STUDIES IN THE BIOSYNTHESIS OF VIRGINIAMYCIN S_1

by

Josephine W. Reed

Dissertation submitted to the faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

CHEMISTRY

APPROVED:

David G. I. Kingston, Chairman

Harold M. Bell

Milos Hudlicky

Norman G. Lewis

Robert H. White

February, 1988

Blacksburg, Virginia

STUDIES IN THE BIOSYNTHESIS OF VIRGINIAMYCIN S_1

by

Josephine W. Reed

Committee Chairman: David G.I. Kingston
Chemistry

(ABSTRACT)

Some aspects of the biosynthesis of three amino acid residues in virginiamycin S_1 have been studied in *Streptomyces virginiae* by the incorporation of amino acid precursors labeled with either radioactive or stable isotopes.

L-Lysine-*U*-^{14}C was incorporated into both the 4-oxo-L-pipecolic acid and 3-hydroxypicolinic acid residues. The formation of the heterocyclic ring of both of these amino acids was shown to occur with retention of the nitrogen from the ε-amino group of lysine, as shown by the incorporation of DL-lysine-*6*-^{13}C-*6*-^{15}N. In addition, the 3-hydroxypicolinic acid residue incorporated deuterium from (2*RS*, 5*R*)-lysine-*5*-d_1, but not from (2*RS*, 5*S*)-lysine-*5*-d_1. This finding indicates that the 5-*pro*-(*R*) hydrogen of L-lysine is retained during the biogenesis of 3-hydroxypicolinic acid.

In the conversion of L-phenylalanine to L-phenylglycine, the amino group moves to the benzylic position. This process could proceed either by an intermolecular mechanism, in which the original nitrogen is lost, or by an intramolecular mechanism, in which that nitrogen is retained. Administration of (*RS*)-phenylalanine-*3*-^{13}C-^{15}N resulted in its incorporation with loss of the labeled nitrogen. The process therefore occurs by an intermolecular mechanism.

ACKNOWLEDGMENTS

Thanks are due to all of those who have helped me achieve this goal, with special thanks to:

my research director ;

the other members of my committee— , , ,

and ;

SmithKline Animal Health Products, for their financial support of my research;

and at SmithKline–RIT, Rixensart, Belgium, for performing several of fermentations;

my parents, for their constant encouragement—and for letting me move in with them at the age of 27 so that I could go back to school;

, for teaching me to be still and see;

, for teaching me to love organic chemistry;

and and their son , for giving me perspective during my graduate studies;

, for showing me the ropes in the lab;

my good friends , , and , who went through all this with me;

, for all the helpful conversations we had;

, for the lessons in focus and self-confidence;

and , most special of all.

TABLE OF CONTENTS

LIST OF FIGURES

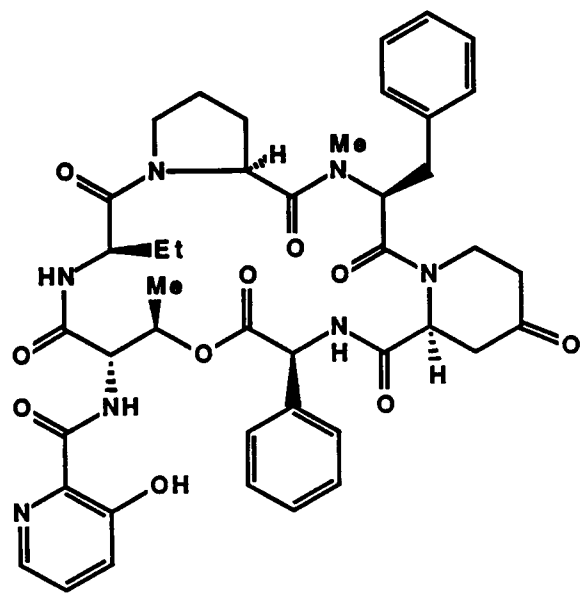
LIST OF SCHEMES

LIST OF TABLES

1. INTRODUCTION

1.1. Purpose

Virginiamycin S_1 (1) is a cyclic peptidolactone antibiotic produced by the soil bacterium *Streptomyces virginiae*. Several of its component amino acids are quite unusual, and their biosynthetic origins are unknown or incompletely understood. It is the aim of this project to determine the origin of the 4-oxo-L-pipecolic acid moiety and to investigate some of the mechanistic aspects of the biosynthesis of the L-phenylglycine and 3-hydroxypicolinic acid residues.



1

1.2. Review of the Literature

Virginiamycin S_1 (**1**) is a member of the virginiamycin family of antibiotics, which is made up of two major types of compounds, type A and type B. Together they have the unique property of exhibiting a synergistic inhibitory action on sensitive organisms. Because of this feature, antibiotics of this family are often referred to as "synergimycins."[1] A number of these antibiotics have been isolated from various sources, almost always as mixtures of A and B components (Table 1); patricins A and B (Figure 2) are chemically synthesized.[21]

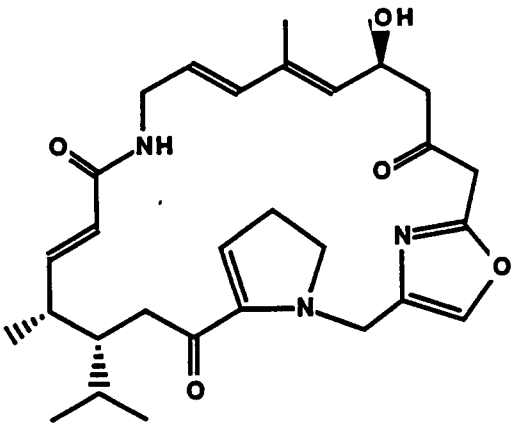
The type A compounds are polyunsaturated cyclic peptolides having a molecular weight of about 500. Characteristic of their structure is a substituted aminodecanoic acid and an unusual oxazole system. Six compounds of this group have been isolated. Their structures are shown in Figure 1, along with different names that have been assigned these structures by various investigators.

The type B antibiotics are cyclic peptidolactones with a molecular weight of about 800. Most of the organisms that produce the virginiamycins provide a mixture of type B components having similar structures. This group of antibiotics is divided into two subgroups: group B-I (Figure 2), which includes those antibiotics (such as virginiamycin S_1) that contain seven amino acid residues, and group B-II (Figure 3), which includes those (such as etamycin) that contain eight amino acid residues.

When virginiamycin was first isolated by De Somer and Van Dijck in 1955 from the culture broth of a bacterial species isolated from a Belgian soil sample thought to be *Streptomyces virginiae*,[51] it was shown to be a mixture of four biologically active components. Infrared spectroscopy suggested that it was similar to streptogramin, which had been isolated in 1953 by Charney et al.[6] By 1957, the individual components had been isolated in pure form and their properties identified.[52] One of these components was

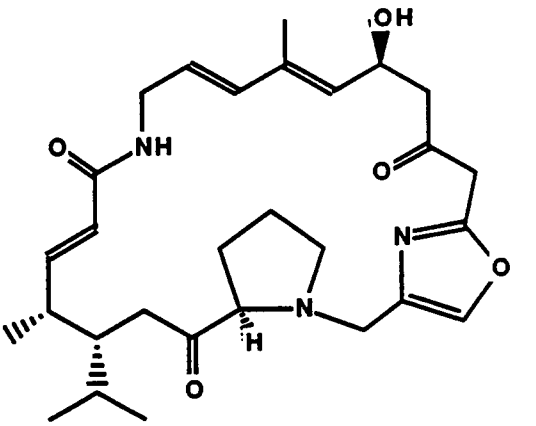
Table 1. Organisms producing the virginiamycin family of antibiotics[2]

Microorganism	Product	Reference
Streptomyces virginiae	virginiamycins (staphylomycins)	3
S. alborectus	virginiamycins	4
S. loidensis	vernamycins	5
S. graminofaciens	streptogramins	6
S. ostreogriseus	ostreogrycins	7
S. mitakaensis	mikamycins	8
S. pristinae	pristinamycins	9
S. olivaceous	PA114A, B (synergistins)	10
S. conganensis	F1370A, B	11
S. griseus *S. griseoviridus*	viridogrisein (etamycin) griseoviridin	12
S. griseoviridus P8648	neoviridogriseins griseoviridin	13
Streptomyces sp.	etamycin	14
Actinoplanes philippinensis	A-2315 A, B, C	15
A. auranticolor	plauricins	16
Actinoplanes sp.	A17002A, B, C, F	17
Actinomadura flava	madumycins	18
Micromonospora sp.	vernamycins	19
Actinomyces daghestanicus	antibiotic 6613 (etamycin)	20



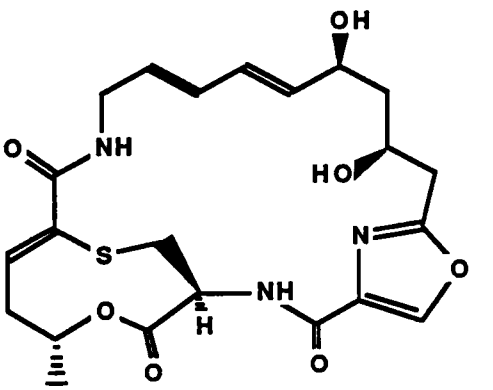
Antibiotic (Ref.)

Virginiamycin M_1 (22)
Vernamycin A (5)
Streptogramin A (23)
Ostreogrycin A (24)
Mikamycin A (25)
Pristinamycin IIA (26)
Synergistin A-I (10)



Antibiotic (Ref.)

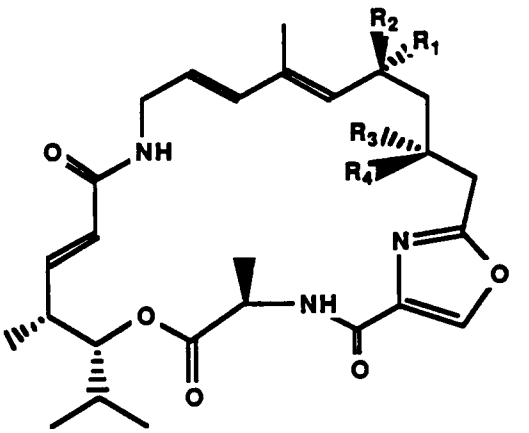
Virginiamycin M_2 (22)
Ostreogrycin G (27)
Pristinamycin IIB (26)



Antibiotic (Ref.)

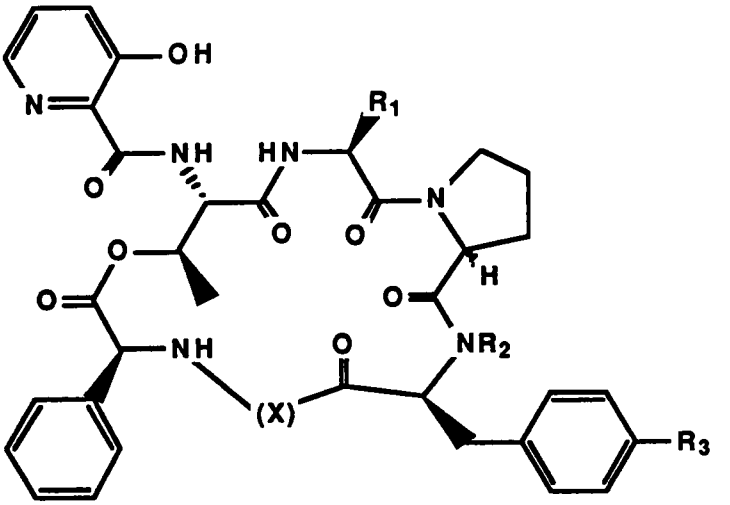
Griseoviridin (28, 29)

Figure 1. Chemical structure of virginiamycin group A antibiotics



Antibiotic (Ref.)	
A-2315A (30) CP35763 (31) Madumycin II (18) A17002F (17)	$R_1 = R_3 = H,\ R_2 = R_4 = OH$
A-2315B (30) CP36926 (31) Madumycin I (18)	$R_1 = H,\ R_2 = OH,\ R_3R_4 = O$
A17002C (17)	$R_1 = R_2 = R_3 = H,\ R_4 = OH$

Figure 1. Continued

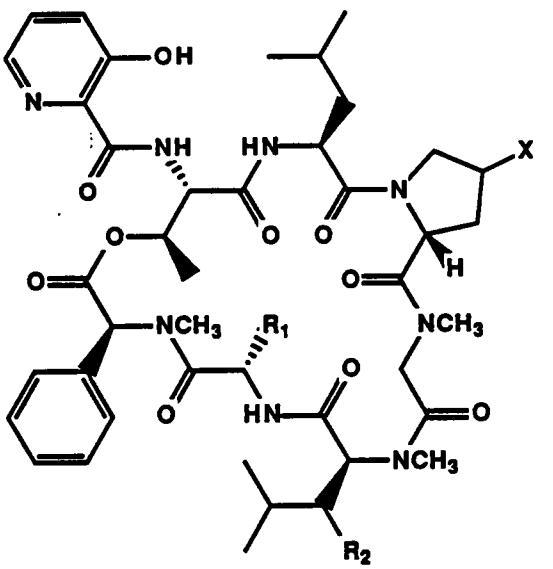


ANTIBIOTIC (REF)	R1	R2	R3	X (other substitutions)
Virginiamycin S_1 (32, 33)	C_2H_5	CH_3	H	4-oxopipecolic acid
Virginiamycin S_2 (34)	C_2H_5	H	H	4-hydroxypipecolic acid
Virginiamycin S_3 (34)	C_2H_5	CH_3	H	5-hydroxy-4-oxopipecolic acid
Virginiamycin S_4 (34)	CH_3	CH_3	H	4-oxopipecolic acid
Virginiamycin S_5 (35)	C_2H_5	CH_3	H	*allo* -4-hydroxypipecolic acid (alanine not phenylglycine)
Streptogramin B (36) Mikamycin IA (37) PA114B1 (38) Pristinamycin IA (26) Vernamycin Bα (39) Ostreogrycin B (40)	C_2H_5	CH_3	$N(CH_3)_2$	4-oxopipecolic acid
Pristinamycin IC (26) Vernamycin Bγ (39) Ostreogrycin B_1 (40)	CH_3	CH_3	$N(CH_3)_2$	4-oxopipecolic acid

Figure 2. Chemical structure of virginiamycin group B-I antibiotics

ANTIBIOTIC (REF)	R1	R2	R3	X (other substitutions)
Pristinamycin IB (26) Vernamycin Bβ (39) Ostreogrycin B_2 (40)	C_2H_5	CH_3	$NHCH_3$	4-oxopipecolic acid
Vernamycin Bδ (39)	CH_3	CH_3	$NHCH_3$	4-oxopipecolic acid
Ostreogrycin B_3 (41)	C_2H_5	CH_3	$N(CH_3)_2$	5-hydroxy-4-oxopipecolic acid
Vernamycin C (42) Doricin	C_2H_5	CH_3	$N(CH_3)_2$	aspartic acid
Patricin A (21)	C_2H_5	CH_3	H	proline
Patricin B (21)	C_2H_5	CH_3	H	pipecolic acid
Plauracin BI (31)	$CH(CH_3)_2$	CH_3	H	pipecolic acid (p-MeO-phenylglycine)

Figure 2. Continued



Antibiotic (Ref.)	R1	R2	X
Neoviridogrisein I (43)	C_2H_5	CH_3	H
Neoviridogrisein II (44) Etamycin (45)	CH_3	CH_3	H
Neovirdogrisein III (43)	C_2H_5	CH_3	OH
Viridogrisein (46) Etamycin (47) 6613 (20) Neoviridogrisein IV (48)	CH_3	CH_3	OH
Neoviridogrisein-MP (49)	CH_3	CH_3	CH_3
Neoviridogrisein-Cl (50)	CH_3	CH_3	Cl
Viridogrisein II (35)	CH_3	H	OH

Figure 3. Chemical structure of virginiamycin group B-II antibiotics

designated factor S (later called virginiamycin S_1) because of its activity against *Bacillus subtilis*. It is a weak acid, with a pKa of 9.0 in ethanol and 7.7 in a 1:2 mixture of dimethylformamide and water. It is insoluble in water and petroleum ether, but soluble in most other organic solvents.

A communication by Vanderhaeghe and Parmentier reported the structure of virginiamycin S_1 (**1**) in 1959.[32] They published a full account of its structure determination the following year.[33] The structure of virginiamycin S_1 was determined by hydrolysis, partial hydrolysis, and Edman degradation; the antibiotic was shown to consist of a lactone ring containing, in order, L-threonine, D-α-amino-*n*-butyric acid, L-proline, *N*-methyl-L-phenylalanine, 4-oxo-L-pipecolic acid, and L-phenylglycine residues. The hydroxyl group of threonine forms the lactone with the carboxyl function of phenylglycine, and the amino group of threonine is acylated with 3-hydroxypicolinic acid. The primary structure proposed by Vanderhaeghe and Parmentier was subsequently confirmed by crystal structure determination of the methanolic solvate.[53] The tertiary structure of the macrocycle was shown to be constrained by a transannular hydrogen bond between the carboxyl oxygen of the *N*-methylphenylalanine residue and the amide nitrogen of the phenylglycine residue. Further confirmation of its structure came from mass spectral studies carried out by Kiryushkin et al.[54] and later by Compernolle et al.[55]

The solution conformation of virginiamycin S_1 and virginiamycin S_4 has been reported.[56] Features of its conformation, determined from ^1H and ^{13}C nuclear magnetic resonance studies, include a bend in the proline–*N*-methylphenylalanine–4-oxopipecolate–phenylglycine region and the aforementioned hydrogen bond between *N*-methylphenylalanine and phenylglycine, as well as a weaker hydrogen bond between the lactone oxygen and the amide nitrogen of α-aminobutyric acid. The benzyl group of *N*-methylphenylalanine is folded over the 4-oxopipecolic acid ring; the 3-hydroxypicolinic acid residue lies outside the region of the peptidolactone ring. Comparison of the results

of a similar study of the solution conformations of allohydroxy- and deoxyvirginiamycin S (obtained by sodium borohydride reduction of the parent antibiotic) to that of virginiamycin S_1 shows that the latter has the most rigid conformation in the series.[57] Comparing the biological activity of these antibiotics (as determined by Janssen et al.),[58] the investigators concluded that virginiamycin S_1 has the optimal conformation for binding to the bacterial ribosome, at least in the cases where the type B antibiotics are active without the presence of type A antibiotics.[57]

Virginiamycin S_1 has recently been synthesized by Kessler et al. by two different routes.[59] In one method the cyclic hexapeptide was constructed and then coupled with 3-hydroxypicolinic acid. In the second, a linear hexapeptide containing the 3-hydroxypipecolate was cyclized. The benzyl ether of virginiamycin S_1 has been synthesized in a similar fashion.[60]

The virginiamycin-like antibiotics inhibit the growth of bacteria. Gram-positive microorganisms are more sensitive (minimum inhibitory concentration, 0.1–5 μg/mL) than gram-negative bacteria (minimum inhibitory concentration, 5–200 μg/mL), although some gram-positive bacteria (*Mycobacteria*) are quite resistant and some gram-negative ones (*Haemophilus* and *Neisseria*) are susceptible. The difference in activity is due to the differences in the permeability of the bacterial cells; ribosomes from gram-negative organisms are just as sensitive as those from gram-positive organisms in cell-free systems.[61]

Individually the A components and the B components stop bacterial growth reversibly; however, a mixture of the two causes a 10- to 100-fold increase in inhibition as well as loss of cell viability, depending on the organism tested. The maximum activity is seen in 1:1 to 2:1 mixtures of components A and B, ratios usually found in nature.[1] Early work with the virginiamycins has shown that a 70:30 mixture is the most active against gram-positive microbes, except in *Bacillus subtilis*, where the effect is reversed.[62]

The virginiamycins have limited effect on eucaryotic organisms. In *Euglena*, type A compounds block chlorophyll synthesis and chloroplast multiplication, causing a reversible bleaching of the cells and inhibition of photoautotrophic growth. Type B compounds alone show no appreciable effect, but a mixture of A and B antibiotics causes permanent bleaching.[1, 61]

The virginiamycins are widely used today as feed additives in animal husbandry as performance promoters for cattle, swine, and poultry. Growth of the animals is enhanced by the inhibition of the intestinal flora, especially gram-positive bacteria.[61] A number of characteristics has led to the success of the virginiamycins in this application:[63] (1) activity confined to gram-positive microorganisms; (2) low solubility and low absorption through the intestinal wall and therefore little accumulation in animal tissues; (3) relative stability at acidic pH; (4) high safety standards related to its synergistic activity; for example, lowered probability of resistance induction and absence of toxicity or residue problems. In addition to their use as feed additives, the virginiamycins are used in veterinary medicine, especially in the treatment of swine dysentery.[64, 65]

Because it is possible to transmit to humans, through meat, bacterial strains carrying plasmids with antibiotic-resistance factors, antibiotics used as growth promoters are not used in the treatment of human infections. For this reason, the virginiamycins are used only in the field of animal husbandry today.[61] For several years, however, they were used successfully in a number of applications in human medicine, including the treatment of whooping cough[66, 67] and staphylococcal infections of the skin.[68]

Some work has been done on the structure–activity relationships of the type B virginiamycins. Nature already provides a variety of analogs, for the most part in the pipecolyl and the *N*-methylphenylalanine residues: all these congeners provide the same antibiotic activity and ability to bind to bacterial ribosomes.[69] A number of derivatives of pristinamycin IA have been prepared by a group of French workers: some of these have

assorted substituents introduced at the 5-position of 4-oxopipecolic acid,[70, 71] and in others the ketone functionality of the same residue was replaced by various amine derivatives.[72] In all cases the activity of the derivatives against *Staphylococcus aureus* was in the range of 0.1-125 μg/mL, comparable to the activity of the naturally occurring virginiamycins. Derivatives at the phenolic functionality have also been prepared.[69] When pristinamycin IA is acetylated, the antibiotic activity is preserved; however, esterification by an allyl moiety leads to complete loss of its biological activity. Apparently the acetylated derivative is hydrolyzed readily to the natural antibiotic. A linear molecule, obtained by hydrolysis of the lactone ring of pristinamycin IA by means of an enzyme isolated from a resistant strain of *Staphylococcus aureus*, is devoid of antibiotic activity.[73]

The virginiamycin-like antibiotics act by inhibition of protein synthesis.[61] In vivo and in vitro studies have shown that both type A and type B antibiotics bind to the 50S subunit of the ribosome, the site of protein synthesis in the cell.[74-76] The type B antibiotics bind to the ribosome in stoichiometric amounts. The type A antibiotics, however, bind in sub-stoichiometric amounts: when these detach from the ribosome, alterations produced in the particle persist.[77] It has been shown that the donor and acceptor sites of peptidyl transferase on the 50S subunit are permanently inactivated on transient incubation with virginiamycin M.[78] In cell-free systems interaction of virginiamycin M with the ribosomes enhances their affinity for virginiamycin S six-fold.[76] Apparently the type A compounds cause a conformational change in the ribosome that increases its affinity for the type B antibiotics. At this time it has not been determined which step of protein synthesis is the specific target of the type B virginiamycins.[79]

Although they are quite different in structure, virginiamycin S_1 and the other type B antibiotics are related to the macrolide antibiotics (eg., erythromycin, leucomycin, spiramycin, tylosin) and the lincosamide antibiotics in their mode of action and in their

cellular targets.[80] All have reversible activity on bacteria and bind to the 50S ribosomal subunit in 1:1 complexes.[79] The macrolides compete with virginiamycin S in the binding reaction to the ribosomes; this suggests that the binding sites of these two groups of antibiotics overlap.[81] When type A virginiamycins are present, the conformational change they trigger was found to lower the affinity of ribosomes for the macrolides while enhancing their affinity for type B antibiotics.[79]

The intrinsic fluorescence of the type B virginiamycins due to the 3-hydroxypicolinyl portion of the antibiotic has allowed investigators to determine the location of the ribosomal binding site by fluorescence energy transfer.[82] In addition, fluorescence quenching studies have shown that the binding site on the surface of the ribosome is in the shape of a well that accommodates the 3-hydroxypicolinyl residue. Access of the antibiotic to this site seems to be controlled by both hydrophobic interactions and electrostatic forces.[83]

Because ions are responsible for the shape of ribosomal particles, the presence of monovalent and bivalent cations affect the activity of the virginiamycin-like antibiotics.[79] The binding of type B antibiotics to the ribosome is dependent on the presence of NH_4^+ or K^+ and also Mg^{2+} if monovalent ions are present. The conformational change of the 50S subunit induced by the type A antibiotics requires either NH_4^+ or K^+ and either Mg^{2+} or Ca^{2+}.[84, 85] The interaction of type B antibiotics with cations (protons and alkaline earth cations) has also been studied.[86,87] These cations form a complex with the 3-hydroxypicolinic acid portion of the antibiotic, which can act as a dibasic acid. Virginiamycin S_1 has also been shown to facilitate the transport of protons and other cations across phospholipid bilayer membranes in much the same fashion as the natural protein cation carriers in membranes.[87] However, the role of this phenomenon in the activity of the type B antibiotics has not been shown.

The biogenesis of a number of medium-sized peptide and depsipeptide antibiotics

has been shown to proceed by a multienzyme thiotemplate mechanism[88] and not by the ribosomal mechanism that produces proteins. Etamycin formation is not inhibited by the presence of chloramphenicol, an antibiotic that inhibits protein synthesis; this finding suggests that it too is formed by the multienzyme thiotemplate mechanism.[89] Presumably the other type B virginiamycins are formed by a similar mechanism. The specificity of these templates are not as strict as that of the messenger ribonucleic acid templates mediating the synthesis of proteins, and similar amino acids can replace each other, so that a variety of antibiotic analogs are produced by a single organism under a variety of growth conditions. This phenomenon is seen in the organisms producing the type B virginiamycins. *Streptomyces virginiae*, for example, produces five related type B antibiotics. (See Figure 2.) Scientists have been able to take advantage of this in a procedure known as directed biosynthesis. For example, the organisms that produce etamycin normally synthesize only that antibiotic, but the addition of a particular amino acid to the culture in high concentration or indeed as the only nitrogen source has resulted in the production of a number of novel congeners where the added amino acid has replaced an amino acid residue (usually 3-hydroxyproline) in the antibiotic.[35, 43, 44, 49, 50] (See Figure 3.)

Before Molinero began the study of the study of the biosynthesis of virginiamycin S_1,[90] the only type B antibiotic whose biosynthesis had been studied was etamycin.[91, 92] The origin of each of the constituent amino acids of that antibiotic was established by the incorporation of radioactively labeled amino acids. Because etamycin bears some resemblance to virginiamycin S_1, the results of these studies provided a starting place for planning a similar study of this antibiotic. Etamycin and virginiamycin S_1 both contain a threonine residue and a 3-hydroxypicolinic acid residue. In addition, the phenylsarcosine residue of etamycin is merely the N-methyl derivative of phenylglycine, found in virginiamycin S_1. And both etamycin and virginiamycin S_1 contain *N*-methyl amino acids.

It is not surprising that radioactively labeled L-threonine was efficiently incorporated in the corresponding portion of both antibiotics. In contrast to the 3-hydroxypicolinic acid residue in the antimycobacterial antibiotic pyridomycin, in which it was shown that L-aspartate, glycerol, and pyruvate were efficient precursors,[93] L-lysine was shown to be the best precursor for the same residue in both etamycin and virginiamycin S_1. The results in etamycin were confirmed in a later biosynthetic study, which also showed that 5-hydroxy-L-pipecolic acid and 5-hydroxy-DL-lysine were incorporated into the 3-hydroxypicolinic acid residue.[94] As in the phenylsarcosine portion of etamycin, the phenylglycine residue of virginiamycin S_1 arises from L-phenylalanine. The *N*-methyl groups of both antibiotics were labeled by L-methionine-*methyl*-^{13}C. Molinero's work also established that the L-proline residue incorporates L-proline-*U*-^{14}C and that the *N*-methyl-L-phenylalanine residue incorporates L-phenylalanine-*U*-^{14}C. The biogenetic origins of the remaining residues of virginiamycin S_1 are less clear. Of the amino acids incorporated into the antibiotic, L-threonine seems to be the best precursor of the D-α-aminobutyric acid residue. And small but significant amounts of labeled L-aspartic acid, L-lysine, and L-methionine seemed to be incorporated into the 4-oxo-L-pipecolic acid. The results of Molinero's research are summarized in Table 2.

Table 2. Distribution of radioactivity in virginiamycin S_1 components[90]

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

*Specific incorporation of the precursor
=(100 X specific activity VS_1/specific activity of precurser)

**Unaccounted radioactivity

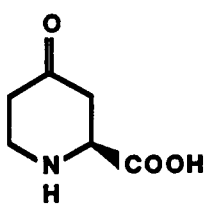
***Not determined

Thr = L-threonine
AmBut = D-α-aminobutyric acid
Pro = L-proline
MePhe = *N*-methyl-L-phenylalanine
Oxo = 4-oxo-L-pipecolic acid
PhGly = L-phenylgylcine
HyPic = 3-hydroxypicolinic acid

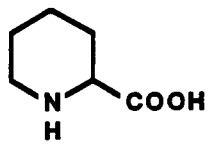
2. RESULTS AND DISCUSSION

2.1. The Origin of the 4-Oxo-L-pipecolic Acid Residue

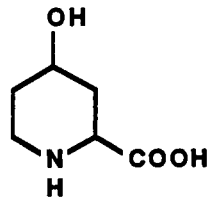
4-Oxo-L-pipecolic acid (2) is an unusual amino acid; to date it has been detected only in the virginiamycin type B antibiotics. The related amino acids pipecolic acid (3) and 4-hydroxypipecolic acid (4), however, are more common. Pipecolic acid is widely distributed in plants and has been isolated from the fungus *Neurospora* and rats.[95] 4-Hydroxypipecolic acid is also found in several plant species;[96] in addition, it replaces 4-oxo-L-pipecolic acid in two of the congeners of virginiamycin S_1—virginiamycins S_2[34] and S_5,[35] both of which are coproduced with virginiamycin S_1.



2

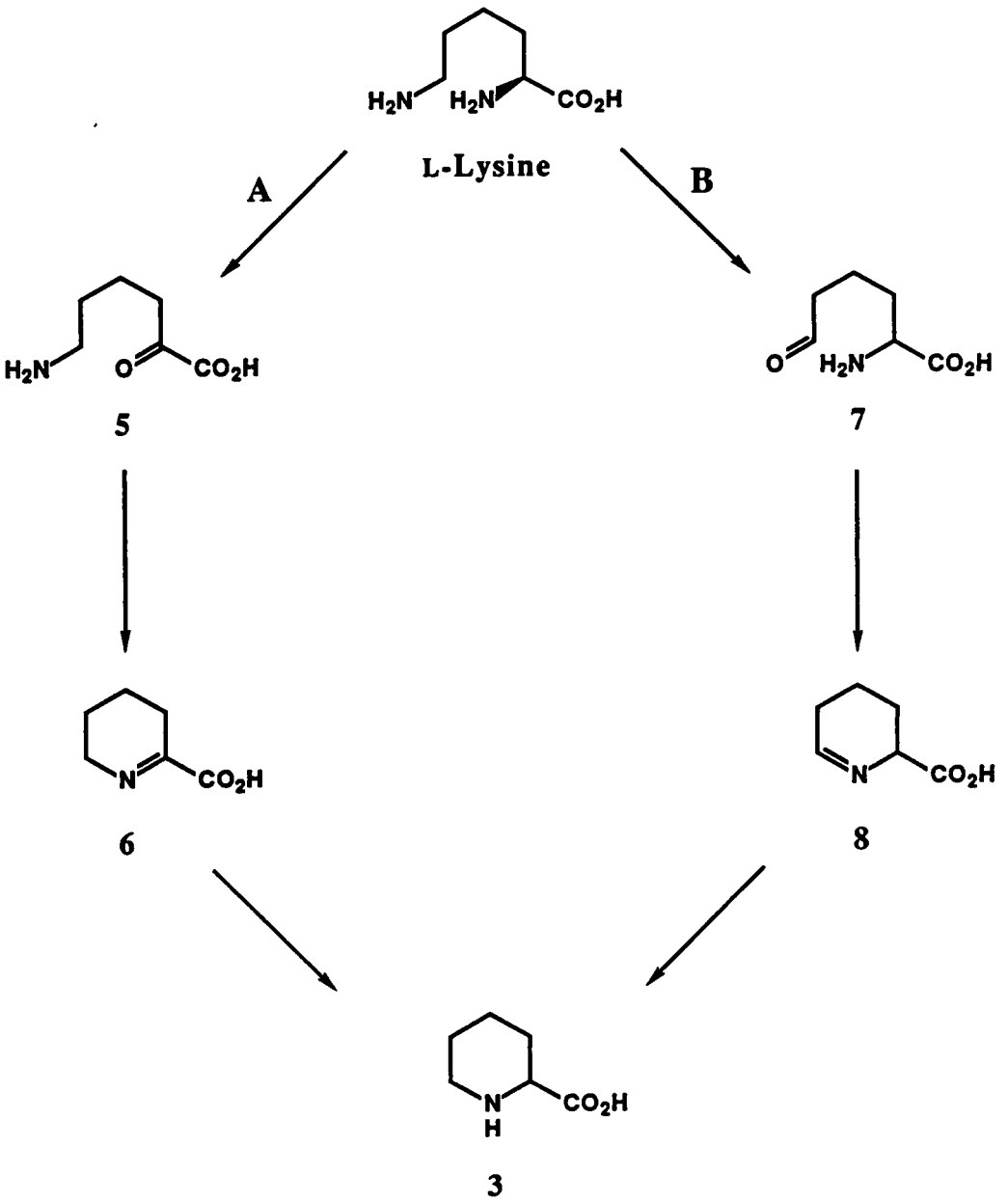


3



4

The biosynthesis of both of these amino acids has been studied. Gupta and Spencer have shown that pipecolic acid in rats, in *Neurospora crassa*, and in two higher plants (*Phaseolus vulgaris* and *Sedum acre*) is formed from lysine with the retention of the ε-amino nitrogen.[97] They have proposed that the conversion of lysine into pipecolic acid proceeds via ε-amino-α-ketocaproic acid (5) and Δ^1-piperideine-2-carboxylic acid (6) (Scheme 1, pathway A). Other researchers have observed similar results in the study of the origin of pipecolic acid.[98,99,100] On the other hand, Fowden has observed that pipecolic acid in *Acacia* arises from the loss of the ε-amino nitrogen: the pathway in this case proceeds by way of α-aminoadipic-δ-semialdehyde (7) and Δ^1-piperideine-6-carboxylic acid (8) (Scheme 1, pathway B).[101] The biosynthesis of 4-hydroxypipecolic acid has also



Scheme 1. Two pathways from L-lysine to pipecolic acid

been studied in *Acacia*.[101,102] Unlike 5-hydroxypipecolic acid, which is produced from 5-hydroxylysine,[103] 4-hydroxypipecolic acid arises from the hydroxylation of pipecolic acid, not from 4-hydroxylysine. Indeed, 4-hydroxylysine is rarely observed in nature. (L-*threo*-γ-hydroxylysine has been isolated as a component of the peptide antibiotics cerexin A and B.)[104]

It is not surprising that the biogenetic origin of 4-oxo-L-pipecolic acid has particularly interested us. Of the seven amino acid components comprising virginiamycin S_1, however, 4-oxo-L-pipecolic acid has been most difficult to study. It is the only one of the seven that is not commercially available; in addition, the amino acid is sensitive to the basic conditions used in many of the traditional amino acid derivatization procedures. The development, therefore, of a suitable derivative for use in its separation by HPLC from the other components of the antibiotic and in its quantification has been tedious. Because of these problems, at the conclusion of Molinero's work, there was still no satisfactory answer to the question of its origin.

In his biosynthetic studies of virginiamycin S_1,[90] Molinero hydrolyzed the radiolabeled antibiotic, then prepared *N*-benzoyl derivatives of the constituent amino acids (except 3-hydroxypicolinic acid, the nitrogen of which cannot be acylated.) These derivatives were separated by reversed-phase HPLC, collected, and the radioactivity of each counted. In the case of 4-oxo-L-pipecolic acid, its derivative was produced in such small yield (4% as compared to 40–50% for the others) that the peak seen on the HPLC chromatogram rises barely above the baseline. In addition, Molinero was unable to obtain the *N*-benzoyl-4-oxopipecolic acid standard in crystalline form.

