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PART 1. THE SYNTHESIS OF SEVERAL STEREOSPECIFICALLY LABELLED AT C-3. PART 2. THE STUDY OF BIOSYNTHESIS USING STABLE ISOTOPE NMR TECHNIQUES.

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THE UNIVERSITY OF ALBERTA

PART 1. The Synthesis of Serine Stereospecifically Labelled at C-3. PART 2. The Study of Biosynthesis Using Stable Isotope NMR Techniques

by

Richard Newton Moore

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY

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External Examiner

Date December 19, 1983

To my Mother for developing a dream,

and to Laurie for helping me fulfil it.

"With the exception of music, we have been trained to think of patterns as fixed affairs. It's easier and lazier that way, but, of course, all nonsense... The right way to begin to think of the pattern which connects, is to think of a dance of interacting parts, pegged down by various sorts of... limits."

Gregory Bateson, 1978.

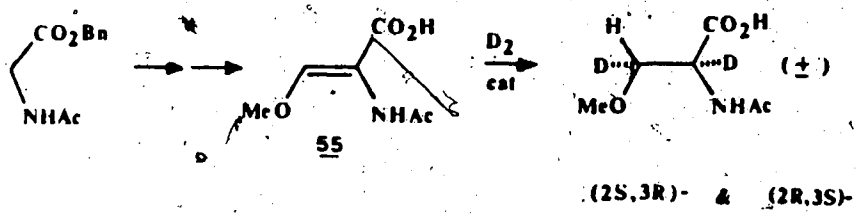
ABSTRACT

This thesis is divided into two parts.

Part 1. The synthesis of serine stereospecifically labelled at the C-3 position.

Approaches to the chemical synthesis of serine stereospecifically labelled at C-3, using catalytic hydrogenation of dehydroserine precursors, were examined. Attempts to prepare the cyclic substrate, 4-(methoxycarbonyl)-1,3-oxazolin-2-one (**7**), by oxidation of 4-(methoxycarbonyl)-1,3-oxazolid-2-one (**1**), modification of 4-(acetoxymethyl)-1,3-oxazolin-2-one (**10**) or oxidation of N-(phenoxycarbonyl)serine methyl ester (**5**) were unsuccessful. Acyclic dehydroserine precursors protected at the carboxyl group (R_1 = Me, Bn, Et), nitrogen functionality (R_2 = Pt, Bn-Ac, Ac, TFA) and hydroxyl moiety of the enol tautomer (R_4 = Me, Ac) **36**, **38**, **41**, **42**, **43**, **44**, **45**, **46**, **47**, **51** and **55** were synthesized by formylation of N-acylated glycine esters **23**, **28**, **24**, **26**, **25**, **27**, **29** followed by enol derivitization. Catalytic hydrogenation with deuterium gas of **36**, **55** and **43** (R_1 = CH_3, H, Et; R_2 = TFA Ac, Ac-Bn; R_4 = CH_3, respectively.) gave serine derivatives, which could be deprotected to form serine labelled at C-3. None of the synthetic routes developed, however, are completely satisfactory throughout the total synthetic sequence.

Compound 55 was found to be the most desirable precursor for future synthetic study.



Part 2. The study of biosynthesis using stable isotope NMR techniques.

Multiple oxygen-18 labelling of acetals induced chemical shift changes (isotope shifts) in the ^{13}C-n.m.r. spectra. These shifts are proportional to the number of atoms isotopically substituted by oxygen-18. A two-bond carbon-oxygen isotope shift (β shift) was also observed which had similar properties. The biosynthetic origin of oxygen atoms and the mechanism of lactone formation of andibenin B (from Aspergillus varicolor), multicolic acid, multicolanic acid and multicolosic acid (from Penicillium multicolor) were investigated by incorporation of ^{18}O-labelled precursors and inspection of the ^{13}C-n.m.r. spectra for isotope shifts. A Spin Echo Fourier Tranform (SEFT) pulse sequence was used to simplify spectra in which two bond carbon couplings obscured the isotope shift. Initial investigation of mevinolin biosynthesis by Aspergillus

terreus involved ^{1}H- and ^{13}C-n.m.r. spectral assignment using homo- and hetero-nuclear decoupling. A modified spin echo pulse sequence (CARPPET) gave the carbon multiplicities. All unknown carbon signals were assigned with [redacted] double quantum coherence (2D-INADEQUATE) n.m.r. expe[redacted]c on mevinolin enriched with carbon-13. This assignment was confirmed by two-dimensional selective Distortionless Enhanced Polarization Transfer (DEPT) n.m.r. experiments which correlate ^{1}H and ^{13}C chemical shift in separate plots for methine, methylene, and methyl groups. Addition of ^{13}C, ^{18}O and ^{2}H labelled precursors to cultures of A. terreus produced isotopically-enriched mevinolin samples. Examination of the n.m.r. spectra for enrichments (^{13}C), α-isotope shifts (^{18}O), β-isotope shifts (^{2}H), and double quantum coherence (adjacent ^{13}C atoms) showed that mevinolin is derived from two chains of intact acetate units and two methyl groups from methionine. One chain consists of nine acetate units linked together in a head to tail manner bearing one methyl group. The 2-methylbutyryl side chain is derived from two acetate units and the other methyl group.

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ABBREVIATIONS

Me	methyl
Et	ethyl
Bn	phenylmethyl
Ph	phenyl
Bz	benzoyl
Ac	acetyl
Pt	phthalimido (see note below)
TFA	trifluoroacetyl
CSI	chlorosulphonyl isocyanate
tlc	thin layer chromatography
MCPBA	meta-chloroperbenzoic acid
n.m.r.	nuclear magnetic resonance
IR	infrared
PLP	pyridoxal phosphate
LHMDS	lithium hexamethyldisilazide
LICA	lithium N-cyclohexyl-N-isopropyl amide
CoA	Coenzyme A
SEFT	Spin Echo Fourier Transform
2D-INADEQUATE	Two-dimensional Incredible Natural Abundance Double Quantum Transfer Experiment
DEPT	Distortionless Enhanced Polarization Transfer
CARPPET	Carbon Parity Partitioning by Echo Technique

DMB	2,4-dimethoxybenzoyl
DNP	2,4-dinitrophenyl
NOE	Nuclear Overhauser Enhancement
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A reductase

Note: The naming of the 1,3-dihydro-1,3-dioxo-2H-isoindol-2-yl (Chem. Abstracts name) group is replaced by phthalimido (IUPAC name) throughout this thesis for simplicity.

PART 1

The Synthesis of Serine Stereospecifically Labelled at C-3.

INTRODUCTION

A. Biological Background of Serine and Dehydroalanine.

Serine is one of the twenty commonly occurring amino acids. It has a hydroxymethyl side chain which renders it a hydrophilic and a polar amino acid.[1]

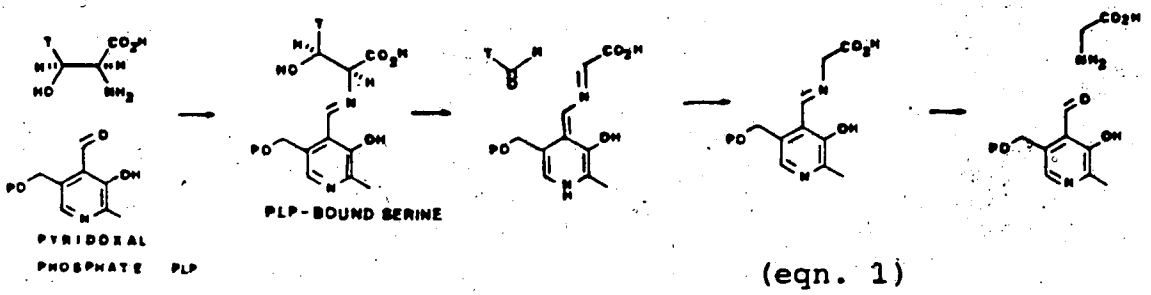
Apart from imparting the required backbone into protein structure, amino acids are involved in a variety of biochemical functions.

In addition to the role played by serine in the serine proteases the control of vision may be regulated by phosphorylation of the hydroxyl group of serine residues at the C-terminal end of the rhodopsin.[2,3]

Serine is also a substrate in a large number of pyridoxal phosphate (PLP) catalyzed reactions.[4] These are divided into three groups:

1. α,β-Cleavage.[5,6,7]

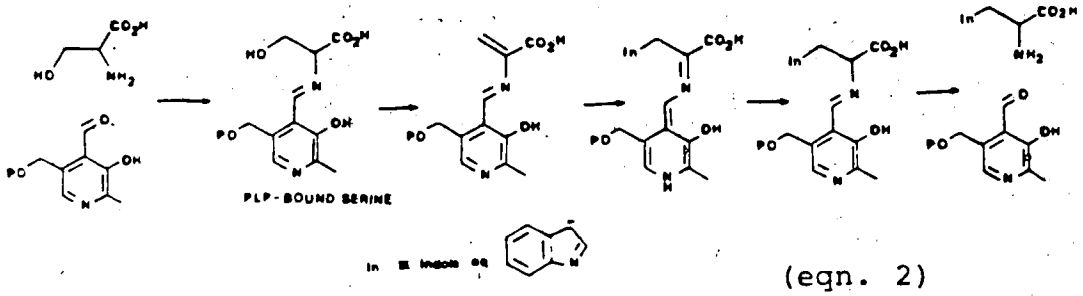
The enzyme serine hydroxymethyltransferase[8] (EC 2.1.2.1) cleaves the 2,3-carbon bond of serine with the formation of glycine and formaldehyde.[4,7,6] The formaldehyde produced acts as the one-carbon donor in the biosynthesis of purines. This process is mediated by the coenzyme tetrahydrofolate, and biologically controls the interconversion of serine and glycine.[9] The cleavage (eqn. 1) is shown below as an example of α, β-cleavage.



(eqn. 1)

2. β-Replacement (displacement at the hydroxyl group of serine).[10,11,12]

The enzyme tryptophan synthetase (EC 4.2.1.20) catalyzes the replacement of the hydroxyl group of serine by an indole equivalent, to yield tryptophan with retention of configuration. This reaction proceeds through a PLP-bound dehydroalanine unit derived from PLP-bound serine by elimination of water. This example of a β-replacement reaction is shown in eqn. 2.

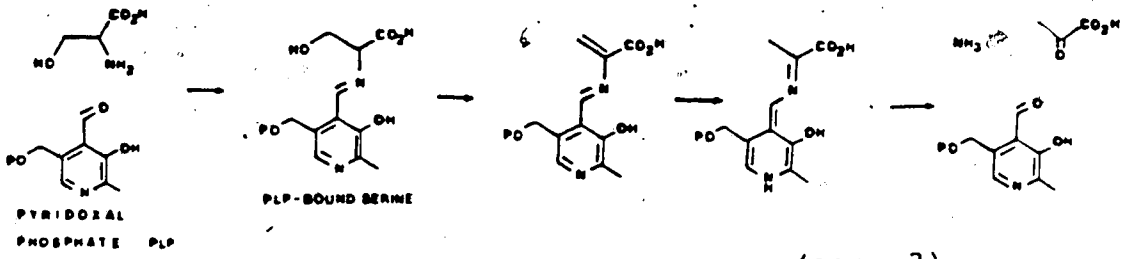


(eqn. 2)

3. α, β-elimination.[11,13]

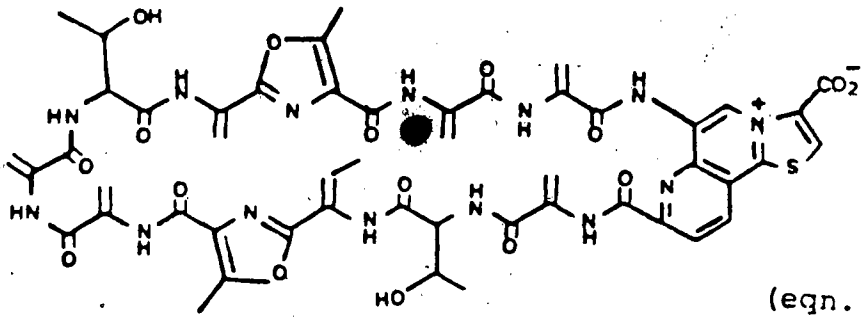
The enzyme serine dehydrase (EC 4.2.1.13) catalyzes the conversion of serine to pyruvic acid. The initial elimination of water from serine gives a bound

dehydroalanine unit as indicated in eqn. 3. Tautomerization and hydrolysis gives pyruvic acid, ammonia and pyridoxal phosphate. This is an example of α, β-elimination.



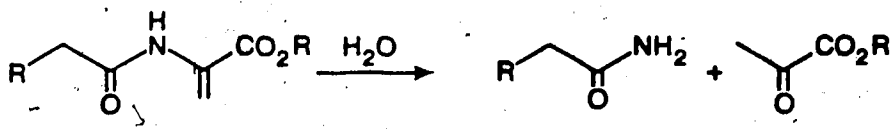
(eqn. 3)

The occurrence of dehydroalanine is not limited to intermediates in PLP processes. Dehydroalanine units have been isolated in several natural products,[14-27] such as berninamycin[28] (eqn. 4), where seven dehydroalanines are present. Lanthionine residues are often found in the same molecule as dehydroalanine residues.[18,20] These are probably formed by a Michael type addition to the dehydroalanine moiety by cysteine.



(eqn. 4)

Gross has suggested[20] that the terminal amide units observed in specific peptides may originate from hydrolysis of dehydroalanine units (eqn. 5).[20] This may be the process by which some pyruvate dependent bacterial decarboxylases such as histidine decaboxylase (EC 4.1.1.22) are activated.[29,30] The hydrolysis also yields a peptide with a modified amino terminus.

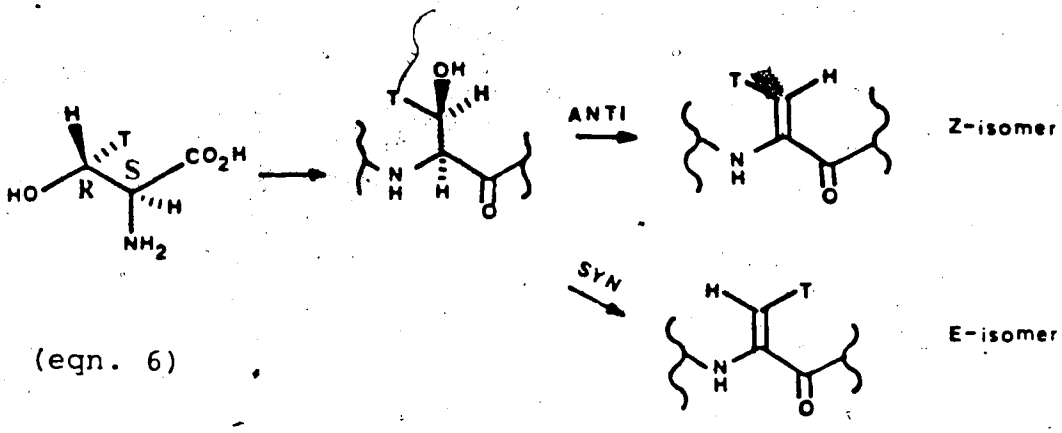


(eqn. 5) Terminal Amide Pyruvyl - Enzyme

Additionally, dehydroamino acids have been synthesized as chiral amino acid synthons, as precursors to β-substituted amino acids, and for insertion into biologically active peptides.[14,16,25,31-39] This substitution of a dehydroalanine moiety into a peptide affords additional hydrophobicity, introduces a sp^2 centre into the polypeptide backbone, and adds an electrophilic centre where modification can occur.

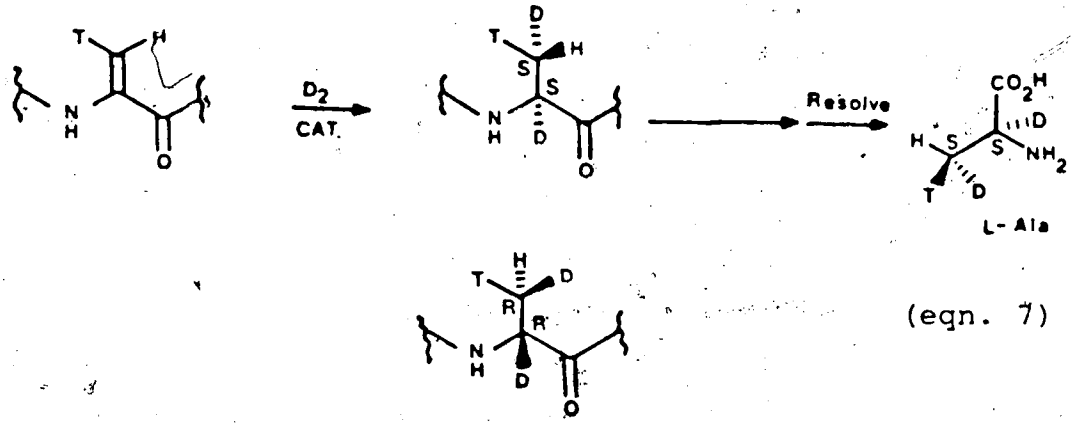
The stereochemistry and mechanism of formation of dehydroalanine is unknown. Rinehart has determined that serine is a precursor of dehydroalanine in berninamycin.[28] Its formation from serine can take place, in principle, by

either syn- or anti- elimination of water (eqn. 6).[40] Since dehydroalanine itself is unstable, this presumably takes place after incorporation of serine into the peptides.



(eqn. 6)

Incorporation of (2S,3R)-[3-$^{3}H_{1}$]-serine into the peptide followed by an anti-elimination of water would give a Z dehydroalanine unit. Alternatively, a syn-elimination of water would yield an E dehydroalanine residue. The stereochemical analysis could be accomplished as shown in eqn 7. Cis hydrogenation[41] of the Z isomer with deuterium gas would result in a (2R,3R)- and (2S,3S)-enantiomeric pair



(eqn. 7)

of labelled alanine residues. The (2S,3S)-labelled alanine would then be isolated by hydrolysis of the peptide to give the racemic mixture followed by enzymatic resolution of the N-acetyl amino acid.[42-44] The enzymatic resolution selectively deacetylates the (2S) N-acetylalanine. In order to establish the chirality of the methyl group of the 2S-alanine obtained (3S in this case), Kuhn-Roth oxidation would give acetic acid with an S configuration in the methyl group. The acetic acid chirality can be determined using the methodology of Cornforth and Arigoni.[45-49] Therefore, if an anti-elimination is involved in the formation of dehydroalanine moieties from (2S,3R)-serine, acetic acid with an S configuration would be obtained.

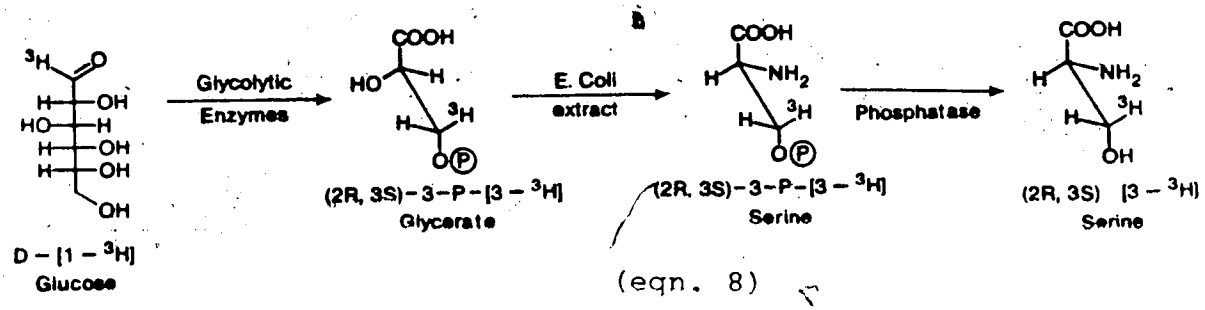
Using the same serine precursor, a syn-elimination would give an E dehydroalanine unit. Reduction would give (2S,3R)- and (2R,3S)-labelled alanines which would result in acetic acid with an R configuration, after degradation and analysis.

The unavailability of this precursor has hindered studies of this type. This thesis examines a number of approaches to developing a useful synthesis of serine stereospecifically labelled at C-3.

B. Syntheses of Serine Stereospecifically Labelled at C-3.

There are two basic requirements for a stereospecifically labelled serine synthesis; a high stereochemical purity (>95%) and a good overall yield. Four syntheses were reported before the initiation of this thesis work. Two do not contain details of yields or stereochemical purity and two additional syntheses have recently been published. These major approaches are discussed below.

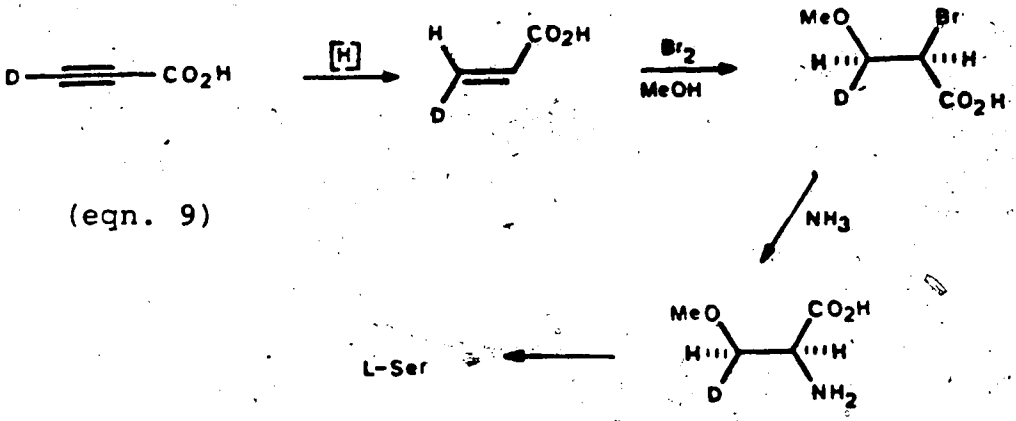
1). Floss and coworkers developed an enzymatic synthesis (eqn. 8) of stereospecifically tritiated samples of L-serine from [1-^{3}H]glucose and [1-^{3}H]mannose.[50,51] The stereochemical purity of these samples was high (>98%); however, the nature of the enzymatic synthesis produces only micromole amounts of labelled compound.



(eqn. 8)

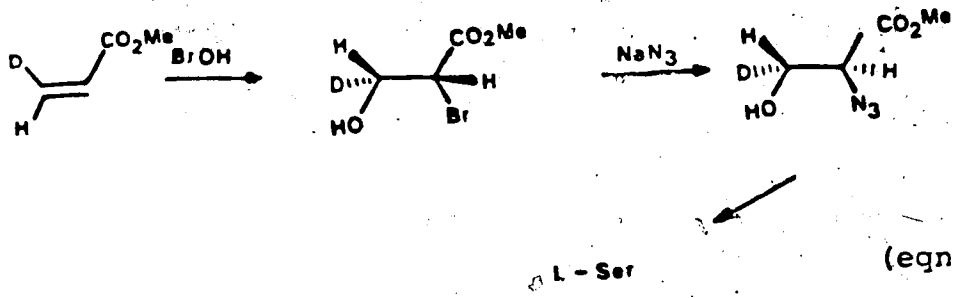
2) Walsh and co-workers established a chemical synthesis (eqn. 9) from labelled acrylic acid.[52a] A bromohydrin reaction fixes the relative stereochemistry at the 2 and 3 positions. Reaction of the bromide with ammonia and subsequent deprotection gives serine. Walsh suggested

that the displacment or bromohydrin reaction introduces a loss of stereochemical purity at the 2 position. An 80% enantiomeric excess of (2S,3R)-[3-$^2H_1$]serine was obtained. The overall yield was 55% over 6 steps from monopotassium acetylene dicarboxylate.



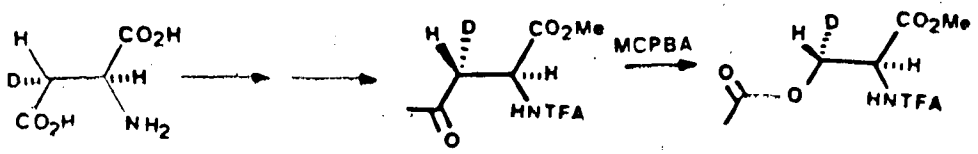
(eqn. 9)

3). Slieker and Benkovic have recently published a modification of the above synthesis.[53] The labelled methyl acrylate was converted to the bromohydrin (eqn 10). This was transformed to L-serine by treatment with sodium azide, catalytic reduction, hydrolysis and enzymatic resolution in a yield of 20%. The enantiomeric excess was 88%.



(eqn. 10)

4). Young and co-workers recently reported a different approach (eqn. 11) to the synthesis of labelled serine.[54] Labelled aspartic acid is converted to labelled serine over five steps in 8% yield. The crucial step involves a Baeyer-Villiger reaction to introduce oxygen at the 3-position.



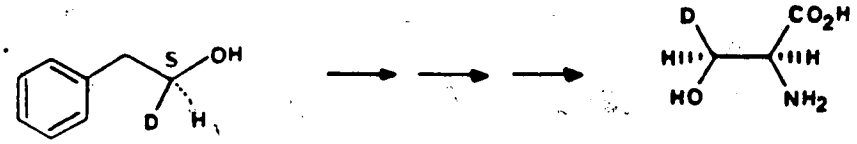
(eqn. 11)

Although a detailed stereochemical analysis is not reported, the enantiomeric excess is claimed to be very high.

5). Stetter reported a synthesis of DL-serine from the condensation of ethyl hippurate and ethyl formate followed by amalgam reduction of the intermediate and workup.[52b] Kainosho and co-workers prepared deuterated serine in an analogous manner using catalytic deuteration of the intermediate double bond (eqn. 12).[52c] The yield or stereochemical purity is not reported.

(eqn. 12)

6). Deuterated serine has also been prepared from 1S-[1-^{2}H]-2-phenylethanol over seven steps in 15% yield with no apparent loss of stereochemical purity (eqn. 13).[52d] The synthesis of the alcohol is complicated by a microbial transformation.

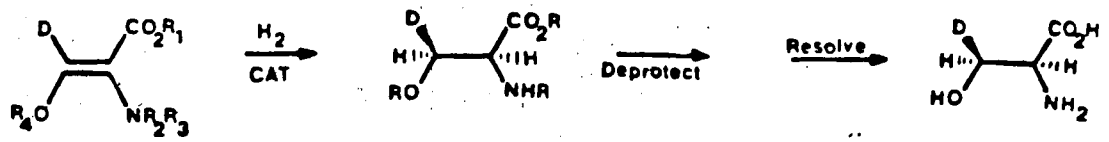


(eqn. 13)

These syntheses have allowed progress to be made in the understanding of PLP dependent processes[4] and in the elucidation of the biosynthesis of norcardicin.[55,56]

SYNTHETIC APPROACH TO SERINE STEREOSPECIFICALLY LABELLED AT C-3.

Serine stereospecifically labelled at the C-3 position has two chiral centres. Our general approach to the synthesis is to fix the relative stereochemistry of the 2 and 3 positions via a single chemical step and in a stereospecific manner (eqn. 14). Since the 2-position can be distinguished enzymatically, the enantiomers produced are separable to give L-serine carrying a stereochemically pure label at C-3. Our methodology relies on stereospecific hydrogenation of a protected "dehydroserine" precursor.



(eqn. 14)

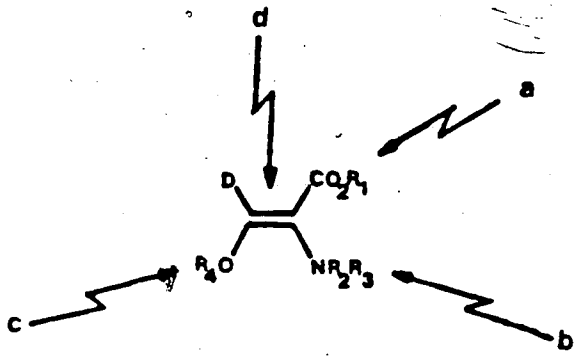
This approach has been used to prepare other labelled amino acids, including cysteine.[57] Two main strategies were investigated; one employs cyclic oxazolinone precursors, whereas the other uses acyclic intermediates. Both syntheses depend upon the ability to prepare a suitable precursor (eqn. 15) with the following features:

a) An ester group easily deprotected without racemization.

b) A suitable protecting group on nitrogen easily convertible to the N-acetyl derivative to allow enzymatic resolution.

c) An enol protecting group stable to reduction conditions and easily removable.

d) A double bond with a known single geometry.

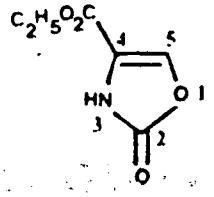


(eqn. 15)

Introduction of label into the vinyl proton position before hydrogenation or reduction of the unlabelled double bond with labelled hydrogen would give rise to the required label at the 3 position of serine.

A. An Oxazolone Approach to Serine.

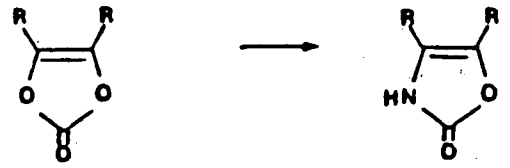
The 4-(ethoxycarbonyl)-1,3-oxazolin-2-one (eqn. 16) has the necessary requirements as a dehydroserine precursor. It has been prepared by Huisgen, in 2% yield, as a side product from photoaddition studies.[58]



(eqn. 16)

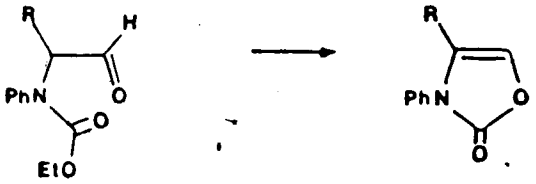
The general approaches to the synthesis of 4-oxazolin-2-ones have been reviewed and are outlined below.[59]

1. From cyclic carbonates.[60]



This method was not considered applicable, since regioselectivity could be poor in the monosubstituted case.

2. From carbamate derivatives.[93]

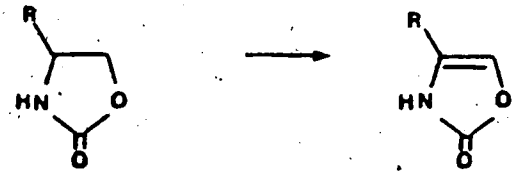


This approach has been investigated with few favourable results.

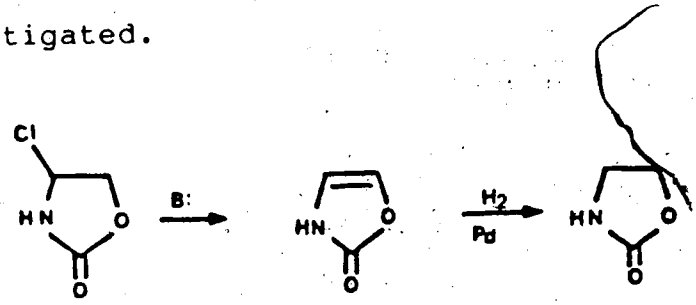
3. From α-hydroxyketone derivatives.[61]

This was considered a viable approach and was utilized at a later stage.

4. From oxazolidones.



The parent compound (R=H) (eqn. 17) was recently prepared by dehydrohalogenation of 4-chloro-1,3-oxazolid-2-one.[62] In addition, the catalytic hydrogenation of the double bond was described. This approach was the first to be investigated.

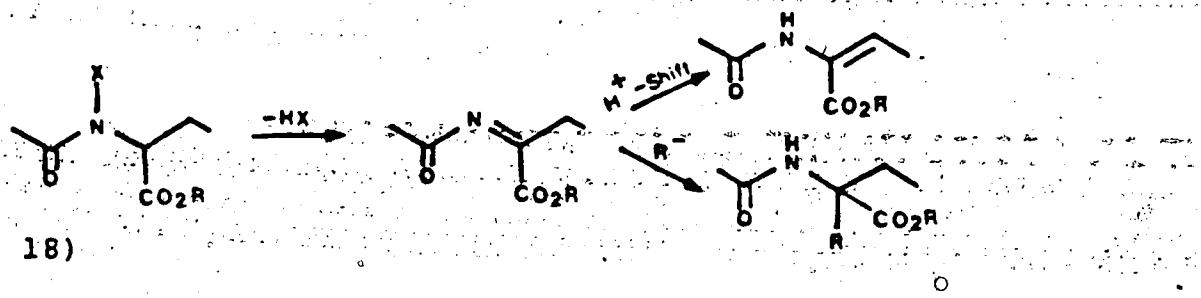


(eqn. 17)

(i) Via Oxazolidones

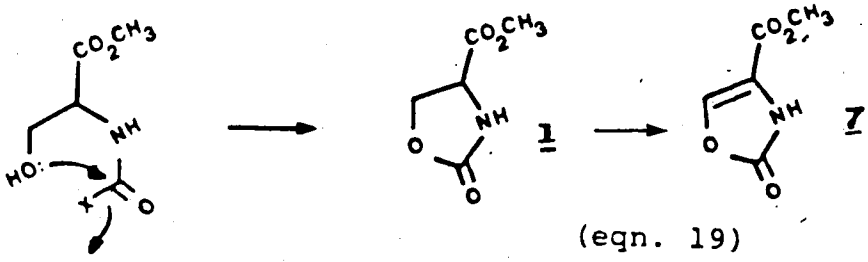
The introduction of a substituent into the 2-position of acyclic amino acids has been achieved by several methods.[31,63,64] For example, an amino acid can be converted to the 2-substituted compound by N-halogenation, elimination to the N-acyl imine and attack by a nucleophile (eqn. 18).

