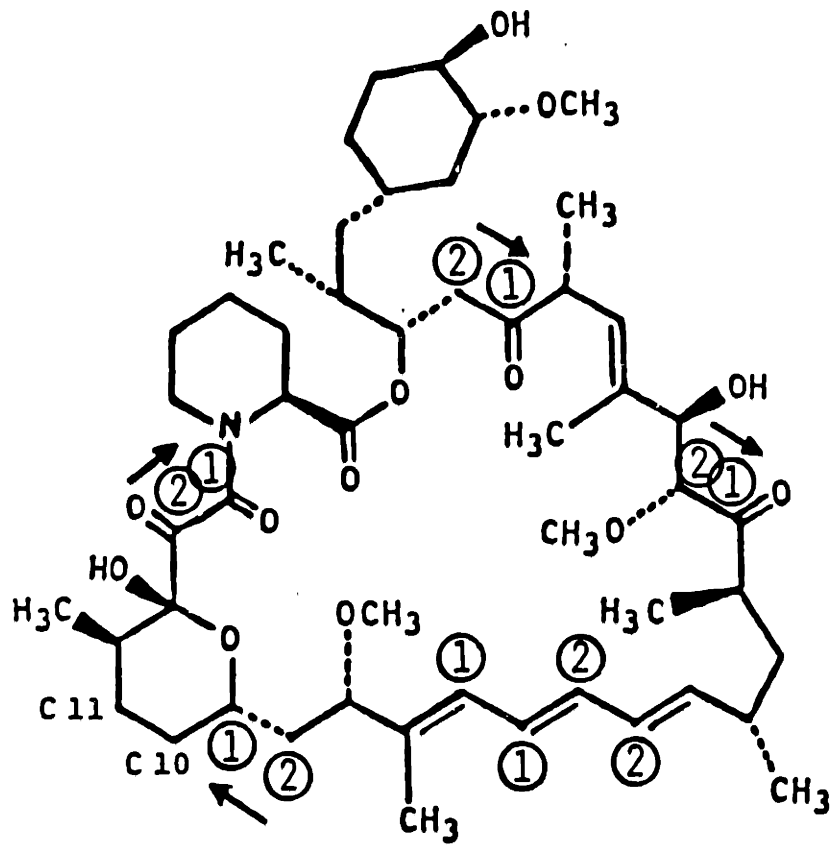
The results of the two experiments are summarized in Figure 27.a. Of the 7 acetate units in the proposed biosynthetic scheme, 4 appear to be present in the predicted locations and in the proper orientations. This supports the idea that the molecule is being synthesized by the polyketide pathway and that the direction of biosynthesis is as predicted.

Carbons 10 and 11 do not appear to be enriched by acetate. The proposed model will have to be modified accordingly, and alternative origins for these two carbons are discussed at the end of the next section.

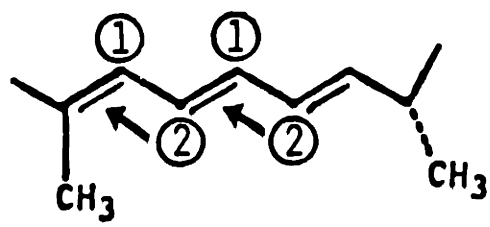
The labelling in the olefinic region is also not in agreement with the proposed scheme. Labelled acetate enriched four of the carbons in this region as predicted but not in the order predicted. (Compare Figure 27.a. with Figure 27.b.) The labelling pattern suggests that either some sort of complex rearrangement or a novel precursor is involved. An alternative explanation is that the published spectral assignments for this region might be incorrect. The chemical shifts of carbons 1 to 5 are very similar and two or more of the assignments may have been confused. The experiment described in the next section using 1,2-$^{13}C_2$- acetate is capable of distinguishing among the above

Figure 27:

COMBINED RESULTS OF 1-^{13}C-ACETATE AND 2-^{13}C-ACETATE INCORPORATION EXPERIMENTS



EXPECTED LABELLING PATTERN OF OLEFINIC REGION



①: ENRICHED BY 1-^{13}C-ACETATE

②: ENRICHED BY 2-^{13}C-ACETATE

→ : $CH_3CO_2^-$; ORIENTATION OF ACETATE UNIT

possibilities.

F.) Incorporation of Doubly Labelled Acetate (1,2-$^{13}C_2$-Acetate)

To confirm the above conclusions and to determine if any error was made in the published ^{13}C-NMR spectral assignments, doubly labelled acetate (1,2-$^{13}C_2$-acetate) was incorporated into rapamycin. If the acetate is incorporated as an intact unit, the nuclei of the two ^{13}C atoms will still experience ^{13}C-^{13}C spin-spin coupling, resulting in the appearance of satellite resonances (coupling patterns) located symmetrically about the two spectral peaks corresponding to the two rapamycin atoms enriched. If the two ^{13}C atoms of the doubly labelled acetate are separated by scrambling reactions, they are most likely to become adjacent to ^{12}C atoms and not another ^{13}C atom. Thus, the satellite resonances will not appear around such carbons and we will be able to distinguish between direct incorporation and incorporation through certain types of scrambling. Because the incorporation of acetate into rapamycin is so low, there is little chance of two ^{13}C-enriched acetates being incorporated adjacent to each other in the same molecule and creating additional coupling. The number of peaks flanked by satellite resonances divided by 2 will give the number of acetate units incorporated intact.

The sample of 1,2-$^{13}C_2$-acetate enriched rapamycin was prepared using the same procedures as for the singly labelled samples. After 2.5 days of incubation, 25 mg sodium 1,2-$^{13}C_2$-acetate (90% ^{13}C enriched; Prochem) was added to each 50 ml culture. The rapamycin was later purified (yield 11 mg), and the spectrum recorded under the same conditions as for the singly labelled samples. The spectrum is shown in Figure 28,

Figure 28: Spectrum from 1,2-$^{13}C_2$-acetate enriched rapamycin compared with the natural abundance ^{13}C-NMR spectrum.

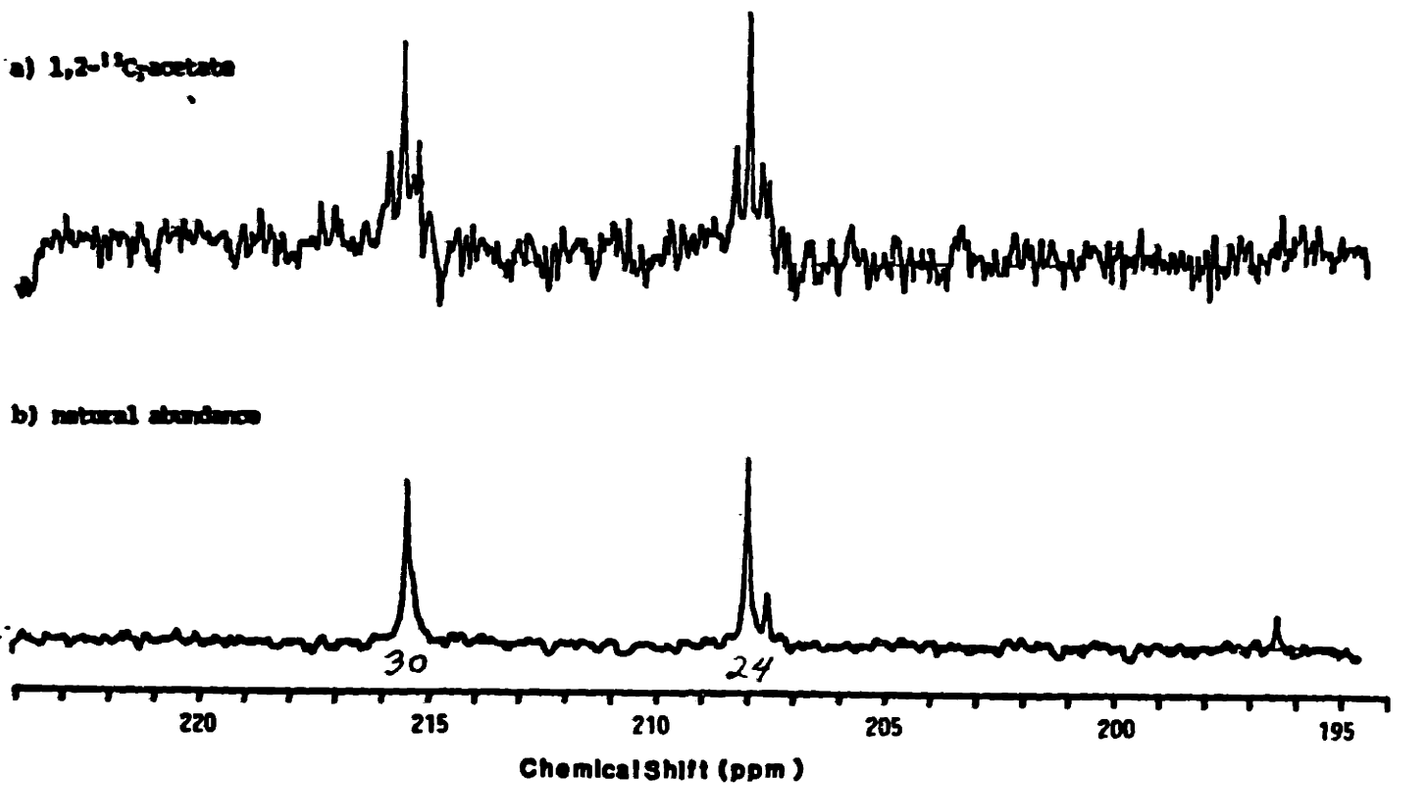


Figure 28 (continued):

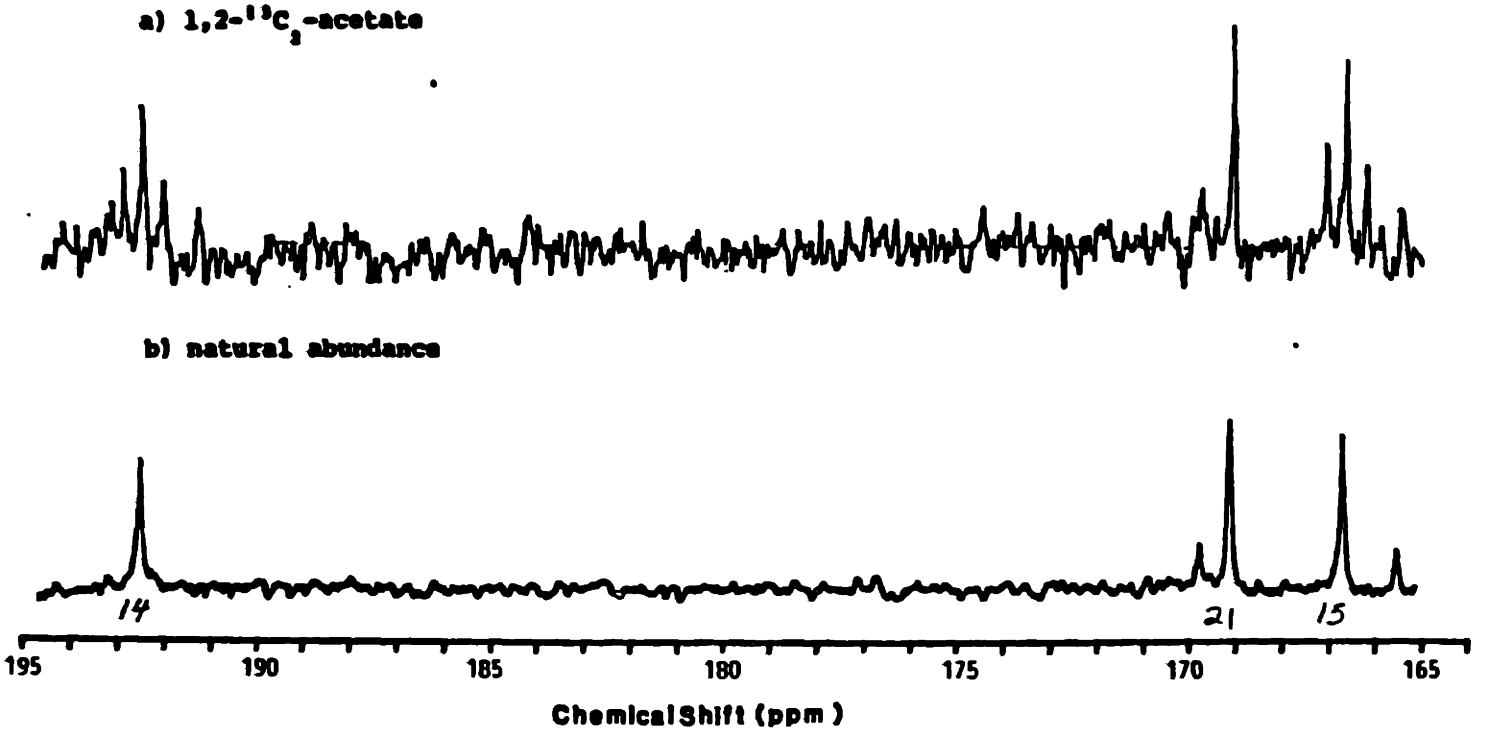


Figure 28 (continued):

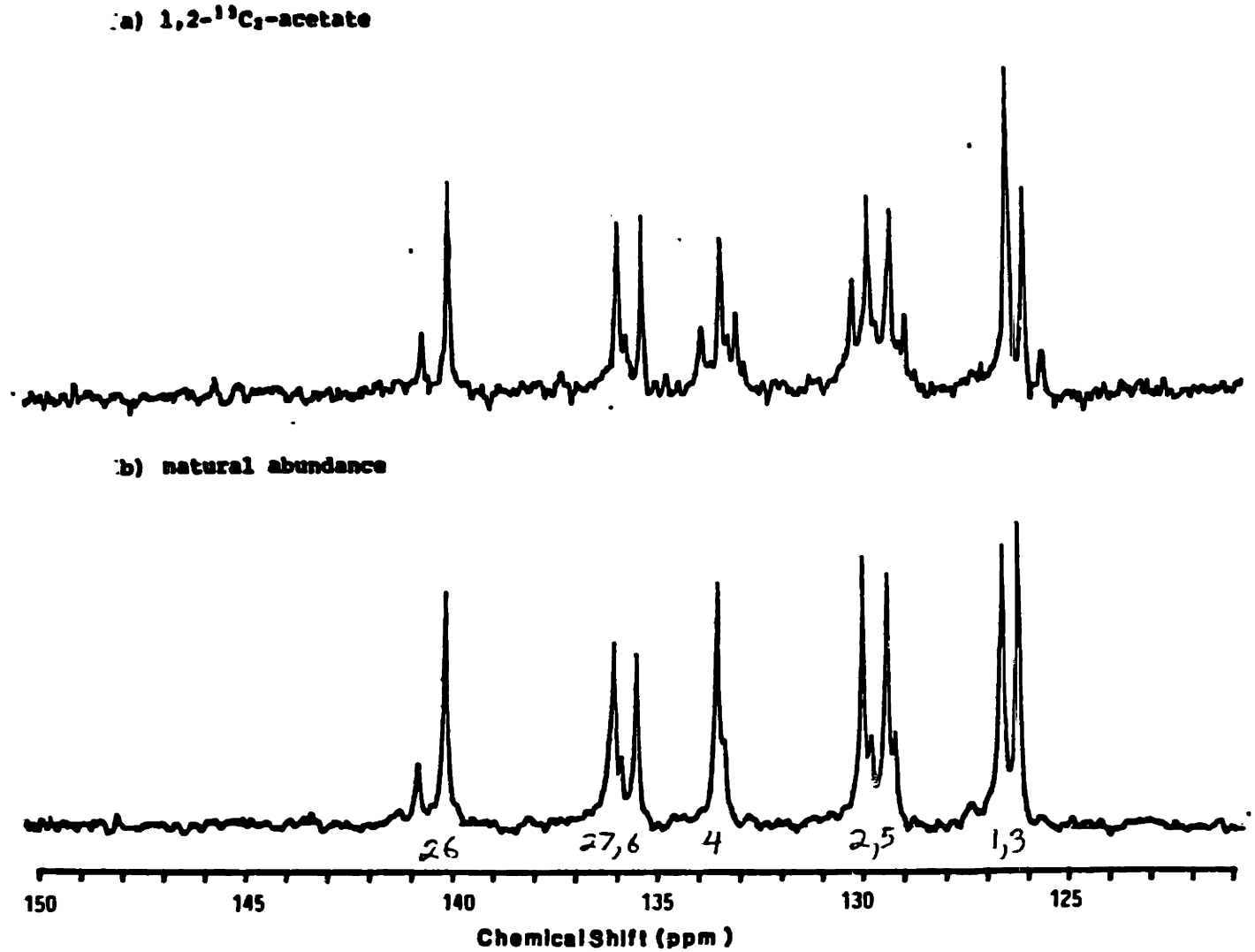


Figure 28 (continued):

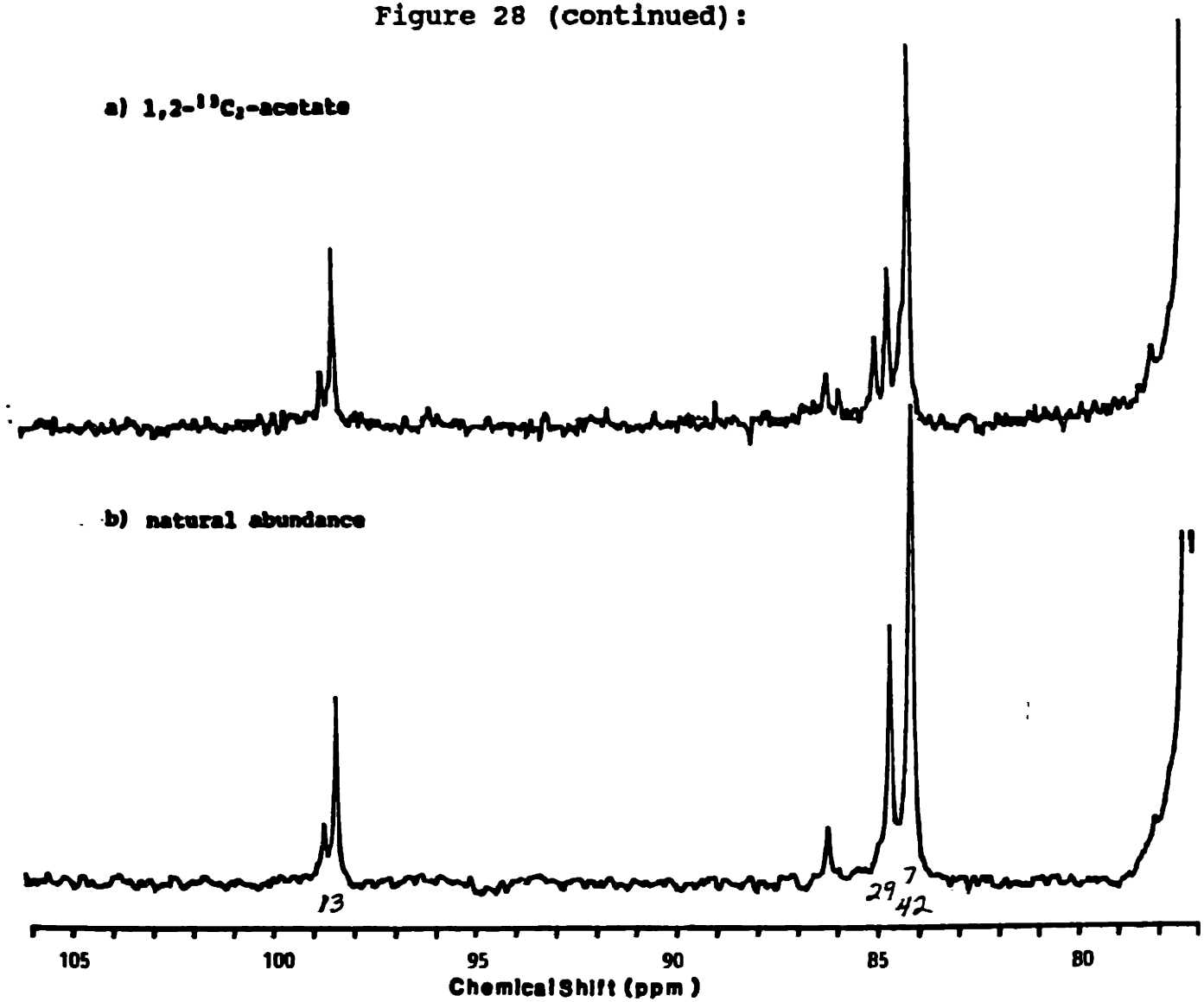


Figure 28 (continued):

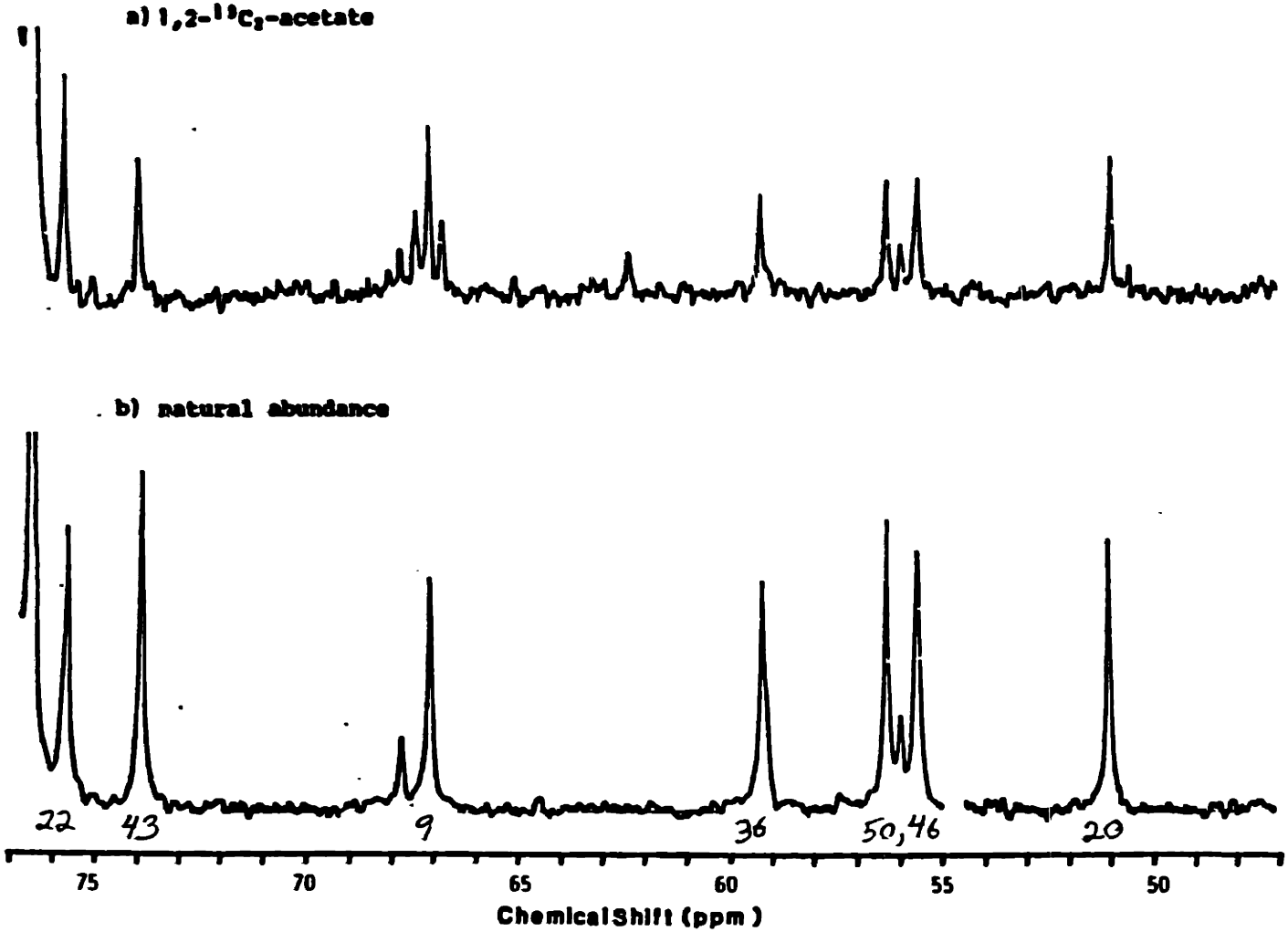


Figure 28 (continued):

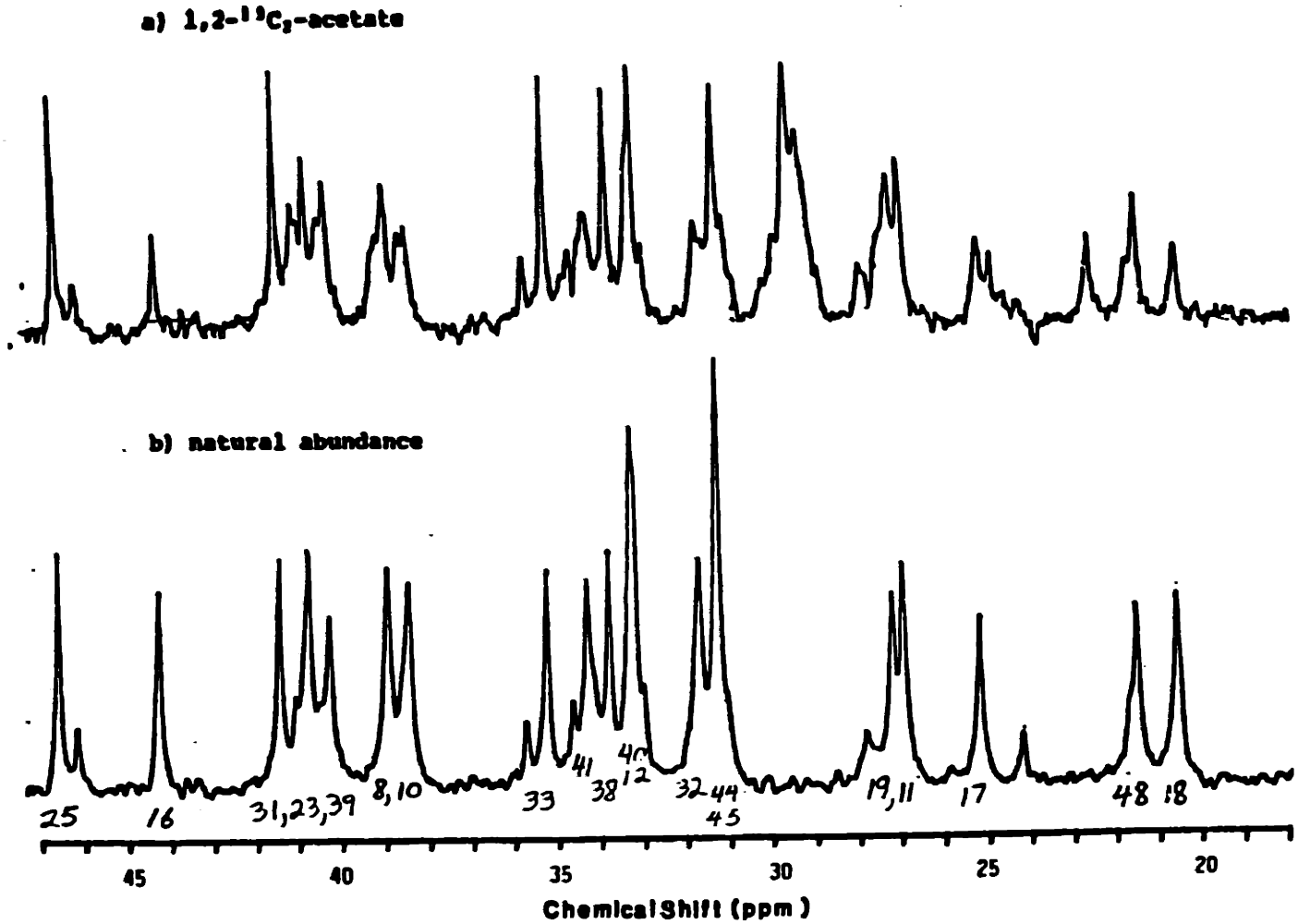
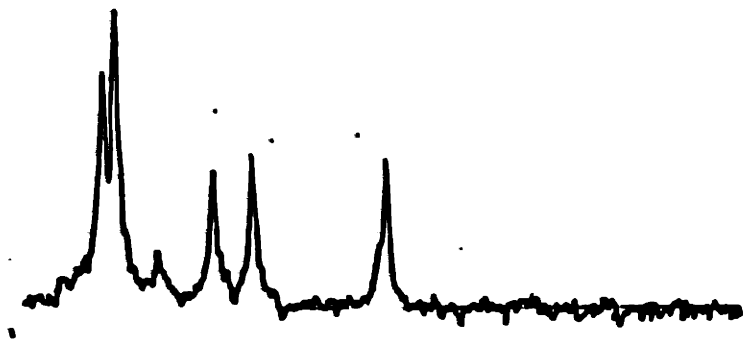
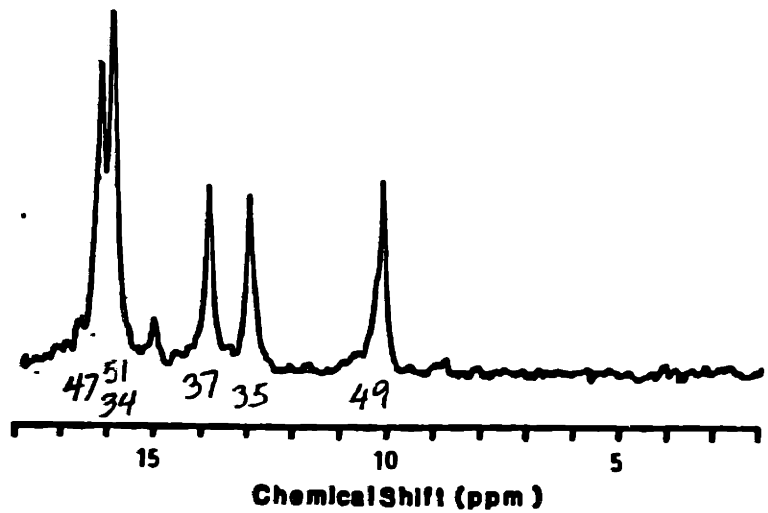


Figure 28 (continued):

a) 1,2-$^{13}C_2$-acetate



b) natural abundance



alongside the natural abundance spectrum. The calculated values (normalized with respect to the average methoxy carbon peak height) for the enrichment of each carbon are given in Table 6 and Figure 29. Note that this enrichment factor is calculated using the peak height at the chemical shift of each carbon, or the center peak of three if a carbon is exhibiting coupling. Only ^{13}C incorporation after scrambling will increase this center peak. Incorporation of intact 1,2-$^{13}C_2$-acetate will only cause an increase in the heights of the satellite peaks.

By comparing the enriched with the natural abundance spectrum, it is easy to find several peaks which are now between two satellite peaks (carbons 8, 9, 14, 15, 30, 4, 23, and 24). In addition to these, there are some carbons which are apparently coupled but only one of the satellite peaks is visible, because the second is hidden underneath a nearby peak (carbons 2, 3, 5, and 29). A total of 12 coupled carbons were identified, implying that 6 acetate units were incorporated intact into rapamycin. The ^{13}C-^{13}C coupling constant (Jcc) was estimated for each of the 12 coupled carbons, and these are listed in Table 7. (Jcc is the distance in Hz between the two satellite peaks, or approximately twice the distance from one satellite peak to the center peak when only one satellite peak is visible.)

The four acetate units labelled in the singly labelled experiments are labelled again here. The Jcc's for each pair agree well. This accounts for 8 of the 12 coupled carbons (4 of the 6 acetate units). The remaining four correspond to carbons 2 to 5, implying the incorporation of two intact acetate units into the olefinic region. The

Table 6: Calculated Enrichment of Rapamycin Carbons by 1,2-$^{13}C_2$-Acetate

Carbon number assigned	Enrichment factor calculated	Carbon number assigned	Enrichment factor calculated
30	1.5	25	2.3
24	1.5	16	1.2
		31	2.7
14	1.5	23	1.7
21	1.5	39	2.0
15	1.4	8	1.6
		10	1.1
26	2.1	33	2.6
27	2.2	41	1.2
6	2.4	38	2.2
4	1.5	40 >	1.6
2	1.7	12 >	
5	1.7	32	1.0
1	2.6	45 >	1.3
3	1.6	44 >	
		19	1.8
13	2.3	11	1.7
29	1.4	17	1.2
7 >	1.9	48	1.6
42 >		18	0.9
(22)	---	47	1.8
43	1.0	51 >	1.9
9	1.7	34 >	
36	1.0	37	1.7
50	0.9	35	2.1
46	1.1	49	1.6
20	1.2		

Figure 29 : Calculated Enrichment of Rapamycin Carbons by 1,2-$^{13}C_2$-acetate

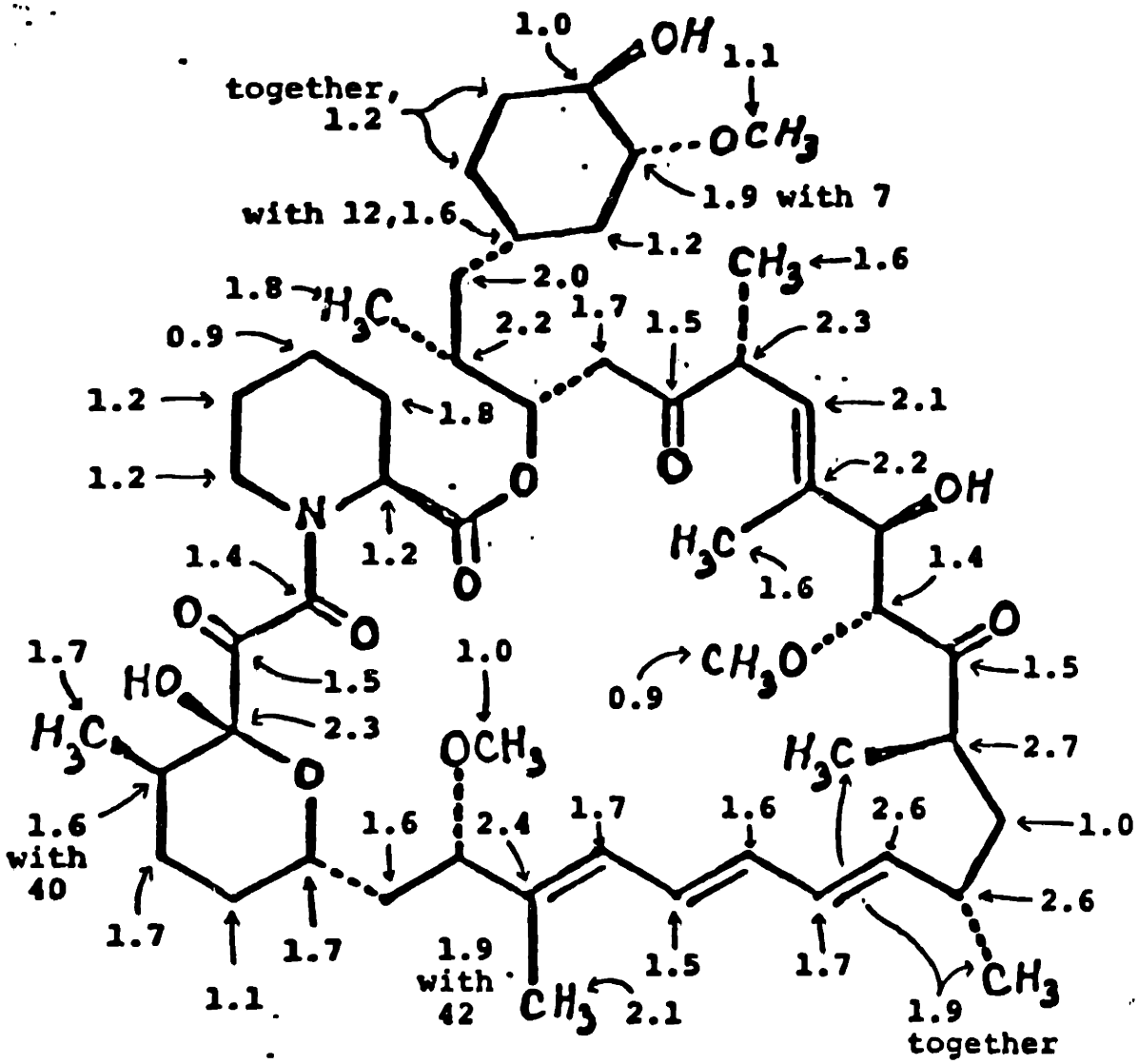


Table 7 : Measured Coupling Constants (Jcc) for Rapamycin Carbons Enriched by 1,2-$^{13}C_2$-Acetate

Carbon number assigned	Jcc (Hz)
30	41.7
29	39.8**
24	37.2
23	40.9
14	60.0
15	61.1
9	41.4
8	40.4
4*	56.2
2*	48.8**
5*	49.8**
3*	67.0**

*: published ^{13}C-NMR spectral assignments questionable

**: Jcc estimated as twice the distance from the only visible satellite peak to the central, natural abundance peak.

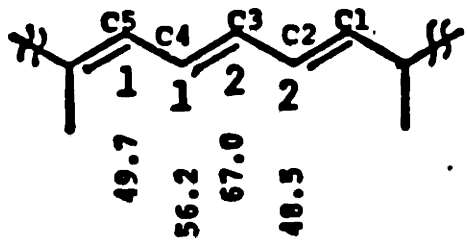
published assignments taken together with the singly labelled acetate incorporation data only allow for the incorporation of one intact acetate unit at C3 and C4 (see Figure 30.a.) Therefore we can conclude that the literature assignments for this region are incorrect. Figures 30.b. to f. illustrate possible reassignments of the carbons to the observed peaks. In Figures 30.b. and c., the assignments of two carbons are switched so that the Jcc's are matched more closely, but taken with the labelling data the orientation of one acetate is the opposite of that predicted by the proposed scheme. In Figure 30.d., carbons 3 and 4 are switched and the orientations of the two acetate units are correct, but the Jcc's do not match well. To have both the Jcc's matched and the orientations correct, three assignments must be interchanged (Figure 30.e. and f.). One of the latter three reassignments is probably the correct one. The actual assignments could possibly be determined by selective irradiation and decoupling of the carbons, or by long range ^{1}H-^{13}C decoupling experiments. At the present, however, it is clear that two acetate units are indeed incorporated into carbons 2 to 5 as predicted; only their orientations are not yet confirmed. These results are summarized in Figure 31.

From the calculated enrichments shown in Table 6 and Figure 29, it appears that carbons attributed to propionate in the proposed scheme are still being enriched by acetate. This apparently takes place only by scrambling, as no coupling patterns were observed around any of the peaks corresponding to these carbons. This eliminates the possibility that some of the predicted propionates are actually derived from acetate

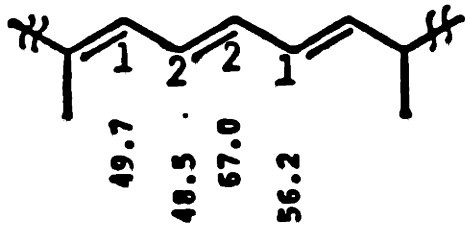
Figure 30:

Possible reassignments of the carbons in the olefinic region of rapamycin.

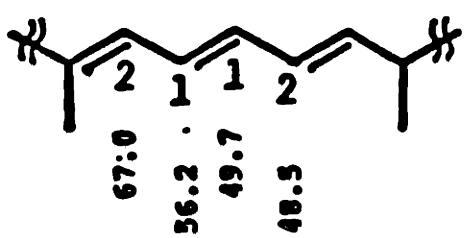
a) results interpreted using published NMR spectral assignments



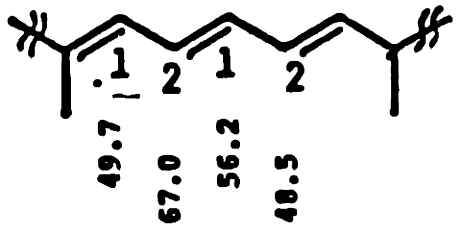
b) assignments of carbons 2 and 4 interchanged



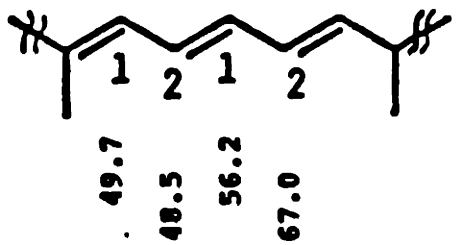
c) asignments of carbons 3 and 5 interchanged



d) assignments of carbons 3 and 4 interchanged



e) assignments of 3 and 4, then 3 and 2 interchanged



f) assignments of 3 and 4, then 4 and 5 interchanged

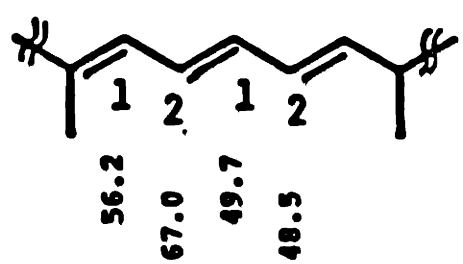
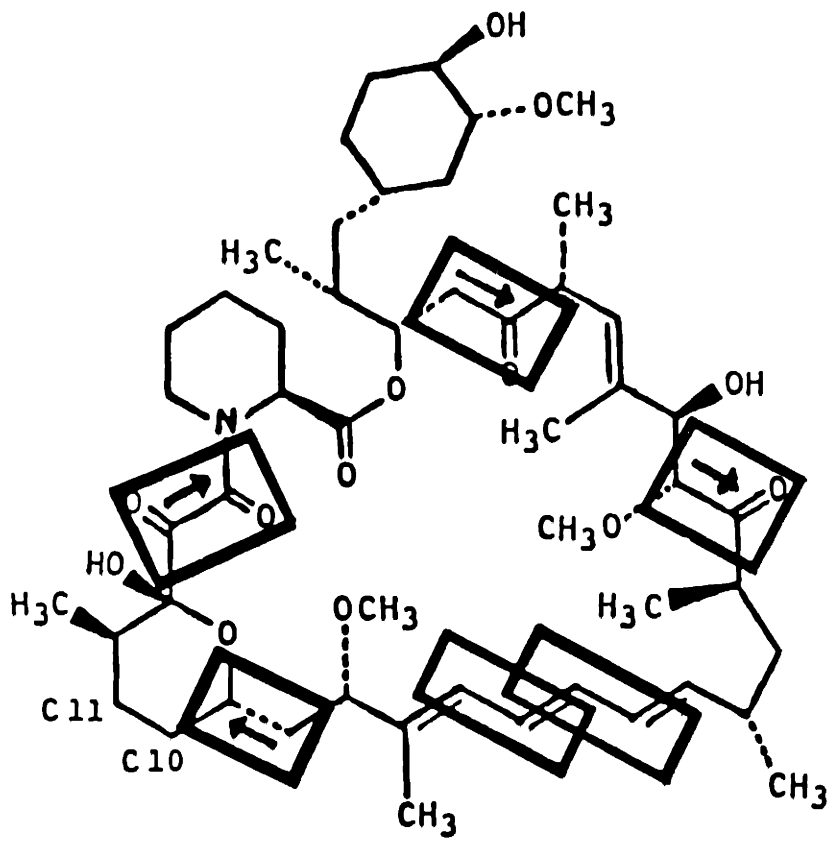


Figure 31:

LOCATIONS OF 6 INTACT ACETATE UNITS INCORPORATED INTO RAPAMYCIN



: ENCIRCLES INTACT ACETATE UNIT

: INDICATES ORIENTATION OF UNIT IF KNOWN

and methionine. No coupling was observed in the regions attributed to pipecolic acid or the shikimic acid pathway intermediate, indicating that these regions are also not derived from acetate.