The results of these studies showed that L-lysine-*U*-^{14}C, L-aspartate-*U*-^{14}C, and L-methionine-*methyl*-^{14}C were incorporated into the 4-oxo-L-pipecolic acid part of virginiamycin S_1, although none of these were incorporated in dramatic proportions. Certainly the lysine result seemed valid on the basis of the studies on pipecolic acid and 4-

hydroxypipecolic acid. And L-aspartate is a reasonable precursor; a number of mechanisms can be imagined that would transform it into 4-oxopipecolic acid. But it seemed unlikely that L-methionine be a precursor, as methionine usually provides methyl groups, and 4-oxopipecolic acid has, of course, no methyl groups.

The first task in the second phase of the study of the biogenesis of virginiamycin S_1 was to devise an improved procedure for the derivatization of 4-oxopipecolic acid, then to repeat the experiments in which radiolabeled L-lysine, L-aspartate, and L-methionine are incorporated into virginiamycin S_1. Because of its relative instability in basic solution, however, the amino acid continued to resist attempts at derivatization. Anxious to reexamine the questionable incorporations, we decided to do the incorporation experiments and to separate the 4-oxo-L-pipecolic acid from the other amino acids in an underivatized form by ion-exchange chromatography.[33]

In the experiment in which L-methionine-*methyl*-^{14}C was fed, the label was, as before, incorporated into the antibiotic with a specific incorporation of 2.4×10^{-2}. (Specific incorporation = 100 X specific activity of VS_1/specific activity of precursor.) (The *N*-methyl-L-phenylalanine portion incorporates labeled methionine; we assume the *N*-methyl group comes from the *S*-methyl of methionine.) The 4-oxo-L-pipecolic acid isolated by ion-exchange chromatography showed virtually no radioactivity: less than 2% of the radioactivity of the intact virginiamycin molecule was located in the 4-oxo-L-pipecolic acid moiety. Virginiamycin S_1 isolated following the incorporation of L-lysine-*U*-^{14}C showed a specific incorporation of 9.8×10^{-3}, a figure comparable to the result of Molinero's experiment. However, when L-aspartate-*U*-^{14}C was administered, the observed specific incorporation (5.9×10^{-4}) differed dramatically from that seen in the earlier experiment and was significantly lower than the incorporations of labeled methionine and lysine. From this experiment, it can be concluded that L-aspartic acid is not a precursor to any part of virginiamycin S_1. Because it is unlikely that there exist two major

biosynthetic routes to 4-oxopipecolic acid and because the results from the labeled lysine incorporations are consistent, one can only conclude that L-aspartic acid is not a precursor to 4-oxo-L-pipecolic acid or any other part of the antibiotic. The results of the above experiments are summarized in Table 3.

A later experiment in which DL-lysine-*6-*^{13}*C-6-*^{15}*N* (**9**) was fed to *S. virginiae* confirmed that L-lysine is indeed a precursor to 4-oxo-L-pipecolic acid. Also this particular experiment answered the question of which nitrogen of lysine is incorporated into 4-oxo-L-pipecolic acid: does the cyclization of lysine proceed by pathway A or pathway B (Scheme 1). The doubly labeled lysine was prepared by M. B. Purvis[105] by a synthetic strategy (Scheme 2) based on the one that was used to prepare (2*RS*, 5*R*)-lysine-*5-d*$_1$ and (2*RS*, 5*S*)-lysine-*5-d*$_1$, which were used in an experiment to study the mechanism of formation of the 3-hydroxypicolinic acid portion of virginiamycin S_1. (See Section 2.2.) The ^{13}C-NMR spectrum of the virginiamycin S_1 isolated following the feeding of DL-lysine-*6-*^{13}*C-6-*^{15}*N* showed that the resonance at 36.8 ppm due to C-6 of the 4-oxo-L-pipecolic acid residue was enhanced and appeared as a doublet ($^1J_{CN}$ = 10 Hz) (Figure 4). This result clearly shows that the the ε-nitrogen of lysine is retained in 4-oxo-L-pipecolic acid in virginiamycin S_1. It was confirmed by GC/MS analysis of the *N*-trifluoroacetyl butyl esters of the amino acids obtained by acid hydrolysis of the labeled virginiamycin S_1. The data shown in Table 4 are the relative abundances of the ions (from the loss of the butoxycarbonyl group) due to the base peak of the 4-oxopipecolic acid derivative. These data show that this peak consists of 1% singly labeled species and 6% doubly labeled species. Incidentally, we did not detect the incorporation of the deuterated lysines (Section 2.2) into 4-oxo-L-pipecolic acid. The deuterium is located at the 5-position, adjacent to the carbonyl group, and the hydrogens at this position apparently exchange in the slightly acidic aqueous fermentation medium or during the derivatization process.

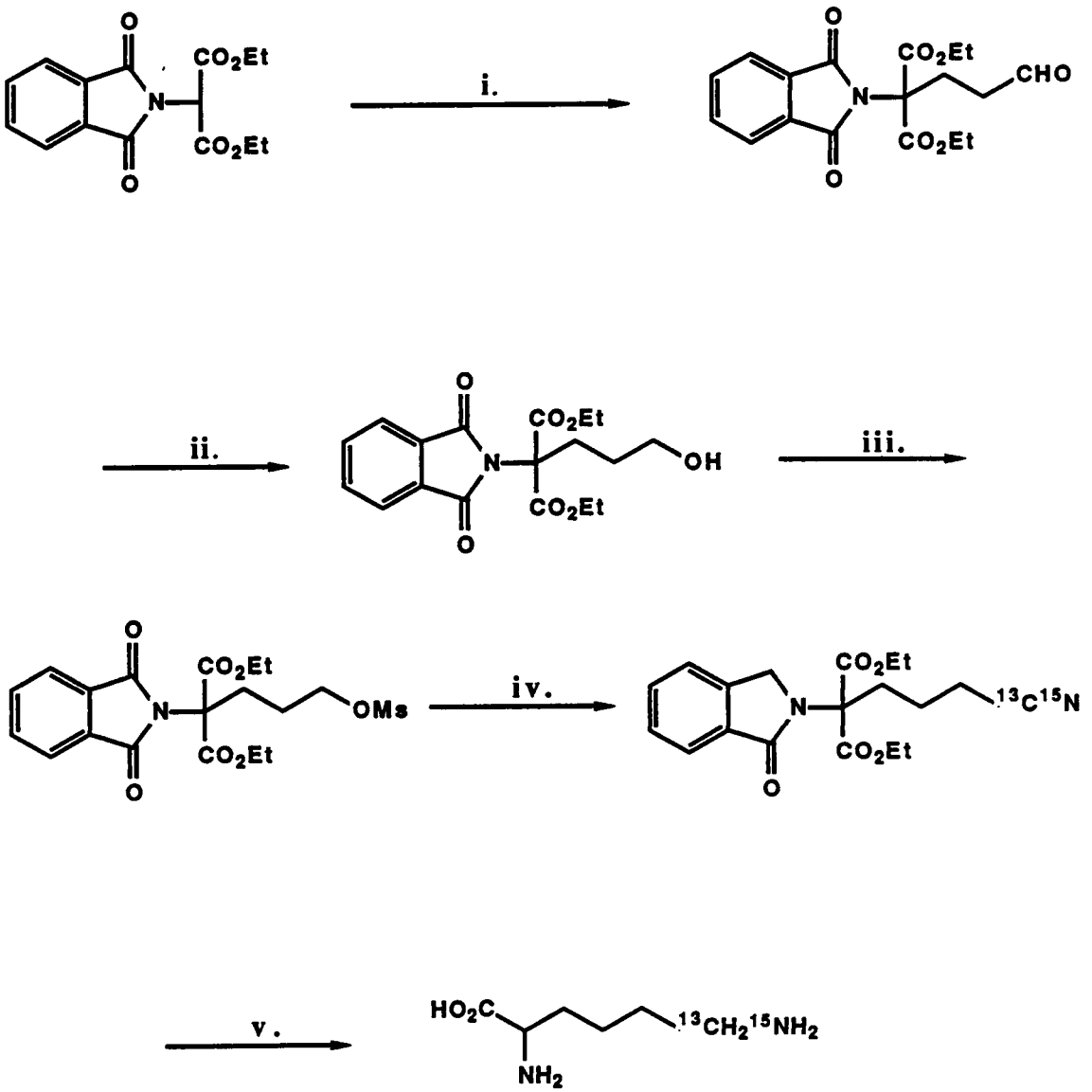
Although the answers to our questions were apparent, it was still our aim to

Table 3. Incorporation of Radioactivity in Virginiamycin S_1

	Production (mg/L)	% Incorp.*	Sp. Act. precursor (mCi/mmol)	Sp. Act. VS_1 (mCi/mmol)	Sp. Inc.**
L-Methionine-*methyl-^{14}C*	9.8	2.0	45.7	0.011	0.024
L-Aspartic-*U-^{14}C acid*	25.6	0.24	184	0.0011	0.00059
L-Lysine-*U-^{14}C*	12.5	3.1	275	0.027	0.0098

* % Incorporation = 100 x DPM VS_1/DPM precursor, where DPM = disintegrations per minute

** Specific Incorporation = 100 x specific activity VS_1/specific activity precursor



9

Scheme 2. Synthesis of DL-lysine-*6-^{13}C-6-^{15}N*.[104] **i.** CH_2=CHCHO, NaOMe; **ii.** 9-BBN; **iii.** MsCl, Et_3N; **iv.** $Na^{13}C^{15}N$, DMSO; **v.** H_2, PtO_2; H_3O^+

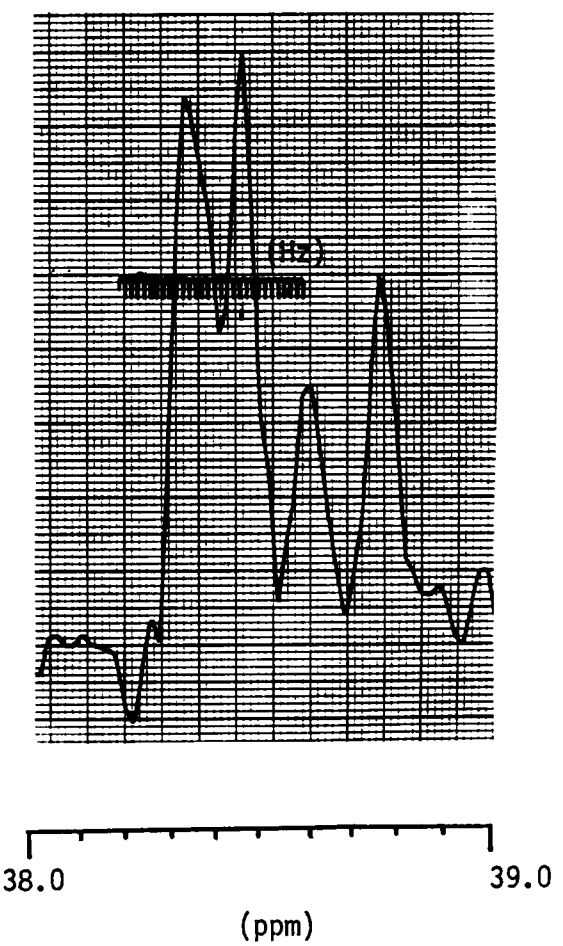


Figure 4. Partial ^{13}C-NMR spectrum of VS_1 labeled with (2*RS*)-lysine-*6*-^{13}*C*-*6*-^{15}*N*: the signal at 38.6 ppm

Table 4. Relative abundance of the ions for the base peak (M–COOBu) of butyl *N*-trifluoroacetyl-4-oxopipecolate from VS_1 labeled with (2*RS*)-lysine-6-^{13}C-6-^{15}N

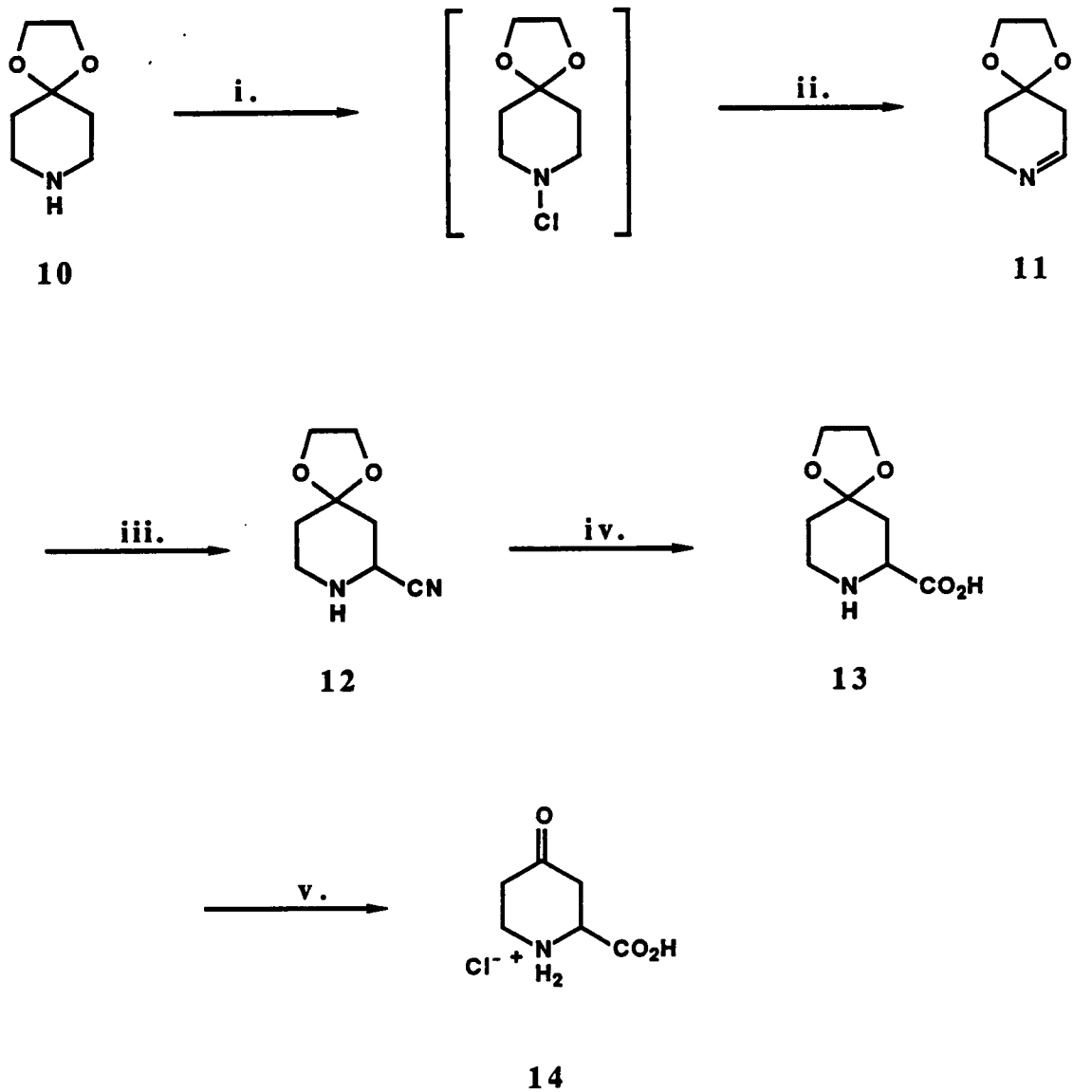
m/z	194	195	196
Labeled	100	12	7
Unlabeled	100	11	1
Corrected labeled*	100	1	6
%**	93	1	6

* Relative abundances of the ions from the labeled compound corrected for the naturally occurring A+1 and A+2 contributions of the unlabeled and singly labeled peaks.

** Mole % unlabeled, singly labeled, and doubly labeled species, respectively

develop a satisfactory method of producing a derivative of 4-oxo-L-pipecolic acid that would allow us to separate it from the other amino acids in the antibiotic by HPLC. It should therefore be a UV-active compound to allow for its detection in our HPLC system. But because we did not have a readily available supply of the amino acid, its development was difficult. At first, we obtained a small supply of by hydrolyzing a portion of virginiamycin S_1, then subjecting the hydrolysate to ion-exchange chromatography. In this manner, we were able to obtain about 220 mg of reasonably pure 4-oxo-L-pipecolic acid hydrochloride. As this supply diminished in what seemed to be endless futile attempts in making a suitable derivative, the appeal of devising a synthesis of this amino acid increased. A search of the literature revealed that 4-oxopipecolic acid had been synthesized previously by Clark-Lewis and Mortimer in low yield (1.5% in 3 steps) from methyl 4-chloropicolinate via 4-hydroxypipecolic acid.[106] The yield was improved by French workers to 16% by the use of different conditions in the step in which the aromatic picolinic acid derivative is reduced to 4-hydroxypipecolic acid.[107] Additional reading of the literature uncovered a synthesis of piperideine **11**, which was used as an intermediate in syntheses of the piperidine alkaloids (+)-4-hydroxysedamine and (+)-4-hydroxyallosedamine.[108] It seemed an ideal starting material for the synthesis of the desired amino acid.

The synthesis of 4-oxo-DL-pipecolic acid hydrochloride was undertaken as outlined in Scheme 3. Piperideine **11** was prepared in 2 steps from the commercially available 4-piperidone ethylene ketal (**10**). Addition of the elements of HCN, followed by hydrolysis of the nitrile provided protected 4-oxopipecolic acid (**13**) in 35% overall yield. Deprotection of the keto group proved to be somewhat troublesome, however. The first attempt at hydrolysis apparently succeeded, but the process of stripping the aqueous acid on a rotary evaporator only caused the ketal to re-form. Neutralization of the hydrolysis reaction mixture followed by ion-exchange chromatography afforded 4-oxo-DL-pipecolic

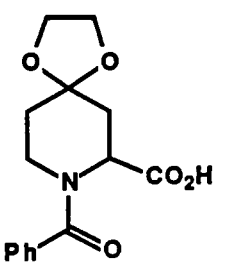


10 11 12 13 14

Scheme 3. Synthesis of 4-oxo-DL-pipecolic acid hydrochloride. i. NCS; **ii.** KO_2, 18-crown-6; **iii.** KCN, pH 5; **iv.** $Ba(OH)_2 \cdot 8H_2O$; **v.** 2 N HCl

acid hydrochloride (14) in modest yield. At the present time the yield of the last step of the synthesis has not been optimized. Because of the poor yield in the last step, the synthesis does not compete favorably with the procedure already existing in the literature. If the isolation of the final product were to be improved, however, this synthesis would provide a convenient source of 4-oxo-DL-pipecolic acid.

Ironically, the problem of isolating the synthetic amino acid provided a solution to the synthesis of a good derivative of that amino acid. The protected 4-oxopipecolic acid underwent acylation with benzoyl chloride to yield its *N*-benzoyl derivative (**15**). Preparation of the derivative in the virginiamycin S_1 hydrolysate was accomplished by the addition of a drop of ethylene glycol to the mixture before removal of the aqueous acid on the rotary evaporator, then acylation with benzoyl chloride by the Schotten-Baumann method. The new derivative separated nicely from the other amino acid derivatives with the same HPLC conditions that had been worked out previously.[90] The magnitude of the peak in the HPLC chromatogram is comparable to those of the other virginiamycin S_1 amino acid derivatives. Figure 5 shows a representative chromatogram.



15

This work establishes that L-lysine is a precursor to 4-oxo-L-pipecolic acid in virginiamycin S_1. It shows that the ε-amino group of the lysine molecule is retained in the cyclization to the heterocyclic compound. Additional biosynthetic studies would further elucidate the mechanism by which lysine is converted to 4-oxo-L-pipecolic acid.

1. *N*-benzoyl-L-threonine
2. *N*-benzoyl-L-proline
3. *N*-benzoyl-D-α-aminobutyric acid
4. *N*-benzoyl-4-oxo-L-pipecolic acid ethylene ketal
5. Benzoic acid
6. *N*-benzoyl-L-phenylalanine

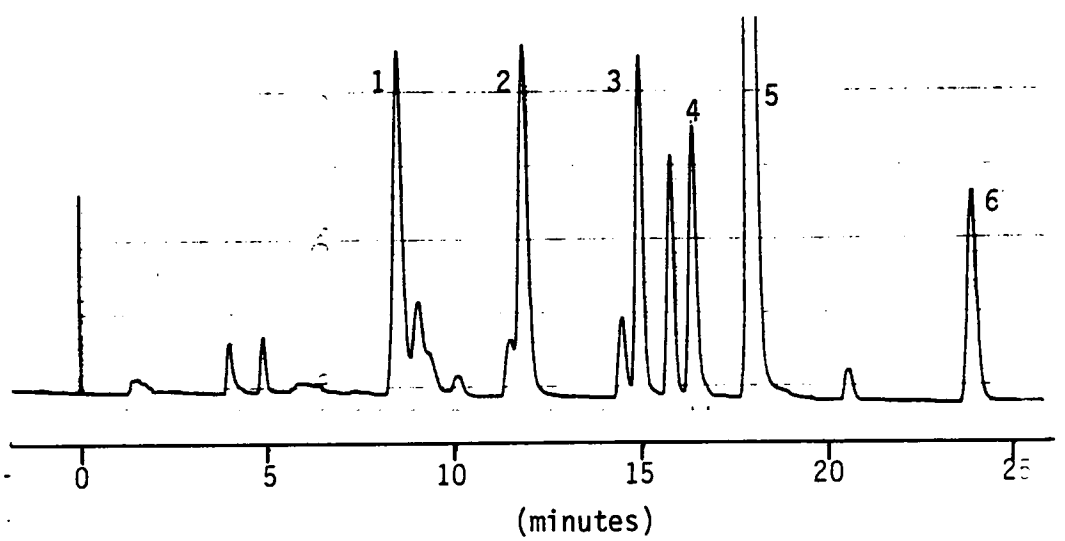
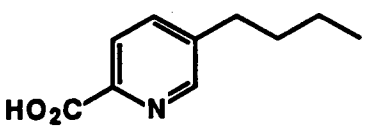


Figure 5. Partial HPLC chromatogram of *N*-benzoyl derivatives from VS_1 hydrolysate

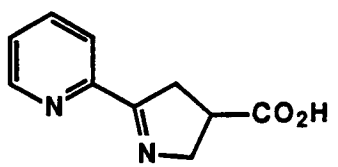
Incorporations of pipecolic acid, 4-hydroxypipecolic acid, and 4-hydroxylysine would establish whether these compounds are intermediates in the process. Also the relationship between the origin of this amino acid and those of its congeners (4-hydroxypipecolic acid and 5-hydroxy-4-oxopipecolic acid) would be an interesting question to examine.

2.2. The Origin of the 3-Hydroxypicolinic Acid Residue

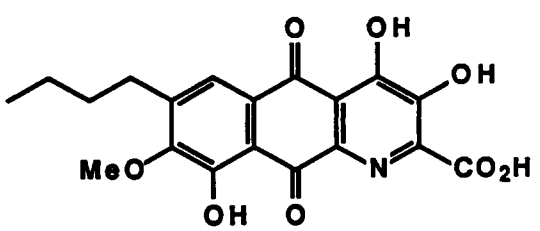
The pyridine nucleus is found in a number of bacterial metabolites having widely disparate origins. For example, fusaric acid (**16**) is derived from aspartic acid and acetate.[109] The pyridine nucleus in proferrorosamine A (**17**) is derived from lysine by way of picolinic acid,[110] whereas phomazarin (**18**) arises from a polyketide chain.[111]



16



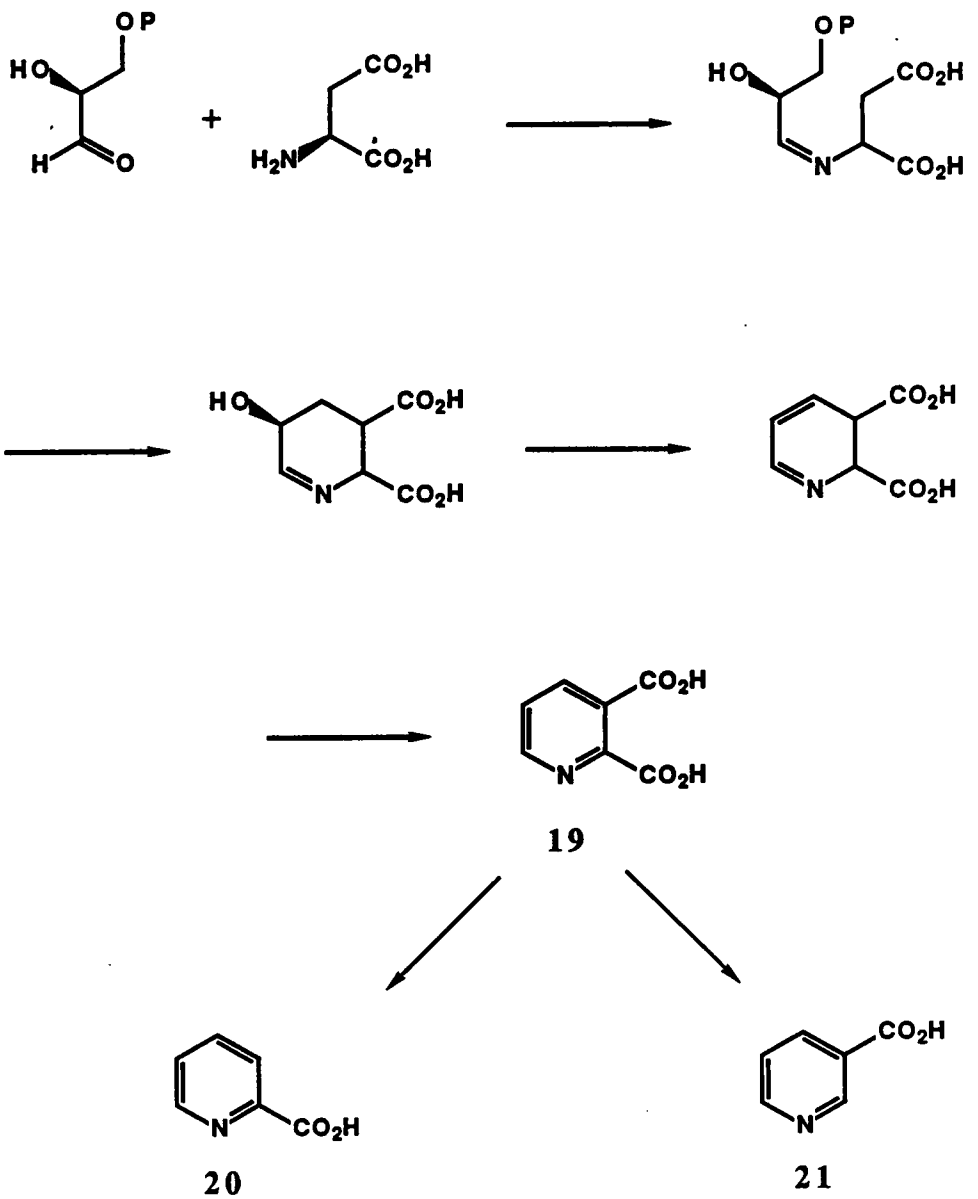
17



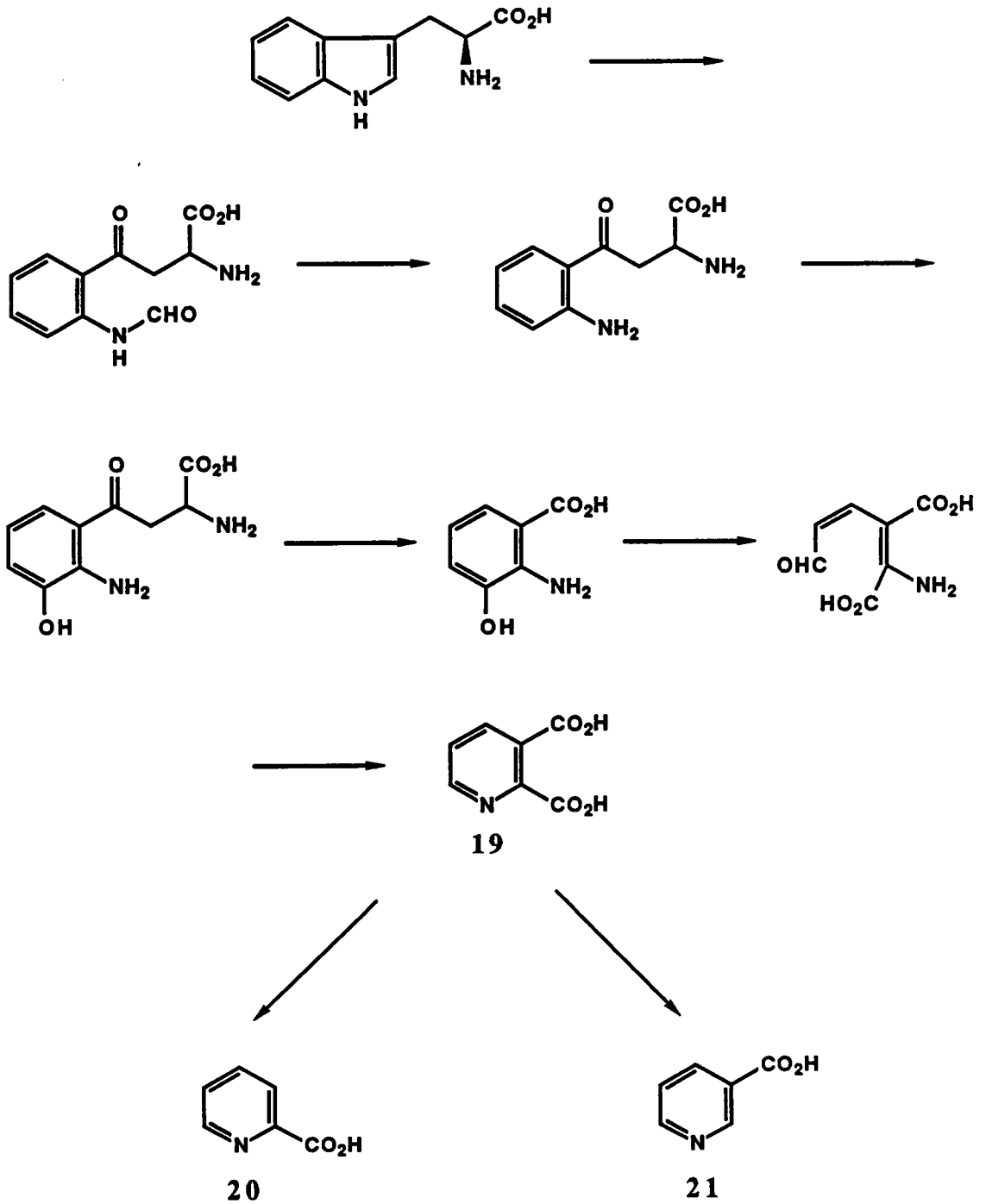
18

There are two biosynthetic pathways to picolinic acid and its isomer nicotinic acid known to operate in different microorganisms.[112] In one pathway (Scheme 4), aspartic acid and glyceraldehyde-3-phosphate are condensed to form a heterocyclic ring, which is then transformed to quinolinic acid (**19**). Quinolinic acid can then be converted to either picolinic acid (**20**) or nicotinic acid (**21**). In the other pathway (Scheme 5), tryptophan undergoes an interesting series of reactions to produce quinolinic acid and then picolinic acid or nicotinic acid.

The biosynthesis of the 3-hydroxypicolinic acid (**22**) has been studied in the antibiotics pyridomycin[93] and in etamycin.[91,92] In pyridomycin, 3-hydroxypicolinic acid

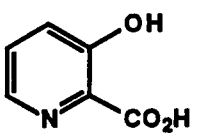


Scheme 4. Pathway to nicotinic acid and picolinic acid from L-aspartatic acid and glyceraldehyde-3-phosphate



Scheme 5. Pathway to nicotinic acid and picolinic acid from L-tryptophan

originates from L-aspartic acid and glycerol or pyruvate by what could be a mechanism similar to that of the biogenesis of picolinic acid. In etamycin, a virginiamycin type B antibiotic, L-lysine is a precursor of the same residue, and L-aspartate is not incorporated in any significant amount. Further studies have shown that 5-hydroxylysine (mixed isomers)[113] and 5-hydroxypipecolic acid (from Sigma, isolated from *Phoenix dactylifera*, dates, and thus of the (*2S, 5R*) configuration)[113] are also precursors.[94]



22

Molinero's work[90] on virginiamycin S_1 showed that both L-aspartic acid and L-lysine are incorporated into 3-hydroxypicolinic acid (Table 1). Molinero also fed labeled tryptophan to *S. virginiae*, and he found no incorporation into the antibiotic. However, the tryptophan he used was labeled at C-3; if it were incorporated into the 3-hydroxypicolinyl portion of virginiamycin S_1 by a mechanism like that shown in Scheme 5, then the labeled carbon would be lost, and no incorporation would be detected. It seems likely though that the mechanism in virginiamycin S_1 is more like that in pyridomycin or etamycin. The more appropriately labeled tryptophan-*ar*-^{14}C was administered in the cases of both pyridomycin and etamycin, and virtually no incorporation was observed.

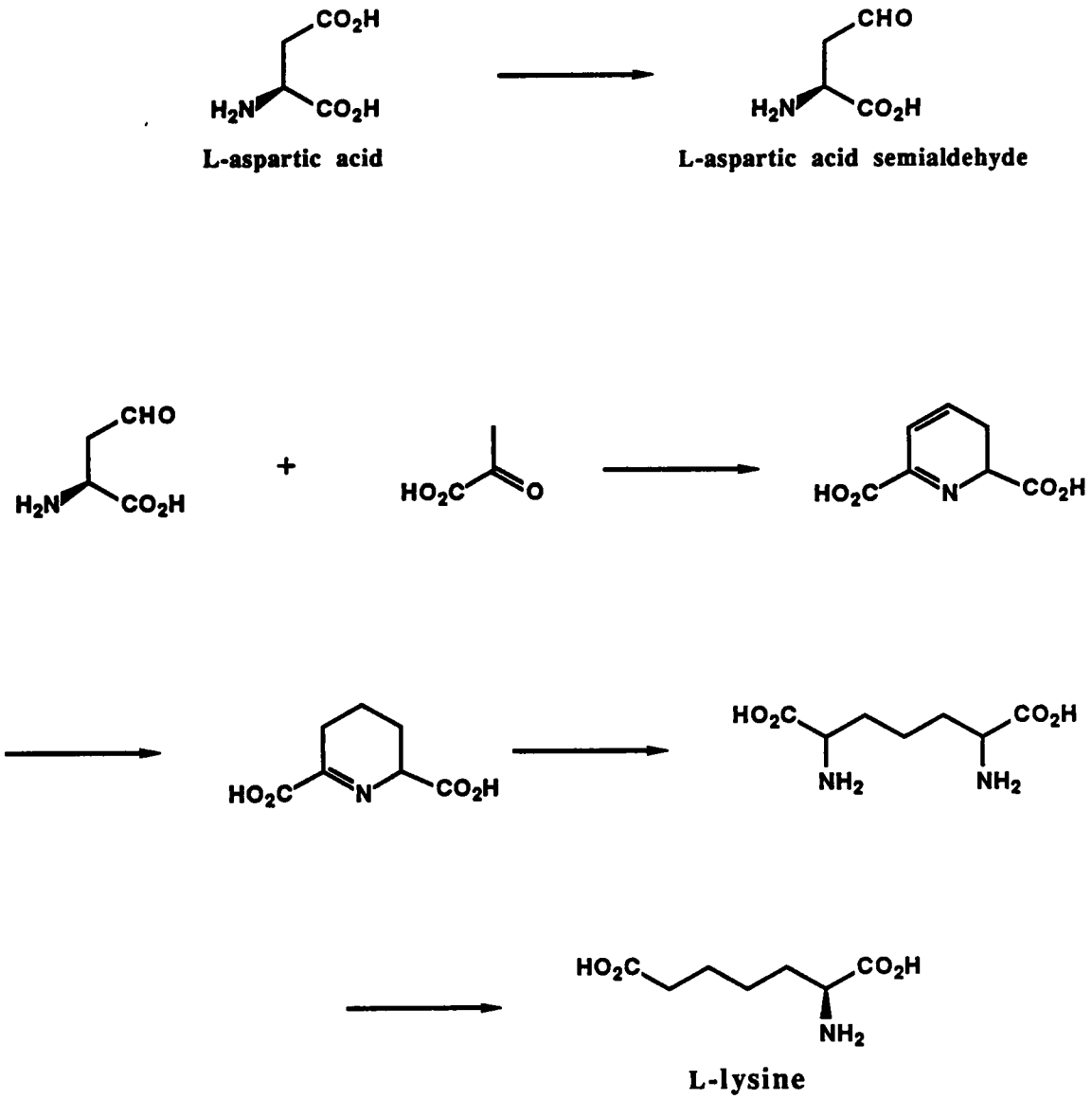
In etamycin, where lysine was incorporated approximately 10 times more efficiently than aspartate, and in pyridomycin, where no incorporation was detected at all, the biogenetic precursors are clear-cut. In virginiamycin S_1, however, both precursors were incorporated into the 3-hydroxypicolinic acid residue in comparable magnitudes. Molinero

attempted to explain the disparity by suggesting that L-lysine was the true precursor and that L-aspartate was being converted to L-lysine by way of the 2,6-diaminopimelate pathway (Scheme 6), known to operate in bacteria.[114] If this were the case, however, one would expect to see a labeling pattern more like the one that is seen in etamycin, where the incorporation of aspartate is significantly less than that of lysine.