Further elimination or rearrangement can occur to produce a dehydroamino acid.[31,65] Our initial strategy was to prepare the oxazolidone **1**, and subject this compound to N-chlorination and elimination to give the oxazolinone 7.



(eqn. 18)

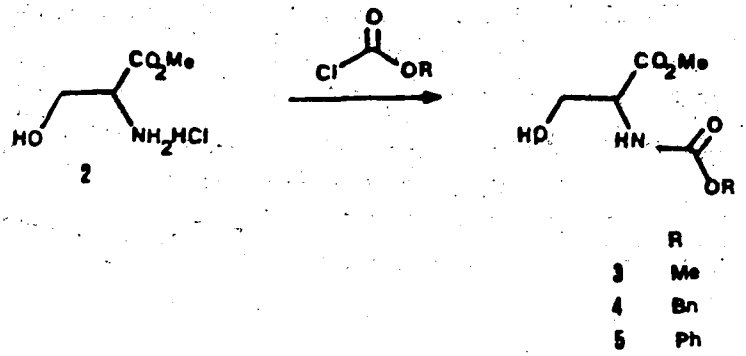
The chemistry of 2-oxazolidones has been extensively reviewed by Swern.[66] The most practical method to prepare **1** appeared to be the ring formation by 5-exo closure[67] onto an activated carbamate (eqn. 19).



(eqn. 19)

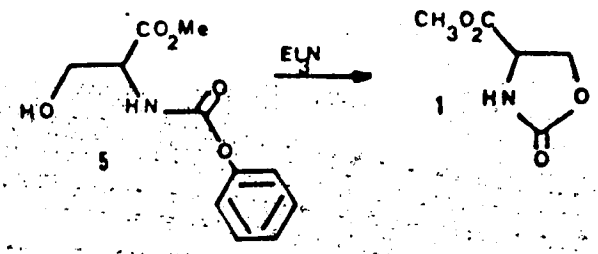
Japanese workers have reported the synthesis of serine derived oxazolidones utilizing phosgene.[68,69]

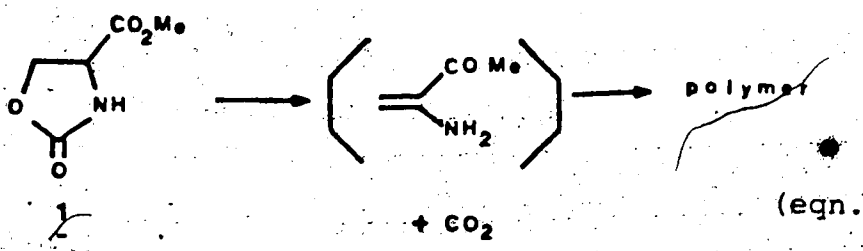
Serine methyl ester hydrochloride (**2**) was prepared by esterification of serine using methanolic hydrochloric acid. All attempts to execute ring closure with phosgene resulted in a failure to produce the desired compound.[68,69]



The activated methoxycarbonyl **3**, (phenylmethoxy)carbonyl **4** and phenoxycarbonyl **5** urethanes were prepared by reaction of serine methyl ester hydrochloride (**2**) and the respective chloroformates in excess of 70% yield.

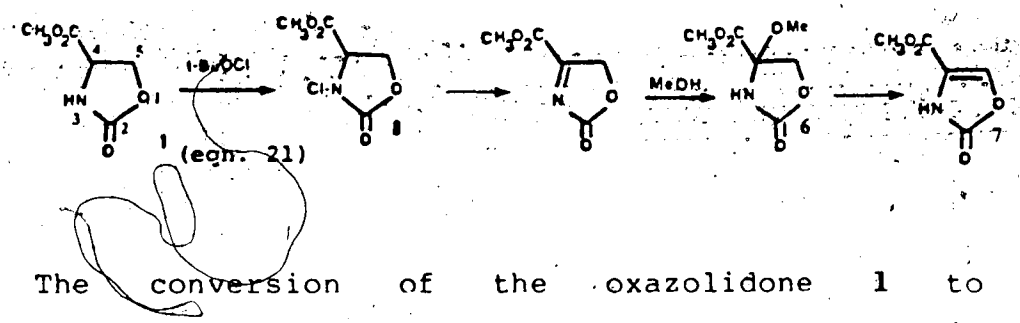
Ring closure attempts on the methoxycarbonyl and (phenylmethoxy)carbonyl compounds (**3** and **4**, respectively) under mild basic conditions (triethylamine), gave no reaction, while stronger basic conditions (sodium methoxide) resulted in the decomposition of starting material. Treatment of the phenoxycarbonyl carbamate **5** under mild conditions (triethylamine in dichloromethane) gave quantitative conversion to **1**.





(eqn. 20)

Many attempts to purify the product resulted in decomposition, but 95% pure oxazolidone **1** was finally obtained in 45% yield by steam distillation (to remove phenol) and neutral extraction of the residue. The instability of **1** may result from elimination of carbon dioxide to give (eqn. 20) an amino acrylic ester followed by polymerization.

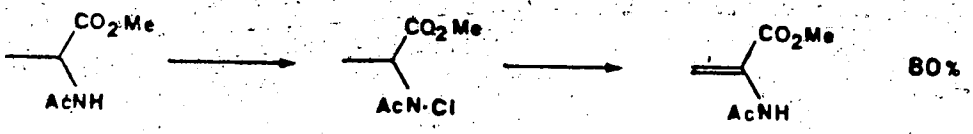


(eqn. 21)

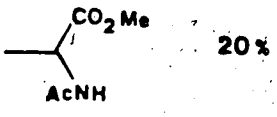
The conversion of the oxazolidone **1** to the 4-(methoxycarbonyl)-2-methoxy-1,3-oxazolid-2-one (**6**) was attempted using Baldwin's procedure.[64] The reaction utilizes t-butyl hypochlorite in methanol in the presence of sodium borate. It is suggested (eqn. 21) that the nitrogen undergoes chlorination followed by elimination to give the N-acyl imine.[64] The N-acyl imine is attacked by methanol to

produce the α-methoxy derivative **6**.[64] The oxazolinone **7** would be obtained by elimination of methanol from **6**. However, reaction under these conditions failed to give either **6** or **7** and resulted in complete decomposition of the oxazolidone **1**.

The intermediate N-chlorooxazolidone **8** was prepared in quantitative yield by reaction of the oxazolidone with t-butyl hypochlorite in a non-nucleophilic solvent, dichloromethane. All attempts to dehydrohalogenate this compound resulted in reversal to oxazolidone **1**. Interestingly, reaction of **8** with potassium t-butoxide produced t-butyl hypochlorite. These observations are in accord with those published by Olsen.[65] The acyclic case (eqn. 22) gives 80% of the dehydroalanine compound and 20% reversal to starting material.[65] This behaviour of N-chloro compounds has also been confirmed by Bach.[70]

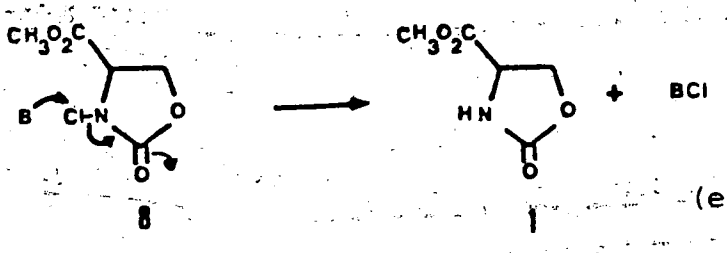


(eqn. 22)



The N-chloro compound **8**, constrained as a five-membered ring, apparently favours behaviour as a positive

chlorinating agent (eqn. 23) over elimination to an N-acyl imine.

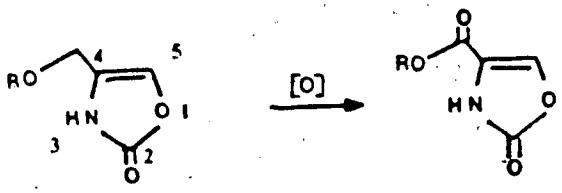


8 1 (eqn. 23)

The instability of **1** and the inability to oxidize the ring system to an oxazolinone led to an alternative approach.

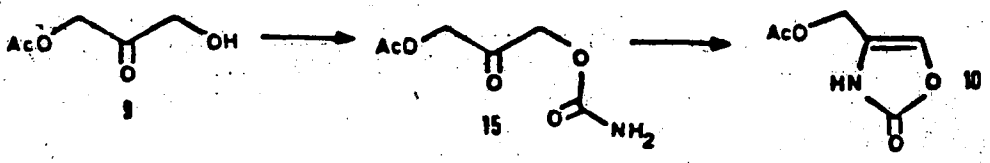
(ii) Via Oxazolinones

There are several examples in the literature of the 4-substituted oxazolinone ring system.[71,72] This alternative approach involves synthesis of a 1,3-oxazolin-2-one with a protected hydroxymethyl group at C-4 followed by side chain oxidation (eqn. 24).

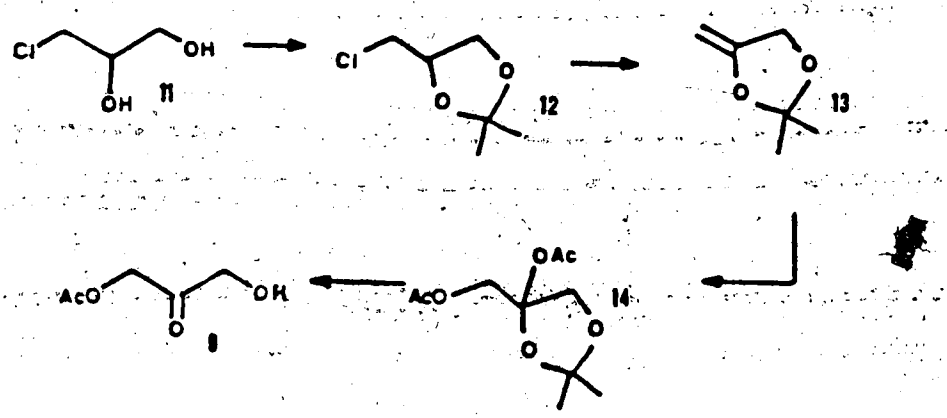


(eqn. 24)

The 1-acetoxy-3-hydroxy-2-propanone (**9**) seemed a suitable precursor for the oxazolinone **10**. The acetoxymethyl side chain would be modified to the desired oxazolinone **7**.

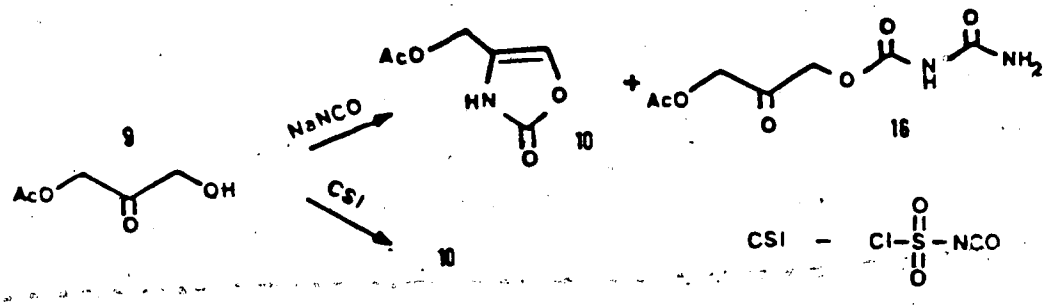


Compound **9** was prepared by a literature procedure.[73] The chloropropanediol **11** was protected as the acetal using 2,2-dimethoxypropane **12** and dehydrohalogenated to the alkene **13** with potassium hydroxide. Compound **13** was treated with lead tetraacetate to give the diacetate **14** which was hydrolyzed in aqueous acetic acid to yield the desired acetoxy compound **9** in 28% overall yield.

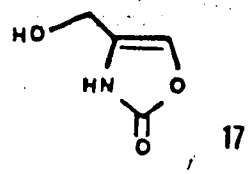


Compound **9** was converted directly to the oxazolinone **10** using two different methods. In an attempt to prepare the primary urethane **15**,[72,74] **9** was transformed to **10** in 14% yield by treatment with trifluoroacetic acid and sodium isocyanate. The major product was the allothanoate **16** obtained in 43% yield. No primary urethane **15** was isolated

from this reaction. The second route employed chlorosulphonyl isocyanate which has been used to convert hydroxyketones into 3-oxazolin-2-ones.[75,76] Treatment of **9** with chlorosulphonyl isocyanate followed by heating in acetic acid gave varying yields of **10** (10-50%) due to extensive formation of side products.

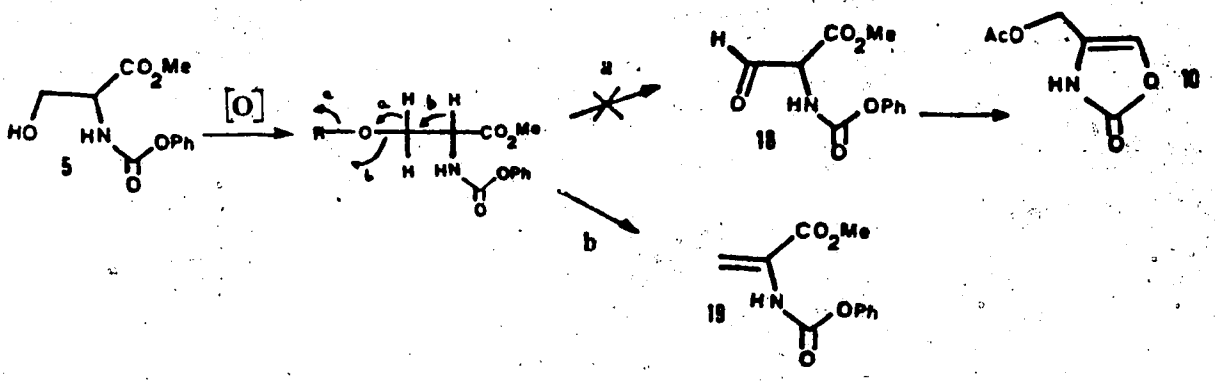


The hydrolysis of **10** to the alcohol **17** was not successful under a variety of conditions. The length of this route and poor yields discouraged further efforts.



(iii) Via Oxidation

Oxidation of the hydroxymethyl side chain of the serine derivative **5** would give the 3-oxoalanine derivative **18**.[77,78] Ring closure of **18** would produce the desired oxazolone **10**. All attempts to oxidize **5** produced **19**. Compound **19** would be formed by β-elimination (path b) of the oxygen

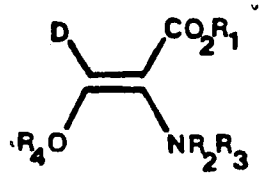


functionality. It would appear that path **b** is preferred over a pathway leading to **18** (path **a**) under oxidising conditions.

Because of the difficulty of synthesizing the desired ring system the research was re-orientated towards the acyclic approach described in the next section.

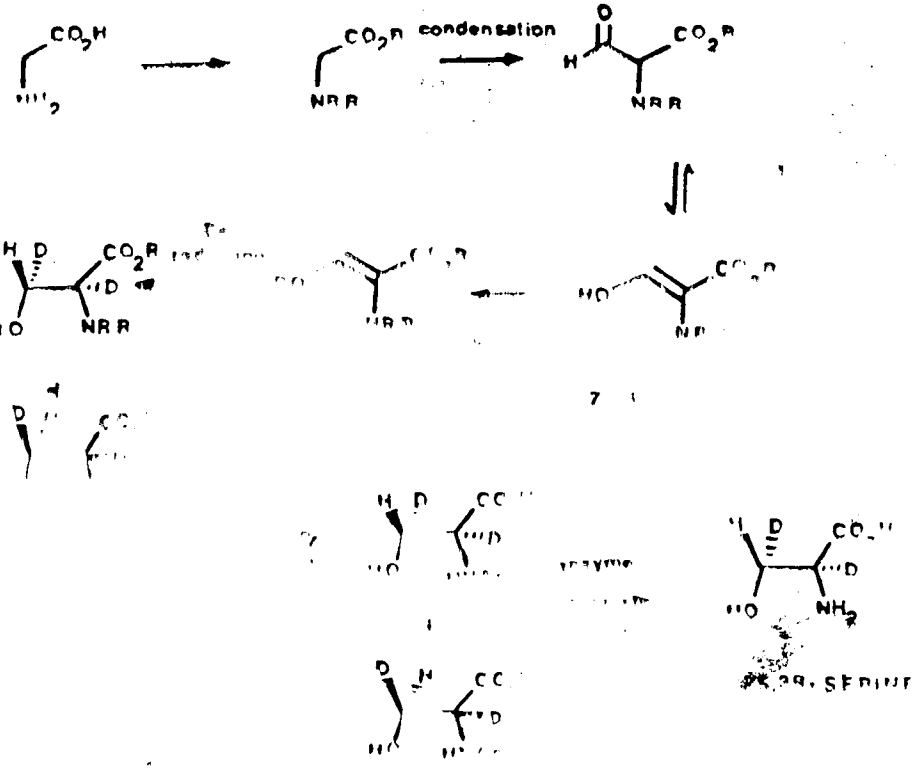
B. ACYCLIC APPROACH TO SERINE SYNTHESIS.

Considerations for the preparation of a suitable acyclic dehydroserine precursor (eqn. 25) are similar to those for an oxazolinone intermediate.



(eqn. 25)

The acyclic approach does have the potential of producing Z and E diastereoisomers which would have to be separated before reduction. A general strategy employing a Z dehydroserine intermediate is outlined below (eqn. 26).



(eqn. 26)

Protected glycine derivatives can be formylated in a Claisen reaction to give 3-oxoalanine derivatives (formylglycines). These compounds are rare natural products

their ease of polymerization and decomposition,[79-81] but a few examples of stable penaldates are known.[79-83] Protection of the 1,3-dicarbonyl system on oxygen is well established. It seemed reasonable to expect the protection to occur on the aldehyde oxygen.[84] This reaction could produce diastereoisomers which would have to be distinguished and separated. Reduction of dehydroamino acid systems using chiral rhodium reagents[85-87] often leads to a large degree of chiral induction,[85,87] but no cases have been reported where the precursor bears a second heteroatom. After reduction of the double bond, the removal of protecting groups to give N-acetylserine must be achieved without epimerisation at C-2 or C-3. Stereoselective enzymatic deacetylation would produce 2S, 3R-serine stereospecifically labelled by deuterium at C-3.

The independent stages of this synthesis are treated separately in the following discussion. Its overall effectiveness by various possible routes is considered in the succeeding summary section.

1. Carboxyl and Amino Group Protection.

Protection on the carboxyl and amino functionalities is found to play a pivotal role in the outcome of various reactions in this synthesis.

The ethyl, phenylmethyl and methyl esters of glycine hydrochloride (**20**, **21** and **22**, respectively) were obtained commercially or prepared by treatment of glycine with thionyl chloride and the corresponding alcohol.

Five different amino protecting groups were used. Reaction of glycine ethyl ester hydrochloride (**20**) with benzoyl chloride (BzCl) and triethylamine produced ethyl hippurate (**23**).[88] The phthalimido (Pt) compounds **24** and **25** were prepared by heating the corresponding ester **21** or **22** with phthalic anhydride and triethylamine in toluene with azeotropic removal of water.[89]

	R^1
20	Et
21	Bn
22	Me

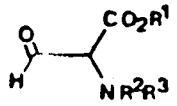
	R^1	R^2	R^3
23	Et	Bz	H
24	Me	Pt	–
25	Bn	Pt	–
26	Me	TFA	H
27	Bn	Ac	H
28	Et	Ac	Bn
29	Bn	Ac	Bn
30	Et	H	Bn
31	Bn	H	Bn

The trifluoroacetyl (TFA) and acetyl (Ac) compounds **26** and **27** were generated in 95% and 53% yield by reaction of **22** or

21 with trifluoroacetyl or acetyl chloride, respectively.[88] N-acetyl-N-(phenylmethyl) compounds **28** and **29** were synthesized by initial conversion of the esters **20** and **21** to the N-(phenylmethyl) compounds **30** and **31**, respectively, by reaction with benzaldehyde, followed by reduction with sodium borohydride in methanol. The N-(phenylmethyl) compounds **30** and **31** were converted to **28** and **29** by treatment with acetyl chloride and triethylamine in dichloromethane at 0°C. The overall yields of **28** and **29** were 75 and 62%, respectively.

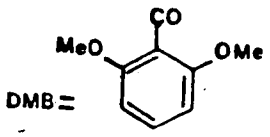
2. Preparation of 3-oxoalanine Derivatives by Condensation

Condensation of the protected glycine derivatives with formylating agents proved to be a crucial step in the synthesis. The few examples[80,81,83] of this reaction (eqn. 27) utilize formate esters in the presence of an alkoxide base. Compound **32** was prepared using sodium ethoxide and ethyl formate.[80,81] The yield was 14% and the product was extremely unstable;[79-81] the decomposition produced ethyl benzoate.



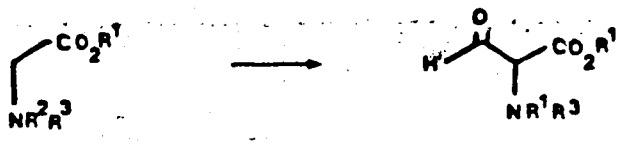
R = alkyl

R^1	R^2	R^3
Et	Bz	H
R	Pt	—
Et	DMB	H
Et	formyl	H



(eqn. 27)

The known phthalimido compounds[82,83] **33** and **34** were prepared, using potassium t-butoxide as the base, in good yields (>70%). In contrast to **32**, these materials were found to be stable crystalline compounds as described.[82,83]



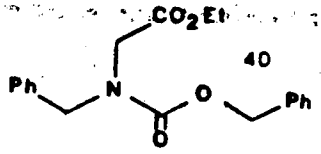
	R^1	R^2	R^3
23	Et	Bz	H
24	Me	Pt	–
25	Bn	Pt	–
26	Me	TFA	H
27	Bn	Ac	H
28	Et	Ac	Bn

	R^1	R^2	R^3
32	Et	Bz	H
33	Me	Pt	–
34	Bn	Pt	–
35	Me	TFA	H
37	Bn	Ac	H
39	Et	Ac	Bn

The previously unreported N-trifluoroacetyl derivative **35** was produced in low yield by reaction of methyl formate and N-(trifluoroacetyl)glycine methyl ester (**26**) in the presence of lithium 1,1,1,3,3,3-hexamethyldisilazide (LHMDS)[90,91] at -30°C. Since the amide proton of an N-trifluoroacetyl group is more acidic than that of an N-arylamide it has a much greater tendency to quench the required base. An excess of strong base, such as LHMDS, appears to be necessary to form the dianion for the condensation step. Because of the previously observed instability of 3-oxoalanine amide esters bearing an amide proton (e.g. **32**), compound **35** was converted directly to the

O-methyl enol ether **36** with diazomethane (see below). The overall yield of **36** from **26** was 22%. Analogously, **27** was transformed to **37** using lithium N-isopropyl-N-cyclohexylamide (LICA) and benzyl formate. Alkylation of **37** gave **38** in 14% overall yield. One of the major problems encountered with the reaction is decomposition of ethyl formate to carbon monoxide by strong amide base. Carbon monoxide was shown to be evolved during the reaction by subjecting a gas sample to a 0.5% solution of $PdCl_2$ in water. A positive test for carbon monoxide was deposition of black palladium metal. Similar observations were reported by Erlenmeyer in 1904.[81] Formate esters also undergo α-proton exchange[90,92] in the presence of strong base. In order to achieve satisfactory condensation, these results indicate that diprotection on nitrogen is required. Thus reaction of the N-(phenylmethyl) compound **28** with potassium t-butoxide and the ethyl formate ester gave **39**. Despite the low initial yield, subjecting the crude reaction mixture to initial reaction conditions several times gave **39** in a yield of 68% as a stable compound. The presence of the N-acetyl group appears to hinder the reaction because it could quench the base. The N-[(phenylmethoxy)carbonyl] compound **40** has been reported to condense more effectively with formate esters than is apparent with the N-acetyl compound **28**.[91] In the presence

of d_4-methyl formate the condensation reaction gives a high incorporation of deuterium into the N-acetyl methyl group demonstrating the formation of an anion at this position.



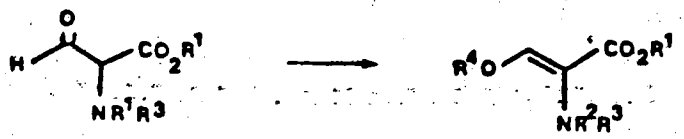
In summary, the condensation reaction gives higher yields if the nitrogen is diprotected with groups containing no exchangeable hydrogens.

3. Protection of the Enol of 3-Oxoalanine Derivatives.

Having obtained the required 3-oxoalanine compounds, it was necessary to protect the enol tautomer to generate the precursor for reduction to a serine derivative. This protection had to meet the following requirements:

* Protection must act exclusively on the formyl oxygen.
* The protecting group must be easily removed, without epmerisation, after introduction of the label.
* If a mixture of diastereomers is produced, these must be separable.
* The protecting group must have minimum steric bulk and allow access of a reducing agent to the double bond.
* The protecting group must withstand the reduction conditions.

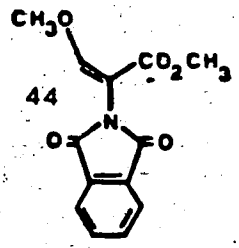
The methyl enol ether or the acetoxy enol ester were considered the most desirable derivatives. The acidity of the enol[94] due to extended conjugation allows alkylation[95] by diazomethane in ether in the presence of a catalytic amount of hydrofluoroboric acid.[94,96] Compounds **36, 38, 41-43** were prepared using this method in excellent yields (> 90%).

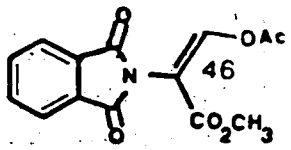


	R^1	R^2	R^3
33	Me	Pt	–
34	Bn	Pt	–
35	Me	TFA	H
37	Bn	Ac	H
39	Et	Ac	Bn

	R^1	R^2	R^3	R^4
41	Me	Pt	–	Me
42	Bn	Pt	–	Me
36	Me	TFA	H	Me
38	Bn	Ac	H	Me
43	Et	Ac	Bn	Me
45	Me	Pt	–	Ac
47	Bn	Pt	–	Ac

Generally, the Z isomers were the only products; however, the Z enol ether **41** was obtained with 5% of its E isomer **44**. These were separated by chromatography and distinguished by different chemical shifts for the vinyl proton. The isomer later shown to have the Z configuration has the vinyl hydrogen signal at a higher chemical shift in the ^{1}H-n.m.r. spectrum than that of the other isomer.[95]





The geometry of the double bond was determined by X-ray crystallography and was confirmed by nuclear Overhauser enhancement (NOE) experiments. The NOE experiments give the following information. In the case of the Z-isomer **41** the vinyl hydrogen and the methyl ester hydrogens are close enough that irradiation at the chemical shift of the methyl ester gives an increase of intensity at the vinyl proton signal. In the E isomer **44** the vinyl proton and ester are not close enough for a direct enhancement; however, irradiation of the methyl group causes a decrease in intensity of the vinyl proton signal (eqn. 28). This is caused by a transfer of magnetization from the irradiated methyl group to the vinyl proton by the methoxy group.

The structural information obtained from the X-ray data (see Appendix 1) confirmed the assignments of the double bond (Fig. 1). Interestingly the plane defined by the enol ether-ester atoms and the plane defined by the pthalimido ring in the Z isomer **42** has a torsion angle (ϕ) between 70° and 80°. These two π systems are therefore aligned such that the pthalimido ring is out of the plane of the enol ether double bond and is nearly orthogonal to it.

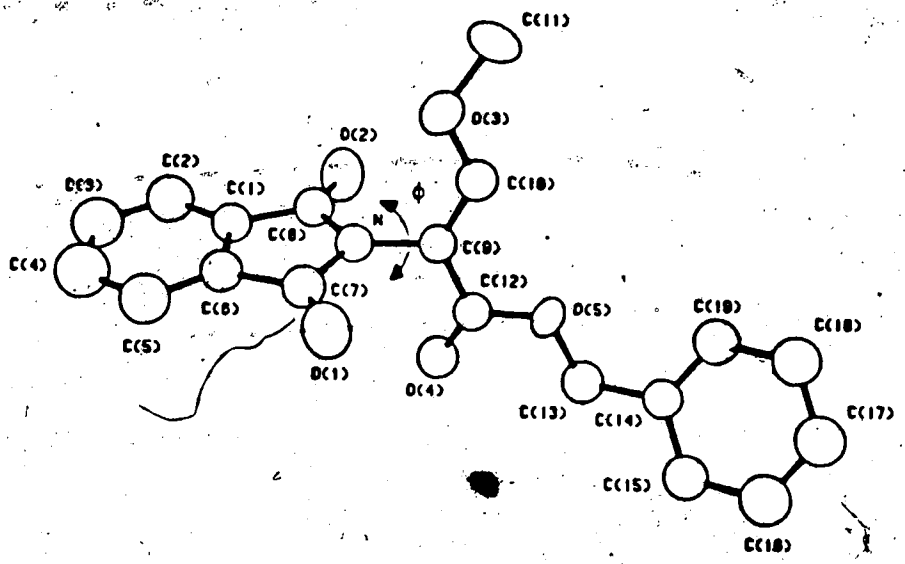
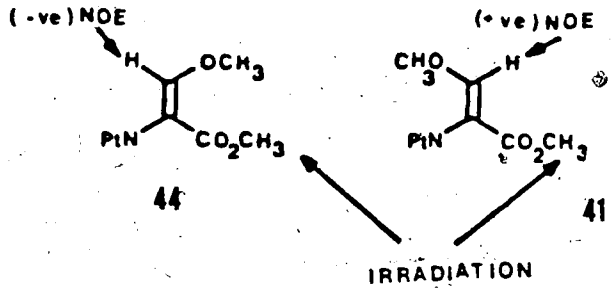


FIGURE 1: X-ray Structure of 2-(N-phthalimido)-3-methoxy-2-propenoic phenylmethyl ester (42).

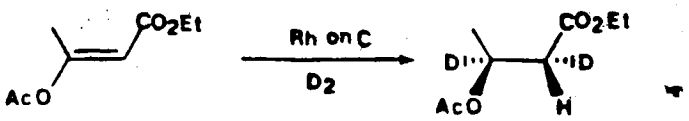


(eqn. 28)

Treatment of **33** with allyl acetate under acidic conditions gave mixtures of Z and E acetoxy enol esters **45** and **46** (the E isomer is favoured). However, **33** was converted exclusively to the Z isomer **45** using triethylamine and acetyl chloride.[97] Similarly, **34** was converted to **47**. Under basic conditions, the enol may be deprotonated to the enolate. A Z configuration allows the largest distance between the enolate oxygen and the ester carbonyl to be obtained. Under acidic conditions the preference for the E isomer may result from complexation of the enol proton by the ester carbonyl group.

4. Hydrogenation of Dehydroserine Derivatives.

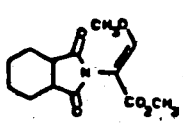
The primary consideration in the reduction of the double bond of dehydroserine derivatives is that hydrogenation is completely stereoselective and proceed in high yield. Cis-addition of hydrogen is favoured using rhodium as a catalyst.[98] There is also considerable interest in the reduction of unsaturated amino acids using chiral rhodium catalysts.[85-87] These reductions proceed quickly with a high degree of enantioselectivity (>95%) in many cases. The presence of an electron withdrawing substituent favours reduction of the double bond.[86] Recently, the stereoselective reduction of 3-acetoxy-2-butenoic acid ethyl ester has been described using rhodium catalyst (eqn. 29).[99]

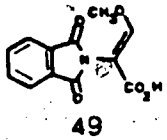


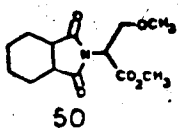
(eqn. 29)

All attempts to hydrogenate the enol ether double bond of **41** were unsuccessful. Hydrogenations with rhodium catalysts gave only reduction of the aromatic ring to give **48**. Palladium hydrogenolysis of **42** gave the free acid **49**.[100] Hydrogenation of **49** over Rh on Al_2O_3 at 45 psi gave a mixture of **48** and **50** after esterification with

diazomethane.


48


49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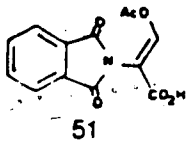
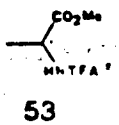

51


53

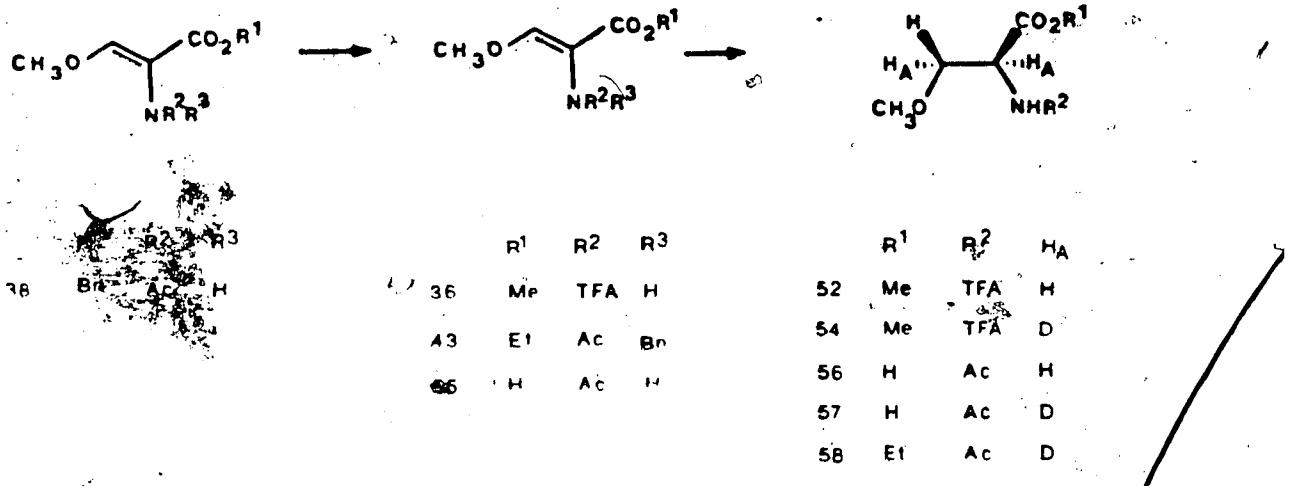
Examination of the X-ray structure suggests the primary reason for lack of reduction of the enol ether double bond may be steric hindrance caused by the phthalimido group which may prevent access of the alkene to the surface of the catalyst. Hydrogenolysis of **47** gave the free acid **51**. Catalytic reduction of **51** gave only a complex mixture of compounds. The problem of steric interaction could be avoided using dehydroserine derivatives which bear monoprotection on nitrogen. This increases the access of catalytic surface to the enol ether double bond.