In contradiction of the proposed scheme, carbons 10 and 11 were not labelled by acetate significantly in any of the experiments. The following are possible alternative origins for these two carbons: 1) The two carbons are still incorporated as malonyl-CoA, but this malonyl-CoA (or acetyl-CoA) is generated from a precursor in a shielded pocket (active site) and then incorporated into rapamycin directly. Labelled acetate (in its activated forms) cannot diffuse into this pocket and thus no labelling is observed when labelled acetate is administered. Malonyl-CoA can be generated from oxaloacetate in one step or from lactate or pyruvate in two or three steps (see Figure 32.a. and Literature Survey, Section II. B.). Enrichment of these two carbons should be observed when the appropriate precursor is added to the culture if the cells can take it up. 2) The two carbons are incorporated as C1 and C2 of a propionate, the C3 of which is later removed by oxidation to CO_2. This has already been shown to occur in rifamycin biosynthesis (see Literature Survey, Section II.B.). A methyl group derived from propionate is oxidized stepwise, while 7 other methyl groups are left unchanged. Enrichment of C10 and C11 should be observed when 1-^{13}C-, 2-^{13}C-, or 1,2-$^{13}C_2$-propionate are added to the cultures. 3) The two carbons are incorporated as part of a branched five carbon acid. If carbons 12, 13, and 37 are not labelled by propionate, all five carbons could be incorporated as an intact unit of the form shown in

Figure 32: Some Possible Origins of Carbons 10 and 11

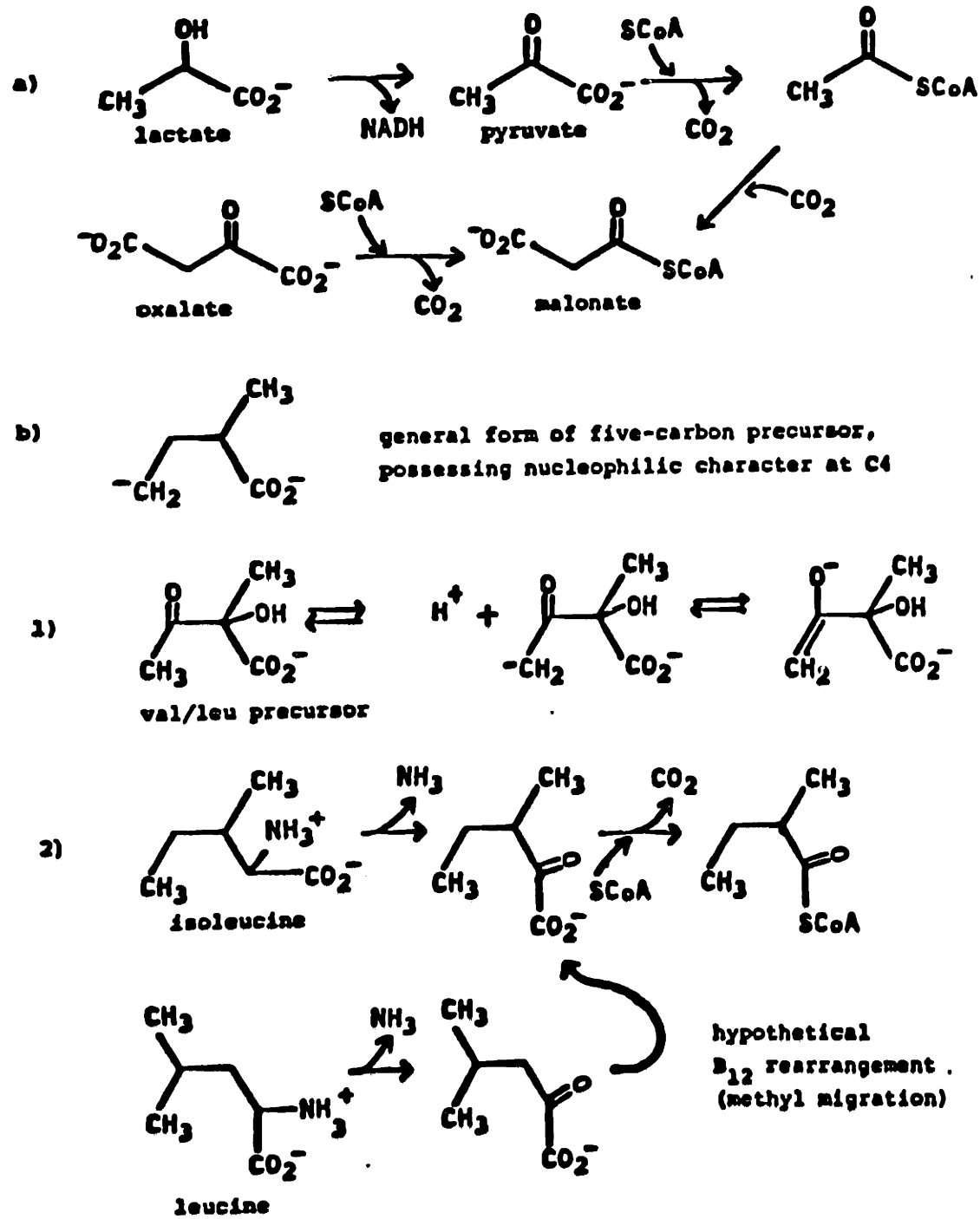


Figure 32.b. Possible sources include leucine biosynthesis and isoleucine and leucine degradation (see Figure 32.b.). Activation of this unit could take place by direct formation of the thioester from the C_5 acid, or by displacement of CO_2 by SCoA from the C_6 α-keto acid, as was demonstrated for valine incorporation into leucomycin (Omura et al., 1983a). The proposed 5 carbon unit would have to be attached to the macrolide ring by C1 and C4, while acetate, propionate, and butyrate are all attached to macrolide rings by C1 and C2 (see Literature Survey, section II.B.). If the five carbon unit is suitably activated, this type of incorporation is theoretically possible, but I am unable to find an example in the literature. Incorporation of ^{13}C-labelled leucine, isoleucine, or five carbon acids should enrich this region. (It may be interesting to note that the defined medium, empirically designed to improve rapamycin production, contains 1.0 g/l leucine.) 4) Glycollate is a two carbon unit which is a precursor of geldanamycin (Johnson et al., 1974; Lancini, 1983; see also Figure 4 in the Literature Survey, Section II.B.). It could be incorporated into rapamycin, but carbons 10 and 11 are fully reduced while glycollate is very oxidized and would have to undergo several rounds of reduction after being incorporated. 5) 2-^{13}C-Glycerol (and U-$^{13}C_6$-glucose) labelled two carbons of leucomycin which were not labelled in previous experiments by acetate, glycollate, propionate, malonate, or several other precursors (Omura et al., 1983b). It was, however, hypothesized that glycerol is first converted to glycollate and then incorporated, and that previous attempts with glycollate failed due to the failure of the compound to

enter the cells. In any case, the carbons derived from glycerol are highly oxidized initially and would have to be reduced several times to get to the oxidation state of rapamycin.

The above possibilities are reevaluated after experiments with labelled propionate are discussed.

G) Incorporation of 1-^{13}C-Propionate

According to the biosynthetic scheme originally proposed, seven propionate units should be incorporated into rapamycin. In addition to labelling these seven propionate moieties, the incorporation of ^{13}C-labelled propionate may also provide information on the origins of carbons 10 and 11. In previous experiments these two carbons were not labelled by acetate, as had been predicted by the original scheme. One explanation was that carbons 10 and 11 may be derived from C2 and C1 of propionate, the methyl group being lost as CO_2. If this is the case then carbons 10 and 11 would be labelled by the appropriately labelled propionates. Another explanation for the lack of acetate incorporation was that C10 and C11 were incorporated as part of an intact C_5 unit, including also C12, C13, and C37. In this case none of these five carbons should be labelled by propionate. 1-^{13}C-propionate was chosen for use in incorporation studies due to its commercial availability and relatively low cost.

Labelled rapamycin was prepared using the same medium, culture conditions, and purification scheme used in the experiments with ^{13}C-acetate. It seemed reasonable to assume that propionate would have similar cell toxicity, membrane permeability, and incorporation levels into rapamycin. In experiments with unlabelled acetate it had been found that the cells could tolerate the addition of 0.5 g/l sodium acetate to the medium at 2.5 days, and another 0.25 g/l could be added at 3.5 days without causing lysis. Sodium propionate added to cultures at the same concentrations and times also had no apparent effect on

growth or rapamycin production. With one addition of 0.5 g/l sodium ^{13}C-acetate at 2.5 days, a two to four-fold increase in peak height had been obtained for acetate-derived carbons. It was reasoned that a higher level of incorporation might be achieved by adding a second pulse of labelled propionate to the producing cultures, thereby maintaining a higher concentration of labelled substrate in the medium during the production phase.

A fermentation was started in fourteen 500 ml flasks, each containing 50 ml of the basal medium. After 2.5 days of incubation, a sterile aqueous solution of sodium 1-^{13}C-propionate (Cambridge Isotope Labs, 90% enriched) was added to each flask to give a final concentration of 0.5 g/l. After 24 hours, more sodium 1-^{13}C-propionate (0.25 g/l) was added to each flask. After 6.5 days of incubation, the contents of the flasks were pooled and the rapamycin was recovered and purified as described earlier. A second fermentation (7 flasks) was started, but before the remainder of the propionate was added, mechanical problems led to an early termination of the fermentation. Since it was unclear when these mechanical problems would be corrected, the ^{13}C-NMR analysis was performed on the material recovered from only 12 flasks, less than 8 mg. This accounts for the relatively poor signal-to-noise ratio in the spectra and the failure of many of the natural abundance peaks to be resolved from the noise. (See Figures 33a and b.)

Despite the roughness of the spectra, five peaks are clearly enriched by 1-^{13}C-propionate. These peaks correspond to carbons 1, 7,

Figure 33: Spectrum of 1-^{13}C-propionate enriched rapamycin compared with the natural abundance spectrum (solvent = $CDCl_3$).

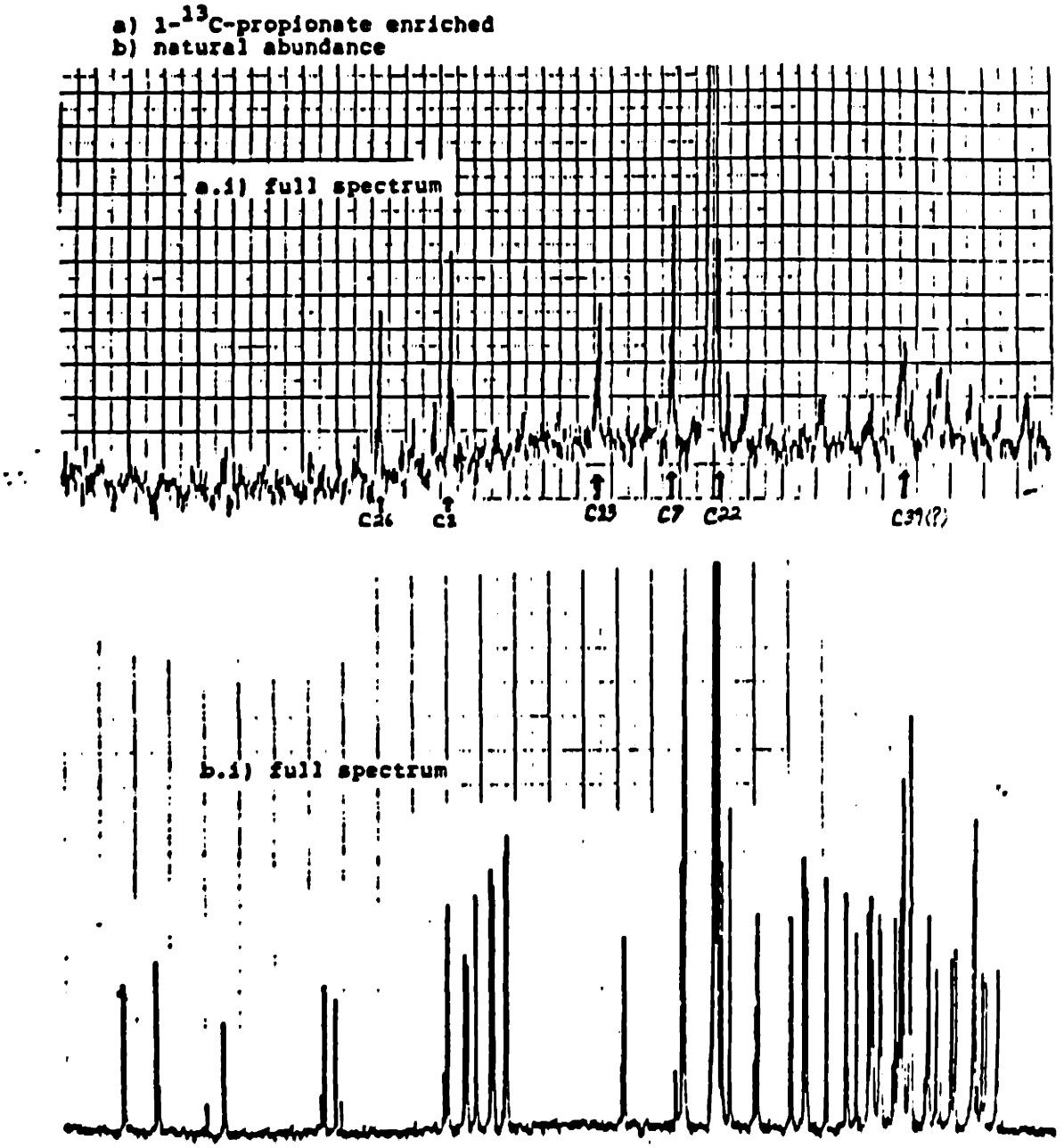
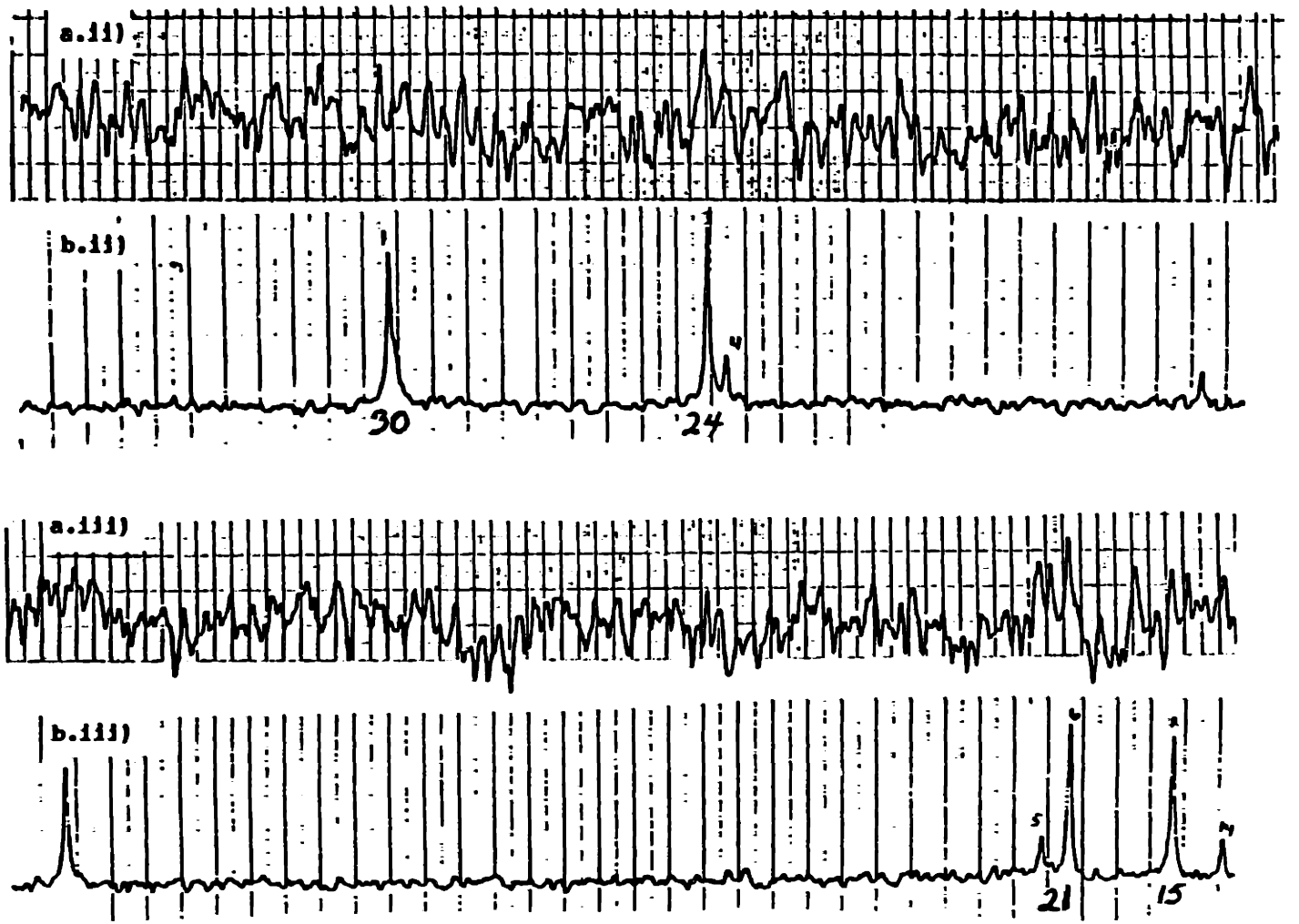


Figure 33 (continued):



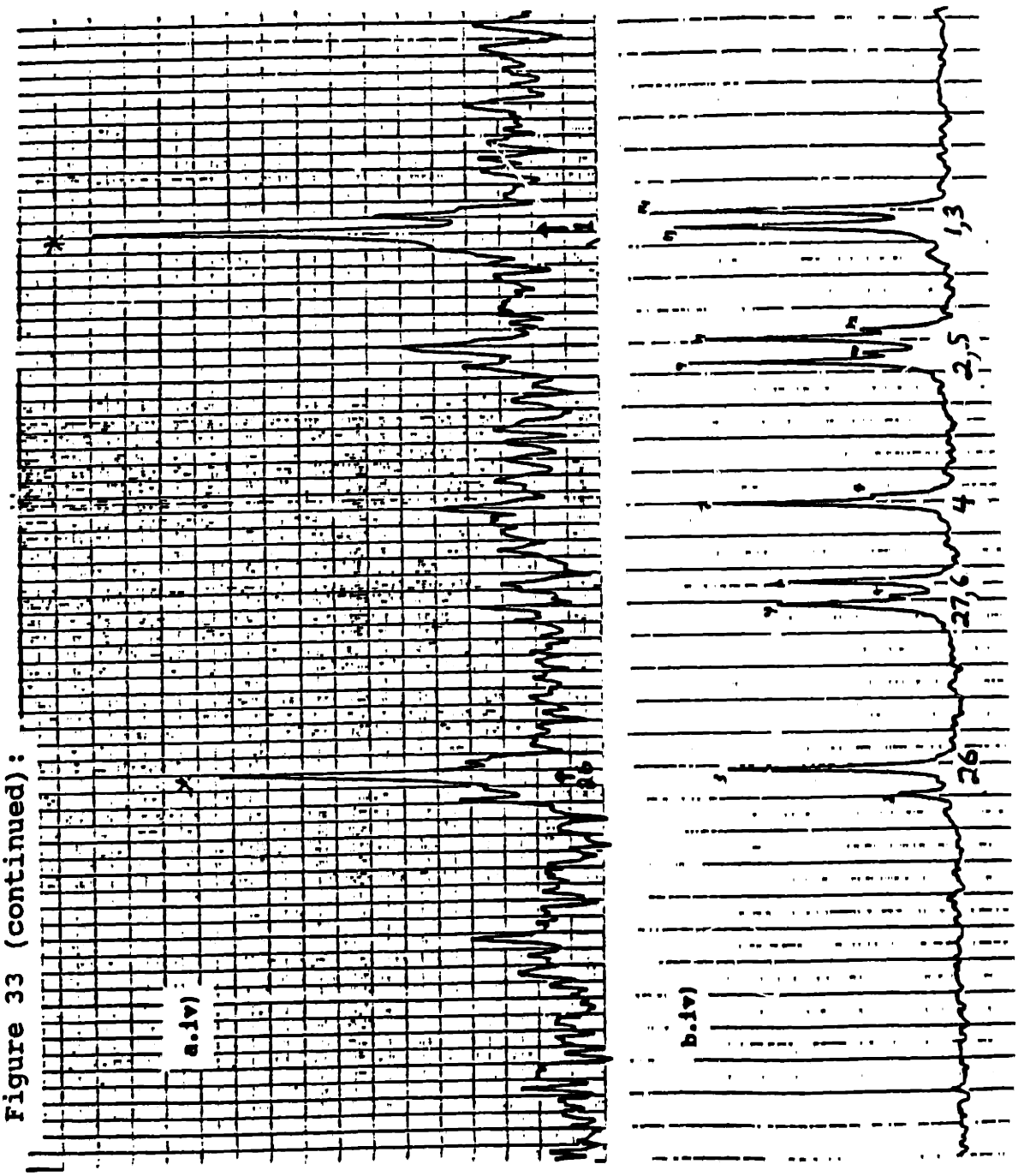


Figure 33 (continued):

Figure 33 (continued):

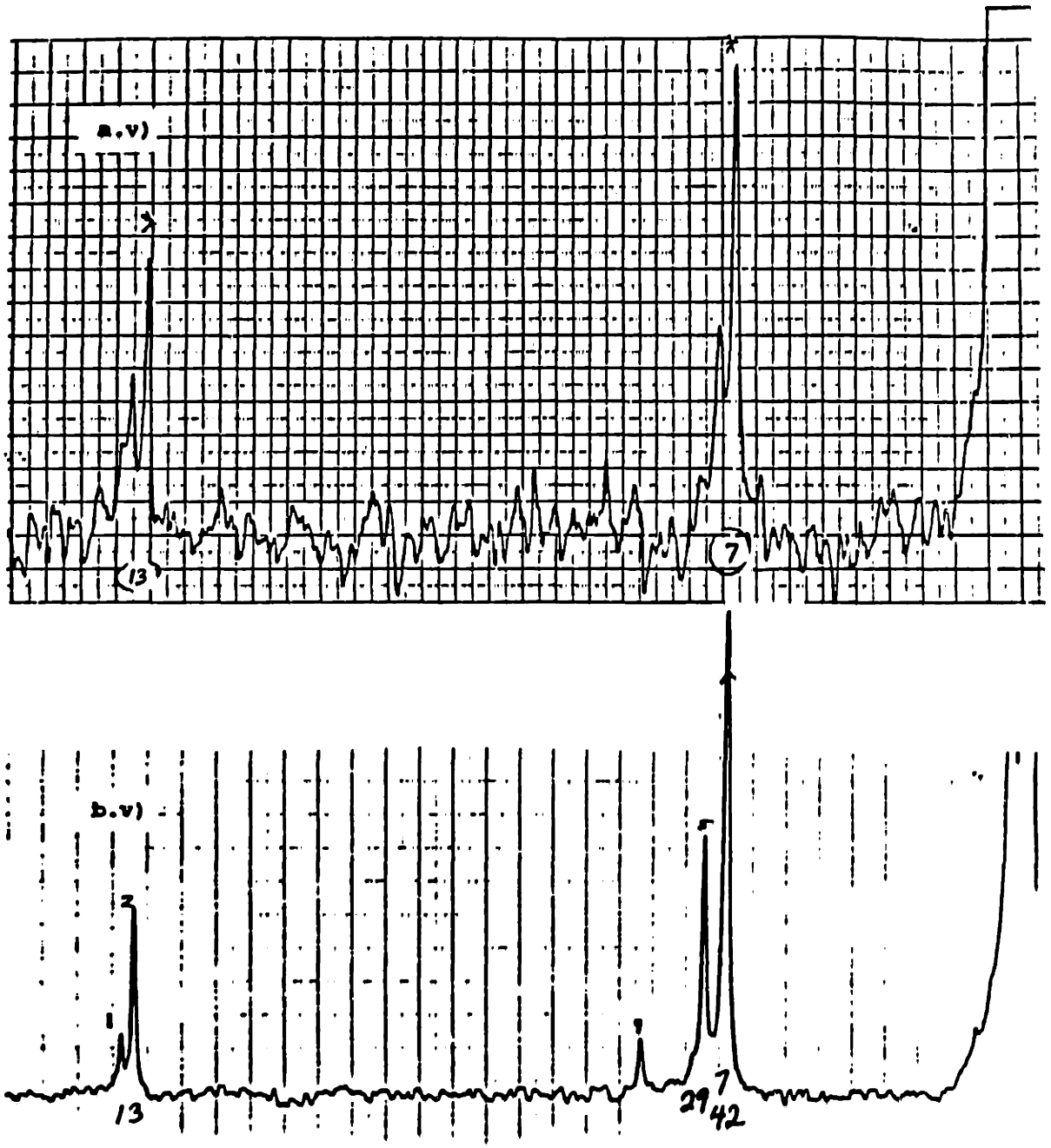


Figure 33 (continued):

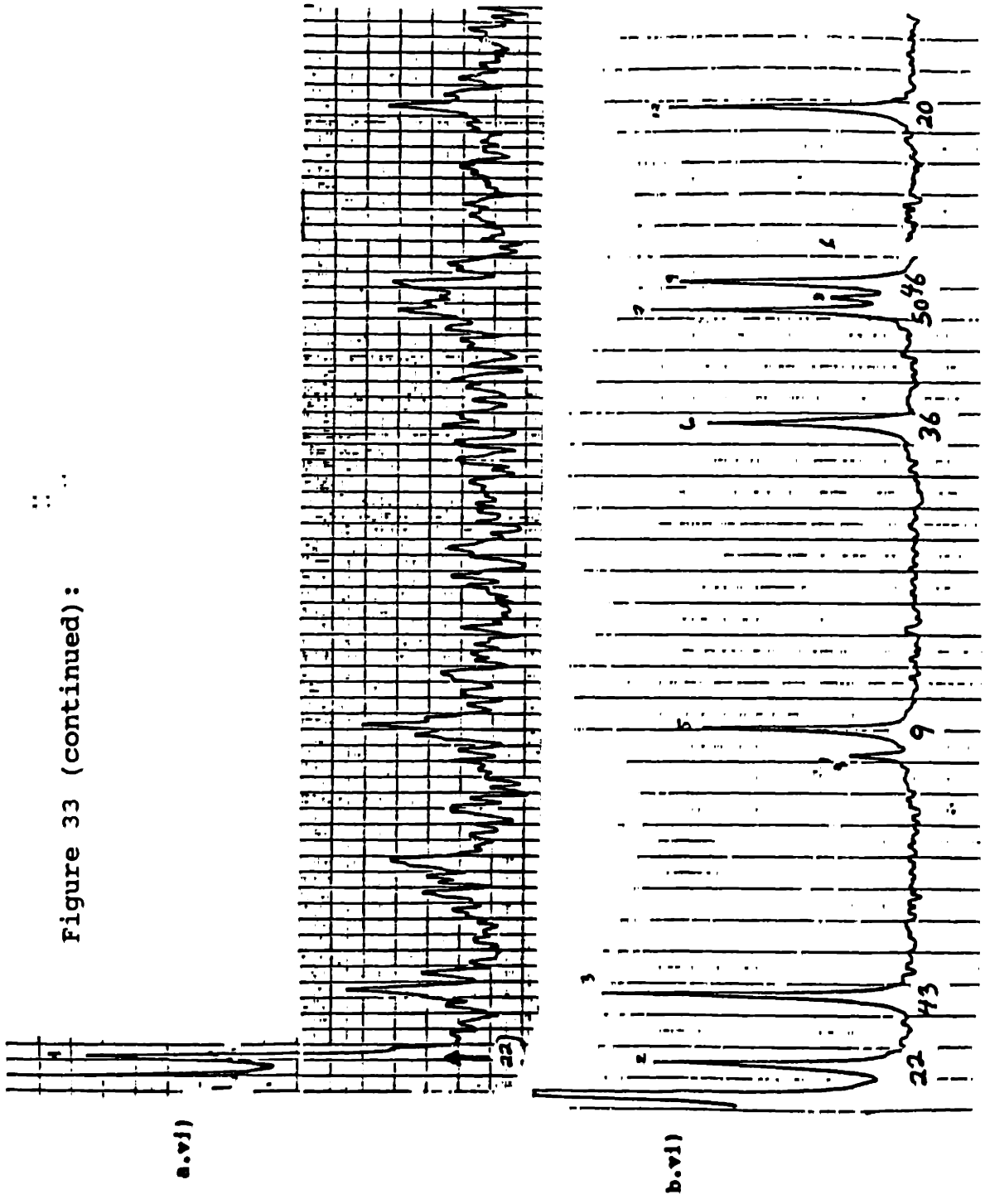


Figure 33 (continued):

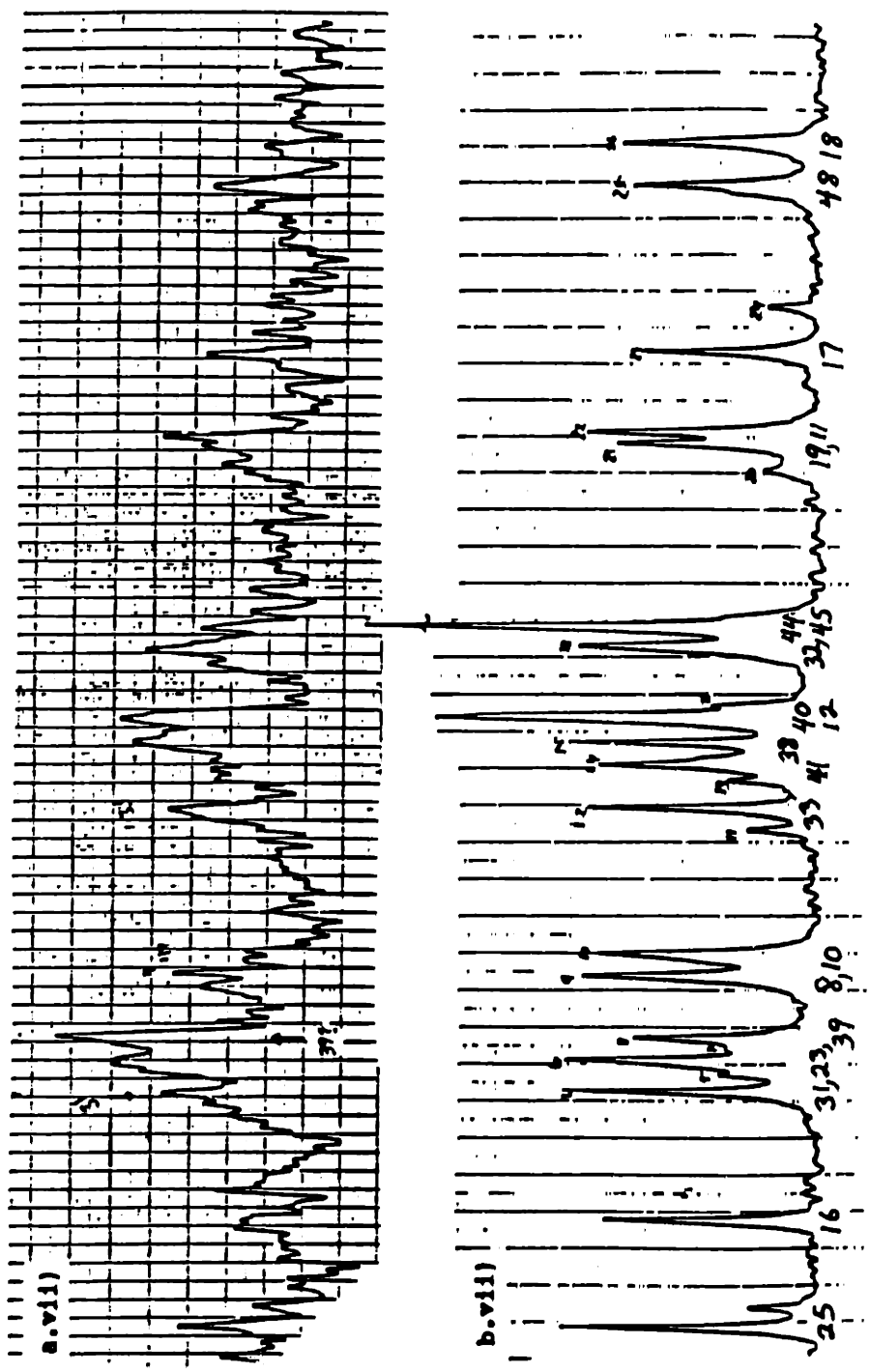
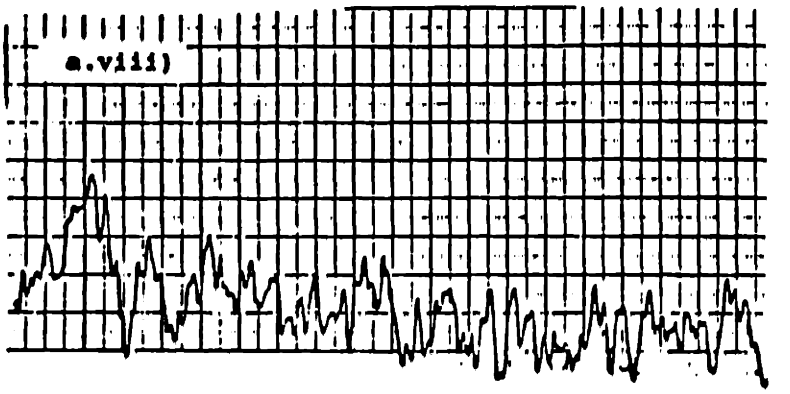
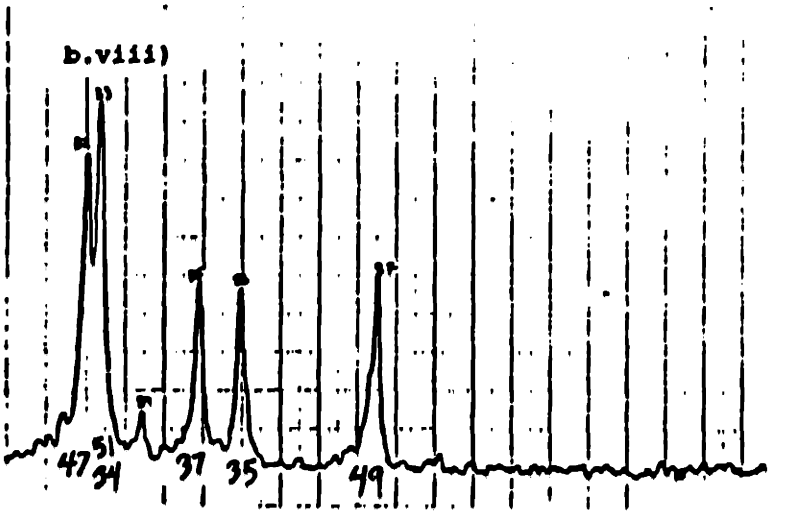


Figure 33 (continued):

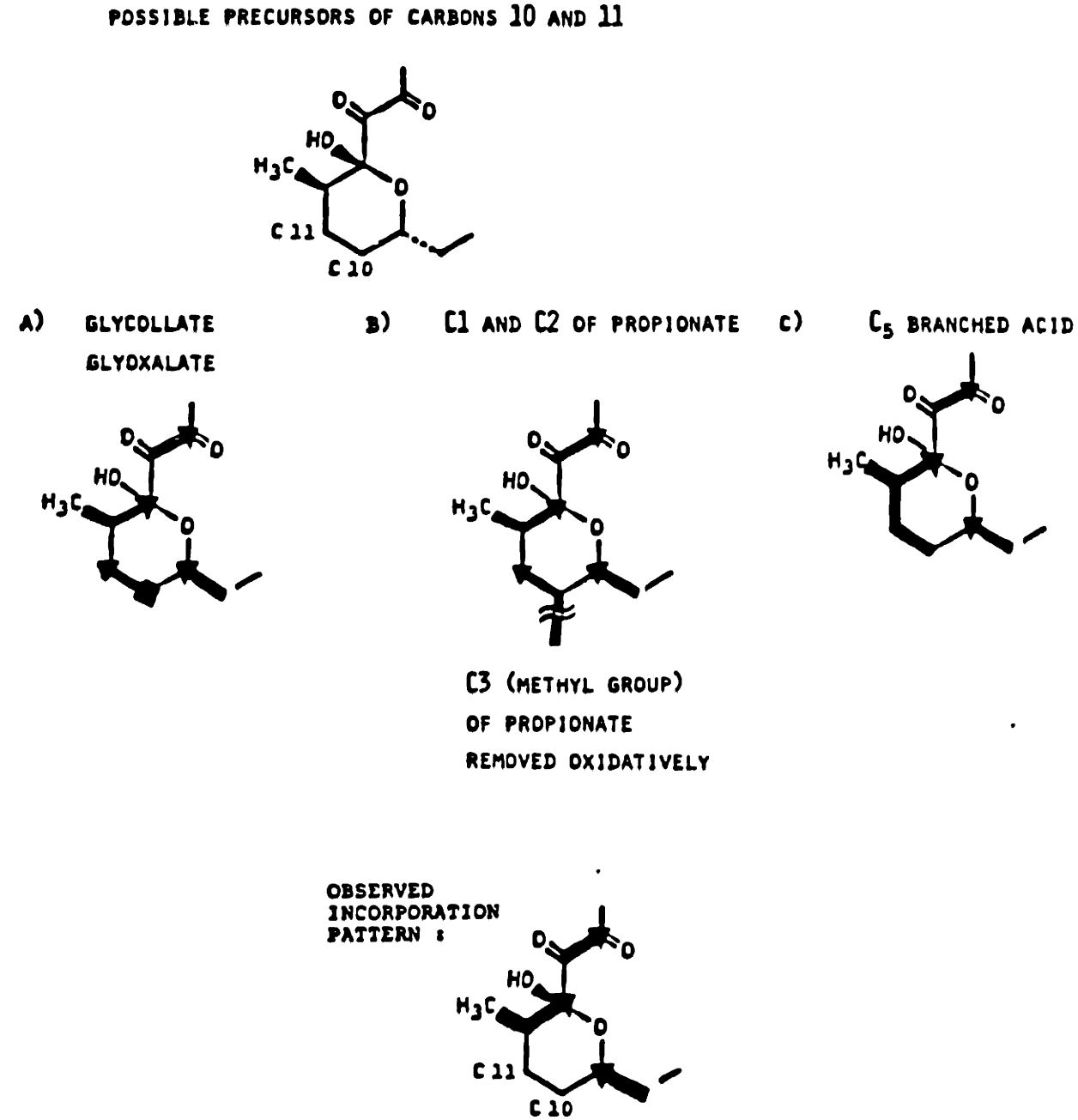




13, 22, and 26. (Carbons 7 and 42 are assigned to the same peak in the spectrum, but it is assumed for the present that the increase in peak height is due to enrichment of C7 and not C42.) These results are in good agreement with the originally proposed scheme, since these carbons were predicted to be derived from the carboxyl group of propionate. Carbon 11 was not enriched, indicating that carbons 10 and 11 are not derived from propionate. Since carbon 13 was clearly enriched by propionate, it is also apparent that carbons 10 and 11 are not incorporated as part of a C_5 unit. (See Figure 34.)

Surprisingly, the peak assigned to carbon 32 did not increase in height. There was a slight increase in the height of the peak assigned to carbon 39, although it is not as significant as the increases in the other five peaks. Carbon 39 is predicted to be derived from the carboxyl group of a shikimate pathway intermediate. It is possible that this carbon could be labelled by 1-^{13}C-propionate via scrambling reactions (propionate --> succinate --> oxaloacetate --> phosphoenolpyruvate --> shikimate), and the observed pattern could be consistent with the proposed shikimate origin of carbon 39. If the assignments are correct, then carbon 32 (and presumably 31 and 51) is not derived from propionate. However, carbons 32 and 39 are both methylene carbons in rather similar environments. They are also not among the few carbons for which Findlay and Radics (1980) established connections by selective ^{13}C-^{1}H decouplings. Further experiments were needed to determine whether or not the assignments were correct. Possibilities included the incorporation of a different form of labelled

Figure 34: Suggested precursors of carbons 10 and 11 and observed incorporation pattern.



propionate (2-^{13}C- or 1,2-$^{13}C_2$-propionate), the incorporation of a precursor known to label specifically the carboxyl group of shikimic acid, and using NMR techniques to reexamine the spectral assignments. The results of such experiments are discussed in Sections R and V.

Assuming the assignments are correct and that carbon 32 is not derived from propionate there are several other possible precursors for the corresponding three carbon unit. One possibility is that, as was suggested for carbons 10 and 11, carbons 31 and 32 are derived from glycollate. The adjacent methyl group (carbon 51) would be donated by methionine. Another alternative is that a C_3 precursor such as glycerate is incorporated in place of the proposed propionate. Finally, a C_4 unit such as succinate or butyrate may be incorporated and then the fourth carbon excised. The first possibility was tested in the next labelling experiment.

The seventh carbon predicted to be enriched by 1-^{13}C-propionate is carbon 28. The peak (77.23 ppm) assigned to this carbon is obscured by the solvent peaks when $CDCl_3$ (triplet, 77.0 ppm) is used as the solvent. In order to see if the carbon 28 is enriched, the $CDCl_3$ was removed from the rapamycin sample enriched with 1-^{13}C-propionate and the sample was redissolved in CD_2Cl_2 (quintet, 53.8 ppm). The spectrum was then recorded using the same conditions used for earlier samples. Data were accumulated over a longer period of time, resulting in a better signal to noise ratio. A natural abundance spectrum of rapamycin in CD_2Cl_2 was also obtained (approximately 100 mg sample). (See Figures 35 a and b.)

From comparisons of the spectra obtained in CD_2Cl_2 it is apparent

Figure 35: Spectrum of 1-^{13}C-propionate enriched rapamycin compared with the natural abundance spectrum (solvent = CD_2Cl_2).