In conjunction with the study of the origin of 4-oxo-L-pipecolic acid (see Section 2.1) the labeled L-aspartic acid experiment was repeated. It was found that the specific incorporation of radiolabeled aspartate was extremely low—5.9 x 10^{-4}. This finding suggests that L-aspartate is **not** a significant precursor to any part of the virginiamycin S_1 molecule. It is in agreement with the findings in the closely related antibiotic etamycin. Thus L-lysine is the principal precursor to 3-hydroxypicolinic acid in virginiamycin S_1.

The questions that we have undertaken to answer in this project concern the mechanism by which L-lysine is transformed into 3-hydroxypicolinic acid. First, which of the two nitrogens of L-lysine is retained in the cyclization to the heterocyclic ring system? Second, is there any stereospecificity in the loss of the hydrogens at C-4, C-5, and C-6 of lysine as the aromatization process takes place? In other words, is the *pro(R)* or *pro(S)* hydrogen retained preferentially at each of the three positions?

The first question is related, of course, to the one asked in the case of the origin of 4-oxo-L-pipecolic acid. The answers to both questions were obtained in the same experiment. Thus (2*RS*)-lysine-*6-*^{13}C-^{15}N[104] was administered to growing cultures of *S. virginiae*, and evidence of incorporation was sought in the 3-hydroxypicolinic acid residue of virginiamycin S_1. The ^{13}C-NMR spectrum of the labeled virginiamycin S_1 showed an intense peak at 139.6 ppm due to the labeled C-6 in the 3-hydroxypicolinic acid residue. Because the one-bond coupling constants are very small between carbon and nitrogen in aromatic systems,[115] it was not expected that any splitting would be observed even if the nitrogen adjacent to this carbon were labeled. Examination of the peak under high



Scheme 6. The 2,6-diaminopimelate pathway of lysine biosynthesis

resolution conditions showed the peak to be broad, with evidence of a shoulder (Figure 6).

During the same GC/MS experiment in which doubly labeled 4-oxo-L-pipecolic acid was studied, we were able to determine the isotopic labeling pattern of 3-hydroxypicolinic acid. The antibiotic was hydrolyzed, and the resulting amino acids were derivatized as the *N*-trifluoroacetyl butyl esters. Under the reaction conditions 3-hydroxypicolinic acid was esterified; because the nitrogen is part of the aromatic system, it is not acylated; neither is the phenolic oxygen. The base peak in the mass spectrum of the butyl ester of 3-hydroxypicolinic acid is at m/z 95, which corresponds to the protonated ion in which the butoxycarbonyl group has been lost. The results are shown in Table 5.

The table shows that 3% of the ions for this peak are singly labeled, and 6% of the peaks are doubly labeled. The results indicate that the mechanism of ring closure of lysine to the heterocyclic ring proceeds with substantial loss of the α-nitrogen and retention of the ε-nitrogen. If the conversion of lysine to 3-hydroxypicolinic acid goes by way of a pipecolic acid intermediate as has been shown in etamycin,[94] the mechanism would be quite similar, up to a point, to that of the formation of 4-oxo-L-pipecolic acid.

The second question posed about the mechanism of the formation of 3-hydroxypicolinic acid in virginiamycin S_1 concerns the stereospecificity involved in the removal of hydrogens in the aromatization process. There are three carbons of lysine that retain hydrogens when it is transformed into 3-hydroxypicolinic acid: C-4, C-5, and C-6. We chose to examine the process at C-5 by administering lysines stereospecifically deuterated at this carbon and observing the incorporation of each isomer.

Because these labeled lysines are not commercially available, it was necessary to devise a synthesis of lysine by which C-5 could be labeled stereospecifically with deuterium. A synthesis of stereospecifically deuterated ornithine by Townsend and coworkers[116,117] provided an intermediate that could be converted into the required compounds. The synthesis of (*2RS, 5R*)-lysine-*5-d_1* is outlined in Scheme 7. Deuterated

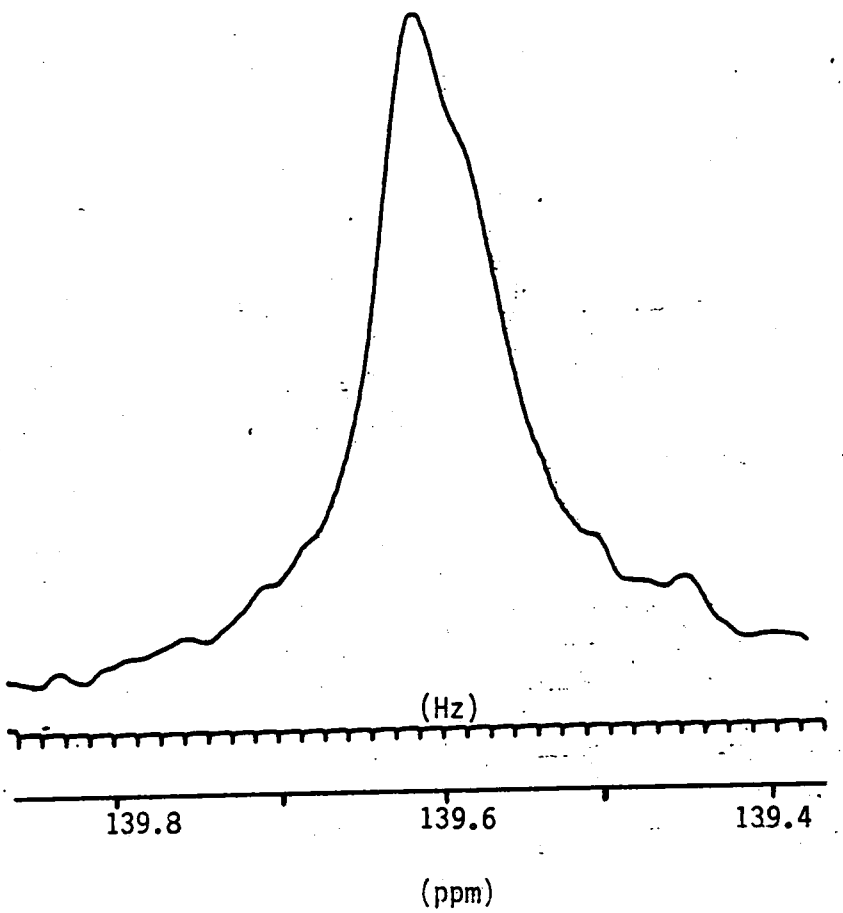


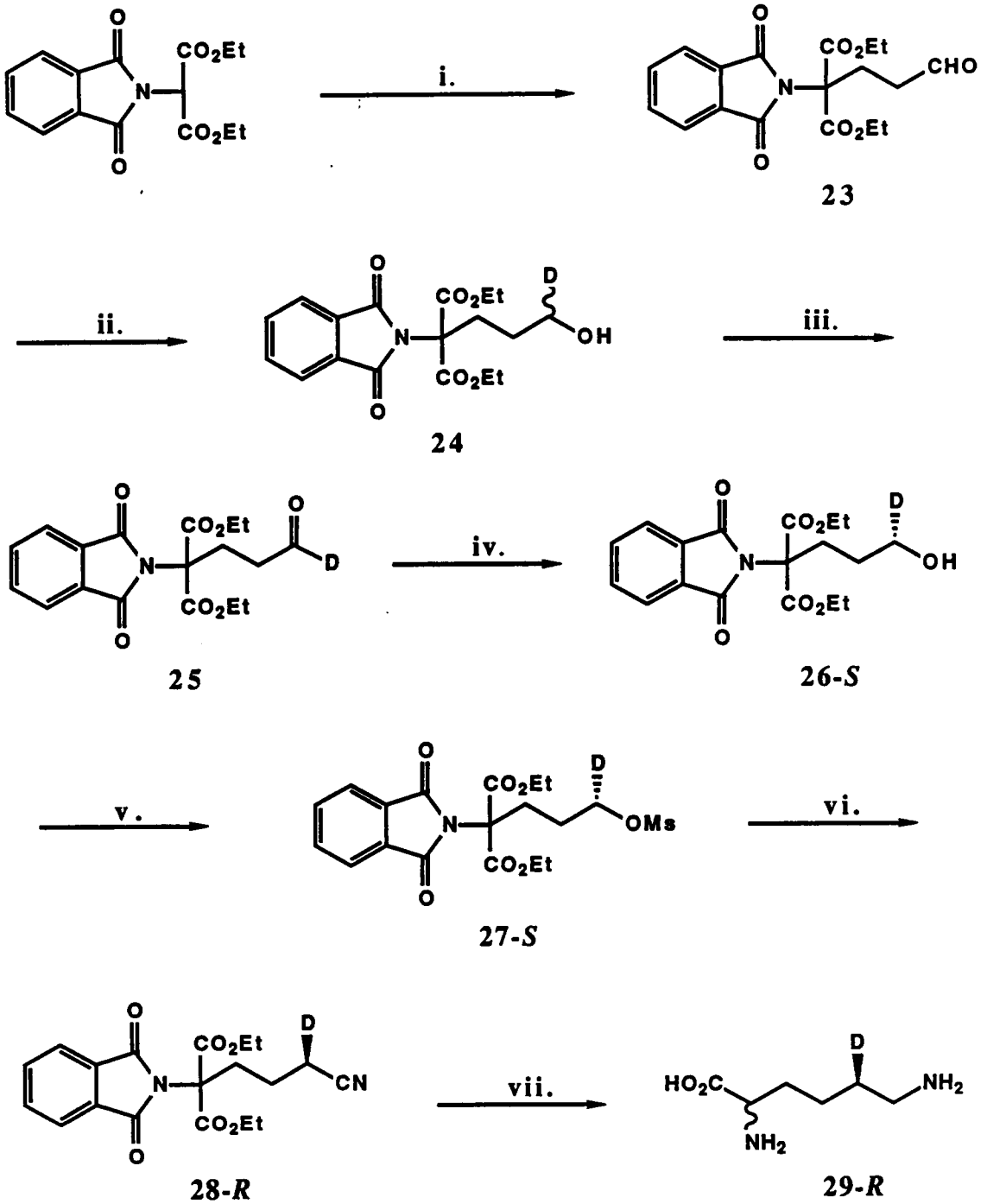
Figure 6. Partial ^{13}C-NMR spectrum of VS_1 labeled with (2*RS*)-lysine-*6*-^{13}*C*-*6*-^{15}*N*: the signal at 139.6 ppm

Table 5. Relative abundance of the ions for the base peak (M–COOBu+H) of butyl 3-hydroxypicolinate from VS_1 labeled with (2*RS*)-lysine-*6*-^{13}C-*6*-^{15}N

m/z	95	96	97
Labeled	100	12.3	7.0
Unlabeled	100	8.8	0.8
Corrected labeled*	100	3.5	6.2
%**	91	3	6

* Relative abundances of the ions from the labeled compound corrected for the naturally occurring A+1 and A+2 contributions of the unlabeled and singly labeled peaks.

** Mole % unlabeled, singly labeled, and doubly labeled species, respectively



Scheme 7. The synthesis of (2*RS*, 5*R*)-lysine-5-d_1: i. CH_2=CHCHO, NaOMe; ii. $NaBD_4$; iii. PCC; iv. (+)-α-pinene–9-BBN; v. MsCl, Et_3N; vi. KCN, DMSO; vii. H_2, PtO_2; H_3O^+

aldehyde **25** was obtained by the reaction of diethyl phthalimidomalonate with acrolein, reduction with sodium borodeuteride, then oxidation with pyridinium chlorochromate. (Use of transition metal oxidants is known to produce large primary kinetic isotopes.)[118,119] The aldehyde obtained in this manner was approximately 85–90% labeled with deuterium (as determined by its NMR spectrum.) Reduction of the labeled aldehyde with Midland's reagent, (+)-α-pinene–9-borobicyclo[3.3.1.]nonane (9-BBN), afforded the (*S*)-alcohol **26-*S***. Reduction of aldehydes with this reagent provides the chiral alcohol with an enantiomeric excess approaching 100%.[120] (*R*)-Nitrile **28-*R*** was obtained by displacement of the mesylate by cyanide ion. Hydrogenation of the nitrile over PtO_2 followed by acid hydrolysis afforded (2*RS*, 5*R*)-lysine-*5-d*$_1$ (**29-*R***). The (*S*)-deuterated amino acid (**29-*S***) was obtained in the same manner, except the reduction of the deuterated aldehyde was effected by (–)-α-pinene–9-BBN.

Virginiamycin S_1, obtained from fermentations in which each of the labeled lysines was administered, was analyzed by GC/MS in the same manner as before. The results of this analysis are shown in Table 6. The results show that the deuterium from (2*RS*, 5*R*)-lysine-*5-d*$_1$ is incorporated into the 3-hydroxypicolinic acid portion of the antibiotic, whereas that from (2*RS*, 5*S*)-lysine-*5-d*$_1$ is not. The *5-pro(S)* hydrogen of lysine is therefore lost in its transformation. If the mechanism proceeds by way of 5-hydroxylysine as has been implicated in the biosynthesis of etamycin,[94] then the *5-pro(R)* hydrogen is retained when C-5 is hydroxylated.

One question that may arise is whether both the D and the L isomers of lysine are incorporated. We know that the L-lysine is incorporated. If the D isomer is incorporated, it is likely that it would be isomerized to the L configuration, as enzymes capable of interconverting D- and L-amino acids are widely distributed in microorganisms. Lysine racemases have been found in *Proteus vulgaris* and a number of other microorganisms.[121]

There are a number of interesting experiments that would further elucidate this

Table 6. Relative abundance of the ions for the base peak (M–COOBu+H) of butyl 3-hydroxypicolinate from VS_1 labeled with (2*RS*,5*R*)-lysine-5-d_1 (I) and (2*RS*,5*S*)lysine-5-d_1 (II)

I.

m/z	95	96
Labeled	100	14.1
Unlabeled	100	8.8
Corrected labeled*	100	5.3
%**	95	5

II.

m/z	95	96
Labeled	100	7.9
Unlabeled	100	8.8
Corrected labeled*	100	–0.9
%**	100	0

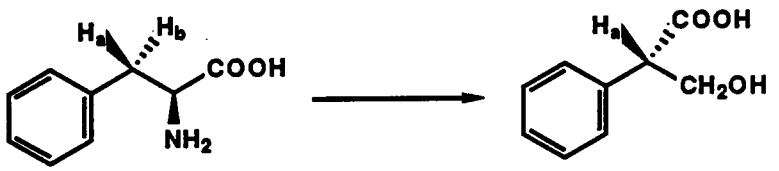
* Relative abundances of the ions from the labeled compound corrected for the naturally occurring A+1 contribution of the unlabeled peaks.

** Mole % unlabeled and labeled species, respectively

interesting process in virginiamycin S_1. Lysines stereospecifically deuterated at C-4 and C-5 could be incorporated. Studies like those done on etamycin could be done in virginiamycin S_1 to establish whether 5-hydroxylysine and 5-hydroxypipecolic acid are precursors. If 5-hydroxylysine is incorporated, then (5*R*)- and (5*S*)-hydroxylysines could be fed to determine which isomer is the preferred precursor. Similar experiments could be performed with labeled 5-hydroxypipecolic acid. A positive result from that experiment coupled with the information obtained from the present study would answer the question of whether the hydroxylation of lysine occurs with retention or inversion of the configuration of the 5-*pro-(R)* hydrogen that is retained from lysine.

2.3. The Origin of the L-Phenylglycine Residue

The mechanism of the conversion of L-phenylalanine to L-phenylglycine has not been previously studied. However, biosynthetic studies of the *N*-methyl derivative of L-phenylglycine, L-phenylsarcosine, were undertaken in the virginiamycin-like antibiotic etamycin.[92] In etamycin, L-phenylsarcosine arises from L-phenylalanine with the loss of the carboxyl group and the preservation of the remainder of the carbon skeleton without rearrangement. This is in contrast with the biosynthesis of tropic acid (**30**), a component of the tropane alkaloids hyoscyamine and scopolamine. Tropic acid also originates from L-phenylalanine, whose carboxyl group migrates to the prochiral C-3 position with retention of configuration.[122]



30

Biosynthetic studies on related systems provide insight into possible mechanisms for the transformation of L-phenylalanine to L-phenylglycine. For example, the β-lactam antibiotic nocardicin A contains L-*p*-hydroxyphenylglycine, which has been shown to be derived from L-tyrosine with clean loss of its carboxyl group.[123] Townsend and Brown report that labeled β-hydroxytyrosine and *p*-hydroxymandelic acid are incorporated into nocardicin A,[124] which suggests that tyrosine is hydroxylated to β-hydroxytyrosine or, if transamination has already occurred, the corresponding β-hydroxyketo acid. Oxidative decarboxylation would then produce *p*-hydroxymandelic acid; finally oxidation and transamination would yield *p*-hydroxyphenylglycine.

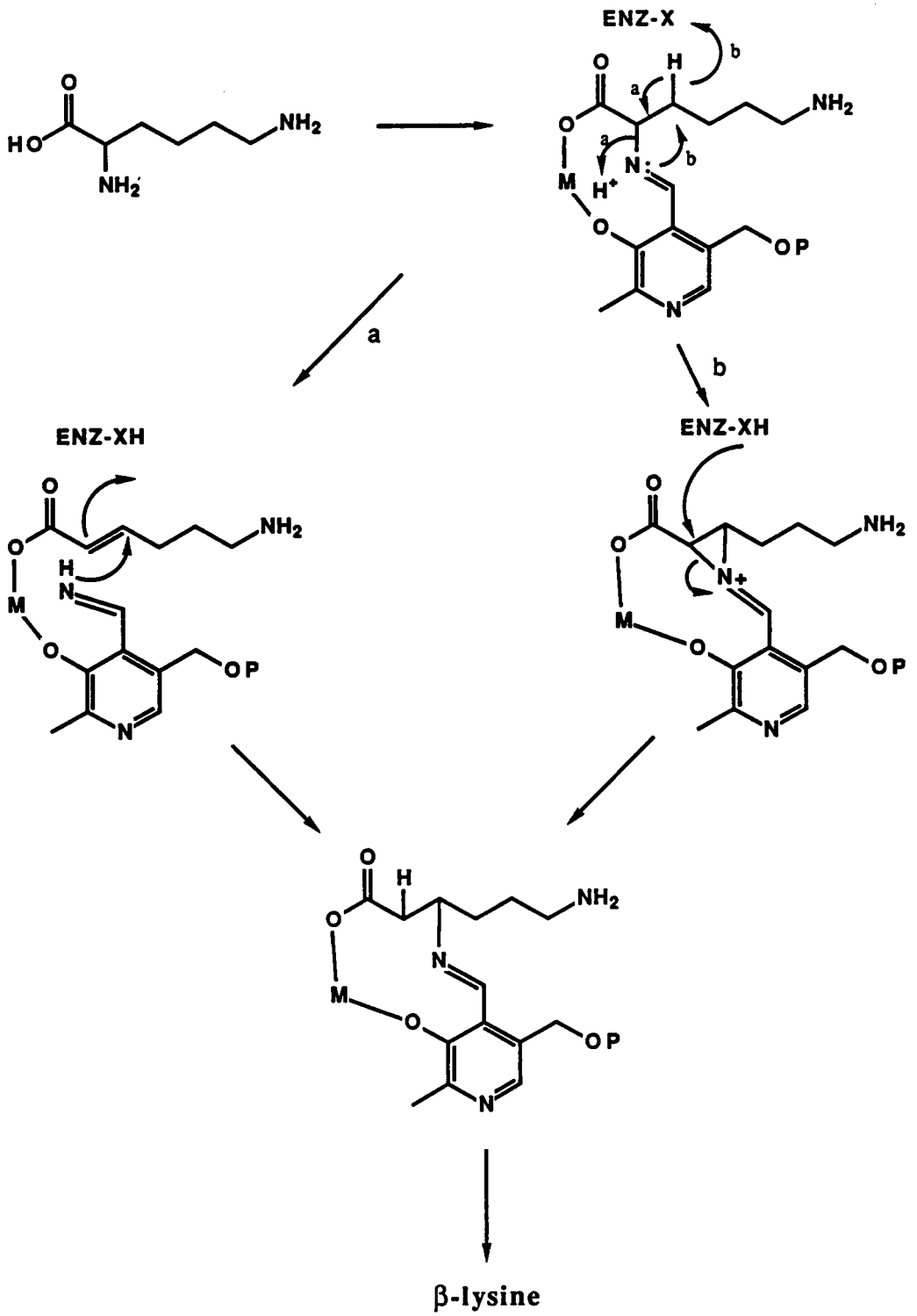
The antibiotic vancomycin, produced by the organism *Streptomyces orientalis*, contains *p*-hydroxyphenylglycine and *m*-chloro-β-hydroxytyrosine residues, both of which

are derived from tyrosine.[125] The related antibiotic avoparcin has a similar origin.[126] The presence of the β-hydroxy compound in these systems adds credence to Townsend's argument that tyrosine is initially hydroxylated in the biosynthesis of these amino acid residues.

None of the above studies broached the question of the nitrogen migration that occurs in the biogenesis of L-phenylglycine and related compounds. However, the 1,2-migration of nitrogen in the formation of some β-amino acids from the corresponding α-amino acids provides some suggestions for possible mechanisms for this process. In the antibiotics edeine A and edeine B,[127] produced in cultures of *Bacillus brevis*, the β-tyrosine residue is formed by the action of an α,β-mutase on tyrosine with the loss of the 3-*pro(S)* hydrogen. The original α-nitrogen is also lost, as is any labeled hydrogen at the 2-position; this suggests an intermediate in which a Schiff base is formed between the amino group of tyrosine and a carbonyl group at the active site of the enzyme. Whereas the loss of the 3-*pro(S)* hydrogen shows a relationship to the mechanism of the ammonia-lyase enzymes (one of which, for example, catalyzes the formation of cinnamic acid from phenylalanine), the latter observation is inconsistent with that kind of mechanism because ammonia-lyase reactions do not involve the exchange of α-hydrogens.

The conversion of lysine to β-lysine, a component of the antibiotic streptothricin F, produced by *Streptomyces* L-1689-23, has been shown to occur by the intramolecular migration of the α-nitrogen to C-3 with inversion of configuration.[128] There is a concomitant migration of the 3-*pro(R)* hydrogen to C-2, also with inversion of configuration, but by an intermolecular process. The same transformation occurs in *Clostridium subterminale* by the enzyme 2,3-aminomutase.[129] Two possible mechanisms are shown in Scheme 8.

The aspect of the transformation of L-phenylalanine to L-phenylglycine that particularly interested us was the migration of the nitrogen. The process could be an

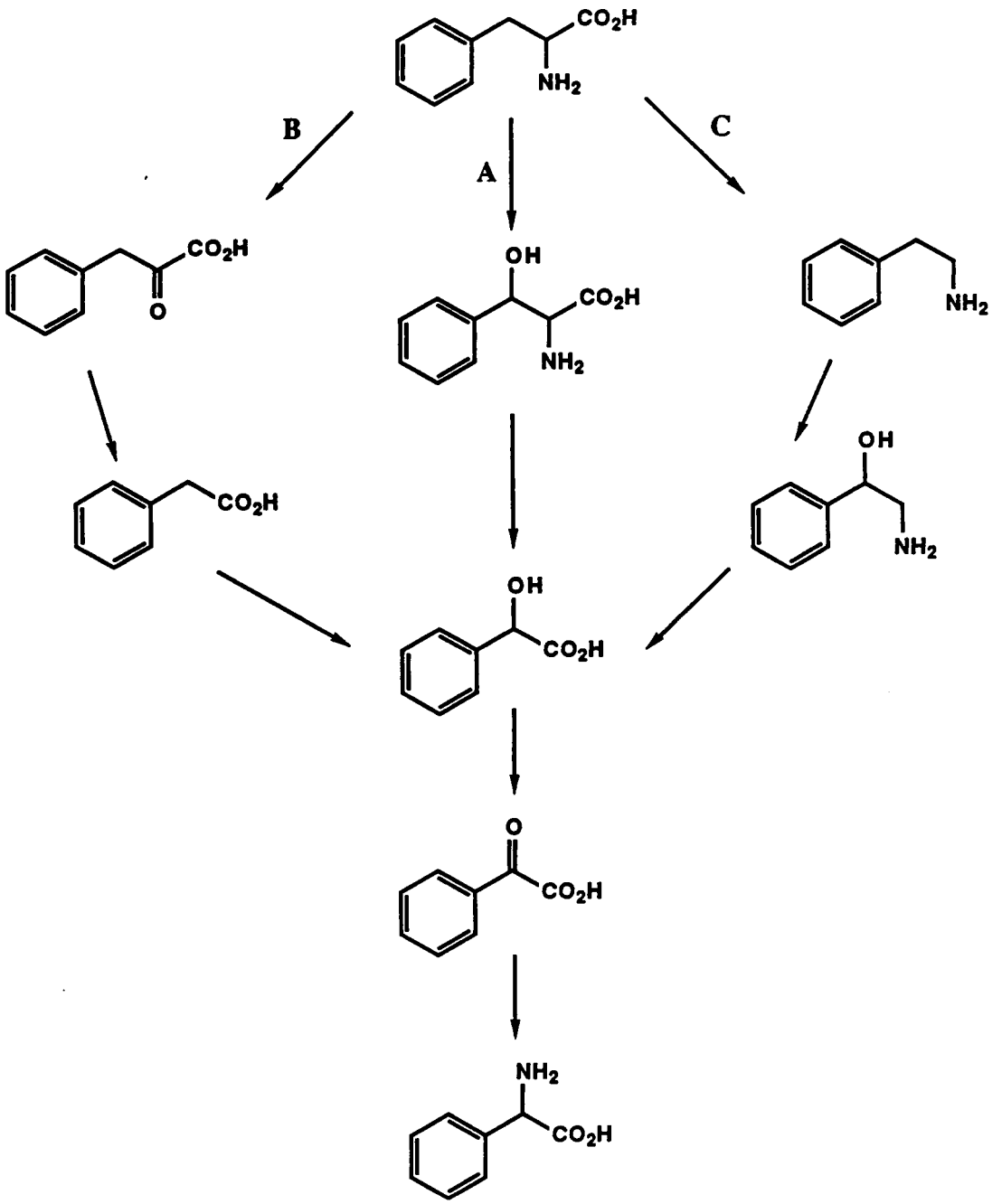


Scheme 8. Possible mechanisms for the conversion of lysine to β-lysine[128]

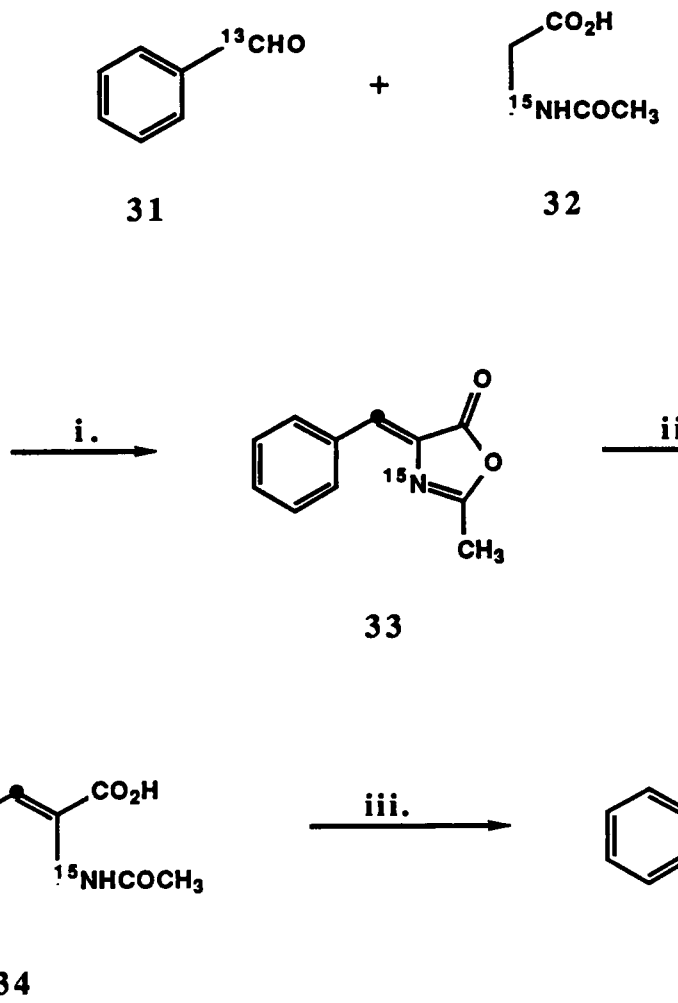
intermolecular process whereby the original nitrogen is lost—a number of mechanisms can be envisioned for an intermolecular process. Some of these are outlined in Scheme 9. In pathway A, phenylalanine is hydroxylated to β-hydroxyphenylalanine, which is degraded to mandelic acid. Phenylglyoxylic acid would arise from further oxidation, and finally transamination would yield phenylglycine. Phenylalanine, in pathway B, would first undergo transamination to phenylpyruvic acid, then oxidative decarboxylation to yield mandelic acid. In the last example, pathway C, phenylalanine would be decarboxylated to phenylethylamine, then hydroxylated, and oxidized to yield mandelic acid. If the nitrogen were transferred by an intramolecular process, a mechanism analogous to that proposed for the conversion of lysine to β-lysine (Scheme 8) could be operating.

We undertook then to determine whether the mechanism proceeds by an inter- or intramolecular mechanism. To this end, we planned to administer phenylalanine-*3-*^{13}*C-*^{15}*N* (**41**) to *S. virginiae*. If the mechanism were an intramolecular process, then the labeled nitrogen would be retained in the phenylglycine molecule, bonded to the labeled carbon. In this case, ^{13}C–^{15}N coupling of a few Hertz would be observed in the ^{13}C-NMR spectrum of the labeled virginiamycin S_1. If the mechanism were intermolecular, on the other hand, no coupling would be seen.

The doubly labeled phenylalanine was synthesized by minor modification of classical methods (Scheme 10). Benzoic-*carboxy-*^{13}*C* acid, prepared from phenyl magnesium bromide and $^{13}CO_2$ obtained from labeled barium carbonate, was reduced and reoxidized[130] to afford benzaldehyde-*carboxy*-^{13}C (**31**). The labeled benzaldehyde was condensed with *N*-acetylglycine-*^{15}N* to produce azlactone **33**.[131] Hydrolysis of the azlactone produced doubly labeled α-acetamidocinnamic acid (**34**), which was subsequently hydrogenated and hydrolyzed[132] to yield the desired DL-phenylalanine-*3-*^{13}*C-*^{15}*N* hydrochloride (**35**).



Scheme 9. Possible mechanisms for the conversion of phenylalanine to phenylglycine



Scheme 10. Synthesis of DL-phenylalanine-3-^{13}C-^{15}N: i. NaOAc, Ac_2O; ii. H_2O, acetone; iii. H_2, 5% Pd/C; H_3O^+

Following fermentation in a medium enriched with the labeled phenylalanine, virginiamycin S_1 (7.6 mg) was isolated. Its ^{13}C-NMR spectrum showed intense resonances at 36.7 ppm and 56.1 ppm due to C-3 of the *N*-methyl-L-phenylalanine residue and C-2 of the L-phenylglycine residue respectively. Examination of the peak at 56.1 ppm under high resolution conditions showed no splitting due to coupling to ^{15}N (Figure 7).

Because it is possible that the nitrogen could be washed out by transamination, as either phenylalanine or phenylglycine, failure to detect labeled nitrogen does not in itself prove that the process of interest proceeds by an intermolecular process. Fortunately the virginiamycin molecule contains an internal standard (*N*-methyl-L-phenylalanine) that can tell us whether any transamination occurs in phenylalanine (although it is not possible to determine to what extent it might occur in phenylglycine). Mass spectrometry was able to give us the required information. The labeled virginiamycin S_1 was hydrolyzed, and the resulting amino acids were converted to their *N*-trifluoroacetyl butyl ester derivatives, which were analyzed by GC/MS. The base peak for *N*-trifluoroacetyl-*N*-methyl-L-phenylalanine butyl ester is due to the ion produced by the loss of the butoxycarbonyl group. The data in Table 7 shows that this ion (therefore the intact amino acid) consists of 14% singly labeled and 8% doubly labeled species. It is apparent that some transamination has occurred, but if comparable transamination had occurred in phenylglycine and rearrangement were intramolecular, it would be possible to see splitting in the peak at 56.1 ppm. In addition, the derivative of phenylglycine was observed in the GC/MS experiment; all of the ions of the base peak (due also the the loss of the butoxycarbonyl group) were either unlabeled (86%) or singly labeled (14%). (See Table 7.) It has been shown, therefore, that the conversion of L-phenylalanine to L-phenylglycine proceeds by an intermolecular mechanism.

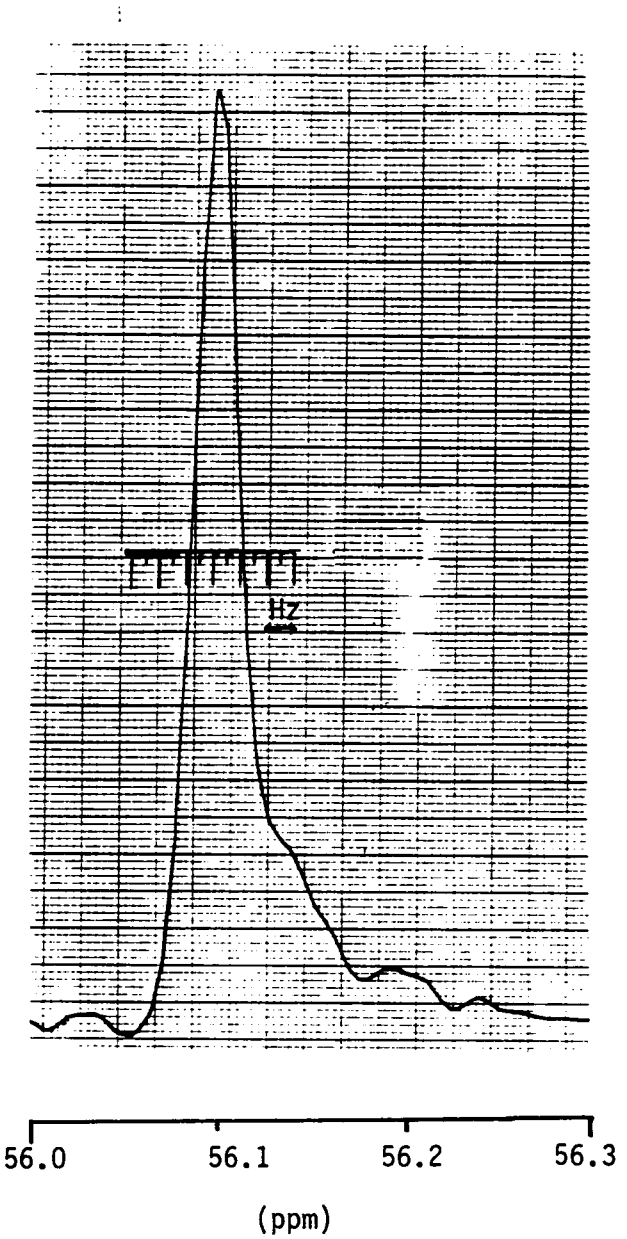


Figure 7. Partial ^{13}C-NMR spectrum of VS_1 labeled with DL-phenylalanine-*3-^{13}C-^{15}N*: the signal at 56.1 ppm

Table 7. Relative abundance of the ions for the base peaks (M–COOBu) of *N*-trifluoroacetyl-*N*-methyl-L-phenylalanine butyl ester (I) and *N*-trifluoroacetyl-L-phenylglycine butyl ester (II) from VS_1 labeled with (2*RS*)-phenylalanine-3-^{13}C-^{15}N

I.

m/z	230	231	232
Labeled	100	31.2	13.6
Unlabeled	100	13.0	1.2
Corrected labeled*	100	18.2	10.0
%**	78	14	8

II.

m/z	202	203	204
Labeled	100	44.9	7.3
Unlabeled	100	28.3	3.4
Corrected labeled*	100	16.6	–0.7
%**	86	14	0

* Relative abundances of the ions from the labeled compound corrected for the naturally occurring A+1 and A+2 contributions of the unlabeled and singly labeled peaks.