Rhodium on carbon catalyzed efficient hydrogenation of the N-trifluoroacetyl compound **36**. However, the product **52** underwent α,β-elimination with further reduction to the alanine derivative **53**.[113] This problem was circumvented by the addition of a catalytic amount of triethylamine to the reaction mixture. This allowed quantitative conversion of **36** to the serine derivative **52**.

The elimination of methanol may be favoured because of catalysis by the N-trifluoroacetyl amide proton. The

addition of triethylamine reduces the acidity of the medium and hinders protonation of the methoxy group. The reduction of **36** using deuterium gas under identical conditions gave the desired deuterated serine derivative **54** as a single diastereoisomer.

The N-acetyl phenylmethyl ester **38** was hydrogenolyzed[100] using palladium on charcoal to the corresponding acid **55**. This gave some premature reduction of the double bond. The reduction was completed using rhodium on carbon to produce the serine derivative **56**. No elimination was observed in this case. Analogous reduction of **38** with deuterium gas gave the desired product **57** as a single diastereoisomer.



	R^1	R^2	R^3
36	Me	TFA	H
43	Et	Ac	Bn
55	H	Ac	H

	R^1	R^2	H_A
52	Me	TFA	H
54	Me	TFA	D
56	H	Ac	H
57	H	Ac	D
58	Et	Ac	D

Unfortunately, the dehydroserine precursors bearing a single protecting group on nitrogen, required for facile hydrogenation, could not be prepared directly in high

yield. The condensation step to form 3-oxoalanine derivatives (penaldates) proceeds well only if there is no amide proton. To overcome this problem the N-acetyl-N-(phenylmethyl) compound **43** was prepared, and was subjected to a variety of hydrogenation conditions.[101] The removal of the N-(phenylmethyl) group was extremely sensitive to reaction conditions.[102,103] The introduction of acid into the reaction mixture aided the removal of the protecting group,[101] but it caused elimination and epimerisation of the desired product **58**. Compound **58** was isolated in a single case in 86% yield with no apparent racemization.

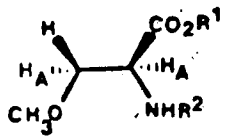
The ability to introduce label into dehydroserine derivatives in a stereoselective manner demonstrates the viability of this approach. However, the requirement for a trisubstituted nitrogen for efficient formation of 3-oxoalanine derivatives and for a disubstituted nitrogen for reduction increases the number of steps and lowers the overall yield.

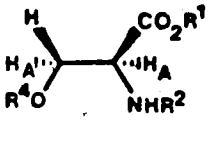
5. Deprotection of serine derivatives.

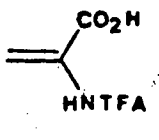
At this stage of the synthesis we have essentially developed three compounds capable of acting as serine precursors **54**, **57**, and **58**.

It had been shown that N-t-butoxycarbonyl protected acid esters could be cleaved to the corresponding amino

acids using boron tribromide.[104-106] Treatment of compound **52** with boron tribromide[105] gave N-(trifluoroacetyl)serine[107] **59** in 60% yield and the dehydroalanine derivative **60** resulting from elimination of water.[107,108]







60

	R^1	R^2	H_A
52	Me	TFA	H
54	Me	TFA	D
56	H	Ac	H
57	H	Ac	D
58	Et	Ac	D

	R^1	R^2	H_A	R^4
59	H	TFA	H	H
61	H	TFA	D	H
62	H	H	D	Me

Similar treatment of the deuterated serine derivative gave deuterated N-(trifluoroacetyl)serine **61**. The deuterated product **61** was analyzed using proton n.m.r. The geminal protons at C-3 of the corresponding 2S unlabelled compound are part of an ABX system and show chemical shifts at 4.21 ppm for the pro-R hydrogen and 4.06 ppm for the pro-S hydrogen. The racemic compound **61** showed that 88% of the signal for the proton at C-3 was in the position corresponding to pro-R for the 2S isomer and 12% of the signal was in the opposite configuration. These results indicate 12% epimerization had occurred at C-2 during the deprotection.

The inability to remove the protecting groups on N-trifluoroacetyl ester **54** without epimerisation led to similar trials on the N-acetyl acid **57**. This compound carries no acid protection, and is suitable for enzymatic resolution (see below). The N-acetyl ester **58** was converted to the corresponding acid **57** in 98% yield by hydrolysis with one equivalent of lithium hydroxide[109] and purified by ion-exchange chromatography. No epimerisation was observed as indicated by ^{1}H-n.m.r.

63 64

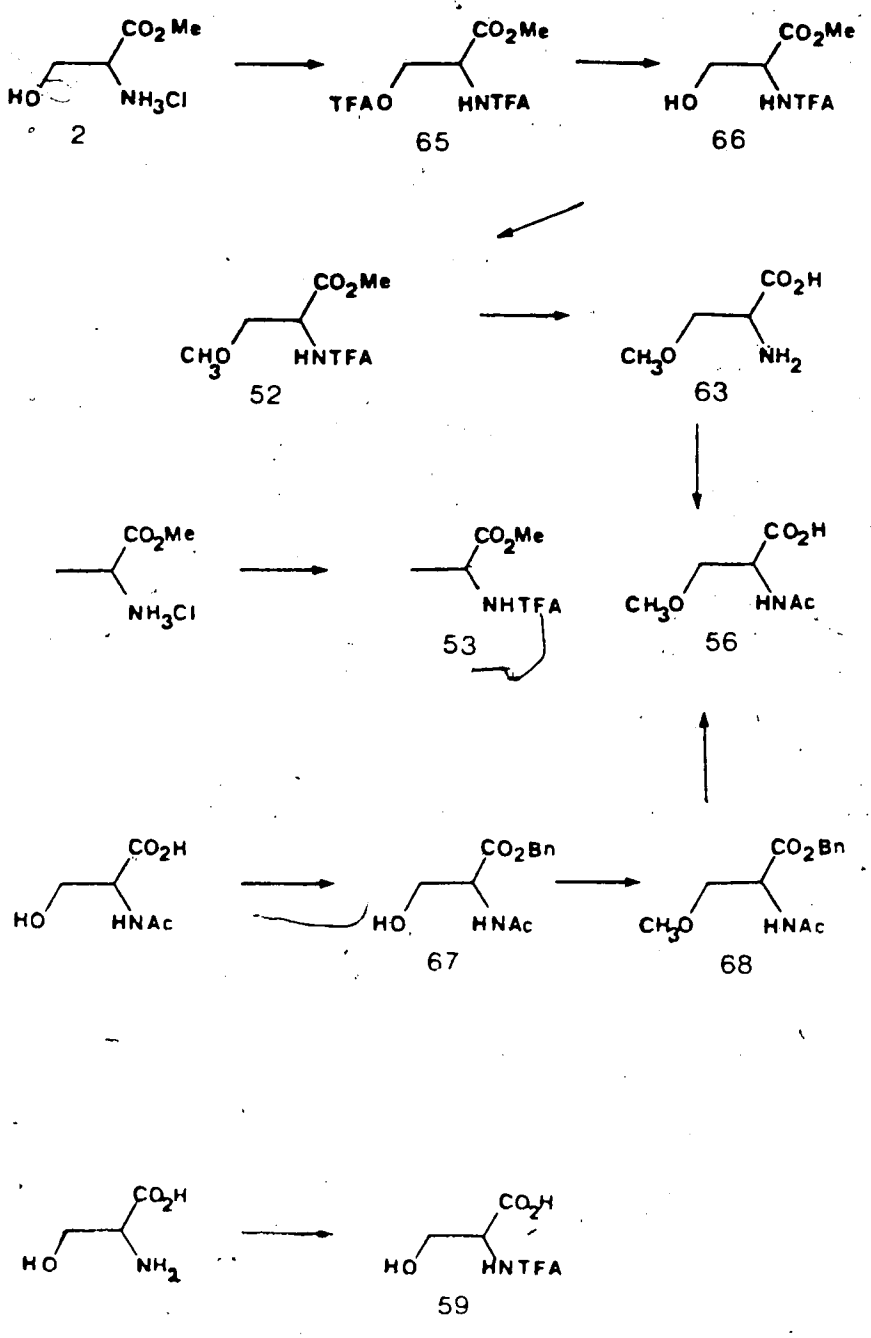
N-Acetyl-O-methylserine **57**[112] is a substrate for enzymatic resolution using hog kidney acylase I (EC 3.5.1.4).[110] This enzyme selectively hydrolyses the L(2S) isomer of racemic **57** at a more rapid rate than the D(2R) isomer to give L(2S,3R)-[2,3-$^2H_2$]-O-methylserine (**62**) directly. Boron tribromide is an excellent reagent for ether cleavage[104-106] because it selectively attacks less hindered centres (e.g. methyl) at a faster rate than secondary centres. O-Methylserine (**63**) was converted to DL-serine (**64**) in 97% yield with no α,β-elimination or bromide formation. The overall effectiveness of the total

synthesis procedure is discussed in the summary section.

6. Preparation of Unlabelled Intermediates

A number of key compounds were synthesized in unlabelled form by alternative routes to provide proof of structure and to generate large quantities of intermediates for maximization of yields.

Unlabelled O-methylserine **63** has been prepared by methylation of N-phthalimidoserine 4'-nitrophenyl ester with diazomethane.[111] An alternative route which did not require large amounts of diazomethane was developed for efficient large scale production. Serine methyl ester hydrochloride (**2**) was converted to the bis-trifluoroacetyl compound **65** with an excess of trifluoroacetic anhydride. The O-trifluoroacetyl group was cleaved under aqueous conditions to N-(trifluoroacetyl)serine methyl ester **66** in 80% yield. Compound **66** was alkylated using iodomethane and silver oxide to form the corresponding O-methyl compound **52** in >99% yield. Hydrolysis of **52** using lithium hydroxide produced **63** in >99% yield.[109] Compound **63** was converted to N-acetyl-O-methylserine **56**, by a modified literature procedure,[112] using acetic anhydride in 95% yield. The overall yield from serine methyl ester was 76%. This synthesis efficiently provided three unlabelled intermediates utilized in our study: N-(trifluoroacetyl)-O-methylserine methyl ester


CO2Me
HO
NH3Cl
2
TFAO
HNTFA
65
66
CH3O
52
CO2H
NH2
63
NHTFA
53
HNAc
56
CO2Bn
67
68
59

(52), N-acetyl-O-methylserine (56) and O-methylserine (63).

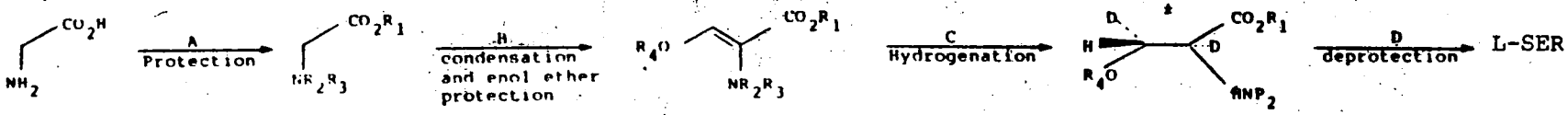
Alternatively, N-acetyl-O-methylserine 56 was prepared in the following manner. N-acetylserine was esterified with benzyl bromide in the presence of triethylamine to give compound 67 in 45% yield,[111] which was O-alkylated using iodomethane and silver oxide to give compound 68 in 50% yield. Hydrogenolysis of 68 using palladium on charcoal afforded N-acetyl-O-methylserine (56) in 75% yield. Large amounts of N-(trifluoroacetyl)serine (59) were prepared by reaction of serine methyl trifluoroacetate and N,N,N',N'-tetramethylguanidine using a modified literature procedure.[107]

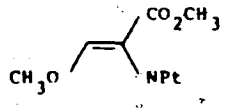
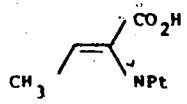
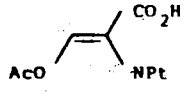
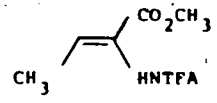
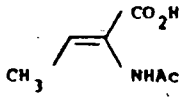
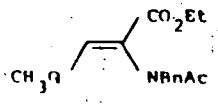
An authentic sample of N-(trifluoroacetyl)alanine (53)[113] was prepared by reaction of alanine methyl ester hydrochloride and trifluoroacetic anhydride.

SUMMARY OF ACYCLIC APPROACH TO STEREOSPECIFICALLY LABELLED SERINE.

The results obtained are summarized in Table 1. The results indicate that each route contains an unsatisfactory reaction during the multistep synthesis. In general, a fully substituted nitrogen (41, 49, 45, and 43) gives excellent yields of the dehydroserine precursor; however, hydrogenation of the double bond is either ineffective or

Table 1



Dehydroserine precursor		Protection R_1	R_2	R_3	R_4	Reaction (% Yield) A	B	C	D
	(41)	CH_3	Pt	-	CH_3	94	77	X	-
	(49)	Bn	Pt	-	CH_3	80	72	25[a]	-
	(45)	Bn	Pt	-	Ac	80	90	X[b]	-
	(36)	CH_3	TFA	H	CH_3	95	22	96	58[c]
	(55)	Bn	Ac	H	CH_3	53	14	95	d
	(43)	Et	Ac	Bn	CH_3	37	67[e]	86[f]	d

[a]Reduction is sensitive and causes reduction of the aromatic ring. [b]Unstable to reduction conditions. [c]12% epimerisation occurs. [d]No deprotection required for enzymatic resolution. [e]After recycling reaction mixture. [f]Sensitive reduction.

results in decomposition. A single protecting group on nitrogen (55 and 36) is necessary for the efficient hydrogenation of the double bond; however, the condensation step gives yields of less than 25%. In order to attain a high overall yield alternative methods for the synthesis of 55 are currently under investigation in our laboratory.

EXPERIMENTAL

Except where stated otherwise, the following procedures were used. Solvents were dried by distillation under static argon atmosphere with the indicated reagents: tetrahydrofuran, ethyl ether, benzene, and toluene (sodium/benzophenone); ethanol and methanol (magnesium and iodine); t-butyl alcohol (sodium); ethyl formate, methyl formate and benzyl formate (phosphorus pentoxide). The following reagents were dried before use: Thionyl chloride and chlorosulphonyl isocyanate (distillation); triethylamine, N,N'-disopropylamine, N-cyclohexyl-N-isopropylamine, and N,N,N',N'-hexamethyldisilazane (distilled from calcium hydride); boron tribromide in dichloromethane (copper dust, calcium sulphate). Diazomethane was prepared by the Diazald method[114] (potassium hydroxide). Experiments requiring an inert dry atmosphere were done under a slight positive pressure of argon in glassware dried at 140°C for two hours. Reagents were added by syringe using a Sage syringe pump. Thermolab cooling baths (0-10°C) or IKA constant temperature baths (25-300°C) were used where possible. Commercial thin layer chromatography (tlc) plates used silica gel (Merck 60F-254). Plates for preparative layer chromatography were 20 cm by 20 cm by 1 mm: silica gel was either Merck 60-PF-254 or Whatman PF254 with a 2 cm preadsorbant layer. UV active spots were detected at

254 nm. Compounds were additionally detected by spraying with one of the following reagents and heating: 10% ninhydrin in acetone, 5% N,N-dimethylaminobenzaldehyde in a 10:90 mixture of concentrated hydrochloric acid and methanol, 10% phosphomolybdic acid in ethanol or 1% bromocresol green in ethanol. Silica gel for column chromatography was Merck type 60 (70-230 mesh). Silica gel for flash chromatography was either Merck type 60 (230-400 mesh) or Whatman LPS-2 (300-400 mesh). Hydrogenation catalysts were removed by filtration through a small bed of Celite. Solvents were removed in vacuo at 30°C on a Buchi rotary evaporator. Samples were dried where possible at 52°C in a Abderhalden vacuum drying apparatus at a pressure of 0.05 mm of Hg in the presence of phosphorus pentoxide. Nuclear magnetic resonance (n.m.r.) spectra were obtained on the Bruker WP80 (continuous wave mode), Varian HA-100, Bruker WH200 or Bruker WH400 (Fourier transform mode) spectrometers with tetramethylsilane (TMS) as an internal standard. Fourier transform infrared (FT-IR) spectra were collected on a Nicolet 7199 FT system, whereas normal infrared (IR) spectra were acquired on a Perkin-Elmer 337 infrared spectrometer. The X-ray data was procured on an Enraf-Nonius CAD4F diffractometer. Microanalyses were done using a Perkin Elmer 240 CHN analyser. High resolution mass spectra were obtained on a Kratos A.E.I. MS50 in the

electron impact (70 eV) mode, and where this was not possible, in the chemical ionization (CI) mode. Melting points were taken by the capillary method using a Thomas Hoover capillary melting point apparatus.

Preparation of serine methyl ester hydrochloride (**2**).

A solution of methanolic hydrogen chloride was prepared by the addition of HCl gas to 100 mL of methanol below 30°C. The solution was cooled to 1°C before the addition of 5.0 g (0.047 mol) of serine. The reaction mixture was allowed to warm to room temperature and was stirred for 16 h. Concentration of the reaction mixture in vacuo in the presence of toluene, produced 6.83 g of **2** as white crystals (yield 92%) (mp. 126°C).

FT-IR (KBr disc) 3000 br, 1730 cm^{-1}; NMR (D_2O, 80 MHz) δ 3.90 (s, 3H, CH_3O), 4.00 (m, 2H, CH_2O), 4.25 (m, 1H, CHN).

Preparation of N-(methoxycarbonyl)serine methyl ester (**3**).

A solution containing 1.00 g of serine methyl ester hydrochloride (**2**) (6.4 mmol) and 1.80 mL of triethylamine (12.9 mmol) in 125 mL of chloroform was stirred at 5°C. A solution containing 0.50 mL of methyl chloroformate (6.5 mmol) in 125 mL of chloroform was added over a 30 minute period and the resulting solution was stirred for 1 h. The reaction mixture was washed with 20 mL of 1 M aqueous hydrochloric acid, 50 mL of aqueous saturated sodium chloride, dried over sodium sulphate, filtered and concentrated to give 0.82 g of **3** as an oil (yield 72%).

FT-IR (film) 3500, 3400, 1820-1740 cm^{-1}; NMR ($CDCl_3$, 80 MHz) δ 3.72 (s, 3H, $C\underline{H}_3OCON$), 3.80 (s, 3H, $C\underline{H}_3O_2C$), 4.02 (m, 2H, $C\underline{H}_2O$), 4.32 (m, 1H, C<u>H</u>N), 6.2 (br, s, 1H, N<u>H</u>). Exact mass calculated for $C_6H_{11}N\ O_5$: 177.0637. Found: 177.0637.

Preparation of N-((phenylmethoxy)carbonyl)serine methyl ester (**4**).

A solution containing 1.55 g of serine methyl ester hydrochloride (**2**) (10.0 mmol) in 15 mL of 30% aqueous potassium hydrogen carbonate was cooled to 5°C. A solution containing 2.0 mL of benzyl chloroformate (14.0 mmol) in 15.0 mL of chloroform and 0.50 mL of pyridine (6.2 mmol) was added and the reaction mixture was stirred for 16 h. The aqueous layer was separated and extracted with 90 mL of ethyl acetate. The organic extracts were combined, dried over sodium sulphate, filtered and concentrated *in vacuo* giving 1.77 g of **4** as a yellow oil (yield 70%).

FT-IR (film) 3600, 3400, 1740, 1720 cm^{-1}; NMR ($CDCl_3$, 80 MHz) δ 2.85 (br s, 1H, O<u>H</u>) 3.77 (s, 3H, $C\underline{H}_3O$), 3.80 (m, 2H, $C\underline{H}_2O$), 4.32 (m, 1H, CHN), 5.13 (s, 2H, $ArC\underline{H}_2$), 5.85 (br d, J = 10 Hz, 1H, N<u>H</u>) 7.1-7.4 (m, 5H, Ar<u>H</u>). Exact mass calculated for $C_{12}H_{15}NO_5$: 253.0950. Found: 253.0950.

Preparation of N-(phenoxycarbonyl)serine methyl ester (**5**).

A solution containing 3.14 g of serine methyl ester hydrochloride (**2**) (20.2 mmol) and 5.90 mL of triethylamine (42.3 mmol) in 100 mL of dichloromethane was cooled to

5°C. A solution containing 2.4 mL of phenyl chloroformate (21.0 mmol) in 30 mL of dichloromethane was added dropwise over a 30 minute period. The reaction mixture was stirred for a further hour. The product was isolated by chromatography on silica gel (3 cm by 30 cm) using 10% ethyl acetate in hexane. Fractions containing product were concentrated to produce 3.80 g of **5** as a colourless oil which crystallized below 5°C (yield 84%).

FT-IR (film) 3400, 1720 cm^{-1}; NMR ($CDCl_3$, 100 MHz) δ 3.79 (s, 3H, $C\underline{H}_3O$), 4.01 (br s, 2H, CH_2O), 4.49 (m, 1H, C<u>H</u>N), 5.30 (s, 1H, O<u>H</u>), 6.03 (br d, 1H, N<u>H</u>), 7.0-7.54 (m, 5H, Ar<u>H</u>). Exact mass calculated for $C_{11}H_{13}NO_5$: 239.0794. Found: 239.0789.

<u>Preparation of 4-(methoxycarbonyl)-1,3-oxazolid-2-one (**1**).</u>

A solution containing 3.66 g of <u>N</u>-(phenoxycarbonyl)serine methyl ester (**5**) (15.3 mmol) and 4.30 mL of triethylamine (31.0 mmol) in 100 mL of dichloromethane was refluxed for 8 h. The reaction mixture was concentrated <u>in vacuo</u> and the phenol produced was removed by steam distillation. The aqueous residue was extracted with 50 mL of ethyl acetate. The organic extract was dried over sodium sulphate, filtered and concentrated, to give 1.01 g of **1** as a yellow oil (yield 45%). Analysis by n.m.r. indicated 5% phenol remained as an impurity. This compound was too unstable to be purified further.

FT-IR (film) 1770, 1740 cm^{-1}; NMR ($CDCl_3$, 80 MHz) δ 3.82 (s, 3H, CH_3O), 4.4-4.7 (m, 3H, CHN, CH_2O) 6.75 (br s, 1H, NH). Exact mass calculated for $C_5H_7NO_4$: 145.0375. Found: 145.0374.

Preparation of N-chloro-4-(methoxycarbonyl)-1,3-oxazolid-2-one (8).

A suspension containing 76.6 mg of 95% 4-(methoxycarbonyl)-1,3-oxazolid-2-one (1) (0.528 mmol) and 60.0 mg of sodium bicarbonate (0.714 mmol) in 10.0 mL of dichloromethane was cooled to 0°C and light was excluded. A 0.1 mL aliquot of freshly prepared t-butyl hypochlorite was added and the reaction mixture was stirred for 18 h. The reaction mixture was filtered and was concentrated to produce 96.1 mg of 8 as a light yellow oil (yield >98%). Compound 8 was susceptible to decomposition and no further attempt at purification was made.

FT-IR (film) 1790, 1750 cm^{-1}; NMR ($CDCl_3$, 80 MHz) δ 3.80 (s, 3H, CH_3O), 4.2-4.8 (m, 3H, CHN, CH_2O).

Preparation of 4-(chloromethyl)-2,2-dimethyl-1,3-dioxolane (12)

A solution containing 90.0 g of 3-chloro-1,2-propanediol (11) (0.814 mol), 160.5 g of 2,2-dimethoxypropane (1.30 mol) and 1.0 g of 4-methylbenzene sulphonate monohydrate (5.3 mmol) in 600 mL of benzene was refluxed with the removal of the methanol-benzene azeotrope at 56°C. After 20 h, no

further azeotrope was obtained. The reaction mixture was washed with 50 mL of 5% aqueous sodium bicarbonate and 50 mL of aqueous saturated sodium chloride. The product was obtained by fractional distillation to give 106.2 g of **12** (Bp. 152-156°C) (yield 86%).

FT-IR (film) 1040, 780 cm^{-1}; NMR ($CDCl_3$, 80 MHz) δ 1.38 (s, 3H, CH_3), 1.41 (s, 3H, CH_3), 3.3-3.7 (m, 2H, CH_2O), 3.8-4.4 (m, 3H, CH_2Cl, CHO). Exact mass calculated for $C_6H_{11}ClO_2$: 150.0448. Found: 150.0415.

Preparation of 4-methylene-2,2-dimethyl-1,3-dioxolane (**13**).

The preparation of the title compound **13** was carried out, according to the procedure in the literature,[73] by the dehydrochlorination of 4-chloromethyl-2,2-dimethyl-1,3-dioxolane (**12**). A reaction time of 5 days was employed to convert 120.0 g (0.797 mol) of **12** to 40.2 g of **13** (yield 45%).

FT-IR (film) 1066, 1372, 845 cm^{-1}; NMR ($CDCl_3$, 80 MHz) δ 1.45 (s, 6H, CH_3), 3.81 (m, 1H, vinyl-H), 4.27 (m, 1H, vinyl-H), 4.40 (m, 2H CH_2O). Exact mass calculated for $C_6H_{10}O_2$: 114.0681. Found: 114.0674.

Preparation of 4-acetoxy-4-(acetoxymethyl)-2,2-dimethyl-1,3-dioxolane (**14**).

The title compound **14** was prepared as described in the literature,[73] by treatment of 4-methylene-2,2-dimethyl-1,3-dioxolane (**13**) with lead tetraacetate. Using this procedure 1.64 g (0.014 mol) of **13** gave 1.93 g of **14** (yield 75%).

FT-IR (film) 1734, 1067 cm^{-1}; NMR ($CDCl_3$, 80 MHz) δ 1.38 (s, 3H, CH_3), 1.41 (s, 3H, CH_3), 2.05 (s, 6H, CH_3CO), 4.20 (d, J = 10 Hz, 1H, CHO), 4.30 (d, J = 10 Hz, 1H, CHO), 4.63 (s, 2H, CH_2O). Exact mass calculated for $C_{10}H_{16}O_6$: 232.0947. Found: 232.0952.

Preparation of 1-acetoxy-3-hydroxy-2-propanone (**9**).

The preparation of the title compound was achieved as described in the literature,[73] by hydrolysis of 4-acetoxy-4-(acetoxymethyl)-2,2-dimethyl-1,3-dioxolane (**14**). Using this procedure 36.3 g (0.156 mol) of **14** gave 19.7 g of **9** (yield 96%).

FT-IR (film) 3450, 1740 cm^{-1}, NMR ($CDCl_3$, 80 MHz) δ 2.12 (s, 3H, CH_3CO), 3.22 (br s, 1H, OH), 4.27 (s, 2H, CH_2OH), 4.71 (s, 2H, CH_2OCO). Exact mass calculated for $C_5H_8O_4$: 132.0423. Found: 132.0417.

Preparation of 4-(acetoxymethyl)-1,3-oxazolin-2-one (**10**)

A solution containing 1.73 mL of freshly distilled chlorosulphonyl isocyanate (19.8 mmol) in 25 mL of chloroform was stirred and cooled to -60°C. A solution containing 1.31 g of 1-acetoxy-3-hydroxy-2-propanone (**9**) (10.0 mmol) in 25 mL of dichloromethane was added to the stirred solution while the temperature was maintained at -60°C. The reaction mixture was stirred for 8 h at -60°C during which time a white precipitate formed. The solvent was removed in vacuo at room temperature before the addition

of 60 mL of acetic acid (107 mmol). This was followed by the addition of 5.1 g of sodium acetate (6.2 mmol) in 5 mL of water and the solution was stirred vigorously. The solution was heated at 125°C until a precipitate formed. The reaction mixture was filtered and the filtrate was neutralized with 10% aqueous potassium carbonate solution. The aqueous solution was extracted with 1 L of chloroform, saturated with sodium chloride and extracted with 1 L of ethyl acetate. The organic extracts were combined, dried over sodium sulphate, and concentrated to give 861 mg of residue. The product was obtained by column chromatography on silica gel (2 cm by 25 cm) using ethyl acetate. Fractions containing product were combined and concentrated yielding 400 mg of **10** as a yellow semicrystalline solid (yield 25%).

(**10**)

FT-IR (film) 3400 br, 1740 1710 cm^{-1}; NMR ($CDCl_3$, 80 MHz) δ 2.14 (s, 3H, CH_3CO), 4.79 (d; J = 1 Hz, 2H, CH_2OCO), 6.80 (br s, 1H, vinyl-H), 8.00 (br s, 1H, NH). Exact mass calculated for $C_6H_7NO_4$: 157.0375. Found: 157.0375.

Preparation of 4-(acetoxymethyl)-1,3-oxazolin-2-one (**10**) from reaction of **9** with sodium isocyanate.

A mixture of 1.60 g of sodium isocyanate (24.6 mmol) and 413 mg of 1-acetoxy-3-hydroxy-2-propanone (**9**) (3.13 mmol) in 10.0 mL of dichloromethane was stirred at 80 rpm and 10.0 mL

of trifluoroacetic acid (130 mmol) was added. After 48 h, the reaction mixture was filtered and the concentrated *in vacuo* to yield 236 mg of the allothanoate **16** (yield 43%). The residue was dissolved in 20.0 mL of water and extracted with 50 mL of dichloromethane. The solution was dried over sodium sulphate, filtered and concentrated to yield 68 mg of **10** (yield 14%) with spectral and chromatographic properties identical to those of **10** obtained by treatment of **9** with chlorosulphonyl isocyanate.

For (**16**): IR (cast) 3400 br, 1740 br cm^{-1}; NMR ($CDCl_3$, 80 MHz) δ 7.21 (br s, 3H, NH), 5.05 (s, 2H, CH_2), 4.80 (s, 2H, CH_2), 2.12 (s, 3H, CH_3CO).

Preparation of 2-(*N*-(phenoxycarbonyl)amino)propenoic acid methyl ester (**19**).

A solution containing 88.0 mg of N-(phenoxycarbonyl)serine methyl ester (**5**) (0.368 mmol), 0.6 mL of dimethyl sulphoxide (0.379 mmol), 0.226 g of N,N'-dicyclohexylcarbodiimide (1.09 mmol), 0.30 mL of pyridine (0.98 mmol) and 0.30 mL of trifluoroacetic acid (3.89 mmol) was stirred for 32 h. The reaction mixture was diluted with 10 mL of ethyl acetate, filtered, dried over sodium sulphate, filtered and concentrated *in vacuo*. Column chromatography on silica gel (1 cm by 10 cm) using ethyl acetate gave 76 mg of **19** as an oil (yield 93%).

NMR ($CDCl_3$, 80 MHz) δ 7.0-7.25 (s, 6H, Ar<u>H</u>, <u>NH</u>), 6.25 (br s, 2H, vinyl-<u>H</u>), 5.85 (br s, 1H, vinyl-<u>H</u>), 3.93 (s, 3H, $C\underline{H}_3$).

Preparation of <u>N</u>-(phenylcarbonyl)glycine ethyl ester (**23**).

A solution containing 11.8 mL (0.111 mol) of benzoyl chloride in 25 mL of dichloromethane was added to a stirred solution containing 14.64 g of glycine ethyl ester hydrochloride **20** (0.105 mol) and 29.2 mL of triethylamine (0.209 mol) in 190 mL of dichloromethane at 5°C over an 80 min period. After 3 h, the solution was washed with 150 mL of 5% aqueous sodium bicarbonate, and with 125 mL of aqueous saturated sodium chloride, and was dried over sodium sulphate. The solution was filtered and solvent was removed <u>in vacuo</u> to give 19.2 g of **23** as a white crystalline product (yield 84%) (m.p. 56-57°C).

FT-IR (cast) 3340, 3000, 1750, 1640, 1530 cm^{-1}; NMR ($CDCl_3$, 80 MHz) δ 7.80 (m, 2H, Ar<u>H</u>), 7.47 (m, 3H, Ar<u>H</u>), 7.00 (br s, 1H, <u>NH</u>), 4.25 (m, 4H, $C\underline{H}_2N$, $C\underline{H}_2CH_3$), 1.32 (t, J = 9.0 Hz, 3H, $C\underline{H}_2CH_3$). Exact mass calculated for $C_{11}H_{13}NO_3$: 207.0895. Found: 207.0895. Anal. Calcd for $C_{11}H_{13}NO_3$: C, 63.77; H, 6.75; N, 6.32. Found: C, 63.55; H, 6.26; N, 6.70.

Preparation of <u>N</u>-(phthalimido)glycine methyl ester (**24**).

In analogy to the preparation of **25**, 87.8 g of glycine methyl ester hydrochloride was converted to 123 g of **24** (yield 94%) (mp. 112.5-113°C).