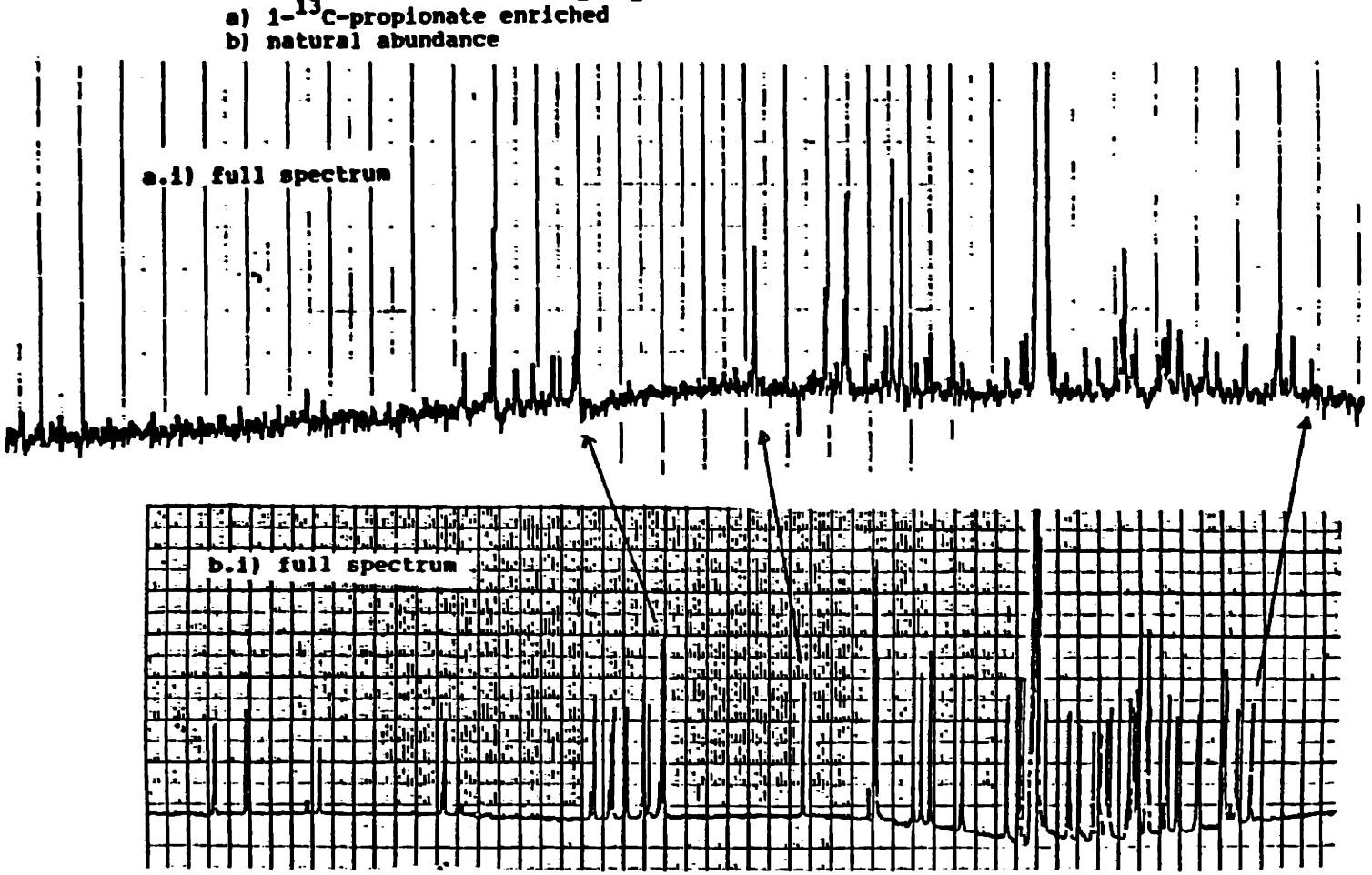
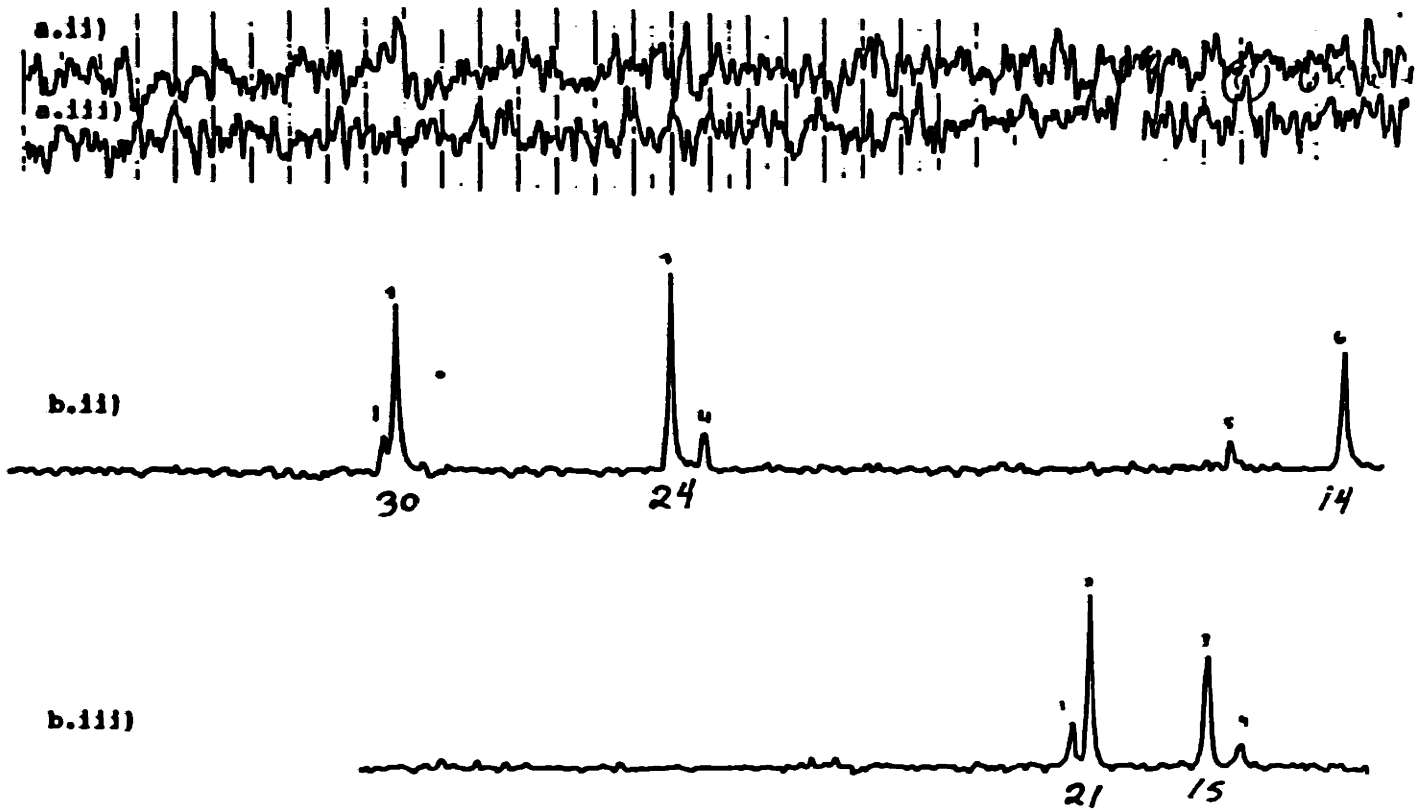


Figure 35 (continued):



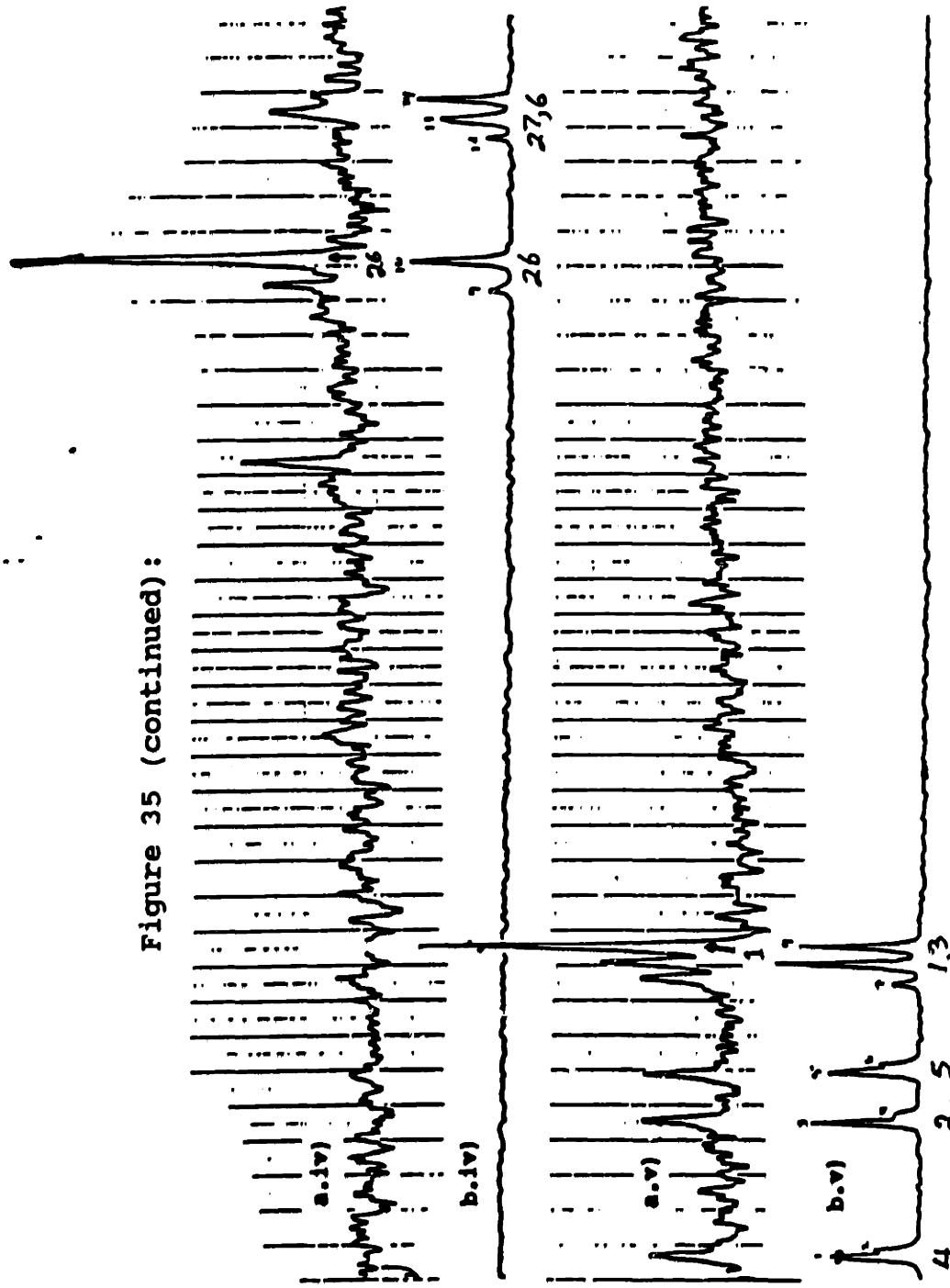


Figure 35 (continued):

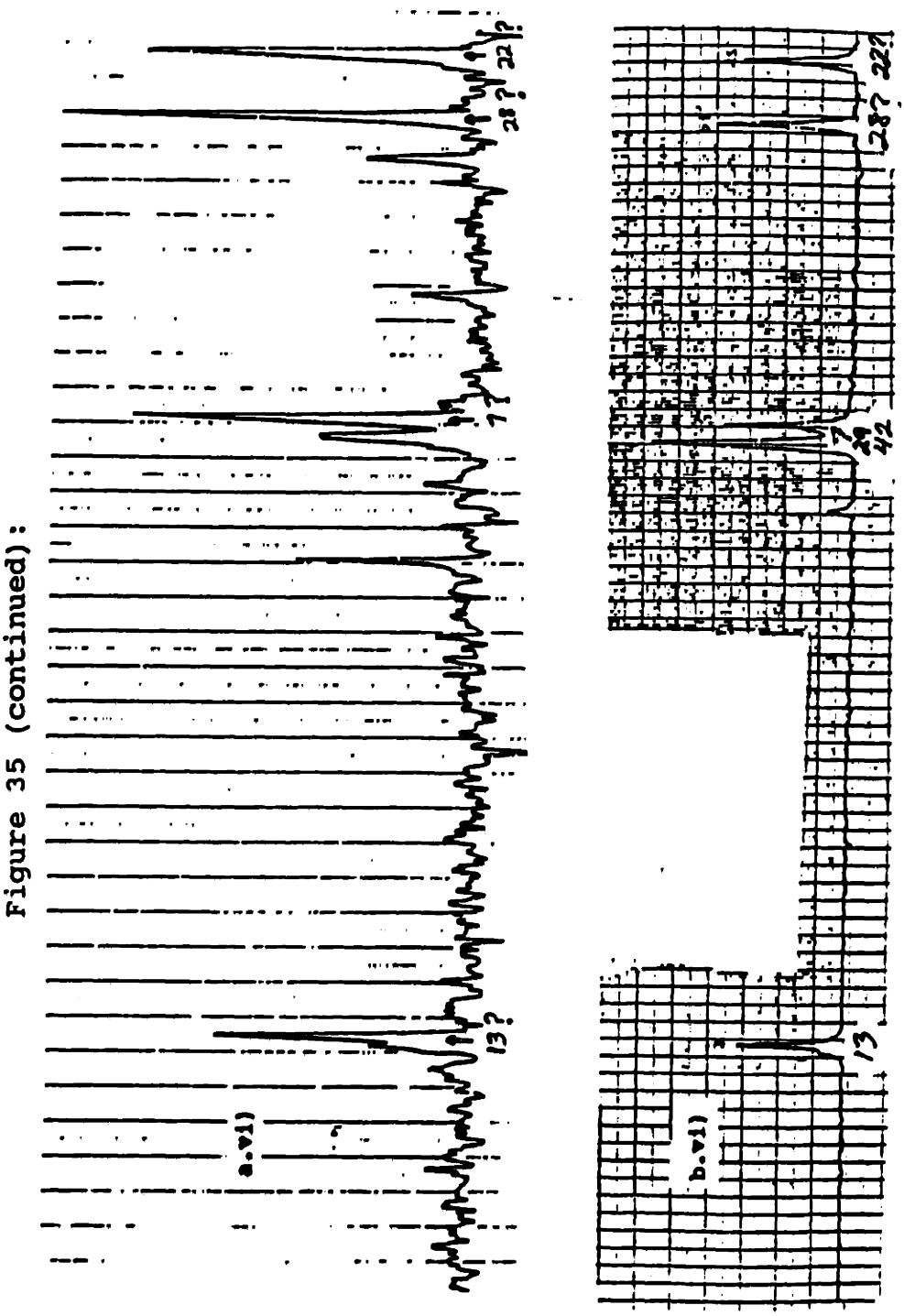


Figure 35 (continued):

Figure 35 (continued):

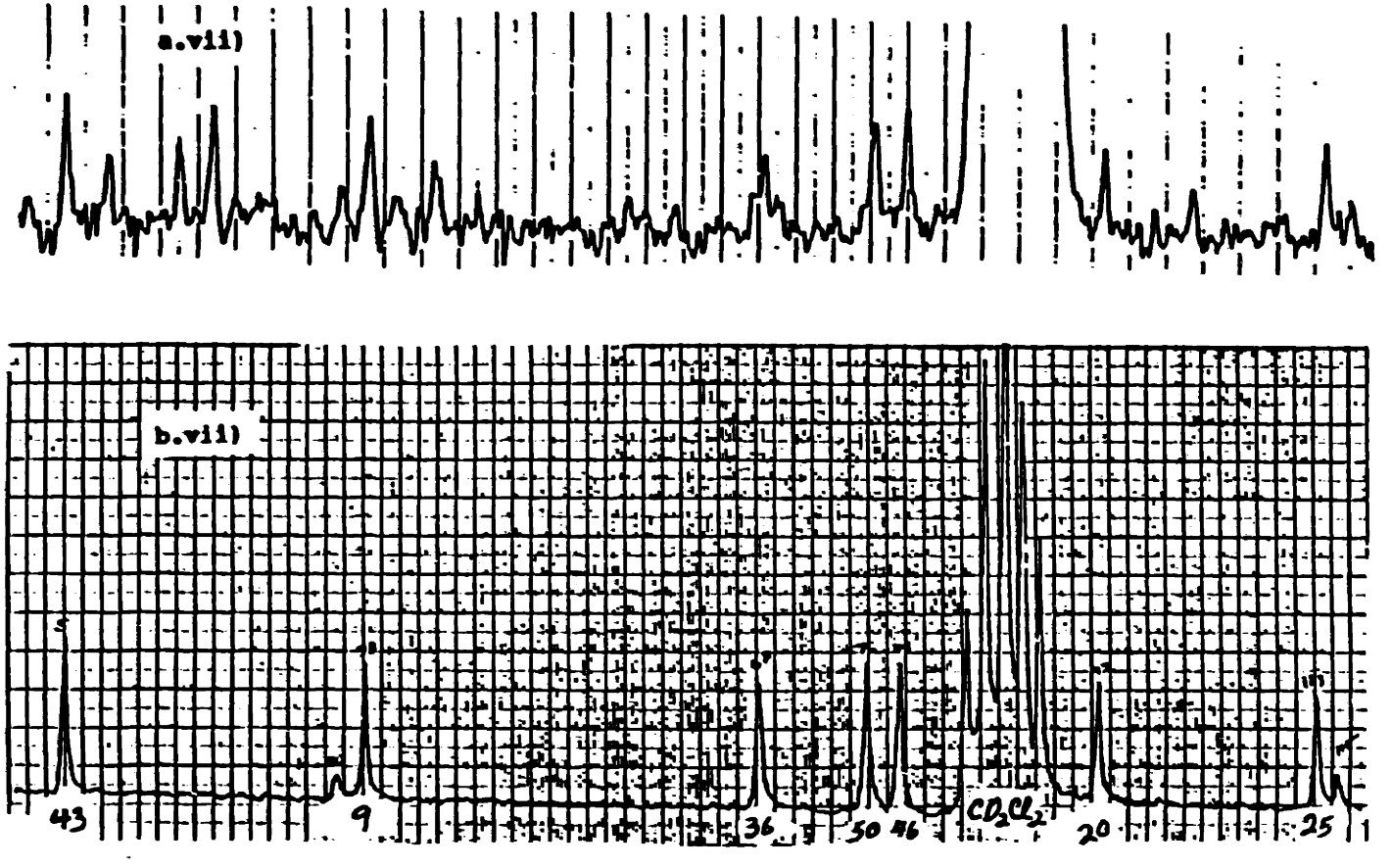
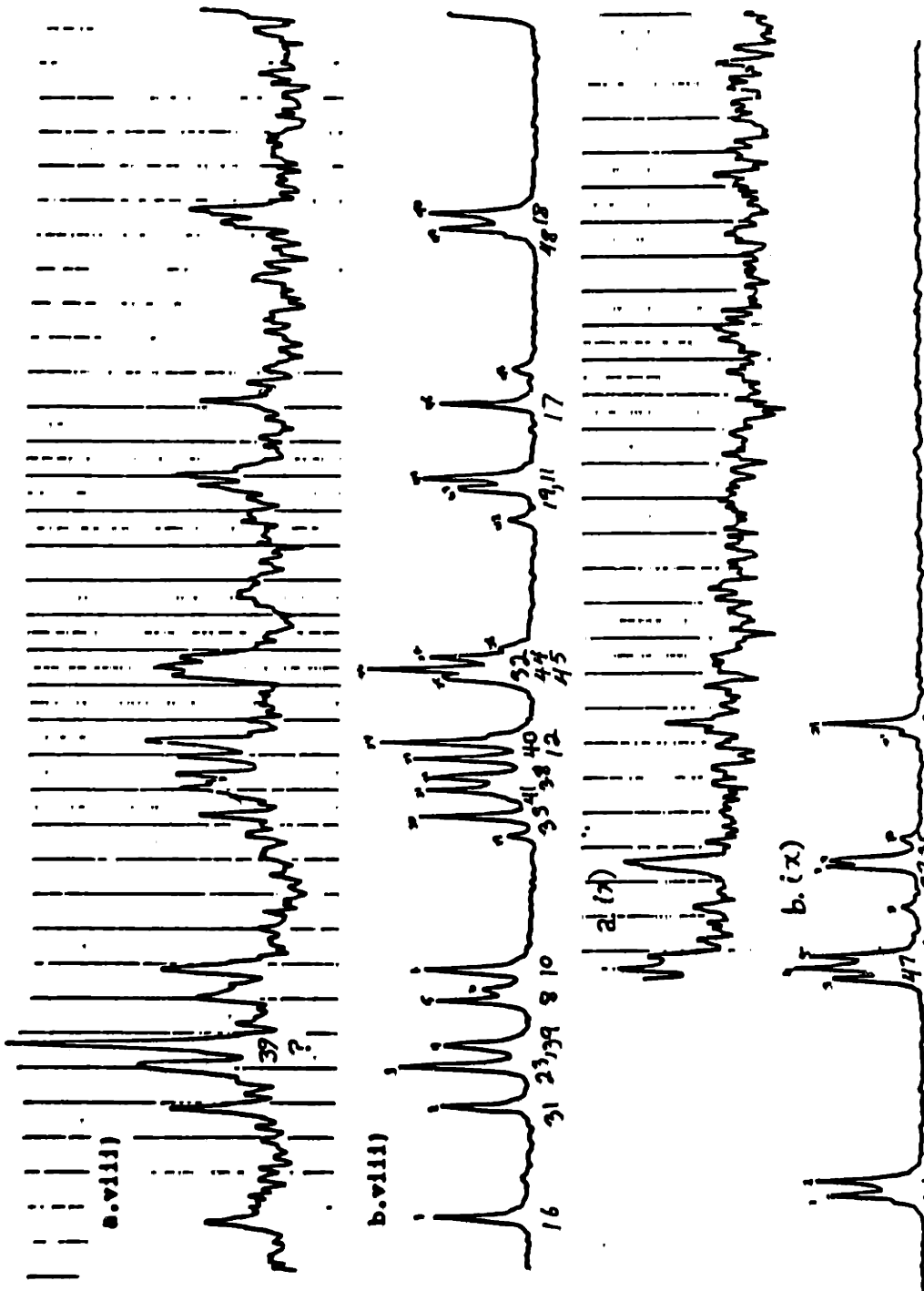


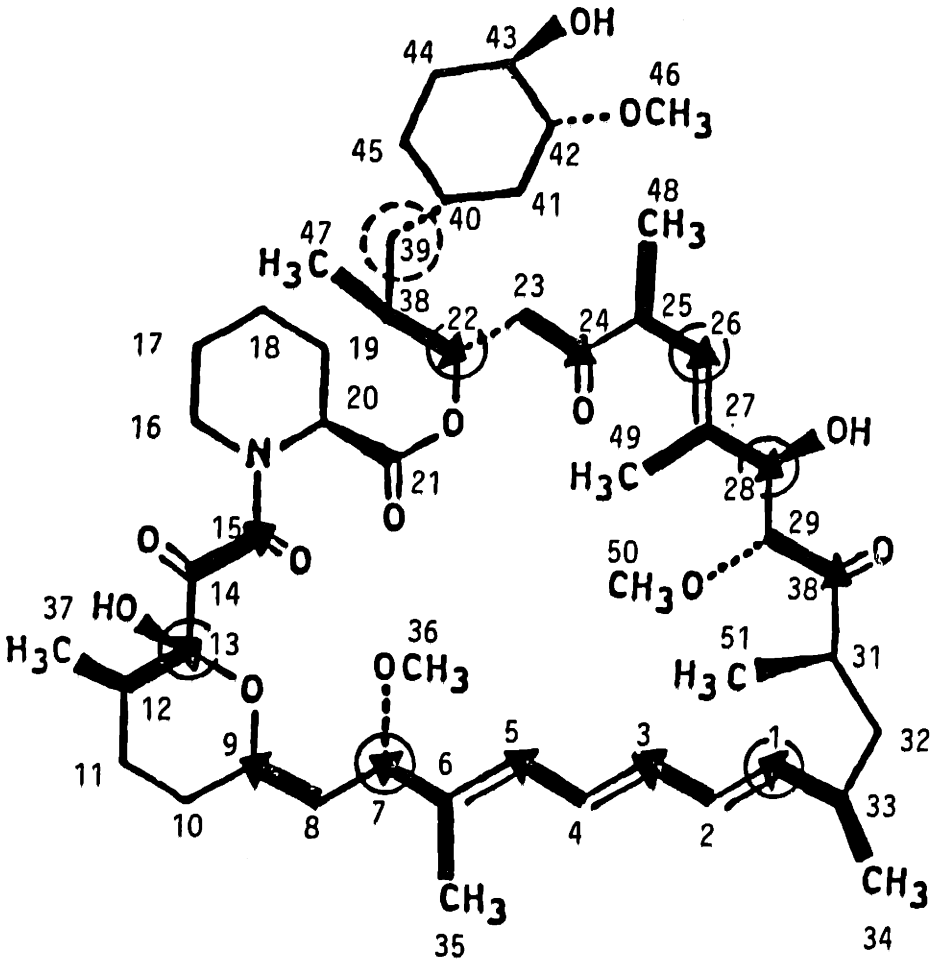
Figure 35 (continued):



that there is an additional peak which has increased in height and was previously hidden by the $CDCl_3$ solvent peaks. The two solvents are very similar in their polarity, lack of hydrogen-bonding, and other solvent properties. As a result, there is very little difference in the two natural abundance spectra. Most peaks appear to have shifted very little or not at all. Therefore it is reasonable to assign the additional labelled peak to carbon 28 of rapamycin, thereby confirming the incorporation of a sixth propionate unit. The possible enrichment of the peak attributed to carbon 39 noted earlier appears much more definite in this "cleaner" spectrum.

The results of the incorporation of 1-^{13}C-propionate are summarized in Figure 36.

Figure36: Locations of propionate and acetate units incorporated.



: $CH_3CO_2^-$, ACETATE

: $CH_3CH_2CO_2^-$, PROPIONATE

: enriched by 1-^{13}C-propionate

: somewhat enriched by 1-^{13}C-propionate, but spectral assignment questionable

H) Effect of the Addition of L-Methionine and D,L-Ethionine

According to the proposed scheme, the three methoxy groups in rapamycin should be derived from the methyl group of methionine (by way of S-adenosylmethionine, or SAM). It is also possible that the methyl group containing carbon 51 is derived from methionine, if it is not derived from propionate. Methionine was added to the fermentation medium to see if it affects rapamycin production.

Eight flasks containing 50 ml of the standard fermentation medium were inoculated. After 3.5 days, 0, 0.5, 1.0, and 2.0 g/l (final concentration) L-methionine was added to duplicate flasks. A sample was taken from two of the flasks at the time of addition. These two samples, along with samples taken from each of the flasks at the end of the fermentation (6.5 days), were assayed for rapamycin. The samples taken at the time of addition showed that significant rapamycin production had already taken place (approximately 30 ug/ml, 50% of the total expected production). The samples taken at the end of the fermentation showed no difference in rapamycin accumulation. This was possibly because the methionine was added too late for it to have a noticeable effect. The cells had probably already synthesized all the enzymes and precursors necessary for rapamycin biosynthesis. Only a very strong inhibition would have been detectable, since the control flasks only increased in rapamycin content from 30 to 55 ug/ml.

Ten flasks each containing 50 ml of the same fermentation medium were inoculated. After 2.5 days incubation, a filter-sterilized solution of L-methionine was added to each flask to give the following

final concentrations: 0, 0.25, 0.5, 1.0, 2.0 g/l. After 6.5 days, the fermentation was stopped and assayed. The dry cell weights obtained for all of the flasks did not differ significantly. However, methionine did have an effect on rapamycin production. At the lowest concentration added (0.25 g/l), methionine may have been slightly stimulatory. However, at concentrations above 0.5 g/l, methionine was clearly inhibitory, causing more than a 40% decrease in rapamycin production at 2 g/l. (See Table 8.)

These findings were at first surprizing since addition of a true precursor is expected to increase product formation if the production of the precursor is rate-limiting. Otherwise the precursor should have no effect, unless it it not the actual precursor and is inhibiting the formation of the true precursor. However, methionine has been proven to be the precursor of the side chain of thienamycin and the methoxy group of cephamycin C and yet inhibits the production of these antibiotics by Streptomyces cattleya (Williamson et al., 1985). Approximately 90% inhibition of total carbapenem biosynthesis was found in resting cells in the presence of 2 g/l methionine. The product distribution among related antibiotics was also changed, as the formation of less methylated products was not inhibited as much. The explanation suggested for the inhibition is that methionine is probably an inhibitor or repressor of SAM-synthetase, as has been shown for E. coli. This is also likely for the case of rapamycin biosynthesis. Another possibility was that methionine was somehow reducing the synthesis or uptake of lysine, the proposed precursor of the pipecolate moiety and a major

Table 8: Effect of L-Methionine Addition on Rapamycin Production

Concentration of Added* L-Methionine (g/l)	Final Rapamycin Concentration (ug/ml)	Rapamycin Production (%)
0	66	100
0.25	72	109
0.5	60	91
1.0	42	64
2.0	35	53

* Addition after 2.5 days of incubation.

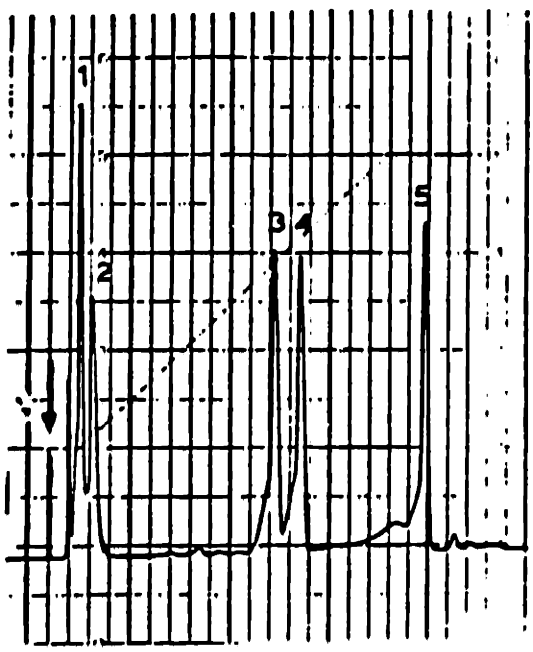
nitrogen source in the medium. Lysine and methionine are both synthesized from aspartate, and may have overlapping regulation effects. Samples from the above fermentation were subjected to amino acid analysis to determine whether lysine was still being utilized in the presence of methionine. When zero or 0.25 g/l methionine was added to the medium, all of the lysine had been removed from the medium by the end of 6.5 days. When 2.0 g/l methionine was added, 26% of the lysine remained in the medium (approximately 1 g/l of the initial 4 g/l L-lysine·HCl) and approximately 15% of the added methionine. (See Figure 37.) The amount of lysine entering the cells is still greatly in excess of the amount theoretically required for rapamycin synthesis, and thus methionine does not appear to act by preventing lysine uptake.

Ethionine, an analog of methionine, often inhibits methylation reactions (Argoudelis et al., 1973). It can also be substituted for methionine, producing the analogous ethylated products or being incorporated into proteins of modified activity. Ethionine was added to fermentation flasks in order to determine its effect on rapamycin production, and to see if it may cause the production of any new rapamycin derivatives. For comparison, similar concentrations of methionine were also added. Twelve flasks were inoculated and incubated 1.3 days. Duplicate flasks received the following additives: nothing, 0.5 or 2.0 g/l L-methionine, and 0.2, 0.5, or 2.0 g/l D,L-ethionine. After a total of 6.5 days, the flasks were sampled and assayed for rapamycin.

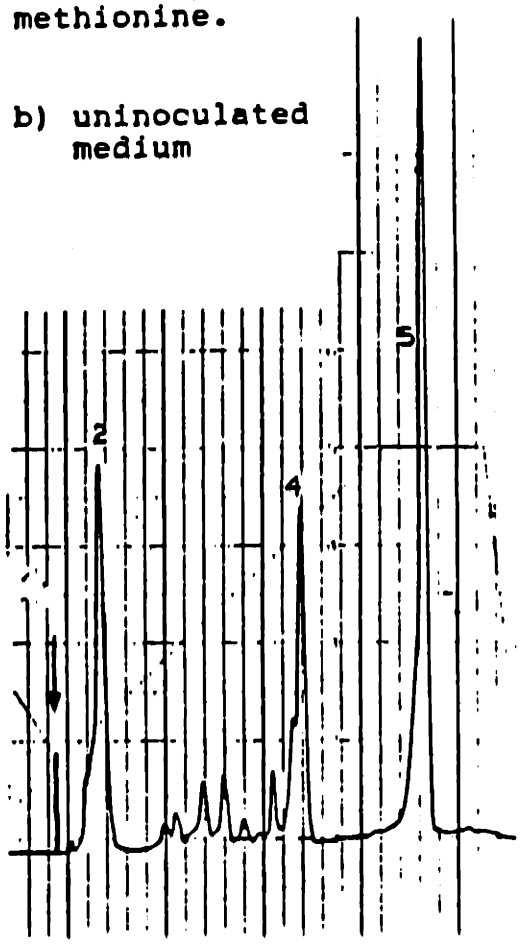
Table 9 shows the final rapamycin concentrations reached and

Figure37: Amino acid profiles of culture supernatants with and without addition of methionine.

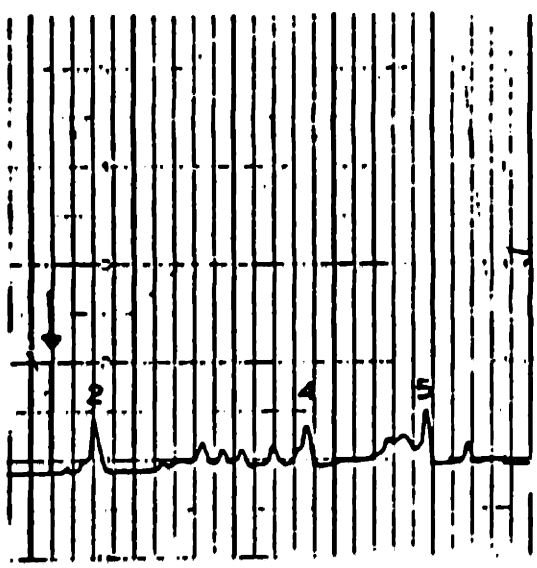
a) standards: (1 mM each)
1 asp
2 glu
3 met
4 leu
5 lys



b) uninoculated medium



c) culture supernatant after 6.5 days incubation, no methionine added



d) culture supernatant after 6.5 days incubation, 2.0 g/l methionine added at 2.5 days

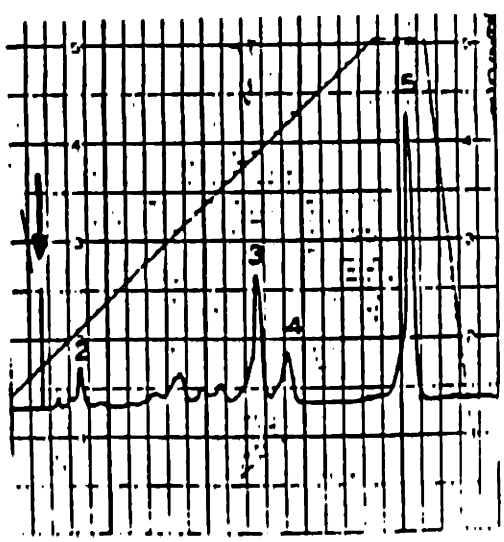


Table 9: Effect of L-Methionine and D,L-Ethionine Addition on Rapamycin Production

Concentration added* (g/l)	Final Rapamycin Concentration (ug/ml)	Rapamycin Production (%)
0 (control)	75	100
0.5 L-methionine	42	56
2.0 L-methionine	28	37
0.2 D,L-ethionine	19	25
0.5 D,L-ethionine	10	13
2.0 D,L-ethionine	7	9

* Added after 1.3 days of incubation

relative production in terms of the percentage of the control flask's final concentration. Methionine had a stronger effect in this experiment (addition at 1.3 days) than in the previous experiment (addition at 2.5 days), 2.0 g/l causing approximately a 60% decrease in final rapamycin concentration. It is clear that D,L-ethionine is a much stronger inhibitor of rapamycin synthesis than L-methionine, since only 0.2 g/l ethionine was necessary to cause a 75% decrease in rapamycin production (with no apparent effect on growth). D,L-Ethionine at 2.0 g/l caused a 90% decrease in rapamycin biosynthesis, but also caused a very visible decrease in growth of the cultures. Ethionine is probably interfering with rapamycin synthesis by inhibiting protein synthesis and other general metabolic processes in the cells, in addition to inhibiting specifically the methylations involved in the biosynthesis of rapamycin.

The addition of ethionine (and sometimes methionine) to production media has caused the accumulation of demethylated or ethylated products by other antibiotic producing strains. The HPLC chromatograms obtained during the analysis of the above samples were examined for the appearance of new peaks eluting near rapamycin. No significant peaks were observed. It is still possible that modified rapamycins were produced, and that they elute much later or are obscured by the other peaks in the rapamycin chromatogroam.

It is clear from the above results that labelling studies with methionine must be conducted with some care. Early addition or high concentrations of methionine will cause a decrease in the amount of

recoverd rapamycin. No significant decrease in production occurs if methionine is added at 0.5 g/l after 2.5 days, so these conditions were used in the following experiments with ^{14}C-methionine.

I) Incorporation of L-^{14}C-[methyl]-Methionine

In order to estimate the degree of incorporation of exogenous methionine into rapamycin, an experiment was performed with radioactive methionine. L-^{14}C-[methyl]-methionine (New England Nuclear, 50 uCi, supplied as a solution in 70% ethanol and assumed sterile) was added to a stock solution of filter-sterilized L-methionine (25 mg/ml). One ml of this solution was added to flasks containing 50 ml medium after 2.5 days of incubation, giving a final concentration of 0.5 g/l methionine in the medium. After 6.0 days of incubation, the contents of the flasks were pooled and the rapamycin extracted as described earlier. The purification was continued to the hexane extraction step. The rapamycin concentration of this extract was determined by HPLC. At the same time an aliquot of this extract was applied to a TLC plate. As was described for experiments with ^{14}C-acetate, the plate was developed and visualized, the rapamycin band was scraped from the plate, and the ^{14}C cpm were measured. The calculated specific activity of the recovered rapamycin and the specific activity of the added methionine were approximately equal. If the proposed scheme is correct and methionine supplies the three methoxy carbons of rapamycin, then 33% of each of the methoxy groups is coming from exogenous methionine under these conditions. In other words, these results indicate that if 0.5 g/l ^{13}C-methionine was added to the medium, we could expect a 33-fold increase in peak height for each of the peaks assigned to these three carbons. If carbon 51 is also derived from methionine, there should still be an average increase of 25%.

J) Incorporation of ^{13}C-[methyl]-Methionine

Since the experiments with radioactive methionine showed very high incorporation, similar conditions were used for the incorporation of ^{13}C-[methyl]-methionine (Merck, Sharp and Dohme, 90% enriched). Fourteen flasks, each containing 50 ml medium, were inoculated and incubated for 2.5 days. A filter-sterilized solution of ^{13}C-methionine was added to the flasks to give a final concentration of 0.3 g/l. The fermentation was stopped after 6.5 days of incubation, the contents of the flasks pooled, and the cells collected, washed, and extracted with methanol as described earlier. This extract was stored at -20 C while another 12 flasks were prepared in an identical fashion. The extracts were pooled and the labelled rapamycin purified by the procedure described in the first progress report. This sample (14 mg) was then subjected to analysis by ^{13}C-NMR.

The spectrum of ^{13}C-[methyl]-methionine enriched rapamycin is shown in Figure 38. Three peaks assigned to the three methoxy carbons (carbons 36, 50, and 46) show a 15 to 20 fold increase in height. No other peaks (including that assigned to carbon 51, the methyl group of the seventh proposed propionate group) increased in height. This eliminates the possibility that the seventh proposed propionate is actually derived from glycollate plus methionine. The three methoxy carbons are the only carbons in rapamycin derived from methionine, as was predicted by the original proposed biosynthetic scheme.

Figure 38: Spectrum of ^{13}C-[methyl]-methionine enriched rapamycin.

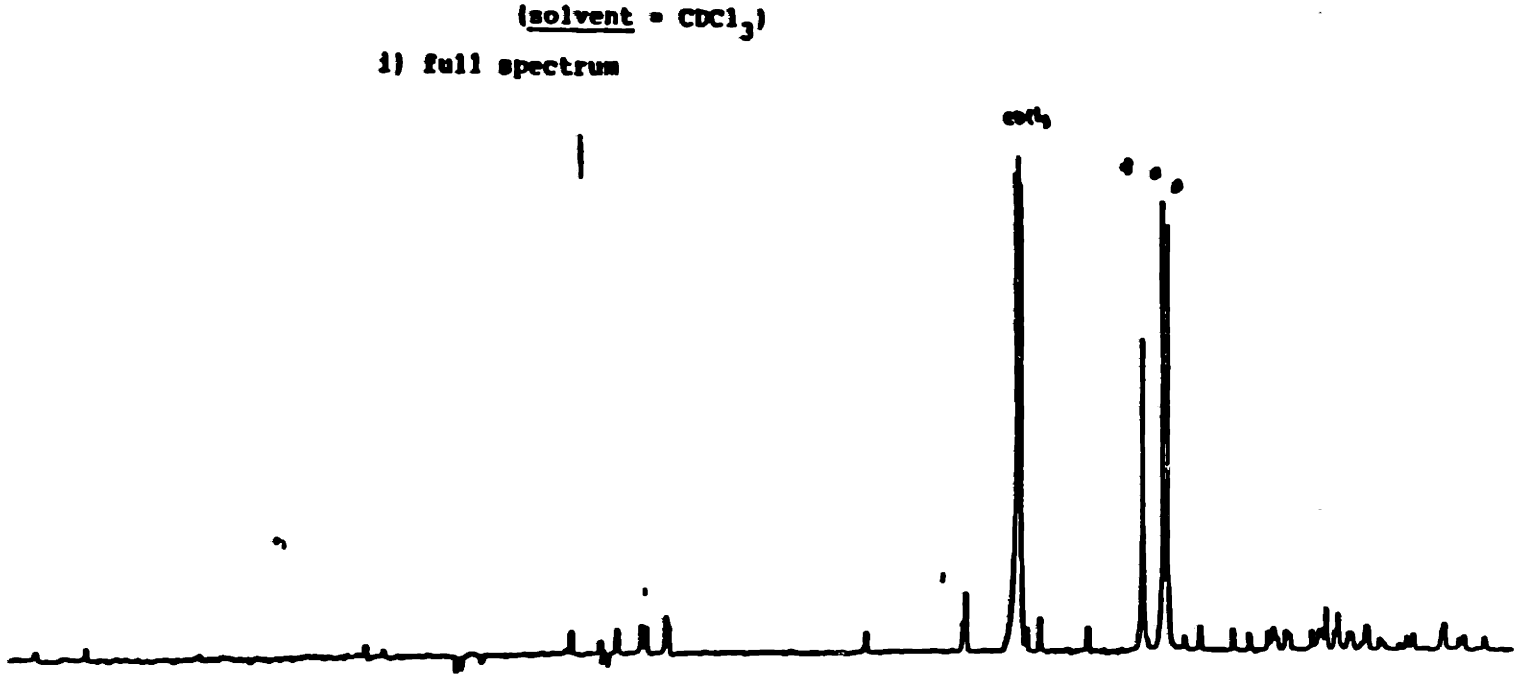


Figure 38 (continued):

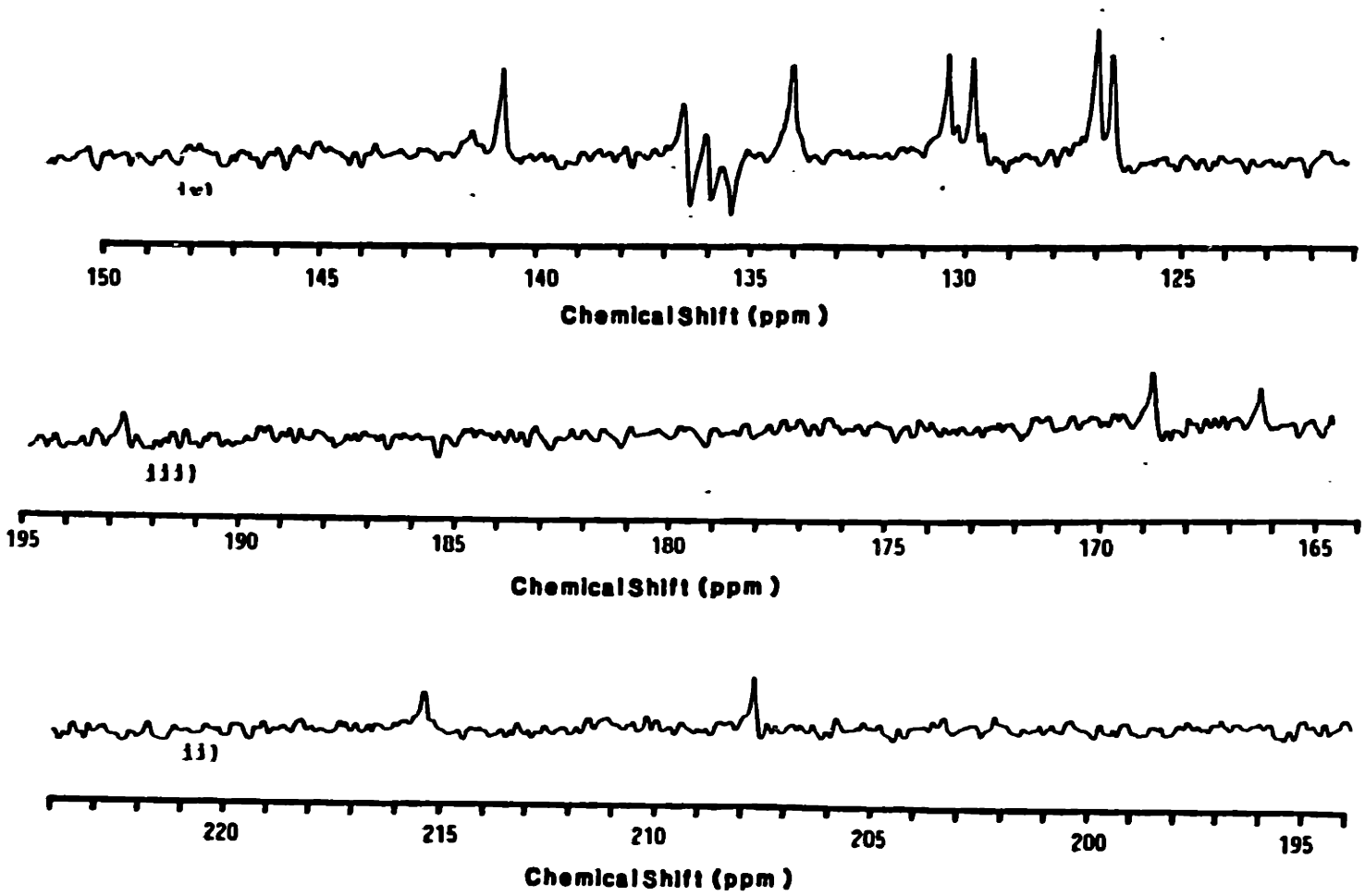


Figure 38 (continued):

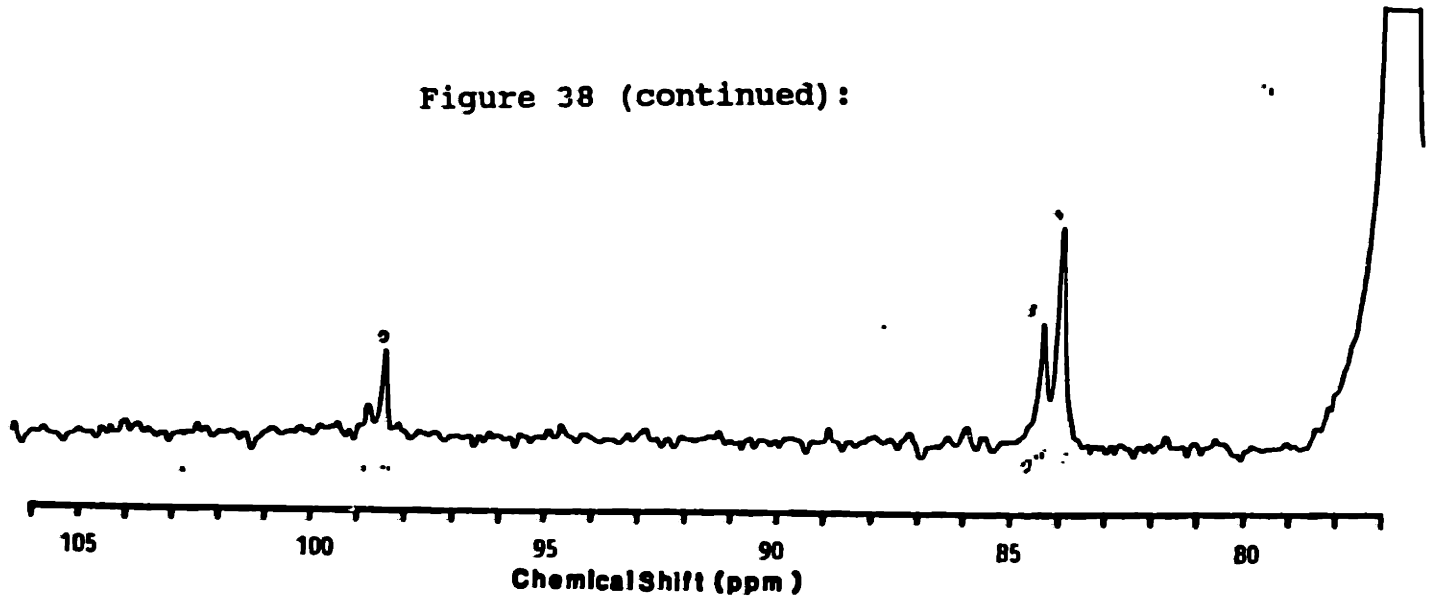


Figure 38 (continued):

(vertical expansion is one half that of the other partial spectra in this series)