** Mole % unlabeled, singly labeled, and doubly labeled species, respectively

3. EXPERIMENTAL

3.1. General

Melting points were determined on a Kofler block and were uncorrected. Radioactivity was determined by means of a Beckman LS-3800 or Beckman LS-100 liquid scintillation counter. The liquid scintillation cocktail was Beckman Readi-Solv MP or a cocktail consisting of 2,5-diphenyloxazole (4 g), *p*-bis(2-(4-methyl-5-phenoxazolyl))-benzene (0.2 g), and Triton X-100 (333 mL) in enough toluene to make 1 L.

The following chromatographic materials were used: for analytical thin-layer chromatography (TLC), Merck silica gel 60 F254 on aluminum, 0.2 mm thickness; for preparative scale TLC, Analtech Uni-Taper silica gel GF plates or Analtech silica gel GF (500 μm); for column chromatography (flash), Merck silica gel 60 (230–400 mesh); for ion-exchange chromatography, Dowex 50W (8% cross-linked, dry mesh 200–400) or Dowex 2 (8% cross-linked, dry mesh 100–200) resin. The following system was employed for high performance liquid chromatography (HPLC): a Waters Associates M6000A pump, a Valco six-port injection valve, and a Waters Associates 440 absorbance detector (at 254 nm). In some cases a Tracor 980 A solvent programmer was utilized. For analytical HPLC, Waters Nova-Pak Radial-Pak columns (C8 or C18) were used with a Waters Associates RCM-100 radial compression module, for preparative scale HPLC, a 30 x 1 cm column packed with LiChrosorb RP-8 (10 μm).

Nuclear magnetic resonance (NMR) spectra were determined on a Bruker WP-270 or a Bruker WP-200 spectrometer. Mass spectra were obtained on a Finnigan–MAT 112 mass spectrometer or a VG Analytical 7070E mass spectrometer. The gas chromatographs used in GC/MS analyses were a Varian 2100 (coupled to the Finnigan instrument) or a Hewlett–Packard 5790A (coupled to the VG instrument). Infrared (IR) spectra were determined on a Perkin–Elmer 710B or a Perkin–Elmer 283B infrared spectrophotometer.

3.2. Culture Conditions

The microorganism *Streptomyces virginiae* strain 1830 was obtained from SmithKline Animal Health Products, West Chester, Pennsylvania, as either a pellet or a slant. The strain was maintained on either soluble-starch–agar or potato–glucose–agar slants. The soluble-starch–agar slants were prepared by combining corn starch (10 g), $(NH_4)_2SO_4$ (2 g), K_2HPO_4 (1 g), $MgSO_4 \cdot 7H_20$ (1 g), NaCl (1 g), and $CaCO_3$ (3 g) in 1 L water. The pH was adjusted to 6.8 with 2 N HCl. Agar (20 g) was added, and the mixture was dispensed into test tubes and sterilized in an autoclave 30 min at 120 °C (15 psi). Each sterile slant was inoculated with a spore suspension (0.2 mL) obtained from either a pellet or another slant and incubated 6–8 days at 25–28 °C. The slants were stored at 4 °C until needed. The potato–glucose–agar slants were prepared as follows: Cut-up potatoes (200 g) were boiled in water for 30 min and the mixture filtered through cheesecloth. After glucose (10 g) was added to the filtrate, water was added to bring the volume to 1 L, and the pH was adjusted to 7.4 with 1 N NaOH. Following the addition of agar (20 g), the mixture was dispensed into test tubes, then sterilized and inoculated as before.

The vegetative inoculum was prepared by transferring a spore suspension from a slant to a baffled 250-mL Erlenmeyer flask containing 30 mL of medium STA-2.[133] STA-2 was prepared as follows: A mixture of corn-steep liquor (36.5 g) in 1 L tap water was adjusted to pH 7.5 with NaOH. After the addition of peanut-oil cake (8 g), the mixture was boiled for 2 min, then filtered. Glucose (50 g), $MnSO_4$ (0.01 g), and $CaCO_3$ (5 g) were added. The medium was dispensed into baffled 250-mL Erlenmeyer flasks and sterilized for 30 min at 120 °C. The inoculum was incubated at room temperature on a rotary shaker (Lab-Line Orbit Environ-Shaker, Lab-Line Instruments) at 330 rpm for 72 h. After this time, the vegetative inoculum was transferred to the production medium STA-14[133] in a fermentor (MultiGen 2-L fermentor, New Brunswick Scientific). Medium STA-

14 was prepared as follows: One liter tap water containing corn-steep liquor (36 g) and yeast autolysate (5 g) was adjusted to pH 7.9 with NaOH. Peanut-oil cake (10 g) was added; the mixture was boiled for 2 min, then filtered. Linseed oil (10 g), glucose (5 g), glycerol (25 g), and $CaCO_3$ (5 g) were added. The mixture was transferred to the fermentor vessel and sterilized in an autoclave for 45 min at 120 °C (15 psi). The broth was aerated at 1.25 L/min and stirred at 450 rpm. Precursors were added after 8–10 h, introduced through a 0.45 μm filter. Fermentation was continued for a total time of 48 h. In some runs, pH was maintained between 6.5 and 6.8 by the addition of sterile 1 N NaOH.

3.3. Isolation of Virginiamycin S_1

Virginiamycin S_1 was isolated from the culture broth after the completion of the incubation period. The broth was filtered with the aid of Hyflo Super Cel in order to remove the mycelia, then the filtrate was extracted twice with a one-third volume of hexane, then three times with a one-half volume of ethyl acetate. The ethyl acetate layers were combined, washed with a one-half volume of water, dried over $MgSO_4$, and evaporated at reduced pressure. Virginiamycin S_1 was isolated by flash chromatography with $CHCl_3$–MeOH (99:1) and shown to be >90% pure by analytical HPLC.

3.4. Analysis of Virginiamycin S_1 Amino Acids by GC/MS

Virginiamycin S_1 to be analyzed by GC/MS was hydrolyzed in 1 mL 6 N HCl at 105 °C for 24 h. *N*-Trifluoroacetyl amino acid butyl esters of the virginiamycin S_1 amino acids were prepared in the following manner: After evaporation to dryness, the residue was dissolved in dry 3–4 N HCl in 1-butanol (prepared either by bubbling dry HCl through dry 1-butanol or by adding acetyl chloride to 1-butanol) and heated at 110 °C for 3

h (15 h in some cases). The excess reagent was evaporated under a stream of N_2, then 1 mL dichloromethane and 1 mL trifluoroacetic anhydride were added. The reaction mixture stood at room temperature for 24 h, then evaporated to dryness under a stream of N_2. The residue was dissolved in a minimal amount of dichloromethane for analysis by GC/MS.

Derivatives of unlabeled amino acids (25-40 mg of each amino acid of interest) to use as standards were prepared in the same way. The derivative of 3-hydroxypicolinic acid was prepared on a larger scale: 3-Hydroxypicolinic acid (110 mg, 0.79 mmol) in 2 mL 4 N HCl in dry 1-butanol was heated overnight at 110 °C. There appeared to be a considerable amount of starting material remaining at the end of this period. The mixture was evaporated to dryness; the residue was dissolved in 2 mL distilled water and brought to pH 6 with NaOH. The product (28 mg, 15% yield) was isolated by extraction of the aqueous mixture with an equal volume of ethyl acetate, evaporation to dryness, then flash chromatography (5 g silica gel; ethyl acetate–hexane, 6:4). The ester was dissolved in 1 mL dichloromethane and 1 mL trifluoroacetic anhydride and allowed to stand for 23 h. Only the ester was detected (TLC; ethyl acetate–hexane, 6:4) at the end of the reaction time. Butyl 3-hydroxypicolinate: ^{1}H NMR ($CDCl_3$) δ 0.98 (t, 3H, J = 7.4 Hz), 1.48 (m, 2H), 1.86 (m, 2H), 4.78 (t, 2H, J = 6.9 Hz), 7.40 (m, 2H), 8.30 (dd, 1H, J = 1.8 Hz, J = 4.0 Hz), 10.80 (s, 1H).

3.5. Incorporation of L-Methionine-*methyl-^{14}C*

S. virginiae 1830 was grown in media STA-2 and STA-14 as previously described. Eight hours after the production medium STA-14 was inoculated, 27 μCi L-methionine-*methyl-^{14}C* (New England Nuclear; specific activity, 45.7 mCi/mmol) in 4.4 mL sterile 0.01 N HCl was added to the fermentation medium. The pH of the medium was maintained in the range of 6.5–6.8. After 48 h, the fermentation was halted, and

virginiamycin S_1 was isolated as previously described. The antibiotic was further purified by HPLC (acetonitrile–water, 1:1; flow rate, 6 mL/min). Pure virginiamycin S_1 (5.9 mg) was obtained in this manner. A portion (10%) of the antibiotic was counted. The specific activity of the virginiamycin S_1 was determined to be 1.1×10^{-2} mCi/mmol. The specific incorporation was then 2.4×10^{-2}. A portion of the antibiotic (3.2 mg) was hydrolyzed in 1 mL 6 N HCl at 104 °C for 24 h. The hydrolysate was evaporated to dryness at reduced pressure; excess HCl was removed by storing the residue over NaOH pellets in an evacuated desiccator overnight. The residue was dissolved in 1 mL water and placed on a column (1 x 44 cm) containing Dowex-50W ion-exchange resin (H^+ form). The column was eluted with 1 N HCl; 1-mL fractions were collected The fractions containing 4-oxo-L-pipecolic acid (52–64) were combined and evaporated to dryness. The isolated compound showed a single spot on TLC and co-chromatographed ($R_f = 0.41$) with authentic 4-oxo-L-pipecolic acid hydrochloride (1-propanol–water, 7:3). It had a specific activity of 1.9×10^{-4} mCi/mmol (on the assumption that 100% of the amino acid was recovered); 1.7% of the total radioactivity of virginiamycin S_1 was located in the 4-oxo-L-pipecolic acid residue.

3.6. Incorporation of L-Aspartic-*U*-^{14}C Acid

This experiment was performed in a similar manner to the one previously described for the incorporation of L-methionine-*methyl*-^{14}C. L-Aspartic-*U*-^{14}C acid (ICN, 49.8 μCi; specific activity, 184 mCi/mmol) and 0.18 g L-threonine (added to enhance production) were dissolved in 5.0 mL 0.01 N HCl and added to medium STA-14 8 h after inoculation. Virginiamycin S_1 (3.6 mg) was isolated by flash chromatography as previously described and had a specific activity of 1.1×10^{-3} mCi/mmol. Specific incorporation was 5.9×10^{-4}. 4-Oxo-L-pipecolic acid obtained as before had a specific activity of 7.9×10^{-5} mCi/mmol; 7.1 % of the radioactivity of the labeled antibiotic was found in the 4-oxo-L-pipecolic acid residue.

3.7. Incorporation of L-Lysine-*U*-^{14}C

The incorporation of L-lysine-*U*-^{14}C was undertaken in a similar manner to those of the other radiolabeled precursors. L-Lysine-*U*-^{14}C•HCl (ICN, 49.8 μCi; specific activity, 275 mCi/mmol) and L-threonine (0.10 g) in 4.5 mL 0.01 N HCl were added to medium STA-14 8 h after inoculation. Virginiamycin S_1 was isolated by flash chromatography as previously described. It had a specific activity of 2.7 x 10^{-2} mCi/mmol; specific incorporation was 9.8 x 10^{-3}.

3.8. Incorporation of DL-Lysine-*6*-^{13}C-*6*-^{15}N

The fermentation and preliminary purification of the antibiotic took place at the laboratories of SmithKline–RIT, Rixensart, Belgium. *S. virginiae* 5722 was grown in a vegetative medium and transferred to a production medium (40 mL in each of 29 250-mL Erlenmeyer flasks) after 2 days. After 24 h, 200 μL of a solution of 65 mg DL-lysine-*6*-^{13}C-*6*-^{15}N,[104] prepared from $Na^{13}C^{15}N$ (Prochem, BOC Limited; 90% ^{13}C, 99% ^{15}N; the intermediate ethyl 2-carbethoxy-2-phthalimido-5-cyanopentanoate-*6*-^{13}C-*6*-^{15}N was determined by MS to be 76.1% doubly labeled and 7.8% singly labeled) and 100 mg L-threonine in 6 mL distilled water, sterilized by passing through a Sartorius membrane (0.22 μm), was added to each flask

After 3 days, the broth was acidified to pH 4.8 with 10% H_2SO_4, then extracted three times with methyl isobutyl ketone. The organic extracts were combined and evaporated to dryness. The residue was dissolved in acetonitrile; the solution was washed twice with *n*-hexane and evaporated to dryness. The new residue was dissolved in 4 mL chloroform. Crude virginiamycin (672 mg) was precipitated by the addition of 40 mL *n*-hexane, then filtered. HPLC analysis indicated that the crude material contained

approximately 360 mg virginiamycin M_2, 80 mg virginiamycin M_1, 16 mg virginiamycin M_3, and 41 mg virginiamycin S_1.

Virginiamycin S_1 was isolated from the mixture as previously described. One-third of the antibiotic was hydrolyzed by heating at 105 °C in 1 mL 6 N HCl for 24 h. *N*-Trifluoroacetyl amino acid butyl esters were prepared as previously described. Because the amount of the proline derivative was much greater than that of the 3-hydroxypicolinic acid derivative, and because separation of the two peaks by GC was unsatisfactory in this case, the mixture of amino acid derivatives was partially purified by preparative TLC (ethyl acetate–hexane, 1:1). The partially purified mixture contained both the 3-hydroxypicolinic acid and 4-oxo-L-pipecolic acid derivatives, but very little of the proline derivative. This mixture was separated by GC (10% SP2100, 6 ft x 2 mm ID, 80 °–300 °C at 10 °/min, or 3% OV1, 3 ft x 2 mm ID, 75 °–200 °C at 10 °/min); the peaks due to butyl *N*-trifluoroacetyl-4-oxo-L-pipecolate and butyl 3-hydroxypicolinate were analyzed by MS. The isotopic composition of the major fragment ion of each derivative was determined on an average of 4–10 spectra by the method of Beimann.[134]

3.9. Synthesis of 4-Oxo-DL-pipecolic Acid Hydrochloride

2-Cyano-4-piperidone Ethylene Ketal (12). To a stirred slurry of *N*-chlorosuccinimide (5.15 g, 38.6 mmol) in freshly distilled dry ether (100 mL) was added dropwise 4-piperidone ethylene ketal (5.01 g, 35.0 mmol). The mixture was stirred at room temperature under N_2 atmosphere for 17.5 h and then filtered. The filtrate was evaporated at reduced pressure to give a colorless oil containing some solid material. An additional portion of ether was added to the residue, and the mixture was filtered.

To the filtrate was added 18-crown-6 (0.107 g, 0.4 mmol) and potassium superoxide (4.99 g, 70.0 mmol). The slurry was stirred at room temperature for 24 h and then filtered. The residue remaining on the filter was washed with a small portion of ether.

The combined filtrate and washings were extracted twice with 100 ml 0.5 N HCl.

A solution of potassium cyanide (11.4 g, 0.175 mol) in 100 ml water was cooled on an ice-water bath, and concentrated HCl was carefully added to bring the solution to pH 7. The aqueous extract was added to the acidified potassium cyanide solution over 0.5 h, after which time the pH of the reaction mixture was adjusted to 5 with potassium hydroxide pellets, and stirring was continued for 7 h, with warming to room temperature. The mixture was cooled in an ice-water bath, and potassium hydroxide pellets were added to adjust the solution to pH 9. The mixture was extracted with ethyl acetate (3 x 200 mL). The combined extracts were dried over $MgSO_4$. Evaporation yielded a pale yellow oil, which after flash chromatography ($CHCl_3$–MeOH, 9:1) afforded 2.75 g of the product (47% overall yield). ^1H NMR ($CDCl_3$) δ 1.72 (t, 2H, J = 5.6 Hz), 1.78 (NH, br s, 1 H), 1.96 (m, 2H), 2.93 (m, 2H), 3.16 (m, 1H), 4.01 (m, 5H); ^{13}C NMR (DMSO) δ 34.9, 37.1, 41.4, 44.4, 63.6, 63.8, 105.4 (OCO), 120.6 (CN); IR (neat) 895, 935, 975, 1025, 1140, 1235, 1290, 1355, 2220, 2885, 2930, 3300 cm^{-1}; mass spectrum m/z (relative abundance) 168 (M^+) (63), 141 (21), 126 (10), 99 (32), 96 (8), 87 (34), 86 (100).

4-Oxo-DL-pipecolic Acid Ethylene Ketal (13). A mixture of 2-cyano-4-piperidone ethylene ketal (0.166 g, 0.99 mmol), barium hydroxide octahydrate (0.176 g, 0.56 mmol), and water (2 mL) was heated at 95 °C for 2.5 h. After the addition of more water (2 mL), small chunks of dry ice were added to the reaction mixture, with the temperature maintained at 90–95 °C. The mixture was stirred for 15 min, and it was then filtered. Water (2 mL) was added to the residue, and this was heated for 10 min, then filtered. The combined filtrates were heated with Norite and filtered. The filtrate was evaporated to give a colorless crystalline residue (0.14 g, 74%). The product was recrystallized from 2-propanol with a trace of water and ethyl ether. MP 246 °C; ^1H NMR

(D_2O) δ 1.78–1.64 (m, 3H), 2.09 (m, 1H), 2.93 (m, 1H), 3.29 (dt, 1H), 3.56 (C-2, dd, 1H), 3.84 (OCH_2, m, 4 H); ^{13}C NMR (D_2O, dioxane = 66.5) δ 30.7, 34.8, 41.1, 57.3, 64.6, 104.9 (OCO), 172.8 (COOH); IR (KBr) 1050, 1105, 1185, 1405, 1600, 2950, 3400 cm^{-1}. As the hydrochloride: mp 210–215 °C; ^1H-NMR (D_2O) δ 1.72–1.89 (m, 3H), 2.18 (m, 1H), 3.01 (dt, 1H), 3.35 (m, 1H), 3.85 (OCH_2, m, 4H), 3.94 (CH, dd, 1H); ^{13}C-NMR (D_2O, dioxane = 66.5) δ 30.7, 33.9, 41.2, 55.5, 64.7, 104.3 (OCO), 170.7 (COOH); IR (KBr) 1050, 1110, 1190, 1380, 1405, 1560, 1760, 2800 (v br) cm^{-1}; mass spectrum m/z (relative abundance) 187 (M^+–HCl) (0.4), 142 (54), 112 (8), 99 (12), 87 (100), 86 (11), 56 (56).

4-Oxo-DL-pipecolic Acid Hydrochloride (14). 4-Oxo-DL-pipecolic acid ethylene ketal hydrochloride (0.16 g, 0.72 mmol) was heated in 5 mL 2 N HCl at 85 °C for 15 h. The mixture was neutralized with 6 N NaOH; then placed on a Dowex 2 (OH^- form) column (2 x 14 cm.) The column was washed with water (100 mL) to remove the sodium ions, then eluted with 1 N aqueous acetic acid. The fractions containing 4-oxo-DL-pipecolic acid (TLC: 1-propanol–water, 7:3) were combined along with 1 mL 6 N HCl and evaporated to dryness to afford 38 mg (30%) of yellowish product, which showed one spot on TLC and co-chromatographed with authentic material (R_f = 0.4). ^1H NMR (D_2O) δ 1.68–1.88 (m, 3H), 2.21 (m, 1H), 3.00 (dt, 1H), 3.29 (dt, 1H), 3.89 (C-2, dd, 1H).

3.10. Synthesis of Virginiamycin S_1 Amino Acid Derivatives for HPLC

N-Benzoyl derivatives of virginiamycin S_1 amino acids were prepared according to standard Schotten–Baumann conditions (benzoyl chloride in ether, 1 N NaOH) or according to a procedure adapted from Pirkle (benzoyl chloride, THF, propylene oxide).[135]

***N*-Benzoyl-L-threonine.** L-Threonine (0.46 g, 3.9 mmol) was dissolved in 1

N NaOH (10 mL). Ether (10 mL) containing 0.50 mL benzoyl chloride (0.61 g, 4.3 mmol) was added, and the two-phase mixture was vigorously stirred for 24 h at room temperature. The ether was evaporated, and the aqueous layer was acidified with 6 N HCl, then extracted with ethyl acetate (2 X 20 mL). The organic extract was dried over $MgSO_4$ and evaporated at reduced pressure. The residue was recrystallized from ethyl acetate to yield 0.33 g (38%) product, mp 148–149 °C (literature 146–148 °C).[136]

***N*-Benzoyl-DL-α-aminobutyric acid.** DL-α-Aminobutyric acid (0.42 g, 4.0 mmol) was dissolved in 1 N NaOH (10 mL). Ether (10 mL) containing 0.5 mL benzoyl chloride (0.61 g, 4.3 mmol) was added, and the two-phase mixture was vigorously stirred for 24 h at room temperature. The ether was evaporated, and the aqueous layer was acidified with 6 N HCl, then extracted with ethyl acetate (2 X 20 mL). The organic extract was dried over $MgSO_4$ and evaporated at reduced pressure. The residue was recrystallized from ethyl acetate–hexane to give 0.42 g (51%) product, mp 143–144 °C (literature 145–146 °C).[137]

***N*-Benzoyl-L-proline.** To a slurry of L-proline (0.50 g, 4.3 mmol) in dry THF was added 0.50 mL benzoyl chloride (0.61 g, 4.3 mmol) and 0.90 mL propylene oxide (0.75 g, 13 mmol). The mixture was stirred under N_2 at room temperature until the solid proline had disappeared (45 min), then for an additional 45 min. The reaction mixture was filtered with suction to remove any unreacted proline, then evaporated at reduced pressure to yield a clear oil (1.12 g) that crystallized on standing. The crude product was recrystallized from ethanol–petroleum ether to give 0.23 g of the product (mp 154–156 °C, literature 156 °C).[138] The mother liquor yielded an additional crop (0.41 g, mp 153–157 °C) for a total yield of 68%.

***N*-Benzoyl-*N*-methyl-L-phenylalanine.** To a slurry of *N*-methyl-L-

phenylalanine (0.125 g, 0.70 mmol) in dry THF (5 mL) was added 81 μL benzoyl chloride (98 mg, 0.7 mmol) and 150 μL propylene oxide (0.125 g, 2.1 mmol). The reaction mixture was stirred at room temperature in a flask surmounted with a drying tube for 24 h, after which time no solid remained. Evaporation of the solvent left 0.21 g crude product, which was subsequently recrystallized from HOAc–water to give 0.108 g (54.5%) of the derivative, mp 136-138 °C.[139]

***N*-Benzoyl-L-phenylglycine.** L-Phenylglycine (0.45 g, 3.0 mmol) was dissolved in 1 N NaOH (10 mL). Ether (10 mL) containing 0.40 mL benzoyl chloride (48 g, 3.4 mmol) was added, and the two-phase mixture was vigorously stirred for 24 h at room temperature. The ether was evaporated, and the aqueous layer was acidified with 6 N HCl, then extracted with ethyl acetate (2 X 20 mL). The organic extract was dried over $MgSO_4$ and evaporated at reduced pressure. The residue was recrystallized from ethyl acetate to provide 0.36 g (47%) product, mp 187–188 °C (literature 192–193 °C).[140]

***N*-Benzoyl-4-oxo-DL-pipecolic Acid Ethylene Ketal (15).** To a slurry of 4-oxopipecolic acid ethylene ketal (13) (0.050 g, 0.27 mmol) in 3 ml dry THF was added 60 μL propylene oxide (0.0498 g, 0.86 mmol) and 40 μL benzoyl chloride (0.048 g, 0.34 mmol). The mixture was stirred at room temperature for 24 h. After filtration, it was evaporated under reduced pressure to yield a crude product still containing traces of benzoyl chloride. The product was purified by preparative TLC to yield an oil (0.056 g, 72%), which had partially decomposed on the silica gel TLC plate.

3.11. Separation of Virginiamycin S_1 Amino Acids by HPLC

A portion of virginiamycin S_1 (up to 50 mg) was hydrolyzed in 1 mL 6 N HCl at 105 °C for 24 h. The mixture was evaporated to dryness, then dissolved in 1 mL 1 N NaOH. To this was added a solution of 1.1 equivalents benzoyl chloride in 1 mL ether.

The two-phase reaction mixture was vigorously stirred at room temperature for 24 h. The ether layer was evaporated, and the aqueous mixture was acidified with 6 N HCl and extracted with ethyl acetate. The aqueous layer contained 3-hydroxypicolinic acid, which remained underivatized under the reaction conditions. The organic extract was evaporated to dryness, redissolved in methanol or acetonitrile , and analyzed by HPLC.

The amino acid derivatives were separated on a C18 column by means of a gradient solvent system. The solvent compositions were as follows: solvent A, water–methanol–THF–formic acid, 85:11.5:2.5:1; solvent B, water–methanol–THF–formic acid, 58:40:1:1. The solvent programmer was programmed for an initial delay of 4 min with solvent A, then a linear gradient (10% per min) until the solvent passing through the system consisted only of solvent B.

3.12. Synthesis of (2*RS*, 5*R*)-Lysine-5-d_1 Dihydrochloride

4,4-Dicarbethoxy-4-phthalimidobutanal (23).[117] To a solution of diethyl phthalimidomalonate (6.11 g, 20 mmol) in benzene (30 mL) was added 0.057 g sodium methoxide (1.1 mmol), and the resulting lemon-yellow solution was cooled to 0 °C in an ice-water bath. A solution of freshly distilled acrolein (1.5 mL, 22 mmol) in benzene (5 mL) was added dropwise to the mixture over 0.5 h, with stirring under N_2 atmosphere. After the addition was complete, the ice-water bath was removed, and stirring was continued for 1 h. The reaction was quenched by the addition of 4 drops of glacial acetic acid. Evaporation of the benzene from the mixture at reduced pressure left a turbid straw-colored oil. Flash chromatography (ethyl acetate–hexane, 3:7) provided 3.89 g (89% yield) pure aldehyde **23** as a colorless oil: R_f = 0.31 (ethyl acetate–petroleum ether, 6:4); ^{1}H-NMR ($CDCl_3$) δ 1.29 (CH_3, t, 6H, J = 7.1 Hz), 2.72 (m, 2H), 2.86 (m, 2H), 4.32 (OCH_2, q, 2H, J =7.1 Hz), 4.33 (OCH_2, q, 2 H, J =7.1 Hz), 7.81(Ar, m, 4H), 9.71

(CHO, t, 1H, J = 0.9 Hz); ^{13}C-NMR ($CDCl_3$) δ 13.6, 25.3, 39.0, 62.8, 66.8, 123.4, 134.4, 165.8, 167.2, 200.5 (CHO).

Ethyl 2-carbethoxy-2-phthalimido-5-hydroxypentanoate-*5*-d_1 (24). Sodium borodeuteride (0.767 g, 18.3 mmol) was added in small portions over 3 h to a vigorously stirred mixture of aldehyde **23** in ether (85 mL) and water (5 mL). The mixture was stirred for an additional 0.5 h, then for another 15 min following the addition of 35 mL water. The aqueous layer was separated from the ether layer and subsequently extracted with additional ether (5 x 50 mL). The combined ether layers were dried over $NaSO_4$ and evaporated in vacuo. Flash chromatography (ethyl acetate–hexane, 1:1) afforded the labeled alcohol as a colorless oil (3.43 g, 53%): ^{1}H-NMR ($CDCl_3$) δ 1.29 (CH_3, t, 6H, J =7.1 Hz), 1.69 (m, 2H), 2.58 (m, 2H), 3.63 (CHDOH, br t, 1H), 4.30 (CH_2O, q, 2H, J = 7.1 Hz), 4.31 (CH_2O, q, 2H, J = 7.1 Hz), 7.81 (Ar, m, 4H); ^{13}C-NMR ($CDCl_3$) δ 13.7, 27.5, 29.5, 61.8 (CHDOH, t, J = 21.4 Hz), 62.5, 67.6, 123.4 (Ar), 131.3 (Ar), 134.3 (Ar), 166.2, 167.3.

4,4-Dicarbethoxy-4-phthalimidobutanal-*1*-d_1 (25). To a mixture of pyridinium chlorochromate (7.30 g, 3.9 mmol) and sodium acetate (1.39 g, 16.9 mmol) in dry CH_2Cl_2 (45 mL) was added labeled alcohol **24** in dry CH_2Cl_2. An additional 30 mL CH_2Cl_2 was used to rinse the flask that had contained the alcohol. The dark brown mixture was stirred at room temperature under N_2 for 4 h. Ether (100 mL) was added to the mixture, and stirring was continued for 5 min. The mixture was filtered through a layer of Florisil (3.5 x 6.5 cm) topped with a 1-cm layer of Hyflo Super Cel. Ether (100 mL) and CH_2Cl_2 (100 mL) were added to the residue remaining in the flask and filtered. The filtrate was evaporated to give a pale yellow oil, which was purified by flash chromatography (ethyl acetate–hexane, 3:7) to provide aldehyde **29** (4.33 g, 73%) as a colorless oil. ^{1}H-NMR (identical to that of the unlabeled aldehyde except for the reduced

intensity of the peak at 9.71 ppm) showed the aldehyde to be approximately 88-90% labeled. ^{13}C-NMR ($CDCl_3$) δ 199.9 (CDO, t, J = 26 Hz), otherwise identical to the unlabeled aldehyde **23**.

Ethyl (5*S*)-2-carbethoxy-2-phthalimido-5-hydroxypentanoate-*5-d*$_1$ (26-*S*). A solution of (1*R*)-(+)-α-pinene (Aldrich, 98% optical purity) (3.69 g, 27.1 mmol) in 49 mL 0.5 M 9-BBN in THF (24.5 mmol) was heated at reflux with stirring under N_2 atmosphere for 4 h. The mixture was cooled and stored under N_2 overnight. Labeled aldehyde (**25**) was dissolved in 25 mL dry THF and transferred by means of a cannula to the pinanyl borane mixture. The reaction mixture was stirred under N_2 for 8 h, then it was placed on a silica gel column (5.5 x 15 cm) and eluted with ether. The fractions containing the alcohol were combined, and the solvent was removed in vacuo. The alcohol was purified further by flash chromatography (ethyl acetate–petroleum ether, 1:1 then 6:4). The chirally deuterated alcohol (3.78 g) was obtained in 84% yield: ^{1}H-NMR and ^{13}C-NMR spectra were identical to those of alcohol **24**.

Ethyl (5*S*)-2-carbethoxy-2-phthalimido-5-methanesulfonyloxypentanoate-*5-d*$_1$ (27-*S*). To an ice-cold stirred mixture of the (*S*)-alcohol **26-*S*** (3.78 g, 10.4 mmol) and triethylamine (2.11 g, 20.8 mmol) in 75 mL CH_2Cl_2 was added dropwise methanesulfonyl chloride (2.40 g, 20.9 mmol) in 15 mL CH_2Cl_2 over 15 min. The ice-water bath was removed, and stirring was continued 2.5 h. The reaction mixture was evaporated at reduced pressure, and to the residue was added 100 mL of 6:4 mixture of ethyl acetate and hexane. The liquid was decanted, and the residue was rinsed again with portions of the ethyl acetate–hexane mixture (4 x 20 mL). The combined washings were evaporated, and the residual yellow oil was purified by flash chromatography (ethyl acetate–hexane, 6:4). The (*S*)-mesylate **27-*S*** (4.11 g) was obtained in 89% yield: ^{1}H-NMR ($CDCl_3$) δ 1.29 (CH_3, t, 6H, J = 7.1 Hz), 1.91 (CH_3, m, 2H), 2.60 (m, 2H),

3.01 (CH_3S, s, 3H), 4.26 (CHDO, m, 1H), 4.30 (CH_2O, q, 2H, J = 7.1 Hz), 4.31 (CH_2O, q, 2H, J =7.1 Hz), 7.82 (m, 4H); ^{13}C-NMR ($CDCl_3$) δ 13.7, 24.2, 29.3, 37.2, 62.7, 67.1, 67.9 (CHDOH, t, J = 58.6 Hz), 123.5 (Ar), 131.2 (Ar), 134.4 (Ar), 165.9, 167.2.

Ethyl (5*R*)-2-Carbethoxy-2-phthalimido-5-cyanopentanoate-*5-d*$_1$ (28-*R*) (*S*)-Mesylate **27-*S*** (4.11 g, 9.3 mmol) and KCN (1.21 g, 18.6 mmol) in Me_2SO (100 mL) were heated at 50 °C under N_2 for 19.5 h. After the mixture had cooled, 100 mL water was added, and the resulting aqueous mixture was extracted with ethyl acetate (4 x 200 mL). The combined organic layers were then washed with brine (2 x 200 mL), dried over Na_2SO_4, and evaporated at reduced pressure. The crude product was purified by flash chromatography (1:1 ethyl acetate–hexane) to afford a crystalline solid (2.40 g, 69%), which was recrystallized from ether–hexane to obtain 2.00 g colorless prisms, mp 89 °C: ^{1}H-NMR ($CDCl_3$) δ 1.30 (CH_3, t, 6H, J = 7.1 Hz), 1.82 (m, 2H), 2.38 (m, 1H), 2.61 (m, 2H), 4.31 (CH_2O, q, 2H, J = 7.1 Hz), 4.32 (CH_2O, q, 2H, J = 7.1 Hz), 7.82 (m, 4H); ^{13}C-NMR ($CDCl_3$) δ 13.7, 16.8 (C-5, t, J =20.2 Hz), 20.7, 32.2, 62.8, 67.0, 119.0 (CN), 123.5 (Ar), 131.2 (Ar), 134.4 (Ar), 165.9, 167.2; mass spectrum (on an unlabeled sample) CI, isobutane m/z (relative abundance) (M+1)+ 374 (100), 373 (15); IR 3020, 2980, 2200, 1800, 1779, 1740, 1385 cm^{-1}. Elemental analysis was performed on an unlabeled sample prepared by the same procedure. Anal. calcd. for $C_{19}H_{20}N_2O_6$: C, 61.29%; H, 5.41%; N, 7.52%; found: C, 61.49%; H, 5.36%; N, 7.56%.

(2*RS*, 5*R*)-Lysine-*5-d*$_1$ Dihydrochloride (29-*R*). (*R*)-Nitrile **28-*R*** (0.434 g; 1.16 mmol) in 5 mL acetic acid and 2 mL concentrated HCl was hydrogenated over PtO_2 (0.162 g, 0.71 mmol). After 5.5 h an additional portion of PtO_2 (0.162 g, 0.71 mmol) was added, and the mixture was stirred under H_2 for an additional 18 h. The catalyst was removed by filtration, and the filtrate was evaporated to give a yellow oil.