FT-IR (film) 3000, 1775, 1750, 1725 cm^{-1}; NMR ($CDCl_3$, 80 MHz) δ 3.76 (s, 3H, $C\underline{H}_3O_2C$), 4.50 (s, 2H, $C\underline{H}_2N$), 7.6-8.0 (m, 4H, Ar$\underline{H}$). Exact mass calculated for $C_{11}H_9NO_4$: 219.0532. Found: 219.0530.

Preparation of N-(phthalimido)glycine phenylmethyl ester (25).

A solution containing 20.0 g of glycine phenylmethyl ester 4'-methylbenzene sulphonate (0.06 moles) and 100 mL of toluene was combined with 11.1 mL of triethylamine. The reaction mixture was stirred for 15 minutes before the addition of 8.80 g of phthalic anhydride (0.06 moles). The reaction mixture was refluxed for 3 h with azeotropic removal of water, cooled to 5°C, and maintained at this temperature for 16 h. A crystalline mass (triethylammonium 4'-methylbenzene sulphonate) was obtained which was removed by filtration and washed with toluene. The filtrate was concentrated in vacuo at 50°C to yield a colourless oil. This oil was stored for 12 h at 0°C yielding 21.4 g 25 a crystalline solid. This product was recrystallized from ethanol/water to give 14.4 g of pure white crystalline 25 (yield 80%) (mp. 105.5-106°C).

FT-IR (film) 1770, 1735, 1720, 1420 cm^{-1}; NMR ($CDCl_3$, 400 MHz) δ 4.50 (s, 2H, $C\underline{H}_2N$), 5.22 (s, 2H, $C\underline{H}_2O$), 7.40 (m, 5H, Ar$\underline{H}$), 7.80 (m, 2H, Ar$\underline{H}$), 7.89 (m, 2H, Ar$\underline{H}$). Exact mass calculated for $C_{17}H_{13}NO_4$: 295.0844. Found: 295.0851.

Preparation of N-(trifluoroacetyl)glycine methyl ester (**26**).

A solution containing 50.0 g of glycine methyl ester hydrochloride (0.40 mol) (**22**), 100 mL of trifluoroacetic anhydride (0.70 mol) and 100 mL of chloroform was stirred for 1 h. The reaction mixture was concentrated in vacuo in the presence of toluene to yield 72.5 g of a yellow oil. This yellow oil was distilled (0.1 mm of Hg, 68°-70°C) to yield 70.1 g of **26** as a colourless liquid (yield 95%). FT-IR (film) 3300, 1760, 1720, 1170 cm^{-1}; NMR ($CDCl_3$, 200 MHz) δ 7.26 (br s, 1H, NH), 4.15 (d, J = 6.0 Hz, 2H, CH_2N), 3.80 (s, 3H, CH_3). Exact mass calculated for $C_5H_6F_3NO_3$: 185.0300. Found: 185.0296. Anal. Calcd for $C_5H_6F_3NO_3$: C, 32.45; H, 3.27; N, 7.56. Found: C, 31.56; H, 3.40; N, 7.49.

Preparation of N-acetylglycine phenylmethyl ester (**27**).

A solution containing 90.8 g of glycine phenylmethyl ester 4'-methylbenzenesulphonate (0.216 mol) and 70.0 mL of triethylamine (0.47 mol) in 300 mL of chloroform was cooled to 5°C. This was followed by the addition of 30 mL of acetyl chloride (0.42 mol) over a 1 h period. After one hour, the reaction mixture was washed with 300 mL of aqueous 5% sodium bicarbonate solution. The organic layer was separated and the aqueous layer was extracted with 300 mL of chloroform. The combined organic extracts were dried over

sodium sulphate for 12 h. The organic extract was filtered, concentrated and distilled (147-150°C, 2 mm of Hg) to give a yellow oil which was contaminated with triethylammonium chloride, which was removed by dissolving the oil in 200 mL of dichloromethane and washing with 100 mL of 5% aqueous sodium bicarbonate solution. The organic layer was separated, filtered, dried and concentrated. The resulting oil was triturated with petroleum ether and dried for four days to give 32.0 g of **27** as a light yellow solid (yield 71%) (mp. 36-37°C).

FT-IR (film) 3300 br, 1750, 1660 cm^{-1}; NMR ($CDCl_3$, 80 MHz) δ 7.32 (m, 5H, ArH), 6.71 (br s, 1H, NH), 5.23 (s, 2H, $ArCH_2$) 3.91 (d, J = 9 Hz, 2H, CH_2N), 1.97 (s, 3H, CH_3CO). Exact mass calculated for $C_{11}H_{13}NO_3$: 207.0895. Found: 207.0900.

Preparation of N-acetyl-N-(phenylmethyl)glycine ethyl ester (**28**).

A solution containing 11.0 mL of acetyl chloride (0.15 mol) in 40 mL of dichloromethane was added to a solution containing 20 g of N-(phenylmethyl)glycine ethyl ester (**30**) (0.104 mol) and 15.0 mL of triethylamine (0.107 mol) in 100 mL of dichloromethane at 5°C over a 30 min period. The solution was warmed to room temperature and stirred for 12 h. The reaction mixture was washed with 400 mL of 5% aqueous sodium bicarbonate solution followed by extraction of the aqueous layer with 100 mL of ether. The organic

extract was dried over sodium sulphate, filtered and concentrated to produce 35 g of crude yellow product. This was purified further by chromatography (3 g on a 5 cm by 15 cm column) on flash silica gel using ether. Fractions containing product were concentrated to give 2.05 g of **28** as a colourless oil (yield 84%).

FT-IR (film) 2980, 1730, 1660 cm^{-1}; NMR ($CDCl_3$, 80 MHz) δ 7.2-7.4 (m, 5H, ArH), 4.05 (s, 2H, CH_2N), 4.61 (s, 2H, $ArCH_2$), 4.1 (q, J = 7 Hz, 2H, CH_2CH_3), 2.21 (s, 3H, CH_3CO), 1.32 (t, J = 7 Hz, 3H, CH_3). Exact mass calculated for $C_{13}H_{17}NO_3$: 235.1208. Found: 235.1202. Anal. Calcd for $C_{13}H_{17}NO_3$: C, 66.36; H, 7.28; N, 5.95. Found: C, 65.52; H, 7.37; N, 5.87.

Preparation of N-acetyl-N-(phenylmethyl)glycine phenylmethyl ester (**29**).

The title compound **29** was prepared in an analogous manner to the preparation of N-acetyl-N-(phenylmethyl)glycine ethyl ester (**28**).

FT-IR (film 3000, 1750, 1655, 1180 cm^{-1}; NMR ($CDCl_3$, 200 MHz) δ 7.32 (m, 10H, ArH), 5.12 (s, 2H, $ArCH_2$) 4.54 (s, 2H, $ArCH_2$), 4.1, 3.9 (s, 2H, CH_2N rotamers), 2.20, 2.08 (s, 3H, CH_3CO rotamers). Exact mass calculated for $C_{18}H_{19}NO_3$: 297.1365. Found: 297.1373. Anal. Calcd for $C_{18}H_{19}NO_3$: C, 72.70; H, 6.44; N, 4.71. Found: C, 72.74; H, 6.41; N, 5.03.

Preparation of N-(phenylmethyl)glycine ethyl ester (30).

N-(Phenylmethyl)glycine ethyl ester (30) was prepared in an analogous manner to 31 by conversion of 60.0 g (0.43 mol) of glycine ethyl ester hydrochloride to give 32.5 g of pure 30 (yield 40%) after distillation (8 mm of Hg, 135-139°C). FT-IR (film) 2920, 1750, 1660 cm^{-1}; NMR ($CDCl_3$, 80 MHz) δ 7.2-7.4 (m, 5H, ArH), 4.21 (q, J = 7 Hz, 2H, CH_2), 3.85 (s, 2H, $ArCH_2$), 3.35 (br s, 2H, CH_2N), 2.20 (br s, 1H, NH), 1.82 (t, J = 7 Hz, 3H, CH_3). Exact mass calculated for $C_{11}H_{15}NO_2$: 193.1103. Found: 193.1105.

Preparation of N-(phenylmethyl)glycine phenylmethyl ester (31).

A solution containing 32.0 g of glycine phenylmethyl ester 4'-methylbenzenesulphonate (0.10 mol), 50 mL of dry methanol, 25 mL of triethylamine (0.18 mol) and 10 mL of freshly distilled benzaldehyde (0.10 mol) was stirred for 55 minutes. Solution was complete after 10 minutes. The reaction mixture was treated carefully with 7.5 g of sodium borohydride (0.2 mol), with the evolution of hydrogen gas and was stirred for 2.5 h. The reaction mixture was poured into 100 mL of a 5% aqueous sodium bicarbonate solution, extracted with 250 mL of ethyl acetate, dried over sodium sulphate and concentrated in vacuo to yield a cloudy oil. This oil was dissolved in 1000 mL of dichloromethane and ether was added until precipitation occurred. The solution

was filtered and concentrated in vacuo giving 30.2 g of crude product. The product was purified by chromatography on flash silica gel (1.6 g on 5 cm by 15 cm column) using 10% ethyl acetate in dichloromethane. Fractions containing product were concentrated in vacuo to give 0.91 g of **31** as a light yellow oil (Yield 74%).

FT-IR (film) br 3300, 3000, 1740 cm^{-1}; NMR ($CDCl_3$, 80 MHz) δ 7.40 (m, 5H, ArH), 5.24 (s, 2H, $ArCH_2$), 3.47 (s, 2H, CH_2N), 3.79 (s, 2H, $ArCH_2N$), 2.40 (br s, 1H, NH). Exact mass calculated for $C_{16}H_{17}NO_2$: 255.1259. Found: 255.1265.

Preparation of N-(phenylcarbonyl)-3-oxoalanine ethyl ester (32)

A solution of sodium ethoxide, prepared by the addition of 5.71 g of sodium (0.248 mol) to 120 mL of dry ethanol at room temperature over 1.5 h, was combined with 20.0 mL of ethyl formate (0.262 mol) and was stirred for 1 h. A solution containing 10.7 g of N-(phenylcarbonyl)glycine ethyl ester (**23**) (0.0914 mol) in 100 mL of ethanol was added and the reaction mixture was stirred for 36 h. The solution was concentrated in vacuo in the presence of 100 mL of benzene. The resulting solid was added to 400 mL of ice water containing 25 mL of aqueous 12 N hydrochloric acid and 260 mL of chloroform. The organic layer was separated and the aqueous layer extracted with 450 mL of chloroform. The combined organic extracts were washed with 20 mL of aqueous

saturated sodium chloride and dried for 1 h over sodium sulphate. The solution was filtered and concentrated *in vacuo* to yield 12 g of crude product which was shown by n.m.r. to contain two major products; **32** and ethyl benzoate. Compound **32** was obtained by chromatography of 11 g of material on flash silica gel (5 cm by 15 cm) using 5% ethyl acetate in toluene. Fractions containing product were rechromatographed, combined and concentrated *in vacuo* to give 1.65 g of **32** as a colourless oil (yield 14%). Compound **32** was found to be unstable decomposing to ethyl benzoate and unidentified products within 12 h.

FT-IR (film) 3300, 1730 cm^{-1}; NMR ($CDCl_3$, 80 MHz) δ 1.30 (t, J = 7.5 Hz, 3H, CH_3), 4.12 (q, J = 7.5 Hz, 2H, CH_2), 7.3 (m, 4H, ArH, vinyl-H), 7.7 (m, 2H, ArH), 8.41 (br, s, 1H, NH), 12.1 (d, J = 14.0 Hz, 1H, OH).

Preparation of N-(phthalimido)-3-oxoalanine methyl ester (33).

A solution containing 47.03 g of N-(phthalimido)glycine methyl ester (**24**) (0.22 moles) in 250 mL of methyl formate (4.00 mol) (dried over phosphorus pentoxide) was cooled to 5°C to give a white suspension. Then, over a 1 h period, 220 mL of a 1 M solution of potassium t-butoxide (prepared by dissolving freshly sublimed potassium t-butoxide in tetrahydrofuran) in tetrahydrofuran were added. The yellow mixture was kept at 5°C for 36 h. The reaction mixture was

quenched at 0°C by the addition of 10 mL of glacial acetic acid (178 mmol) in 250 mL of dry benzene. After 30 min, the reaction mixture was added to 250 mL of 0.5 M aqueous hydrochloric acid and extracted with 4 L of benzene. The combined benzene extracts were concentrated in vacuo to yield 41.0 g of **33** white fluorescent compound (yield 77%) (mp. 140-142°C).

FT-IR (film) 1790, 1720, 1680, 1640, 1410, 3200 cm^{-1}; NMR ($CDCl_3$, 200 MHz) δ 3.81 (s, 3H, $C\underline{H}_3O_2C$), 7.32 (br s, 1H, vinyl-H), 7.5-8.0 (m, 4H, ArH), 11.5 (br, d, J = 15.0 Hz, 1H, OH). Exact mass calculated for $C_{12}H_9NO_5$: 247.0481. Found: 247.0478. Anal. Calcd for $C_{12}H_9NO_5$: C, 58.30; H, 3.67; N, 5.67. Found: C, 58.24; H, 3.71; N, 5.55.

Preparation of N-(phthalimido)-3-oxoalanine phenylmethyl ester (**34**).

The synthesis of **34** was done using the procedure described for the corresponding methyl ester **33** with the replacement of methyl formate by benzyl formate to give 26.1 g of **34** as a yellow crystalline product from 30.0 g (0.101 mol) of starting material **25** (yield 80%) (mp. 136.5-138°C). FT-IR (film) 1790, 1720 cm^{-1}; NMR ($CDCl_3$, 200 MHz) δ 5.20 (s, 1H), 5.38 (s, 2H), 7.2-7.4 (m, 7H), 7.85-7.95 (m, 2H). Exact mass calculated for $C_{18}H_{13}NO_5$: 323.0794. Found: 323.0794. Anal. Calcd for $C_{18}H_{13}NO_5$: C, 66.87; H, 4.05; N, 4.33. Found: C, 67.09; H, 4.22; N, 4.27.

Preparation of N-(trifluoroacetyl)-3-oxoalanine methyl ester (35).

A solution containing 7.0 mL of 1,1,1,3,3,3-hexamethyldisilazane (33 mmol) in dry tetrahydrofuran was cooled to -30°C. The amide base was prepared by the addition of 22.0 mL of 0.77 M n-butyl lithium in hexane over a 30 minute period. The solution was stirred for 1 h. After cooling the solution to -78°C, a solution containing 1.52 g of N-(trifluoroacetyl)glycine methyl ester (**26**) (8.2 mmol) in 250 mL of dry tetrahydrofuran was added over a 20 min period and the resulting solution was stirred for 1 h. A solution containing 2.0 mL of methyl formate (32 mmol) was added over a 25 min period, to form a white precipitate. The solution was warmed to -30°C and was maintained within 10°C of this temperature for 5 h, before it was maintained at 5°C for 12 h. The solution was cooled to -65°C and 5 mL of glacial acetic acid (87 mmol) in 15 mL of tetrahydrofuran were added. A white precipitate formed as the reaction mixture was stirred for 20 minutes. The solution was poured into 80 mL of 1 N aqueous hydrochloric acid and was extracted with 300 mL of chloroform. The solution was dried over sodium sulphate, filtered and concentrated in vacuo in the presence of toluene to give 1.62 g of crude **35**. Because of its instability, no attempt was made to isolate pure **35**; however, analysis by n.m.r. indicated the presence of **35**.

This crude product was directly alkylated to form the corresponding enol ether **36**.

Preparation of Z-2-(N-acetylamino)-3-methoxypropenoic acid phenylmethyl ester (**38**).

A solution of lithium cyclohexylisopropylamide was prepared by the dropwise addition of 50.0 mL of 1.2 M n-butyl lithium in hexane (60.0 mmol) to 10.0 mL of N-isopropyl-N-cyclohexylamine (68.0 mmol) in 1560 mL of dry tetrahydrofuran at -78°C. The resulting solution was stirred for 1 h at this temperature, before the addition of 8.6 g of TMEDA (74.0 mmol). A solution containing 2.0 g of N-acetylglycine phenylmethyl ester (**27**) (9.6 mmol) in 10 mL of tetrahydrofuran was added dropwise over a 20 min period. The reaction mixture was stirred for 40 min to form a deep orange solution. The anion was then quenched by the addition of 20 mL of methyl formate (325 mmol) to give a yellow solution. A solution containing 10 mL of glacial acetic acid (175 mmol) in 30 mL of tetrahydrofuran was added after 45 min and was stirred for a further 15 min. The reaction mixture was poured into 140 mL of 1 N aqueous hydrochloric acid and extracted with 300 mL of chloroform. The organic extract was dried over sodium sulphate, filtered and concentrated *in vacuo* to give 2.51 g of crude product as an orange oil. This material was dissolved in ether, filtered and treated with excess diazomethane solution in

the presence of 0.05 mL of 50% aqueous hydrofluoroboric acid. After stirring for 1 h, the solution was concentrated in vacuo. The desired product was separated by chromatography on flash silica gel (2 cm by 15 cm) using 10% ether in dichloromethane. Fractions containing product were concentrated in vacuo to give 160 mg of **38** as a colourless oil (yield 14%).

FT-IR (film) 3300, 2900, 1740, 1710, 1250, 1120 cm^{-1}; NMR ($CDCl_3$, 200 MHz) δ 7.38 (m, 5H, ArH), 7.35 (s, 1H, vinyl-H), 6.75 (s, 1H, NH), 5.20 (s, 2H, $ArCH_2$), 3.81 (s, 3H, CH_3O), 2.10 (s, 3H, CH_3CO). Exact mass calculated for $C_{13}H_{15}NO_4$: 249.1001. Found: 249.1005. Anal. Calcd for $C_{13}H_{15}NO_4$: C, 62.64; H, 6.07; N, 5.62. Found: C, 62.61; H, 6.12; N, 5.32.

Preparation of N-acetyl-N-(phenylmethyl)-3-oxoalanine ethyl ester (**39**).

A solution containing 2.28 g (9.70 mmol) of N-acetyl-N-(phenylmethyl)glycine ethyl ester (**28**) in 12.0 mL (148 mmol) of ethyl formate was cooled to 5°C. Over a period of 1 h, 14.0 mL (10.8 mmol) of 0.77 M potassium t-butoxide in tetrahydrofuran were added to form an orange solution. The reaction mixture was stirred for 48 h with the evolution of carbon monoxide. The reaction was quenched by the addition of a solution of 1.0 mL of acetic acid in 9 mL of benzene. A white precipitate was formed. After the reaction mixture

was stirred for 5 min, it was poured into 50.0 mL of 1 M aqueous hydrochloric acid. The solution was extracted with 200 mL of ethyl acetate, dried over sodium sulphate and concentrated in vacuo with toluene to give 3.1 g of crude product. N.m.r. analysis showed that the starting material had undergone 30% conversion to product. The crude product was subjected to the initial reaction conditions for an additional 24 h. This step was repeated 3 times to give conversion to 80% of **39**. Work up gave 2.21 g of crude product. The product was isolated by chromatography on silica gel (500 mg on a 3 cm by 30 cm column) using ethyl acetate. Fractions containing product were concentrated to produce 394 mg of **39** as a colourless oil (68% yield) which was crystallized by dissolving in a minimum amount of ether at 5°C (mp. 94-96°C). Compound **39** exists as an enol-keto tautomer. Integral values are given in the n.m.r. relative to the acetyl peak.

FT-IR (film) 3000, 1710, 1400, 1220 cm^{-1}; NMR ($CDCl_3$, 200 MHz) δ 1.20 (t, 2.2H), 1.30 (t, 0.8H), 2.02 (s, 3H, CH_3CO), 4.0-4.3 (m, 2H), 4.75 (m, 1.5H), 5.25 (d, J = 10.0 Hz, 0.4H), 6.85 (s, 0.2H), 7.2-7.4 (m, 5.2H), 7.75 (m, 0.8H), 11.3 (br s, 1.2H). Exact mass calculated for $C_{14}H_{17}NO_4$: 263.1157. Found: 263.1155. Anal. Calcd for $C_{14}H_{17}NO_4$: C, 63.87; H, 6.51; N, 5.32. Found: C, 63.25; H, 6.56; N, 5.30.

Preparation of Z-2-(N-phthalimido)-3-methoxypropenoic acid methyl ester (41) and its E isomer (44).

A solution containing 5.0 g (20 mmol) of N-(phthalimido)-3-oxoalanine methyl ester (35), 0.5 mL of 1% aqueous hydrofluoroboric acid and 300 mL of ether was cooled to 0°C. The reaction mixture was treated with a solution of diazomethane in ether until a clear yellow colour persisted. It was then warmed to room temperature and was stirred for 15 h. The mixture was filtered and was concentrated to yield 5.1 g of a yellow solid. Two products were separated by chromatography on silica gel (5 cm by 45 cm) using 32% ethyl acetate in chloroform followed by gradient elution to 100% ethyl acetate. Fractions containing each product were concentrated in vacuo to give 4.91 g of 41 (yield 95%) and 150 mg of 44 (yield 0.6%).

For 44: (mp. 185-190°C)

FT-IR (film) 1780, 1760, 1720, 1675, 1650 cm^{-1}; NMR ($CDCl_3$, 200 MHz) δ 3.71 (s, 3H, $C\underline{H}_3O_2C$), 4.02 (s, 3H, $C\underline{H}_3O$), 6.82 (s, 1H, vinyl-H), 7.75 (m, 2H, ArH), 7.90 (m, 2H, ArH). Exact mass calculated for $C_{13}H_{11}NO_5$: 261.0637. Found: 261.0634. Anal. Calcd for $C_{13}H_{11}NO_5$: C, 59.77; H, 4.24; N, 5.36. Found: C, 59.64; H, 4.33; N, 5.44.

For 41: (mp. 140-142°C)

FT-IR (film) 1790, 1721, 1650 cm^{-1}; NMR ($CDCl_3$, 200 MHz) δ 3.62 (s, 3H, $C\underline{H}_3O$), 3.78 (s, 3H, $C\underline{H}_3O_2C$), 7.5-8.9 (m, 6H

ArH, vinyl-H). Exact mass calculated for $C_{13}H_{11}NO_5$: 261.0637. Found: 261.0632. Anal. Calcd. for $C_{13}H_{11}NO_5$: C, 59.77; H, 4.24; N, 5.36. Found: C, 59.70; H, 4.21; N, 5.26..

Preparation of Z-2-(N-phthalimido)-3-methoxypropenoic acid phenyl methyl ester (42).

A solution containing 1.02 g (3.15 mmol) of **34** was converted to **42** by treatment with diazomethane in ether in analogy to the preparation of **41**. After chromatography, 0.93 g of **42** was obtained (yield 87%) (mp. 116-117°C).

FT-IR (film) 1790, 1760, 1720, 1650 cm^{-1}; NMR ($CDCl_3$, 200 MHz), 3.89 (s, 3H, CH_3O), 5.21 (s, 2H, $ArCH_2$), 7.32 (m, 5H, ArH), 7.71 (m, 3H, ArH, vinyl-H), 7.87 (m, 2H, ArH). Exact mass calculated for $C_9H_5NO_5$: 337.0950. Found: 337.0953. Anal. Calcd for $C_9H_5NO_5$: C, 67.65; H, 4.48; N, 4.15. Found: C, 67.58; H, 4.50; N, 4.11.

Preparation of Z-2-(N-phthalimido)-3-methoxypropenoic acid (49).

A solution containing 1.48 g (4.39 mmol) of Z-2-(N-phthalimido)-3-methoxypropenoic acid phenylmethyl ester (**42**) in 15 mL of chloroform was added to a suspension containing 20 mg of 5% palladium on carbon in 20 mL of methanol which had been prehydrogenated at 1 atmosphere of hydrogen for 40 min. The resulting suspension was stirred under an atmosphere of hydrogen for 4 days. The solution

was filtered through Celite and was concentrated *in vacuo* producing 1.01 g of **49** as a white crystalline solid (yield 93%) (mp. 234-235°C).

FT-IR (cast) 4000-2900, 1790, 1760, 1670, 1640 cm^{-1}; NMR ($CDCl_3$, 200 MHz) δ 3.89 (s, 3H, CH_3O), 7.70 (m, 2H, ArH), 7.79 (s, 1H, vinyl-H), 7.85 (m, 2H, ArH). Exact mass calculated for $C_{12}H_9NO_5$: 247.0481. Found: 247.0475. Anal. Calcd for $C_{12}H_9NO_5$: C, 58.30; H, 3.67; N, 5.67. Found: C, 58.29; H, 3.68; N, 5.50.

Preparation of Z-2-(N-trifluoroacetyl)amino-3-methoxy propenoic acid methyl ester (**36**).

A solution containing 1.60 g of crude **35** in 50 mL of ether was filtered and was cooled to 10°C. After the addition of 0.02 mL aqueous 50% hydrofluoroboric acid, a 30 mL solution of diazomethane in ether was added over a 2 hour period with the formation of a new spot on tlc at Rf = 0.8 (EtOAc). The solvent was removed *in vacuo* and the residue chromatographed upon flash silica gel (15 cm by 3 cm) using 8% ethyl acetate in hexane. Fractions containing product were concentrated *in vacuo* giving 252 mg of pure **36** (yield 14%). Additionally, 600 mg of N-(trifluoroacetyl)glycine methyl ester (**26**) were obtained.

FT-IR (film) 3300, 1730, 1660, 1530, 1260, 1160 cm^{-1}; NMR ($CDCl_3$, 400 MHz) δ 7.32 (br s, 1H, NH), 7.42 (s, 1H, vinyl-

H), 3.95 (s, 3H, CH_3CO), 3.78 (s, 3H, CH_3O). Exact mass calculated for $C_7H_8F_3NO_4$: 227.0405. Found: 227.0400. Anal. Calcd for $C_7H_8F_3NO_4$: C, 37.01; H, 3.55; N, 6.18. Found: C, 36.83; H, 3.53; N, 6.20.

Preparation of Z-2-(N-acetyl-N-(phenylmethyl)amino-3-methoxy propenoic ethyl ester (43).

A solution containing 300 mg of N-acetyl-N-(phenylmethyl)-3-oxoalanine ethyl ester **39** (1.08 mmol) and 0.05 mL of 50% hydrofluoroboric acid in 100 mL of ether was titrated with a solution of diazomethane in ether in 10 mL portions. After addition of 110 mL of diazomethane solution, the conversion to product was complete. The reaction mixture was stirred for 1 h, concentrated *in vacuo* and flash chromatography of the residue on silica gel (2 cm by 15 cm) using 6% ether in dichloromethane. Fractions containing product were concentrated *in vacuo* to produce 301 mg of **43** as a colourless oil (yield 98%).

FT-IR (film) 2900, 1710, 1660, 1260 cm^{-1}; NMR ($CDCl_3$, 200 MHz) δ 7.25 (m, 6H, ArH, vinyl-H), 4.91 (d, J = 15 Hz, 1H, ArCH), 4.35 (d, J = 15 Hz, 1H, ArCH), 4.13 (q, J = 7.5 Hz, 2H, CH_2CH_3), 3.72 (s, 3H, CH_3O), 2.05 (s, 3H, CH_3CO), 1.25 (t, J = 7.5 Hz, 3H, CH_3CH_2). Exact mass calculated for $C_{15}H_{19}NO_4$: 277.1314. Found: 277.1315. Anal. Calcd for $C_{15}H_{19}NO_4$: C, 64.97; H, 6.91; N, 5.05. Found: C, 64.77; H, 7.02; N, 5.43.

Preparation of Z-2-(N-phthalimido)amino-3-acetoxypropenoic acid methyl ester (45) and its E isomer (46).

A solution containing 1.32 g of N-(phthalimido)-3-oxoalanine methyl ester (33) (5.3 mmol), 0.60 g of allyl acetate (0.60 mmol) and 128 mg of 4'-methylbenzenesulphonic acid in 100 mL of benzene was refluxed with the removal of acetone for 1 h. The solution was washed with 25 mL of 5% aqueous saturated sodium bicarbonate and dried over sodium sulphate. Concentration in vacuo gave 1.50 g of 45 and 46 as a colourless oil. The n.m.r. analysis on this compound showed it to be a 60:40 mixture of E/Z isomers.

NMR ($CDCl_3$, 80 MHz) δ 2.26 (s, 3H, E-isomer), 2.70 (s, 3H, Z-isomer), 3.75 (s, 3H, Z-isomer), 3.80 (s, 3H, E-isomer), 7.7-8.0 (m, 9H, ArH, E-isomer-vinyl-H), 8.65 (s, 1H, Z-isomer-vinyl-H).

Preparation of Z-2-(N-phthalimido)-3-acetoxypropenoic acid methyl ester (45) using acetyl chloride.

A solution containing 1.55 g of 2-(N-phthalimido)-3-oxoalanine methyl ester (33) (6.3 mmol) 1.0 mL of triethylamine (7.0 mmol) in 100 mL of chloroform was cooled to 0°C. To this cooled solution, 0.50 mL of acetyl chloride (7.0 mmol) was added dropwise over a 30 min period to form a yellow-brown precipitate. After an additional 30 min, the mixture was washed with 100 mL of 5% aqueous sodium bicarbonate solution, dried over sodium sulphate, filtered

and concentrated *in vacuo* to give a light yellow oil. N.m.r. analysis showed this to contain only the crude Z-isomer **45**. Chromatography on silica gel (2 cm by 30 cm) using ethyl acetate gave 1.62 g of **45** as a colourless oil (yield 89%).

FTIR (film) 3000, 1720 cm^{-1}; NMR ($CDCl_3$, 80 MHz) δ 2.22 (s, 3H, CH_3CO), 3.71 (s, 3H, CH_3O_2C), 7.4-8.0 (m, 4H, ArH), 8.62 (s, 1H, vinyl-H). Exact mass calculated for $C_{14}H_{11}NO_6$: 289.0586. Found. 289.0582.

Preparation of Z-2-(N-phthalimido)-3-acetoxypropenoic acid phenylmethyl ester (**47**).

Using the procedure described for the methyl compound **45**, 0.81 g of 2-(N-phthalimido)-3-oxoalanine phenylmethyl ester (**34**) (2.5 mmol) was converted to 0.79 g of **47** (yield 86%).

FT-IR (film) 3000, 1725 cm^{-1}; NMR ($CDCl_3$, 80 MHz) δ 2.25 (s, 3H, CH_3CO), 5.35 (s, 2H, CH_2Ar), 7.35 (m, 5H, ArH), 7.4-8.1 (m, 4H, ArH), 8.70 (s, 1H, vinyl-H). Exact mass calculated for $C_{20}H_{15}NO_6$: 365.0899. Found 365.0899.

Preparation of Z-2-(N-phthalimido)-3-acetoxypropenoic acid (**51**).

A suspension containing 0.71 g of the phenylmethyl ester **47** (2.0 mmol) and 70 mg of 5% palladium on charcoal in 50 mL of ethyl acetate was stirred in the presence of hydrogen gas at atmospheric pressure. After 4 h, the reaction mixture was concentrated to give 0.52 g of pure **51** (yield 98%) (mp. 139-140°C).

FT-IR (cast) 3100 br, 1710, 1740 cm^{-1}; NMR ($CDCl_3$, 400 MHz) δ 2.21 (s, 3H, CH_3CO); 7.80 (m, 2H, ArH), 7.94 (m, 2H, ArH), 8.74 (s, 1H, vinyl-H). Unable to obtain M^+ peak using electron impact or chemical ionization.

Reductions of N-phthalimido dehydroserine derivatives. Preparation of **48** and **50** from Z-2-(N-phthalimido)-3-methoxy propenoic acid (**51**).

All reactions were carried out on a ratio of 1.0 mmol of either **50** or **51** as a substrate to 20 mL of methanol. A weight of catalyst 5% of that of the substrate was employed. Work up employed simple filtrations through Celite. High field (200 MHz or 400 MHz) n.m.r. analysis was used to determine the product ratio. The reductions on **51** were worked up by filtration and concentration to give a crude oil. The acids formed from this reaction were treated with diazomethane for conversion to their methyl esters and were separated by chromatography. Using this procedure reduction with Rh on C gave 53% of **48**, and reduction with Rh on C gave 40% of **48** and 25% of **50** (see Table 2).

For 2-(1,3-dioxooctahydro-2-isoindol-2-yl)-3-methoxypropenoic acid methyl ester: (**48**)

FT-IR (film) 2940, 1785, 1720, 1655 cm^{-1}; NMR ($CDCl_3$, 200 MHz) δ 1.49 (m, 4H), 1.83 (m, 4H), 1.95 (m, 2H), 3.72 (s,

TABLE 2

Reductions of protected phthalimido derivatives

Compound No.	Catalyst	Time (Days)	Pressure	Result
41	$(Ph_3P)_3RhCl$	4	1 atm	no rxn
41	Rh on Al_2O_3	7	45 psi	48
41	Pd on C	4	1 atm	no rxn
49	Pd on C	4	1 atm	no rxn
49	PtO_2	4	45 psi	no rxn
49	Rh on C	4	45 psi	48
49	Rh on Al_2O_3	4	45 psi	48 + 50
49	$(Ph_3P)_3RhCl$	4	1 atm	no rxn
49	RaNi	4	1 atm	no rxn*

All the above reactions were carried out in methanol using similar mole concentrations. *Results from J.G. Hill.

3H), 3.89 (s, 3H), 7.50 (s, 1H). Exact mass calculated for $C_{13}H_{17}NO_5$: 267.1107. Found: 267.1108. Anal. Calcd for $C_{13}H_{17}NO_5$: C, 58.41; H, 6.41; N, 5.24. Found: C, 58.28; H, 6.25; N, 4.79.