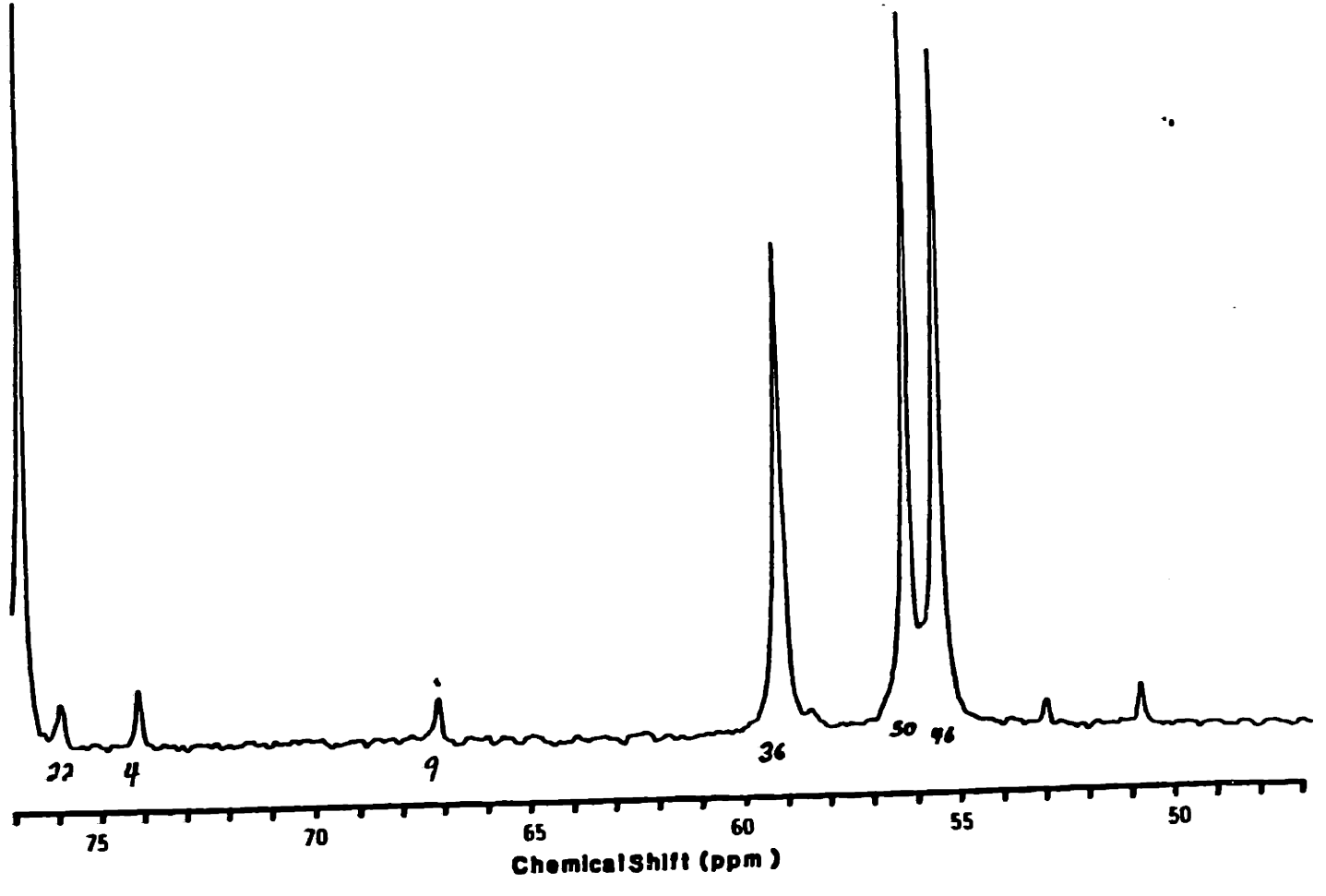
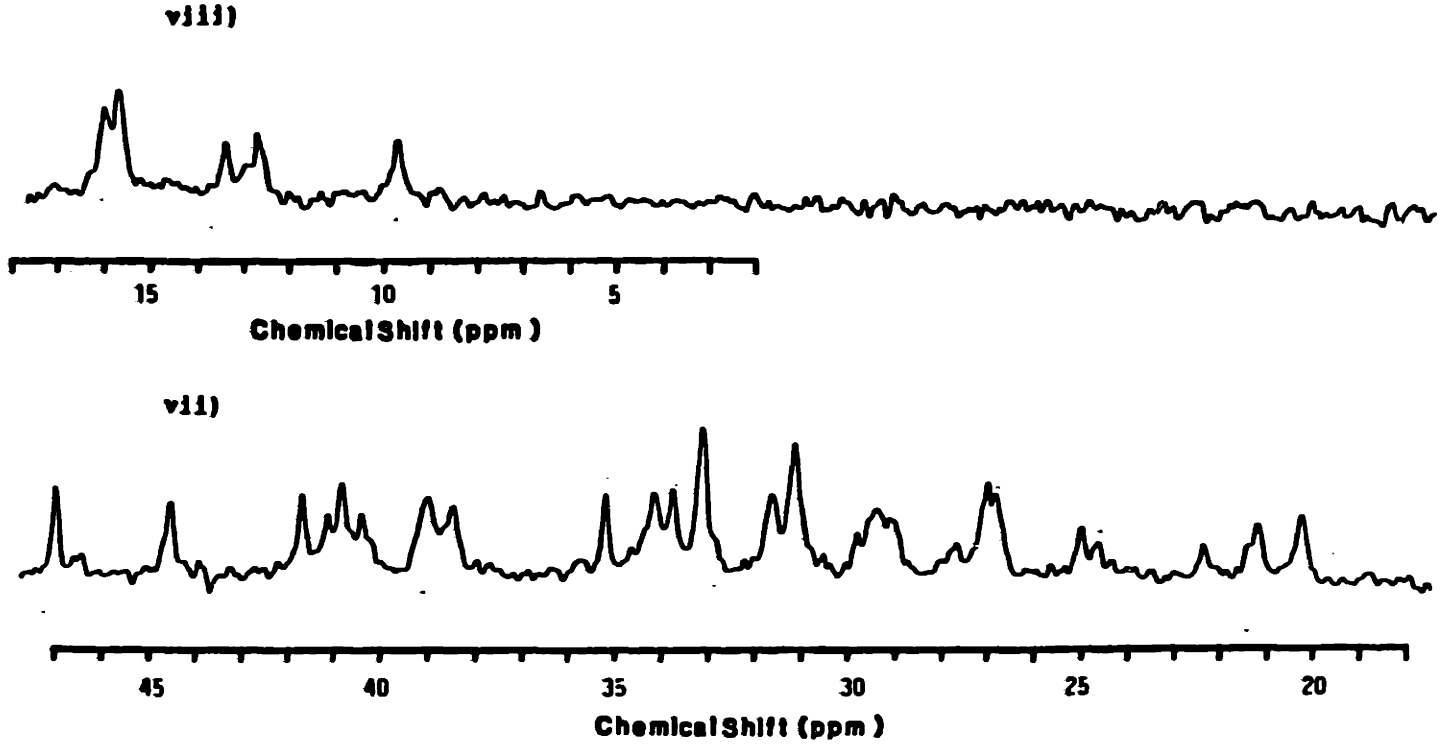


Figure 38 (continued):



K) Effect of Lysine and Pipecolic Acid Addition; Development of a Lysine-Free Fermentation Medium

The medium used in all the previous experiments included 4.0 g/l L-lysine HCl. This medium was developed from one recommended by workers at Ayerst Laboratories (personal communication, S.N.Sehgal; see Results, Section V. A.), who also stated that the addition of lysine to their original medium stimulated rapamycin production. To test this claim, medium was prepared, omitting the 4 g/l L-lysine HCl. Duplicate flasks were prepared, containing 50 ml medium, plus an appropriate weight of L-lysine HCl, resulting in concentrations of 0, 2, 4, 6, and 8 g/l. The flasks were autoclaved, inoculated, and incubated for 6.0 days. The final pH, dry cell weight, and rapamycin concentrations were determined for each flask. There was an increase in volumetric rapamycin production when the lysine content was increased from 0 to 2 g/l, but this was accompanied by an increase in dry cell weight such that the specific production of rapamycin was constant. Above 2 g/l there was no significant effect from the addition of lysine. It is likely that when the lysine is omitted from the medium, the growth of the cultures becomes nitrogen-limited, and some other factor is controlling the level of rapamycin production.

In order to do labelling studies without substantially diluting the labelled lysine, it was necessary to develop a medium containing reduced amounts of lysine. (The 5 g/l yeast extract in the medium surely contains some lysine, either free or in peptides, but no attempt was made to remove this component from the medium.) In early studies I

found that adding NH_4Cl to the medium caused the pH of the culture to drop to very low values (pH 4 or lower), decreasing growth and rapamycin production. In other experiments it was found that additional K_2HPO_4 in the medium did not affect rapamycin production. (Lowering the concentration of phosphate to growth-limiting concentrations slightly increased specific rapamycin production, but substantially increased the height of UV_{254nm} peaks eluting before and after rapamycin in the HPLC chromatograms.) A new medium (AmP medium) was therefore formulated, in which the 4.0 g/l L-lysine HCl was replaced by 5.0 g/l $NH_4H_2PO_4$ (monobasic ammonium phosphate). Thus the nitrogen content of the medium was kept the same but the buffer capacity of the medium was increased. There was no substantial difference in growth extent, pH profile, or rapamycin production after 6 days when AmP medium was compared to the medium previously used. Therefore, AmP medium was used in all subsequent labelling studies with labelled lysine and pipecolic acid.

The addition of up to 4.0 g/l L-lysine HCl or D,L-pipecolic acid to AmP medium caused no increase in rapamycin production. Apparently, the strain of Streptomyces hygroscopicus used in these experiments is not limited by the availability of lysine for the production of rapamycin. (A different strain of S. hygroscopicus was used in the experiments at Ayerst.) Also, the syntheses of many antibiotics are inhibited or repressed by NH_4^+ or PO_4^{3-}, but these compounds do not appear to have a large effect on rapamycin production in this medium.

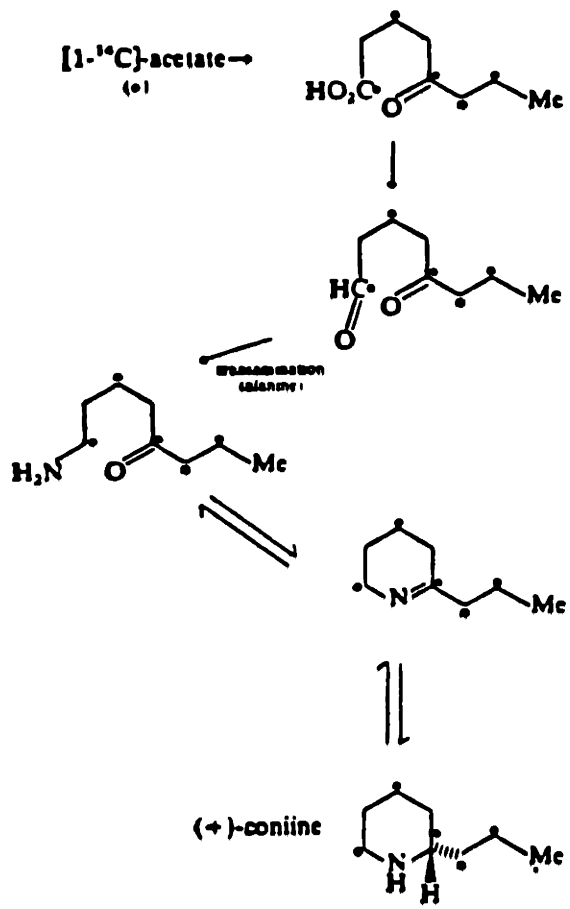
L) Incorporation of L-^{14}C-U-Lysine and D,L-^{3}H-U-Pipecolic Acid

Rapamycin contains an L-pipecolic acid moiety (carbons 16 to 21 plus the nitrogen). This imino acid occurs widely in bacteria, plants, and animals and has often been found to be derived from lysine. D-lysine is usually the preferred precursor and can actually induce the synthesis of the pipecolate-forming enzymes (see Literature Survey, Section II. B.), but in at least one case, L-lysine is the preferred precursor for L-pipecolate (Herbert, 1981). Some metabolites (coniine from hemlock and nigrifactin from a strain of Streptomyces) containing moieties resembling pipecolic acid were found not to be derived from lysine but from acetate via a polyketide pathway (Mann, 1978; Herbert,1981) (see Figure 39). Since ^{13}C-acetate did not significantly label the pipecolic acid moiety in earlier experiments, the possibility of a polyketide origin for this moiety is eliminated. A remaining possibility is that pipecolic acid is formed from a precursor of lysine, in which case labelled lysine would not label the pipecolate moiety. If this moiety is indeed derived from lysine, it is also of interest whether the rapamycin-synthesizing enzymes incorporate lysine which is then cyclized, or they incorporate free pipecolate directly. Experiments were performed to determine the levels at which lysine and pipecolic acid are incorporated into rapamycin.

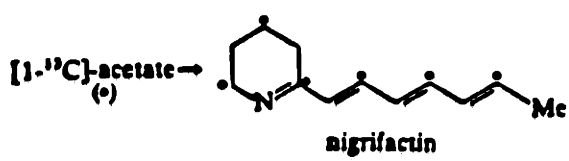
In a preliminary experiment, L-^{14}C-U-lysine (2 uCi; New England Nuclear, biosynthesized from U-^{14}C-glucose) was mixed with a filter-sterilized solution of L-lysine HCl (4 ml, 5 mg/ml) and added to cultures after 2.5 days of incubation, giving a final concentration of

Figure 39: The biosynthesis of coniine and nigrifactin from acetate (Mann, 1978).

a) coniine



b) nigrifactin



0.1 g/l. Using the same procedures described for the experiments with radioactive acetate and methionine, the incorporation of lysine was estimated to be approximately 1 lysine molecule per 6 rapamycin molecules, assuming no incorporation through scrambling. Although incorporation through scrambling could not be ruled out, this high level of incorporation is consistent with the pipecolate moiety being derived from lysine.

Radioactive pipecolate is not listed in the catalogs of several radioisotope suppliers. However, Amersham had available D,L-^{3}H-U-pipecolate (prepared by the catalytic reduction of picolinate by $^{3}H_2$ gas) left over from a custom synthesis order and we did obtain 1 mCi of this material.

The incorporation of L-lysine and D,L-pipecolic acid was compared, both by themselves and in competition with each other. Sixteen 50 ml cultures in AmP medium were incubated 2.5 days. The following stock solutions were prepared:1) 9 ml of 0.5 mg/ml L-lysine HCl plus 2 uCi L-^{14}C-U-lysine, 2) 9 ml of 0.5 mg/ml D,L-pipecolic acid plus 100 uCi D,L-^{3}H-U-pipecolic acid, 3) 25 mg/ml L-lysine HCl, and 4) 25 mg/ml D,L-pipecolic acid. The flasks were divided into four groups. The first group received 1 ml of the radioactive lysine solution. The second group received 1 ml of the radioactive pipecolate solution. The third group received both 1 ml radioactive lysine solution and 1 ml unlabelled pipecolate solution. The fourth group received radioactive pipecolate and unlabelled lysine. In all flasks, the concentration of radioactive precursor was 0.01 g/l while the cold precursors were added

at 50 times this amount, or 0.5 g/l. It was hoped that by keeping the concentrations of the radioactive precursors low (at least in the first and second groups of flasks), induction of catabolic pathways might be avoided. At the end of 5.5 days of incubation, the flasks were harvested, groups pooled, and rapamycin extracted. Since these samples were going to be subjected to hydrolysis (see next section) and therefore had to be very pure, the hexane extracts containing rapamycin were evaporated to dryness and the residue redissolved in a small amount of methanol. The rapamycin from these samples was then purified by semi-preparative HPLC as had been described earlier for ^{13}C-labelled samples. The specific activity of each of the samples was determined and the level of incorporation of the precursors calculated for the first two groups of flasks. The results are summarized in Tables 10 and 11.

When each is added at the level of 0.01 g/l, L-lysine is incorporated at a rate of approximately 1 lysine per 50 rapamycin molecules, while D,L-pipecolate is incorporated at a rate of 1 in 24 rapamycin molecules. (If only one isomer of pipecolate is used, it would not affect the above calculations. It would simply imply that the effective concentration of the precursor was 0.005 g/l.) This result implies that both pipecolate and lysine are readily incorporated into rapamycin, and that pipecolate is incorporated with a somewhat greater efficiency than lysine.

The level of L-lysine incorporation was decreased more than 90% by the addition of 0.5 g/l D,L-pipecolic acid. The incorporation of

Table 10: Relative Incorporation of L-^{14}C-Lysine and D,L-^{3}H-Pipecolate.

Added to culture	L-^{14}C-Lysine 0.01 g/l (54.8 umole/l)	D,L-^{3}H-Pipecolate 0.01 g/l (77.4 umole/l)
Specific activity of Additive	1.8×10^{11} dpm/mole	6.4×10^{12} dpm/mole
Specific activity Recovered Rapamycin	3.6×10^{9} dpm/mole	2.6×10^{11} dpm/mole
Number of Molecules of Additive Incorporated	$\frac{1 \text{ lysine}}{50 \text{ rapamycin}}$	$\frac{1 \text{ pipecolate}}{24 \text{ rapamycin}}$

Table 11: Effect of the Addition of Unlabelled Pipecolate and Lysine on the Incorporation of ^{3}H-Pipecolate and ^{14}C-Lysine

Added to Culture	Specific Activity of Recovered Rapamycin (cpm/ug)	Relative Specific Activity (%)
L-^{14}C-Lysine alone	3.83	100
L-^{14}C-Lysine and D,L-Pipecolate	0.28	7
D,L-^{3}H-Pipecolate alone	283	100
D,L-^{3}H-Pipecolate and L-Lysine	187	66

Radioactive precursors were added at a concentration of 0.01 g/l. Unlabelled precursors were added at a concentration of 0.5 g/l. All precursors were added after 2.5 days of incubation.

D,L-pipecolate was only decreased by about one third when 0.5 g/l L-lysine was added. These results cannot be explained by a difference in cell permeability to lysine and pipecolic acid since in the data discussed above, these two compounds labelled rapamycin at approximately the same level. Pipecolic acid appears to be more readily accepted by the rapamycin synthesizing enzymes, and appears to be the closer precursor. The above conclusions are based on the assumption that lysine and pipecolate are incorporated specifically into the pipecolate moiety and not through scrambling. An attempt to justify this assumption is described in the next section.

M) Hydrolysis of Rapamycin Enriched with L-^{14}C-Lysine, ^{3}H-Pipecolate, and ^{14}C-Acetate

The pipecolic acid moiety of rapamycin had been separated from the rest of the molecule by acid and base hydrolysis during the determination of the structure of rapamycin by Findlay and Radics (1980). It was thought that this hydrolysis procedure could be scaled down and used to determine whether the pipecolate moiety is specifically labelled in rapamycin. The initial appraoch was to first determine the specific activity of a labelled rapamycin sample, hydrolyze it, recover and purify the pipecolate by TLC, and then determine the specific activity of the pipecolate. This last step requires a sensitive method for determining the concentration of pipecolic acid recovered from the hydrolysate. Hill (1979) published a now-popular technique for the quantitative detection of amino acids using orthophthalaldehyde (OPA) derivatization, separation on a C18 column, and fluorescence detection (see Materials and Methods). As published, this method is specific for primary amines. Applications scientists from Waters (Milford, MA; personal communication) reported that it was possible to detect proline using this method if the samples were first treated with NaHOCl (bleach) at high pH, as they had done using automatic injectors to control reaction times. Several attempts were made to confirm this observation, using a wide range of reaction times with bleach and with the derivatization reagents. While the primary amino acids were detected with the usual sensitivity, no peaks were ever observed for proline or pipecolate. Another technique which is becoming popular is precolumn

derivatization with phenylisothiocyanate, separation on a C18 column, and detection by absorbance at 254 nm. The derivatization is more laborious than with OPA, but the derivatives are stable indefinitely if stored in a dried state in the cold. This method will also detect primary as well as secondary amino acids. When tested, proline along with several primary amino acids reacted and eluted as published. However, no reproducible peak could be assigned to pipecolate. Possibly the derivative is unstable or the pipecolate peak elutes under the reagent peak at approximately 12 min. After several attempts, this procedure was also abandoned.

Without a method for determining the pipecolate concentration in the hydrolysate, the approach was modified slightly. It was found that ninhydrin could be used to detect pipecolate on TLC plates, although not at the low levels likely to be generated in the hydrolysis mixtures. Samples of labelled rapamycin could be hydrolyzed, the products separated by tlc, and the distribution of label among these products determined by scraping off bands of silica from each of the plates. The identification of the pipecolate band was made by the simultaneous treatment of an unlabelled pipecolate sample, which is applied to a TLC plate, developed alongside the radioactive samples, and then sprayed with ninhydrin and heated. (Ninhydrin cannot be used directly on the radioactive plates because it strongly quenches the flourescence necessary for scintillation counting.) It is assumed that hydrolysis efficiencies would be identical for rapamycin samples treated under identical conditions. Previous experiments showed that acetate was

incorporated into the pipecolate moiety to only a low degree, apparently through scrambling. A sample of ^{14}C-acetate enriched rapamycin was therefore prepared using the previous conditions (basal fermentation medium including 4.0 g/l lysine and 0.5 g/l ^{14}C-acetate added at 2.5 days). It was purified by HPLC in the same manner that the lysine and pipecolate enriched samples had been. This sample was hydrolyzed and treated in an identical fashion as the other radioactive samples, to provide a background value for the amount of radioactivty incorporated through scrambling. Also, a sample of ^{3}H-pipecolate was subjected to the hydrolysis conditions and TLC procedure to serve as a measure of the recovery of radioactivity. Exact details of the hydrolysis and TLC procedure are given in the Materials and Methods section.

The radioactive profiles of the hydrolysates are shown in Figures 40 a to d. The radioactivity for each band of silica removed is expressed in terms of the percentage of total recovered counts for each sample. The total number of counts recovered range from approximately 35% for tritiated samples to approximately 65% for ^{14}C-enriched samples, probably due to the different levels of quenching caused by the presence of silica gel in the scintillation vials. Approximately 80% of the counts recovered from the ^{3}H-pipecolate sample were located in bands 6 and 7a, at approximately the same R_f value as the cold pipecolate. Apparently there is little degradation of the pipecolate under the reaction conditions. The profile of the ^{14}C-acetate enriched rapamycin sample shows a small peak at the R_f of pipecolate, containing perhaps 3% of the recovered counts. This small amount is likely to come from

Figure 40

Distribution of radioactivity recovered after hydrolysis of rapamycin samples.

Note: Pipecolate is located in the region of bands 6 and 7a.

a) ^{3}H-pipecolate control

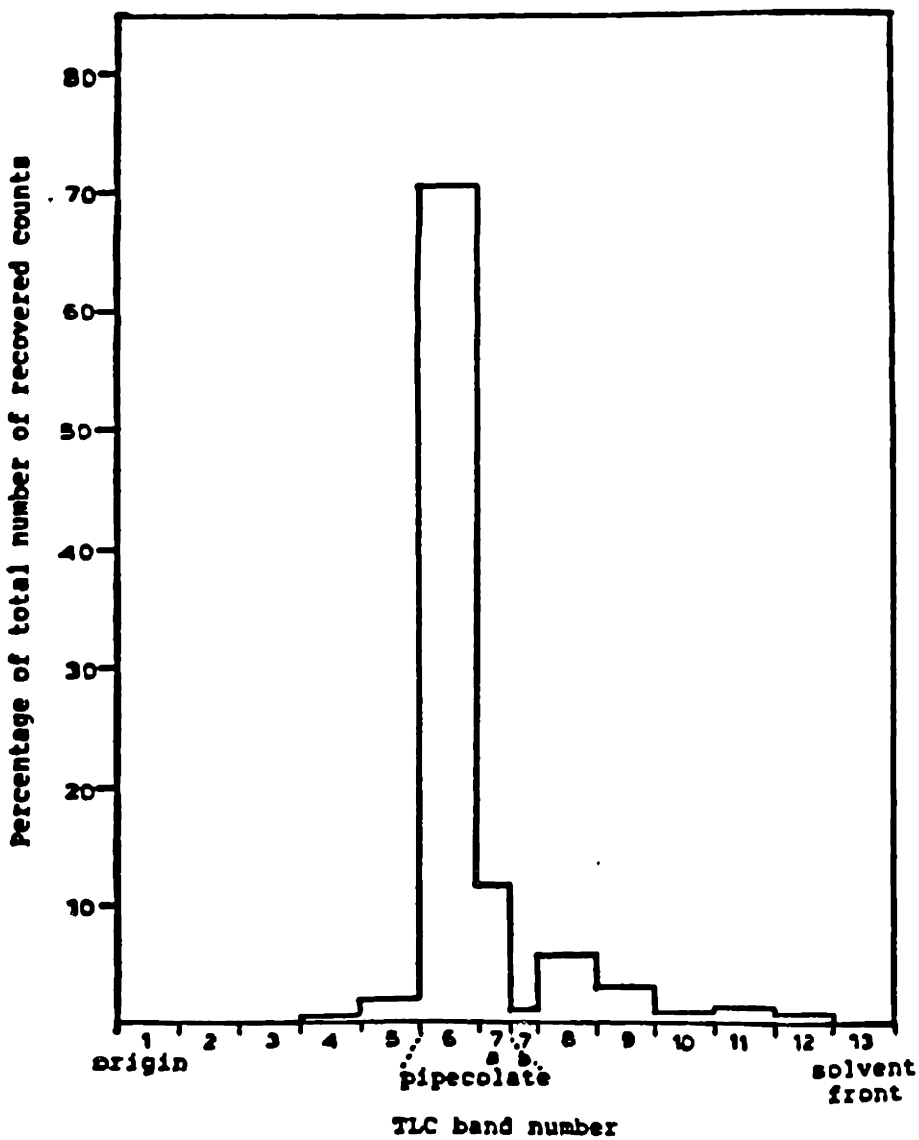
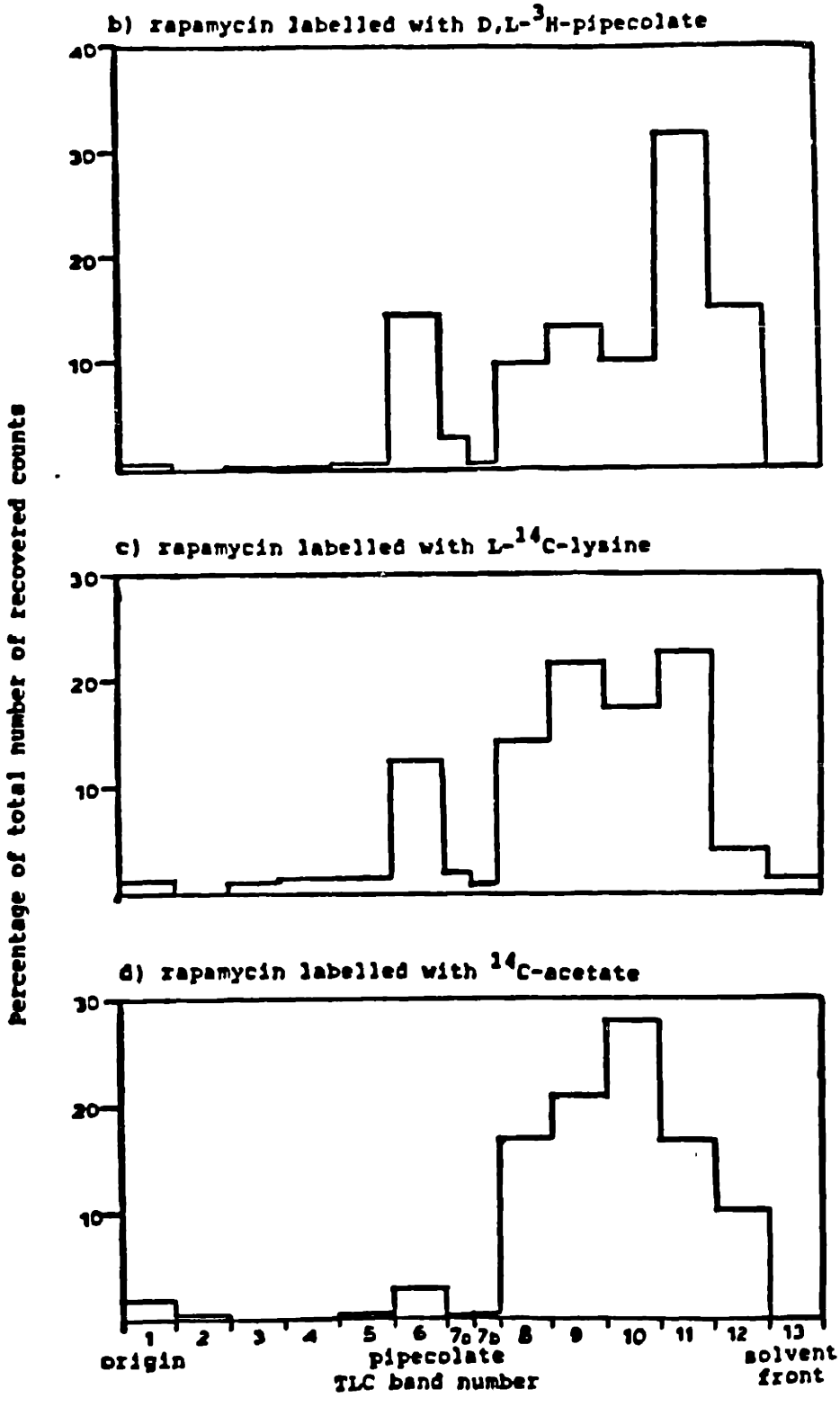


Figure 40 (continued):



metabolic scrambling of acetate into the pipecolic acid moiety. In comparison, the rapamycin samples enriched with ^{14}C-lysine and ^{3}H-pipecolate gave profiles where 10 and 14% respectively of the counts were contained in the bands corresponding to pipecolic acid. These values are three to five times the value obtained for the ^{14}C-acetate enriched sample, implying that the main mode of incorporation of lysine and pipecolate into these samples was other than scrambling via acetate. Thus, lysine and pipecolate appear to label this pipecolate moiety more specifically than does acetate.

Taken together with the results in the previous section, these results imply that pipecolate is the more direct precursor of rapamycin, lysine being converted to pipecolate and free pipecolate being directly incorporated into rapamycin.

The evidence for direct incorporation of lysine and pipecolate would be stronger if a higher percentage of the recovered counts were found at the pipecolate R_f. Over 80% of the recovered counts migrated near the solvent front. One explanation is that the hydrolysis was incomplete and pipecolate was bound to less polar fragments of rapamycin, causing it to move farther than free pipecolate. Another possibility is that the pipecolate was hydrolyzed away from the rest of the molecule and then condensed with a reactive fragment, again causing it to migrate at a higher R_f. This latter possibility was investigated by subjecting two samples of ^{3}H-pipecolate to the hydrolysis conditions, one in the usual fashion and one in the presence of 300 ug rapamycin (a higher concentration than is usually used in the hydrolysis

experiments). The two samples had identical profiles of their recovered counts, demonstrating that free pipecolate does not condense with rapamycin fragments to a significant degree. One final and very likely explanation for the presence of a large number of counts at the solvent front is biological scrambling of lysine into acetate and incorporation of this acetate into the lactone ring. Rapamycin is produced as the culture is running out of easily utilized substrates and the formation of many catabolic enzymes may be induced. Pipecolate is also an intermediate of lysine degradation in many organisms, and therefore could be quickly degraded as it enters the cells.

N) Effect of the Addition of Shikimic Acid and Aromatic Amino Acids

In my original biosynthetic scheme, an early intermediate of the shikimic acid pathway has been proposed as the source of the C_7 starter unit of rapamycin. None of the previous labelling experiments have contradicted this proposal. Shikimic acid was added to cultures to see if it would have an effect on rapamycin production. When added to cultures at the time of inoculation and after 2.5 days incubation at a concentration of 1.0 g/l, shikimic acid had no effect on either the growth extent or rapamycin production. Possible explanations include: 1) shikimic acid is a precursor, but its formation is not rate-limiting for the biosynthesis of rapamycin; 2) shikimic acid is the precursor of the C_7 unit, but is not entering the cells; 3) shikimic acid is not a precursor of rapamycin, the C_7 unit being derived from an earlier intermediate in the shikimic acid pathway, or from an unrelated pathway.

In many cases the addition of an end product of a pathway causes inhibition and/or repression of early enzymes of the pathway. If an intermediate of the pathway is a precursor of another metabolite, synthesis of this metabolite may also be decreased. This type of effect was observed when lysine was added to penicillin-producing cultures of Penicillium chrysogenum (Demain et al., 1983). Aminoadipic acid is a precursor of both penicillin and lysine in this organism, and lysine inhibited the synthesis of penicillin by inhibiting the formation of aminoadipic acid. By the same logic, it was hypothesized that the addition of aromatic amino acids might decrease the production of rapamycin by decreasing the availability of shikimic acid pathway

intermediates.

Aromatic amino acids were added at the level of 1.0 g/l, both individually and in combination. Due to the limited solubility of tyrosine, it was weighed directly into the fermentation flasks. For addition at the time of inoculation, medium was added to the flask containing tyrosine and the flask was autoclaved and inoculated as usual. For addition at 2.5 days, a flask containing the correct amount of tyrosine was autoclaved. A separate flask was used to start the fermentation. After 2.5 days of incubation the contents of the fermentation flask were aseptically transferred to the tyrosine flask, and then this flask was placed in the incubator. Since phenylalanine is also thermostable, it was treated in the same fashion. Tryptophan can be degraded by autoclaving, and was added as a filter-sterilized 25-fold concentrated stock solution. Two types of control cultures were also prepared: those which were transferred to sterile, empty flasks after 2.5 days and those which received sterile water in place of an amino acid solution. After 6 days of incubation the flasks were assayed for rapamycin. No significant differences in production were observed.

While these results do not support the proposed shikimate origin of the starter unit of rapamycin, they do not eliminate the possibility either. The strict system of feedback inhibition and repression by aromatic amino acids on their synthesis is indeed found in *E. coli*. However, there are several other patterns of control for DAHP synthetase, each pattern being tightly conserved among members of a given genus (Jensen and Stenmark, 1970). The lack of effect of aromatic

amino acids on rapamycin production is consistent with findings for another streptomycete, Streptomyces sp.3022a (Lowe and Westlake, 1971). This latter organism produces chloramphenicol in amounts approaching the total production of aromatic amino acids during growth. Shikimic acid is incorporated into chloramphenicol to a high degree. However, DAHP (3-deoxy-D-arabino-heptulosonate 7-phosphate) synthetase from this organism was not inhibited by aromatic amino acids, shikimic acid pathway intermediates, chloramphenicol, or several other aromatic compounds. Synthesis of DAHP synthetase was repressed 50% by 40 mg/l p-hydroxybenzoic acid, but this did not decrease chloramphenicol production. Thus, failure of the aromatic amino acids to decrease rapamycin production does not eliminate the possibility that a shikimate pathway intermediate is a precursor of rapamycin.

O) Incorporation of ^{14}C-Shikimic Acid

Shikimic acid has been found to enter some cells but not others (See Literature Survey, Section II. B.). Also, shikimic acid may not label compounds which have an earlier shikimate pathway intermediate as their precursor. For example, the starter unit of rifamycin is derived from DAHP, 3-dehydroshikimic acid, or 3-dehydroquinic acid, but ^{14}C-shikimic acid did not label rifamycin, even though the early reactions in the shikimic acid pathway are reversible (Ghisalba and Nuesch, 1981; Karlsson et al., 1974). In order to determine whether or not shikimic acid is entering the cells and if shikimic acid is incorporated into rapamycin, ^{14}C-shikimic acid was added to cultures.

Fifty microliters of ^{14}C-U-shikimic acid (1.0 uCi, 19.7 mCi/mmol; Amersham, biosynthesized from ^{14}C-U-glucose) were added to 4 ml of a filter-sterilized solution of shikimic acid (25 mg/ml, pH 5.0). One ml of this solution was added to each of three 50 ml cultures after 2.5 days of incubation (final concentration of 0.5 g/l shikimic acid). After 6 days of incubation the contents of the flasks were pooled and the rapamycin extracted. A portion of the hexane extract was applied to a TLC plate while another portion was used to determine by HPLC the concentration of rapamycin in the solution. The plate was developed in 50/50 acetone/hexane, and the rapamycin band scraped off and counted. From the specific activity of the recovered rapamycin, it appeared that one out of every three rapamycin molecules had incorporated a shikimic acid molecule. The whole experiment was repeated and an incorporation of one shikimic acid molecule per 4.4 rapamycin molecules was

calculated. (These calculations assume no incorporation through scrambling and that all incorporation of shikimate is directly into the C_7 unit.)

Shikimic acid is obviously getting into the cells since it is being metabolized. The very high incorporation of shikimate into rapamycin strongly supports the idea that the starter unit is derived from a shikimic acid pathway intermediate. However, the possibility that the incorporation is through scrambling cannot be ruled out, since the sites of incorporation have not been identified. Shikimic acid can be converted to aromatic amino acids,which are catabolized to form fumarate and acetoacetate. Shikimate itself can be converted to protocatechuate which is degraded to acetyl-CoA and succinate (Gottschalk, 1979). (See Figure 41 a and b.) These products could be incorporated into rapamycin after conversion to malonyl- and methylmalonyl-CoA. Shikimic acid labels rapamycin at a much higher efficiency than does acetate, even though it is added at a lower molar concentration. This suggests that it is being incorporated directly, through a route different from that of acetate incorporation. However one might argue that shikimate enters the cells more easily or that shikimate degradation products are more easily converted to methylmalonyl- and malonyl-CoA derivatives when compared to acetate.

^{14}C-Shikimic acid is being incorporated into compounds other than rapamycin, although it is unclear whether this is via direct or indirect routes. Silica gel from the region of the solvent front was removed from the TLC plates which had been used in determining the specific

Figure 41: Conversion of shikimate to succinate and acetyl-CoA. (Gottschalk, 1979)

a) conversion of shikimate and other compounds to protocatechuate

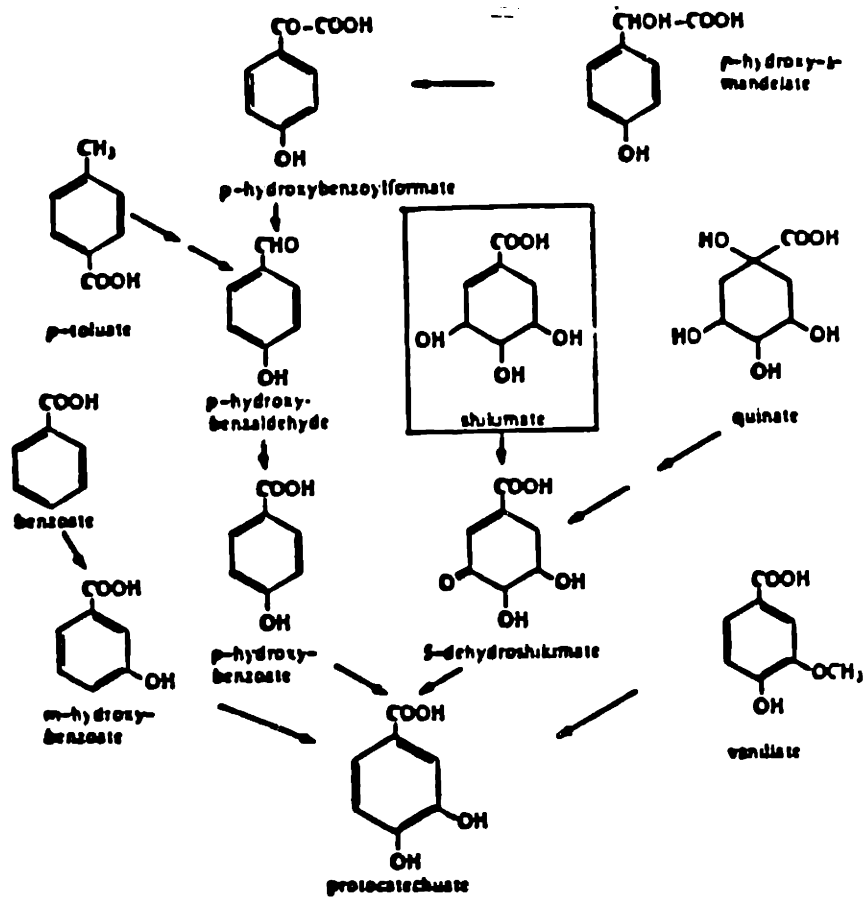
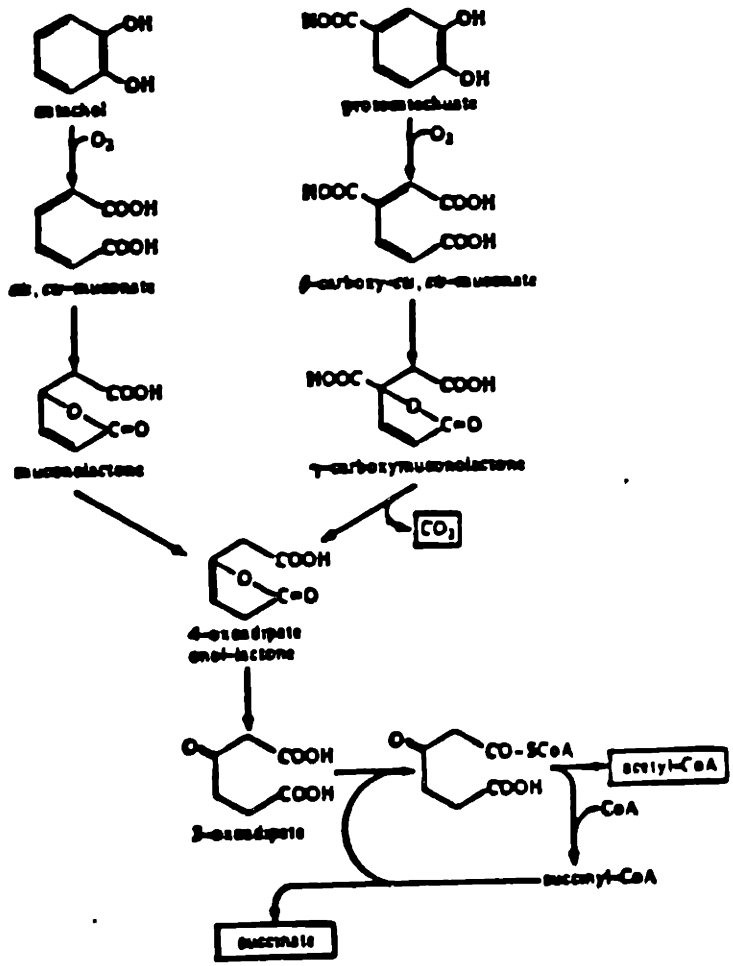


Figure 41 (continued):

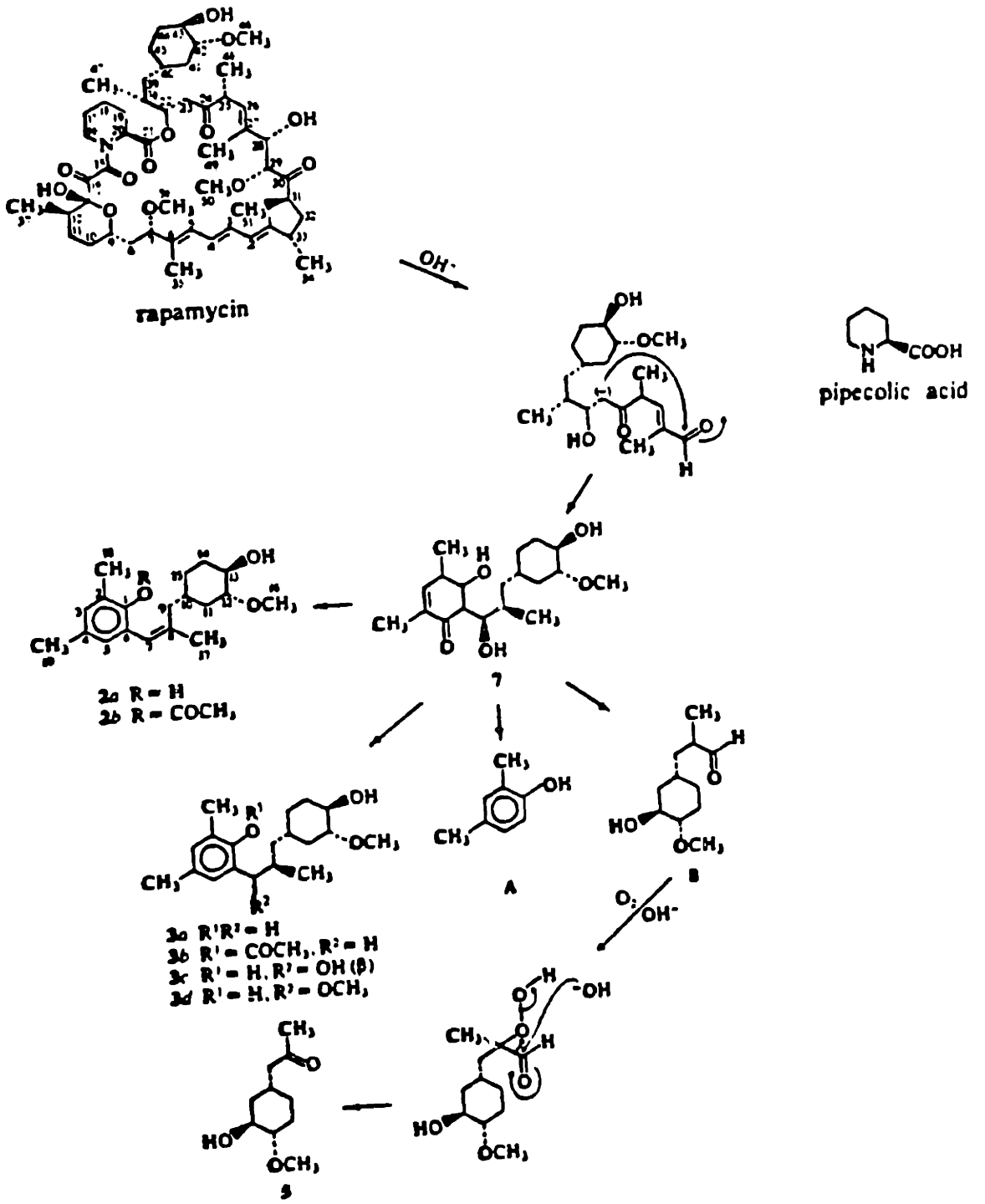
b) conversion of protocatechuate and catechol to succinate and acetyl-CoA



activity of rapamycin. This region was found to contain more ^{14}C counts (roughly 5 times more) than the rapamycin band. Fatty acids migrate at the solvent front in this solvent system, and the identification of highly labelled fatty acids would imply that significant scrambling and incorporation of generated ^{14}C-acetate had taken place. However, unidentified aromatic compounds and rapamycin derivatives may also migrate at the solvent front, and these could account for the high number of counts. Shikimic acid, phenylalanine, tyrosine, and tryptophan all remain near the origin in this solvent system (data not shown).