Thin-layer chromatography showed no starting material to be present. The crude product was heated in 5 mL 6 N HCl at 115 °C for 15 h. An equal portion of water was added, and the mixture was evaporated at reduced pressure. An additional portion of water was added, and the mixture was evaporated to remove any excess acid. The solid residue was dissolved in 20 ml water, and the mixture was extracted with ether (3 x 20 mL) to remove the phthalic acid, then treated with activated carbon. After evaporation of the water, the residue was recrystallized from ethanol–ether to yield 0.063 g (25%) of the labeled lysine dihydrochloride, which on TLC co-chromatographed with authentic lysine (R_f = 0.20, 1-propanol–concentrated NH_4OH, 7:3). ^1H NMR (D_2O, DSS) δ 1.45 (m, 2H), 1.69 (m, 1H), 1.89 (m, 2H), 3.00 (d, 2H, J = 7.5 Hz), 3.74 (t, 1H, J = 6.0 Hz) ^{13}C NMR (D_2O, *p*-dioxane = 66.5) δ 21.2, 25.9 (C-5, t, J = 19.5 Hz), 29.5, 38.9 (C-6), 54.4 (C-2), 173.1 (COOH).

3.13. Synthesis of (2*RS*, 5*S*)-Lysine-*5*-d_1 Hydrochloride

Ethyl (5*R*)-2-Carbethoxy-2-phthalimido-5-hydroxypentanoate-*5*-d_1 (26-*R*). To 52 ml (*S*)-(–)-Alpine Borane (Aldrich, 81% optical purity) (0.5M solution in THF, 26 mmol) was added labeled aldehyde **25** (4.72 g, 13.1 mmol) in 25 mL dry THF. After the mixture had been stirred at room temperature under N_2 for 5.5 h, it was placed on a silica gel column and eluted with ether. Fractions containing the alcohol were combined and evaporated at reduced pressure. The crude product was further purified by flash chromatography (ethyl acetate–petroleum ether, 4:6, then 6:4) to yield 4.30 g (90%) of the labeled alcohol. ^1H and ^{13}C NMR spectra were identical to those of the (5*S*)-alcohol.

Ethyl (5*R*)-2-Carbethoxy-2-phthalimido-5-methanesulfonyloxypentanoate-*5*-d_1 (27-*R*). To an ice-cold, stirred mixture of (*R*)-alcohol **26-*R*** (3.89 g, 10.7 mmol) and triethylamine (2.17 g, 21.4 mmol) in 75 ml CH_2Cl_2 was added

methanesulfonyl chloride (2.45 g, 21.4 mmol) in 20 mL CH_2Cl_2 dropwise over 1 h. The ice-water bath was removed, and stirring was continued for 2 h. The reaction mixture was evaporated at reduced pressure, and 100 mL ethyl acetate–hexane (6:4) was added to the residue. The liquid was decanted, and the residue was rinsed with portions of the ethyl acetate–hexane mixture (4 x 20 mL). The crude product obtained after evaporation of the combined washings was purified by flash chromatography (ethyl acetate–hexane, 6:4) to afford 4.34 g (92%) of (*R*)-mesylate (**27-*R***). ^1H and ^{13}C NMR spectra were identical to those of the (5*S*)-mesylate.

Ethyl (5*S*)-2-Carbethoxy-2-phthalimido-5-cyanopentanoate-*5-d*$_1$ (28-*S*). (*R*)-Mesylate **27-*R*** (4.26 g, 9.6 mmol) and KCN (1.26 g, 19.3 mmol) in Me_2SO (100 mL) were heated at 45-50 °C under N_2 for 23 h. Water (100 mL) was added to the cooled reaction mixture, and the resulting mixture was extracted with ethyl acetate (4 x 200 mL). The extract was washed with brine (2 x 200 mL), dried over Na_2SO_4, and evaporated under reduced pressure. The crude product was purified by flash chromatography (1:1 ethyl acetate–hexane) to afford a crystalline solid (2.69 g, 72%), which was recrystallized from ether–hexane to yield 2.41 g of (*S*)-nitrile **28-*S*** as colorless prisms, mp 89 °C. ^1H and ^{13}C NMR spectra were identical to those of the (5*R*)-nitrile.

(2*RS*, 5*S*)-Lysine-*5-d*$_1$ Hydrochloride (29-*S*). (*S*)-Nitrile **28-*S*** (0.75 g, 2.0 mmol) was hydrogenated at an initial pressure of 61 psi over PtO_2 (0.225 g) in acetic acid (10 mL) and concentrated HCl (94 mL) over 23.5 h. The reaction mixture was filtered to remove the catalyst, and the filtrate was evaporated under reduced pressure to leave a yellow oil. TLC (6:4, ethyl acetate–hexane) showed no remaining starting material. The crude product in 6 N HCl (20 mL) was heated at 115 °C for 20 h. After that time the reaction mixture was cooled, water (30 mL) was added, and the resulting mixture was evaporated under reduced pressure. Additional portions of water were added, and the

mixture was evaporated to remove excess acid. The residue was dissolved in water (20 mL), and the aqueous mixture was extracted with ethyl acetate (2 x 20 mL) to remove phthalic acid. The aqueous layer was treated with activated charcoal; however, it remained quite yellow. The mixture was placed on a Dowex 50W column (3 x 15 cm). The column was washed with water (400 mL), then eluted with 2 N NH_4OH. The fractions containing lysine (TLC: 7:3, 1-propanol–concentrated NH_4OH) were combined, brought to pH 4 with HCl, treated with activated charcoal, and evaporated at reduced pressure. The residue was recrystallized from H_2O–ethanol–ether to yield 98.6 mg lysine monohydrochloride, which on TLC co-chromatographed with authentic lysine (R_f = 0.19, 1-propanol–concentrated NH_4OH). The mother liquor yielded an additional 38.2 mg of slightly less pure material, for a total yield of 37%. ^1H and ^{13}C NMR spectra were identical to those of (2*RS*,5*R*)-lysine.

3.14. Incorporation of (2*RS*, 5*R*)-Lysine-5-d_1

The fermentation and preliminary purification of the antibiotic took place at the laboratories of SmithKline–RIT, Rixensart, Belgium. After *S. virginiae* 5277 was grown for 3 days in a vegetative medium, ten 250-mL Erlenmeyer flasks each containing 40 mL of the production medium were inoculated. After growth for 24 h, 250 μL of a solution consisting of 50 mg (2*RS*, 5*R*)-lysine-5-d_1 and 100 mg L-threonine in 2.5 mL deionized water, sterilized by passing the solution through a Millipore filter, was added to each flask. The fermentation continued for an unspecified amount of time (2–3 days), then the broth was brought to pH 4.7 with H_2SO_4. The acidified broth was extracted three times with methyl isobutyl ketone; the combined extracts were centrifuged for 10 min (5000 rpm), then concentrated in vacuo to 5 mL. *n*-Hexane (200 mL) was added to the concentrated extract, and the mixture was allowed to stand overnight at 4 °C. Only part of the crude antibiotic precipitated; it was filtered and dried. The supernatant was therefore concentrated

to dryness. The residue was dissolved in acetone–hexane; the mixture was extracted with water. The water phase was lyophilized to yield an additional portion of crude virginiamycin. A total of 650 mg material containing approximately 42 mg (HPLC) undefined virginiamycins was obtained.

The crude material was purified as previously described to yield 7 mg virginiamycin S_1. A portion of the labeled antibiotic was hydrolyzed, and the resulting mixture of amino acids was derivatized as the *N*-trifluoroacetyl butyl esters as previously described. The derivatives were analyzed by GC/MS as previously described.

3.15. Incorporation of (2*RS*, 5*S*)-Lysine-5-d_1

The fermentation and preliminary purification of the antibiotic took place at the laboratories of SmithKline–RIT, Rixensart, Belgium. *S. virginiae* 5277 was grown for 2 days in a vegetative medium, then 15 250-mL Erlenmeyer flasks each containing 40 mL of a production medium were inoculated with the vegetative broth. A solution of 65 mg (2*RS*, 5*S*)-lysine-5-d_1 and 100 mg L-threonine in 3 mL distilled water was sterilized by passing through a 0.45 μm filter; 200 μL of this solution was added to each of the production flasks after 24 h fermentation. After 48 h, The broth was acidified, then extracted three times with methyl isobutyl ketone. The combined extracts were concentrated, diluted with water, then washed three times with *n*-hexane. The aqueous phase was lyophilized to yield 480 mg of a material containing 30 mg virginiamycin M_2, 9 mg virginiamycin M_1, and 6 mg virginiamycin S_1. Virginiamycin S_1 was isolated, hydrolyzed, and prepared for GC/MS analysis as described previously.

3.16. Synthesis of DL-Phenylalanine-3-^{13}C-^{15}N Hydrochloride

Benzoic-*carboxy*-^{13}C acid. Bromobenzene (46 mL, excess) in dry, freshly

distilled THF was added dropwise to a flask containing magnesium turnings (4.57 g, 0.19 mol) that had been activated by heating in the presence of I_2 vapors. When the reaction was complete, the flask was attached to a vacuum line and immersed in liquid N_2. When the mixture had solidified, the system was evacuated. Carbon dioxide-*^{13}C* was produced by the addition of concentrated H_2SO_4 dropwise into a flask containing barium carbonate-*^{13}C* (34.6 g, 0.176 mol). The carbon dioxide was condensed into the flask containing the frozen phenylmagnesium bromide solution, then the flask was slowly brought to room temperature. The mixture was extracted with ether. The ether layer was extracted with 1.5 N NaOH; the extract was acidified to pH 1 and extracted with ether. The ether layer was dried over $MgSO_4$, filtered, and evaporated to yield 18.7 g benzoic acid (88% yield), mp 120-121 °C. ^{13}C NMR ($CDCl_3$) δ 128.5 (C-3, d, $^3J_{CC}$ = 4.4 Hz), (129.4, C-1, not seen), 130.2 (C-2, d, $^2J_{CC}$ = 2.6 Hz), 133.8 (C-4, 172.3 (COOH, intense singlet).

Benzyl-α-*^{13}C* alcohol. Benzoic-*carboxy-^{13}C* acid (3.20 g, 26.0 mmol) was dissolved in 150 mL freshly distilled dry ether. Lithium aluminum hydride (2.19 g, 57.6 mmol) was added in small portions over 30 min, during which time the mixture was stirred at room temperature under N_2. The reaction mixture was then heated at reflux for 3.5 h. The flask was cooled in an ice bath, and the reaction was quenched by the dropwise addition of 2 mL H_2O, followed by 4 mL 10% aqueous NaOH and 6 mL H_2O. The mixture was filtered and the residue rinsed with several small portions of ether. The combined filtrate and washings were dried over $MgSO_4$. Evaporation of the solvent left 3.24 g of the crude product. Vacuum distillation (2.5 torr) afforded 2.58 g of benzyl alcohol (87.6% yield).

Benzaldehyde-α-*^{13}C* (31). Benzyl-α-*^{13}C* alcohol (2.58 g, 24.0 mmol) in 50 mL of dimethyl sulfoxide was heated to 180 °C for 6 h with a stream of air bubbling through a fine frit into the reaction mixture. To the cooled mixture was added 100 mL

H_2O, and the aqueous mixture was extracted with ether (3 x 100 mL). The combined ether layers were washed with H_2O (2 x 100 mL), dried over $MgSO_4$, and evaporated at reduced pressure to yield 2.13 g benzaldehyde (83%), which was shown to be relatively pure by TLC (hexane–ether, 3:2), but gave low yields in subsequent reactions. Distillation did not improve the quality of the product. The crude product was therefore purified as its bisulfite addition product. Crude benzaldehyde from 7.74 g benzyl alcohol was dissolved in 2 mL ether, treated with saturated aqueous $NaHSO_3$ (2 mL), and vigorously stirred for 30 min. The resulting heavy precipitate was filtered from the mixture, rinsed with a little ether, and dried. The bisulfite addition product was dissolved in 50 mL saturated aqueous $NaHCO_3$, and the solution was extracted with ether (4 x 50 mL). The combined ether layers were dried over $MgSO_4$ and evaporated to give 1.20 g (16%) pure benzaldehyde (**31**).

***N*-Acetylglycine-^{15}N (32).** To a stirred solution of glycine-^{15}N (MSD Isotopes) (1.04 g, 13.7 mmol) in 4 mL H_2O was added acetic anhydride (2.9 g, 28 mmol). The mixture became warm, and a white precipitate formed within 5 min. Stirring was continued for 30 min, then the mixture was stored at 4 °C overnight to maximize crystallization. The product was filtered from the mixture, washed with a small volume of ice-cold water, and dried to give 1.12 g (69%) product, mp 206-208 °C (literature 207–208 °C).[141]

α-Acetamidocinnamic-3-^{13}C-^{15}N Acid Azlactone (33). Benzaldehyde-α-^{13}C (1.20 g, 11.2 mmol), *N*-acetylglycine-^{15}N (0.906 g, 7.68 mmol), anhydrous sodium acetate (0.470 g, 5.7 mmol), and acetic anhydride (1.8 mL) were heated at reflux for 2 h under N_2. After cooling, the flask was stored at 4 °C overnight, then water (2 mL) was added. The solid mass was broken up, filtered, and rinsed with two 1-mL portions of cold water to yield 1.06 g (73%) product, which was used in the next step without further purification.

α-Acetamidocinnamic Acid-*3-^{13}C-^{15}N* (34). The crude azlactone 33 (1.06 g) in 10 mL acetone and 4 mL H_2O was heated at reflux for 6 h. The acetone was evaporated at reduced pressure, 25 mL H_2O was added, and the mixture was brought to a boil. The hot solution was filtered, and the residue was rinsed with a small portion of boiling water. The filtrate was boiled with Norit A , and the hot mixture was filtered. The filtrate was reduced to 20 mL in vacuo and stored at 4 °C overnight to allow for the development of crystals. The white crystalline product was filtered from the mixture and dried to give 0.977 g (84%) product, mp 193-194 °C (literature 193-194 °C).[131]

DL-Phenylalanine-*3-^{13}C-^{15}N* Hydrochloride (35). α-Acetamidocinnamic acid **34** (0.878 g, 4.24 mmol) was hydrogenated in glacial acetic acid (10 mL) over 5% palladium on carbon (0.25 g) at room temperature for 2.5 h. The catalyst was removed by filtration, and the filtrate was evaporated to dryness. The residue was heated at reflux for 11 h in 20 mL 1 N HCl. The mixture was evaporated to dryness at reduced pressure, and the residue was dried further in vacuo over NaOH pellets to give 0.645 g (75%) of the doubly labeled phenylalanine hydrochloride, identical by TLC and co-TLC with an authentic sample of phenylalanine. ^{1}H-NMR: (D_2O, DSS) δ 2.93–3.16, 3.42–3.65 (CH_2, dm, 2H, $^1J_{CH}$ = 132 Hz, $^3J_{NH}$ = 3 Hz), 4.36 (CH, m, 1H), 7.32–7.48 (Ar, m, 5H); ^{13}C-NMR (D_2O) δ 35.6 (C-3, intense singlet), 54.1 (C-2, dd, $^1J_{CC}$ = 33 Hz, $^1J_{CN}$ = 6.5 Hz), 127.9, 129.2 (d, J_{CC} = 3.7 Hz), 129.4 (d, J_{CC} = 3.1 Hz), 134.0 (Ar-1, d, $^1J_{CC}$ = 43 Hz), 171.5 (COOH); mass spectrum m/z (relative abundance) 167 (M^+–HCl) (1.9), 120 (5), 121 (8), 122 (60), 104 (13), 92 (70), 93 (19), 75 (100). The ions 120, 121, and 122 represent the ions (M^+–COOH) that are unlabeled, singly labeled, and doubly labeled, respectively. From the relative abundances of these ions it can be deduced that 35 is 7% unlabeled, 10% singly labeled (with either ^{13}C or ^{15}N), and 83% doubly labeled.

3.17. Incorporation of DL-Phenylalanine-*3-^{13}C-^{15}N* Hydrochloride

The fermentation was carried out as previously described. After 8 h growth, a filter-sterilized (0.45 μm) solution of 290 mg DL-phenylalanine-*3-^{13}C-^{15}N* hydrochloride and 230 mg L-threonine in 10 mL distilled water was added to the production broth (1.5 L). Virginiamycin S_1 (7 mg) was isolated as described previously, then analyzed by ^{13}C NMR. It was hydrolyzed and prepared for GC/MS analysis as described previously.

4. CONCLUSION

Some details of the biosynthesis of three unusual amino acid residues in the antibiotic virginiamycin S_1 have been studied. 4-Oxo-L-pipecolic acid was shown to arise from L-lysine and not from L-aspartic acid or L-methionine as was previously suggested. Incorporation of DL-lysine-*6-*^{13}C-^{15}N demonstrated that its nitrogen originates from the ε-amino group of lysine.

Like 4-oxo-L-pipecolic acid, 3-hydroxypicolinic acid is biosynthesized from L-lysine; the ε-nitrogen of L-lysine is also retained in this amino acid. (2*RS*, 5*R*)-lysine-*5-*d_1 and (2*RS*, 5*S*)-lysine-*5-*d_1 were synthesized and incorporated into virginiamycin S_1. The deuterium from the 5-(*R*) isomer was detected in the 3-hydroxypicolinyl residue of the antibiotic. No incorporation was seen of the 5-(*S*) isomer. This finding indicates that the *5-pro(S)* hydrogen of lysine is lost at some point during the biogenesis of 3-hydroxypicolinic acid.

L-Phenylglycine had previously been shown to originate from L-phenylalanine. In order to study the mechanism of the migration of the amino group to the benzylic position during the transformation, DL-phenylalanine-*3-*^{13}C-^{15}N was synthesized and incorporated into virginiamycin S_1. The labeled nitrogen was not incorporated in L-phenylglycine; therefore, it can be concluded that the process is an intermolecular one.

In addition, a synthesis of 4-oxo-DL-pipecolic acid was devised. A new derivative of this amino acid, *N*-benzoyl-4-oxopipecolic acid ethylene ketal, was developed, which allowed its separation from the other virginiamycin S_1 amino acids by a previously worked-out procedure.

5. REFERENCES

1. Cocito, C. In *Antibiotics*, Hahn, F. E., Ed.; Springer–Verlag: Berlin, 1983; Vol.6, pp 296-332.

2. Okumura, Y. In *Biochemistry and Genetic Regulation of Commercially Important Antibiotics*; Vining, L. C., Ed.; Addison–Wesley: Reading, MA, 1983; Chapter 6, p 147.

3. De Somer, P.; Van Dijck, P. *Antibiot. Chemother.* **1955**, *5*, 632.

4. Ogata, K.; Matsuura, M.; Irie, H.; Ueno, T.; Tani, Y.; Yamada, H. *J. Antibiot.* **1978**, *31*, 1313.

5. Donovick, R.; Dutcher, J. D.; Heuser, L. J.; Pagano, J. F. U. S. Patent 2 990 325, 1961; *Chem. Abstr.* **1962**, *56*, 3923b.

6. Charney, J.; Fisher, W. P.; Curran, C.; Machlowitz, R. A.; Tytell, A. A. *Antibiot. Chemother.* **1953**, *3*, 1283.

7. Ball, S.; Boothroyd, B.; Lees, K. A.; Raper, A. H.; Smith, E. L. *Biochem. J.* **1958**, *68*, 24P.

8. Arai, M.; Karasawa, S.; Yonehara, H.; Umezawa, H. *J. Antibiot.* **1958**, *11*, 14.

9. Benazet, F.; Cosar, C.; Dubost, N.; Julou, L.; Mancy, D. *Semaine Thérapeutique* **1962**, *38*, 13.

10. Celmer, W. D.; Sobin, B. A. *Antibiot. Annual 1955/56* **1956**, 437.

11. Linder, F.; Walhausser, K. H.; Weidenmüller, H. L. German Patent 1 072 773, 1960; *Chem. Abstr.* **1961**, *55*, 13764c.

12. Bartz, Q. R.; Standiford, J.; Mold, J. D.; Johannessen, D. W.; Ryder, A.; Maretzki, A.; Haskell, T. H. *Antibiot. Annual 1954/55* **1955**, 777.

13. Okumura, Y.; Okumura, K.; Takei, T.; Kouno, K.; Lein, J; Ishikura, T.; Fukagawa, Y. *J. Antibiot.* **1979**, *32*, 575.

14. Heinemann, B.; Gourevitch, A.; Lein, J.; Johnson, D. L.; Kaplan, M. A.; Vans, D.; Hooper, I. R. *Antibiot. Annual 1954/55* **1955**, 728.

15. Hamill, R. L.; Stark, W. M. U. S. Patent 3 932 980, 1975; *Chem. Abstr.* **1976**, *84*, 87963x.

16. Celmer, W. D.; Cullen, W. P.; Routien, J. B.; Moppet, C. E.; Shibakawa, R.; Tone, J. German Patent 2 516 020, 1975; *Chem. Abstr.* **1976**, *84*, 87960u.

17. Martinelli., E.; Zerilli, F.; Volpe, G.; Pagani, H.; Cavalleri, B. *J. Antibiot.* **1979**, *32*, 108.

18. Brazhnikova, M. G.; Kudinova, M. K.; Potapova, N. P.; Filippova, T. M.; Borowski, E.; Zielinski, J.; Golic, J. *Bioorg. Khim.* **1975**, *1*, 1383.

19. Liu, W.-C.; Seiner, V.; Dean. L. D.; Trejo, W. H.; Principe, P. A.; Meyers, E.; Sykes, R. B. *J. Antibiot.* **1981**, *34*, 1515.

20. Kruglyak, E. B.; Kostantinova, N. V.; Sulavko, L. A. *Antibiotiki* **1961**, *6*, 298.

21. Bodanszky, M.; Ondetti, M. A.; Sheehan, J. T. U. S. Patent 3 373 151; *Chem. Abstr.* **1968**, *69*, 59563p.

22. Durant, F.; Evrard, G.; Declercq, J.P.; German, G. *Cryst. Struct. Commun.* **1974**, *3*, 503.

23. Vasquez, D. *Biochem. Biophys. Acta* **1962**, *61*, 849.

24. Delpierre, G. R.; Eastwood, F. W.; Gream, G. E.; Kingston, D. G. I.; Sarin, P. S.; Todd, A. R.; Williams, D. H. *J. Chem. Soc. C* **1966**, 1653.

25. Okabe, K. *J. Antibiot. Ser. A* **1959**, *12*, 86.

26. Preud'homme, J.; Tarridec, P; Belloc, A. *Bull. Soc. Chim. Fr.* **1968**, 585.

27. Kingston, D. G. I.; Sarin, P. S.; Todd, A. R.; Williams, D. H. *J. Chem. Soc. C* **1966**, 1856.

28. Fallona, M. C.; Mayo, P. D.; McMorris, T. C.; Money, T.; Stossel, A. *Can. J. Chem.* **1964**, *42*, 371.

29. Birnbaum, G. I.; Hall, S. R. *J. Am. Chem. Soc.* **1976**, *98*, 1926.

30. Chamberlin, J. W.; Chen, S. *J. Antibiot.* **1977**, *30*, 197.

31. Moppet, C. E.; Whipple, E. B. Abstracts of the Seventeenth Interscientific Conference on Antimicrobial Agents and Chemotherapy, 1977, p 243.

32. Vanderhaeghe, H.; Parmentier, G. *Bull. Soc. Chim. Belg.* **1959**, *68*, 716.

33. Vanderhaeghe, H.; Parmentier, G. *J. Am. Chem. Soc.* **1960**, *82*, 4414.

34. Vanderhaeghe, H.; Janssen, G.; Campernolle, F. *Tetrahedron Lett.* **1971**, 2687.

35. Oberbäumer, I.; Grell, E.; Raschdorf, F.; Richter, W. J. *Helv. Chim. Acta* **1982**, *65*, 2280.

36. Verwey, W. F.; West, M. K.; Miller, A. K. *Antibiot. Chemother.* **1958**, *8*, 500.

37. Watanabe, K. J. *Antibiot. Series A* **1961**, *14*, 1.

38. Hobbs, D. C.; Celmer, W. D. *Nature* **1960**, *187*, 598.

39. Bodanszki, M.; Ondetti, M. A. *Antimicrob. Agents Chemother. 1963* **1964**, 360.

40. Eastwood, F. W.; Snell, B. K.; Todd, A. R. *J. Chem Soc.* **1960**, 2286.

41. Cox, B. R.; Eastwood, F. W.; Snell, B. K.; Todd, A. R. *J. Chem. Soc., Chem. Commun. 1970*, 1623.

42. Bodanszky, M.; Sheehan, J. T. *Antimicrob. Agents Chemother. 1963* **1964**, 38.

43. Okumura, Y.; Takei, T.; Sakamoto, M.; Ishikura, T.; Fukagawa, Y. *J. Antibiot.* **1979**, *32*, 1002.

44. Okumura, Y.; Takei, T.; Sakamoto, M.; Ishikura, T.; Fukagawa, Y. *J. Antibiot.* **1979**, *32*, 584.

45. Chopra, C.; Hook, D. J.; Vining, L. C.; Das, B. C.; Shimizu, S.; Taylor, A.; Wright, J. L. C. *J. Antibiot.* **1979**, *32*, 392.

46. Haskell, T. H.; Maretzki, A.; Bartz, Q. R. *Antibiot. Annual 1954/55* **1955**, 784.

47. Sheehan, J. C.; Zachan, H. G.; Lawson, W. B. *J. Am. Chem. Soc.* **1958**, *80*, 3349.

48. Okumura, Y.; Okamura, K.; Takei, T.; Kouno, K.; Lein, J.; Ishikura, T.; Fukagawa, Y. *J. Antibiot.* **1979**, *32*, 575.

49. Okumura, Y.; Fukagawa, Y.; Okamoto, R.; Ishikura, T. *Agri. Biol. Chem.* **1982**, *46*, 731.

50. Okumura, Y.; Okamoto, R.; Ishikura, T. *Agric. Biol. Chem.* **1984**, *48*, 543.

51. De Somer, P.; Van Dijck, P. *Antibiot. Chemother.* **1955**, *5*, 632.

52. Vanderhaeghe, H.; Van Dijck, P.; Parmentier, G.; De Somer, P. *Antibiot. Chemother.* **1957**, *7*, 606.

53. Declercq, J. P.; Germain, G.; Van Meerssche, M.; Hull, S. E.; Irwin, M. J. *Acta Crystallogr.* **1978**, *B34*, 3644.

54. Kiryushkin, A. A.; Burikov, V. M.; Rosinov, B. V. *Tetrahedron Lett.* **1967**, 2675.

55. Compernolle, F.; Vanderhaeghe, H.; Janssen, G. *Org. Mass Spectrom.* **1972**, *6*, 151.

56. Anteunis, M. J. O.; Callens, R. E. A.; Tavernier, D. K. *Eur. J. Biochem.* **1975**, *58*, 259.

57. Callens, R. E. A.; Anteunis, M. J. O. *Biochem. Biophys. Acta* **1979**, *577*, 324.

58. Jannsen, G.; Anne, J.; Vanderhaeghe, H. *J. Antibiot.* **1977**, *30*, 141.

59. Kessler, H.; Kühn, M.; Löschner, T. *Leibigs Ann. Chem.* **1986**, 21.

60. Kessler, H.; Kühn, M.; Löschner, T. *Liebigs Ann. Chem.* **1986**, 1.

61. Cocito, C. *Microbiol. Rev.* **1979**, *43*, 145.

62. Van Dijck, P.; Vanderhaeghe, H.; De Somer, P. *Antibiot. Chemother.* **1957**, *7*, 625.

63. Biot, A. M. In *Biotechnology of Industrial Antibiotics*; Vandamme, E.J., Ed.; Marcel Dekker: New York, 1984; Chapter 25.

64. Danielson, D. M. *J. Anim. Sci.* **1972**, *35*, 187.

65. Olson, L. D.; Rodabaugh, D. E. *Am. J. Vet. Res.* **1977**, *38*, 1485.

66. Chassagne, P.; Curveiller, J.; Bocquet, L.; Guibert, C. *Thérapie* **1963**, *18*, 969.

67. Fossiez, M. *Ann. Pediatr.* **1963**, *39*, 633.

68. Prinquet, R. *Presse Med.* **1962**, *70*, 2573.

69. Le Goffic, F. *J. Antimicrob. Chemother.* **1985**, *16 (Suppl. A)*, 13.

70. Corbet, J. P.; Cotrel, C.; Farge, D.; Paris, J. M. French Patent 2 549 063, 1985; *Chem. Abstr.* **1985**, *103*, 160862f.

71. Corbet, J. P.; Cotrel, C.; Farge, D.; Paris, J. M. French Patent 2 549 064, 1985; *Chem. Abstr.* **1985**, *103*, 196421a.

72. Corbet, J. P.; Cotrel, C.; Farge, D.; Paris, J. M. French Patent 2 549 062, 1985; *Chem. Abstr.* **1985**, *103*, 160860d.

73. Le Goffic, F.; Capmau, M. L.; Abbé, J.; Cerceau, C.; Dublanchet, A.; Duval, J. *Annales de Microbiologie (Institut Pasteur)* **1977**, *128B*, 471.

74. Ennis, H. L. *Arch. Biochem. Biophys.* **1974**, *160*, 394.

75. Contreras, A.; Vasquez, D. *Eur. J. Biochem.* **1977**, *74*, 549.

76. Parfait, R.; DeBethune, M. P.; Cocito, C. *Mol. Gen. Genet.* **1978**, *166*, 45.

77. Parfait, R.; Cocito, C. *Proc. Natl. Acad. Sci. USA* **1980**, *77*, 5492.

78. Chinali, G.; Moureau, P.; Cocito, C. *J. Biol. Chem.* **1984**, *259*, 9563.

79. Cocito, C.; Chinali, G. *J. Antimicrob. Chemother.* **1985**, *16 (Suppl. A)*, 35.

80. Vasquez, D. *J. Antimicrob. Chemother.* **1985**, *16 (Suppl. A)*, 225.

81. Di Giambattista, M.; Vannuffel, P.; Sunazuka, T.; Jacob, T.; Omura, S.; Cocito, C. *J. Antimicrob. Chemother.* **1986**, *18*, 307.

82. Di Giambattista, M.; Thielen, A. P. G. M.; Maassen, J. A.; Moeller, W.; Cocito, C. *Biochemistry* **1986**, *25*, 3540.

83. Di Giambattista, M.; Ide, G.; Engelborghs, Y.; Cocito, C. *J. Biol. Chem.* **1984**, *259*, 6334.

84. Di Giambattista, M.; Cocito, C. *Biochim. Biophys. Acta* **1983**, *757*, 92.

85. Ennis, H. L. *Biochemistry* **1971**, *10*, 1265.

86. Grell, E.; Krause, G.; Lewitzki, E.; Mager, G.; Ruf, H. In *Prog. Bioorg. Chem. Mol. Biol., Proc. Intl. Symp. Front. Bioorg. Chem. Mol. Biol.*; Ovchinnikov, Y.A., Ed.; Elsevier: Amsterdam, 1984; pp 239.

87. Grell, E.; Oberbäumer, I.; Ruf, H.; Zingsheim, H. P. In *Biochemistry of Membrane Transport*; Semenza, G.; Carafoli, E., Eds.; Springer–Verlag: Heidelberg, 1977; p 147.

88. Kurahashi, K. In *Antibiotics*; Corcoran, J. W., Ed.; Springer–Verlag: Berlin, 1981; Vol. 4, pp 325-352.

89. Kamal, F.; Katz, E. *J. Antibiot.* **1976**, *29*, 944.

90. Molinero, A. A. Master's Thesis, Virginia Polytechnic Institute and State University, 1982.

91. Hook, D. J.; Vining, L. C. *J. Chem. Soc., Chem. Commun.* **1973**, 185.

92. Hook, D. J.; Vining, L. C. *Can. J. Biochem.* **1973**, *51*, 1630.

93. Ogawara, H.; Maeda, K.; Umezawa, H. *Biochemistry* **1968**, *7*, 3296.

94. Nagato, N.; Okumura, Y.; Okamoto, R.; Ishikura, T. *Agric. Biol. Chem.* **1984**, *48*, 3135.

95. Rodwell, V. W. In *Metabolic Pathways*, 3rd ed.; Greenberg, D. M., Ed.; Academic Press: New York, 1969; Vol. 3, Chapter 15 (Part II), and references therein.

96. Clark-Lewis, J. W.; Mortimer, P. I. *J. Chem. Soc.* **1961**, 189; and references therein.

97. Gupta, R. M.; Spencer, I. A. *Phytochemistry* **1970**, *9*, 2329.

98. Grobbelaar, N.; Steward, F. C. *J. Am. Chem. Soc.* **1953**, *75*, 4341.

99. Rothstein, M.; Miller, L. L. *J. Biol. Chem.* **1954**, *211*, 851.

100. Meister, A.; Padhakrishnan, A. N.; Buckley, S. D. *J. Biol. Chem.* **1957**, *229*, 789.

101. Fowden, L. *J. Exp. Botany* **1960**, *11*, 302.

102. Meyer, J. J. M.; Grobbellaar, N. *Phytochemistry* **1986**, *25*, 1469.

103. Boulanger, P.; Osteux, R.; Bertrand, J. *Biochim. Biophys. Acta* **1958**, *29*, 534.

104. Shoji, J.; Hinoo, H. *J. Antibiot.* **1975**, *28*, 60.

105. Purvis, M. B. Ph.D. Dissertation, Virginia Polytechnic Institute and State University, 1988.

106. Clark-Lewis, J. W.; Mortimer, P. I. *J.Chem. Soc.* **1969**, 189.

107. Jollès, G.; Poiget, G.; Robert, J.; Terlain, B.; Thomas, J.-P. *Bull. Soc. Chim. Fr.* **1965**, 2252.

108. Halin, F.; Slosse, P.; Hootelé, C. *Tetrahedron* **1985**, *41*, 2891.

109. Dobson, T. A.; Desaty, D.; Brewer, D.; Vining, L. C. *Can. J. Biochem.* **1967**, *45* 809.

110. Helbling, A. M.; Viscontini, M. *Helv. Chim. Acta* **1976**, 2284.

111. Birch, A. J.; Simpson, T. J. *J.Chem. Soc. Perkin I* **1979**, 816.

112. Luckner, M. *Secondary Metabolism in Microorganisms, Plants, and Animals*, 2nd ed.; Springer–Verlag: Berlin, 1984.

113. Personal communication from Dr. Yasushi Okumura.

114. Rodwell, V. W. In *Metabolic Pathways*, 3rd ed.; Greenberg, D. M., Ed.; Academic Press: New York, 1969; Vol.3, Chapter 16 (Part II).

115. Levy, G. C.; Lichter, R. L. *Nitrogen-15 Nuclear Magnetic Resonance Spectroscopy*; John Wiley: New York, 1979; Chapter 4.

116. Townsend, C. A.; Ho, M.; Mao, S. *J. Chem. Soc., Chem. Commun.* **1986**, 638.

117. Ho, M. Ph.D. Dissertation, The Johns Hopkins University, 1983.

118. Kwart, H.; Nickle, J. H. *J. Am. Chem. Soc.* **1976**, *98*, 2881.

119. Kwart, H.; George, T. J. *J. Org. Chem.* **1979**, *44*, 162.

120. Midland, M. M.; Greer, S.; Tramontano, A.; Zderic, S. A. *J. Am. Chem. Soc.* **1979**, *101*, 2352.

121. Sallach, H. J.; Fahien, L. A. In *Metabolic Pathways*, 3rd ed.; Greenberg, D. M., Ed.; Academic Press: New York, 1969; Vol. 3, Chapter 14, and references therein.

122. Leete, E. *Can. J. Chem.* **1987**, *65*, 226.

123. Townsend, C. A.; Brown, A. M. *J. Am. Chem. Soc.* **1981**, *103*, 2873.

124. Townsend, C. A.; Brown, A. M. *J. Am. Chem. Soc.* **1983**, *105*, 913.

125. Hammond, S. J.; Williamson, M. P.; Williams, D. H.; Boeck, L. D.; Marconi, G. G. *J. Chem. Soc., Chem. Commun.* **1982**, 344.

126. McGahren, W. J.; Martin, J. H.; Morton, G. O.; Hargreaves, R. T.; Leese, R. A.; Lowell, F. M.; Ellestad, G. A.; O'Brien, E. O.; Holker, J. S. E. *J. Am. Chem. Soc.* **1980**, *102*, 1671.