For 2-(1,3-dioxooctahydro-2H-isoindol-2-yl)-3-methoxypropanoic acid methyl ester: (50)

FT-IR (film) 2940, 2840, 1750, 1710 cm^{-1}; NMR ($CDCl_3$, 200 MHz) δ 1.45 (m, 4H, CH), 1.90 (m, 4H), 2.89 (m, 2H), 3.35 (s, 3H), 3.77 (s, 3H), 3.91 (m, 1H), 4.08 (m, 1H, CHO), 5.00 (m, 1H). Exact mass calculated for $C_{13}H_{19}NO_5$: 269.1263. Found: 269.1275.

Preparation of N-(trifluoroacetyl)-O-methylserine methyl ester (52).

A suspension of 20 mg of 5% rhodium on carbon in 6 mL of ether was stirred under an atmosphere of hydrogen gas, for 16 h. A solution containing 40 mg of 2-[N-(trifluoroacetyl)amino]-3-methoxypropenoic acid methyl ester (36) (0.17 mmol) and 0.005 mL of triethylamine (0.03 mmol) in 4 mL of ether was added. The reaction mixture was stirred for 3.5 h under an atmosphere of hydrogen gas, filtered and concentrated in vacuo to give 38 mg of 52 as a colourless oil (yield 95%).

FT-IR (cast) 3300, 1750, 1720, 1550, 1260, 1220 cm^{-1}; NMR ($CDCl_3$, 200 MHz) δ 7.45 (m, 1H, NH), 4.78 (m, 1H), 3.81 (s,

4.0, 10.0 Hz, 1H). Exact mass calculated for $C_7H_{10}F_3NO_4$: 229.0562. Found: 229.0561. Anal. Calcd for $C_7H_{10}F_3NO_4$: C, 36.69; H, 6.11; N, 4.40. Found: C, 36.90; H, 6.08; N, 4.40.

Preparation of a racemic mixture of (2R,3S)- and (2S,3R)-[2,3-$^2H_2$]-N-(trifluoroacetyl)-O-methylserine methyl ester (**54**).

In an analogous manner to the preparation of **57**, 200 mg (0.88 mmol) of 2-(N-trifluoroacetyl)amino-3-methoxypropenoic acid methyl ester (**36**) was catalytically hydrogenated using deuterium gas (99.5% purity) to yield 185 mg of **54** as a colourless oil (yield 93%).

FT-IR (cast) 3200, 1750 cm^{-1}; NMR ($CDCl_3$, 200 MHz) δ 3.83 (br s, 1H, CHD), 3.81 (s, 3H, CH_3CO), 3.32 (s, 3H, CH_3O), 7.04 (br s, 1H, NH). Exact mass calculated for $C_7H_8D_2F_3NO_4$: 231.0685. Found: 231.0669.

Preparation of N-acetyl-O-methylserine (**56**).

A solution containing 10 mg of 5% palladium on charcoal in 10 mL of ethyl acetate was stirred for 1 h under an atmosphere of hydrogen. A solution containing 20 mg of 2-(N-acetylamino)-3-methoxypropenoic acid phenylmethyl ester (**38**) (0.091 mmol) in 4 mL of ethyl acetate was added. After being stirred for 3 h under an atmosphere of hydrogen the ester **38** had been completely hydrogenolised (tlc: Rf = 0.4 (EtOAc)). The reaction mixture was filtered through celite

and concentrated and added to a suspension of 5 mg of 5% rhodium on carbon, 5 mL of ether and 5 mL of ethyl acetate. The reaction mixture was stirred for 1.5 h under an atmosphere of hydrogen filtered through celite and concentrated in vacuo to give 12 mg of **56** as a white crystalline solid (yield 82%).

FT-IR (cast) 3320, 2900, br 1730, 1640, 1580, 1120 cm^{-1}; NMR ($CDCl_3$, 400 MHz) δ 6.82 (d, J = 8.0 Hz, 1H, NH), 4.75 (m, 1H, CHN), 3.85 (dd, J = 9.0, 3.0 Hz, 1H, $CHOCH_3$), 3.65 (dd, J = 9.0, 4.0 Hz, 1H, $CHOCH_3$), 3.43 (s, 3H, CH_3OCH_2), 2.1 (s, 3H, CH_3CO). Exact mass calculated for $C_6H_{11}NO_4$: 161.0688. Found: 161.0657. Anal. Calcd for $C_6H_{11}NO_4$: C, 44.80; H, 6.85; N, 8.70. Found: C, 45.10, H, 6.38; N, 8.36.

Preparation of a racemic mixture of (2R,3S)- and (2S,3R)-[2,3-$^2H_2$]-N-acetyl-O-methylserine **57** from **38**.

The preparation of **57** was carried out in an analogous manner as for **56**, under an atmosphere of deuterium gas. This procedure gave 29 mg (0.15 mmol) of **57** from 18 mg of **38** (yield 97%).

FT-IR (cast) 3320, 2900 br, 1730, 1640, 1580, 1120 cm^{-1}; NMR (D_2O, 400 MHz) δ 3.85 (br s, 1H, $CHDOCH_3$), 3.43 (s, 3H, CH_3OCH), 2.1 (s, 3H, CH_3CO). Exact mass calculated for $C_6H_9D_2NO_4$: 163.0811. Found: 163.0755.

Preparation of a racemic mixture of (2R,3S)- and (2S,3R)-[2,3-$^2H_2$]-N-acetyl-O-methylserine ethyl ester (58).

A solution containing 700 mg of a 60:40 mixture of **43** and **28** was subjected to catalytic deuteration in 110 mL of ethyl acetate and 100 mg of 5% palladium on carbon for 2 days. N.m.r. analysis showed 20% conversion to product had occurred. The oil was resubjected to deuteration conditions using 50 mL of ethyl acetate, 0.1 mL of acetic acid and 100 mg of palladium catalyst. The reaction mixture was stirred for 24 h. Removal of solvent gave 440 mg an inseparable mixture of **58** and N-acetylglycine ethyl ester was obtained (yield 86%). N.m.r. analysis showed complete conversion of starting material to product had been achieved without elimination or racemisaztion.

NMR ($CDCl_3$, 200 MHz) δ 7.74 (m, 1H, NH), 4.16 (2H, CH_2CH_3), 3.76 (t, s, 1H, $CHDOCHN$), 3.34 (s, 3H, CH_3OCHD), 2.02 (s, 3H, CH_3CO), 1.2 (3H, CH_3CH_2). Exact mass calculated for $C_8H_{13}D_2NO_4$: 191.1124. Found: 191.1124.

Preparation of (2R,3S)- and (2S,3R)-[2,3-$^2H_2$]-N-(trifluoroacetyl)serine (**61**).

This compound was prepared in an identical manner to **59** from 52 [illegible] was obtained from 75 mg of **61** (yield [illegible] give relative to NH equal to one

FT-IR (film) 3100 br, 1750 cm^{-1}; NMR ($CDCl_3$, 400 MHz) 7.24 (s, 1H, N<u>H</u>), 4.82 (m, 0.1 H, CHN), 4.21 (br s, 0.88 H, CHD), 4.06 (0.12H, CHD) 2.62 (br s, 1H, O<u>H</u>). Exact mass calculated for $C_5H_4D_2F_3NO_3$: 187.0423. Found: 187.0421

<u>Preparation of a racemic mixture of (2*R*,3*S*)-.and (2*S*,3*R*)-[2,3-$^2H_2$]-*N*-acetyl-*O*-methylserine from (**58**).</u>

A solution containing 100 mg of a 60:40 mixture of **58** and N-acetylglycine ethyl ester in 3 mL of tetrahydrofuran, was cooled to 5°C. A 6.30 mL aliquot of 0.095 M aqueous lithium hydroxide solution was added and the reaction mixture stirred at 5°C for 24 h. The product was isolated as the lithium salt by removal of the tetrahydrofuran *in vacuo*, followed by lyophilization of the aqueous solution to give 82 mg of a 60:40 mixture of **57** and N-acetylglycine (y[illegible] 98%). Compound 57 could be identified by comparison [illegible] prepared from **38**.

Preparation of (2*S*,3*R*) [2,3-$^2H_2$] *O*-methylserine (**62**) by enzymatic resolution of (2S,3R) and (2R,3S) N-acetyl-O-methylserine (**57**).

40 mg of a 60:40 mixture of 57 and N-acetylglycine were hydrolyzed with hog kidney [illegible] described in the literature.[110] Isolation [illegible] column [illegible] analogous [illegible] 11 mg of a 40:60 [illegible] serine (**62**)

NMR (D_2O,, 400 MHz) 3.90 (br s, 1H, CHDOH), 3.49 (s, 3H, OCH_3).

Preparation of unlabelled DL-serine (**64**).

A solution containing 1.0 g of O-methylserine (**63**) (8.4 mmol) in 20 mL of dichloromethane was stirred for 5 minutes. A 30 mL aliquot of 1 M boron tribromide (30.0 mmol) in dichloromethane was added dropwise to the reaction mixture and was stirred for 3 days. The reaction was quenched by the addition of 50 mL of water, and a vigourous evolution of gas (presumably HBr) was observed. This two phase system was stirred for 30 min. The phases were separated, and the organic layer was washed with a further 50 mL of water. The aqueous phase was concentrated and applied to an ion-exchange column containing AG50-X8(H^+) resin (1 cm by 20 cm) in the proton form. After washing the column with 300 mL water, the product was eluted with 150 mL of 0.4 M pyridinium acetate. The fractions containing product were lyophilised, neutralized and crystallized (EtOH) to produce 0.85 g of **64** as a solid (yield 97%) with spectral properties identical to DL-serine.

Preparation of N-(trifluoroacetyl)serine methyl ester (**66**).

A solution containing 4.81 g of serine methyl ester hydrochloride (31.21 mmol), 30 mL of chloroform and 14.0 mL of trifluoroacetic anhydride (99.1 mmol) was refluxed for 1 hour. The reaction mixture was

concentrated *in vacuo* to give a colourless oil containing *N,O*-bis-(trifluoroacetyl)serine methyl ester (**65**). Compound **65** was dissolved in water and extracted with 400 mL of ethyl acetate. The organic layer was dried over sodium sulphate, filtered and concentrated to give 5.33 g of **66** as a colourless oil (yield 80%).

For *N,O*-bis(trifluoroacetyl)serine methyl ester (**65**):

FT-IR (film) 3400, 1760 cm^{-1}; NMR ($CDCl_3$, 80 MHz) δ 7.40 (br, s, 1H, NH), 4.91 (m, 1H, CHN), 4.75 (m, 2H, CH_2O), 3.81 (s, 3H, CH_3O).

For *N*-(trifluoroacetyl)serine methyl ester (**66**):

FT-IR (film) 3100 br, 1750, 1720 cm^{-1}; NMR ($CDCl_3$, 80 MHz) δ 7.70 (br s, 1H, NH), 4.82 (m, 1H, CHN), 4.05 (dd, J = 11.5, 4.0 Hz, 1H, $CHOCH_3$) 4.30, (br s, 1H, OH), 4.21 (dd, J = 11.5, 3.5 Hz, 1H, $CHOCH_3$). Exact mass calculated for $C_6H_8F_3NO_4$: 215.0405. Found: 215.0402.

Preparation of *N*-(trifluoroacetyl)-*O*-methylserine methyl ester (**52**) from **66**.

A solution containing 5.23 g of *N*-(trifluoroacetyl)serine methyl ester (**66**) (24.3 mmol) in 200 mL of chloroform was combined with 1.50 g of silver oxide (64.7 mmol). The reaction flask was sealed with a rubber septum and 10 mL of iodomethane (161 mmol) were added. The solution was stirred in order to form a light green precipitate. After 21 h

contained only 40% product. This oil was combined with 1.61 g of silver oxide (69.4 mmol), 200 mL of chloroform and 10.0 mL of iodomethane (161 mmol). After 24 h, the solution was filtered, concentrated and decolourized with charcoal to yield 5.6 g of pure **52** as a colourless oil (yield >99%), which possessed identical properties to those of **52** obtained from **36**.

Preparation of O-methyl-serine (**63**).

A solution containing 5.60 g of N-(trifluoroacetyl)-O-methylserine methyl ester (**52**) (24.4 mmol), 60 mL of tetrahydrofuran and 60.0 mL of 0.95 M aqueous lithium hydroxide (57.0 mmol) was stirred for 2 days at room temperature. The solution was concentrated in vacuo at 50°C in the presence of toluene to yield a semisolid. This was dissolved in a minimum amount of water and placed upon a 15 cm by 5 cm Ag50-X8(H^+) ion exchange column (prepared by eluting with 1 M HCl, water, 1 M NaOH, water, 1 M HCl and water with pH equilibration at each stage). The product was eluted from the column using 0.40 M pyridinium acetate. Fractions containing product (ninhydrin active) were lyophilised, neutralized and crystallized (EtOH-H_2O) to yield 4.30 g of (**63**) as a crystalline solid (yield >99%). FT-IR (cast) 2980 br, 1570, 1410 cm^{-1}; NMR (D_2O, 200 MHz) δ 4.03 (t, J = 3.8 Hz, 1H, CHN), 3.91 (m, 2H, CH_2O), 3.52 (s, 3H, CH_3O).

Preparation of N-acetyl-O-methylserine (**56**).

A solution containing 121 mg of O-methylserine (**63**) (1.02 mmol), 2.0 mL of water and 2.0 mL of acetic anhydride (21 mmol) was stirred for 2 h. Heat was evolved during the first 30 minutes of the reaction. The reaction mixture was cooled to 5°C for 15 h. The solvent was removed by lyophilisation to give 155 mg of (**56**) as a yellow crystalline product (yield 95%) which had identical properties to **56** obtained from **38**.

FT-IR (cast) 3320, 2900 br, 1730, 1640, 1580, 1120 cm^{-1}; NMR (D_2O, 400 MHz) δ 6.82 (d, J = 8.0 Hz, 1H, NH), 4.75 (m, 1H, CHN), 3.85 (dd, J = 9.0, 3.0 Hz, 1H, $CHOCH_3$), 3.65 (dd, J = 9.0, 4.0 Hz, 1H, $CHOCH_3$), 3.43 (s, 3H, CH_3OCH_2), 2.1 (s, 3H, CH_3CO). Exact mass calculated for $C_6H_{11}NO_4$: 161.0688. Found: 161.0693.

Preparation of N-acetyl serine phenylmethyl ester (**67**).

A solution containing 1.00 g of N-acetylserine (6.8 mmol), 1.12 mL of triethylamine (8.0 mmol) and 0.81 mL (6.8 mmol) of 1-(bromomethyl)benzene was refluxed for 5 h. After 5 h, an additional 1.00 mL of 1-(bromomethyl)benzene (8.4 mmol) and 1.00 mL of triethylamine (7.2 mmol) were added. This process was repeated after a further 3 h. After a total reflux time of 24 h, the reaction mixture was concentrated to a yellow oil. This oil was dissolved in 100 mL of ethyl

filtration through a silica gel pad (2 cm by 1 cm). The filtrate was concentrated in the presence of toluene *in vacuo* to a yellow oil. The residue was dissolved in 200 mL of ethyl acetate and a layer of 100 mL of hexane was placed on the surface of the solution and cooled at 5°C for 15 h to produce long white crystals. The crystals were filtered, washed with hexane and dried in vacuum to give 0.77 g of pure **67** (yield 45%) (mp. 69-70°C).

FT-IR (film) 3300 br, 1740, 1650 cm^{-1}; NMR ($CDCl_3$, 400 MHz) δ 6.52 (br d, J = 7.0 Hz, 1H, NH), 7.4 (m, 5H, ArH), 5.25 (s, 2H, CH_2CO), 4.85 (dd, J_{AV} = 7.0, 3.7 Hz, 1H, CHN), 4.05 (dd, J = 3.2, 10.5 Hz, 1H, CHOH), 3.96 (dd, J = 4.0, 10.5 Hz, 1H, CHOH), 2.62 (br s, 1H, OH), 2.12 (s, 3H, CH_3CO). Exact mass calculated for $C_{12}H_{15}NO_4$: 237.1001. Found: 237.1004. Anal. Calcd for $C_{12}H_{15}NO_4$: C, 60.75; H, 6.37; N, 5.90. Found: C, 60.54; H, 6.31; N, 5.88.

Preparation of N-acetyl-O-methyl-serine phenylmethyl ester (**68**).

A solution containing 0.30 g of N-acetylserine phenylmethyl ester (**67**) (1.26 mmol), 0.40 g of silver oxide (1.73 mmol) and 80 mL of dichloromethane was sealed from the atmosphere with a rubber septum. The reaction mixture was stirred and 0.1 mL of iodomethane (1.6 mmol) was added via syringe. Over a two-day period, four aliquots of iodomethane were added (0.5 mL each). The reaction mixture was filtered and

concentrated to an oil. Only 15% conversion to product was observed. The oil was recombined with 2 g of silver oxide (8.65 mmol), 1.50 mL of iodomethane (24.0 mmol) and 2 mL of DMF. After a further 2 h, an additional 0.20 mL of iodomethane (2.5 mmol) were added. After 30 min, the reaction mixture was filtered and concentrated in vacuo.

The desired product was obtained by chromatography on flash silica gel (2 cm by 15 cm) using ether as eluant. Fractions containing product were concentrated to give 160 mg of **68** as white crystals (yield 50%) (mp. 60-61°C). FT-IR (film) 3300, 1740, 1660, 1200 cm^{-1}; NMR ($CDCl_3$, 200 MHz) δ 6.51 (br d, J = 8.0 Hz, 1H, NH), 5.30 (d, J = 12.0 Hz, 1H, ArCH), 5.20 (d, J = 12.0 Hz, 1H, ArCH), 4.8 (m, J_{AV} = 8.0, 3.7 Hz, 1H, CHN), 3.85 (dd, J = 4.0, 11.0 Hz, 1H, $CHOCH_3$), 3.62 (dd, J = 3.5, 11.0 Hz, 1H, $CHOCH_3$), 3.30 (s, 3H, CH_3OCH_2), 2.01 (s, 3H, CH_3). Exact mass calculated for $C_{13}H_{17}NO_4$: 251.1157. Found: 251.1144. Anal. calcd for $C_{13}H_{17}NO_4$: C, [illegible]; H, 6.82; N, [illegible]. Found: C, [illegible]; H, 6.78; N, 5.45.

Preparation of N-acetyl O-methyl serine (56) from **68**.

A solution containing 1[illegible] mg of N-acetyl O-methyl serine phenylmethyl ester (**68**) ([illegible] mmol), 10.0 mg of 5% palladium on charcoal and 1 mL of ethyl acetate was stirred under an atmosphere of [illegible] for 3 min. The

concentrated *in vacuo* to give 70 mg **56** as a white crystalline product (yield 75%) (mp. 109-109.5°C). The product was identical to **56** prepared by acetylation of *O*-methylserine **63**.

Preparation of *N*-(trifluoroacetyl)-serine (**59**) from DL-serine.

Over a 15 minute period, 1.725 g of *N,N,N',N'*-tetramethylguanidine (15.0 mmol) was added dropwise to a solution at 5°C containing 1.05 g of DL-serine (10.0 mmol) in 6.41 g of methyl trifluoroacetate (50.0 mmol). After 1 h, concentrated hydrochloric acid was added dropwise until the reaction mixture became acidic. The solution was diluted with 20 mL of water and the aqueous phase was extracted with 500 mL of ethyl acetate. The organic extracts were combined, dried over magnesium sulphate, filtered and concentrated *in vacuo*. The product was isolated by chromatography on silica gel (35 cm by 3 cm) using 20% acetic acid in ethyl acetate as eluant. Fractions containing product were concentrated *in vacuo* in the presence of toluene to give 1.93 g of **59** as a colourless oil (yield 95%).

FT IR (film) 3100 br, 1750, 1720 cm^{-1}; NMR ($CDCl_3$, 200 MHz) δ 11.7 (br s, 1H, OH) 7.24 (s, 1H, NH), 4.82 (m, 1H, CHN), 4.07 (dd, J = 11.5, 4.0 Hz, 1H, $CHOCH_3$), 4.21 (dd, J = 11.5, 3.5 Hz, 1H, $CHOCH_3$) 2.42 (br s, 1H, OH). Exact mass calculated for $C_5H_6F_3NO_4$: 185.0249. Found: 185.0261

Preparation of N-(trifluoroacetyl)serine (59) from 52.

A solution containing 96.2 mg of N-(trifluoroacetyl)-O-methylserine methyl ester (52) (0.42 mmol) in 8 mL of dry dichloromethane was cooled to 3°C. A 0.8 mL aliquot of 1 M boron tribromide in dichloromethane was added and the mixture was stirred at 5°C for 1.5 h. The reaction was quenched by the addition of 5 mL of aqueous saturated sodium chloride and 30 mL of ethyl acetate. The organic layer was separated and the aqueous layer washed with 30 mL of ethyl acetate. The organic extracts were combined and dried over sodium sulphate. The organic extract was filtered and was concentrated to give 55 mg of crude product which was purified by chromatography on flash silica gel (1 cm by 15 cm) using 30% methanol and 0.25% acetic acid in ether. Fractions containing product were concentrated to give 40 mg of 59 as a light yellow oil (yield 50%) which had identical properties to 59 obtained from DL serine. A small amount (5 mg) of N-(trifluoroacetyl)dehydroalanine (60) was isolated.

For N-(trifluoroacetyl)dehydroalanine (60):

FT-IR (film) 3200, 1715 cm^{-1}; NMR ($CDCl_3$, 200 MHz) 12.0 (br s, 1H, OH), 8.51 (s, 1H, NH), 6.3 (br s, 1H, vinyl H), 6.82 (br s, 1H, vinyl H). Exact mass calculated for $C_5H_4F_3NO_3$ 183.0144, found 183.0132

Preparation of N-(trifluoroacetyl)alanine methyl ester (53) from alanine methyl ester hydrochloride.

A solution containing 502 mg of alanine methyl ester hydrochloride (2.52 mmol) in 25.0 mL of trifluoroacetic anhydride (177 mmol) was refluxed for 1.5 h. After removal of the solvent in vacuo, 710 mg of 77 was obtained as a colourless oil (yield 99%).

FT-IR (film) 3320 br, 1750, 1720, 1160 cm^{-1}; NMR ($CDCl_3$, 80 MHz) δ 7.20 (br s, 1H, NH), 4.6 (br q, J = 8.0 Hz, 1H, CHN), 3.82 (s, 3H, CH_3O_2C), 1.51 (d, J = 8.0 Hz, 3H, CH_3CH). Exact mass calculated for $C_6H_8F_3NO_3$: 199.0456. Found: 199.0469.

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APPENDIX I

STRUCTURAL REPORT

ON THE REFINEMENT OF

$O_5NC_{19}H_{15}$

FOR

J.C. VEDERAS

EXPERIMENTAL

Data Collection

A clear crystal of $O_5NC_{19}H_{15}$ was mounted in a non-specific orientation on an Enraf-Nonius CAD4 automated diffractometer. All intensity measurements were performed using Mo Kα radiation (λ = 0.71073 Å) with a graphite crystal, incident beam monochromator.

The automatic peak search and reflection indexing programs [1] in conjunction with a cell reduction program showed the crystal to be monoclinic and from the systematic absences of

$h0l$, $h+l$ odd.

$0k0$, k odd

the space group was determined to be $P2_1/c$ or an alternative setting of $P2_1/c$ (No. 14) [2].

Cell constants were obtained from a least squares refinement of the setting angles of 18 reflections in the range $12 < 2\theta < 27°$. The various crystal parameters are given in Table 1.

The intensity data were collected at room temperature using an ω-2θ scan ranging in speed from 6.7 to 1.1 deg/min (in omega). The variable scan rate was chosep to give $\sigma(I)/I \leq 0.02$ within a time limit of 75 s in order to achieve improved counting statistics for both intense and weak reflections in a minimum time. The scan range and aperture width were determined as a function of θ to compensate for the α_1-α_2 wavelength dispersion:

$$\text{omega scan width(deg)} = 0.75 + 0.35\tan(\theta)$$

$$\text{apertue width(mm)} = 2.00 + 0.50\tan(\theta)$$

Backgrounds for the peaks were measured by extending the scan 25% on either side of the calculated range to give a peak to background counting time of 2:1. Intensity measurements were made out to a maximum 2θ of 50.00 deg. There were 3 reflections which were chosen as standard reflections and these were remeasured every 60 min of exposure time to check on crystal and electronic stability over the course of data collection. The maximum, minimum and mean change in intensity of these standards over the course of data collection was 4.4, 0.1, and 0.2 %, respectively

Data Reduction

A total of 3254 reflections were collected and these were corrected for Lorentz, polarization and background ef-

fects according to the following formulae:

$$I = SR(SC-R*B)/ Lp$$

$$\sigma^2(I)=\{ SR(SC+R^2B) + (pI)^2\}/ Lp^2$$

where SR is the scan rate, SC is the total scan count, R is the ratio of scan time to background time, B is the total background count, p is a factor to down weight intense reflections (chosen as 0.040 in this experiment) and Lp is the Lorentz and polarization correction term. After rejecting any systematically absent and symmetry equivalent data there were 2936 unique reflections which were used for the structure solution and refinement

Structure Solution and Refinement

The structure was solved using the direct methods program MULTAN 4 which gave the positional parameters for all the non-H atoms

Refinement of atomic parameters was carried out by using full matrix least squares techniques on Fo minimizing the function

$$\Sigma w(|Fo| - |Fc|)^2$$

where |Fo| and |Fc| are the observed and calculated struc-

ture factor amplitudes respectively, and the weighting factor w is given by

$$w=4\underline{F}o^2/\sigma^2(\underline{F}o^2)$$

The neutral atom scattering factors were calculated from the analytical expression for the scattering factor curves [5]. The $\underline{f}'$ and $\underline{f}''$ components of anomalous dispersion [6] were included in the calculations for all non-hydrogen atoms.

The contributions to the structure factors from the fifteen H atoms were included in the calculations with these atoms fixed at their calculated positions assuming an 'ideal' geometry (Csp^2 or Csp^3 hybridization, the non-methyl H's having C-H =0.95 A and the methyl H atoms having C-H =1.05 A) and fixed isotropic thermal parameters of 5.0 A^2

In the final cycle 131 parameters were refined using 1003 observations having $I > 2.0\sigma(I)$. The final agreement factors were

$$R_1 = \Sigma||\underline{F}o|-|\underline{F}c||/\Sigma|\underline{F}o| = 0.056, \text{ and}$$

$$R_2 = (\Sigma w(|\underline{F}o|-|\underline{F}c|)^2/\Sigma w\underline{F}o^2)^{0.5} = 0.066$$

The largest shift in any parameter was 0.02 times its esti-

mated standard deviation and the error in an observation of unit weight was 2.06 e. The highest peak in the final difference Fourier was 0.28(5) eA^{-3}. An analysis of R_2 in terms of F_o, $\lambda^{-1}\sin\theta$, and various combinations of Miller indices showed no unusual trends.

References and Notes

1. The diffractometer programs are those supplied by Enraf-Nonius for operating the CAD4F diffractometer.

2. "International Tables for X-Ray Crystallography", Kynoch Press, Birmingham, England, 1969, Vol. I.

3. The computer programs used in this analysis include the Enraf-Nonius Structure Determination Package by B.A. Frenz ("Computing in Crystallography", Delft University Press, Delft, Holland, 1978, pp. 64-71) and several locally written or modified programs.

4. P. Main, L. Lessinger, M.M. Woolfson, G. Germain and J.P. Declercq, MULTAN 78. A System of Computer Programs for the Automatic Solution of Crystal Structures from X-Ray Diffraction Data.

5. "International Tables for X-Ray Crystallography", Kynoch Press, Birmingham, England, 1974, Vol. IV, Table 2.2B.

6. Ibid, Table 2.3.1.

Table of Experimental Details

A. Crystal Data

$O_5NC_{19}H_{15}$

F.W. = 337.31

Monoclinic space group $P2_1/n$

a = 7.810 (2) b = 24.119 (1) c = 7.071 (2)

β = 105°

V = 1670 Z = 4

D = 1.34 g/cm³

Table of Experimental Details

B. Intensity Measurements

Radiation:	Mo Kα (λ = 0.71073 A)
Monochromator:	Incident beam, graphite crystal
Take-off angle:	2.45 deg
Detector aperture:	2.00 + 0.50tan(θ) mm horizontal
	4.0 mm vertical
Crystal-to-detector distance:	205 mm
Scan type:	ω-2θ
Scan rate:	6.7 - 1.1 deg/min
Scan width:	0.75 + 0.35tan(θ) deg
Data collection 2θ limit:	50.00 deg
Reflections measured:	2936 unique, 1003 with I> 3.0σ(I)
Corrections applied:	None

ble of Positional and Thermal Parameters and Their Estimated Standard Deviations.

tom				U_{11}	U_{22}	U_{33}	U_{12}	U_{13}	U_{23}
1)	30.9(5)	4.8(2)	745.8(6)	55(3)	46(2)	110(4)	12(2)	1(3)	6(3)
2)	-40.6(6)	92.8(2)	530.1(5)	54(3)	46(2)	115(3)	11(2)	13(3)	3(3)
3)	-57.0(5)	93.2(2)	363.4(4)	61(3)	53(3)	54(2)	-9(2)	17(2)	4(2)
4)	-49.6(6)	62.5(2)	971.3(4)	80(3)	58(3)	50(2)	-15(2)	11(2)	-4(2)
5)	206.3(5)	5.5(1)	690.9(4)	60(2)	45(2)	51(2)	-19(2)	13(2)	5(2)
1)	-159.6(9)	96.0(3)	208.8(7)	76(5)	72(4)	49(4)	3(4)	13(3)	4(4)

om				), A2	Atom				U, A2
	2.9(6)	110.4(2)	667.9(5)	42(1)	1(18)	-510.5(8)	-140.7(3)	568.4(7)	64(2)
1)	78.5(8)	188.3(2)	734.1(6)	48(2)	1(19)	-444.0(8)	-87.6(3)	609.8(7)	62(2)
2)	41.3(9)	242.8(3)	755.3(7)	69(2)	HC(2)	266	274	727	63
3)	21(1)	248.9(3)	815.9(8)	76(2)	HC(3)	569	285	832	63
4)	29(1)	205.0(3)	855.4(8)	91(2)	HC(4)	751	212	899	63
5)	59.0(9)	50.5(3)	835.3(7)	68(2)	HC(5)	545	120	863	63
3)	99.4(7)	44.2(2)	772.8(7)	50(2)	HC(10)	-202	40	439	63
1)	84.2(8)	92.5(2)	731.4(7)	49(2)	H1C(11)	-283	106	190	63
9)	3.8(7)	68.5(2)	671.0(6)	49(2)	H2C(11)	-95	103	131	63
9)	-32.8(7)	72.6(2)	617.7(6)	42(1)	H3C(11)	-184	44	182	63
10)	-108.1(7)	65.9(2)	470.1(6)	48(2)	H1C(13)	-362	0	327	63
12)	-94.1(7)	47.0(2)	738.4(6)	43(2)	H2C(13)	-178	-25	899	63
13)	-272.4(8)	-21.1(2)	306.3(7)	54(2)	HC(15)	-223	-116	755	63

Table of Positional and Thermal Parameters and Their Estimated Standard Deviations (continued).

Atom	x	y	z	U, A²	Atom	x	y	z	U, A²
C(14)	-837.8(7)	-77.9(2)	753.5(6)	43(2)	HC(16)	-331	-205	882	63
C(15)	-295.8(8)	-122.0(2)	855.1(7)	56(2)	HC(17)	-509	-219	641	63
C(16)	-360.7(9)	-174.5(3)	811.5(8)	68(2)	HC(18)	-586	-147	470	63
C(17)	-466.6(9)	-182.8(3)	670.1(7)	67(2)	HC(19)	-473	-58	538	63

The atomic positional parameters have been multiplied by 10^3. The anisotropic thermal parameters have been multiplied by 10^3
The isotropic thermal parameters have been multiplied by 10^2.

The form of the anisotropic thermal parameter is:
$\exp[-2\pi^2(h^2a^{*2}U_{11} + k^2b^{*2}U_{22} + l^2c^{*2}U_{33} + 2hka^*b^*U_{12} + 2hla^*c^*U_{13} + 2klb^*c^*U_{23})]$

Estimated standard deviations in the least significant digits are shown in parentheses. Those parameters without an error were not refined.

Table of Anisotropic and Equivalent Isotropic Thermal Parameters

Atom	U_{11}	U_{22}	U_{33}	U_{12}	U_{13}	U_{23}	Bequiv
O(1)	55(3)	46(2)	110(4)	12(2)	1(3)	6(3)	5.9(1)
O(2)	54(3)	46(2)	115(3)	11(2)	13(3)	3(3)	5.8(1)
O(3)	61(3)	63(3)	54(2)	-9(2)	17(2)	4(2)	4.6(1)
O(4)	80(3)	58(3)	50(2)	-15(2)	11(2)	-4(2)	5.0(1)
O(5)	60(2)	45(2)	51(2)	-19(2)	13(2)	5(2)	4.1(1)
C(11)	76(5)	72(4)	49(4)	3(4)	13(3)	4(4)	5.2(2)

The anisotropic thermal parameters have been multiplied by 10^3.

The form of the anisotropic thermal parameter is:
$\exp[-2\pi^2(h^2a^{*2}U_{11} + k^2b^{*2}U_{22} + l^2c^{*2}U_{33} + 2hka^*b^*U_{12} + 2hla^*c^*U_{13} + 2klb^*c^*U_{23})]$

Estimated standard deviations in the least significant digits are shown in parentheses. Those parameters without an error were not refined.

Table of Root-Mean-Square Amplitudes of Thermal Vibration in Angstroms.