Unfortunately, it would not be easy to prove that the cyclohexane moiety is preferentially labelled by ^{14}C-shikimic acid. When subjected to base or acid hydrolysis, the cyclohexane ring is converted to several different products, each of which includes other rapamycin carbons (Findlay and Radics, 1980; see Figure 42). It would be difficult to separate these compounds from other degradation products and recover them quantitatively. The double bond between carbons 26 and 27 could perhaps be cleaved by ozonolysis and the lactone linkage hydrolyzed under mild acidic conditions, releasing a fragment including the cyclohexane moiety and carbons 22, 23, 24, 25, 26, 47, and 48 (derived from one acetate and two propionate units). Without standards it would be difficult to identify and separate this moiety from the other products. If resolved, a comparison could be made of the distribution of the radioactivity in rapamycin samples labelled with ^{14}C-acetate and ^{14}C-shikimate. Such a system would be difficult to develop and may not

Figure 42 : Several products of the base catalyzed hydrolysis of rapamycin. (Findlay and Radics, 1980)



conclusively demonstrate the direct incorporation of shikimic acid.

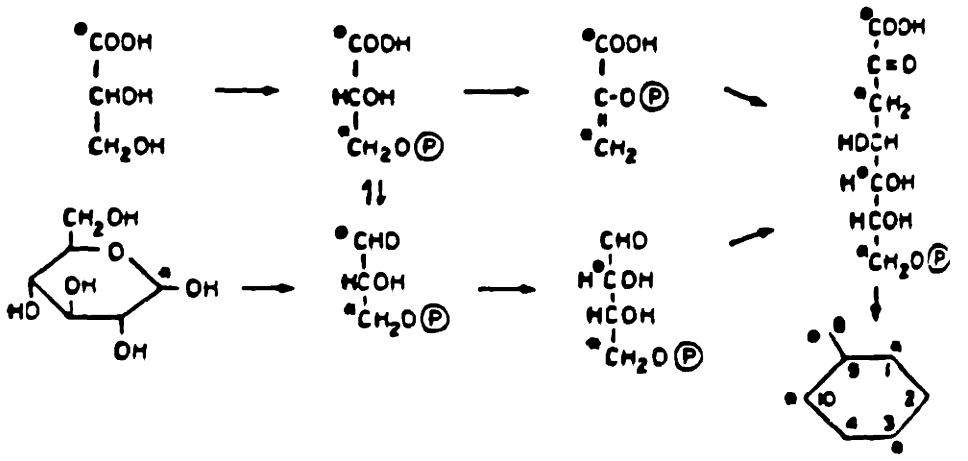
More conclusive and more easily analyzed results may be obtained from ^{13}C-labelling experiments or from work with shikimate pathway mutants, such as those discussed in the Literature Survey for analogous systems. Briefly described below are the possible experimental approaches suggested by the literature for establishing a shikimate origin for the C_7 unit in rapamycin. Some advantages and disadvantages of each approach are also briefly described.

1) 1-^{13}C-Glycerate: White and Martinelli (1974) demonstrated the shikimate origin of the starter unit of rifamycin by the successful incorporation of 1-^{13}C-glycerate, and similar results were obtained by Haber et al. (1977) (Figure 43) in studies on geldanamycin biosynthesis. Glycerate did not, however, seem to enter the Streptomyces producer of leucomycin (Omura et al., 1983).

1-^{13}C-Glycerate is not commercially available. However, it can be very easily synthesized from glycolaldehyde and $Na^{13}CN$ (Ashworth, 1966). With the published 70% yield (based on NaCN), one should be able to synthesize approximately 2 grams of D,L-1-^{13}C-glycerate from 1 g of $Na^{13}CN$ ($175/g).

It is unknown whether glycerate can enter cells of the rapamycin producer and how much the label would be diluted by the metabolism of unlabelled glycerol in the medium. ^{14}C-Glycerate is also not commercially available and would have to be synthesized before preliminary radioactive experiments could be performed. If $^{13}C_2$-glycine

Figure 43: Incorporation of 1-^{13}C-glycerate and 1-^{13}C-glucose into rifamycin chromophore. (Vining, 1983, p.242)



does not label carbons 10 and 11 of rapamycin, it may be necessary to synthesize and incorporate 1-^{13}C-glycerate as a means of labelling these two carbons also. 1-^{13}C-glycerate should label the carboxy group of a shikimic acic moiety, corresponding to C39 of rapamycin, plus one of the ring carbons, C43. Successful incorporation of 1-^{13}C-glycerate would help confirm or disprove the published spectral assignments of carbons 39 and 32.

2) ^{13}C-Glucose: Singly and multiply labelled forms of glucose have been used to label the shikimate-related starter units of rifamycin and geldanamycin. 1-^{13}C- and 6-^{13}C-Glucose both symmetrically label the ring of the starter units, while U-$^{13}C_6$-glucose is converted to C_3 and C_4 units which asymmetrically label the moiety, revealing the orientation of the incorporated shikimate pathway intermediate (Haber et al., 1977; White and Martinelli, 1974; Rinehart, 1982).

At the time these experiments were performed, labelled glucose was not commercially available and was donated by the Los Alamos Stable Isotope Research Center (supported by National Institutes of Health) or private labs. Labelled glucose has become commercially available, but the current prices may prohibit its use. For the incorporation of 6-^{13}C-glucose into geldanamycin, Haber et al. (1977) used a concentration of 5 g labelled glucose/l and only obtained a six to seven-fold increase in peak height. Lower concentrations could possibly be used but may result in insignificant increases in peak height and waste of label. Even at 1 g glucose/l, an experiment with 1-^{13}C-glucose

would cost approximately $700. Four of the seven carbons of the starter unit of rapamycin correspond to peaks in the spectrum which overlap with peaks from other carbons, thereby complicating the analysis of possible results from the incorporation of U-$^{13}C_6$-glucose.

3) Shikimate-requiring mutants: If the starter unit of rapamycin is derived from shikimic acid, then mutants of Streptomyces hygroscopicus which cannot make shikimic acid would also fail to make rapamycin. Such mutants could grow in the presence of shikimate or five or six aromatic supplements (phenylalanine, tyrosine, tryptophan, p-aminobenzoate, p-hydroxybenzoate, and possibly 2,3-dihydroxybenzoic acid). If shikimic acid is the precursor of rapamycin, then mutants grown in the presence of the aromatic supplements alone should not produce rapamycin. Addition of shikimic acid to these cultures should restore rapamycin production.

The main drawback of this method is the amount of time required, mainly due to the low growth rate of the producing strain of Streptomyces hygroscopicus. Killing curves using ultraviolet light as the mutagen have been obtained and culture conditions for growth of single colonies have been found (data not shown). It takes a minimum of four days after spores are plated before single colonies can be seen well enough to be counted or transferred for screening. Two additional transfers are necessary to screen each colony. These transfers are normally done one colony at a time with Streptomyces; replica plating with sterile velvet does not work well. If a suitable mutant were found,

it would take several weeks to produce enough spores to do fermentation studies. Luck plays a role in such a search; suitable mutants may be found immediately, or thousands of colonies may be screened without finding any. Despite these and several other drawbacks, the results of such studies with blocked mutants often offer the best proof for the identity of a precursor, often allowing exact identification of the immediate precursor of the antibiotic.

4) ^{13}C-Shikimic acid: ^{13}C-Shikimic acid is not commercially available and a custom synthesis for carboxy labelled shikimic acid was quoted at $10,000 by one company. One alternative is to use a blocked mutant of Klebsiella pneumoniae to biosynthesize ^{13}C-shikimic acid from ^{13}C-glucose. One mutant (ATCC # 25597) has been reported to produce 1020 mg/l shikimic acid phosphate in a defined medium containing 5 g/l glucose (Knowles and Sprinson, 1970). If all of the glucose is not utilized, the yield (based on glucose) may be improved by lowering the glucose concentration. Shikimic acid phosphate can be converted to shikimic acid by acid hydrolysis or by the action of alkaline phophatase. Shikimic acid can be purified from the medium by ion exchange chromatography in greater than 80% yield. If these literature values are correct, then I should be able to obtain 70 to 150 mg shikimic acid from 0.5 to 0.75 g glucose ($350 to $500 if 1- or 2-^{13}C-glucose). From the results of the experiments with ^{14}C-shikimate, 0.1 g/l ^{13}C-shikimate should cause a very visible (4%) enrichment of the starter unit, assuming no scrambling has taken place.

This approach has the advantage that shikimic acid has already been shown to enter the cells and be readily incorporated into rapamycin. No scrambling reactions are necessary to achieve incorporation. While the cost of labelled precursor is within reason, a significant amount of time must be devoted to testing each step of the production procedure. After the strain is obtained from the American Type Culture Collection, the level of production must first be verified. If it is not what was published, the feasibility of this approach will be decreased.

In preliminary experiments with suitably blocked E. coli mutants, I observed the accumulation of approximately 400 mg/l shikimic acid. The Klebsiella mutant is said to accumulate shikimic acid phosphate at a higher level than similar E. coli mutants, but exact numbers are not given (Weiss and Mingioli, 1956). A chemical assay specific for shikimic acid can be used to follow the production during fermentation and purification (Yoshida and Hasegawa, 1957). An HPLC method and bioassay have also been described (Mousdale and Coggins, 1985; Weiss and Mingioli, 1956).

From the above alternatives, the approach of synthesizing and incorporating ^{13}C-shikimate was chosen. The actual procedures used are described in a later section.

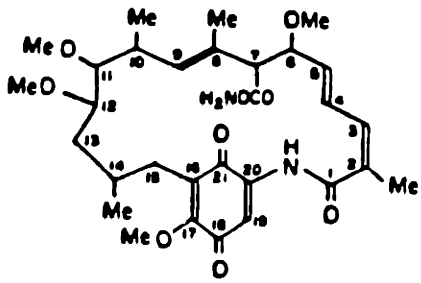
P) Effect of Glycine Addition; Rationale for Choosing Glycine as a Possible Source of Carbons 10 and 11.

Since labelled acetate and propionate had not been incorporated into carbons 10 and 11, I suggested that glycollate (hydroxyacetate) or glycerate was the source of this two carbon unit. This suggestion is based on results described in the Literature Survey as well as some more recent studies that were published after this project was begun. The relevant results are summarized below.

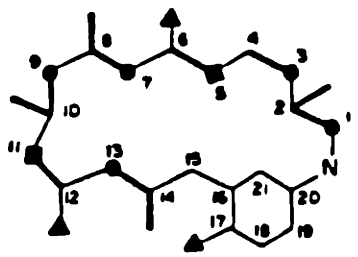
In studies on leucomycin and geldanamycin biosynthesis, "glycollate" was found to enrich two-carbon units which had not been enriched by acetate. Calcium [carboxy-^{13}C]- glycollate and [carboxy-^{13}C]-glycerate both labelled C5 and C11 of geldanamycin when added to cultures of Streptomyces hygroscopicus var. geldanus var.nova (Haber et al., 1977) (see Figure 44). The interpretation of the authors was that glycollate was incorporated directly (probably as the hydroxymalonate derivative), and C1 and C2 of glycerate are incorporated after being converted to glycollate. An alternative theory which has not been ruled out is that glycerate is incorporated intact and the hydroxymethyl group is later removed. Glycollate would be incorporated after conversion into glycerate. In the case of leucomycin biosynthesis in Streptomyces kitasatoensis, 1-^{13}C-glycine, 1-^{13}C-glycollate, and 1-^{13}C-glycerate did not label C3, corresponding to the carboxyl group of the unlabelled two-carbon unit (see Figure 45) (Omura et al., 1983). This failure was attributed to the inability of the compounds to enter the cells, since later 2-^{13}C-glycerol labelled C3, presumably after

Figure 44: Geldanamycin Biosynthesis (Vining, 1983)

Geldanamycin



Origin of the geldanamycin carbon skeleton



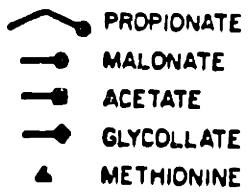
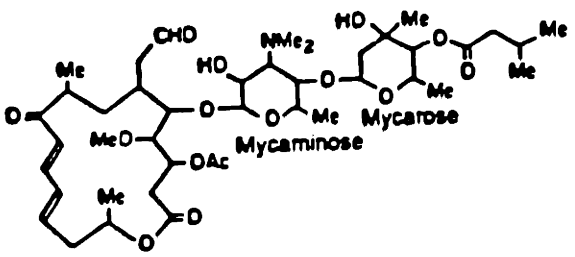
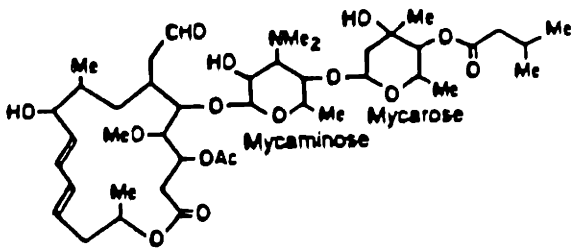


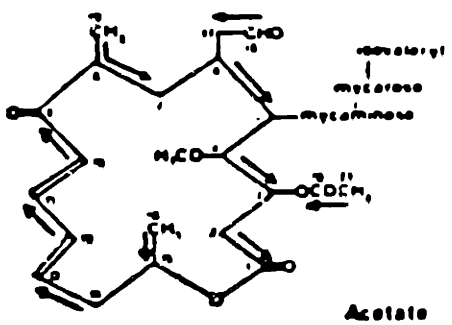
Figure 45 : Comparison of the biosynthesis of Leucomycin A3 and Magnamycin B. (Omura et al., 1975 and 1983)



Magnamycin B

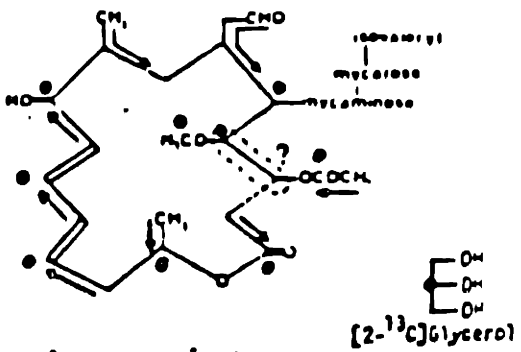


Leucomycin A_3



Magnamycin B

Acetate
Propionate
Butyrate



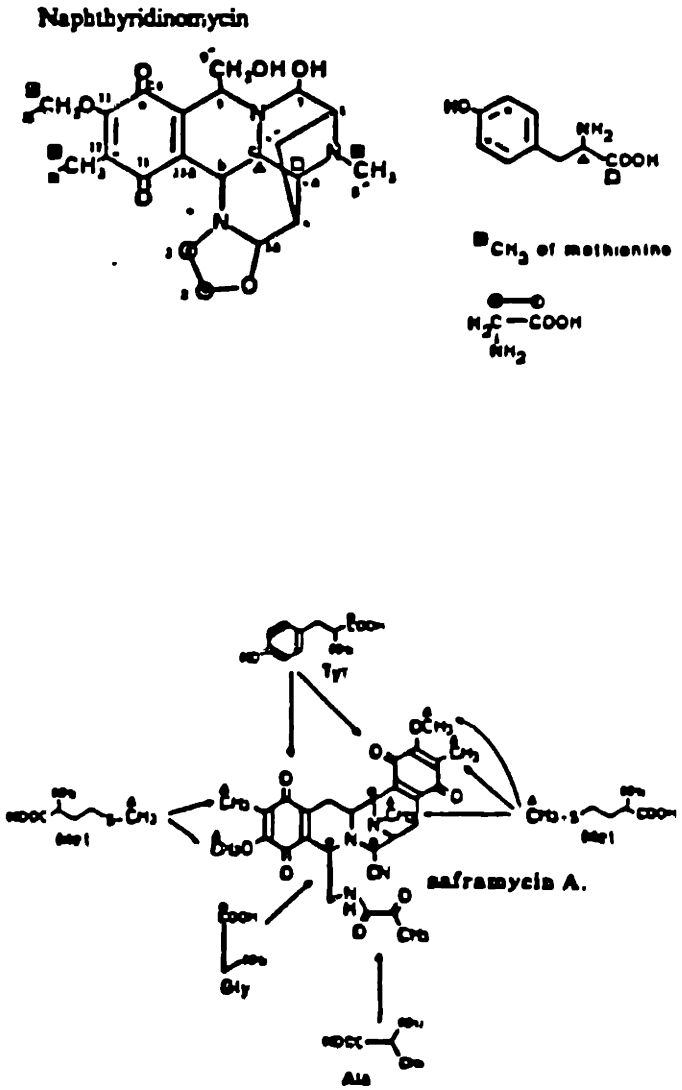
Leucomycin A_3

[2-^{13}C]Glycerol

being converted to glycerate or glycollate. (It is interesting to note that the corresponding carbons in magnamycin B are readily labelled by acetate (Omura et al., 1975).) 1-^{13}C-Glycine was found to label saframycin A, a heterocyclic quinone antibiotic synthesized by Streptomyces lavendulae from tyrosine, glycine, methionine, and alanine (Mikami et al., 1985). (See Figure 46.) Naphthyridomycin is a structurally similar antibiotic produced by Streptomyces lusitanus, but the analogous carbons (9 and 9') were not labelled by $^{13}C_2$-glycine (Zmijewski et al., 1982). Another two-carbon fragment in the molecule was highly enriched by $^{13}C_2$-glycine, proving that glycine was entering the cells. It appears that very similar structures produced by somewhat related organisms can be derived from different precursors.

The possibility that carbons 10 and 11 of rapamycin are derived from glycollate was examined by feeding $^{13}C_2$-glycine to the producing cultures. There are several advantages to this approach. $^{13}C_2$-Glycine is commercially available at reasonable cost, while labelled glycerate and glycollate are not. The coupling patterns generated by the incorporation of covalently linked ^{13}C atoms allow us to discriminate between direct incorporation and incorporation through scrambling, as was shown in the earlier experiments with $^{13}C_2$-labelled acetate (see Results, Section V. F.). The use of a doubly labelled compound also to some extent increases the sensitivity of the NMR technique, as it is easier to detect the appearance of a triplet in place of singlet as opposed to a slight increase in the height of a single peak. It is also likely that glycine will enter the cells.

Figure 46: Biosynthesis of Naphthyridinomycin and Saframycin A.
(Zmijewski et al., 1982; Mikami et al., 1985)



Glycine is only two steps (deamination and reduction) from glycollate (see Figure 47), the suspected direct precursor.

Labelled glycine can also be converted to glycerate via serine. Also, two molecules of glyoxalate can be condensed and decarboxylated to form glycerate by a pathway found in E. coli and pseudomonads (Gottschalk, 1979). (See Figure 48.) Thus, if glycerate is the actual precursor of C10 and C11, labelled glycine is likely to enrich these carbons. However, incorporation through glycerate may be very low since the medium contains 20 ml glycerol. Glycerol is metabolized by way of 3-phosphoglycerate, a potential source of unlabelled glycerate which would isotopically dilute any labelled glycerate formed (Figure 49.).

Since ^{13}C-glycollate and ^{13}C-glycerate are not available commercially, and because there are known pathways leading from glycine to glycollate and glycerate, $^{13}C_2$-glycine was selected for use in an incorporation experiment. Prior to work with labelled compound, unlabelled glycine was added to a fermentation to see if it affects rapamycin production.

Six flasks containing 50 ml of the standard fermentation medium were inoculated. After 2.5 days, 0, 1.0, and 2.0 g/l (final concentration) glycine was added to duplicate flasks. After 6.5 days the fermentation was stopped and the flasks were sampled. There was no significant difference in the concentration of rapamycin in the samples. Therefore glycine has no effect on rapamycin production when added at concentrations up to 2.0 g/l.

Figure 47: Conversion of glycine to glycollate.

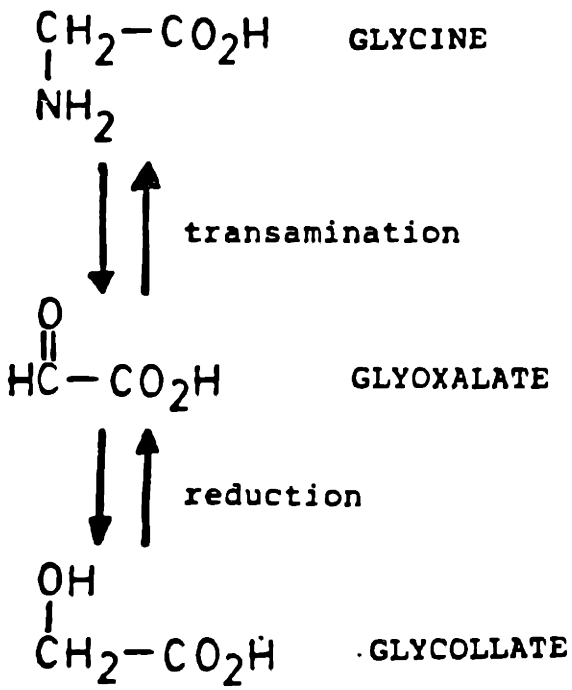


Figure 48: Conversion of glycine to glycerate. (Gottschalk, 1979)

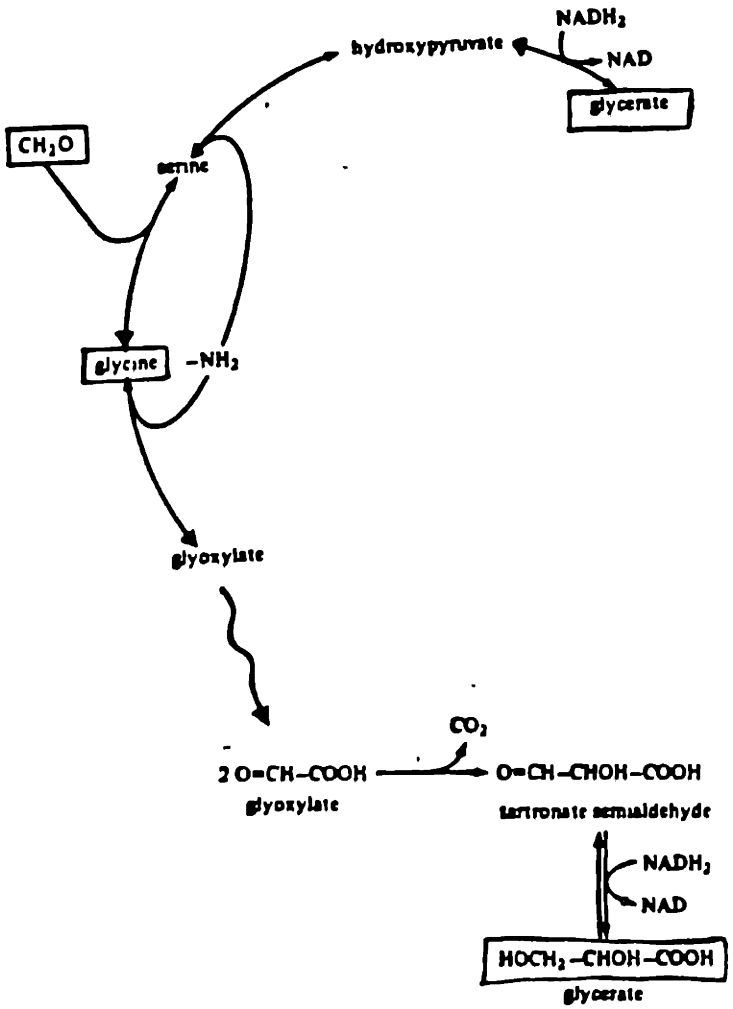
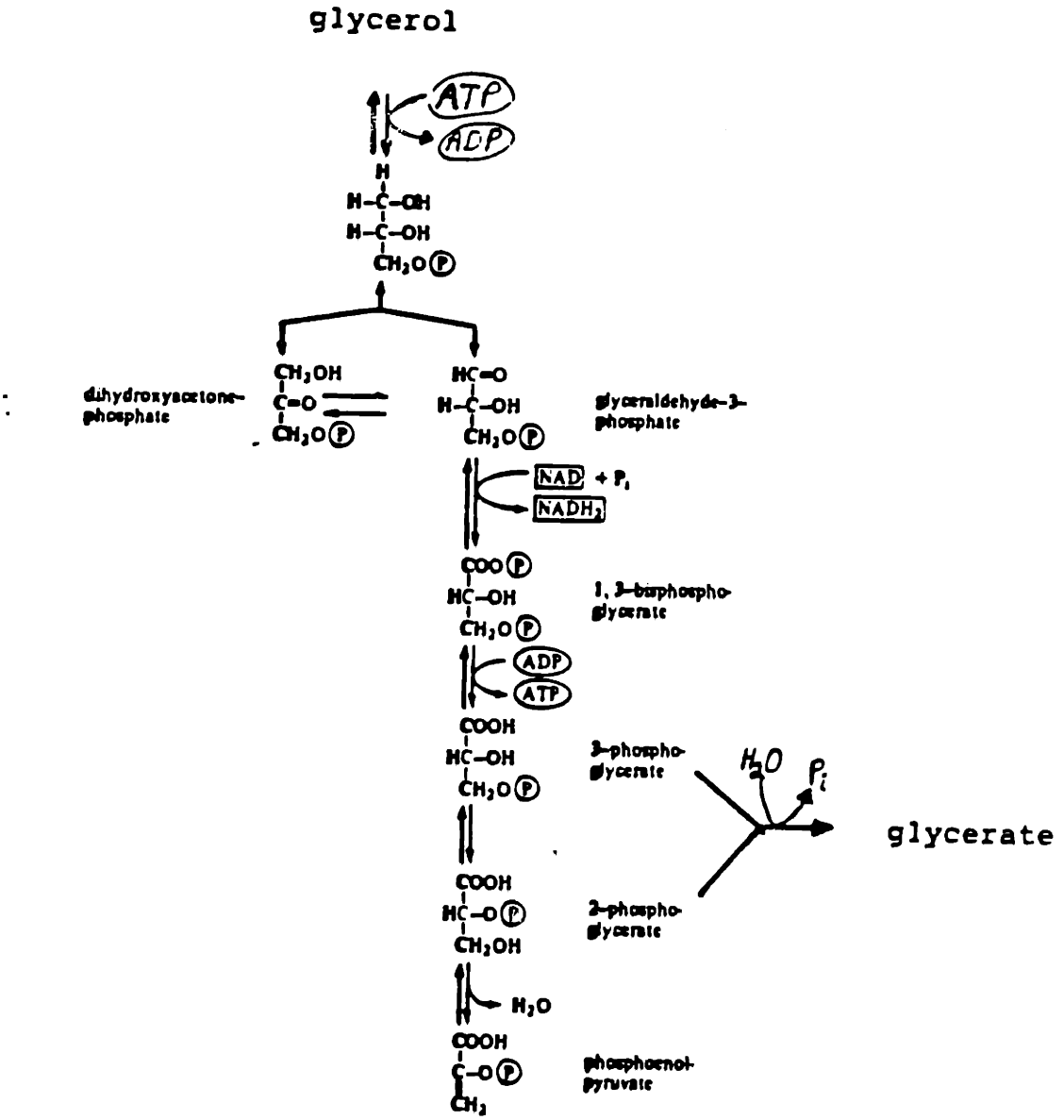


Figure 49 : Conversion of glycerol to glycerate and phosphoenol pyruvate. (Gottschalk, 1979)



Q) Incorporation of $^{13}C_2$-Glycine

Since glycine had not interfered with rapamycin production, and it was reasonable to assume that a small molecule like glycine should be able to enter the producing cells, an experiment with $^{13}C_2$-glycine (Sigma Chemical Co., 99% enriched) was conducted. Twelve flasks each containing 50 ml medium were inoculated and incubated 2.5 days. A filter-sterilized solution of $^{13}C_2$-glycine was added to the flasks to give a final concentration of 0.75 g/l. After another 24 hours of incubation a second dose of 0.25 g/l $^{13}C_2$-glycine was added. The fermentation was stopped after 6.5 days total incubation, the contents of the flasks were pooled, and the cells collected, washed, and extracted with methanol as described earlier. This extract was stored at -20 C while another 7 flasks were prepared and treated in an identical fashion. The extracts were pooled and the labelled rapamycin purified by the procedure described in the Materials and Methods (Section III). Instead of freeze-drying the HPLC fractions, however, the rapamycin was absorbed from the fractions onto C-18 silica cartridges and then eluted with pure methanol. This methanol was removed using a rotary evaporator at room temperature and the final traces of water were removed by freeze-drying. This rapamycin sample (10 - 11 mg) was then analyzed by ^{13}C-NMR on the 360 MHz NMR spectrometer. The spectrum of $^{13}C_2$-glycine enriched rapamycin is shown in Figure 50, and the natural abundance spectrum from unlabelled rapamycin from the same spectrometer is shown in Figure 51. The three peaks assigned to the three methoxy carbons (carbons 36, 50, and 46)

Figure 50: Spectrum of rapamycin enriched with $^{13}C_2$-glycine (360 MHz NMR).

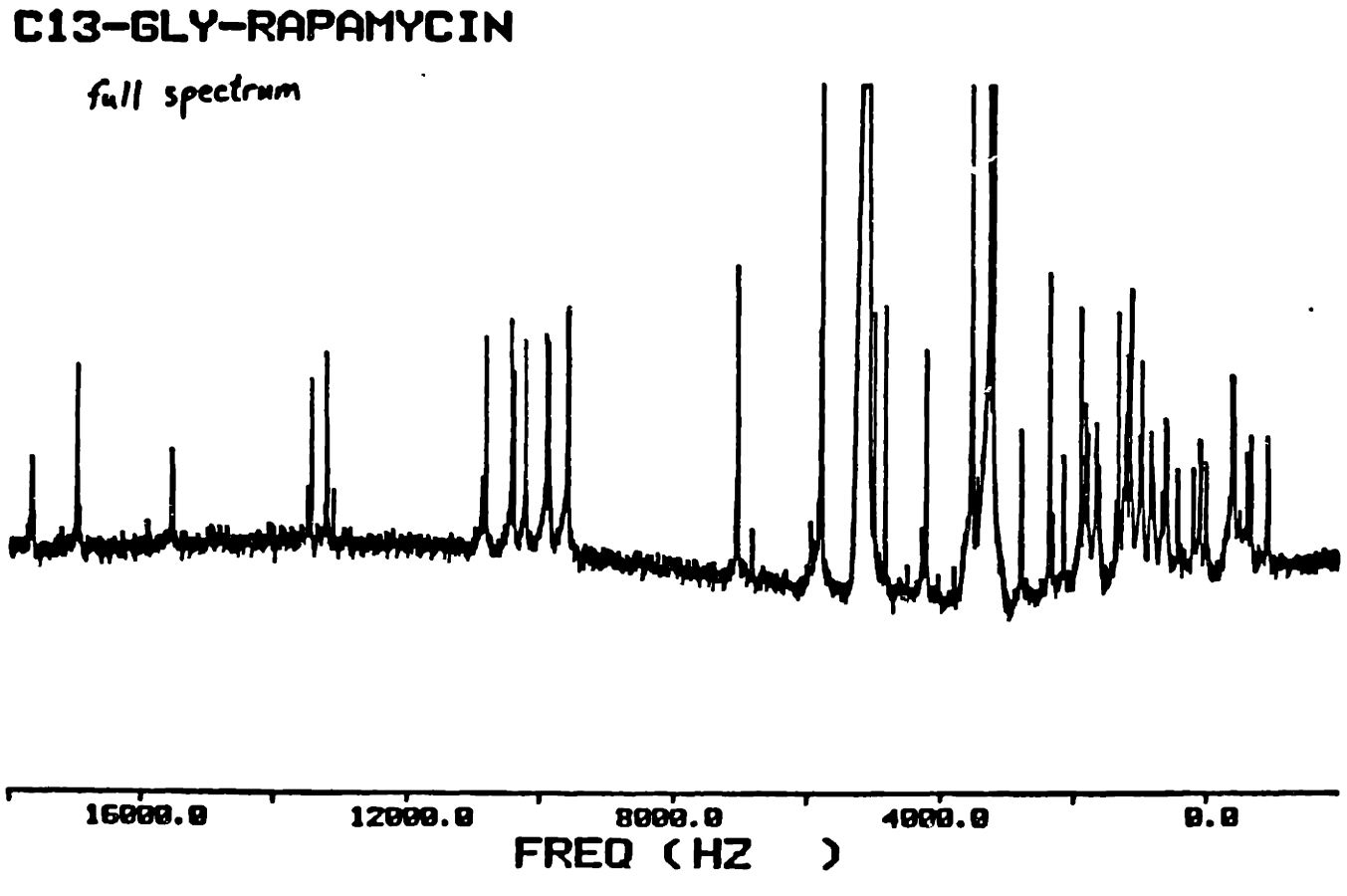


Figure 50 (continued):

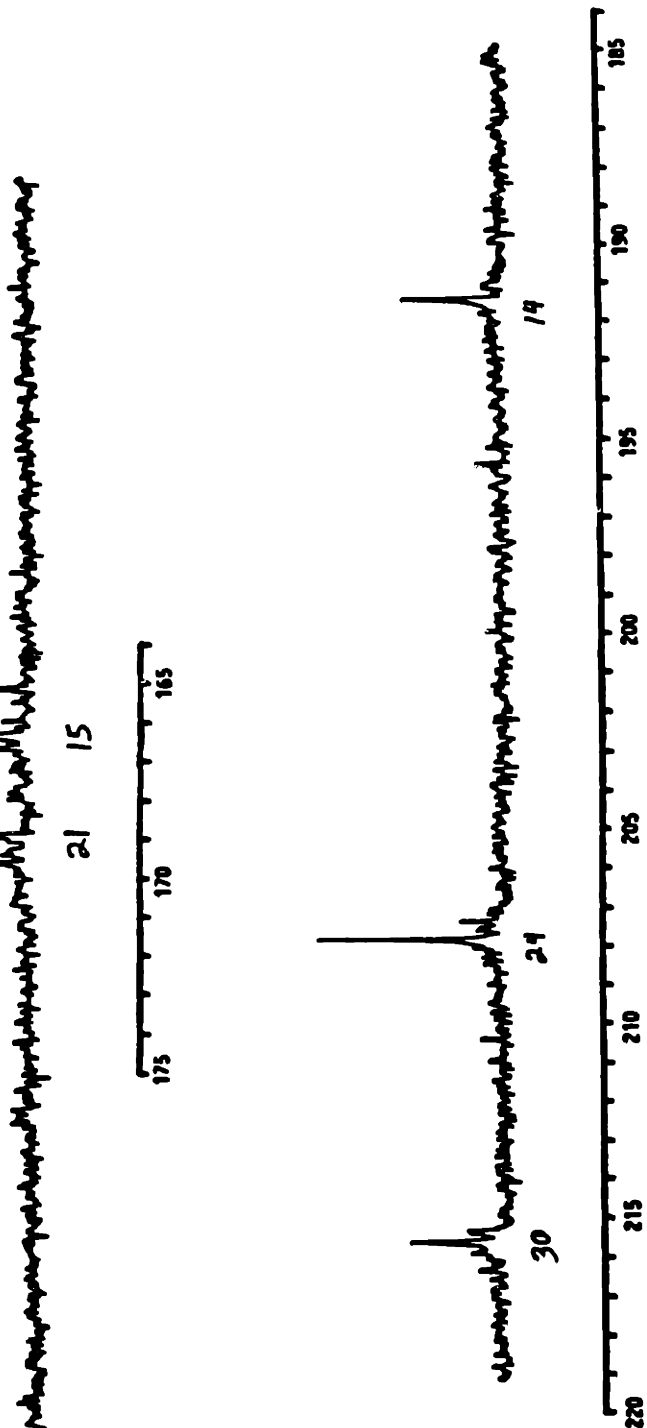


Figure 50 (continued):

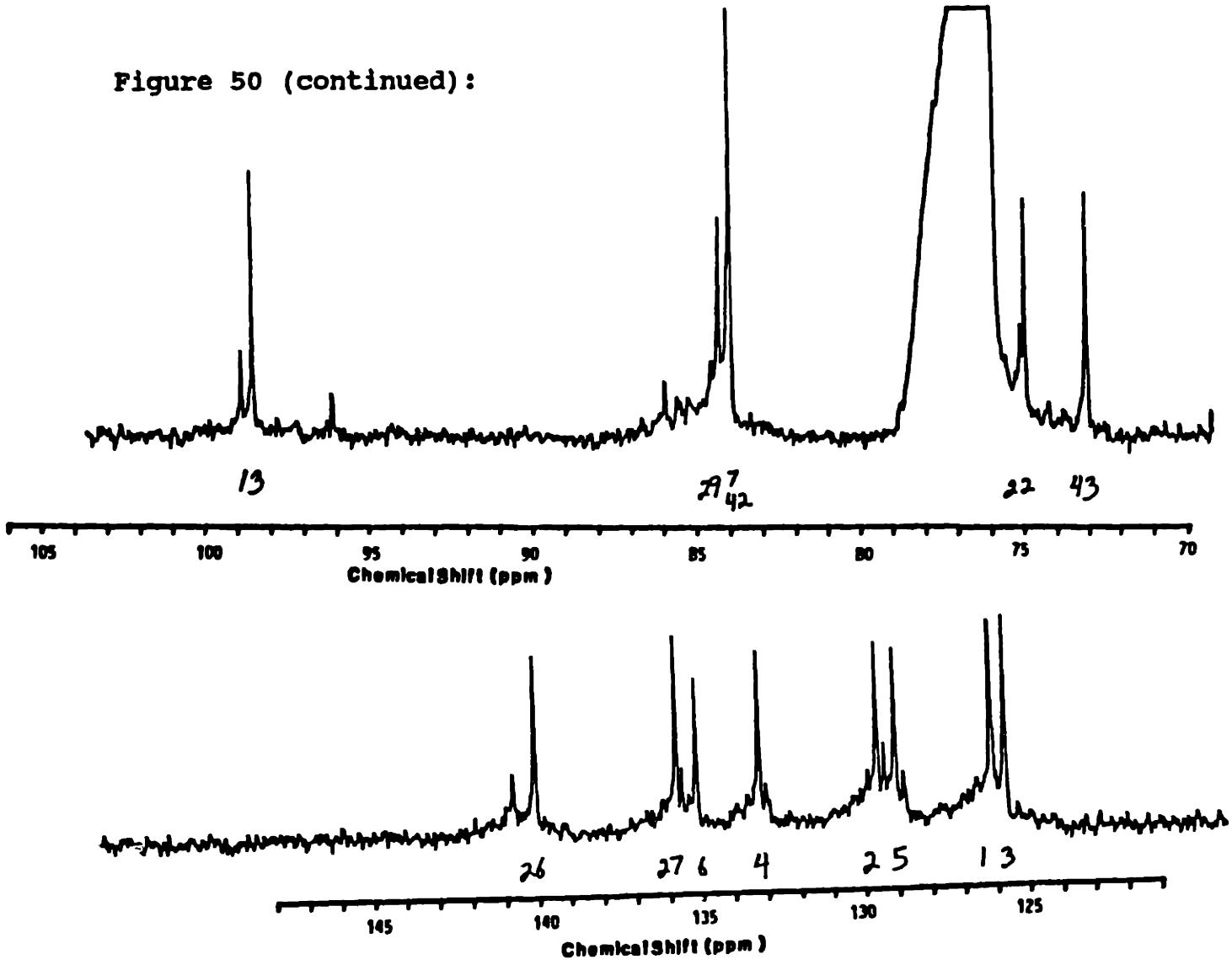


Figure 50 (continued):

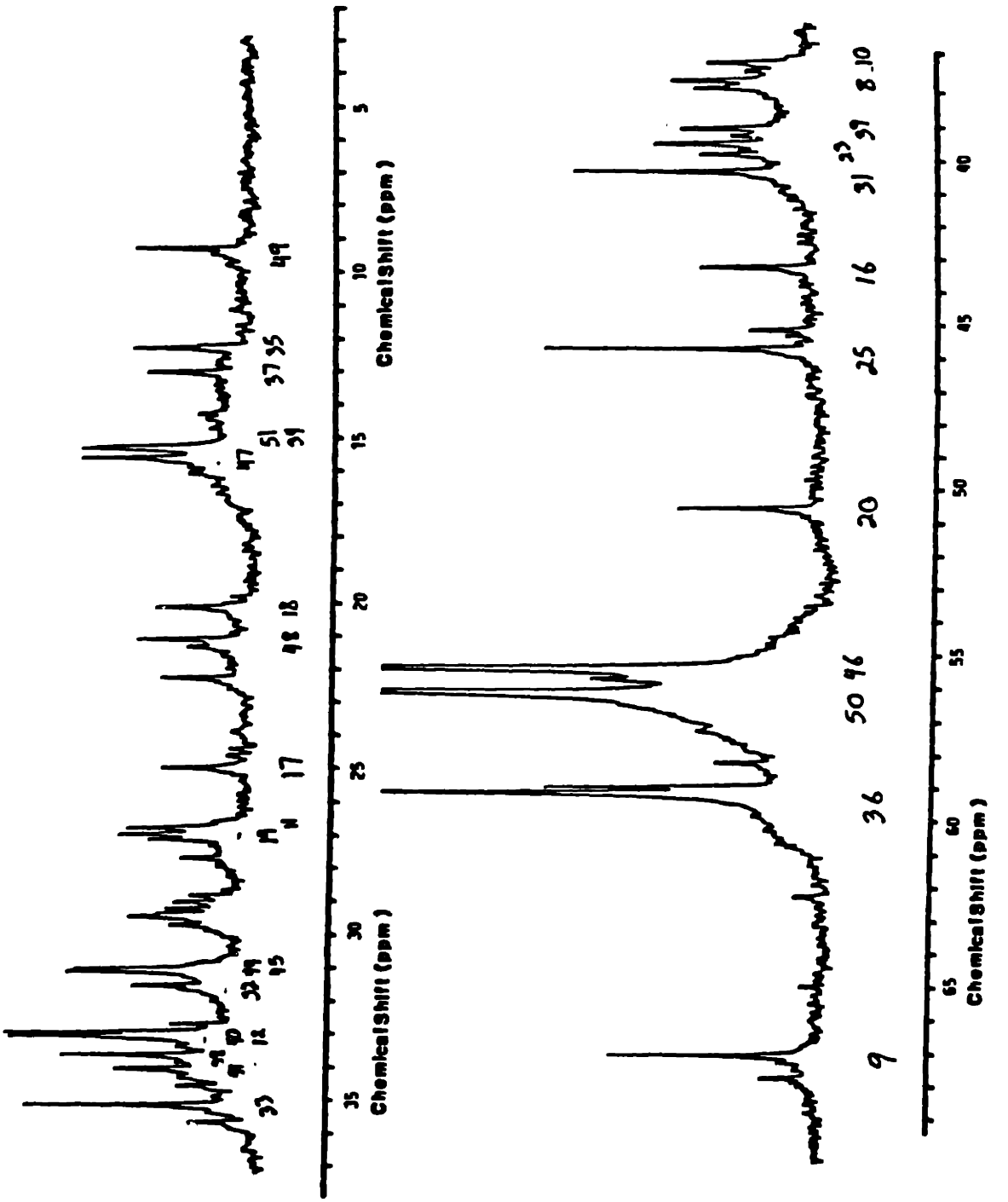


Figure 51: Spectrum of rapamycin natural abundance sample (360 MHz NMR).