127. Parry, R. J.; Kurylo-Borowska, Z. *J. Am. Chem. Soc.* **1980**, *102*, 836.

128. Thiruvengadam, T. K.; Gould, S. J.; Aberhart, D. J.; Lin, H.-J. *J. Am. Chem. Soc.* **1983**, *105*, 5470.

129. Aberhart, D. J.; Gould, S. J.; Lin, H.-L.; Thiruvengadam, T. K.; Weiller, B. H. *J. Am. Chem. Soc.* **1983**, *105*, 5461.

130. Yoshitake, A.; Makari, Y.; Kawahara, K.; Endo, M. *J. Labelled Comp.* **1973**, *9*, 537.

131. Herbst, R. M.; Shemin, D. In *Organic Syntheses*; Blatt, A. H., Ed.; John Wiley: New York, 1943; Coll. Vol. 2, p 1.

132. Gillespie, H. B.; Snyder, H. R. In *Organic Syntheses*; Blatt, A. H., Ed.; John Wiley: New York, 1943; Coll. Vol. 2, p 489.

133. Personal communication from Dr. André Biot, SmithKline–RIT.

134. Beimann, K. *Mass Spectrometry: Organic Chemical Applications*; McGraw–Hill: New York, 1962.

135. Pirkle, W. H.; Hyun, M. H. *J. Org. Chem.* **1984**, *49*, 3043.

136. Elliot, D. F. *J. Chem. Soc.* **1950**, 62.

137. Mozingo, R.; Wolf, D. E.; Harris, S. A.; Folkers, K. *J. Am. Chem. Soc.* **1943**, *65*, 1013.

138. Abderhalden, E.; Heyns, K. *Chem. Ber.* **1934**, *67*, 530.

139. Although *N*-benzoyl-*N*-methyl-L-phenylalanine is a known compound (Jones, G. Eur. Pat. Appl. 19,411, 1980; *Chem. Abstr.* **1981**, *95*, 62696b), we were unable to locate a reference to its melting point. Molinero reported in his notes a melting point of 138–140 °C for the same compound. The racemic compound has a melting point of 121 °C (Deulofeu, V. *Chem. Ber.* **1934**, *67*, 1542).

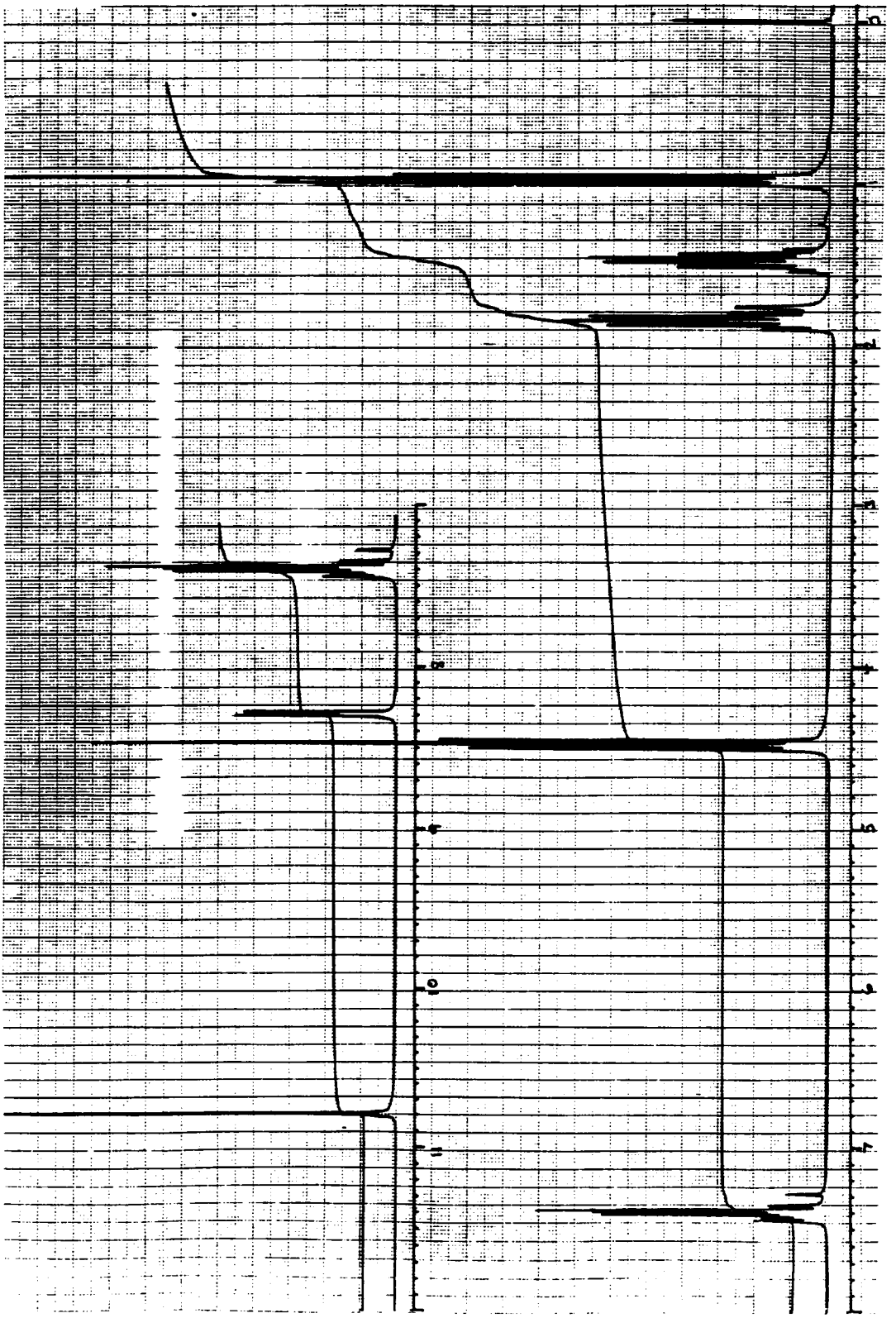
140. Balog, A.; Breasu, D.; Voinescu, V.; Herman, M.; Vargha, E.; Ramontian, E. *Rev. Roum. Chim.* **1973**, *18*, 123.

141. Herbst, R. M.; Shemin, D. In *Organic Syntheses*; Blatt, A.H., Ed.; John Wiley: New York, 1943; Coll. Vol. 2, p 11.

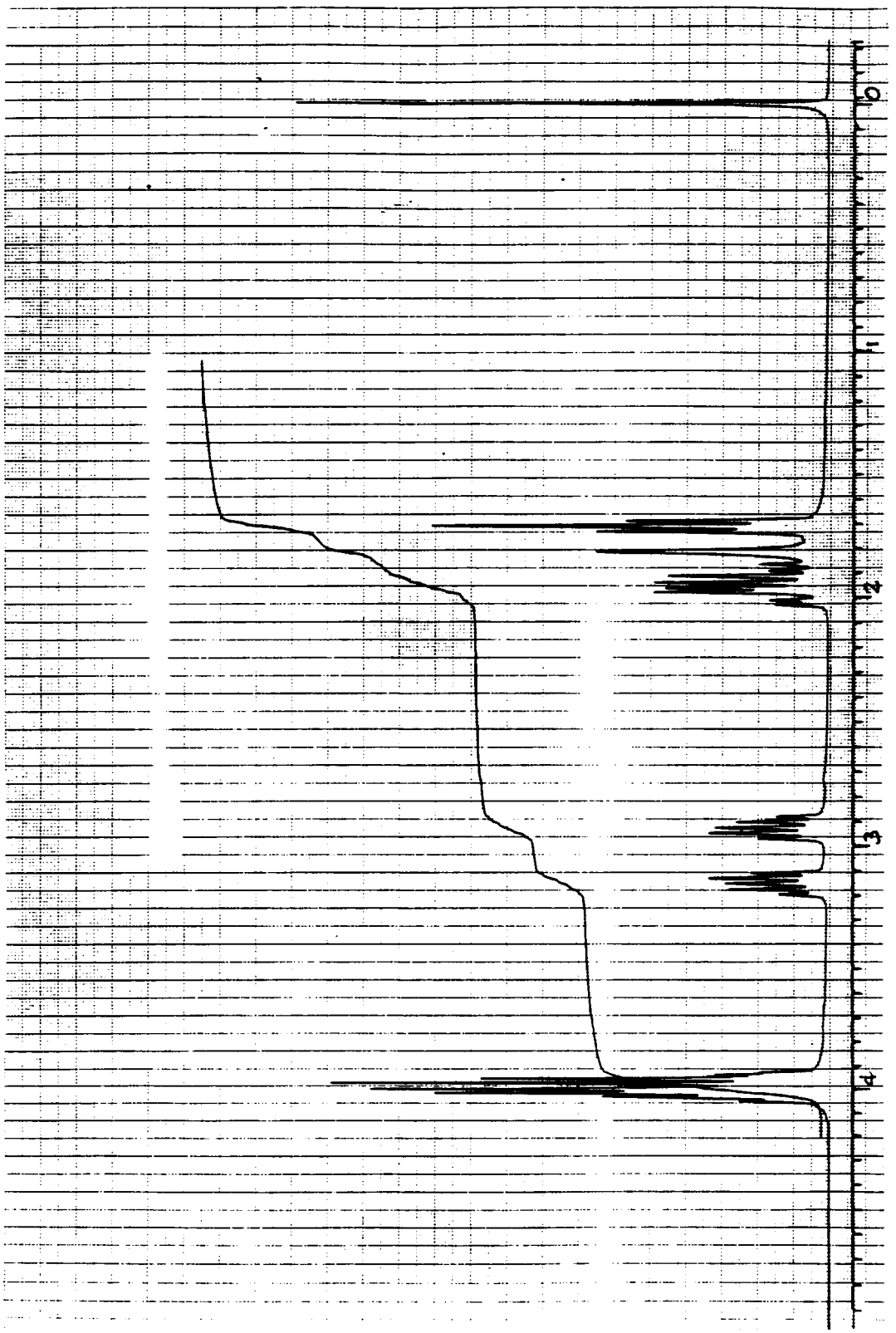
APPENDIX

LIST OF SPECTRA

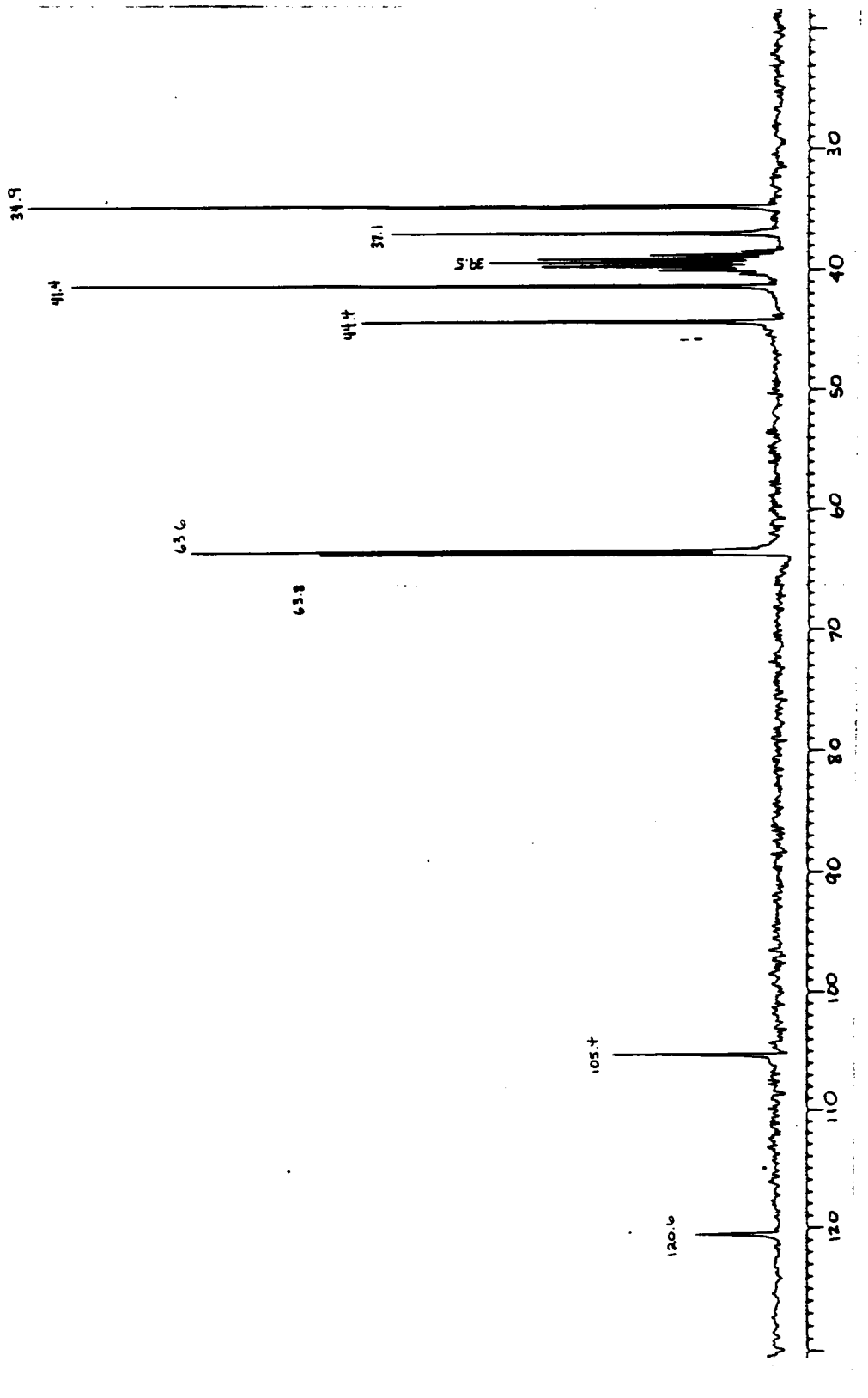
19. ^{1}H-NMR spectrum of (2*RS*, 5*R*)-lysine-5d_1 dihydrochloride (**29**)
20. ^{13}C-NMR spectrum of (2*RS*, 5*R*)-lysine-5d_1 dihydrochloride (**29**)
21. ^{13}C-NMR spectrum of virginiamycin S_1 standard
22. ^{13}C-NMR spectrum of virginiamycin S_1 from lysine-6-^{13}C-6-^{15}N incorporation
23. ^{1}H-NMR spectrum of benzoic-*carboxy*-^{13}C acid
24. ^{13}C-NMR spectrum of benzoic-*carboxy*-^{13}C acid
25. ^{13}C-NMR spectrum of benzyl-α-^{13}C alcohol
26. ^{1}H-NMR spectrum of DL-phenylalanine-3-^{13}C-^{15}N hydrochloride (**35**)
27. ^{13}C-NMR spectrum of DL-phenylalanine-3-^{13}C-^{15}N hydrochloride (**35**)
28. ^{13}C-NMR spectrum of virginiamycin S_1 from phenylalanine-3-^{13}C-^{15}N incorporation



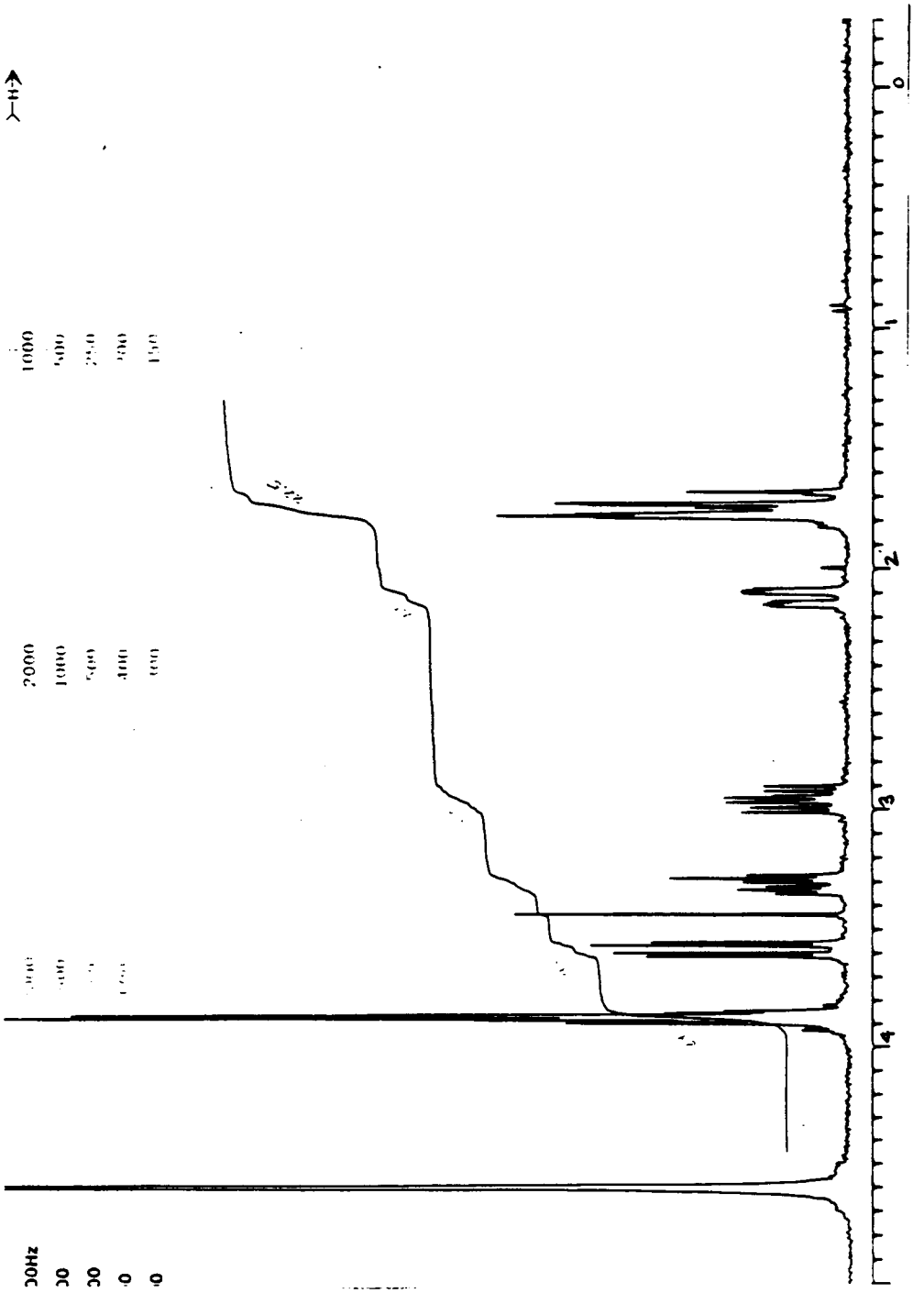
1. ^{1}H-NMR spectrum of butyl 3-hydroxypicolinate



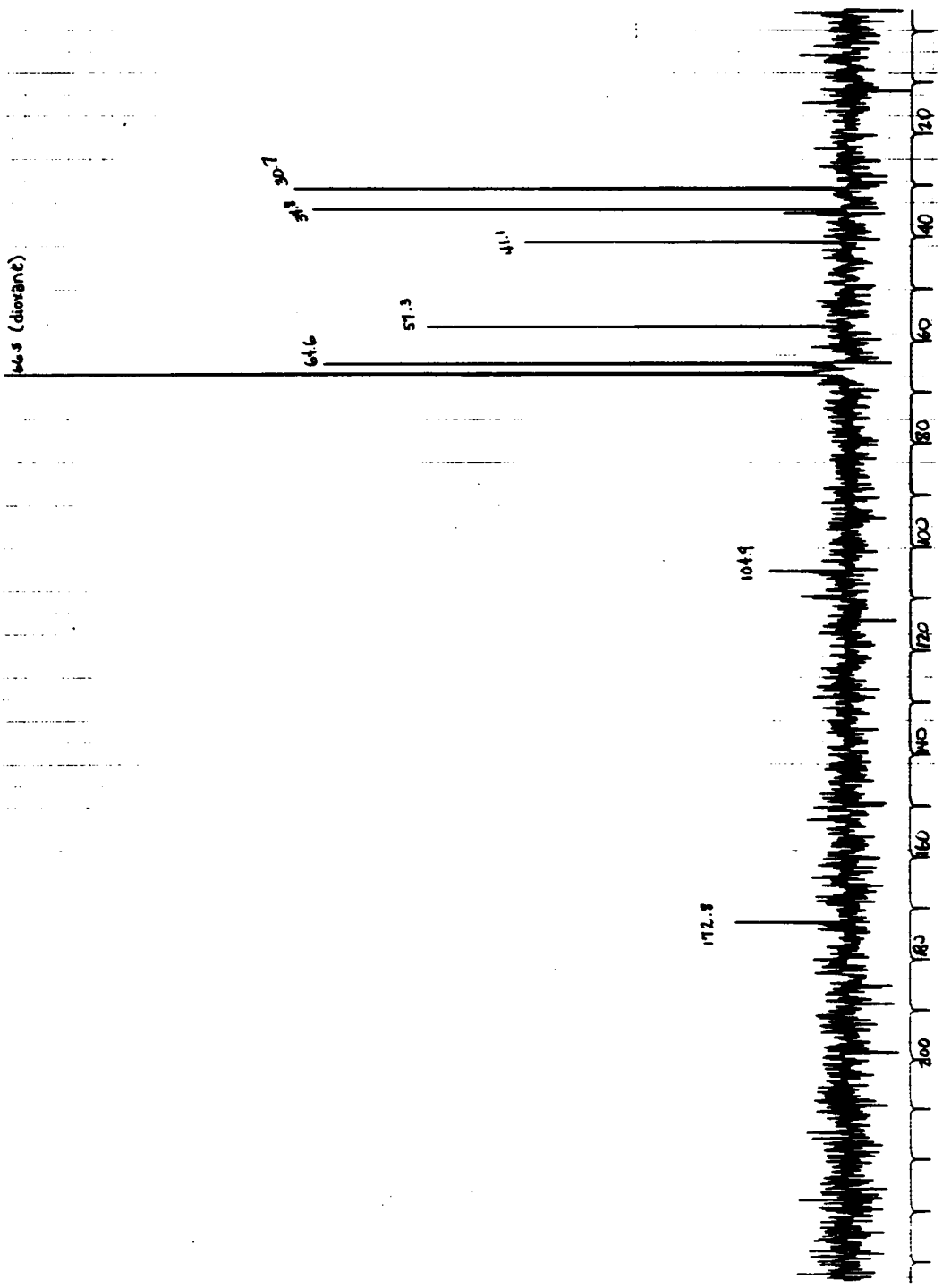
2. ^{1}H-NMR spectrum of 2-cyano-4-piperidone ethylene ketal (12)



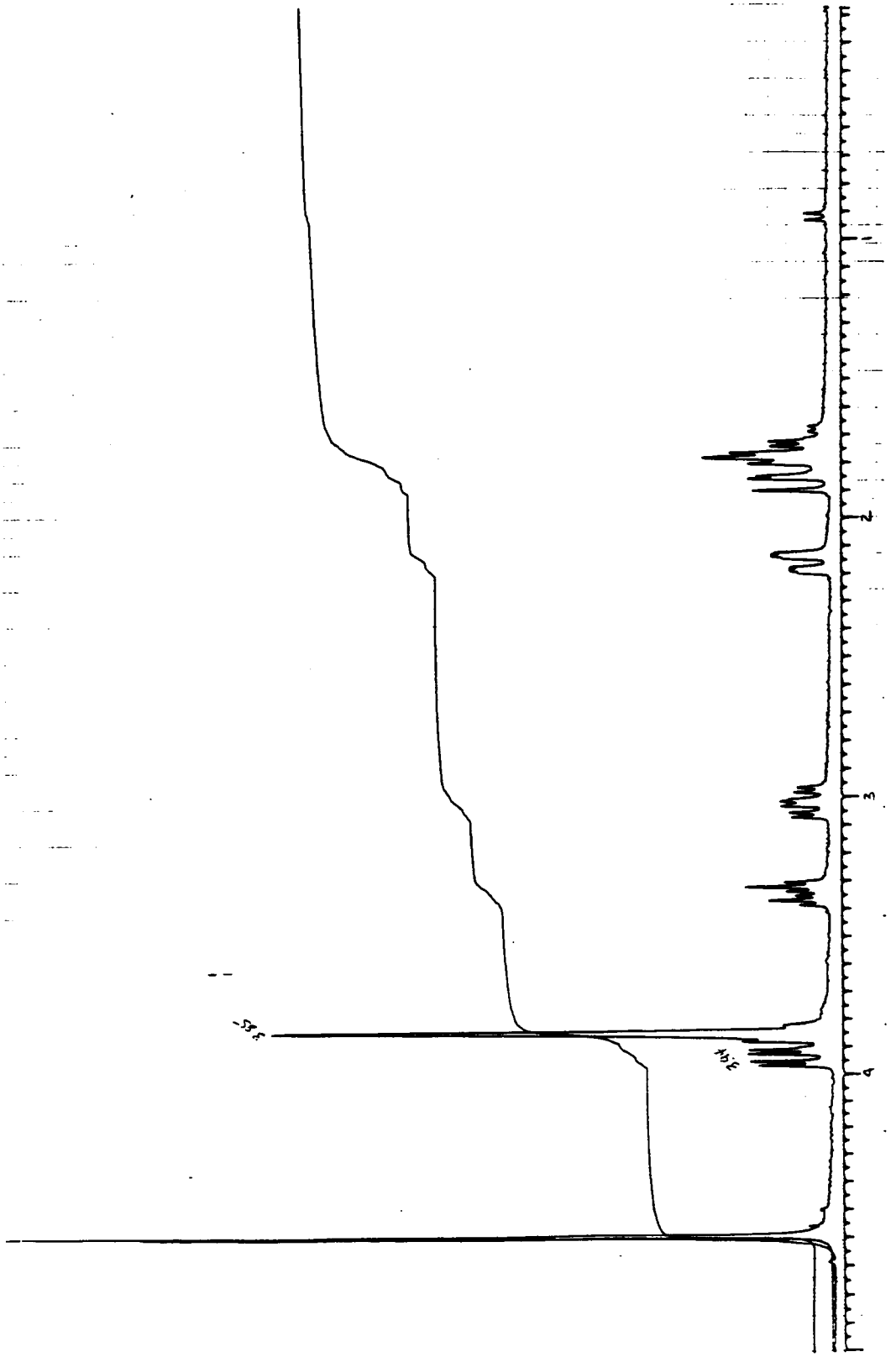
3. ^{13}C-NMR spectrum of 2-cyano-4-piperidone ethylene ketal (12)



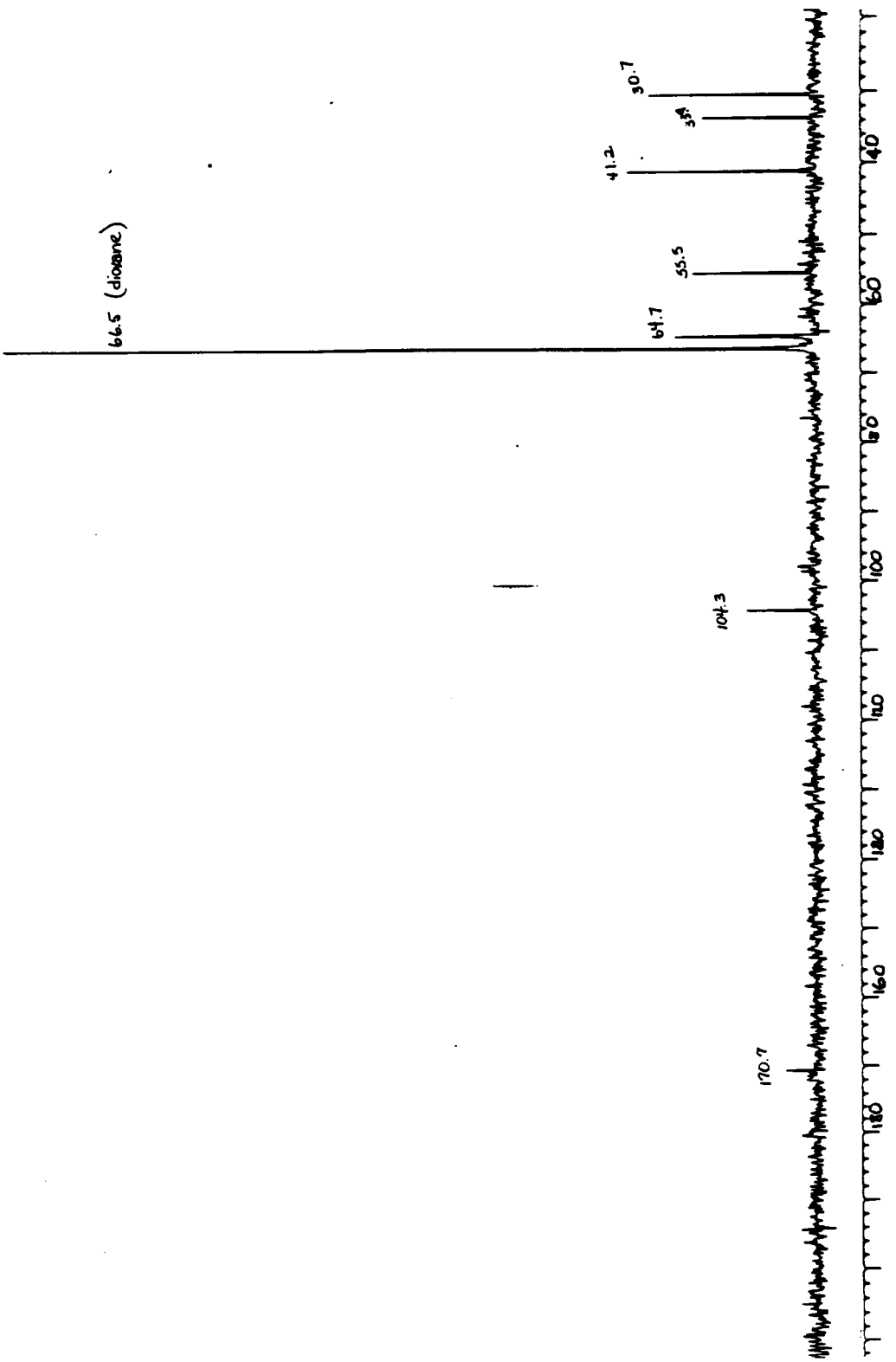
4. ^{1}H-NMR spectrum of 4-oxo-DL-pipecolic acid ethylene ketal (13)



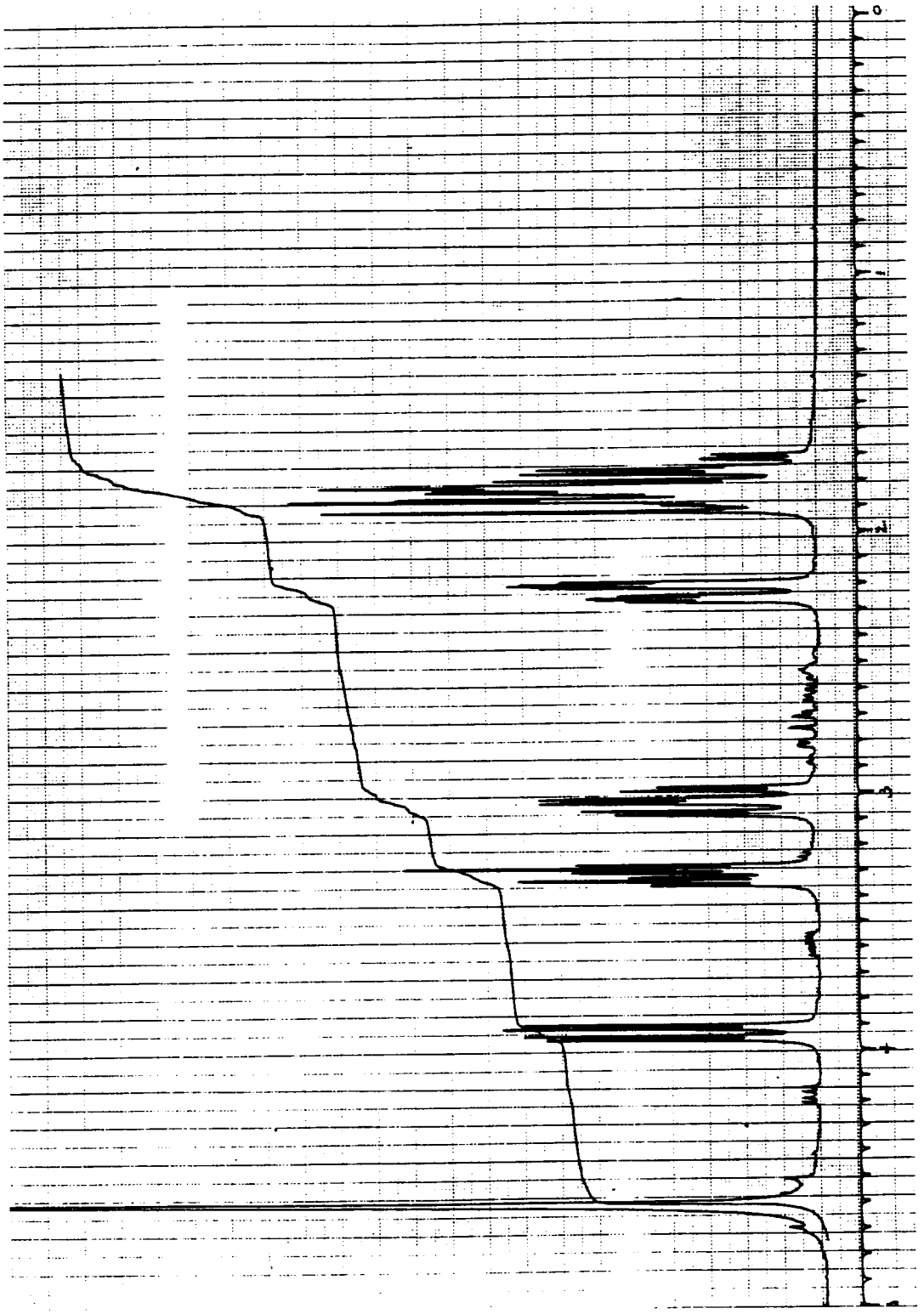
5. ^{13}C-NMR spectrum of 4-oxo-DL-pipecolic acid ethylene ketal (13)



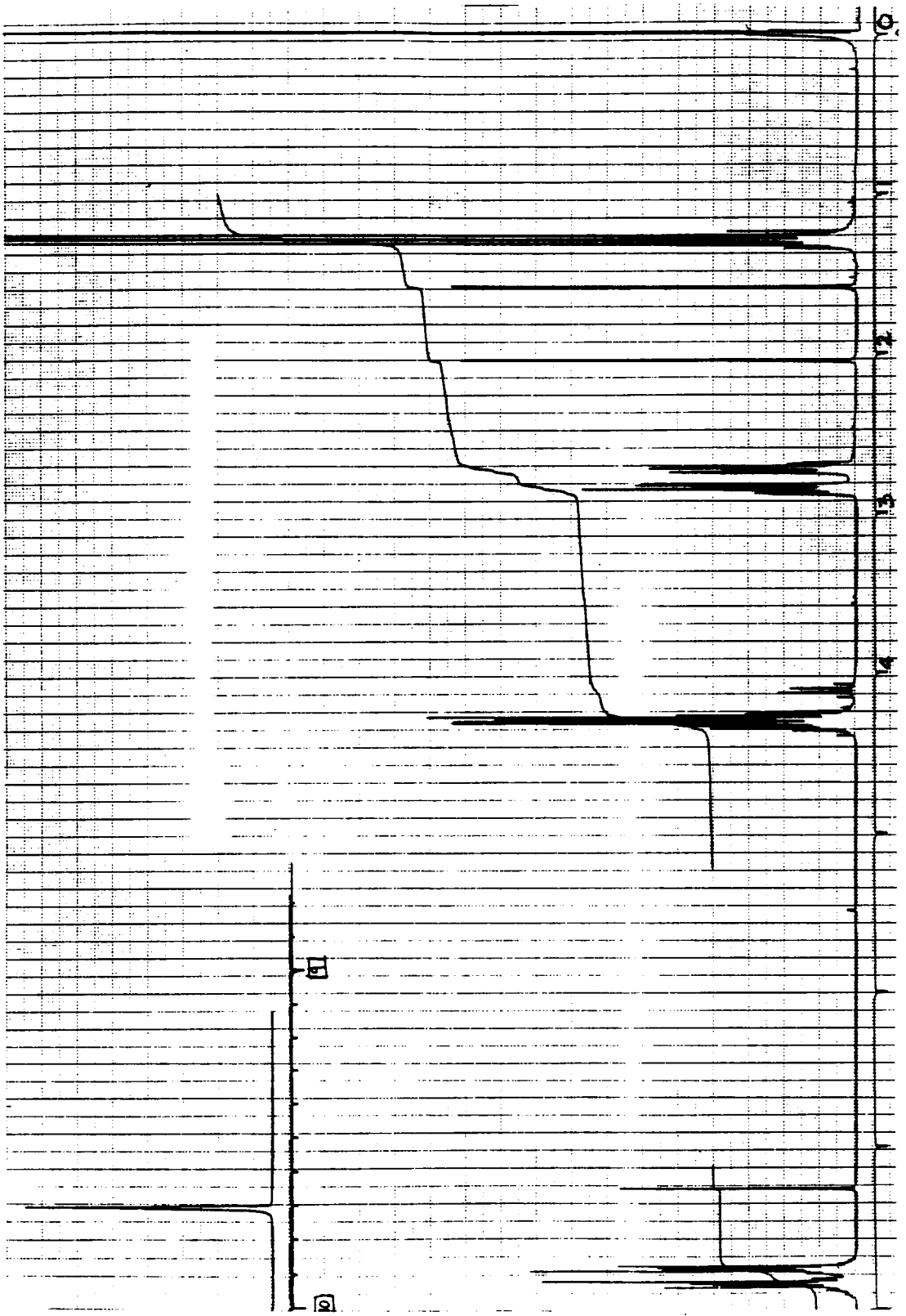
6. ^{1}H-NMR spectrum of 4-oxo-DL-pipecolic acid ethylene ketal hydrochloride (13)