Atom	Min.	Int'med.	Max.	Atom	Min.	Int'med.	Max.
O(1)	0.190	0.249	0.355	C(11)	0.219	0.269	0.281
O(2)	0.196	0.248	0.348	O(4)	0.215	0.236	0.300
O(3)	0.218	0.239	0.269	O(5)	0.171	0.226	0.276

Table of Selected Bond Distances (A)

C(1)	C(2)	1.399 (6)	C(9)	C(12)	1.452 (6)
C(1)	C(6)	1.364 (6)	C(10)	O(3)	1.323 (5)
C(1)	C(8)	1.496 (6)	O(3)	C(11)	1.431 (5)
C(2)	C(3)	1.388 (7)	C(12)	O(4)	1.223 (5)
C(3)	C(4)	1.347 (7)	C(12)	O(5)	1.330 (5)
C(4)	C(5)	1.395 (6)	O(5)	C(13)	1.444 (5)
C(5)	C(6)	1.389 (6)	C(13)	C(14)	1.496 (5)
C(6)	C(7)	1.488 (6)	C(14)	C(15)	1.390 (5)
C(7)	N	1.390 (5)	C(14)	C(19)	1.369 (6)
C(7)	O(1)	1.206 (5)	C(15)	C(16)	1.385 (6)
N	C(8)	1.411 (5)	C(16)	C(17)	1.347 (6)
N	C(9)	1.442 (5)	C(17)	C(18)	1.356 (6)
C(8)	O(2)	1.183 (5)	C(18)	C(19)	1.396 (6)
C(9)	C(10)	1.324 (5)			

Table of Selected Bond Angles (deg)

C(2)	C(1)	C(6)	121.2 (4)	N	C(9)	C(10)	120.0 (4)
C(2)	C(1)	C(8)	128.8 (4)	N	C(9)	C(12)	115.5 (4)
C(6)	C(1)	C(8)	110.0 (4)	C(10)	C(9)	C(12)	124.3 (4)
C(1)	C(2)	C(3)	116.3 (5)	C(9)	C(10)	O(3)	122.7 (4)
C(2)	C(3)	C(4)	122.1 (5)	C(10)	O(3)	C(11)	117.1 (4)
C(3)	C(4)	C(5)	122.4 (5)	C(9)	C(12)	O(4)	123.4 (4)
C(4)	C(5)	C(6)	115.7 (5)	C(9)	C(12)	O(5)	113.9 (4)
C(1)	C(6)	C(5)	122.4 (4)	O(4)	C(12)	O(5)	122.7 (4)
C(1)	C(6)	C(7)	108.1 (4)	C(12)	O(5)	C(13)	116.1 (3)
C(5)	C(6)	C(7)	129.5 (4)	O(5)	C(13)	C(14)	109.6 (4)
C(6)	C(7)	N	105.2 (4)	C(13)	C(14)	C(15)	119.1 (4)
C(6)	C(7)	O(1)	129.6 (5)	C(13)	C(14)	C(19)	122.2 (4)
N	C(7)	O(1)	125.2 (4)	C(15)	C(14)	C(19)	118.6 (4)
C(7)	N	C(8)	113.4 (4)	C(14)	C(15)	C(16)	120.5 (4)
C(7)	N	C(9)	122.8 (3)	C(15)	C(16)	C(17)	119.7 (5)
C(8)	N	C(9)	123.5 (4)	C(16)	C(17)	C(18)	121.2 (5)
C(1)	C(8)	N	103.1 (4)	C(17)	C(18)	C(19)	119.7 (5)
C(1)	C(8)	O(2)	131.5 (4)	C(14)	C(19)	C(18)	120.3 (4)
N	C(8)	O(2)	125.4 (4)				

Table of Selected Torsional Angles

Atom 1	Atom 2	Atom 3	Atom 4	Angle
C(9)	C(10)	O(3)	C(11)	-174.4
C(10)	C(9)	C(12)	O(4)	-163.2
C(7)	N	C(9)	C(10)	-108.7
C(8)	N	C(9)	C(10)	77.5

Table of Weighted Least-Squares Planes

The equation of the plane is of the form: $Ax + By + Cz - D = 0$

where A, B, C & D are constants and x, y & z are orthogonalized coordinates

Plane No.	A	B	C	D
1	-0.6897	0.7176	-0.0966	2.0424
2	0.4093	0.0332	-0.9118	-5.5659
3	-0.7422	0.6437	-0.1863	1.4273
4	0.9228	-0.2028	-0.3277	-5.9515

Atom	x	y	z	Distance	Esd
Atoms in Plane					
C(9)	-1.7759	1.7516	5.4090	-0.083	0.006
C(10)	-2.0095	1.5898	4.1163	0.087	0.006
C(12)	-2.5573	1.1336	6.4655	-0.090	0.006
O(4)	-2.5318	1.5083	7.6294	0.049	0.004
Other Atoms					
O(3)	-1.3432	2.2487	3.1819	0.190	0.004
N	-0.7468	2.6618	5.8485	-0.182	0.005
O(5)	-3.3283	0.1333	6.0494	-0.236	0.004
Atoms in Plane					
C(1)	0.4002	4.5420	6.4283	0.019	0.006
C(6)	1.1824	3.4771	6.7669	-0.005	0.006
C(7)	0.4527	2.2321	6.4039	-0.014	0.006
N	-0.7468	2.6618	5.8485	0.016	0.004
C(8)	-0.9054	4.0632	5.8758	-0.028	0.006
Other Atoms					
C(2)	0.8455	5.8558	6.6132	0.076	0.007
C(3)	2.1189	6.0037	7.1443	0.118	0.007
C(4)	2.8775	4.9453	7.4900	0.078	0.007
C(5)	2.4498	3.6295	7.3141	0.020	0.007
O(1)	0.7863	1.0800	6.5300	-0.031	0.005
O(2)	-1.8680	4.6503	5.5175	-0.075	0.005
C(9)	-1.7759	1.7516	5.4090	-0.035	0.005
Atoms in Plane					
C(12)	-2.5573	1.1336	6.4655	-0.004	0.006
O(4)	-2.5318	1.5083	7.6294	0.001	0.004
C(13)	-4.1346	-0.5094	7.0606	-0.002	0.006
O(5)	-3.3283	0.1333	6.0494	0.002	0.004
Other Atoms					
C(9)	-1.7759	1.7516	5.4090	0.010	0.006
C(10)	-2.0095	1.5898	4.1163	0.321	0.006
C(14)	-4.5222	-1.8777	6.5976	-0.509	0.006
Atoms in Plane					
C(14)	-4.5222	-1.8777	6.5976	-0.003	0.006
C(15)	-4.4394	-2.9420	7.4876	-0.002	0.007
C(16)	-4.8456	-4.2100	7.1061	0.005	0.007
C(17)	-5.3361	-4.4100	5.8675	-0.001	0.007
C(18)	-5.4334	-3.3924	4.9769	-0.005	0.007
C(19)	-5.0094	-2.1124	5.3396	0.008	0.007
Other Atoms					
C(13)	-4.1346	-0.5094	7.0606	-0.074	0.007

Chi-Squared Values

Plane No	Chi-Squared
1	814
2	53
3	1
4	3

Dihedral Angles Between Planes:

Plane No	Plane No	Dihedral Angle
1	2	99.8
1	3	7.3
1	4	138.6
2	3	96.5
2	4	48.0
3	4	139.0

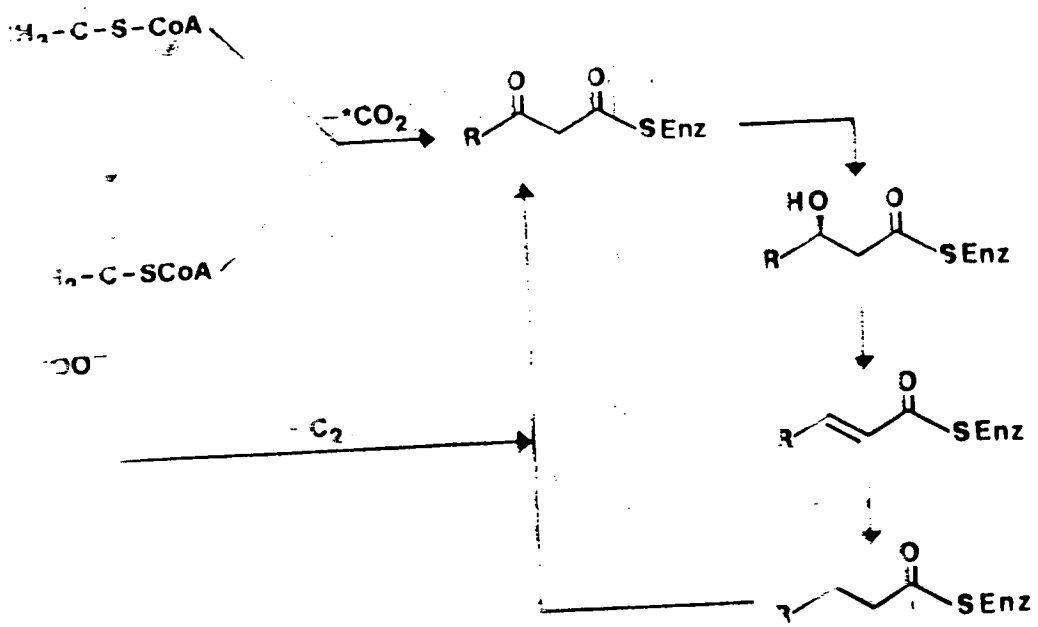
PART 2.

THE STUDY OF BIOSYNTHESIS USING STABLE ISOTOPE NMR TECHNIQUES

INTRODUCTION

Metabolism produces a wide variety of natural products. The biochemical origin of these materials and the mechanisms by which they are transformed are the major objectives of biosynthetic study.[1] Primary metabolites are natural products ubiquitous in nature whereas secondary metabolites are compounds found to occur in only a limited number of organisms.[2] Secondary metabolites can be divided into several groups on the basis of their biological origin. Polyketides and fatty acids are derived from acetyl-CoA metabolism by related pathways.

Lynen and Cornforth showed that fatty acids are produced by the continuous linkage of a two-carbon unit from acetyl - CoA.[3] Fatty acid biosynthesis occurs on the multisubunit enzyme complex fatty acid synthetase (eqn. 1). Initially, acetyl-CoA is carboxylated by carbon dioxide to form malonyl-CoA, a thioester anion equivalent, which condenses with a two carbon fragment derived from another unit of acetyl-CoA with the loss of carbon dioxide to form a β-ketobutyryl thioester. The original "acetate" units from acetyl CoA are thereby linked in a head to tail manner. The β-keto group is reduced to the R hydroxy derivative. Syn-elimination of water occurs followed by Michael addition of a hydride from NADPH to the unsaturated system. The

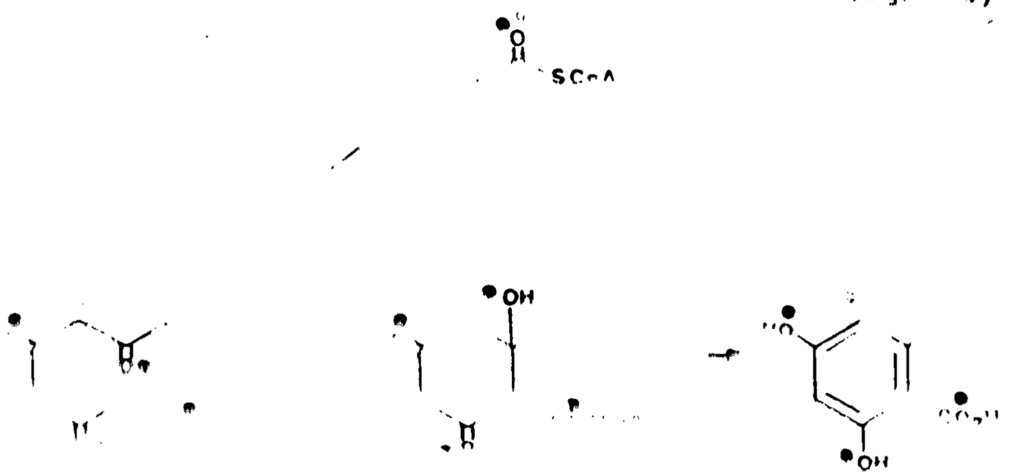

-C-S-CoA
-CO2
R
SEnz
HO
-C-SCoA
-C2

product is an enzyme-bound saturated thioester. The cycle of reactions is repeated with the continuous addition of "acetate" units by further condensation of malonyl-CoA onto the growing chain. Commonly seven or eight "acetate" units from malonyl-CoA are attached to the starter unit (derived directly from acetyl CoA) before the fatty acid (C_{16} or C_{18}) is released from the enzyme complex.

In the 1950's Birch expanded the hypothesis of Collie (1893) and proposed that polyketides are formed by a process analogous to that for fatty acids.[4,5] Failure of complete reduction before the next two carbon fragment is added can result in incorporation of carbonyl, hydroxyl or double bond functionality into the growing chain. The functionalized chain can then react further. Little direct evidence for such intermediates was present since these species are enzyme bound and transitory.[2] A consequence of the Birch-Collie hypothesis is that the carbon-oxygen bonds of acetate would be expected to be incorporated directly into the final metabolites. The intact ratio of carbon and oxygen labels derived from acetate [illegible] acid has been demonstrated (eqn. 2).[7,8]

This thesis presents results which demonstrate the use of high field n.m.r. to provide evidence for the Birch [illegible] problems.

(eqn. 2)



orsellinic acid

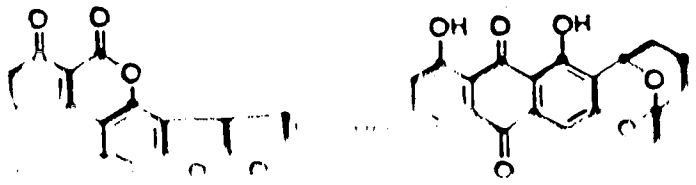
Oxygen-18 Isotope Shifts in ^{13}C NMR Studies with Acetals

Two oxygen isotopes are commonly available, oxygen-18 and oxygen-17. Both ... spectroscopy. Although ... labelling ... to define ... position ... highly ... labelling.

... 17 ... indicated; however, it ... greatly restricted ... quadrupolar ... nucleus ...

Oxygen-18 has a nuclear spin of zero and cannot be observed directly by n.m.r. However, oxygen-18 isotopic substitution has been found to cause an upfield shift in the ^{13}C-n.m.r. signals[8] of directly attached carbon atoms in agreement with theoretical predictions.[9] An oxygen-18 isotope shift has been detected with other nuclei,[10] but the small size of this carbon-oxygen shift (0.01 to 0.06 ppm) meant that it was unobservable until the development of high field n.m.r. spectrometers. This isotope shift provides an easy non-degradative method for determining the location and extent of oxygen label in complex oxygenated compounds.[11] This method has been used in metabolic and mechanistic studies[12] and as a structural probe.[13]

Since the acetal structure is found in many natural products such as aflatoxin B_1[14] and averufin[15], (eqn. 3)



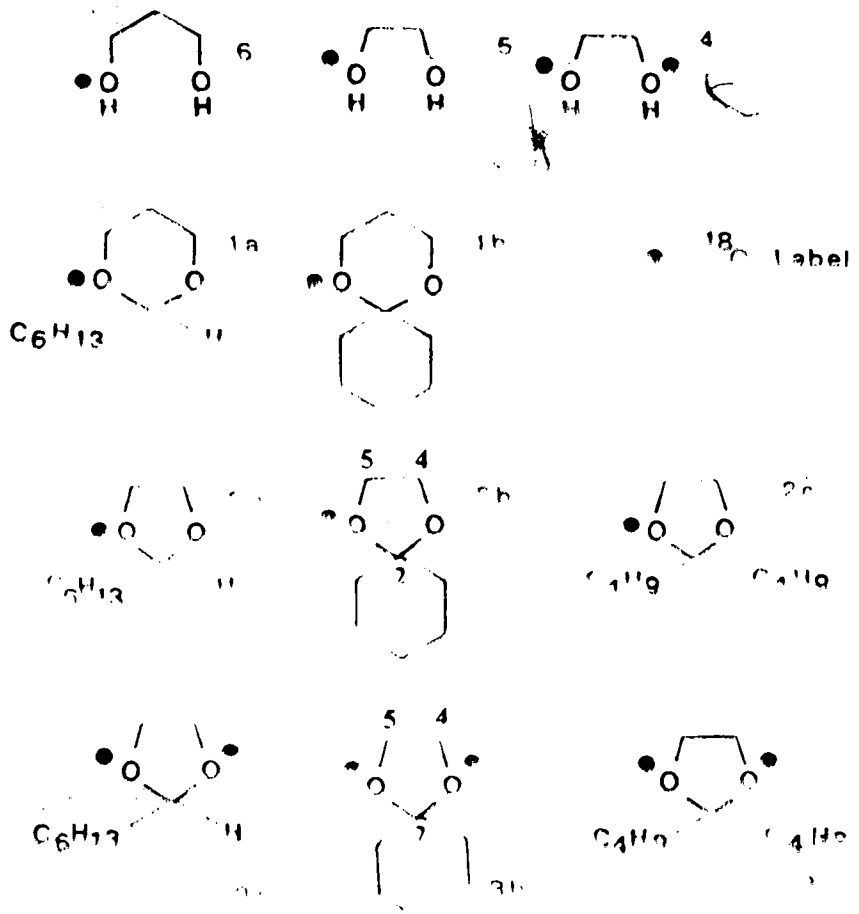
AFLATOXIN B_1 AVERUFIN

(eqn. 3)

the effect of multiple oxygen 18 substitution on the isotope shift in acetal systems was examined.

[illegible] were prepared by small scale [illegible] 5-nonanone and

cyclohexanone.[16] The required dilabelled ethylene glycol (**4**) was prepared by exchange of oxalic acid with [^{18}O] water[17] followed by reduction of the dried acid with diborane.[18] Monolabelled ethylene glycol (**5**) was obtained by acidic hydrolysis of ethylene oxide in the presence of oxygen-18 water.[19] The monolabelled 1,3-propanediol (**6**) was generated by the displacement of 1-bromo-3-hydroxy-propane with oxygen-18 labelled sodium acetate.[20] Hydrolysis yielded the diol.[20]



The isotope shifts were measured by observation of ^{13}C signals at 100 MHz. These results are listed in Table 1.

TABLE 1. ISOTOPE SHIFTS OF OXYGEN-18 LABELLED ACETALS

	Isotope Shift[b] ppm x 100			
Compound No.	C(2)	C(4)	C(5)	C(6)
(1a)	2.1	-	-	2.0
(1b)	2.6	-	-	2.2
(2a)	2.3	0.8	2.0	-
(2b)	2.8	0.7	2.3	-
(2c)	2.7	0.6	2.1	-
(3a)	4.6	2.8	c	-
(3b)	5.5	2.9	c	-
(3c)	5.5	2.8	c	-

[a]Shifts are ± 0.1 (ppm x 100). [b]All shifts are upfield. [c]C(4) and C(5) are identical because of symmetry. Ratios were confirmed by mass spectroscopy.

The results indicate that replacement of a hydrogen by an alkyl group at the acetal carbon causes an increase in the magnitude of the isotope shift. This has been observed with alcohols and esters.[8i,8d,8h]

Risley and Van Etten have shown that for orthocarbonates and orthoesters sequential replacement of ^{16}O-atoms by ^{18}O-atoms causes the isotope shift to increase in an additive manner (i.e. an orthoester containing two ^{18}O-atoms and one ^{16}O-atom will show an isotope shift at the central carbon that is twice as large as the similar orthoester containing one ^{18}O-atom and two ^{16}O-atoms).[8d] The present study shows that this additivity is also seen in acetals. The shifts at C(2) of the dilabelled acetal compounds (**3a-c**) are twice the isotope shifts that are observed in the monolabelled compounds (**2a-c**) (Fig. 1).

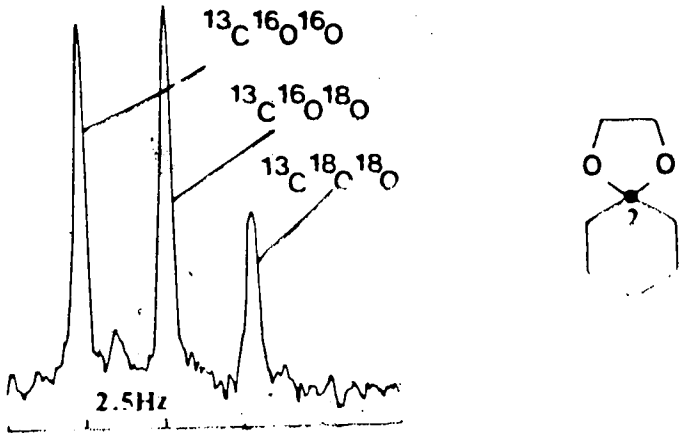


Fig. 1. 100 MHz ^{13}C n.m.r. spectrum of C(2) of a mixture of un-, mono- and di-[^{18}O]labelled acetal of cyclohexanone (**2b** and **3b**).

These shifts are referred to as alpha shifts because the isotope is directly attached to the carbon atom observed in the ^{13}C-n.m.r. spectrum. Isotope shifts are also induced through two bonds at the carbon atom beta to the isotope substitution.[8h,i] This effect is normally obscured except in favourable cases.[8i] The isotope shifts observed for C(4) and C(5) of acetals **2b** and **3b** demonstrate this effect (Fig. 2).

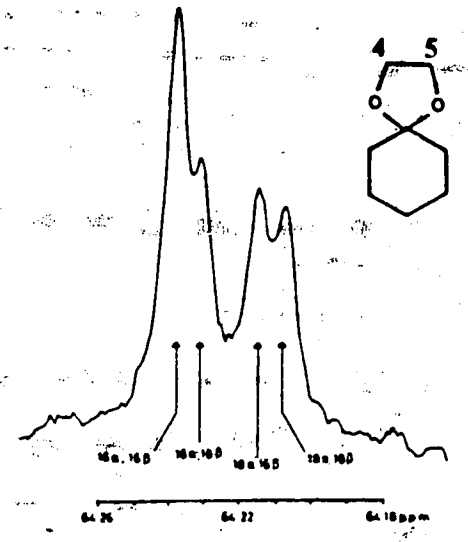


Fig. 2. 100 MHz ^{13}C-n.m.r. spectrum for C(4) and C(5) in a mixture of **2b**, **3b** and unlabelled compound showing β-shifts.

Four peaks are observed at 64 ppm in a mixture of **2b**, **3b** and unlabelled compound. The large intense downfield signal is due to completely unlabelled material; the next upfield signal is due to a beta isotope shift (^{13}C-C-^{18}O); the next signal is an alpha shift due to compound **2b** where the

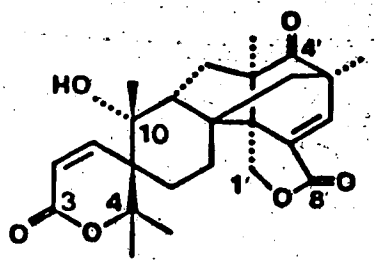
isotope is directly attached to the observed carbon (^{13}C-^{18}O); and finally the most upfield peak is due to the combination of an alpha and a beta shift on the observed carbon from the disubstituted material **3b** (^{18}O-^{13}C-C-^{18}O). It can be seen from the data that beta shifts are additive in the same manner as the alpha shifts.

This work also supports the observation that a lone pair of electrons adjacent to the oxygen-18 substituted carbon reduces the isotope shift.[8d,8h] If this lone pair is conjugated with an electron-withdrawing group an increase in isotope shift is observed.

The use of this technique in biosynthetic studies for the determination of oxygen labelling in secondary metabolites will now be described.

BIOSYNTHETIC STUDIES ON ANDIBENIN B.[21]

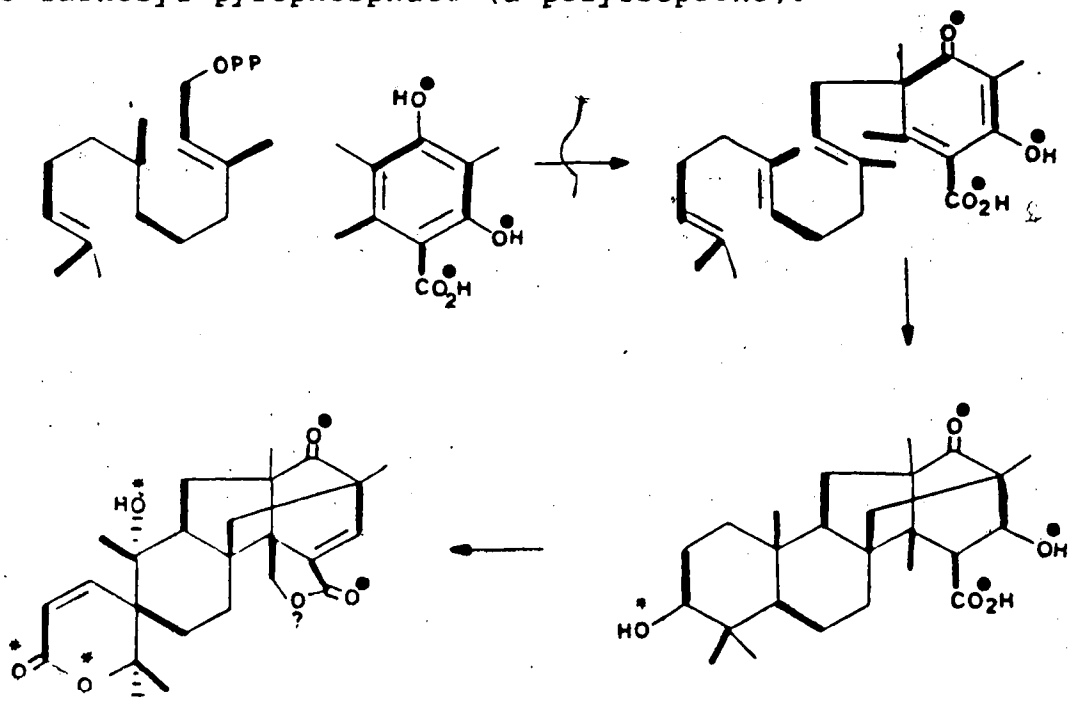
Andibenin B (eqn. 4) is a major fungal metabolite produced by Aspergillus variecolor.[22] The origin of the carbon atoms from acetate and the S-methyl group of methionine has been determined by Holker and Simpson.[22] The arrangement of intact acetate units incorporated into this metabolite was established by incorporation of doubly labelled [1,2-$^{13}C_2$]acetate. This provided evidence for the unusual mixed biosynthesis (eqn.5). Dimethyl orsellinic acid has also been shown to be a precursor.



ANDIBENIN B

(eqn. 4)

It is proposed that the initial condensation takes place between dimethyl orsellinic acid (a polyketide bearing two methyl groups from methionine) and a sesquiterpene, such as farnesyl pyrophosphate (a polyisoprene).[22a]



(eqn. 5)

● O from Acetyl CoA

✱ O from Atmospheric oxygen

The initially condensed product can undergo further oxidation and rearrangement to yield andibenin B. The partial isoprene origin accounts for its steroidal-type appearance.

Oxygen labelling studies can provide a clearer understanding of the mechanistic details involved in the formation of the unusual ring system of andibenin B, and confirm its mixed origin. Since ^{13}C-n.m.r. spectroscopy allows rapid identification of labelled sites in small quantities of metabolites,[23,32] its use in the location of oxygen-18 by induced isotope shift appeared to be an ideal technique.

Andibenin B was produced from A. variecolor in the presence of sodium [1-^{13}C, $^{18}O_2$]acetate. In a separate experiment the fungus was grown in an atmosphere of oxygen-18 gas with an unlabelled carbon source. The results are listed in Table 2.

On the basis of the proposed biosynthesis (eqn. 5), oxygen label from acetate would incorporate into dimethyl orsellinic acid. No incorporation from acetate would be possible into the reduced polyisoprene. Since the extent of acetate incorporation is low (3%), any cleavage of oxygen-18 from labelled precursor will dilute the label and an isotope shift would not be detectable (due to low intensity). This implies that for an "acetate" isotope shift to be observed the carbon-oxygen bond must remain intact throughout the biosynthesis. The results show that the retention of label from sodium [1-^{13}C, $^{18}O_2$]acetate throughout the remaining

TABLE 2

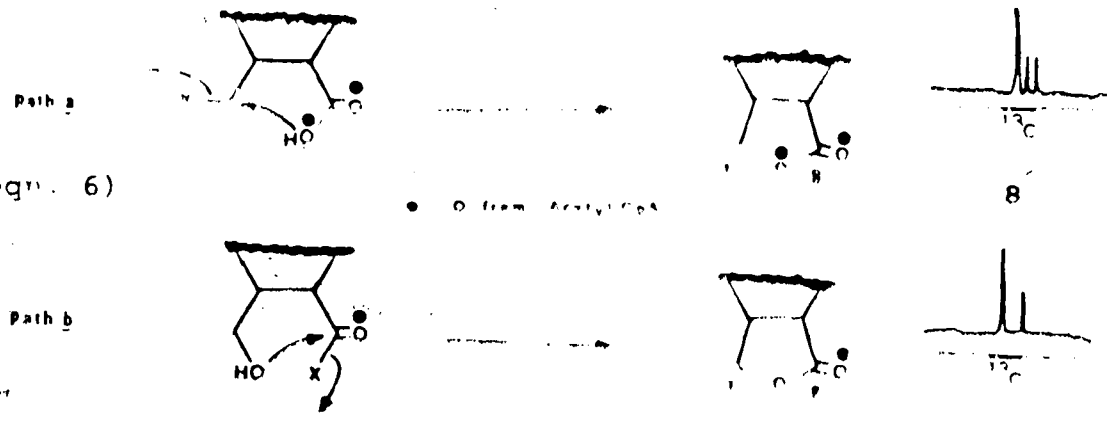
Isotope Shifts of andibenin B after incorporation of sodium [1-^{13}C, $^{18}O_2$] acetate and [$^{18}O_2$] gas.

Carbon No.	Shift (ppm)	Isotope Shift (ppm x 100)	Isotope Ratio $^{16}O:^{18}O$
3*	164.2	5.0*	32:68
4*	85.3	4.2*	34:66
10*	77.2	3.2*	32:68
1'*	68.7	2.9*	26:74
4'	213.9	5.0	73:17
8'	167.4	1.6;3.5	85:7.5:8
8'*	167.4	3.5*	32:68

*Labelled by $^{18}O_2$ gas.

steps gives oxygen-18 at C-4' and at both oxygens attached to C-8'. These results provide evidence for the Birch hypothesis since oxygen label from acetate is introduced into the final metabolite. Oxygen-18 originating from

atmospheric oxidation is found at C-3, C-4, C-10, C-8' and C-1' (the lactone ring oxygen). The alkoxy oxygen in the five-membered lactone ring could arise from acetate by attack of carboxylate on a leaving group at C-1' (path a) (eqn. 6). Alternatively, aerobic oxidation at C-1' followed by lactonization involving attack of the C-1' oxygen onto the carboxylate carbonyl would give an alkoxy oxygen in the lactone ring arising from the atmosphere (path b). If path a operates two isotope shifts would be observed at C-8' after [$^{18}O_2$]acetate incorporation, a large shift from molecules containing label in the carbonyl oxygen and a smaller shift from molecules incorporating label into the alkoxy carbon. If path b operates an isotope shift would be observed at C-1' and C-8' after the oxygen-18 gas experiment. The results indicate both paths are operative. Because the level of oxygen-18 at C-1' is comparable to that at other labelled sites, it appears that the major portion of andibenin B is formed by path b, and a minor amount may be formed by ring opening and lactonization by path a.



(eqn. 6)

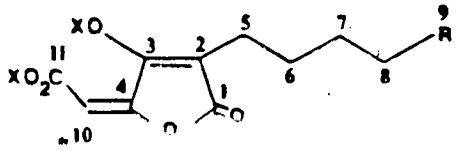
8

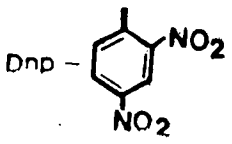
Another example of the use of oxygen labelling to determine the mechanism of ring closure is considered in the next section.