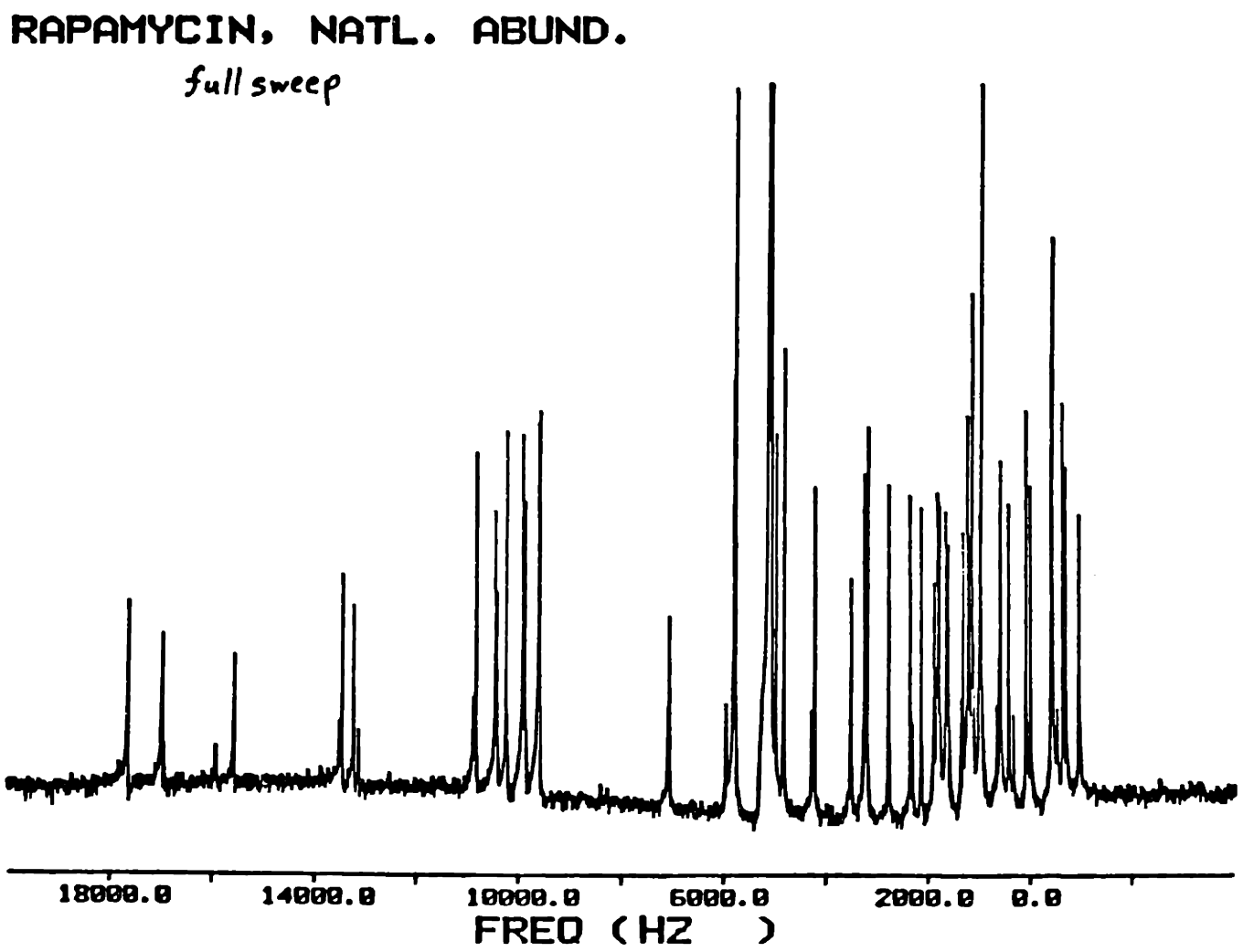


Figure 51 (continued):

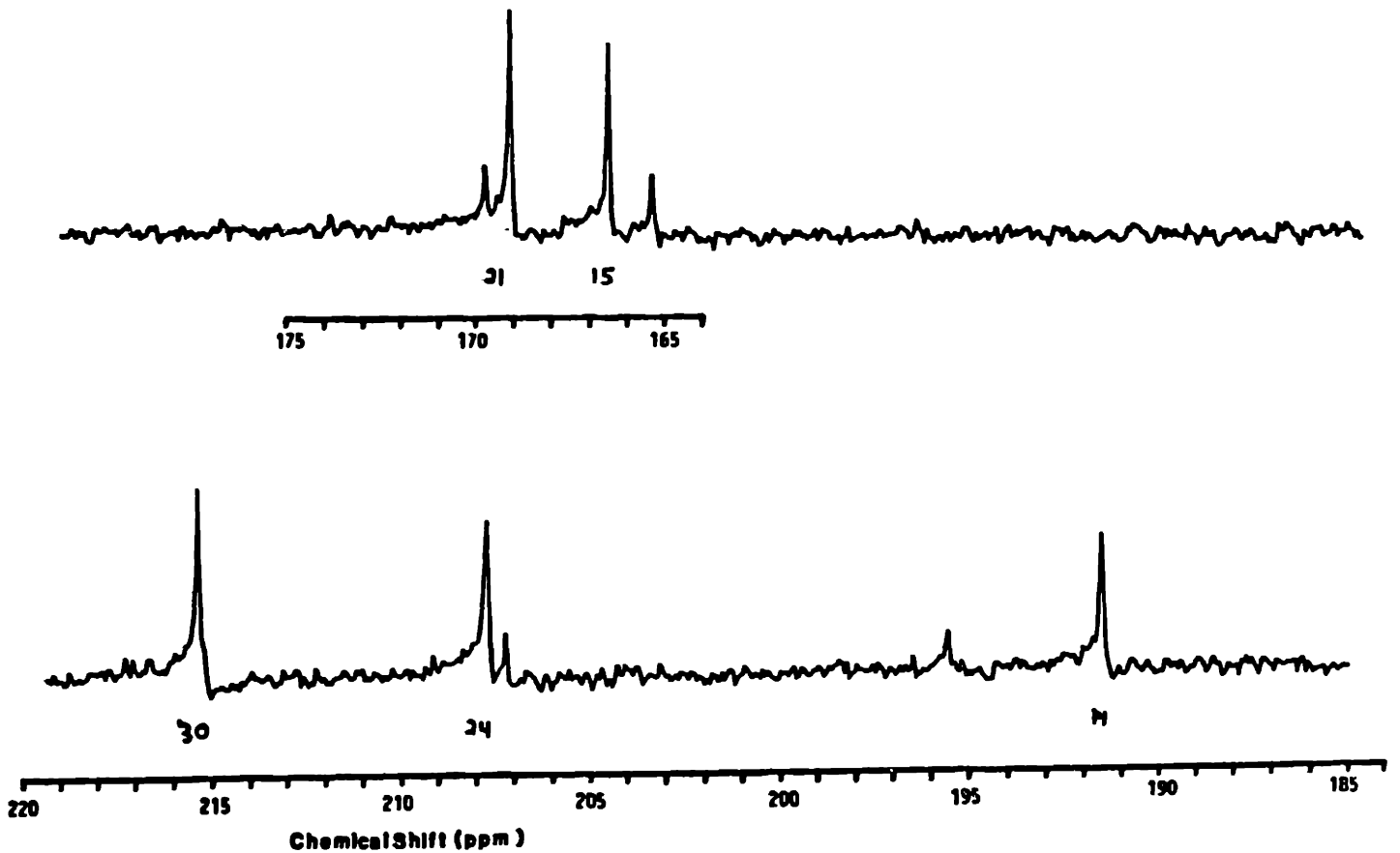


Figure 51 (continued):

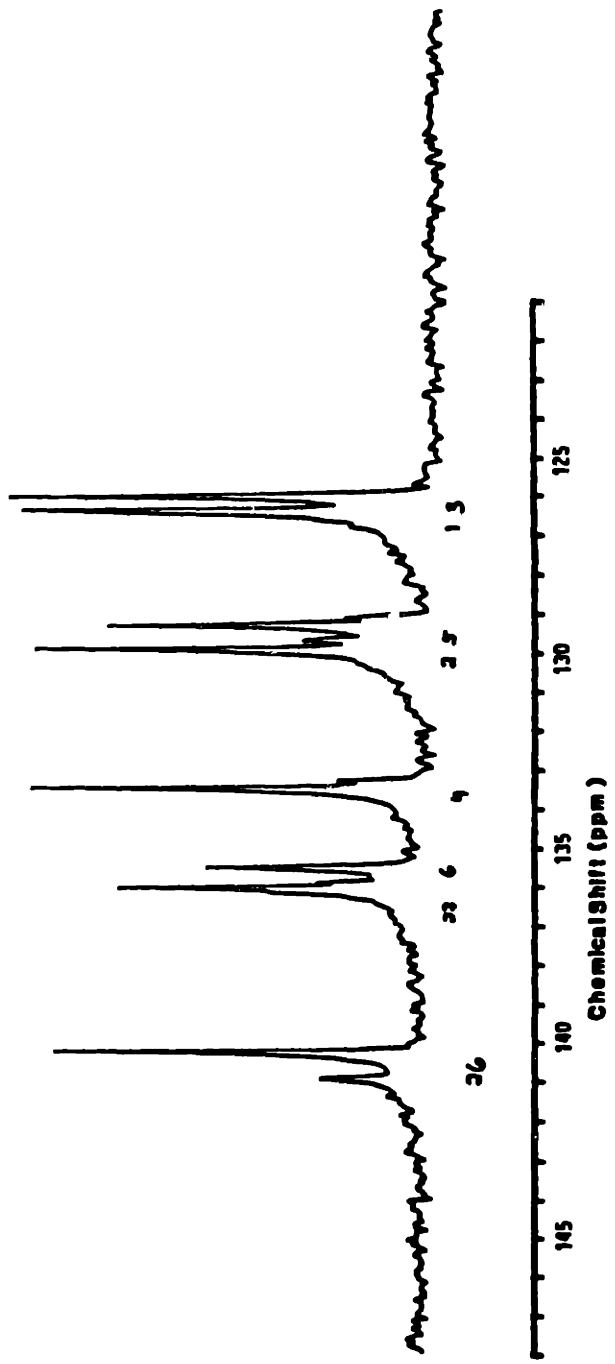


Figure 51 (continued):

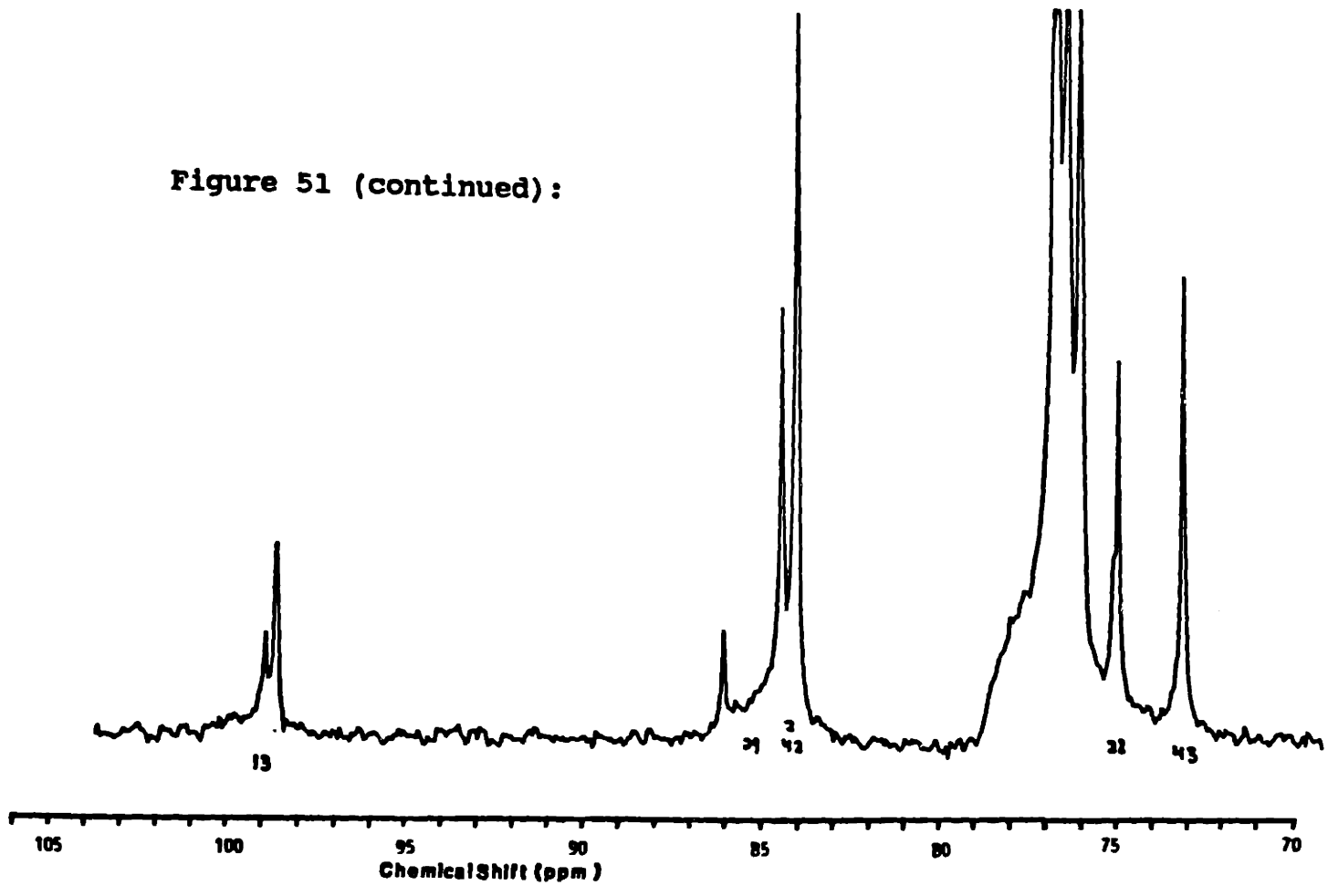


Figure 51 (continued):

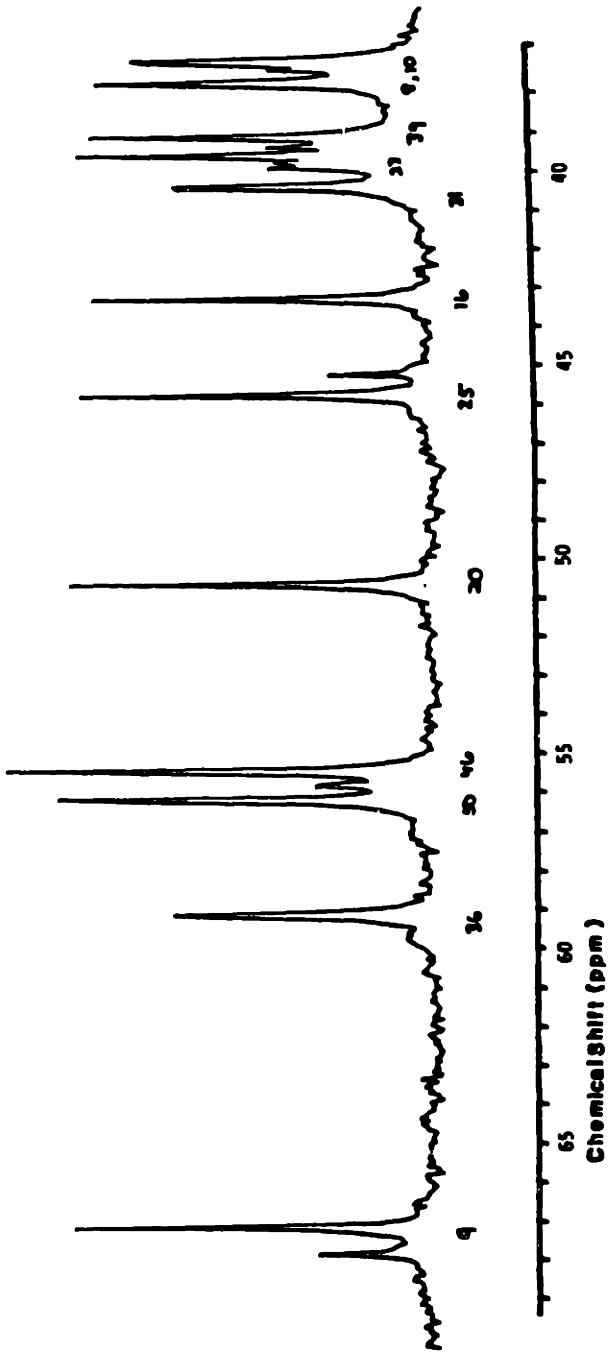
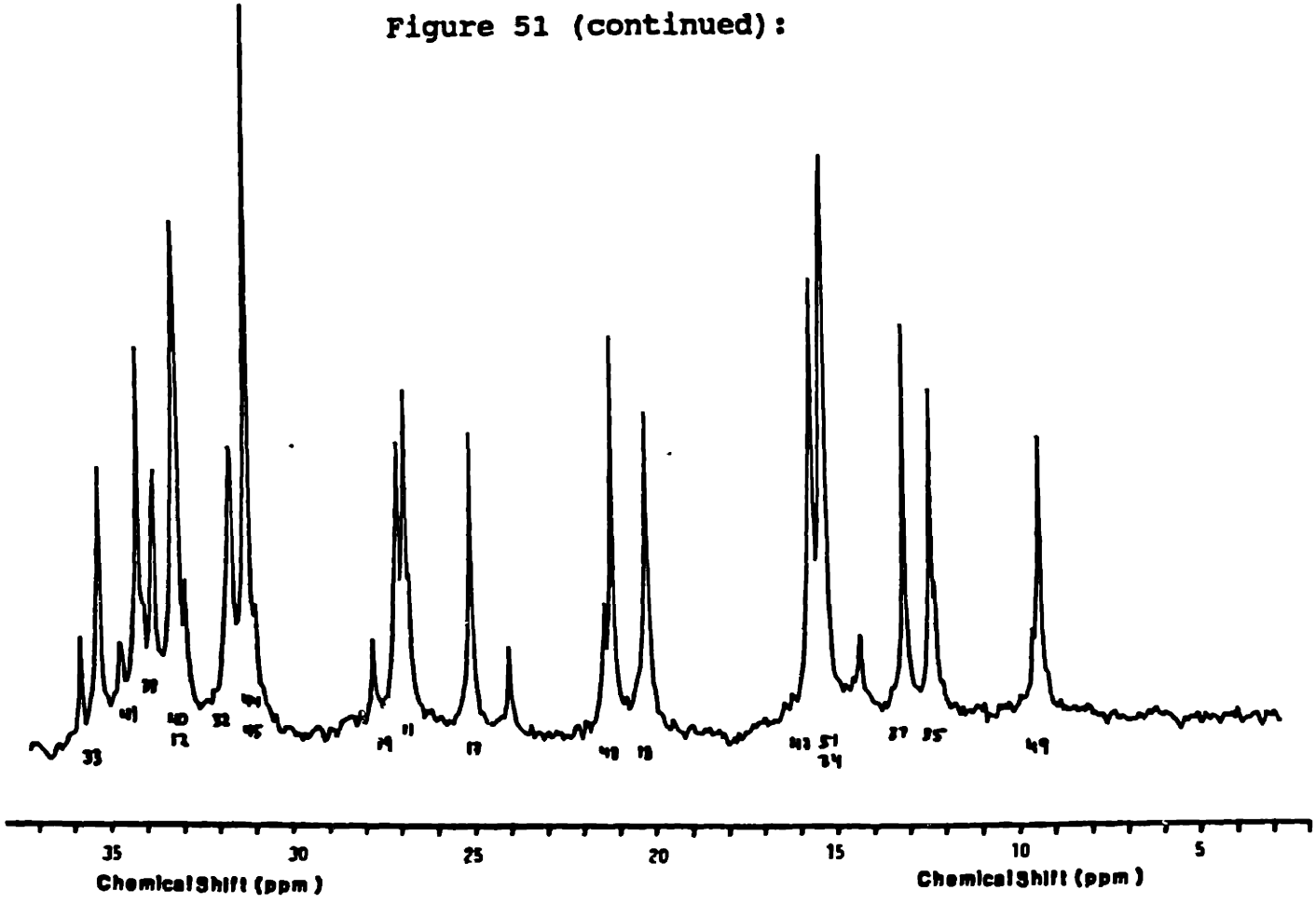


Figure 51 (continued):



show a 9 to 10-fold increase in height. Near the peaks corresponding to carbons 10 and 11 there was no significant evidence of any coupling patterns as would be produced by the successful incorporation of this doubly labelled substrate. (An initial spectrum of the sample showed possible satellite peaks but upon rerunning the sample and obtaining a sample with better a signal to noise ratio, it was found that these satellite peaks were indistinguishable from noise peaks.) No other carbons showed a significant increase in height or evidence of any coupling patterns. (The $^{13}C_2$-glycine was checked by ^{13}C-NMR, and the spectrum clearly showed the expected coupling patterns between the two carbons.)

From the high enrichment of the methoxy carbons it is apparent that the glycine was indeed getting into the cells. Labelled glycine is probably cleaved by glycine synthase, yielding a tetrahydrofolate-bound ^{13}C-methyl group and $^{13}CO_2$. The methyl group is then transferred to become the methyl group of methionine, and later transferred to the methoxy groups of rapamycin. The failure of $^{13}C_2$-glycine to label carbons 10 and 11 could be explained in two ways. The first possibility is that little or no glycine was converted to glycollate or glycerate. The conversion of glycine to methionine might be so highly favored over these other fates for glycine that insufficient label would be available for incorporation into other sites in rapamycin. A second possibility is that glycollate or glycerate is not the source of carbons 10 and 11, and no labelling could possibly be observed by this method. One possible way to further test these possibilities would be to repeat the

experiment with $^{13}C_2$-glycine addition, but this time add a low concentration of methionine simultaneously in an effort to suppress the conversion of glycine to methionine, but avoiding supression of rapamycin production. Another approach would be to chemically synthesize ^{13}C-glycollate or ^{13}C-glycerate and feed these directly to the cultures.

Although in the above experiment it did not appear that glycine was converted to glycollate, rapamycin was still synthesized, and therefore the cells would have to have a source of glycollate other than glycine if glycollate is indeed the precursor of C10 and C11. Glycollate can be obtained from the catabolism of certain compounds, and there is evidence that the glyoxalate bypass is active in the strain of Streptomyces which produces chloramphenicol (Vining and Westlake, 1964).

R) Incorporation of 2-^{13}C-Propionate

The incorporation of 1-^{13}C-propionate into rapamycin enriched seven carbons, six of these corresponding to the carboxyl groups of predicted propionate groups, while the seventh (carbon 39) corresponded to the carboxyl group of the predicted shikimate group. Carbon 32 (corresponding to the carboxyl group of a seventh predicted propionate group) was not enriched.

While this labelling pattern could possibly be explained by scrambling and other factors, an error in the published ^{13}C-NMR spectral assignments could also account for the observed results. Carbons 32 and 39 are methylene carbons in very similar environments and the data presented by Findlay and Radics (1980) do not conclusively distinguish between the two. The incorporation of 2-^{13}C-propionate was chosen as an indirect method to check the assignments of carbons 32 and 39. If 2-^{13}C-propionate labels carbon 31 (in addition to the six other corresponding propionate carbons) but not carbon 40, then this will show that there are seven propionate groups in rapamycin and that a mistake had been made in the assignments.

Twelve 500 ml flasks each containing 50 ml medium were inoculated. After 2.5 days of incubation a sterile aqueous solution of sodium 2-^{13}C-propionate was added to each flask to give a final concentration of 0.5 g/l. After 24 hours, more sodium 2-^{13}C-propionate (0.25 g/l) was added to each flask. After a total of 6.5 days incubation, the contents of the flasks were pooled and extracted as described earlier. A second fermentation (7 flasks) was started and treated in an identical fashion.

The extracts were pooled and purified using the procedure described for the $^{13}C_2$-glycine sample. The enriched sample was then analyzed by ^{13}C-NMR.

The resulting NMR spectra are shown in Figure 52. It is clear that seven peaks have increased in height. These peaks are assigned to carbons 38, 25, 27, 31, 33, 6, and 12. These are the same seven carbons that the proposed scheme predicts should be enriched by 2-^{13}C-propionate, confirming that there are indeed seven propionate units incorporated into rapamycin and that there had been an error in the published spectral assignments. Since carbon 31 was labelled and carbon 40 was not, it appears that the seventh carbon labelled by 1-^{13}C-propionate was actually carbon 32 and not carbon 39. The peak assigned to carbon 39 should actually be reassigned to carbon 32. It is very likely that the converse is true also, and that the peak assigned to carbon 32 should actually be reassigned to carbon 39, although it is possible that other methylene carbons are also misassigned and C32 has been confused with one of these. (See also discussion in Results, Sections U. and V.)

Figure 52: Spectrum of rapamycin enriched with $_2$-^{13}C-propionate (360 MHz NMR).

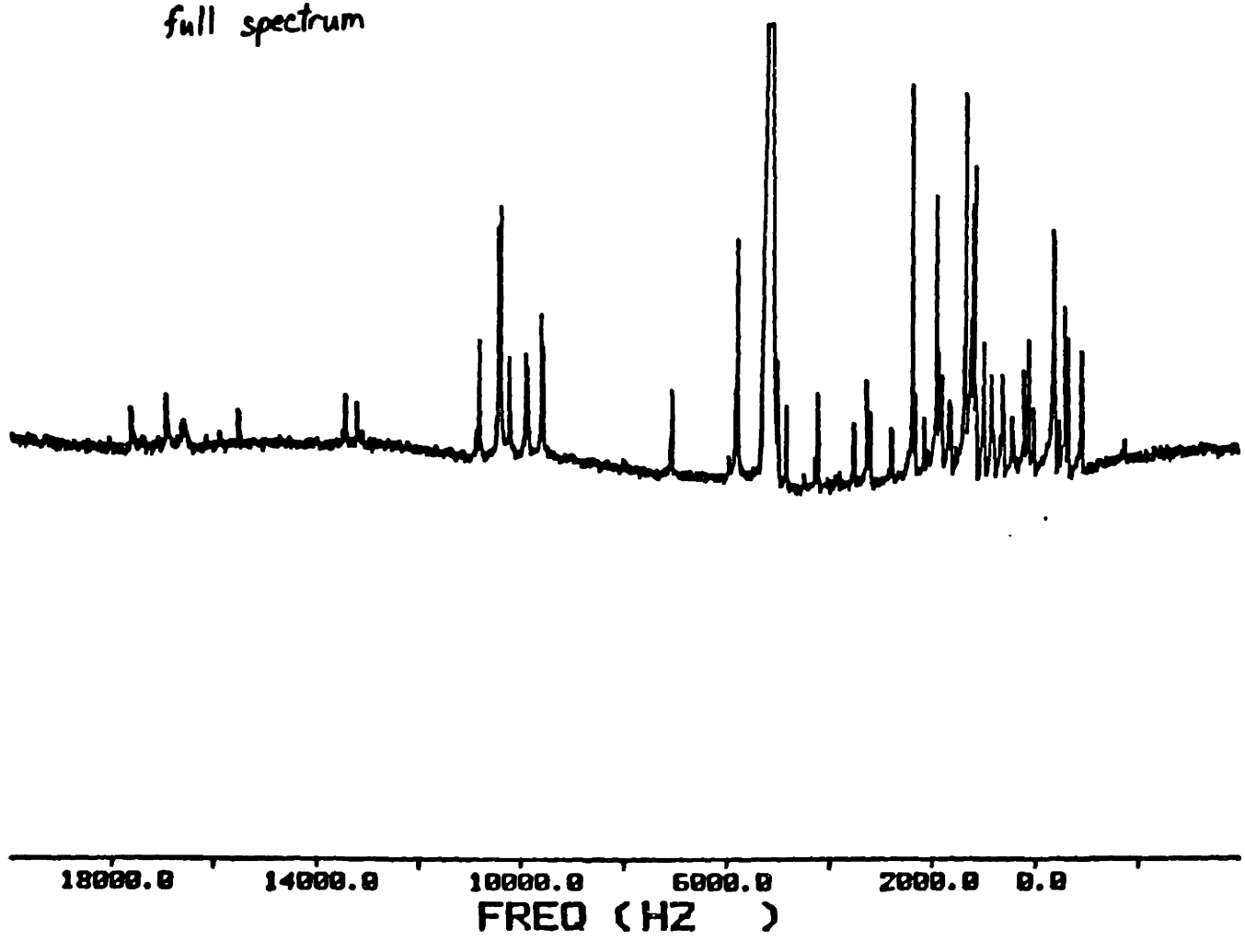


Figure 52 (continued):

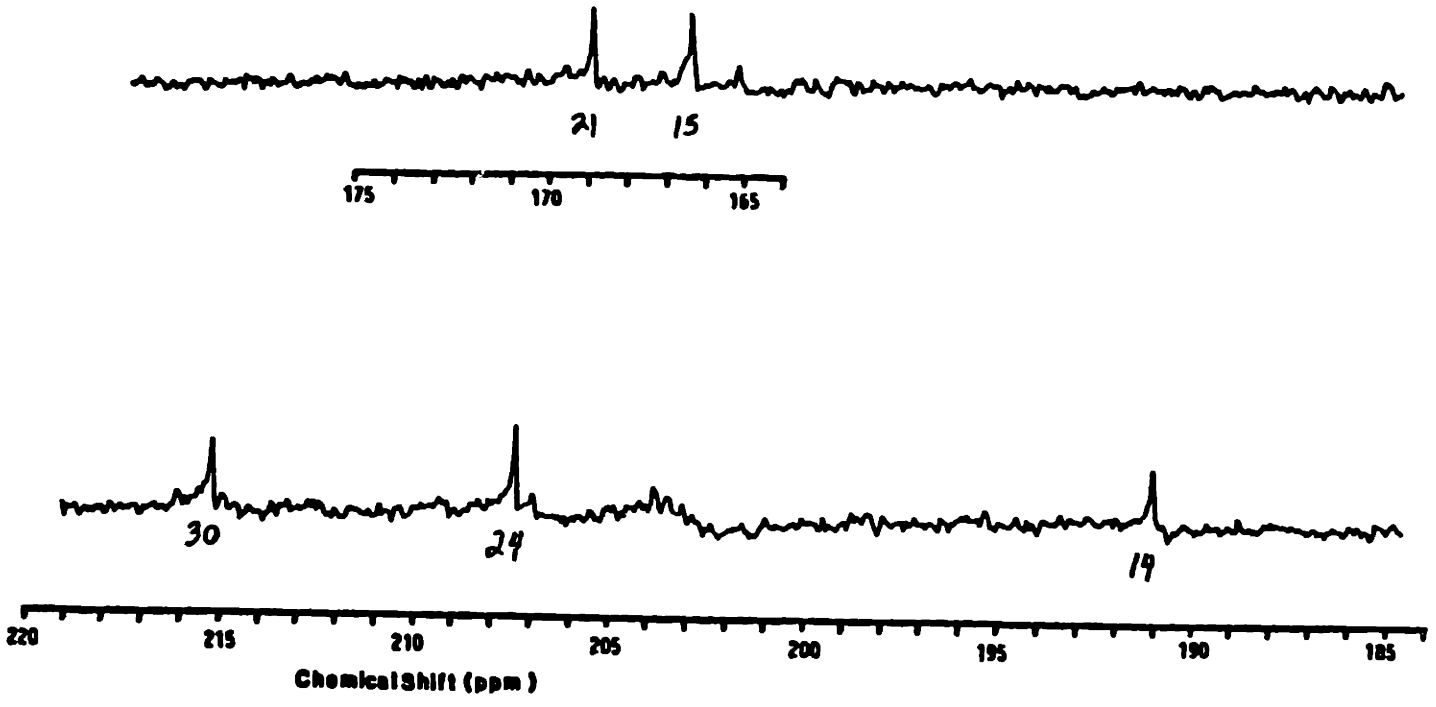


Figure 52 (continued):

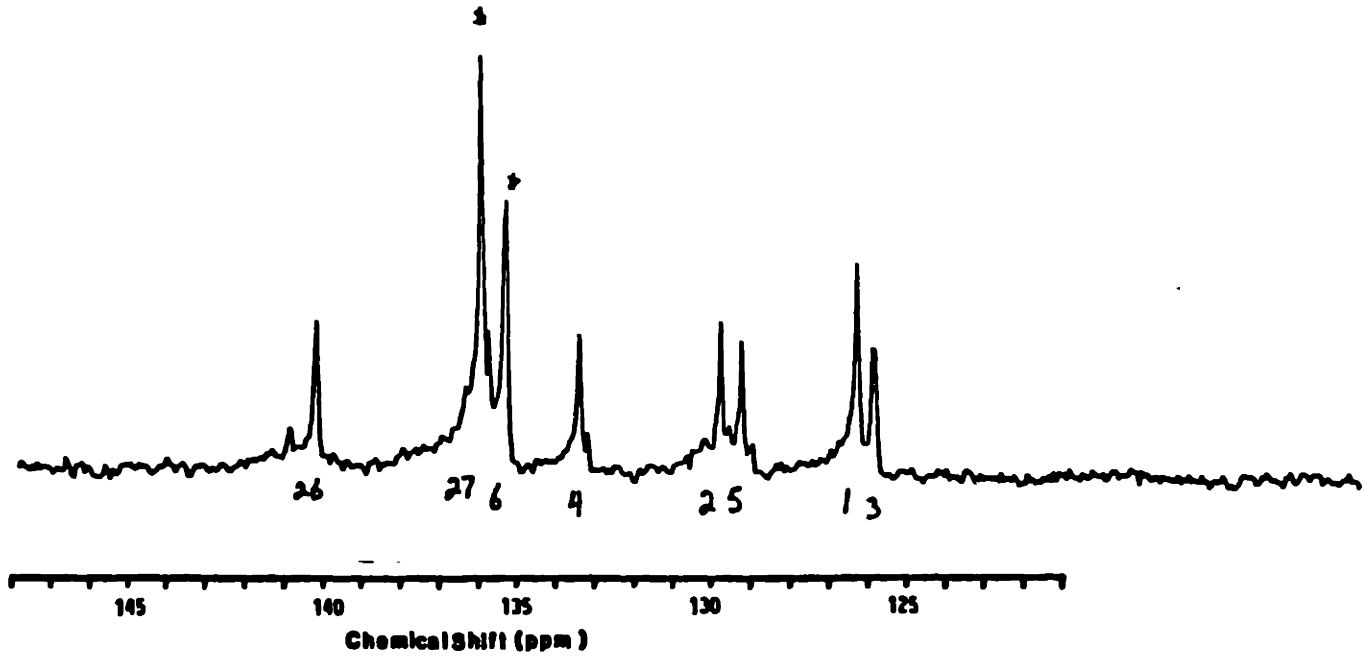


Figure 52 (continued):

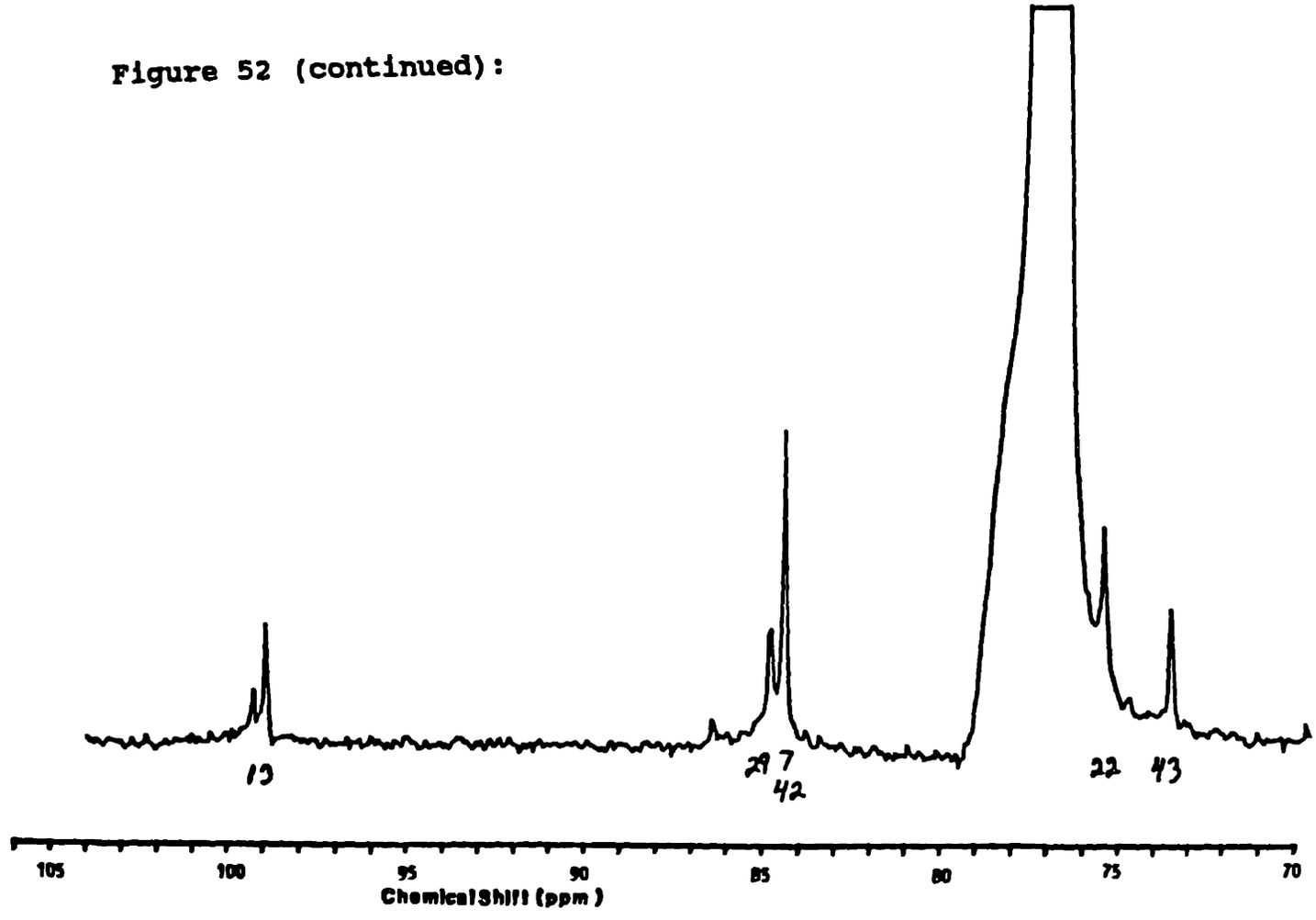


Figure 52 (continued):

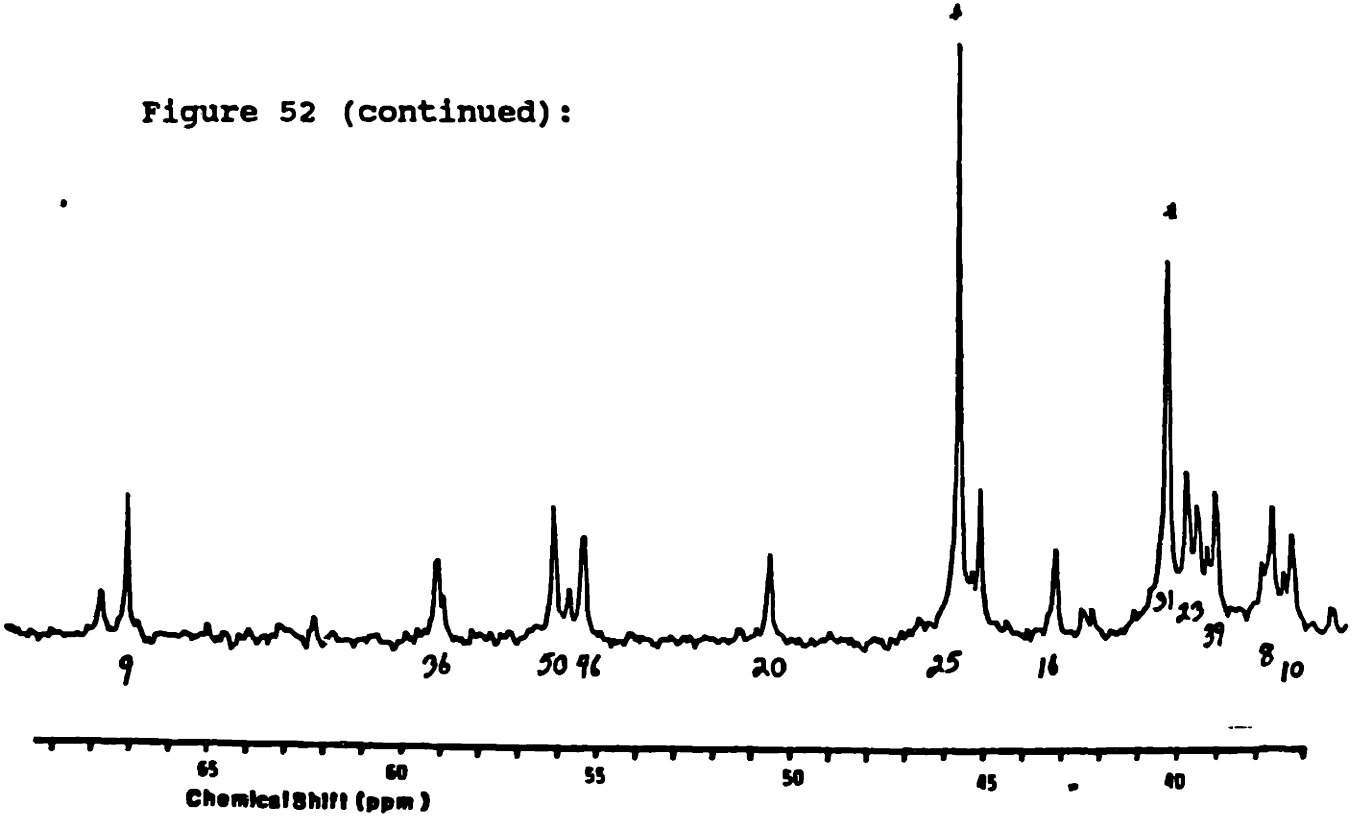
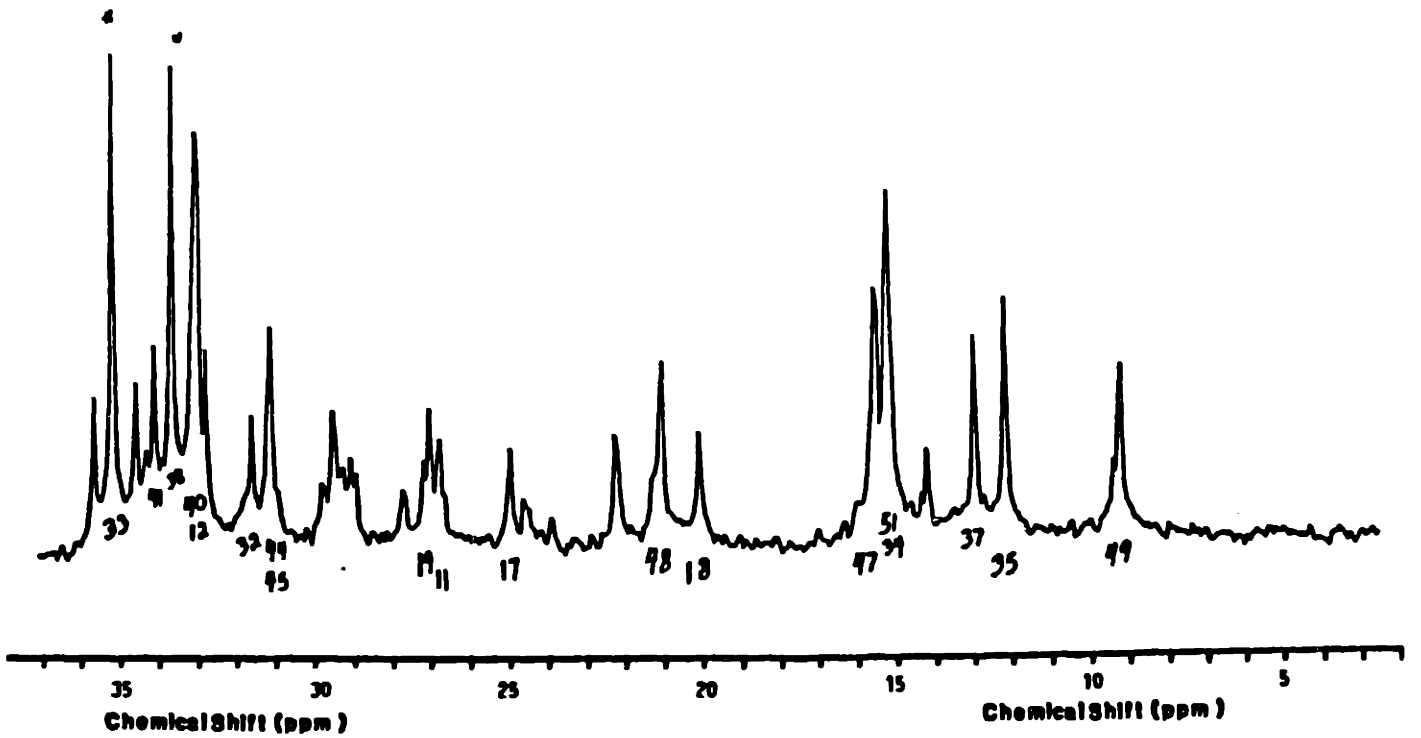


Figure 52 (continued):

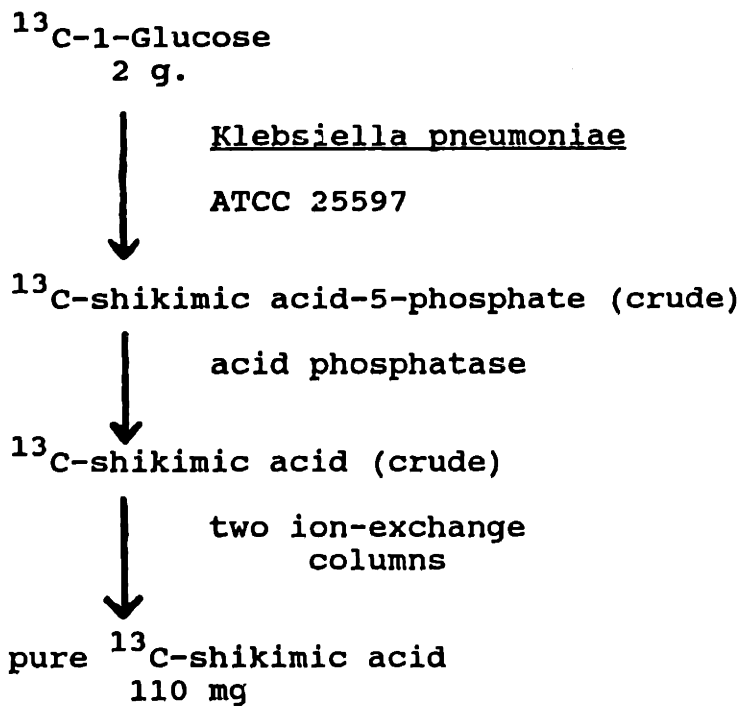


S) Preparation of ^{13}C-Shikimic Acid

At the end of the section describing the incorporation of ^{14}C-shikimic acid (see Results Section O.), several approaches were outlined for establishing the shikimate origin of the cyclohexane ring of rapamycin. From these possible alternatives, the method chosen was that of producing ^{13}C-shikimic acid from 1-^{13}C-glucose using a mutant of Klebsiella pneumoniae, and then adding this ^{13}C-shikimic acid to a rapamycin fermentation. The following procedure for the preparation of ^{13}C-shikimic acid is a modification of published procedures for the production and purification of ^{14}C-shikimic acid from E. coli (Knowles and Sprinson, 1964; Millican, 1962). The final procedure is summarized in Figure 53. All the procedures were optimized using unlabelled glucose prior to work with 1-^{13}C-glucose. A full-scale trial with unlabelled glucose produced approximately 125 mg of shikimic acid. Concentrations of shikimic acid were estimated by the assay method developed by Yoshida and Hasegawa (1957), which is specific for shikimic acid. Shikimic acid-5-phosphate does not react positively in this assay system and therefore must be hydrolyzed to shikimic acid by heating in acid or by treating with acid phosphatase prior to assay if its concentration is to be determined. (See Materials and Methods for assay procedure.) Heating in acid is fast and convenient for processing many samples, but will not release 100% of the shikimate no matter how long the sample is heated (Weiss and Mingioli, 1956). Acid phosphatase is slower but should produce a higher yield of shikimic acid.