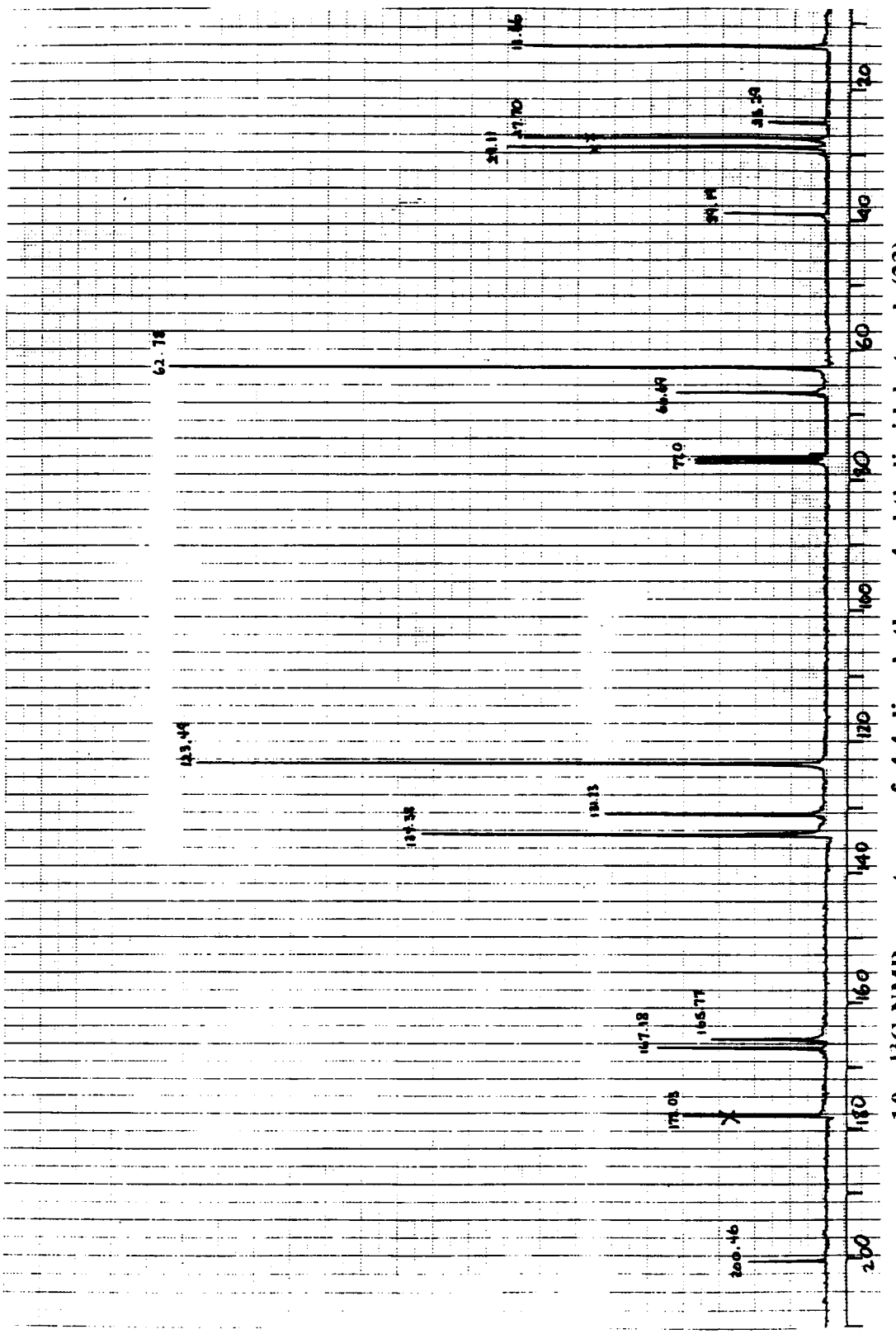
7. ^{13}C-NMR spectrum of 4-oxo-DL-pipecolic acid ethylene ketal hydrochloride (13)



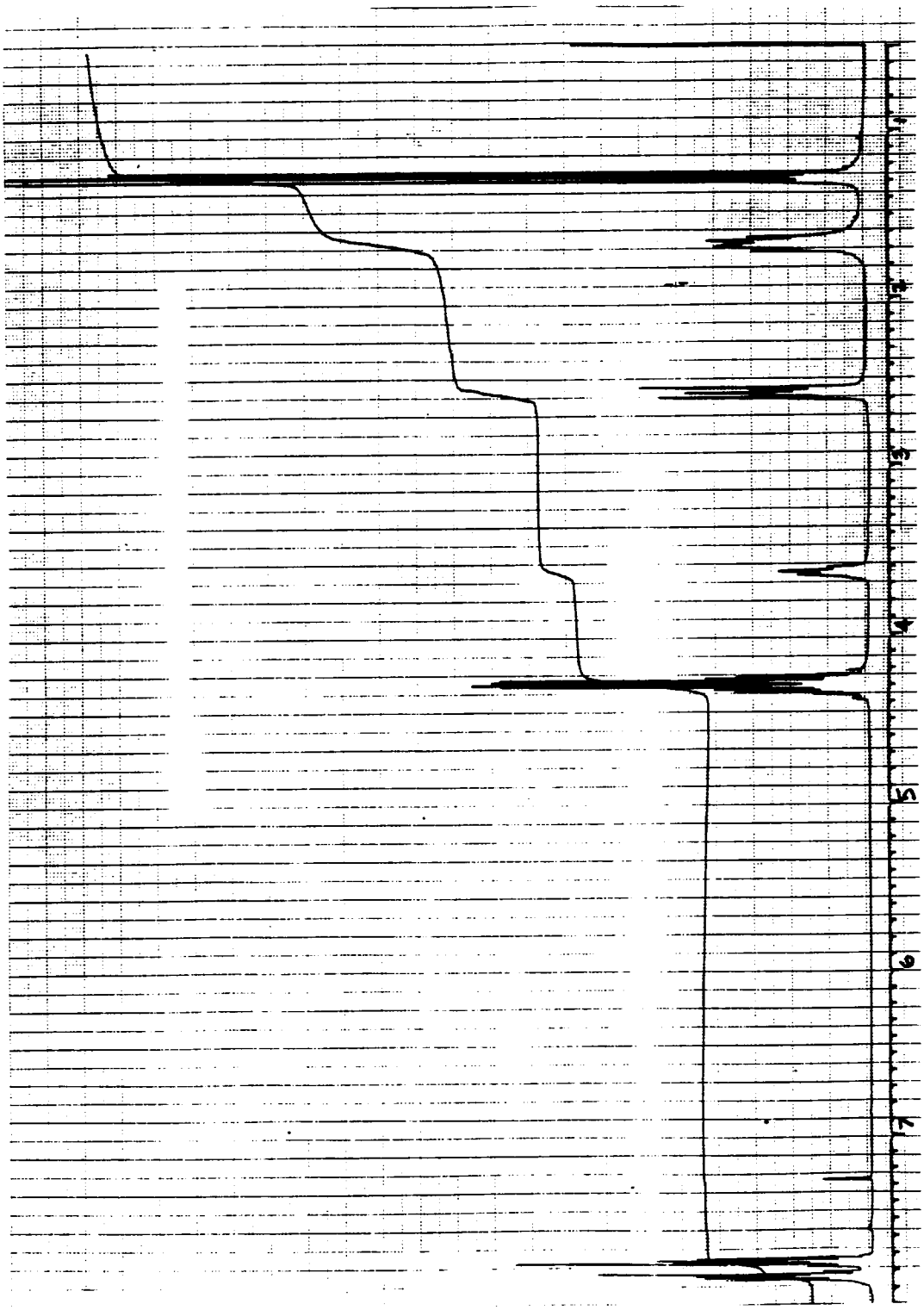
8. ^{1}H-NMR spectrum of 4-oxo-DL-pipecolic acid hydrochloride (14)



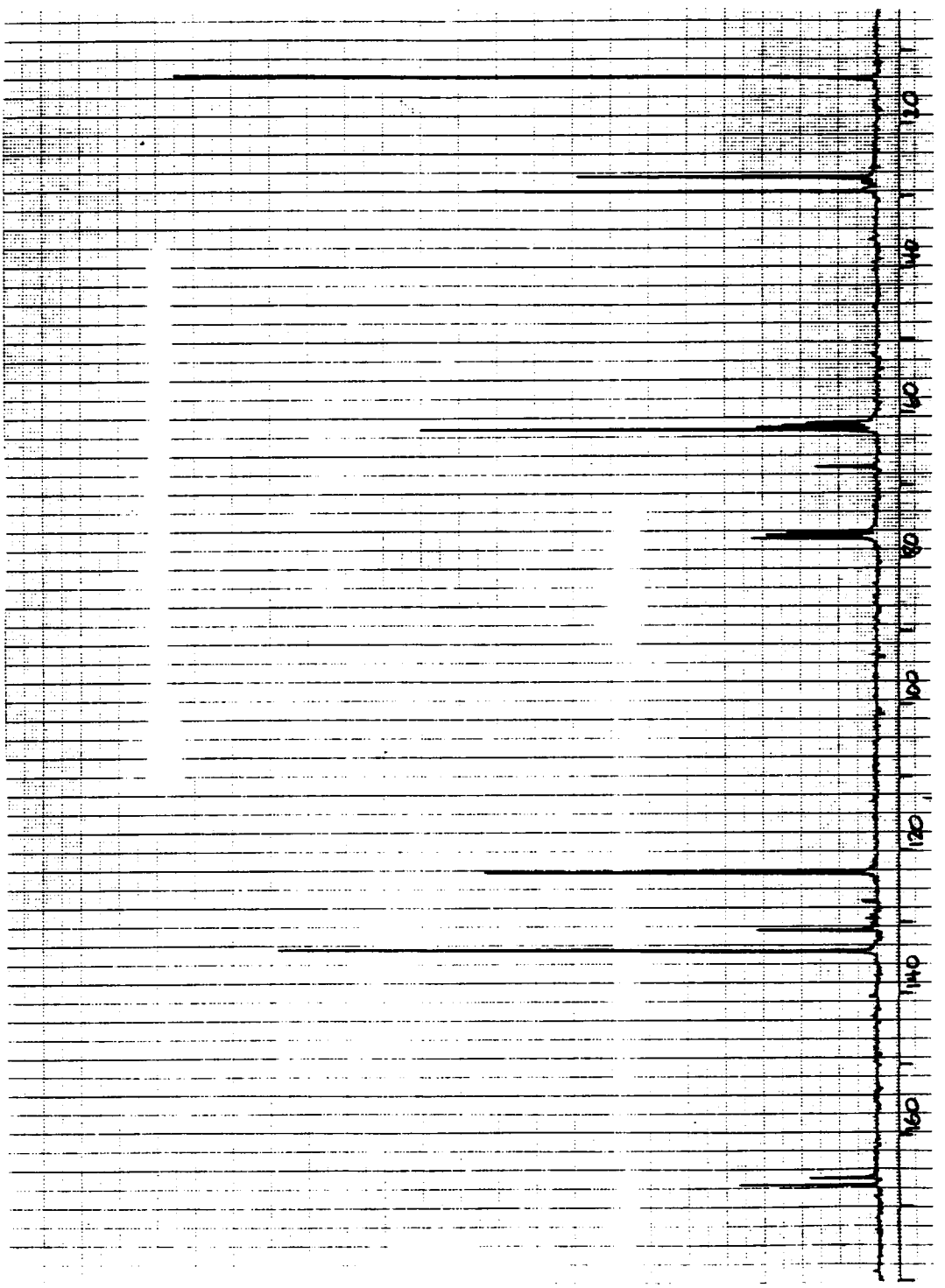
9. ^{1}H-NMR spectrum of 4,4-dicarbethoxy-4-phthalimidobutanal (23)



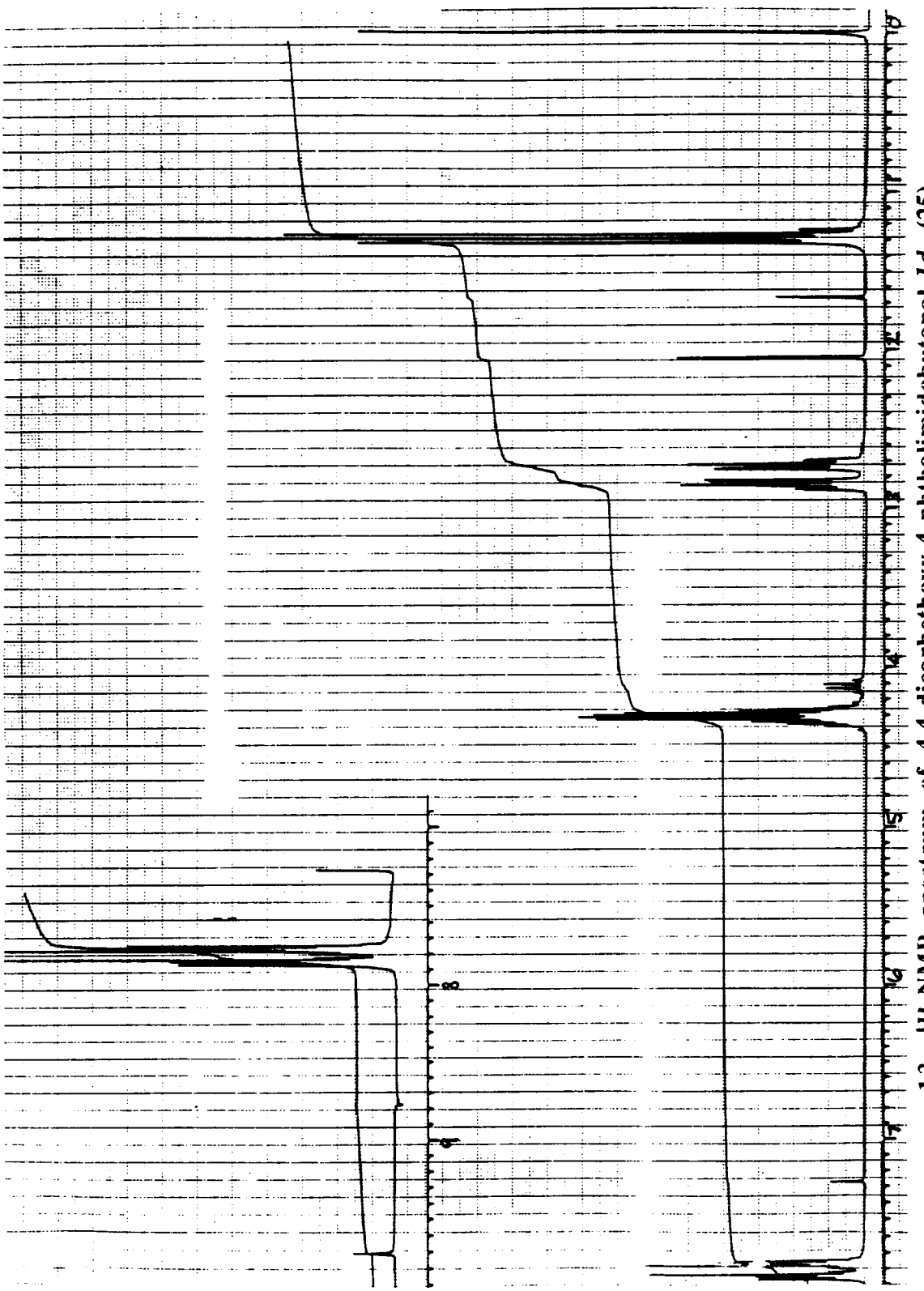
10. ^{13}C-NMR spectrum of 4,4-dicarbethoxy-4-phthalimidobutanal (23)



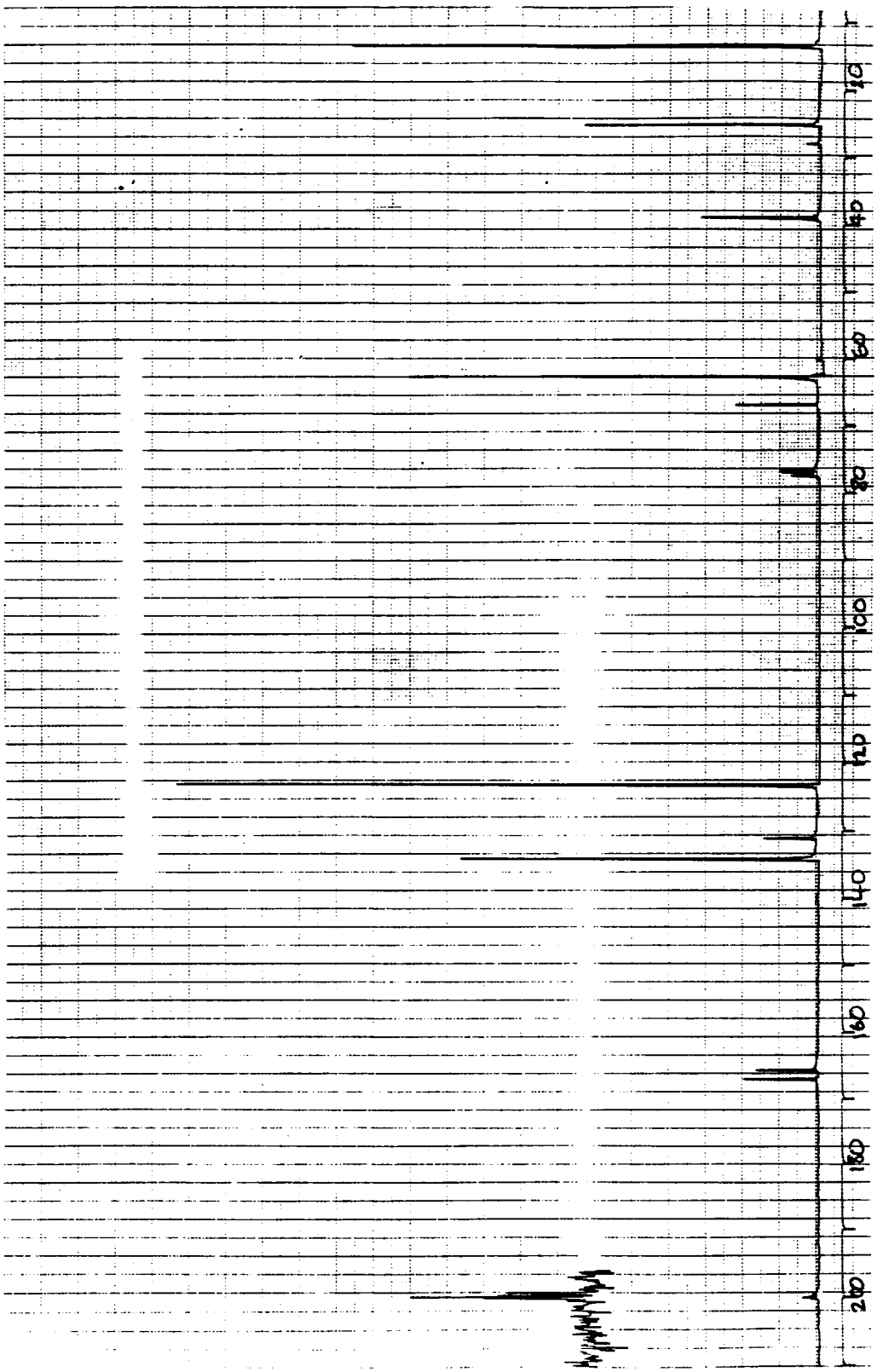
11. ^{1}H-NMR spectrum of ethyl 2-carbethoxy-2-phthalimido-5-hydroxypentanoate-5d_1 (24)



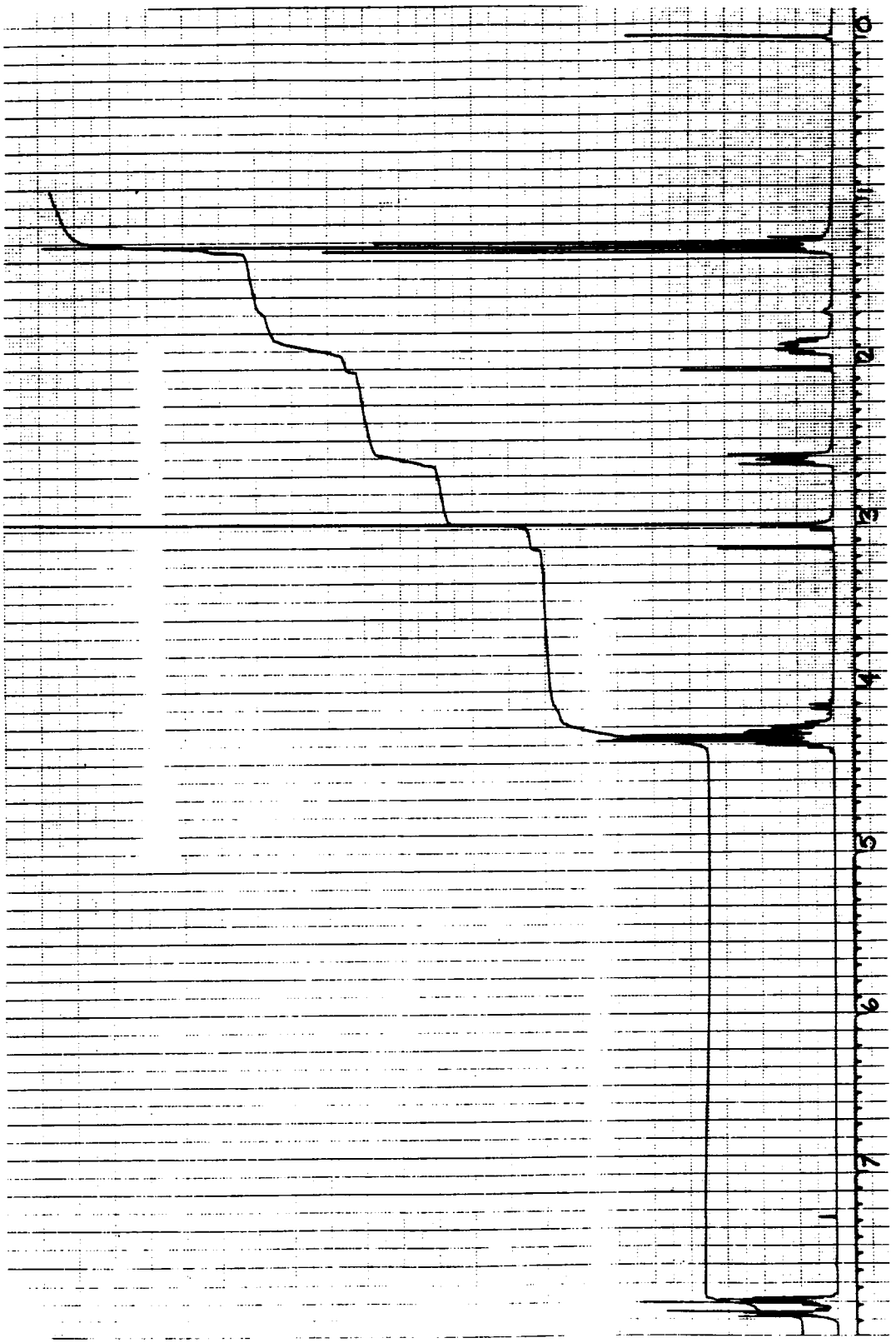
12. ^{13}C-NMR spectrum of ethyl 2-carbethoxy-2-phthalimido-5-hydroxypentanoate-$5d_1$ (24)



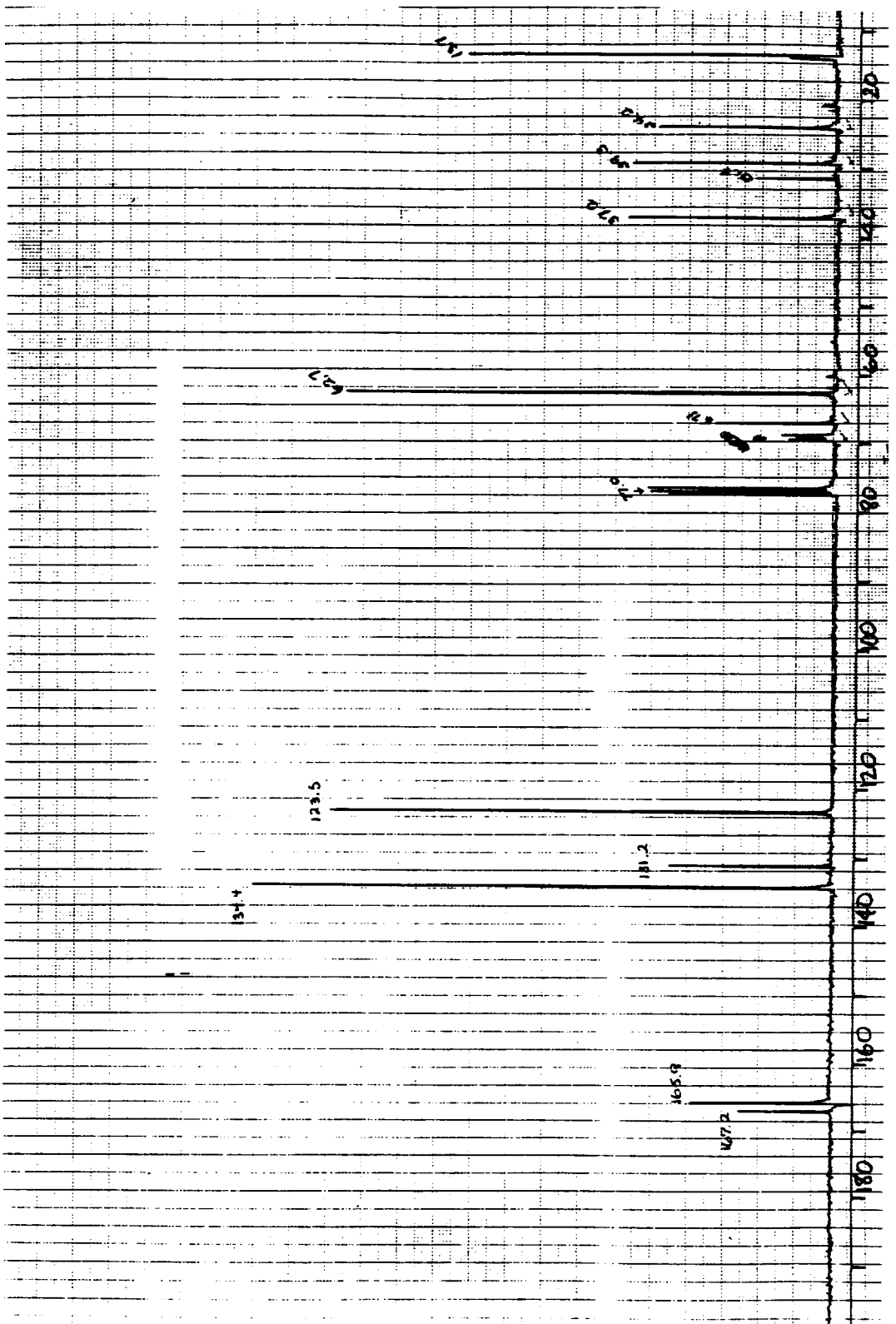
13. ^{1}H-NMR spectrum of 4,4-dicarbethoxy-4-phthalimidobutanal-$1d_1$ (25)



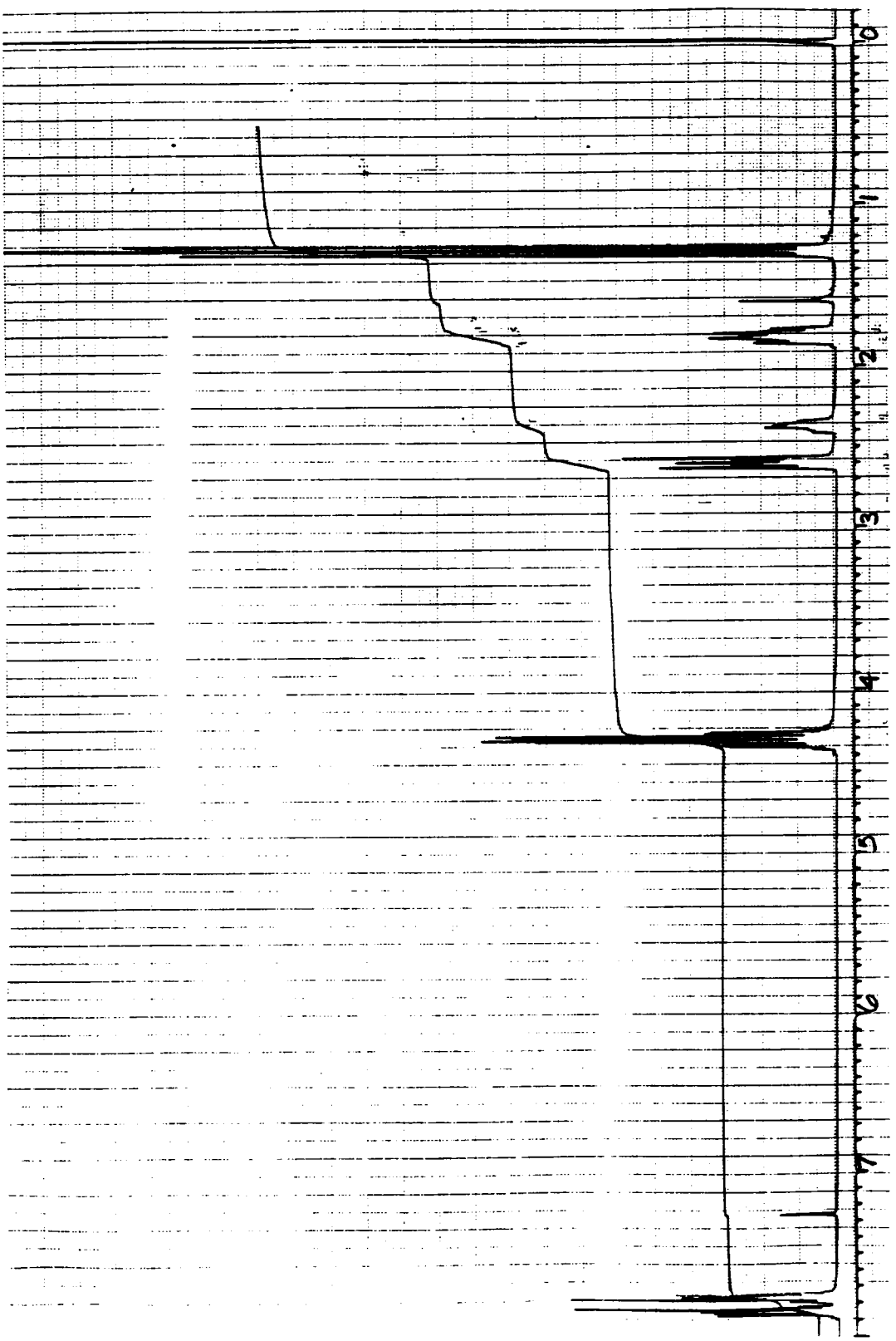
14. ^{13}C-NMR spectrum of 4,4-dicarbethoxy-4-phthalimidobutanal-$1d_1$ (25)



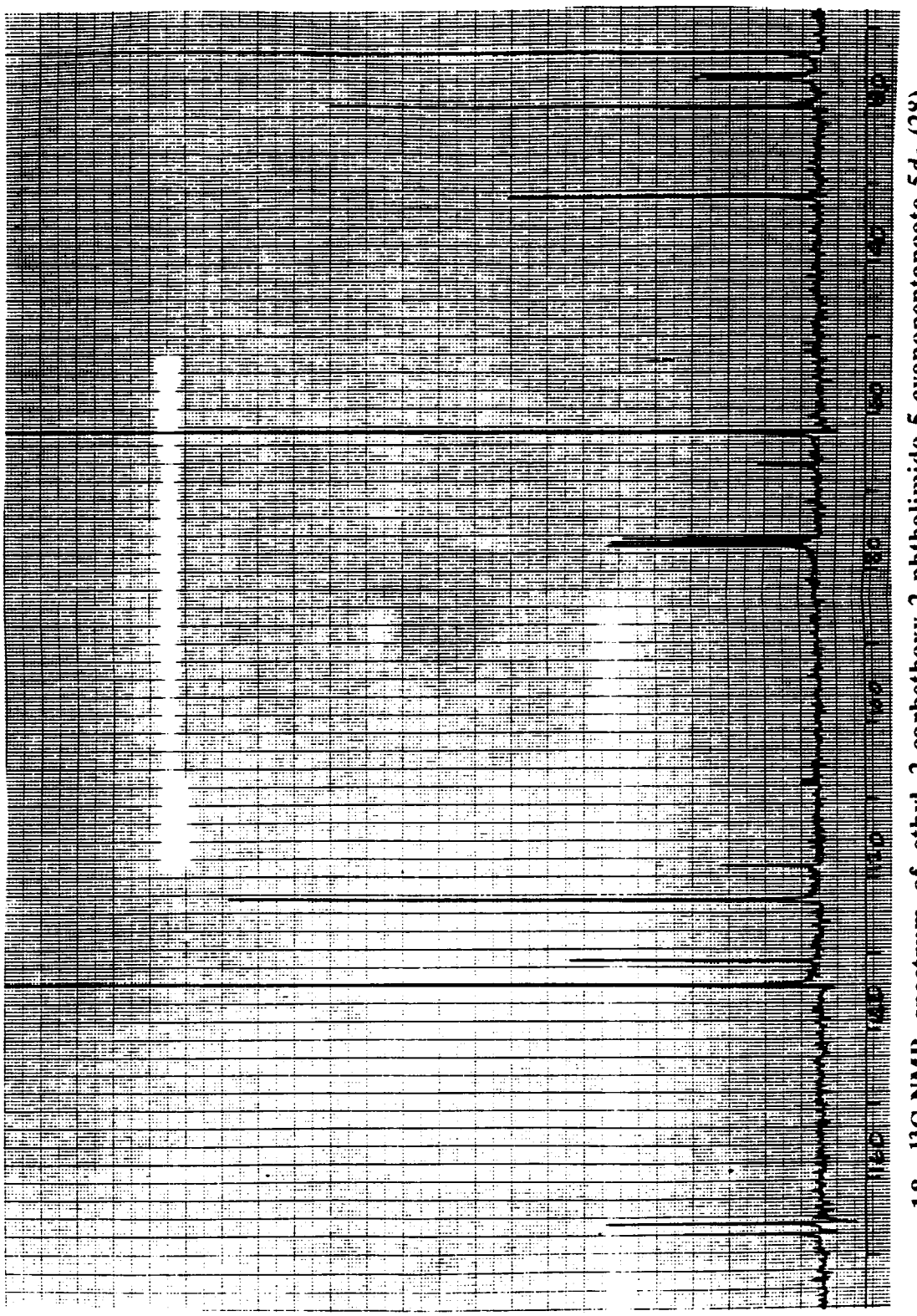
15. ^{1}H-NMR spectrum of ethyl 2-carbethoxy-2-phthalimido-5-methanesulfonyloxypentanoate-$5d_1$ (27)