BIOSYNTHESIS OF TETRONIC ACID DERIVATIVES.[25]

The tetronic acid derivatives, multicolanic (**7**), multicolosic (**8**) and multicolic (**9**) acids, were isolated in 1974 from strains of Penicillium multicolor.[26]

Carbon labelling studies gave the origin of all the carbon atoms present in these metabolites.[26a] It was proposed that a hexaketide chain cyclizes to a resorcinol derivative (eqn 7).[26a]

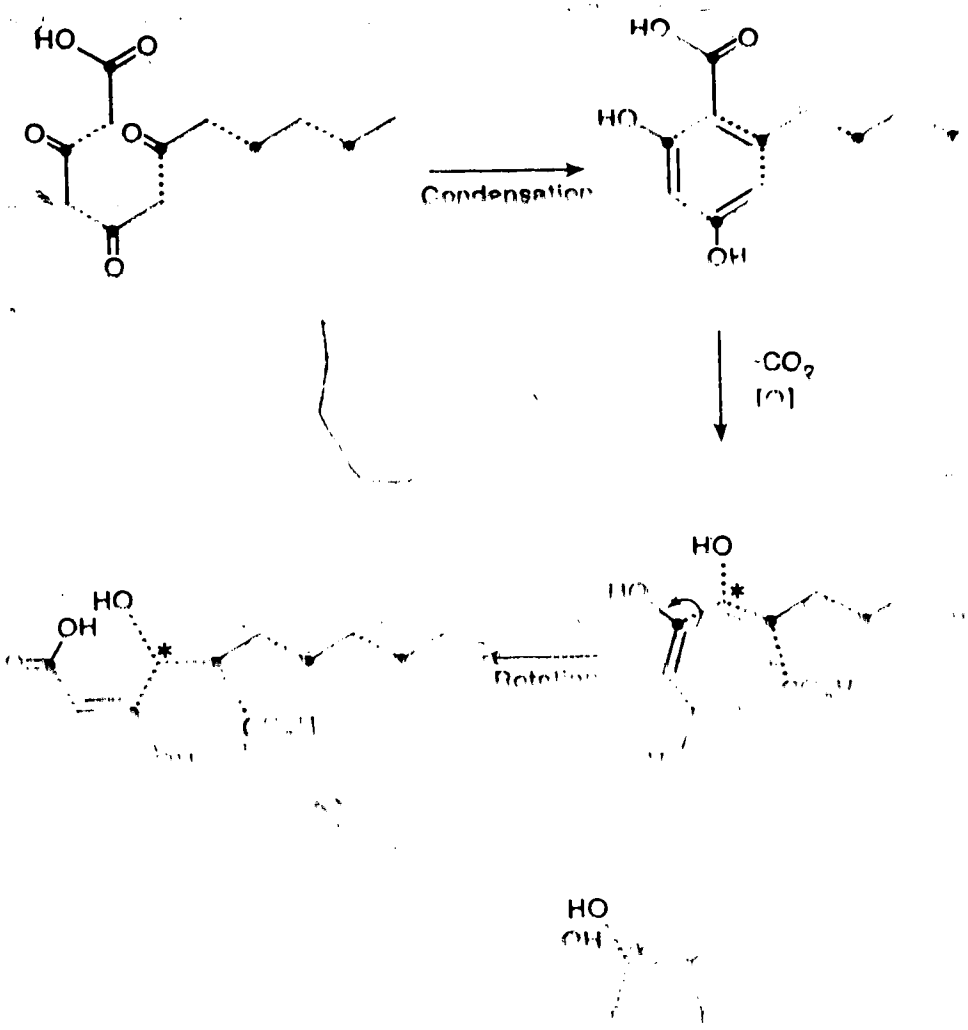




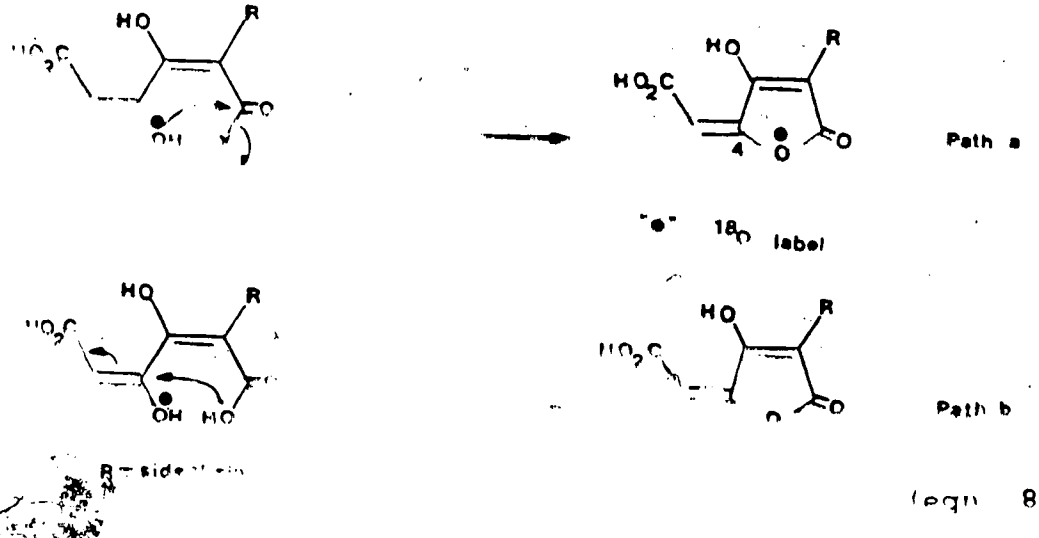
	X	R
7	H	Me
8	H	CO_2H
9	H	CH_2OH

	X	R
10	Me	Me
11	Me	CO_2Me
12	Me	CH_2OH
13	Me	CH_2O Dnp

This aromatic compound apparently undergoes oxidative decarboxylation and ring cleavage to a diacid. Rotation of the diacid and ring closure produces the tetronic acids.


HO
O
O
O
O
Condensation
HO
HO
O
OH
-CO2
[O]
HO
OH
HO
HO

The ring closure by one of two routes seemed likely (eqn. 8). Path a implicates a displacement on an activated carboxyl by an enol oxygen. This pathway leaves the C-4 oxygen bond intact. Since C-4 of the tetronic acid is derived from C-1 of acetate, the incorporation of sodium [1-^{13}C, $^{18}O_2$]acetate would give rise to an isotope shift at the ^{13}C n.m.r. signal of C-4. This assumes that acetate oxygens would be incorporated in agreement with the Birch hypothesis.



(eqn 8)

Alternatively, ring closure could occur by path b. This would result in cleavage of the acetate derived C-4 carbon-oxygen bond. No isotope shift in the ^{13}C n.m.r. spectrum would be observed [illegible] incorporation of [1-^{13}C, $^{18}O_2$]acetate.

Tetronic acids obtained from fermentation [illegible] sodium

TABLE 3

Isotope Shift Results of Methyl O-methylmulticolanate (**10**), Dimethyl O-methylmulticolosate (**11**) and Methyl O-methylmulticolate 2,4 dinitrophenyl ether (**13**).

	No.	Shift (ppm)	Isotope Shift (ppm x 100)	Isotope Ratio $^{16}O:^{18}O$
(10)	1	161.1		-
	3	168.8		
	4	151.1	1.9	59:41
	1	164.6	[illegible]	[illegible]
	1	160.[illegible]		
	3	168.1		
	4	150.4		
	1	172.9		
	1	164.3	[illegible]	
	1	161.5		
	3	168.8		
	1	[illegible]		
		4		
		6		

[1-^{13}C, $^{18}O_2$]acetate were methylated to form **10**, **11** and **12** which are more easily purified.

The ^{13}C-n.m.r. data was obtained on methyl derivatives **10** and **11**; however, pure methyl O-methylmulticolate (**12**) could not be obtained for analysis. This compound was converted to its 2,4-dinitrophenyl ether **13** by treatment with Sanger's reagent (2,4-dinitrofluorobenzene).[27] Compound **13** was purified on preparative layer chromatography before spectral analysis.

In addition to this problem, the isotope shifts were obscured by the presence of long range carbon-carbon coupling (2-bond) caused by multiple carbon-13 enrichment into the same molecule.[11b,11c] The coupled signals were reduced to the base line by applying the spin echo Fourier transform technique (SEFT) which inverts uncoupled singlets, but phase modulates the coupled signals.[11b,c,28] This allowed the isotope shifts (Fig. 3) to be measured and observed without interference. These results (Table 3) indicate that the C-4 carbon-oxygen bond remains intact through all steps of the biosynthesis from acetate to the tetronic acid derivatives **7**, **8** and **9**. Therefore ring closure must occur by a mechanism resembling path a (enol attack on the carbonyl oxygen) rather than path b. In addition, the labelling pattern is consistent with the Birch hypothesis[4] and the proposed general biosynthetic sequence

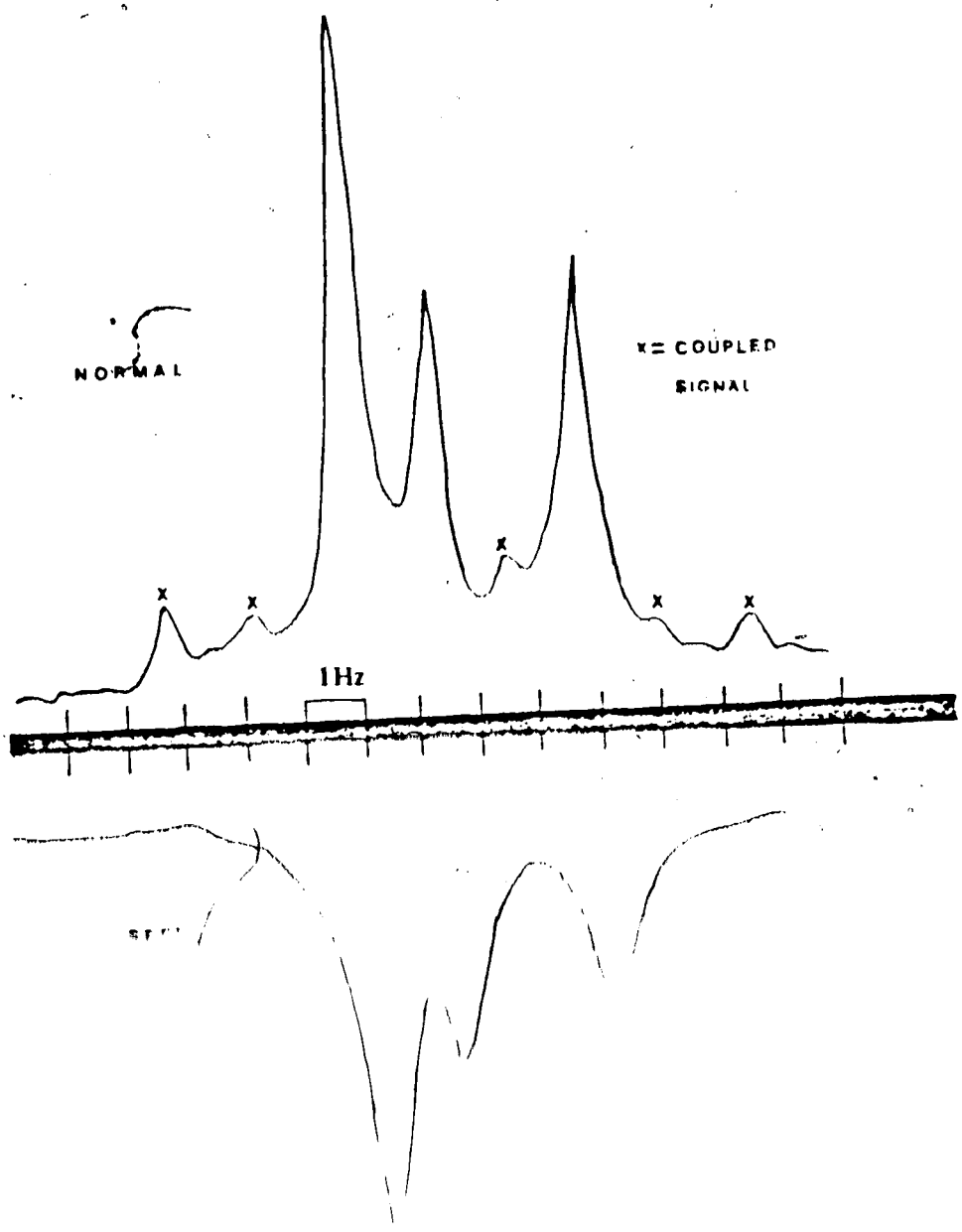
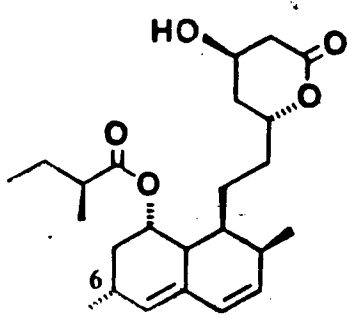


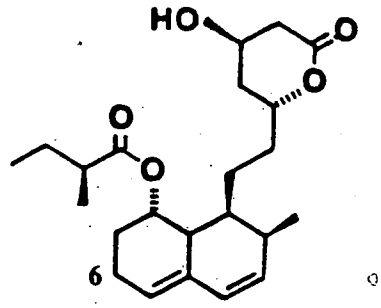
Figure 3: 100 MHz ^{13}C-n.m.r. spectrum of methyl O-methyl multicolanate (11) at 161.6 ppm (C-11), in normal acquisition mode and FNT acquisition mode

BIOSYNTHESIS OF MEVINOLIN.

Recent reports have appeared on the isolation of the tricyclic compounds, mevinolin (13),[29] compactin (14)[30] and the 4a,5-dihydro derivatives.[31]



Mevinolin

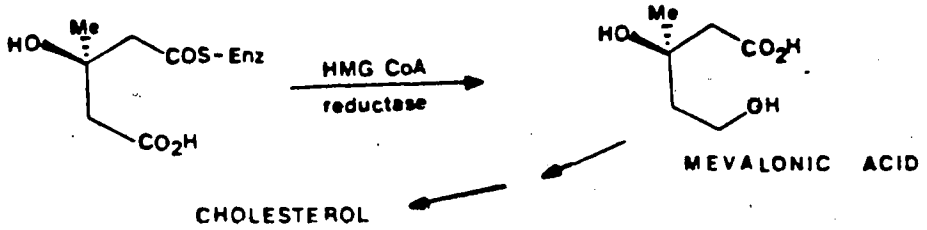


Compactin

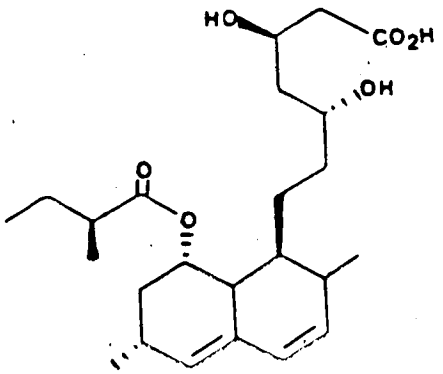
Mevinolin is a fungal metabolite isolated from Aspergillus terreus[32] and is similar to compactin, except that it contains a methyl group at the 6-position. Both the lactone ring form and the open hydroxy acid form are produced by A. terreus. These metabolites have been isolated as part of screening for compounds useful in the treatment of heart disease.[33,34] Mevinolin is very potent in lowering the plasma cholesterol level[33,34] in mammals, including man (hypocholesterolemic agent). The presence of the methyl group at C-6 increases the potency by a factor of three.[33]

These compounds competitively inhibit 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase, , EC

1.1.1.34), which is the rate controlling enzyme in the *in vivo* synthesis of cholesterol. Since the enzyme converts 3-hydroxy-3-methylglutaryl-CoA to mevalonic acid, the inhibition may occur because the open hydroxy acid of mevinolin is a structural analogue of mevalonic acid (eqn. 9). These compounds also inhibit production of other isoprene-derived natural products.[35]



MEVINOLIN IN FREE ACID FORM



(eqn. 9)

Although these compounds may not prove to be clinically useful, they have been increasingly used to study the role of HMG-CoA reductase in cholesterol regulation.[33,34,36]

The search for more active compounds has resulted in synthetic approaches[37] to these molecules and four total syntheses of compactin have been reported.[38] Structural modifications have also been studied.[39]

The unusual bicyclic ring system attracted our attention from a biosynthetic perspective.[40]

A complete assignment of the ^{1}H-n.m.r. and ^{13}C-n.m.r. spectra is required to carry out a biosynthetic study using stable isotopes. The proton n.m.r. spectrum of mevinolin was therefore assigned using homonuclear decoupling and chemical shift analogy.[41] The high field region (1.0 to 3.5 ppm) contains a large number of overlapping signals (Fig. 4). This complexity in the proton n.m.r. is apparent in the ^{13}C-spectrum (Fig. 5). The methyl, methylene and methine region has a high concentration of carbon signals in the 10 to 50 ppm range.

The multiplicities of the carbon signals (Fig. 6) were determined using a CARPPET pulse sequence.[42] Selective heteronuclear decoupling of protons gave unambiguous assignments to the low field vinyl and alkoxy carbon signals, but a definite assignment could not be obtained for the high field region.

A complete assignment was made by obtaining a 2D-INADEQUATE spectrum.[43] This spectrum gives rise to signals based on adjacent ^{13}C-nuclei (adjacent ^{13}C nuclei have identical double quantum transitions). Using this method the chemical shift of all unassigned carbons could be obtained because of the adjacent nature of unassigned carbons to assigned carbons. The major drawback with this

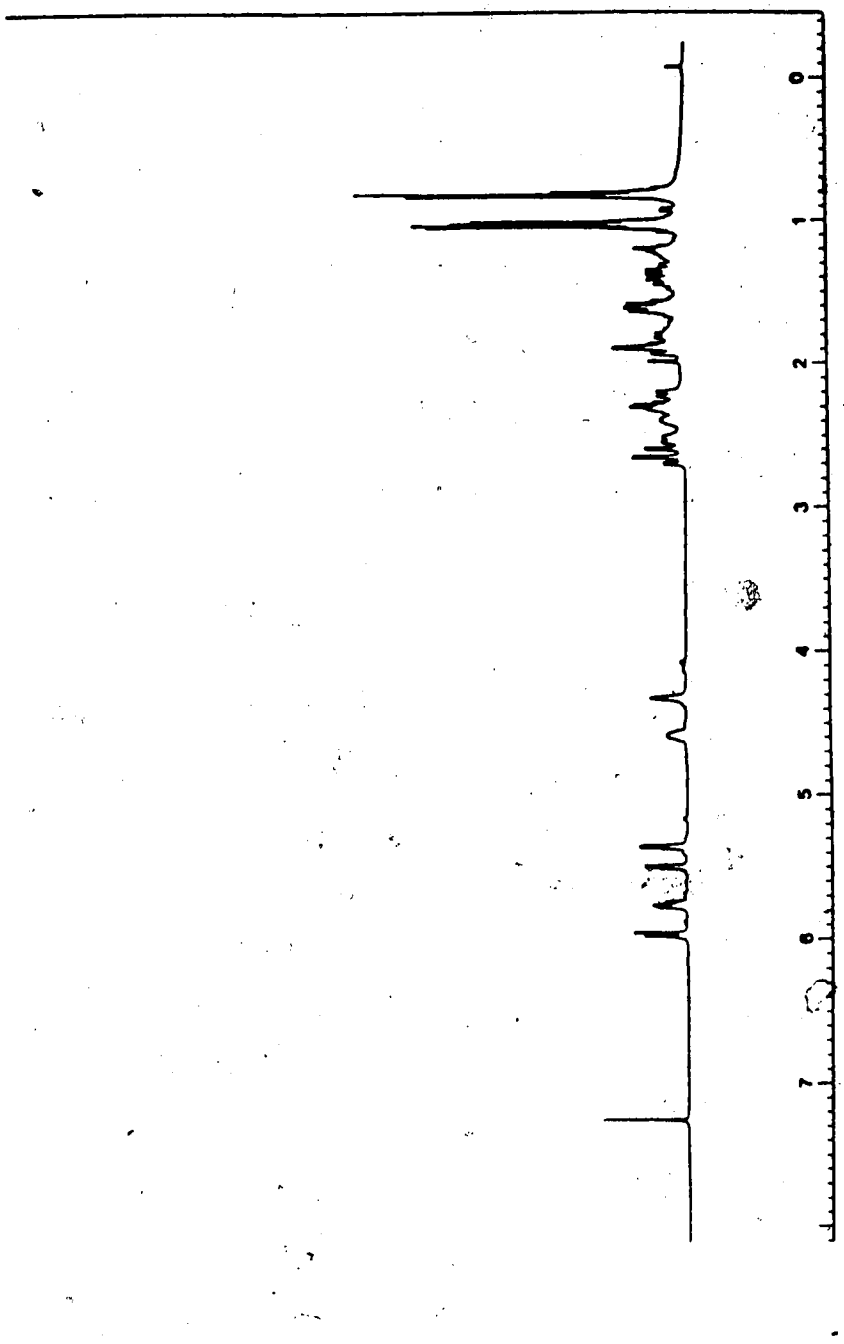


FIGURE 4: 400 MHz Proton n.m.r. spectrum of mevinolin (0-10 ppm).

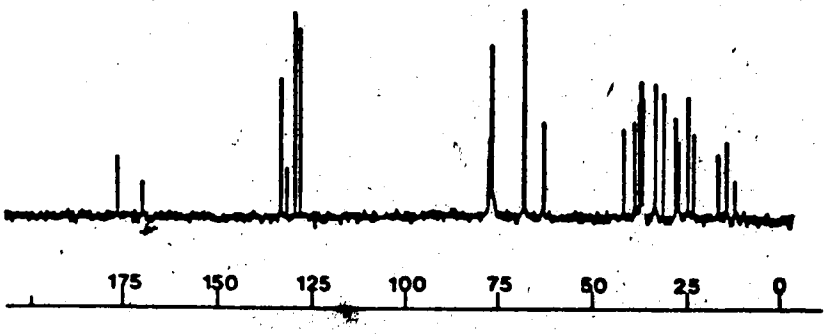


FIGURE 5: 100.16 MHz ^{13}C-n.m.r. spectrum of mevinolin (0-250 ppm).

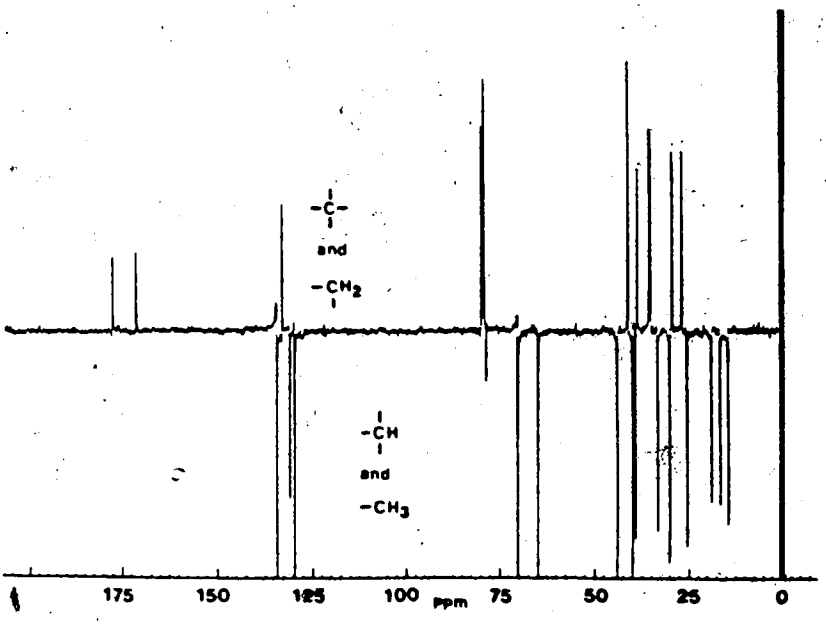
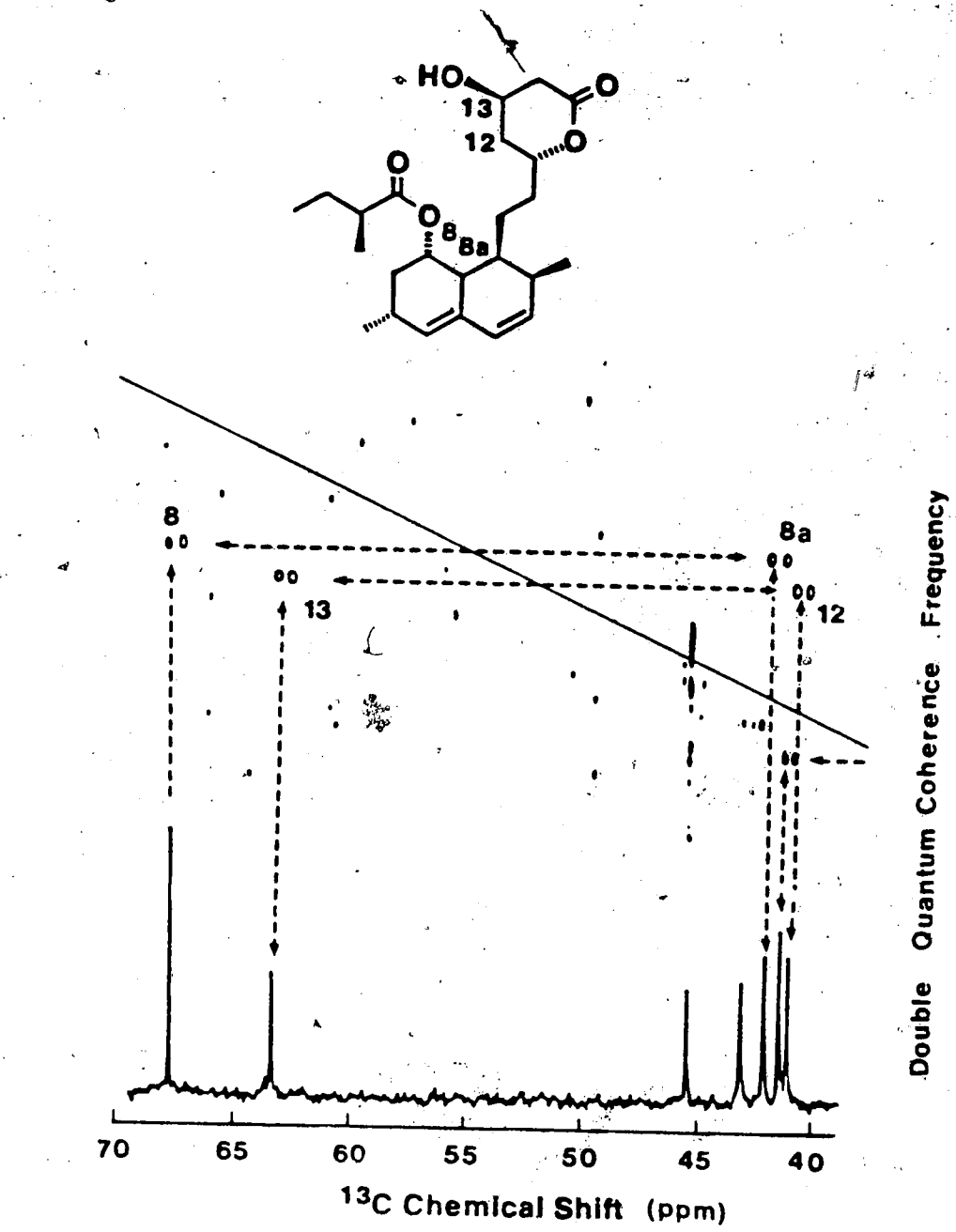


FIGURE 6: 100.16 MHz ^{13}C-n.m.r. CARPPET spectrum of mevinolin (0-250 ppm).

technique is its dependence on the abundance of adjacent ^{13}C-nuclei (0.011% at natural abundance). This problem may be circumvented using large amounts of compound (ca. 1 g). This amount of material is generally unavailable to the biosynthetic chemist. The problem was overcome by increasing the intensity of carbon-13 coupled signals in mevinolin. Incorporation of sodium [1,2-$^{13}C_2$]acetate into mevinolin by A. terreus raised the level of adjacent carbon-13 nuclei up to 2% (an increase of 100-fold). The 2D-INADEQUATE spectrum that was obtained on 50 mg of enriched material permitted a complete assignment of the ^{13}C-n.m.r. spectrum (Fig. 7). A similar strategy has been recently reported by Floss and Bacher using [U-^{13}C]glucose[45] to raise the ^{13}C level in riboflavin.[44]

Once the spectral assignments were complete, the origin of carbon atoms was determined. Mevinolin was isolated after separate incorporations of sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate and [methyl-^{13}C]methionine. These three experiments show that all carbon atoms originate from acetate except for C-6 and C-2' which come from methionine. The preservation of the carbon-carbon bonds of the acetate units was obtained from the 2D-INADEQUATE spectrum of mevinolin derived from [1,2-$^{13}C_2$]acetate. Normally measurement of ^{13}C-^{13}C coupling constants is used in such experiments;[46] however, the doubly labelled meviolin



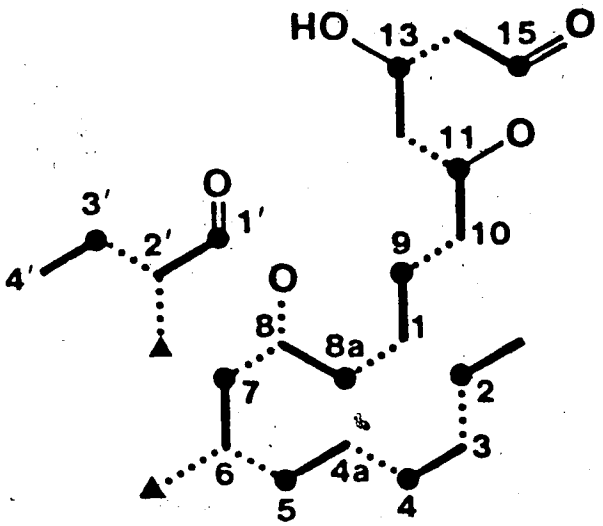
Overlay of Normal Spectrum on 2D-Inadequate Spectrum.

FIGURE 7: 2D-INADEQUATE spectrum between 40 and 70 ppm of mevinolin enriched with sodium [1,2-$^{13}C_2$]acetate.

15 possessed multiple labelled-acetate units in the same molecule which led to long range coupling. This made normal measurement of $J_{^{13}C-^{13}C}$ difficult. The 2D-INADEQUATE experiment is advantageous in this respect since values of $J_{^{13}C-^{13}C}$ are not required.

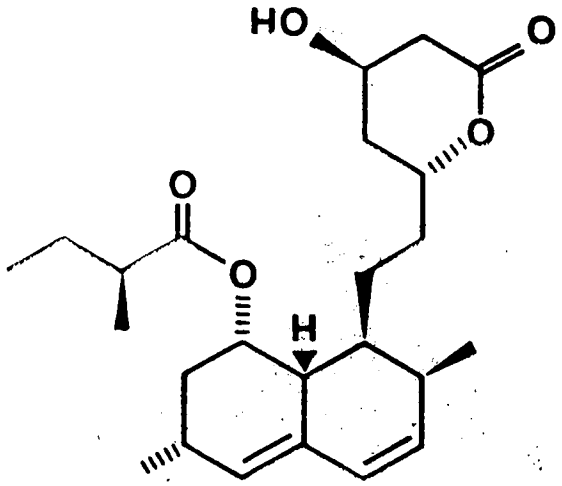
The results show that the main portion of mevinolin consists of nine intact acetate units (eqn. 10), bearing a methyl group derived from methionine at C-6. The 2-methylbutyryl side chain is constructed analogously and consists of two acetate units and one carbon from methionine at the 2-position. This eliminates propionate[48] or isoleucine[47] as possible precursors.

The fate of acetate carbon-oxygen bonds was determined by incorporation of sodium [1-^{13}C, $^{18}O_2$]acetate, and ^{13}C-n.m.r. spectral analysis of the resulting mevinolin using the SEFT technique. Excellent incorporation of ^{13}C label was clearly detected, however, only a single isotope shift at C-1' was observed. The level of oxygen label at C-11, C-13, and C-15 was less than 5% of the carbon-13 labelling. This loss of oxygen may arise from solvent exchange during the biosynthesis and is currently under investigation. It is interesting to note that the stereochemistry of all singly bonded oxygens at carbons derived from C-1 of acetate is that which would be obtained by failure of the elimination step in fat biosynthesis.



— CH_3COONa

▲ $CH_3SCH_2CH_2CHCOOH$
NH_2



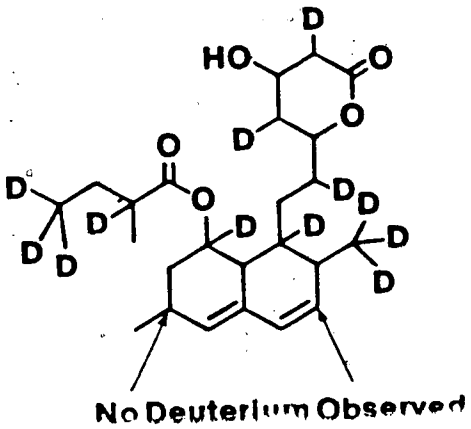
(eqn. 10)

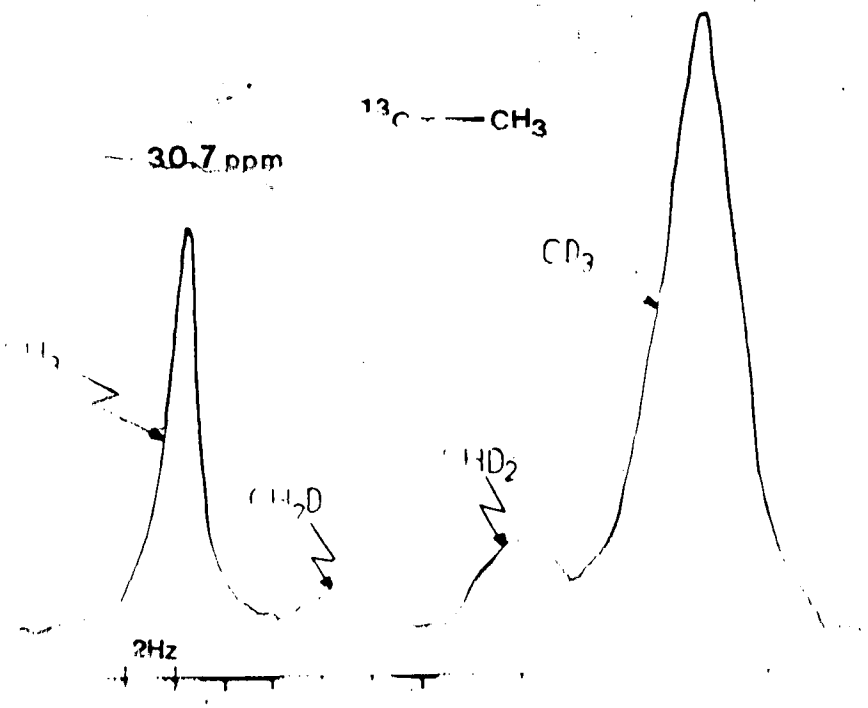
The biosynthetic fate of carbon-hydrogen bonds has not been easy to determine until recently. The line-broadened signals and small spectral width characteristic of deuterium n.m.r.[23] have not made this technique popular for the investigation of complex compounds. Alpha substitution of deuterium on a carbon-13 atom causes a large isotope shift to be observed in the carbon-13 n.m.r. spectrum.[23,49] This detection is complicated by the appearance of the carbon signal as a broadened triplet due to quadrupolar relaxation, coupling (ca. 20-40 Hz), and reduced NOE. These effects decrease the observed signal intensity and complicate the spectrum with many additional signals unless broad band deuterium decoupling is employed. Such an experiment requires a triple resonance n.m.r. probe with a fluorine lock.