Four 500 ml flasks each containing 100 ml Davis A medium

Figure 53: Process for the production of ^{13}C-Shikimic Acid from 1-^{13}C-Glucose.



supplemented with five aromatic supplements (see Materials and Methods) were inoculated with Klebsiella pneumoniae ATCC 25597. The flasks contained 5 g/l ^{13}C-1-glucose (Cambridge Isotope Laboratories). A set of four control flasks were also prepared, containing 5 g/l unlabelled glucose. The flasks were incubated at 37 C, 250 rpm, 2 inch stroke for 3.5 days. The cultures were centrifuged, the supernatants were pooled (400 ml) in screw-cap media bottles, small samples were removed, and the supernatants were frozen. The samples were acidified and autoclaved for 2 hours as described in the assay procedure (see Materials and Methods). The control supernatant contained at least 300 mg/l free shikimic acid while the ^{13}C fermentation contained at least 250 mg/l shikimate. The supernatants were then thawed, acidified to pH 4.9 with acetic acid, and sampled. Fifty mg of acid phosphatase was added to both supernatants, a few drops of toluene were added to the bottles, then the bottles were sealed and incubated at 37 C. After 14 hrs the bottles were sampled, 50 mg acid phosphatase was added, additional toluene was added, and the bottles were resealed and returned to 37 C. This procedure was repeated periodically, adding 40 mg phosphatase at 23, 48, 64, and 110 hours. Between 64 and 110 hours there was no significant increase in free shikimic acid in either of the supernatants. The final concentrations were 375 mg/l (150 mg total) in the ^{13}C fermentation and 500 mg/l in the control fermentation. Previous trials with unlabelled glucose had also produced approximately 500 mg/l. It is not known why the yield was lower in the ^{13}C fermentation.

The ^{13}C-1-glucose fermentation supernatant was loaded onto a column

containing 180 ml AG1-x8 ion exchange resin (acetate form; Biorad). The effluent was assayed for glucose and for shikimic acid but neither was detected. Apparently all ^{13}C-1-glucose was utilized and the shikimic acid was successfully bound to the column. The column was then washed with 300 ml dH_2O. No shikimic acid was detected in this wash effluent. Elution with 0.1 M ammonium acetate (pH 6.5) was then started. Shikimic acid was detected after about 1100 ml had passed through the column. Elution was continued until no more shikimic acid was detected in the effluent. The shikimic acid-containing fractions were combined (425 ml total). The assay showed approximately 250 mg/l (106 mg total).

The combined effluents were loaded onto a second ion-exchange column (100 ml AG1-x8 resin, acetate form). The column was washed with 100 ml dH_2O. No shikimic acid was detected in either the load or wash effluents. Elution with 0.1 M acetic acid was begun. The shikimic acid started to elute after approximately 600 ml had passed through the column. The fractions containing shikimic acid were pooled (1200 ml). The pool was then freeze-dried. The final product contained light brownish to off-white crystals. The product was collected by dissolving the crystals in dH_2O and pooling the solutions. The total volume was brought to 20 ml and the pool was assayed. By the chemical assay, the yield was approximately 110 mg. A sample was also diluted and scanned in a UV spectrophotometer. A standard solution was also scanned and found to have the same UV profile, with a maximum absorbance at 205 nm. From the relative absorbances at 205 nm, it was also calculated that the final yield of ^{13}C-shikimic acid was 110 mg.

T) Determination of the Labelling Pattern of ^{13}C-Shikimic Acid

In order to interpret the incorporation of ^{13}C-shikimate into rapamycin it is necessary to know the sites of ^{13}C incorporation in the shikimic acid. ^{13}C-NMR was used to determine the relative enrichments of the carbons. An attempt was made to quantitate the total enrichment by GC/MS, but these results were found to be unreliable.

Figure 54 shows the ^{13}C-NMR spectrum of the ^{13}C-labelled shikimic acid. The spectrum contained seven peaks, corresponding to the seven carbons. Spectral assignments were made based on the relative chemical shifts of the peaks, except for the 3- and 5-hydroxyl groups which could not be absolutely assigned from the information available. Figure 55 shows the ^{13}C-NMR spectrum of the commercial shikimic acid (Sigma Chemical Co.). Eleven peaks appear in this spectrum, the four additional peaks apparently arising from a splitting of four of the seven expected peaks. This type of spectrum may be due to isomerization of the shikimic acid during its processing or the presence of a strongly interacting metal. (The commercial acid has a slight brownish color.) This spectrum was unsuitable for a direct comparison with the spectrum of the labelled sample, so an attempt was made to purify the commercial shikimic acid by passing it through the same column that had been used to purify the labelled sample. Fractions which reacted positively in the assay for shikimic acid were pooled and freeze-dried as described for the preparation of the labelled sample. The ^{13}C-NMR spectrum of the resulting shikimic acid is shown in Figure 56. The spectrum now contains only seven peaks, in one-to-one correspondence with those in

Figure 54: 13C-NMR Spectrum of 13C-Shikimic Acid.

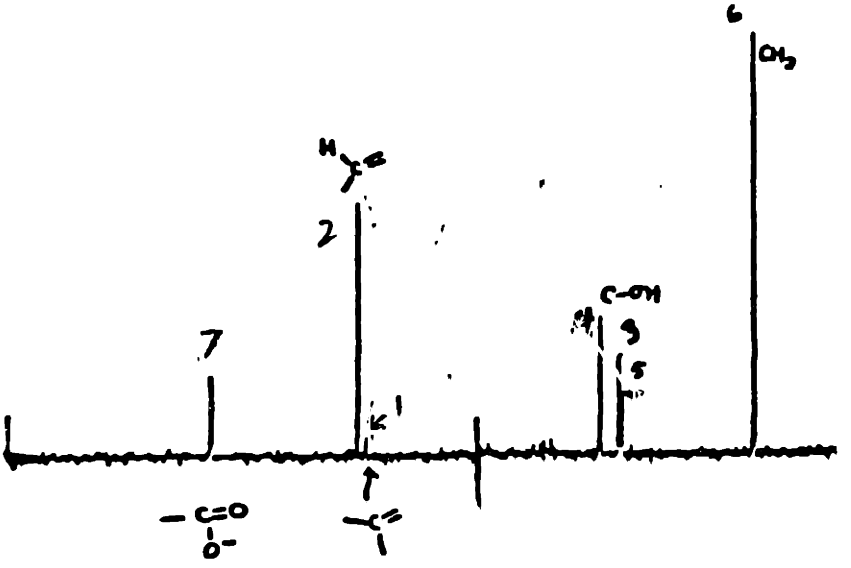


Figure 55: 13C-NMR Spectrum of Commercial Shikimic Acid (Natural Abundance).

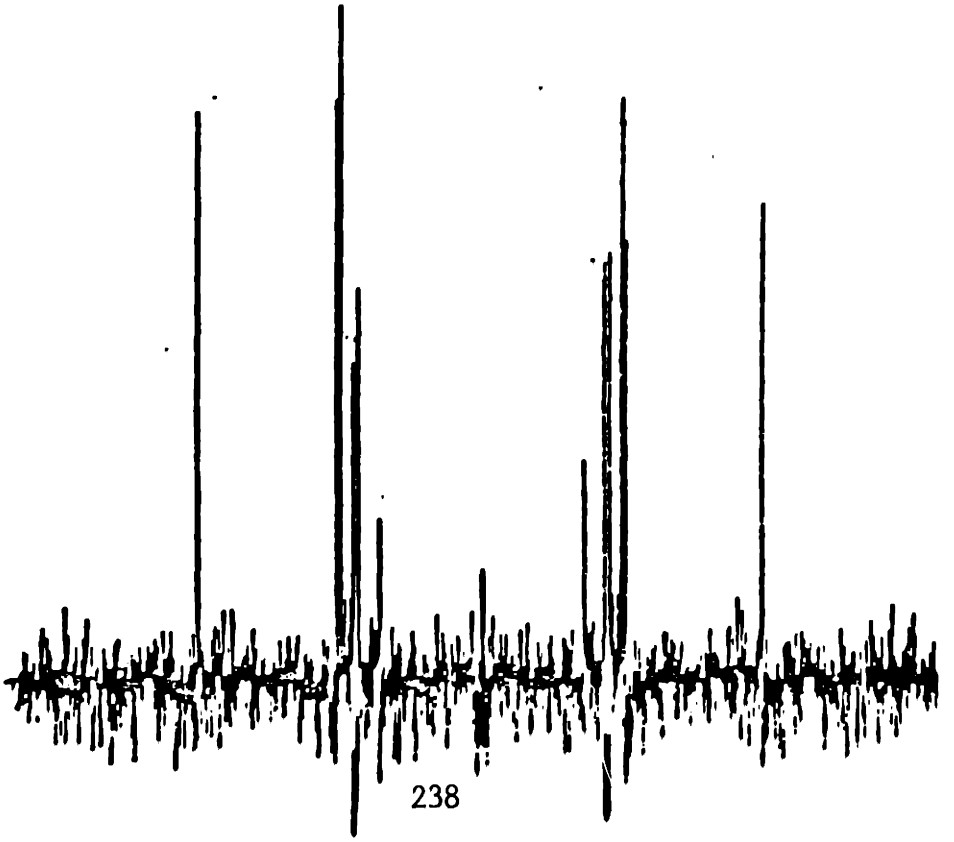
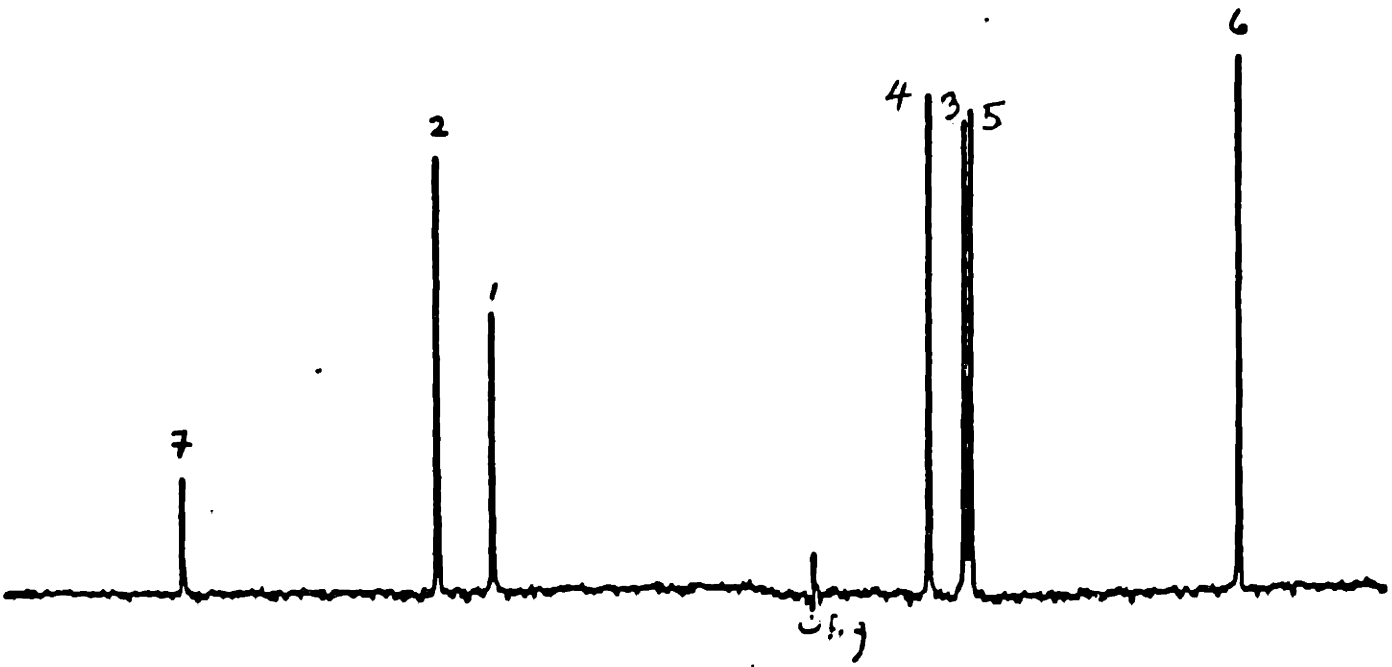
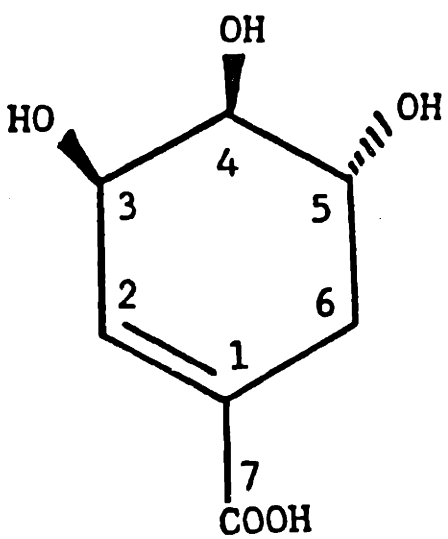


Figure 56: 13C-NMR Spectrum of Commercial Shikimic Acid (Natural Abundance) after purification.





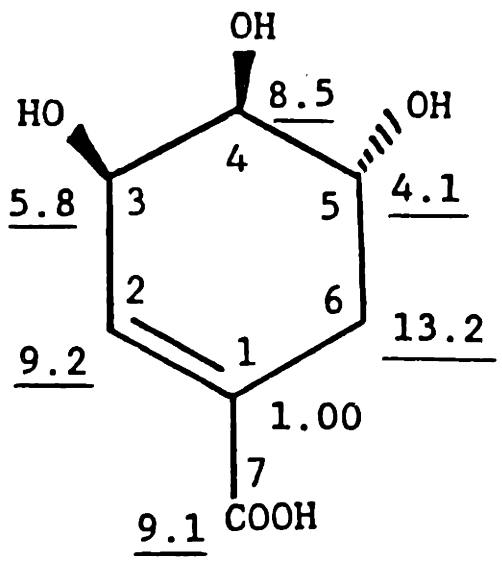
the labelled sample. Apparently, the isomer or metal was removed by the purification procedure.

Figure 57 shows the calculated enrichments for the seven carbons based on the ^{13}C-NMR data. These were estimated by normalizing the peak heights in each spectrum by the C1 peak height, then dividing the normalized peak heights of the ^{13}C-shikimic acid by those of the unlabelled sample. These calculated relative enrichments actually represent the minimum possible enrichments, assuming no enrichment of C1. If there is some incorporation at C1, then the actual enrichments would be higher for each carbon, although the relative enrichments would stay the same. There is also some uncertainty (+/- 20%) in the measurement of the height of the peak for C1, since the peak is small relative to the magnitude of the noise in the spectrum.

It had been predicted that C2 and C6 would be the most intensely labelled carbons, based on known pathways for glucose metabolism. These carbons are enriched (9.2- and 13.2-fold respectively), but C7 and C4 are also very enriched (9.1- and 8.5-fold), and C3 and C5 are slightly enriched (5.8- and 4.1-fold). It appears that modes of scrambling other than those predicted must be active in this strain of Klebsiella pneumoniae.

It has been estimated that 20 to 30% of the glucose utilized by enteric bacteria is assimilated by the oxidative pentose phosphate cycle. In this pathway, glucose is converted to 6-phosphogluconate, and then the C1 of glucose is lost as CO_2, yielding ribulose-5-phosphate, which is further metabolized by transketolase and transaldolase

Figure 57: Calculated ^{13}C-Enrichments of the Prepared ^{13}C-Shikimic Acid.



(Enrichment value underlined and expressed as ^{13}C content relative to the ^{13}C content of C1.)

reactions. Since 1-^{13}C-glucose was used to produce the ^{13}C-shikimic acid, some of the label was lost as $^{13}CO_2$. The maximum labelling possible at C6 (methylene carbon) of shikimic acid would be 35 to 40% (35- to 40-fold enrichment) if the Embden-Meyerhof (EM) pathway was the only other pathway used for glucose utilization. (If processed through the EM pathway, each molecule of ^{13}C-glucose generates two molecules of phosphoenolpyruvate, only one of which is labelled.) This maximum level might not be reached if the oxidative pentose phosphate cycle is more active than normal, which is quite likely considering the type of Klebsiella mutant used. By stimulating the demand for erythrose-4-phosphate for the production of shikimic acid, the normal balance of the cell may have been shifted. The maximum enrichment may not have been reached if yet another route of glucose assimilation was also active, namely the Entner-Doudoroff (ED) pathway. This pathway is not normally used by enteric bacteria for glucose accumulation, but is induced by the presence of gluconate. Stimulation of the oxidative pentose cycle might also induce the ED pathway enzymes. The relatively high enrichments of C4 and C7 are evidence that the ED pathway is probably operating. The ED pathway would produce pyruvate labelled in the carboxyl group, which would label C4 and C7 of shikimate, in contrast to the EM pathway which only produces pyruvate labelled in the methyl group.

GC/MS was used in an attempt to determine the total enrichment of the shikimic acid and thereby establish an upper limit on the absolute enrichment of the carbons. Prior to GC/MS, samples of both repurified

commercial (unlabelled; Sigma Chemical Co.) and purified ^{13}C-labelled shikimic acid were converted to the tetra-substituted trimethylsilane derivative (see Materials and Methods). The samples were subjected to GC/MS under identical conditions. In Figures 58 a and b are shown the GC chromatograms and MS patterns for the samples. (These GC profiles were produced using the MS as the detector, monitoring the total ions from 50 to 500 amu.) The GC profiles for the two samples were almost identical, with a major peak at 7.8 min, a slight shoulder peak at 8.0 min, and a few very small peaks at other times.

The mass spectrum of each sample was taken at approximately the maximum of the major peak (retention time 7.7 to 7.8 minutes). The overall MS pattern appeared to match that reported in the Eight Peak Index of Mass Spectra (1983):

m/z:	73	204	147	205	75	74	206	45	462
% :	100	88	25	17	10	9	8	7	3.10

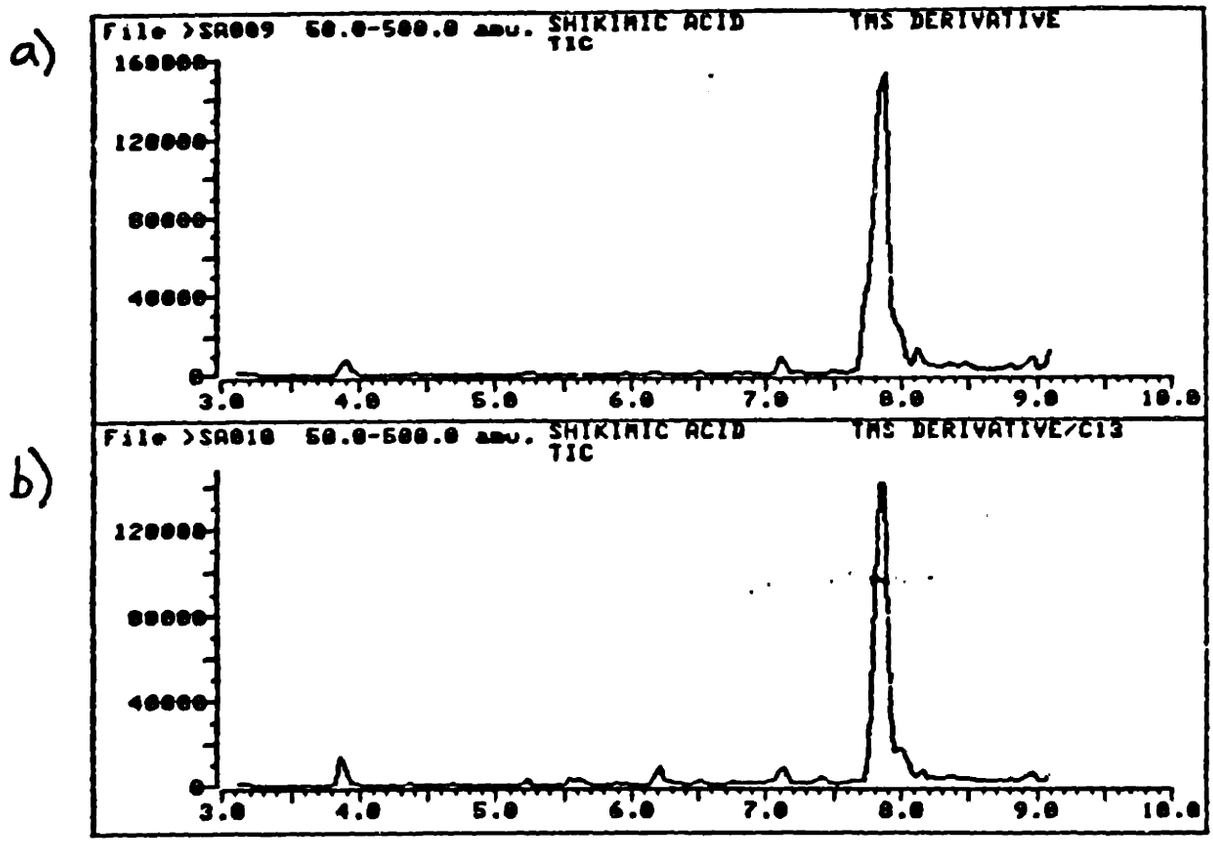
where % = relative peak height expressed as the percent of the peak height of the most abundant peak (m/z = 73) and m/z = 462 is the parent peak. While the exact experimental conditions used to obtain these published values may have been different from those used in this study, electron impact ionization was used in both cases.

The integrated peak areas for the parent peak (m/z = 462) and the m + 1, m + 2, and m + 3 peaks were measured. In the unlabelled sample, the m + 1 and m + 2 peaks were very noticeable due to the high natural abundance of ^{29}Si and ^{30}Si and the large numbers of Si and C atoms in the derivative molecule. In the ^{13}C-shikimate samples, these peaks were

Figure 58: GC Chromatograms of Shikimic Acid Samples.

a) purified commercial shikimic acid

b) 13C-shikimic acid



more intense, indicating some enrichment had taken place. Following procedures outlined in texts on mass spectrometry calculations (Biemann, 1963; Millard, 1968), it was calculated that the sample was approximately 18% $^{13}C_1$ and 2 to 3% $^{13}C_2$-shikimic acid. The total enrichment from these calculations would be approximately 22 to 24%. This is much lower than the 44% +/- 8% predicted by the ^{13}C-NMR calculations. The MS data was then re-examined more closely. The signals recorded for the peaks in the parent peak region were very low, so much so that probably not enough data had been accumulated in these channels to be statistically significant. Until the GC/MS work is repeated with higher sample loading or with different data accumulation parameters, the total ^{13}C incorporation cannot be absolutely determined. However, the GC profiles (recorded at 50 to 500 amu) showed that the compounds are very similar, if not identical.

It may be possible at some point to solve the problems associated with the GC/MS analysis, but it is not absolutely necessary for this project. Although the absolute enrichment of each of the carbons is not known, the relative enrichments from the NMR data provided sufficient information for the interpretation of the incorporation of ^{13}C-shikimic acid into rapamycin.

U) Incorporation of ^{13}C-Shikimic Acid

The proposed biosynthetic scheme predicts that a shikimic acid pathway intermediate should provide the seven-carbon cyclohexane moiety of rapamycin (carbons 39 to 45). None of the ^{13}C-labelled precursors previously incorporated into rapamycin labelled this region. 1-^{13}C-Propionate had enriched the peak originally assigned to carbon 39, but it was later concluded that there had been an error in the assignments, and that this peak actually corresponds to carbon 32. It is very likely that the peak assigned to C32 actually corresponds to C39. ^{14}C-Shikimic acid labelled rapamycin to a high degree (see Results, Section O.), but there was no easy way to determine if this incorporation was specific for the cyclohexane moiety or if it was through the incorporation of breakdown products from ^{14}C-shikimate. If the incorporation of shikimate was specific for the cyclohexane moiety, the ^{13}C-shikimate described in the previous sections should label certain carbons in this moiety.

Sixteen 500 ml flasks each containing 50 ml medium and six 250 ml flasks each containing 25 ml medium were inoculated. After 2.5 days of incubation, a sterile aqueous solution of ^{13}C-shikimic acid was added to each flask to give a final concentration of 0.075 g/l. After an additional 24 hours, a second dose of shikimic acid (0.025 g/l) was added to each flask. After a total of 6.5 days of incubation, the contents of the flasks were pooled and extracted as described earlier. The rapamycin was purified using the procedures described earlier, yielding approximately 9 to 10 mg. The enriched sample was then

analyzed by ^{13}C-NMR using the 360 MHz spectrometer.

The resulting NMR spectra are shown in Figure 59. (The sample was analyzed twice, the first spectrum being noisier than that shown, but having the same relative peak heights.) Visual inspection of the spectrum and comparison with the natural abundance and $^{13}C_2$-glycine spectra revealed three carbons that were significantly enriched and presumably related to the C_7 unit, namely carbons 41, "32", and 43. Surprisingly, C10, part of the unlabelled C_2 unit, also showed some incorporation. (Because the enrichment was low, the $^{13}C_2$-glycine spectrum was also used for comparison. The $^{13}C_2$-glycine sample was prepared in the same fashion as the ^{13}C-shikimate sample and should contain the same trace impurities which might affect peak heights. Some relative peak heights in the ^{13}C-shikimate sample were different in the natural abundance spectrum but were the same in the $^{13}C_2$-glycine spectrum. For example, the peak corresponding to carbon 13 was decreased in height in both the ^{13}C-shikimate and the $^{13}C_2$-glycine spectra relative to the natural abundance spectrum. Also, the double peak corresponding to carbons 44 and 45 was more resolved in the two ^{13}C-labelled samples than in the natural abundance samples, resulting in a shorter, broader peak in the ^{13}C-labelled samples.) Enrichment factors were also calculated for all of the carbons in the spectrum, compared to both the natural abundance spectrum and the $^{13}C_2$-glycine spectrum. The average height of six methylene carbon peaks was used as an internal standard to normalize each spectrum. The six peaks (carbons 16, 23, 8, 19, 17, and 18) correspond to carbons which were labelled by

Figure 59: Spectrum of rapamycin enriched with ^{13}C-shikimic acid.

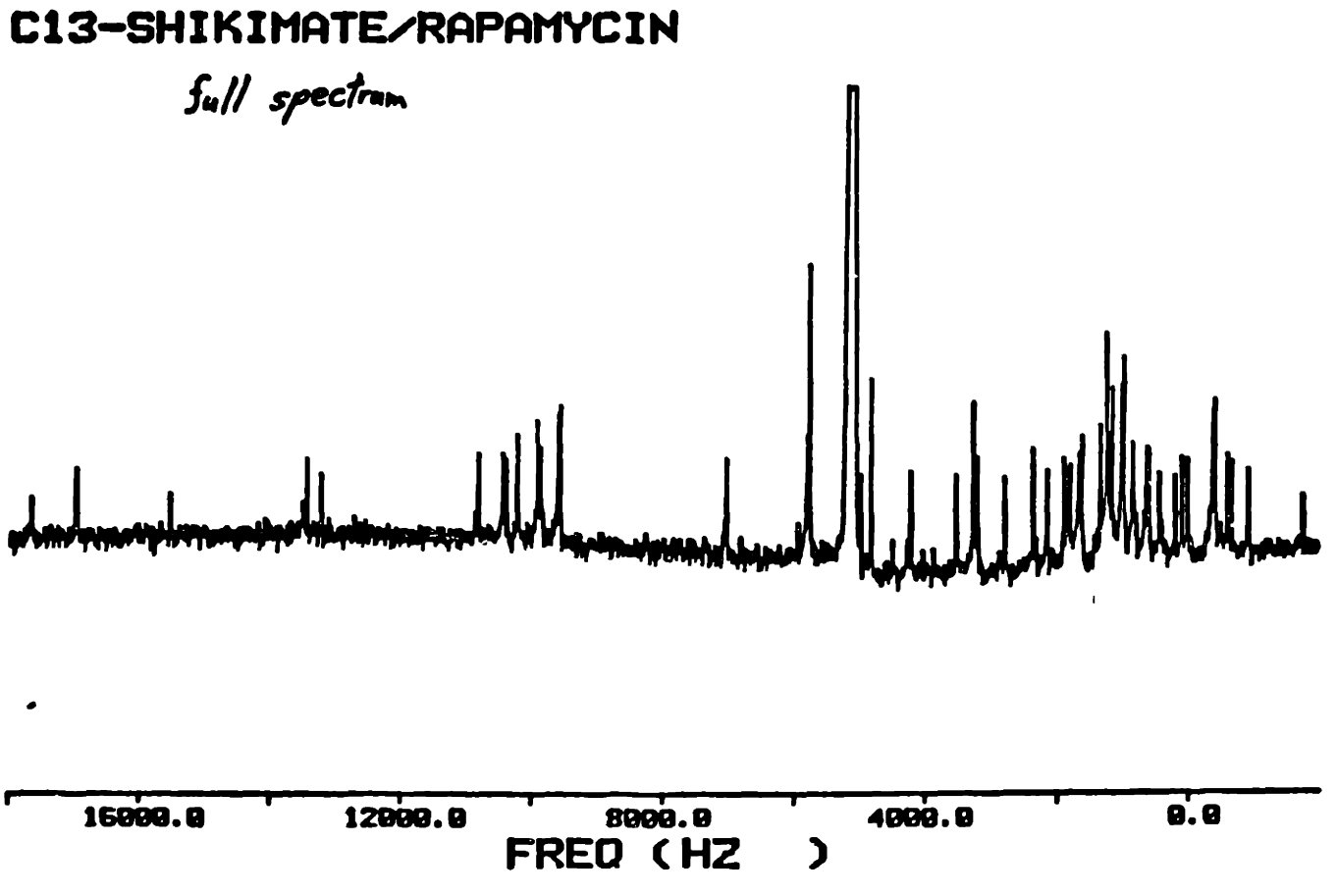


Figure 59 (continued):

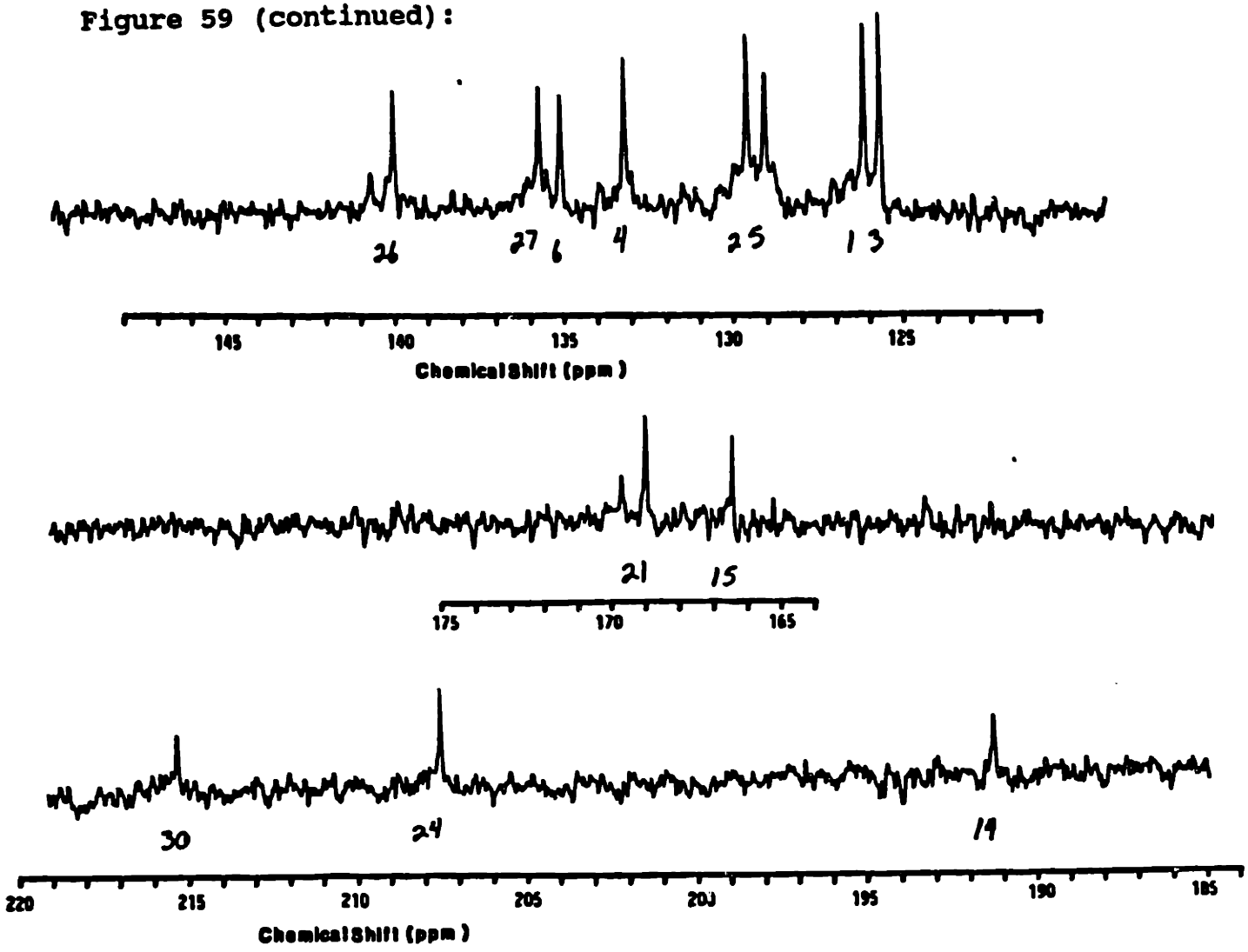


Figure 59 (continued):

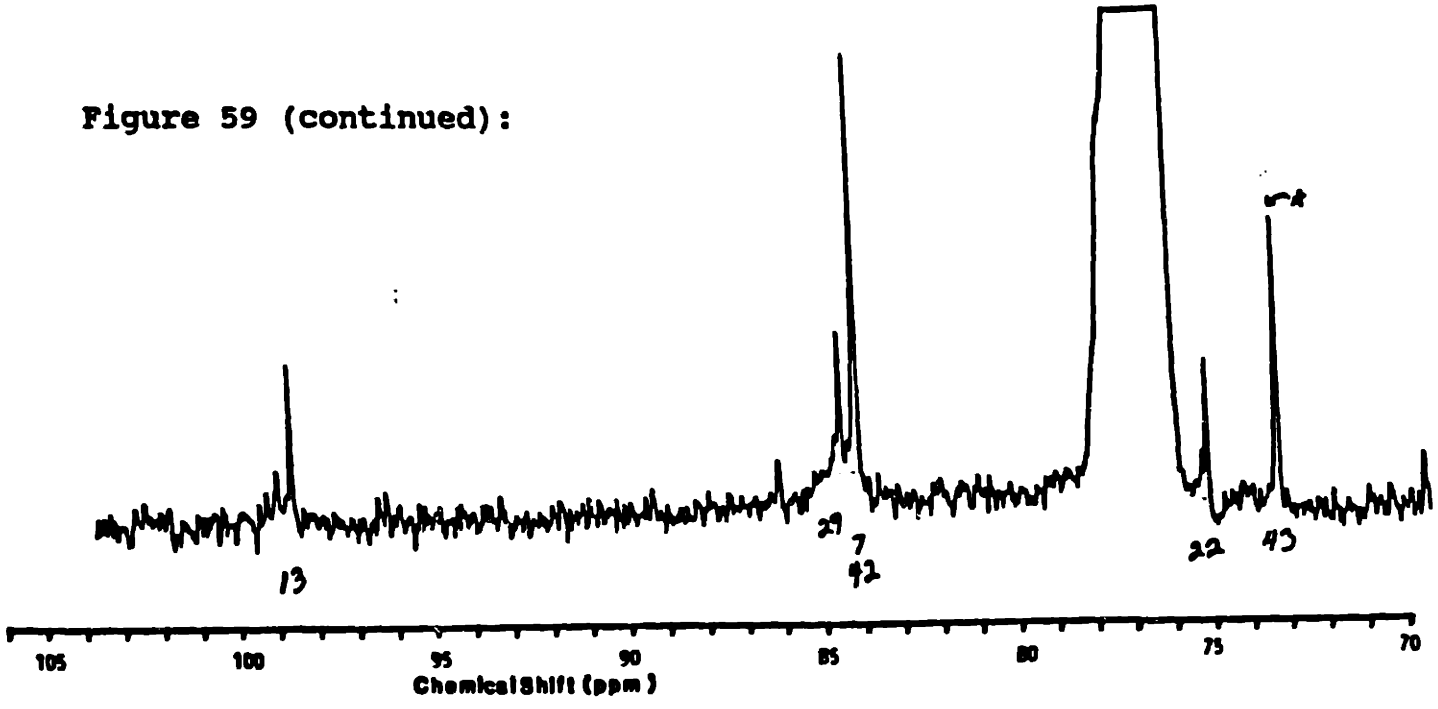
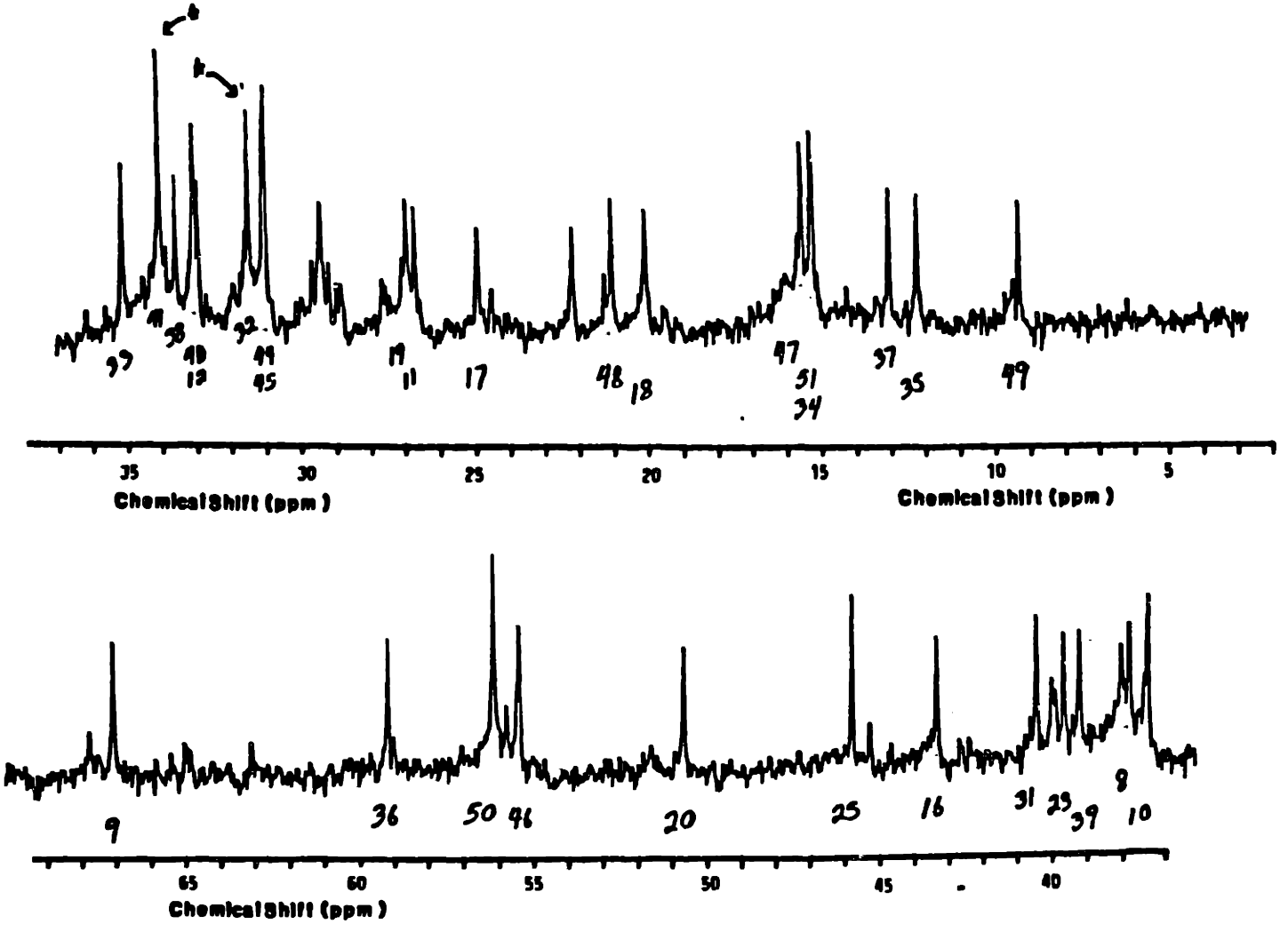


Figure 59 (continued):



^{13}C-acetate or were assigned to carbons of the pipecolic acid moiety, and should therefore not be labelled by ^{13}C-shikimic acid. For the calculations involving the glycine spectrum, the peak heights were measured two ways, first where a baseline was drawn based on the base noise immediately on either side of the peaks (A), and secondly where an average baseline was drawn for all the peaks in a given region (B). The former method will cause larger values to be calculated for the enrichment of peaks in crowded regions of the spectrum. All of these enrichment factors are shown in Table 12. In Table 13, selected data are grouped according to the type of carbon (methylene, methyne, or hydroxy and methoxy substituted), to account for any possible differences in the tuning of the spectrometer at the different times of analysis. It can be seen that among the methylene carbons, the peaks assigned to carbons 41 and 32 have increased in height at least 70 to 90%, and C10 at least 60%, according to the values calculated by method B. The peak corresponding to carbon 43 also appears to have increased in height 60% to 100% compared to similar carbons. (Method A gives higher values for the enrichments of carbons 32, 41, and 43, and approximately the same value for C10. By method A the enrichment of C10 seems questionable but by method B it appears much more significant in comparison to the enrichment of other carbons. Method B is probably the more reliable method for calculating the enrichments.)

It was shown earlier that the peak assigned to carbon 39 should actually be reassigned to carbon 32. Conversely, it is very likely that the peak assigned to carbon 32 should be reassigned to carbon 39. The

Table 12: Calculated relative enrichment factors for certain classes of rapamycin carbons.
A: Local baseline drawn for each peak.
B: Straight baseline drawn for entire spectrum.

Carbon # assigned	^{13}C-Shik / $^{13}C_2$-Gly		^{13}C-Shik / nat. abun.
	A	B	
30	0.66	.68	0.83
24	0.58	.62	1.85
14	0.69	.75	1.25
21	0.71	.75	1.40
15	0.50	.51	1.33
26	0.65	.62	0.99
27	0.60	.61	1.20
6	0.71	.73	1.50
4	0.77	.81	1.09
2	0.83	.89	1.21
5	0.67	.72	1.13
1	0.82	.85	1.26
3	0.85	.88	1.29
13	0.52	.51	2.12 ?
29	0.57	.66	0.81
7 and 42	0.84	.93	1.40
solvent	----		----
22	0.58	.54	0.92
43	1.02 *	1.02 *	1.49 *
9	0.60	.62	1.13
36	----	----	1.59
50	----	----	1.64
46	----	----	0.94
20	0.85	.86	1.02
25	0.59	.61	1.43
16	1.37	1.13	1.05
31	0.58	.59	1.52
23	0.82	.80	0.94

Table 12 (continued):

Carbon # assigned	^{13}C-Shik / $^{13}C_2$-Gly A	^{13}C-Shik / $^{13}C_2$-Gly B	^{13}C-Shik / nat. abun.
39 (32?)	1.00	.98	1.00
8	0.91	1.00	0.97
10	1.66 *	1.63 *	1.36
33	0.73	.71	1.56
41	2.64 *	1.89 *	1.72 *
38	0.81	.79	1.22
40 & 12	0.91 & 0.64	1.00 & 0.717	
		0.80 & .59	
32 (39?)	2.27 *	1.74 *	2.10 *
44 & 45 **	1.25 & 1.49''		0.78/0.92
		1.10 & 1.34''	
19	0.95	.96	1.13
11	0.95	.96	1.13
17	1.12	1.05	0.88
48	1.26	1.08	0.90
18	1.37	1.20	1.03
47	0.96	1.06	0.82
51 and 34	1.19	1.12	0.80
37	1.21	1.29	0.89
35	1.21	1.08	1.01
49	1.04	1.04	1.13

* = enriched carbon; see text for explanation.
** = unresolved in natural abundance spectrum, but partially resolved in rapamycin samples enriched with ^{13}C-shikimate and $^{13}C_2$-glycine.