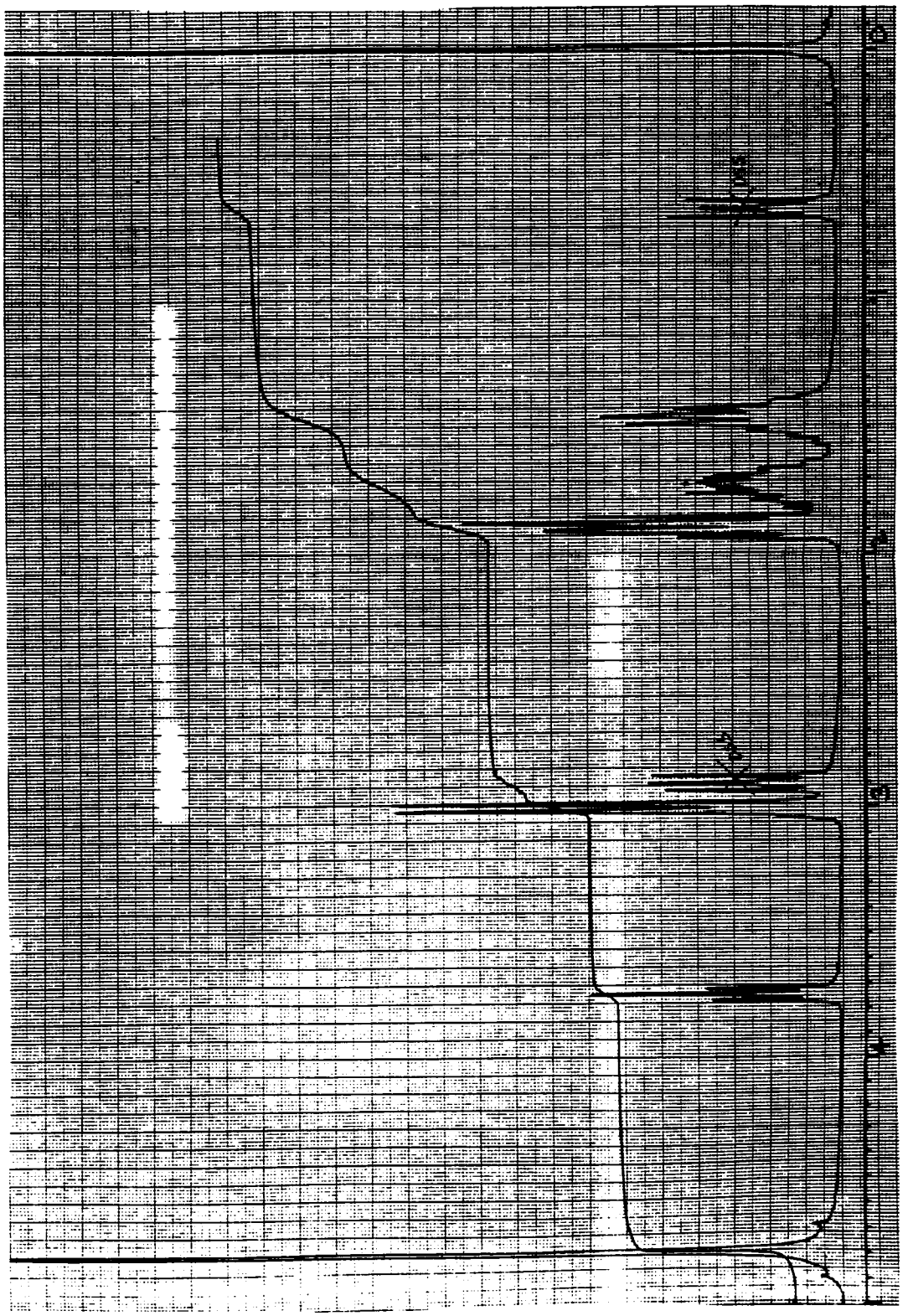
16. ^{13}C-NMR spectrum of ethyl 2-carbethoxy-2-phthalimido-5-methanesulfonyloxypentanoate-5d_1 (27)



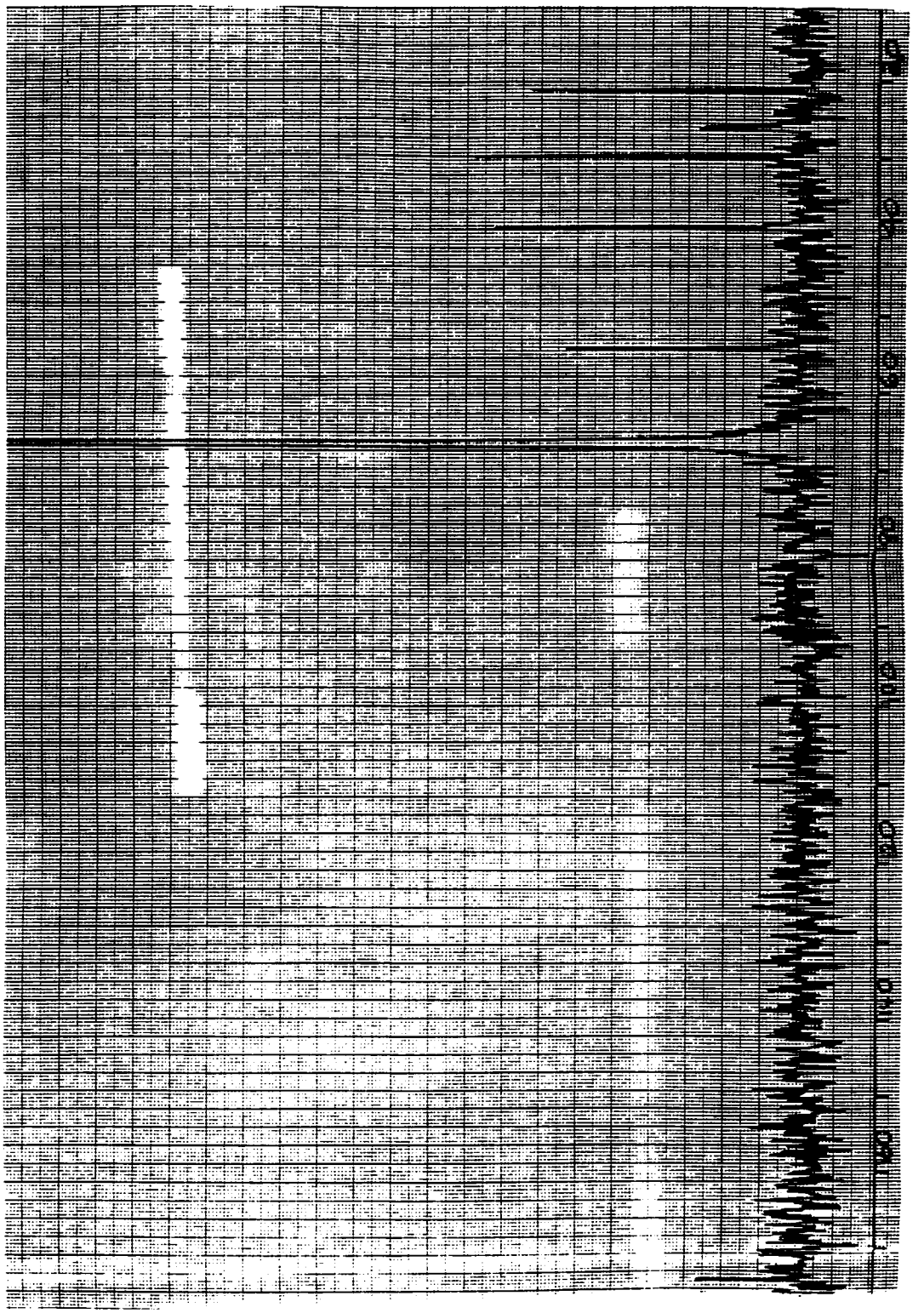
17. ^{1}H-NMR spectrum of ethyl 2-carbethoxy-2-phthalimido-5-cyanopentanoate-$5d_1$ (28)



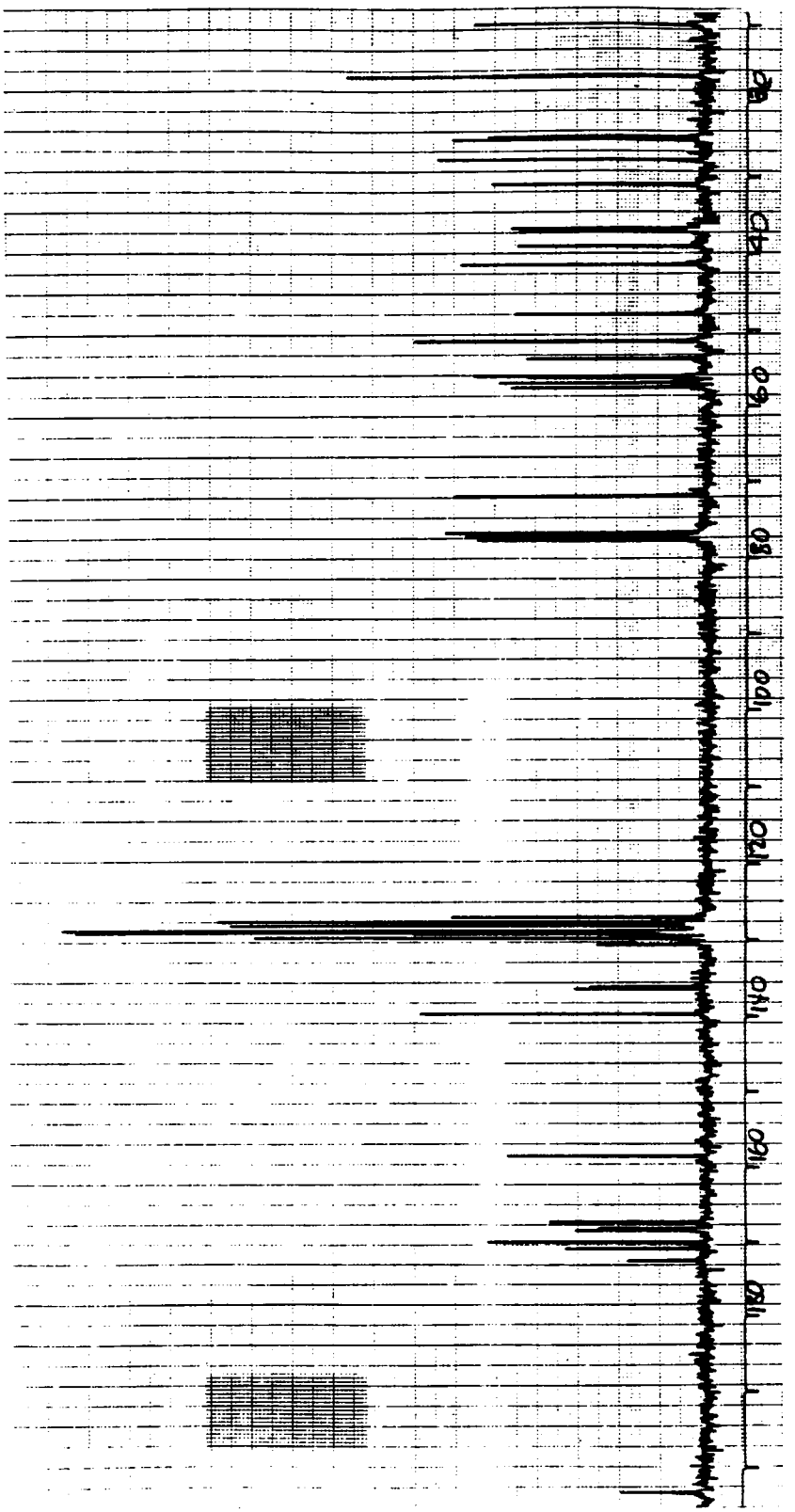
18. ^{13}C-NMR spectrum of ethyl 2-carbethoxy-2-phthalimido-5-cyanopentanoate-$5d_1$ (28)



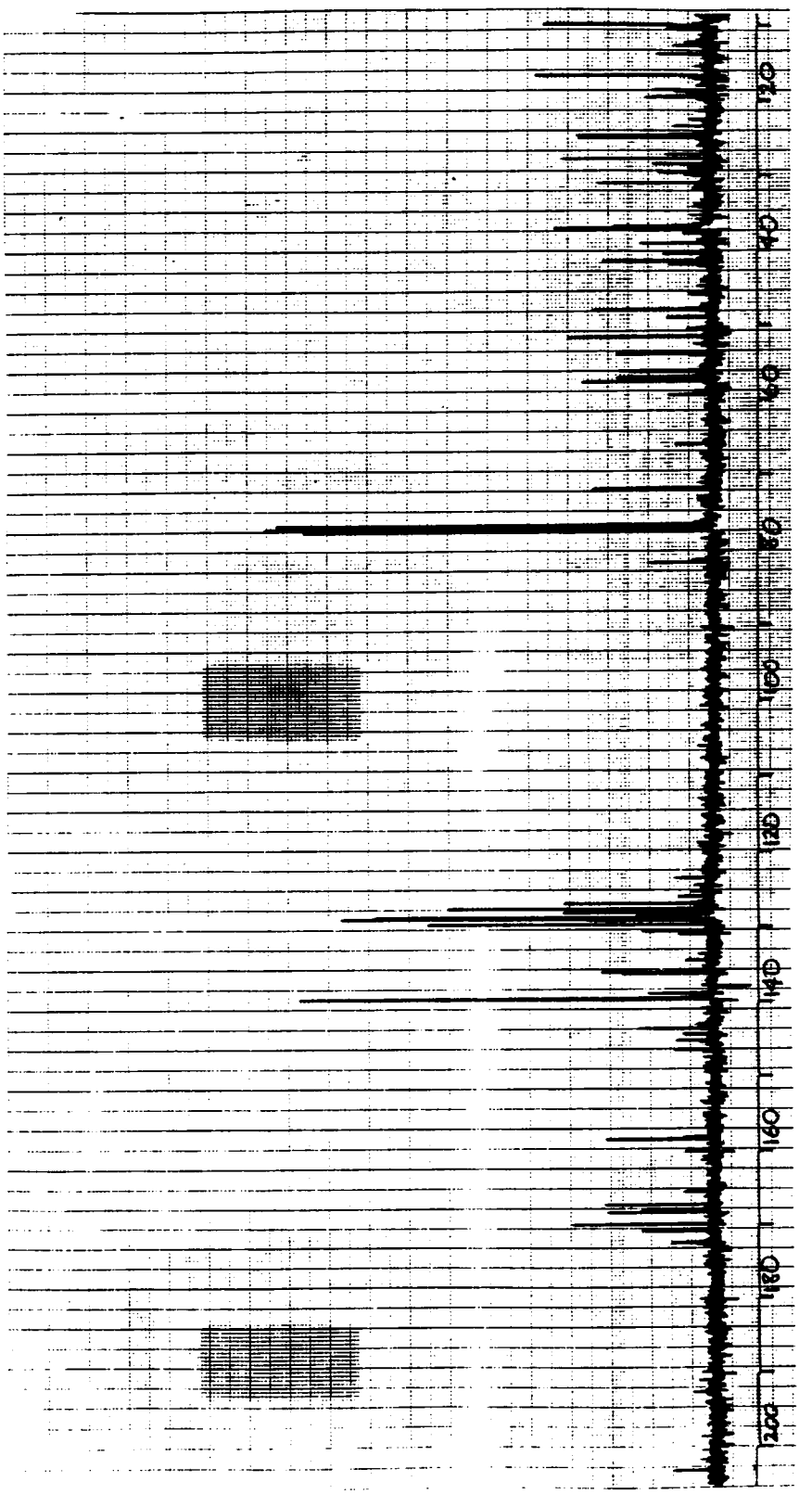
19. ^{1}H-NMR spectrum of (2*RS*, 5*R*)-lysine-5d_1 dihydrochloride (29)



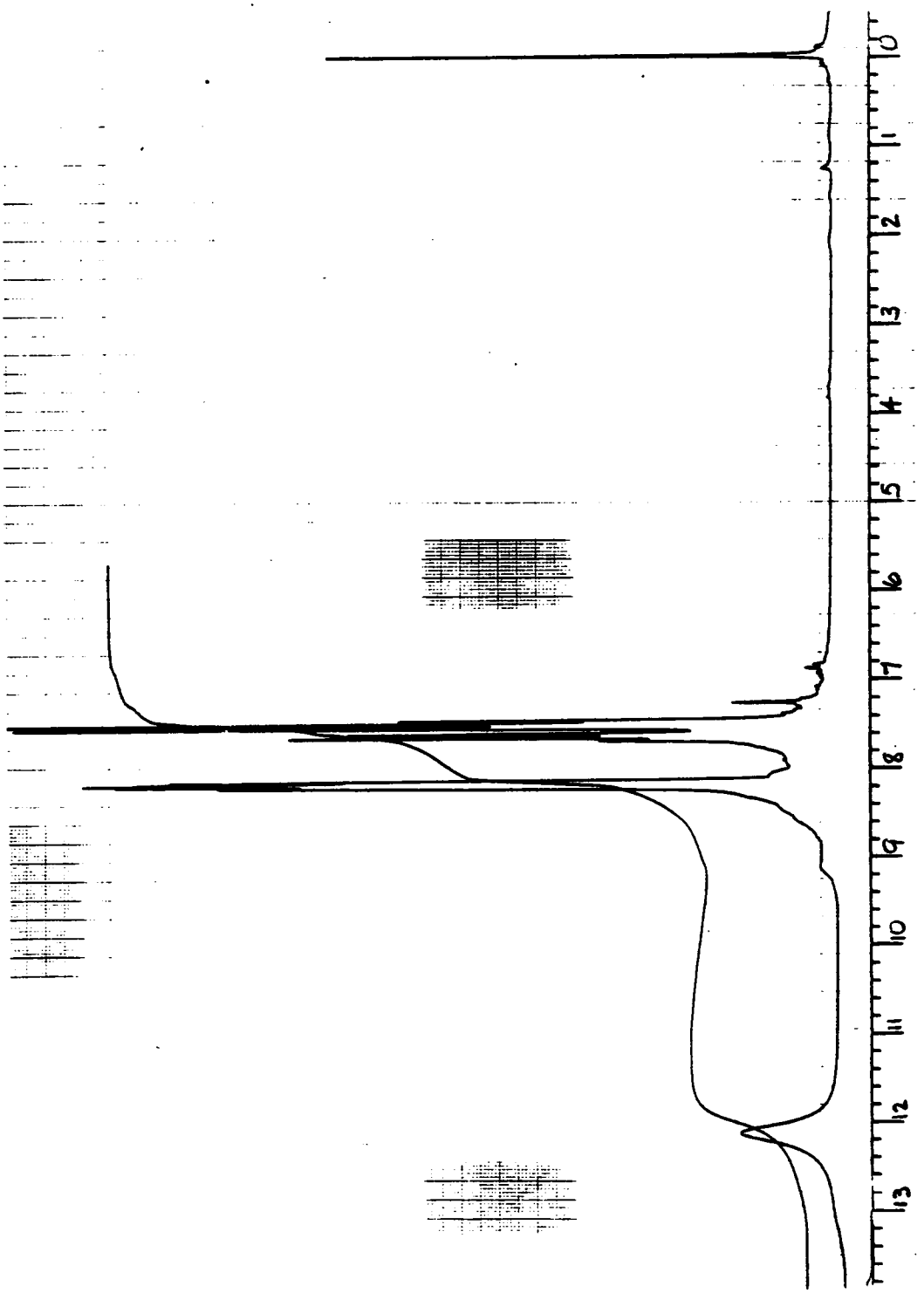
20. ^{13}C-NMR spectrum of (2*RS*, 5*R*)-lysine-5d_1 dihydrochloride (29)



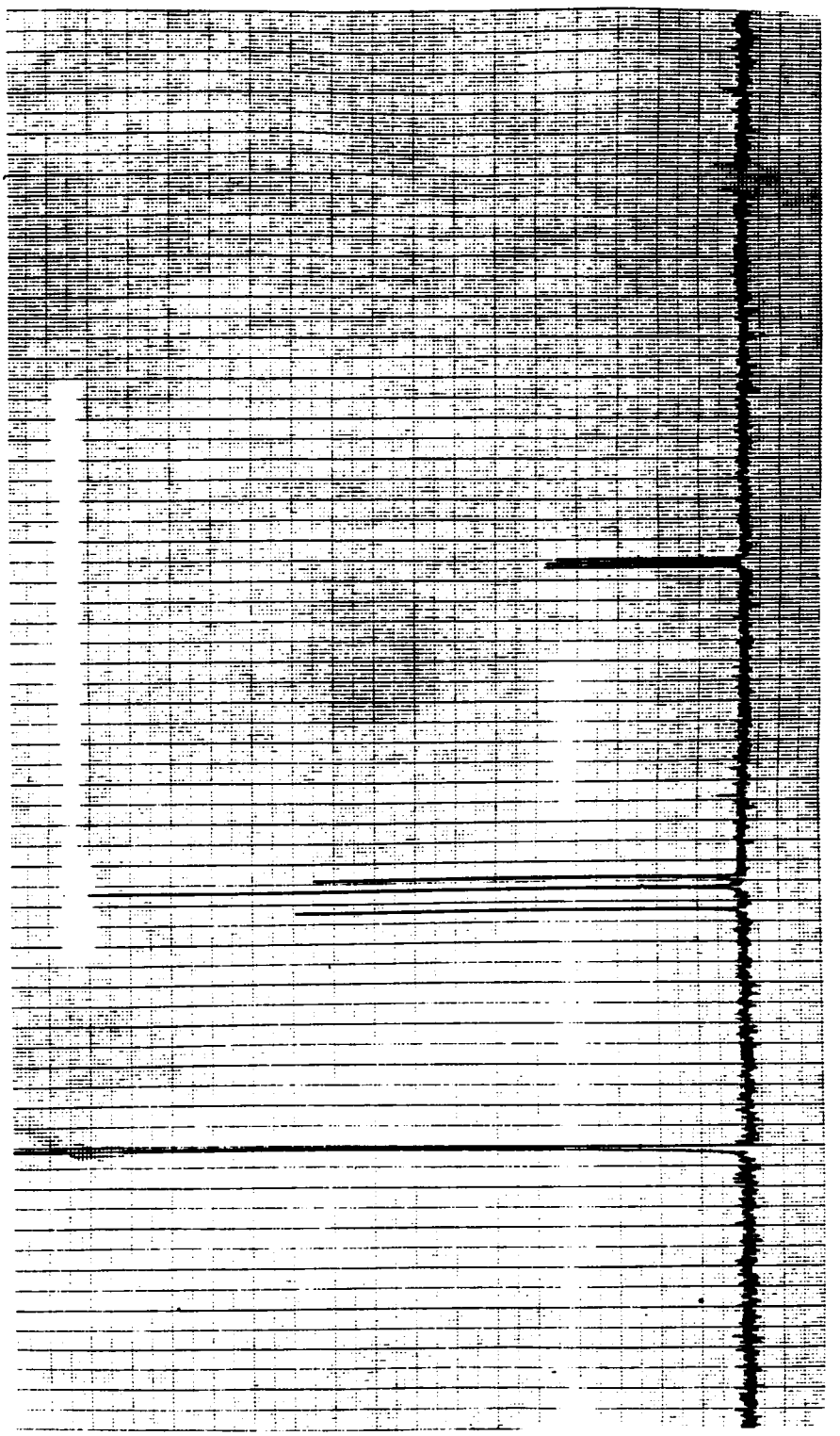
21. ^{13}C-NMR spectrum of virginiamycin S_1 standard



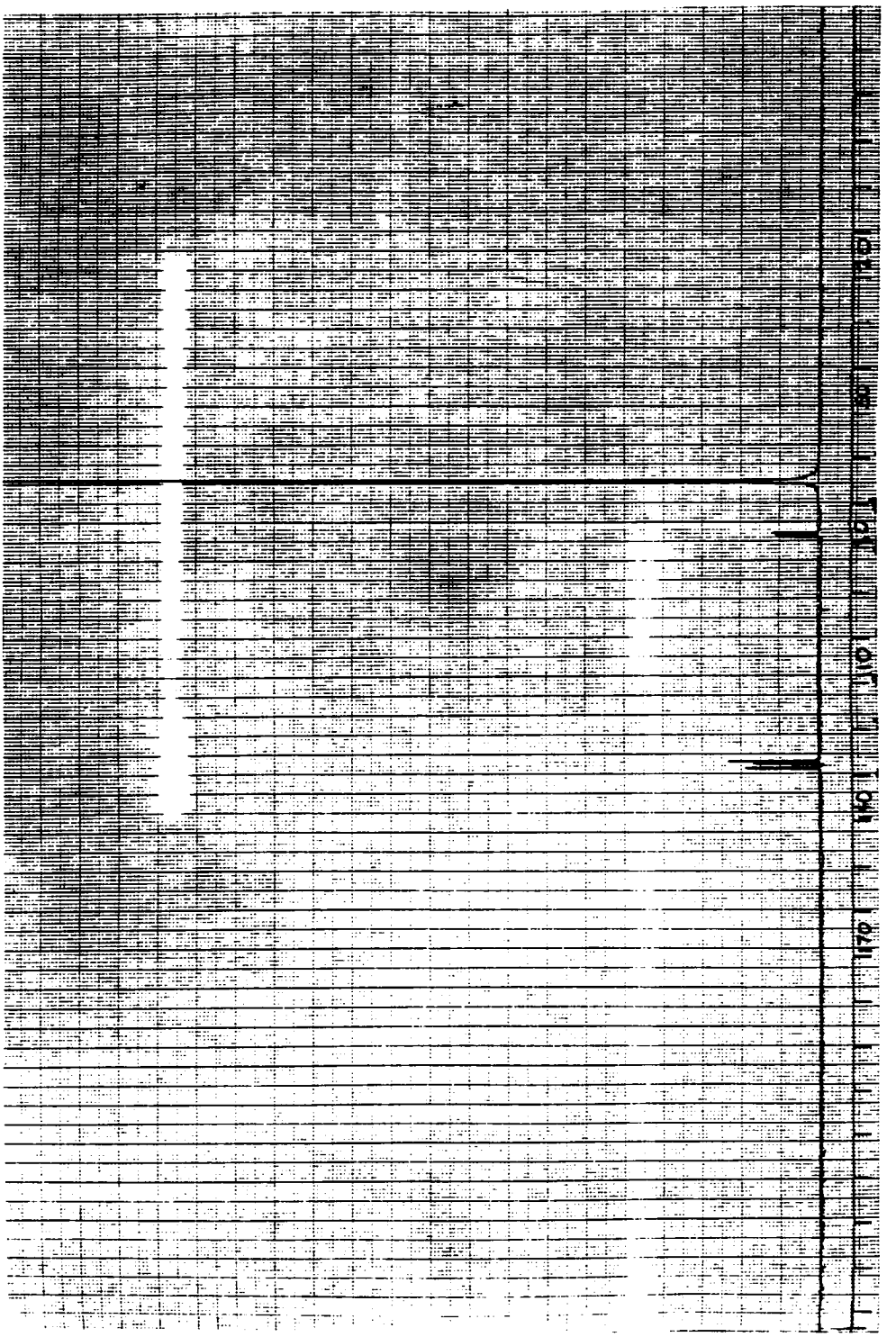
22. ^{13}C-NMR spectrum of virginiamycin S_1 from lysine-6-^{13}C-6-^{15}N incorporation



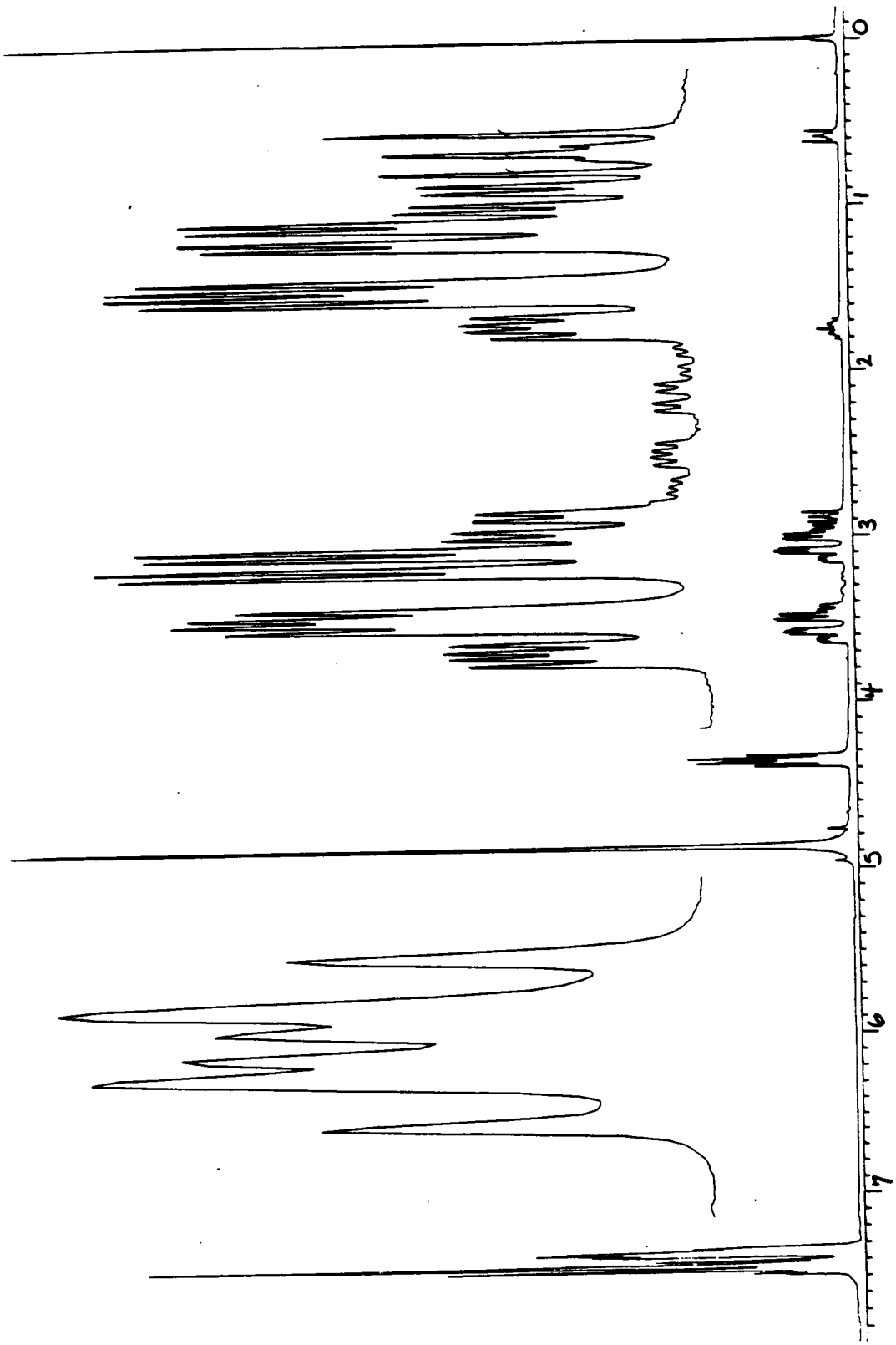
23. ^{1}H-NMR spectrum of benzoic-*carboxy*-^{13}*C* acid



24. ^{13}C-NMR spectrum of benzoic-*carboxy*-^{13}C acid

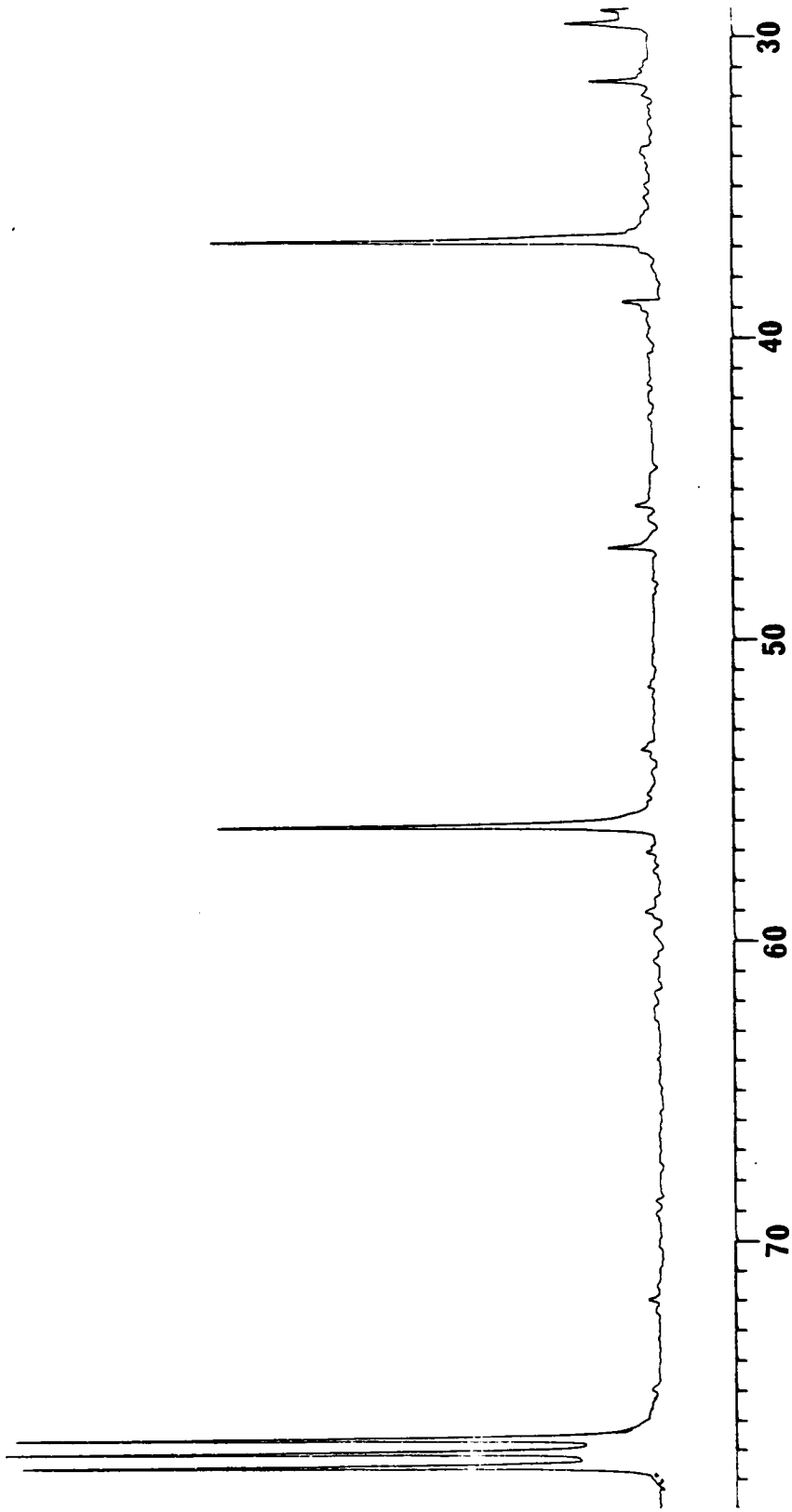


25. ^{13}C-NMR spectrum of benzyl-α-^{13}C alcohol



26. ^{1}H-NMR spectrum of DL-phenylalanine-3-^{13}C-^{15}N hydrochloride (35)

27. ^{13}C-NMR spectrum of DL-phenylalanine-3-^{13}C-^{15}N hydrochloride (35)



28. ^{13}C-NMR spectrum of virginiamycin S_1 from phenylalanine-3-^{13}C-^{15}N incorporation

The vita has been removed from the scanned document