Table 13: Calculated relative enrichment factors for certain classes of rapamycin carbons.
A: Local baseline drawn for each peak.
B: Straight baseline drawn for entire spectrum.

Carbon # assigned	^{13}C-Shikimate / $^{13}C_2$-Glycine	
	Methylene carbons	
	A	B
16	1.37	1.13
23	0.82	0.80
39 (32?)	1.00	0.98
8	0.91	1.00
10	1.66 *	1.63 *
41	2.64 *	1.89 *
32 (39?)	2.27 *	1.74 *
44 & 45**	1.25 & 1.49 u	1.10 & 1.34 u
19	0.95	0.96
11	0.95	0.96
17	1.12	1.05
18	1.37	1.20
	Methyne carbons	
40 & 12	0.91 & 0.64	0.80 & 0.59
25	0.59	0.61
38	0.81	0.79
31	0.58	0.59
33	0.73	0.71
	Single proton/oxygen bearing carbons	
9	0.60	0.62
29	0.57	0.66
7 & 42	0.84 u	0.93 u
22	0.58	0.54
43	1.02 *	1.02 *

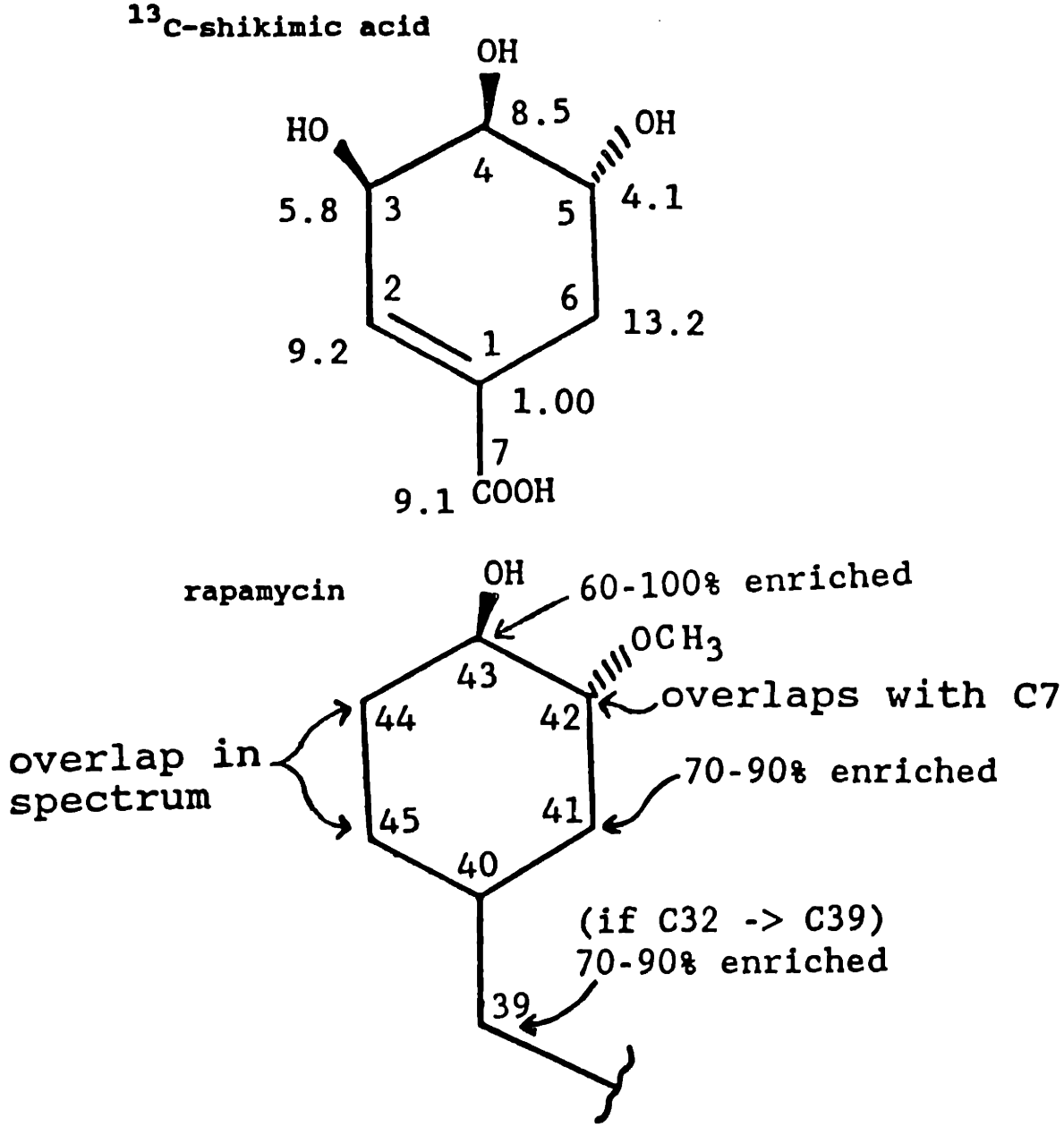
* = enriched carbon; see text for explanation.
** = unresolved in natural abundance spectrum, but partially resolved in rapamycin samples enriched with ^{13}C-shikimate and $^{13}C_2$-glycine.
u = uncertain enrichment; slight enrichment possible.

enrichment factors observed for the ^{13}C-shikimate spectrum are consistent with this reassignment, since there is no reason for C32 to be enriched by shikimic acid, while it is very likely that C39 should be enriched at about the same magnitude as C41 if the ^{13}C-shikimate was incorporated. Allowing for the exchange in the peak assignments of C32 and C39, the labelling pattern observed for the cyclohexane region of rapamycin is shown in Figure 60, alongside that found for the ^{13}C-shikimate. The levels of incorporation are consistent with those predicted by the experiments with ^{14}C-shikimate (see Results, Section O.).

Three of the four more intensely labelled carbons in the shikimic acid correspond to three of the carbons observed to be enriched in rapamycin. The fourth carbon labelled in the ^{13}C-shikimic acid would correspond to C45 in rapamycin, but it is difficult to say whether or not this carbon is enriched since the peaks assigned to carbons 44 and 45 overlap. This peak is broader and split at the top in the ^{13}C-shikimate and $^{13}C_2$-glycine spectra, and there appears to be some increase in the height on one side of the peak, but it is questionable whether or not this increase is significant.

These results indicate that ^{13}C-shikimate was successfully incorporated into the cyclohexane moiety of rapamycin, and that this moiety is derived from a shikimate pathway intermediate, perhaps shikimic acid itself. However, the data are not completely consistent with what should be expected. It is surprising that C10 should be labelled by ^{13}C-shikimate, although this could have occurred through

Figure 60: Comparison of the ^{13}C-enrichment patterns of ^{13}C-shikimic acid and the cyclohexane region of rapamycin.



scrambling reactions. It is also slightly surprising that there is no observable enrichment of C45, considering the level of enrichment observed for the other carbons. While these concerns have been answered above, there is still a possibility that there is still an error in the ^{13}C-NMR assignments which would account for these inconsistencies. This is discussed further in the next section.

V) Reassignments of the Chemical Shifts of Rapamycin: Reevaluation of Incorporation Data.

The results of several ^{13}C incorporation experiments suggested that there might be errors in the ^{13}C-NMR spectral assignments published by Findlay and Radics (1980). Incorporation of 1,2-$^{13}C_2$-acetate demonstrated that there was an error in the assignments of two or three olefinic carbons, but did not reveal what the correct assignments are. Incorporation of 1-^{13}C-propionate suggested that the NMR peak assigned to C39 should be reassigned to C32. Finally, the incorporation of ^{13}C-shikimic acid suggested that C39 and perhaps C10 were also misassigned, but again the correct assignments could not be absolutely determined from the available incorporation data. Findlay and Radics (1980) had established connectivities between certain carbons using selective ^{13}C-^{1}H decouplings, but none of the doubtful carbon assignments was supported by these decoupling experiments.

NMR instrumentation and data acquisition programs have evolved considerably since the time the original rapamycin assignments were made. There now exist commercially available instruments and programs which can map ^{13}C-^{1}H couplings for molecules even as complex as rapamycin. A sample (300 mg) of rapamycin was used to generate HECTOR (^{13}C-^{1}H heteronuclear 2D), COSY, and NOESY (^{1}H-^{1}H homonuclear 2D) maps of rapamycin in $CDCl_3$ using a Varian 400 spectrometer. The preliminary results for ^{13}C are summarized in Table 14. (Further experiments are necessary to check the assignments of the remaining carbons.)

All of the methyl carbons were reassigned. However, the seven

Table 14: Revised Methyl, Vinyl, and Methylene Carbon 13 Assignments in Rapamycin.

Carbon #	Chemical Shift (PPM)	Carbon Formerly Assigned to by Findlay and Radics
methyl:		
34	21.5	48
35	10.2	49
37	16.3	47
47	15.9	51
48	16.0	34
49	13.1	35
51	13.8	37
vinyl:		
1	140.1	26
2	130.2a	
3	133.6	4
4	126.4	3
5	129.6a	
26	126.7	1
methylene:		
16	44.2a	
23	40.7a	
32	40.2	39
8	38.9a	
39	38.3	10
41	34.2a	
45	31.7	32
44	31.3a	
10	31.3	45
19	27.3a	
11	27.1a	
17	25.3a	
18	20.6a	

a: Assignments agree with those of Findlay and Radics (1980).

methyl group assignments were simply interchanged amongst themselves. This does not affect the interpretation of the incorporation studies, since no attempt was made to label the methyl groups and no previously labelled carbon was reassigned as a methyl group. All methyl groups in rapamycin are believed to be derived from C3 of propionate. 2-^{13}C-Acetate labelled methyl groups, but apparently after the label was scrambled via the TCA cycle into propionate, and the reassignments have no effect on these conclusions.

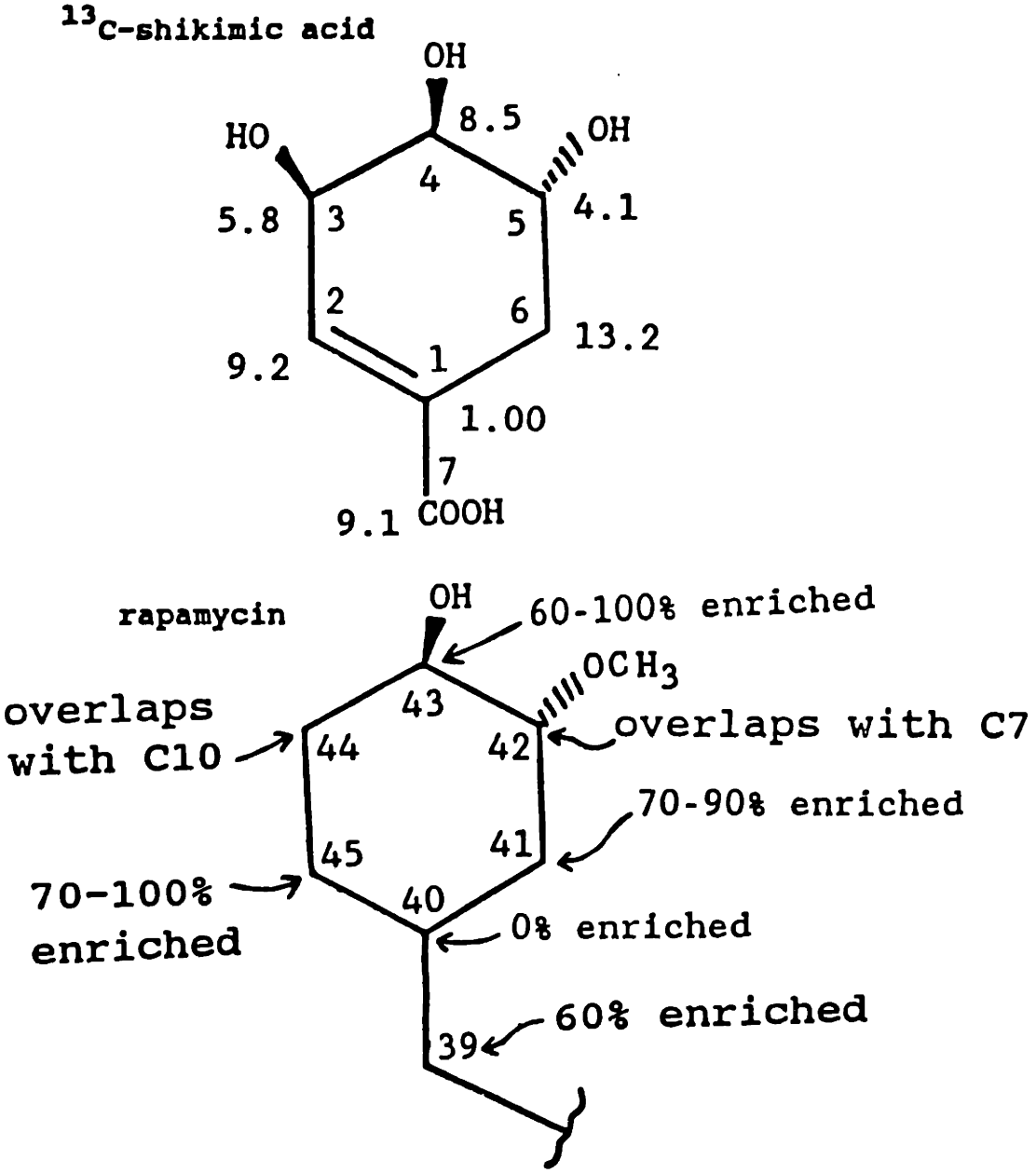
Four of the six vinyl carbons were reassigned. Assignments for C1 and C26 were interchanged, which is still in agreement with the labelling data, since both were labelled by 1-^{13}C-propionate, as predicted. The assignments for C3 and C4 were also interchanged. This is also consisitent with the incorporation data. This reassignment is one of the three possible reassignments as proposed in Figure 30, allowing for the incorporation of two intact acetate units into the triene region of rapamycin with the same orientation as the other acetate and propionate units.

Four of the 13 methylene carbons were reassigned. As had been suggested by the incorporation of 1-^{13}C-propionate, the peak assigned to C39 was reassigned to C32, thus confirming the conclusion that seven propionate units were incorporated into rapamycin. (Successful incorporation of 2-^{13}C-propionate into the seven predicted sites in rapamycin had independently confirmed that seven propionate units were incorporated into rapamycin, and that C32 had most likely been misassigned.)

The reassignments of the other three methylene carbons greatly strengthens the interpretation of the incorporation of ^{13}C-shikimate. The peak previously assigned to C10 was reassigned to C39, that assigned to C45 was reassigned to C10, and that assigned to C32 was reassigned to C45. Previously, during the interpretation of the spectrum of the ^{13}C-shikimate-enriched rapamycin sample, concerns had been raised about why the relative incorporation levels in the corresponding rapamycin carbons did not match the relative enrichments in the ^{13}C-shikimate. According to the published assignments, C45 showed little or no enrichment, while C39 and C41 showed close to a 100% enrichment. The lack of apparent enrichment of C45 was tentatively attributed to differences in peak width for the peak assigned to C44 and C45. Again according to the old assignments, C10 had been mysteriously enriched by ^{13}C-shikimate, although slightly less than the other enriched carbons. It was suggested that this might be due to scrambling of the labelled shikimate, but a possible misassignment was also considered. The new assignments alter the data for the incorporation of ^{13}C-shikimate as shown in Figure 61.

According to the new assignments, C41 was enriched approximately 90%, C45 70 to 100%, C43 80 to 100%, and C39 60% (after normalization with respect to carbon type). C40 overlaps with C12 and showed no increase, and C42 overlaps with C7 and showed perhaps some slight increase, both as expected. Thus the four most highly enriched carbons in ^{13}C-shikimate correspond to the four most highly enriched carbons in ^{13}C-shikimate-enriched rapamycin, and the relative enrichments

Figure 61: Comparison of the ^{13}C-enrichment patterns of ^{13}C-shikimic acid and the cyclohexane region of rapamycin.



correspond. The reassignments allow a straightforward conclusion that shikimic acid is the source of the C_7 starter unit of rapamycin. The new assignments imply that C10 is not signifcantly labelled by ^{13}C-shikimate. Any increase in the peak (partially resolved) corresponding to C44 and C10 is probably due to the enrichment of C44 by ^{13}C-shikimate.

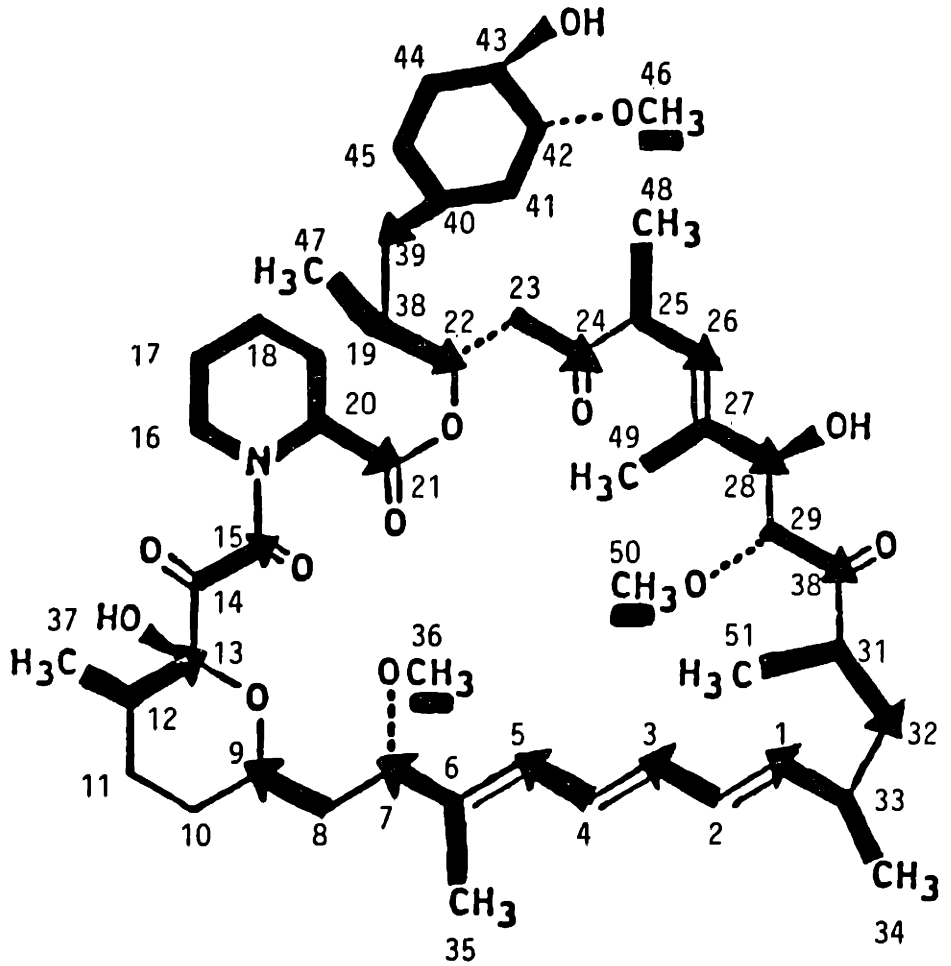
The reassignment of C10 to a different ^{13}C-NMR peak caused the reexamination of the previous incorporation data. The peak now reassigned to C10 and C44 had slightly increased in height in the spectrum of 2-^{13}C-acetate-enriched rapamycin, but did not show any of the characteristic satellite peaks in the spectrum of 1,2-$^{13}C_2$-acetate-enriched rapamycin. Also, the peak assigned to C11 did not increase in height significantly when 1-^{13}C-acetate was incorporated nor did it show satellite peaks when 1,2-$^{13}C_2$-acetate was incorporated. Thus the conclusion that C10 and C11 are not derived from acetate still holds. The spectrum of the rapamycin sample after incorporation of 2-^{13}C-propionate also shows no increase in the height of the peak now assigned to C10 and C44, and the spectrum after incorporation of 1,2-^{13}C-glycine shows no satellite peaks near the C10/C44 peak. Therefore the earlier conclusions about the source of C10 and C11 hold; namely, they are not derived from C1 and C2 of propionate and while they might be derived from glycollate, there is no evidence available to support this suggestion.

VI. SUMMARY AND CONCLUSIONS

The origins of 49 of the 51 carbons of rapamycin have been identified. These results are summarized in Figure 62. The findings match those of the predicted scheme quite well, except for the lack of acetate incorporation into C10 and C11.

The majority of the carbons in the lactone ring of rapamycin are derived from acetate and propionate, condensed in a "head to tail" fashion. Although the incorporation of singly-labelled forms of acetate suggested that some sort of strange rearrangement was occurring in the triene region of rapamycin, incorporation of doubly-labelled acetate proved that no rearrangement had taken place and that there must be an error in the published ^{13}C-NMR assignments (Findlay and Radics, 1980). This conclusion was later supported by the independent reassignments of the vinyl carbons of rapamycin, using ^{1}H-^{1}H and ^{13}C-^{1}H decoupling. I am now able to conclude that rapamycin contains six of the seven predicted acetate units in their predicted locations and orientations. The seventh predicted acetate unit was not labelled by any form of acetate, even when the new assignments were taken into account. Of note is the fact that the rare diketo functionality (C14 and C15) is indeed derived from acetate, while the high oxidation state of these two carbons and the adjacent carbon might have suggested a carbohydrate origin. The incorporation of 1-^{13}C-propionate indicated that six of the seven propionate groups were present as expected in rapamycin. A seventh peak was labelled, but not the one predicted, implying either a deviation from the expected biosynthetic pathway, or that another misassignment

Figure 62: Summary of the incorporation of labelled precursors into rapamycin.



: $CH_3CO_2^-$, ACETATE

: $CH_3CH_2CO_2^-$, PROPIONATE

$\underline{CH_3}$: METHYL GROUP FROM METHIONINE

: PIPECOLIC ACID

: SHIKIMIC ACID PATHWAY INTERMEDIATE ("STARTER UNIT")

had been made. Incorporation of 2-^{13}C-propionate into the seven sites predicted by the proposed biosynthetic scheme confirmed the hypothesis that an error had been made in the ^{13}C-NMR assignments, and that the peak assigned to C39 should be reassigned to C32. This was later confirmed by the independent reassignments of the ^{13}C-NMR spectra. Thus I conclude that rapamycin contains seven three-carbon units derived from propionate, in the locations and orientations that I originally proposed. Based on this incorporation of acetate and propionate, it appears that the biosynthesis of the majority of the lactone ring of rapamycin resembles that of many macrolides and polyenes, implying a polyketide pathway mechanism.

The three methoxy carbons were clearly labelled by ^{13}C-[methyl]-methionine. This is consistent with the proposed pathway, and the notion that these carbons are transferred to hydroxyl groups from methionine activated in the form of S-adenosyl-methionine. No methyl groups were labelled by ^{13}C-methionine, indirectly confirming that the seven methyl groups are derived from C3 of propionate.

Two carbons, C10 and C11, were not labelled by any precursor tested, and their origins remain unknown. Acetate and propionate have been eliminated as possible precursors. In an attempt to determine whether these two carbons are derived from glycollate (hydroxyacetic acid), 1,2-^{13}C-glycine was added to rapamycin-producing cultures to serve as a source of $^{13}C_2$-glycollate. The only carbons to be enriched by 1,2-^{13}C-glycine were the methoxy carbons, implying that the only observable mode of incorporation under these conditions was the

conversion of the methyl group of glycine to the methyl group of methionine, which was then incorporated into the methoxy groups of rapamycin. This does not eliminate glycollate as a possible precursor but does not support the hypothesis either. Possible ways to determine the origin of the unlabelled C_2 unit are discussed in the Suggestions for Future Work section (following). There are other examples of C_2 units of undetermined origin in the literature. While 1-^{13}C-glycine was found to label saframycin A, a heterocyclic quinone antibiotic synthesized by Streptomyces lavendulae, $^{13}C_2$-glycine failed to label the analogous carbons (9 and 9') of naphthyridomycin, a structurally similar antibiotic produced by Streptomyces lusitanus (Mikami et al., 1985; Zmijewski et al., 1982). For years the origin of a two carbon unit in leucomycin went undetermined, despite the fact that analogous carbons in magnamycin were readily labelled by acetate. Finally the carbons were labelled by glycerol, presumably after being converted to glycerate or glycollate (Omura et al., 1975; Omura et al., 1983).

Studies with radioactive lysine and pipecolate showed that both were readily incorporated into rapamycin. In competition studies, pipecolic acid was more effective in decreasing radioactive lysine incorporation than was lysine at decreasing radioactive pipecolate incorporation. A method was developed to separate the pipecolate acid moiety from the rest of the rapamycin molecule, thus allowing the determination of the level of incorporation of radiactivity into the pipecolate moiety in addition to the total incorporation into rapamycin. In comparing the incorporation profiles of rapamycin labelled with

radioactive acetate, lysine, and pipecolate, it was apparent that lysine and pipecolate were incorporated into the pipecolate moeity more specifically than acetate, implying that lysine and pipecolate were not simply incorporated through scrambling. Taken together, these data show that the pipecolate moiety is derived from lysine and pipecolate, and that pipecolate is the closer precusor. In other words, pipecolate is incorporated directly into rapamycin, and that lysine is incorporated after first being cyclized to pipecolate. Alternative origins for this moiety including the direct incorporation of lysine followed by cyclization, or the incorporation of a lysine precursor have been eliminated by these results.

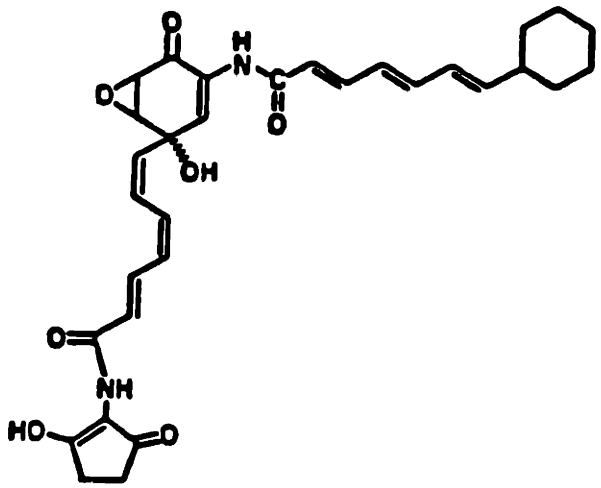
^{13}C-Shikimic acid was prepared from 1-^{13}C-glucose and, after characterization, was successfully incorporated into the cyclohexane moiety of rapamycin. Initially, the incorporation data were somewhat confusing, since the relative enrichments of the shikimate carbons were slightly different from the corresponding shikimate-labelled rapamycin carbons, and C10 was also possibly enriched. However, when the ^{13}C-NMR reassignments became available, the peak assigned to C10 was reassigned to C39, and three other carbons were also reassigned, allowing a much better match of the relative enrichments. I therefore conclude that the seven-carbon starter unit of rapamycin arises from an intermediate of the shikimic acid pathway, perhaps shikimic acid itself. This intermediate could be incorporated directly, or modified before incorporation. To my knowledge this is the first instance of ^{13}C-shikimate being used to establish the origin of such a biosynthetic

unit. It is also one of the first times that the origin of a saturated cyclohexane ring such as the one in rapamycin has been conclusively determined.

The nearly symmetrical labelling of the ^{13}C-shikimate does not allow an absolute determination of the orientation of this unit when incorporated into rapamycin. However, as discussed in the Literature Survey, based on the relative stereochemistry of the hydroxy and methoxy substituents it is most likely that carbons 1, 2, 3, 4, 5, 6, and 7 of shikimic acid correspond to rapamycin carbons 40, 45, 44, 43, 42, 41, and 39, respectively. In ^{13}C-shikimate, the enrichment of C6 is slightly higher than that of C2, and in rapamycin the enrichment of C41 appears slightly higher than C45, supporting this hypothesis.

As was discussed in the Literature Survey, saturated cyclohexane rings are rare among known natural products, especially those linked through carbon-carbon bonds to the rest of a molecule. Recently there was a preliminary report that "radioactive precursors" were used to establish the shikimate origin of the cyclohexane moiety of asukamycin (Floss et al., 1986) (Figure 63). (In this paper, no data are given, but the authors claim to have shown that shikimic acid is converted into cyclohexane carboxylic acid, which is then incorporated into the cyclohexane ring and adjacent carbon atom of asukamycin. It is likely that these authors were able to identify the sites of incorporation fairly easily, since the seven carbons of interest can be separated from the rest of the molecule by cleaving a double bond.) The only other example which I could find of terminal cyclohexane rings whose

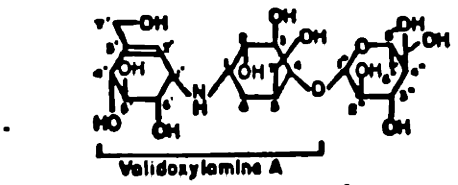
Figure 63: Structure of asukamycin.



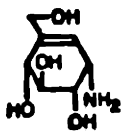
biosynthetic origins are known is the fatty acids of acidophilic-thermophilic bacteria. The fatty acids of one such species, Bacillus acidocaldarius, contain as much as 95% omega-cyclohexyl fatty acids (11-cyclohexyl undecanoate and 13-cyclohexyl tridecanoate) (Oshima et al., 1978). The cyclohexyl ring was also found to be derived from shikimate via cyclohexane carboxylic acid. In contrast to these two metabolites, the cyclohexane ring of rapamycin retains two oxygen substituents in the same stereochemical relationship as in the original shikimic acid, and thus does not appear to be converted all the way to cyclohexane carboxylic acid before incorporation.

The incorporation of ^{13}C-shikimate into rapamycin is also significant in light of the results recently published for other "cyclohexane carboxylic acid" type antibiotics. Valienamine is a component of both validamycins and acarbose (see Figure 64), and other (hydroxymethyl)cyclitols, including validamine, have been found in related antibiotics. When work on rapamycin was begun, it was proposed by several authors that these C_7 units were derived from the shikimic acid pathway or from 3-amino-5-hydroxybenzoic acid (known to be a product of the shikimic acid pathway and a precursor of several antibiotics) (Herlt et al., 1981; Rinehart et al., 1982). Studies on the biosynthesis of validamycins have now shown that both validamine and valienamine are not derived from the shikimate pathway, but instead from a seven-carbon sugar related to the pentose phosphate pathway (Toyokuni, et al., 1987). Independently, studies on acarbose provided almost identical results for the biosynthesis of valienamine in this antibiotic

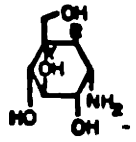
Figure 64: Structures of validamycin A, related (hydroxymethyl)cyclitols, and acarbose.



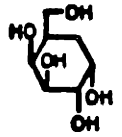
Validamycin A



Valienamine

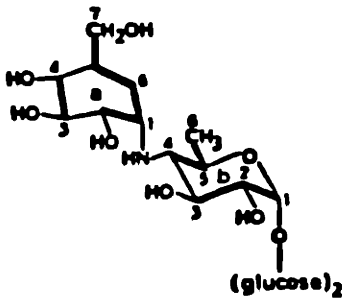


Validamine: R=R'=H
Hydroxy-validamine: R=OH, R'=H
Valiolamine: R=H, R'=OH



Pseudo-α-galactose

acarbose

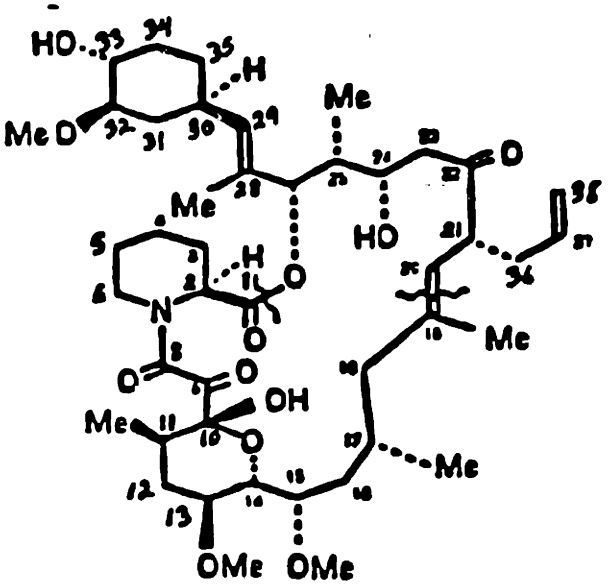


(Degwert et al., 1987). In both cases the C_7 unit is formed from a three-carbon unit (derived from glycerol or glyceraldehyde-3-phosphate) to which two C_2 units are successively added, via transketolase reactions. These conclusions are based on the observed ^{13}C-^{13}C coupling patterns after incorporation of D-$^{13}C_6$-glucose and $^{13}C_3$-glycerol, as well as the incorporation of singly-labelled precursors. The uniformly labelled substrates gave a $C_3 + C_2 + C_2$ pattern, rather than the $C_3 + C_4$ pattern characteristic of the shikimic acid pathway. These results are consistent with earlier observations that ^{14}C-shikimate, glycerate, and glycollate were not readily incorporated into validamycin.

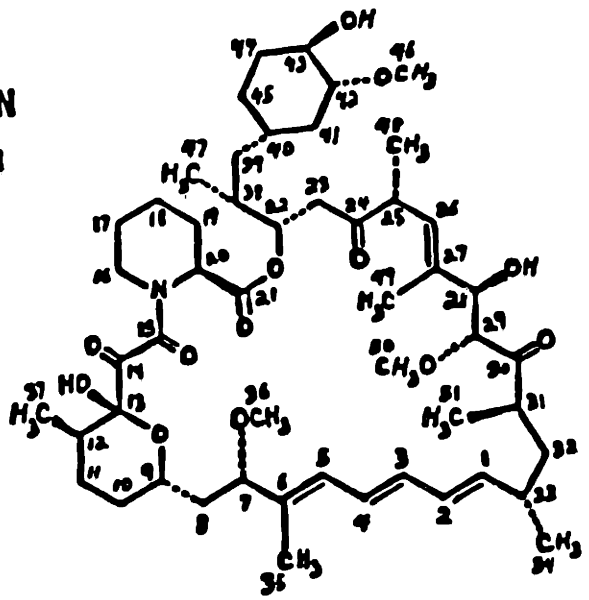
As stated in the Introduction, one benefit of biosynthetic studies is that often information gained about one natural product is often applicable to structurally similar compounds. At the time this work was begun, rapamycin was a unique combination of some rare structural features, including a saturated cyclohexane moiety linked to the molecule through a C-C bond, a diketo group forming a peptide bond to a pipecolic acid molecule, and a triene region. (Demethoxyrapamycin had been isolated from the same culture but was clearly biosynthetically related to rapamycin.) Within the last few months a surprisingly similar antibiotic, FK506, has been isolated from Streptomyces tsukubaensis (Tanaka et al., 1987). Approximately one half of this molecule is nearly identical to a large portion of rapamycin, as shown in Figure 65. The similarities include the substitutions and stereochemistry of their substituted cyclohexane rings, the locations of the L-pipecolic acid and diketo units, and the presence of a

Figure 65: Structures of FK506 and rapamycin.

FK506
$C_{44}H_{69}NO_{12}$



RAPAMYCIN
$C_{51}H_{79}O_{13}N$



hemiketal ring. They differ in the fact that FK506 has a double bond between what corresponds to C39 and C38 of rapamycin (derived from the carboxyl group of shikimic acid and C2 of a propionate unit), that there is a methoxy group on the hemiketal ring of FK506 on what corresponds to C10 of rapamycin, and in the remainder of their lactone rings. It is likely that the shared structural features are derived from the same biosynthetic precursors. The biosynthetic information described herein could reduce the number of experiments necessary to determine the biosynthetic origins of FK506. Also, information on the origins of C13 and C12 of FK506 could also indicate the origins of the corresponding C10 and C11 of rapamycin. The presence of the methoxy group on C13 of FK506 could be taken as support of the idea that C10 and C11 of rapamycin are derived from an oxidized precursor like glycollate or glycerate. However, as was previously noted for the case of leucomycin and magnamycin and that of nigrafactin and pipecolic acid, structural similarities do not guarantee similar biosynthetic origins.

Rapamycin was originally isolated as an antifungal agent, but no antifungal or antibacterial activity is reported as having been determined for FK506. FK506 is reported as a "novel" immunosupressant agent, demonstrating much greater activity than cyclosporin A, one of the main immunosuppressant drugs in use. Rapamycin was also reported to have immunosuppressant activity, about equal to that of cyclophosphamide and lower than that of methotrexate, but was not compared to cyclosporin. In terms of structure/function relationships it would be interesting to compare directly rapamycin and FK506 in their antifungal,

antitumor, and immunosuppressant activity.

All precursors were tested for effects on rapamycin production. For the media used, no precursor caused an increase in production, implying that none of those tested are rate-limiting for the biosynthesis. Methionine was found to decrease rapamycin production, especially when added early in the fermentation. While it was initially surprising that addition of a precursor would be detrimental, methionine has been found to have the same effect on several other antibiotics for which it is a precursor, possibly by inhibiting or repressing the enzyme which activates methionine by converting it to S-adenosyl-methionine. Ethionine, a methionine analog, was found to be even more inhibitory. Addition of ammonium or phosphate ions to the medium had no effect on the production of rapamycin, even though these are often detrimental to the production of other antibiotics. Lowering the phosphate concentration to growth-limiting levels only slightly increased rapamycin levels. It is possible that rapamycin is not regulated by these nutrients, or that some other component of the medium is suppressing the production, or that some required factor is missing from the medium. While the medium developed herein was sufficient for the biosynthetic work, development of a completely defined medium or a medium allowing higher production might make future studies easier.

VII. Suggestions for Future Research

The origins of almost all of the carbons in rapamycin have been identified. Labelling experiments have shown that my initially proposed scheme is correct for the most part, the only exception being that carbons 10 and 11 are not derived from acetate. Additional experiments are necessary to determine the origins of these two carbons. Other experiments are suggested to answer other important questions regarding rapamycin biosynthesis.

Carbons 10 and 11 are not derived from acetate as had been predicted in the proposed scheme, and their origins remain unknown. They are also not derived from propionate or a branched C_5 acid as had been suggested after the failure of acetate to enrich these carbons, nor are they derived from methionine. $^{13}C_2$-Glycine failed to label these carbons, but this does not eliminate the possibility that these carbons are derived from glycine or the closely related two-carbon unit, glycollate. From the high enrichment of the methoxy groups of rapamycin, it is apparent that the glycine was entering the cells but was readily converted to methionine. Perhaps the conversion of glycine to methionine occurs at too great a rate to allow utilization of glycine for rapamycin biosynthesis. One way to possibly determine the origins of C10 and C11 is to repeat the above feeding experiment with $^{13}C_2$-glycine, but this time add low levels of methionine to the medium at the same time the labelled glycine is added. The presence of methionine may suppress the conversion of glycine to methionine, thereby making glycine available to other pathways, including the conversion of

glycine to glycollate and/or glycerate and the possible incorporation into rapamycin.

Another possible approach to identifying the source of C10 and C11 would be to feed ^{13}C-labelled glycollate or glycerate directly to the cultures. ^{13}C-Labelled glycollate and glycerate are not available commercially. However, 1-^{13}C-glycerate can be easily synthesized from glycoaldehyde and Na^{13}CN (Ashworth, 1966). With the published 70% yield (based on NaCN), one should be able to synthesize approximately 2 grams of D,L-1-^{13}C-glycerate from 1 g of NaCN (currently $175/g). 1-^{13}C-Glycollate can be formed from 1-^{13}C-glycerate by permanganate oxidation. White and Martinelli (1974) demonstrated the shikimate origin of the starter unit of rifamycin by the successful incorporation of 1-^{13}C-glycerate. Similar results were obtained by Haber et al. (1977) (Figure 6) in studies on geldanamycin biosynthesis. Glycerate did not, however, seem to enter the Streptomyces producer of leucomycin (Omura et al., 1983). It is unknown whether glycollate or glycerate can enter the cells of the rapamycin producer and how much the label would be diluted by the presence of unlabelled glycollate or glycerate in the cells. ^{14}C-Glycerate and ^{14}C-glycollate are also not commercially available and would have to be synthesized before preliminary radioactive experiments could be performed. ^{13}C-Labelled glycollate and glycerate may also label certain carbons of the shikimate-derived starter unit of rapamycin, as has been found in other studies on shikimate-derived products. Such incorporation could serve as a measure of how well the labelled compound is utilized by the cells. For

example, if the shikimate moiety is labelled by glycerate and carbons 10 and 11 are not, then one can conclude that these two carbons are not derived from glycerate and that the lack of incorporation was not simply due to the failure of the labelled compound to enter the cells.

Other compounds which might label C10 and C11 include glucose and glycerol, although these would also be expected to label many of the other carbons of rapamycin as well and also undergo scrambling reactions. Use of uniformly labelled glucose or glycerol ($^{13}C_6$-glucose or $^{13}C_3$-glycerol) may show the incorporation of an intact C_2 unit. Singly labelled sugars may preferentially label C10 or C11, yielding clues as to their origins. Carbons 10 and 11 could also be derived from C1 and C2 of betahydroxypropionate, by a mechanism similar to that proposed for the labelling of C_2 units by propionate (see Results, Section V. F.). Betahydroxypropionate is known to be a product of glycerol metabolism in some organisms (Sobolov and Smiley, 1960; Slinginger et al., 1983) and has recently been found to be a precursor of clavulanic acid in Streptomyces clavuligerus (Gutman et al., 1985). If the betahydroxypropionate is incorporated, the presence of the hydroxyl group would prime C3 for oxidative removal.

The incorporation of ^{13}C-shikimic acid into rapamycin clearly indicates that the C_7 starter unit is derived from the shikimic acid pathway. However, it is unknown where rapamycin biosynthesis branches away from the shikimic acid pathway. It is also unknown whether shikimic acid is incorporated directly into rapamycin and then modified, or modified first and then incorporated. A stereochemically-specific

synthesis of the hydroxy- and methoxy-substituted acid corresponding to the C_7 starter unit moiety of rapamycin has recently been published (Tanaka et al., 1987) and could possibly be used to synthesize labelled derivatives of hypothetical rapamycin precursors. These could then be checked for incorporation into the starter unit region of rapamycin. Successful incorporation of these derivatives would demonstrate that shikimic acid is modified before incorporation into rapamycin. If the derivatives are not incorporated readily, it may be worthwhile to retest the derivatives after conversion to their thioesters. Two independent studies on erythromycin and tylosin have recently shown that intact incorporation of chain-elongation intermediates required conversion to the N-acetyl-cysteamine thioesters (Cane and Yang, 1987; Yue et al., 1987). The units incorporated in these studies were six- or nine-carbon branched acids which correspond to the first two or three propionate units of these macrolides, condensed and modified to match their final oxidation states and stereochemistries. A series of mutants of the rapamycin-producer blocked in the shikimic acid pathway should identify where the rapamycin pathway branches away from the shikimic acid pathway. Also, they may help in the determination of the route from the shikimic acid pathway to rapamycin. For example, mutants unable to synthesize any products of the shikimate pathway would only be able to synthesize rapamycin when correct precursors of the C_7 moiety are added to the fermentation. These mutants might also incorporate the above-mentioned labelled cyclohexane derivatives to a higher degree than the wild type because they would not contain endogeneous precursors to

dilute the label.

Studies with FK506 might provide information on both the missing C_2 unit and on the above questions regarding the C_7 starter unit (see Summary and Conclusions). Especially in the case of studies involving labelled cyclohexane derivatives, FK506 may prove easier to work with. The presence of the double bond between what corresponds to the C_7 starter unit and the first propionate unit provides a simple means of separating the C_7 unit from the rest of the molecule. Potential precursors could be synthesized, labelled with ^3H or ^{14}C, and tested for specific incorporation into the cyclohexane moiety. Those which showed the most incorporation could be retested with the corresponding ^{13}C-labelled derivative.

It was found that pipecolate is incorporated directly into rapamycin. It would be interesting to see if new rapamycin derivatives could be produced by feeding compounds similar to pipecolate ("directed biosynthesis"). Likely candidates for incorporation include nipecotic acid (a structural isomer), hydroxypipecolate, picolinate (the aromatic analog), and proline. These compounds are either available radioactively labelled or could be produced fairly easily. Incorporation could be monitored using the procedure developed for the studies on incorporation of ^{14}C-lysine and ^3H-pipecolate. Such rapamycin derivatives could have improved or altered antifungal, antitumor, or immunosuppressant activity and thus could provide clues as to which moieties are important for the different activities. Results in the rapamycin system could also be applicable to FK506.

Methionine and ethionine greatly decreased rapamycin production. One way to eliminate this effect and perhaps increase rapamycin production would be to obtain mutants of the producer resistant to ethionine. Large-scale production of rapamycin would probably involve the use of complex media, but cheap protein hydrolysates could contain inhibitory levels of methionine.

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