Recently, a technique for determining the fate of hydrogens during biosynthesis[50] was reported by Staunton.[50a] Deuterium substitution [illegible] label gives an isotopically shifted [illegible] signal. Whereas oxygen-18 beta isotope shifts have only been observed upfield of the unlabelled signal, the deuterium substitution can cause ^{13}C resonances to be [illegible] upfield, downfield or not at all.[50a] The line width is appreciably affected because the coupling [illegible] ation

the ^{13}C-C-D bonds must remain intact for the isotope shift to be observed (unless a 1,3-migration occurs) the deuterium atom must be incorporated at the C-2 position of the intact acetate unit. Stothers has used this approach to study 1,2 migration of hydrogens.[50g]

This technique was used to determine the fate of carbon-hydrogen bonds during mevinolin biosynthesis. Incorporation of sodium [1-^{13}C,$^{2}H_3$]acetate and examination of the carbon n.m.r. spectrum for β-shifts showed high deuterium retention at all expected sites except at C-3 and at C-6. Since compactin bears no methyl group at C-6 the incorporation of the same precursor may provide an interesting result. The signals at C-2 and C-4' are composed of four peaks resulting from CD_3, CD_2H, and CDH_2 incorporation (Fig. 8) into the 2'-methyl group. The ratios of these peaks indicate high retention of all three deuteriums. This confirms the role of these units as the starter acetyl units of the polyketide chains since they are the only [illegible] not converted to malonyl-CoA with loss of one hydrogen. This result was supported by the ^{2}H-n.m.r. spectrum of mevinolin derived from sodium [$^{2}H_3$]acetate. Examination of the ^{2}H labelling results and the stereochemistry at C-[illegible] C-8, [illegible] 2' and C-6 suggest that a single deuterium label always occupies the same relative [illegible] istry on [illegible] ing polyketide chain. Recently,



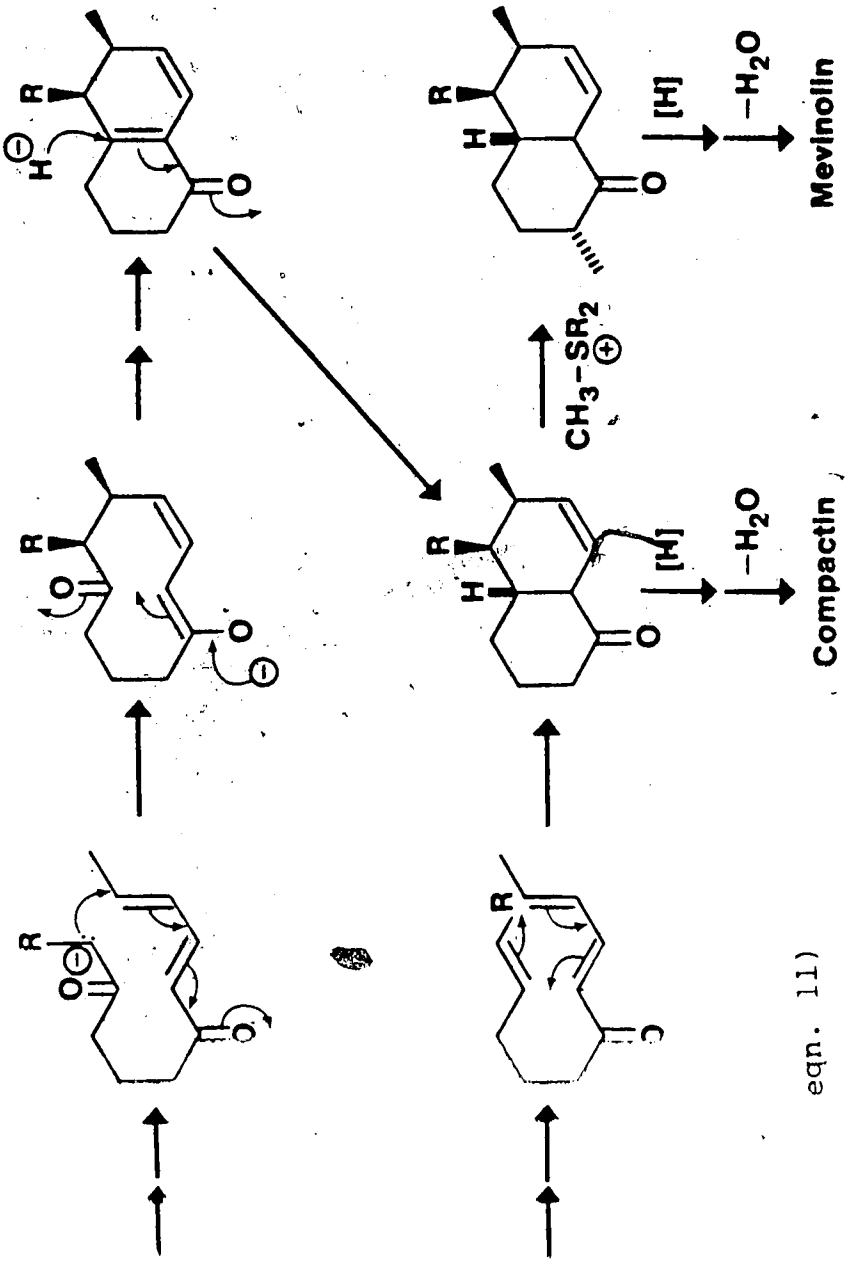


100 MHz ^{13}C n.m.r. spectrum of C(2) of mevin
ched ith sodium [1-^{13}C, $^{2}H_3$]lactate.

new n.m.r. pulse techniques have allowed deuterium directly attached to carbon to be observed by polarization transfer.[51] Such approaches are currently under investigation in our laboratory using samples of mevinolin derived from sodium [2-^{13}C, $^{2}H_3$]acetate.

The results obtained so far support biogenesis of mevinolin either by a Diels-Alder reaction of a polyunsaturated C-18 fatty acid, or by a series of intramolecular condensations of a polyketide precursor (eqn 11). Either route could give a single intermediate which would be transformed to mevinolin after methylation, or converted to compactin directly.

Initial difficulty in determining the ^{13}C-n.m.r. assignment for mevinolin encouraged the examination of the carbon-proton correlation spectrum.[52] The complexity and signal overlap of the carbon and proton n.m.r. spectra of mevinolin hinders assignment by this method. However, a technique for correlating carbon signals of specific multiplicity with the proton signals has been developed[53a] and applied to cholesterol.[53b] This technique is known as a 2D selective DEPT correlation. Three spectral plots are obtained (Fig. 9): one for methine (CH) signals, one for methylene (CH_2) resonances and one for methyl (CH_3) peaks. In each case, the proton chemical shift (0 to 2.5 ppm) is plotted on the vertical axis against the carbon chemical



eqn. 11)

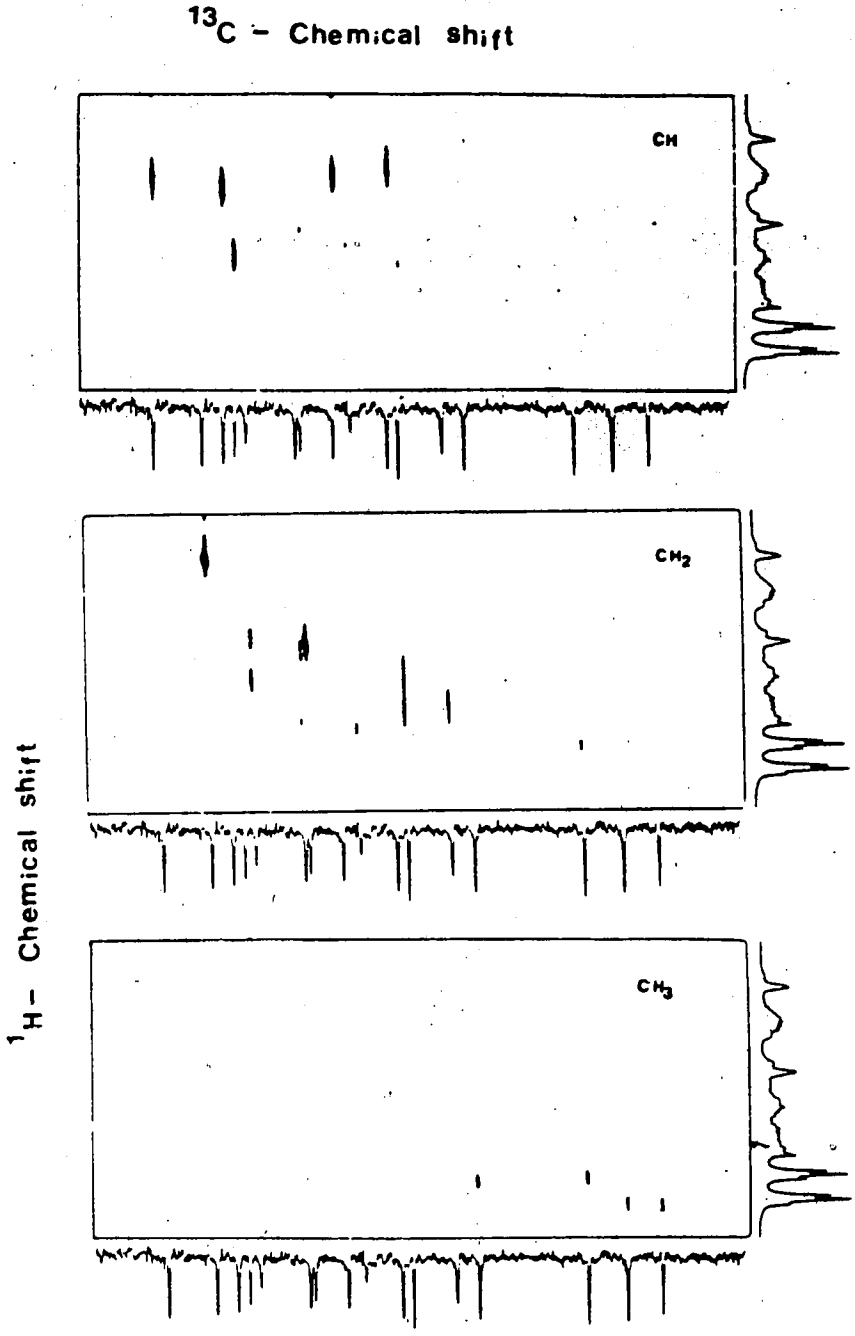


FIGURE 9: 2D-Selective DEPT plots obtained correlating CH, CH_2 and CH_3 carbon signals with the proton spectrum, respectively.

shift (10 to 50 ppm) on the horizontal axis in the form of a contour plot. The CH_3 plot illustrates the large degree of separation of overlapping proton signals that can be achieved by this method. Further modifications of this technique are being developed to decrease the line width of signals on the proton axis and to suppress "break-through" signals (demonstrated by a CH_3 signal of low intensity in the CH_2 plot). It should also be noted that the CH_2 spectrum gives rise to two signals where the methylene protons are of differing chemical shift. The determination of the stereochemistry of deuterium and the assignment of large complex molecules by such techniques is presently under study in our laboratory.

EXPERIMENTAL

The general experimental procedures described in Part 1 were employed along with the following additional techniques.

Fermentations of Aspergillus terreus were done in New Brunswick Psychrotherm G-25 shakers using conical flasks which were sealed with cotton batten or foam plugs using aseptic technique. All media was sterilized for 20 minutes in an autoclave at 120°C. Diluted precursors were administered, using a Hamilton 1 mL syringe (sterilized with ethanol), through the plug on each flask. Constant temperature was employed for fermentations. Visualization of silica tlc plates was accomplished using ultraviolet (UV) for mevinolin from A. terreus and for tetronic acids from P. multicolor projects. A solution of N,N-dimethyl-aminobenzaldehyde in 90:10 methanol-concentrated hydrochloric acid spray was used for tlc visualization of acetals.

Carbon-13 spectra were obtained on the Bruker WH-400 cryospectrometer unless otherwise indicated. Spectral widths and windows were adjusted in all expansions to give a resolution of < 1 Hz per point. All full spectra were run using a 32K data block over 250 ppm and employing tetramethylsilane as an internal standard in deuterated

chloroform. All n.m.r. samples were filtered through a cotton plug directly into a 5 mm thin-walled n.m.r. tube. Each sample was allowed to equilibrate to probe temperature for 30 minutes. A 5 mm carbon probe with an internal lock on deuterium was employed in all cases.

Pulse sequences and delay times are given in appendix 1. Isotope ratios were obtained by expansion of signals to 0.5 Hz/cm plot on paper followed by peak area analysis on a DuPont curve resolver. Two-dimensional spectra were transformed on an Aspect 2000 computer using the Bruker DISNEWP program to allow zerofilling in both directions. Labelled precursors such as [^{18}O]water, [^{18}O]gas, and sodium [^{13}C]acetate were purchased from Cambridge Isotopes (Cambridge, Massachusetts).

PREPARATION OF LABELLED DIOLS AND ACETALS

Preparation of [1,2-$^{18}O_2$]ethylene glycol (4)

[^{18}O]water (0.9 mL, 97% isotopic purity) and dry benzene (20 mL) were added to anhydrous oxalic acid (275 mg, 3 mmol) under an inert atmosphere. The mixture was refluxed for 18 h with the dissolution of oxalic acid. The water was then recovered using a Dean-Stark apparatus and the benzene was removed by distillation (1 atm). After drying under vacuum (0.1 mm of Hg), a quantitative yield of anhydrous

oxalic acid was obtained. Tetrahydrofuran (10 mL) was added to the anhydrous [$^{18}O_4$]oxalic acid (3 mmol) under an inert atmosphere. The solution was cooled to 0°C and freshly prepared 1 M diborane in tetrahydrofuran (10 mL)[18] was added dropwise over 20 min. The solution was warmed to 20°C. A white precipitate formed within 30 min. Afer 12 h a 1:1 solution of water/tetrahydrofuran was added dropwise until hydrogen evolution ceased. Anhydrous potassium carbonate was added until the formation of ethylene glycol was observed by tlc (THF). The solid residue was removed by filtration and was extracted with dry tetrahydrofuran (2 x 10 mL). Concentration under reduced pressure gave a viscous liquid. Distillation at 196°C yielded [1,2-$^{18}O_2$]ethylene glycol (**4**) (180 mg, 94%) with the expected chromatographic and spectral properties. Mass spectrum, m/e (relative intensity) 66 (32), 64 (48), 62 (20).

Preparation of [^{18}O]acetals

The corresponding ketone (0.5 mmol) in benzene (25 mL) was added to the appropriate diol (0.5 mmol to 1.5 mmol) under an inert atmosphere. The solution was refluxed and the water was continuously removed using a Soxhlet extractor containing calcium hydride until tlc showed the complete disappearance of ketone (6 h to 18 h). The solution was washed with 1 M potassium carbonate (10 mL) and was back

extracted with chloroform (2 x 10 mL). The combined organic extracts were dried over sodium sulphate. Concentration under reduced pressure yielded the acetal (50-90%). The acetals showed the expected chromatographic and spectral properties.

2,2-Dibutyl-1,3-[$^{18}O_2$]dioxalane (**3c**)

2,2-Dibutyl-1,3-[$^{18}O_2$]dioxolane (**3c**) was prepared as described above using [1,2-$^{18}O_2$]-ethylene glycol (1.5 mmol) and 5-nonanone (0.5 mmol). The solution was refluxed for 18 h to give 86 mg of **3c** (91%) as a liquid. NMR ($CDCl_3$) δ 0.9 (t, 6H), 1.15-1.75 (m, 12H), 3.9 (s, 4H); mass spectrum m/e (relative intensity) 133 (31), 131 (48), 129 (20). CAS. reg. no. 54661-97-1.

1,5-[^{18}O]Dioxoaspiro[5,6]undecane (**1b**).

1,5-[^{18}O]Dioxoaspiro[5,6]undecane (**1b**) was prepared as described above using [1-^{18}O]-1,3-propanediol (1 mmol) and cyclohexanone (0.5 mmol). The solution was refluxed for 12 h to give 71 mg (70%) of **1b** as a liquid. NMR ($CDCl_3$) δ 1.2-2.0 (m, 12H), 3.88 (t, 4H); mass spectrum, m/e (relative intensity) 158 (31), 156 (69). CAS. reg. no. 780-93-8.

1,4-[$^{18}O_2$]Dioxoaspiro[4.5]decane (**3b**)

1,4-[$^{18}O_2$]Dioxoaspiro[4.5]decane (**3b**) was prepared as described above using [1,2-$^{18}O_2$] ethylene glycol (1 mmol) and cyclohexanone (0.7 mmol). The solution was refluxed for 6 h to give 83 mg (77%) of **3b** as a liquid. NMR ($CDCl_3$) δ 1.3-1.8 (m, 10 H), 3.9 (s, 4H); mass spectrum, m/e (relative intensity) 146 (20), 144 (40), 142 (40). CAS reg. no. 177-10-6.

2-Hexyl-1,3-[$^{18}O_2$]dioxolane (**3c**)

2-Hexyl-1,3-[$^{18}O_2$]dioxolane (**3c**) was prepared as described above using [1,2-$^{18}O_2$] ethylene glycol (1.5 mmol) and heptaldehyde (0.5 mmol). The solution was refluxed for 6 h to give 80 mg (90%) of **3c** as a liquid. NMR ($CDCl_3$) δ 0.85 (t, 3H), 1.0-1.75 (m, 10H), 3.50-4.05 (m, 4H), 4.85 (t, 1H); mass spectrum, m/e (relative intensity) 161 (31), 159 (48), 157 (20). CAS reg. no. 1708-34-5.

2-Hexyl-1,3-[^{18}O]dioxane (**1a**)

2-Hexyl-1,3-[^{18}O]dioxane (**1a**) was prepared as described above using [1-^{18}O]-1,3-propanediol (1 mmol) and heptaldehyde (0.5 mmol). The solution was refluxed for 17 h to give 45 mg (50%)of **1a** as a liquid. NMR ($CDCl_3$) δ 0.9 (t, 3H), 1.1-1.8 (m, 12H), 3.5-4.2 (m, 4H), 4.4 (t, 1H); mass spectrum, m/e (relative intensity) 173 (30), 171 (70).

BIOSYNTHESIS OF TETRONIC ACIDS

Preparation of Methyl-O-methylmulticolate-2,4-dinitro phenylether (13).

A 5 mm n.m.r. tube containing 10.0 mg (0.058 mmol) of 2,4-dinitrofluorobenzene, 12.0 mg of (0.042 mmol) impure methyl-O-methylmulticolate and 0.020 mL (0.14 mmol) of triethylamine in 0.32 mL of deuterodichloromethane was prepared. The reaction mixture was left in the dark at room temperature and checked periodically by ^{1}H-n.m.r. at 200 Mz and by tlc. After six days, the reaction was complete. Compound **13** was purified by chromatography on a 20 cm by 20 cm by 2 mm silica gel plate using 3% ethyl acetate in chloroform. Elution of the silica gel with 250 mL of dichloromethane and removal of solvent *in vacuo* gave 9 mg of **13** as a white crystalline solid (yield 46%).

NMR ($CDCl_3$, 400 MHz) δ 8.66 (d, J = 4.0 Hz, 1H, ArH), 8.45 (dd, J = 4.0, 10.0 Hz, 1H, ArH), 7.05 (d, J = 10.0 Hz, 1H, ArH), 4.80 (s, 1H, vinyl-H), 4.15 (t, J = 6.0 Hz, 2H, CH_2OAr) 4.05 (s, 3H, CH_3O), 3.70 (s, 3H, CH_3OC(O)), 2.50 (m, 2H, CH_2-vinyl), 1.9 (m, 2H, CH_2CH_2OAr), 1.55 (m, 4H, vinyl-$CH_2CH_2CH_2$). Exact mass calc for $C_{19}H_{20}N_2O_{10}$: 436.11. Found: 436.11. 2,4-Dinitrophenol fragmentation observed at 184.01[20] (Fragmentation pattern checked on 2,4-dinitrophenylethyl ether) contained no ... adjacent peak. Rf

= 0.9 in 3% ethyl acetate in chloroform. ^{13}C-n.m.r. ($CDCl_3$, 400 MHz) δ 161.5 (C1), 110.0 (C2), 168.3 (C3), 150.7 (C4), 23.3 (C5), 29.2 (C6), 25.4 (C7), 28.3 (C8), 70.4 (C9), 101.5 (C 10), 164.4 (C 11), 59.8 (C3-MeO), 52.2 (C 11-MeO), 156.7 (C 1'), 134.0 (C2'), 121,8 (C3'), 147.0 (C4'), 129.0 (C5'), 114.3 (C6').

BIOSYNTHESIS OF MEVINOLIN FROM ASPERIGILLUS TERREUS

Fermentation of Aspergillus Terreus

Aspergillus terreus ATCC 20542 was grown as described[29b] with the following modifications. Twelve day old agar slants were used to innoculate 500 mL Erlenmeyer flasks containing 100 mL of sterile medium A (appendix II) which were incubated on a rotary shaker (180 rpm) for 48 h. Each flask was partitioned across six similar flasks containing sterile medium B (plate 1) (appendix II). Each flask was injected with 1.0 mL of aqueous labelled sodium acetate solution (1.0 g/18 mL) every 24 h for three days. After a further 24 h, extraction (plate 2) and purification as described[29b] gave pure mevinolin whose spectral and physical properties were compared to an authentic unlabelled sample (Merck Sharp Dohme).

FT-IR (film) 3200, 1725, 1280 cm^{-1}; NMR ($CDCl_3$, 400 MHz) δ 0.89 (d, J = 8.0 Hz, 3H, H-4'), 0.91 (d, J = 7.0 Hz, 3H, 2-

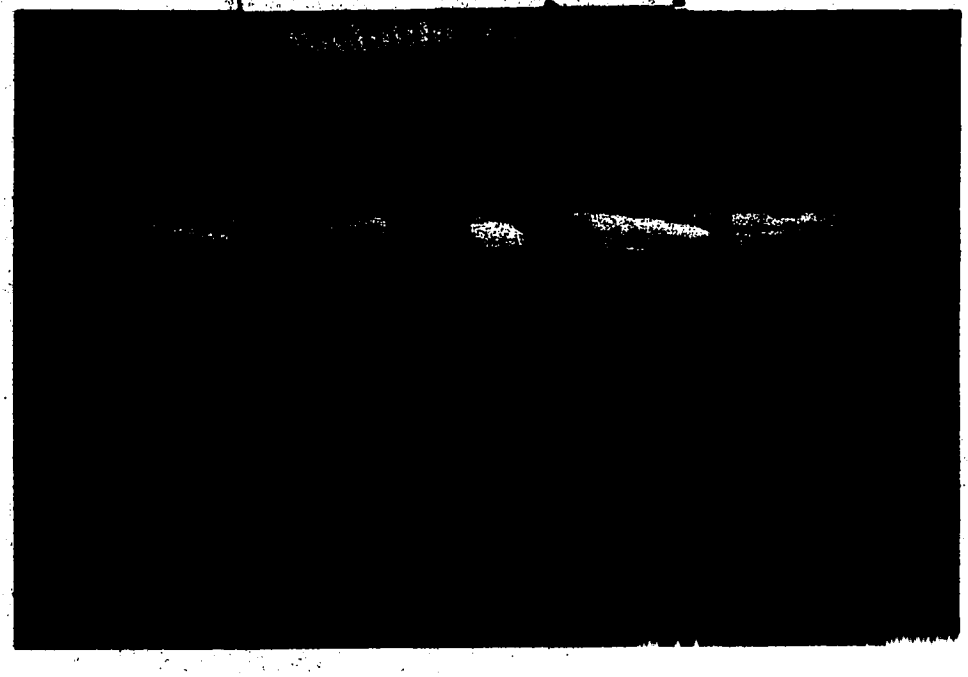


Plate 1. Aspergillus terreus cultures after 4 days growth in medium B.

Plate [illegible] Extraction of mevinolin from fermentation [illegible] ethyl acetate

Me), 1.08 (d, J = 8.0 Hz, 3H, 6-Me), 1.12 (d, J = 8.0 Hz, 3H, 2'-Me), 1.30 (m, 1H, Ha-10), 1.35-1.40 (m, 3H, H-9, Ha-3'), 1.5-1.75 (m, 1H, Hb-3', H-1, Ha-12), 1.8-2.0 (m, 4H, Hb-10, Hb-12, H-7), 2.16 (br s, 1H, OH), 2.2[illegible] (dd, J = 12.0, 3.0 Hz, 1H, H-8a), 2.3-2.4 (m, 2H, H-2, H-2'), 2.45 (m, 1H, H-6), 2.62 (m, J = 18, 4, 2 Hz, 1H, Hb-14), 2.74 (dd, J = 18.0, 5.0 Hz, 1H, Ha-14), 4.38 (m, J = 4 Hz, 1H, H-12), 4.62 (m, 1H, H-11), 5.40 (q, J = 3.0 Hz, 1H, H-8), 5.54 (m, 1H, H-5), 5.[illegible] (dd, J = 10.0, 6.0 Hz, 1H, H-3), 6.00 (d, J = 10.0 Hz, 1H, H-4). Exact mass calculated for $C_{24}H_{36}O_5$: 404.25. Found: 404.25.

<u>Data obtained from 2-D INADEQUATE experiment on compound</u> **19**).

Paired signals were obtained at: 30.7 (C 2) and 13.9 ($2-CH_3$); [illegible]7.5 (C 6) and 32.8 (C 7); 68.0 (C8) and 37.4 (C 8a); 36.[illegible] (C 1) and 24.[illegible] (C 9); 33.0 (C 10) and 75.7 (C 11); [illegible] (C 12) and 62.6 (C 13); 26.8 (C 3') and 11.[illegible] (C 4').

[illegible]), (C 15), (C [illegible] (C 2'), ([illegible]) and ($2'-CH_3$) were [illegible] coupling.

Table 4.

Yields Obtained

Compound No.	Sodium acetate Incorporated	Fermentation Size	Yield (Isolated)
(13)	[1-^{13}C]	600 mL	17 mg
(14)	[2-^{13}C]	800 mL	11 mg
(15)	[1,2-$^{13}C_2$]	900 mL	56 mg
(16)	[$^{2}H_3$]	900 mL	50 mg
(17)	[1-^{13}C,$^{2}H_3$]	500 mL	20 mg
(18)	[1-^{13}C,$^{18}O_2$]	600 mL	26 mg
(19)	[2-^{13}C,$^{2}H_3$]	600 mL	22 mg
Non-acetate incorporations			
(20)	[^{13}C]methyl methionine	600 mL	8 mg
(21)	18% D_2O in medium B	1000 mL	10 mg

Table 5.

Carbon-13 Enrichment Data on Labelled Compounds

Carbon	Shift (ppm)	(13)	(14)	(20)	(17)
1	36.7	-	2.4	-	-
2	30.7	4.1	-	-	5.1
3	133.1	-	3.1	-	-
4	128.4	3.3	-	-	5.8
5	129.6	3.0	-	-	4.7
6	27.5	-	5.2	-	-
7	32.8	3.2	-	-	6.2
8	68.0	-	1.4	-	-
9	24.3	4.1	-	-	3.6
10	33.0	-	2.2	-	-
11	75.7	4.6	-	-	4.0
12	36.2	-	2.4	-	-
13	62.6	7.7	-	-	4.5
14	38.7	-	3.4	-	-
15	170.7	2.8	-	-	2.8
1'	176.9	2.4	-	-	5.6
2'	41.6	-	2.9	-	-
3'	26.8	4.4	-	-	6.5
4'	11.7	-	2.9	-	-
2'-CH_3	16.3	-	-	45	-
6-CH_3	22.9	-	-	29	-
4a	131.6	-	4.4	-	-
8a	37.4	4.6	-	-	3.4
2-CH_3	13.9	-	2.3	-	-

Compound (**15**) gave enrichment (0.5-2%) of ^{13}C at all sites except 6-CH_3 and 2'-CH_3.

Table 6

Isotope Shifts of Mevinolin Obtained after Incorporation of Sodium [1-^{13}C, $^{18}O_2$]acetate (**18**).

No.	Shift (ppm)	Isotope Shift (ppm x 100)	Isotope Ratio $^{16}O:^{18}O$	Carbon-13 enrichment
8	68.0	-	-	-
11	75.7	-	-	3.4
13	62.6	-	-	7.1
15	170.7	-	-	2.1
1'	176.9	3.75	85:15	1.9

Table 7

^{13}C-NMR Data of Mevinolin (17) Derived from Sodium [1-^{13}C, $^{2}H_3$]acetate.

Carbon	$^{2}H:^{1}H$[a]	Δδ(ppm)[b]
1	24:76	
2		-0.25,-0.17,-0.082[c]
2-Me	76:8:8:8[c]	
3	0:100	
4		
4a		
6	0:100	
6-Me		
7		
8	45:55	
8a		+0.013
9		-0.096
10	80:20	
11		-0.06
12	50:50	
13		-0.12
14	50:50	
15		+0.04
1'		+0.018
2'	75:25	
2'-Me		
3'		-0.21,-0.14,-0.071[c]
4'	76:8:8:8[c]	

a) Ratio of carbon peak areas for β-isotope shifted signals.

b) β-isotope shifts due to ^{2}H.

c) $CD_3:CHD_2:CH_2D:CH_3$.

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APPENDIX II

APPENDIX I

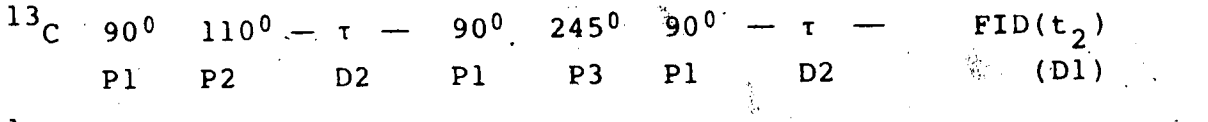
Pulse sequences used in the acquisition of spectra are listed below with corresponding parameters.

1. Normal aquisition:

```
ZE
2BB ; for carbon-13
GO=2
EXIT
```

LB is set to the Hz/Pt.

2. Carbon Parity Partitioning by Echo Technique (CARPPET) CARPPET PULSE SEQUENCE



CARPPET PULSE PROGRAM for ASPECT 2000 (DISNMRP)

```
1    ZE
2    BB
3    (D1 P1: A P2: A A3 D2)
4    (P1 PH4 P3 P1 PH4 D2)DO
5    GO = 3 BB
```

P1; 90° carbon pulse

P2; 110° carbon pulse

P3; 245° carbon pulse

D1; Relaxation delay (3-4 s)

D2; $1/J_{C-H} \equiv 0.007$ s for carbon-proton system

3. Spin Echo Fourier Transform (SEFT)

SEFT PULSE SEQUENCE

13 C: 90° - τ - 180° - τ - FID(t_2)

P1 D2 P2 D2 (D1)

SEFT PULSE PROGRAM FOR ASPECT 2000 (DISNMRP).

ZE

2BB

D1 ; carbon relaxation delay (3-10 s)

P1 ; a 90° carbon pulse

D2 ; Delay =$1/2J_{CC}$ for carbon-carbon system (0.111-0.090-S)

P2 ; a 180° refocusing carbon pulse.

D2

GO=2

EXIT

4. 2D-INADEQUATE (Incredible Natural Abundance DoublE QUAtum Transfer Experiment.)

2D-INADEQUATE PULSE SEQUENCE

^{13}C: 90° - τ - 180° - τ - 90° - t_1 - 90° FID(t_2)

P1 D2 P2 D2 P1 DØ P1 D1

^{13}C: $90° - \tau - 180° - \tau - 45°90° - t_1 - 90°FID(t_2)$

P1 D2 P2 D2 P3D3P1 DØ P1 D1

2D-INADEQUATE PULSE PROGRAM FOR ASPECT 2000 (DISNMRP)

```
1    ZE
2    D1 BB S1
3    D4 S2
4    P1 PH1 D2)
5    (P2 PH2 D2)
6    (P1 PH3 D5)
7    LO TO6, TIMES I
8    P1 PH4
9    60 = 2 PH5
10   D1 BB S1
11   D4 S2
12   (P1 PH1 D2)
13   (P2 PH2 D2)
14   (P3 PH6 D3)
15   (P1 PH5 D5)
16   LO TO 15, TIMES 4
17   P1 PH4
18   60 = 10 PH7
19   IPHA
20   LO TO 2, TIMES 4
21   WR FN•SER
```

```
22   IF FN•SER
23   IN=1
24   DO
25   EXIT
PH1 A0
PH2 = A0A2
PH3 = A0
PH4 = A0 A0 A1 A1 A2 A2 A3 A3
PH5 = R0 R0 R3 R3 R2 R2 R1 R1
PH6 = A3
PH7 = R1 R1 R0 R0 R3 R3 R2 R2
```

P1, P2 and P3 are 90°, 180°, and 45° carbon pulses, respectively.

D1 is the recycle delay (5.0 s).

D2 is 1/4J where J is the mean carbon-carbon coupling constant (J = 40 Hz).

DØ is the initial time delay and is set to 0.000005 s.

D4 is an additional delay to raise power levels and is set to 0.010 s.

The 2D-INADEQUATE was obtained on a 70 ppm sweep width covering the region from 10 to 80 ppm.

D5 = IN = 0.5/SW is the increment.

5. 2D-Selective Distortionless Enhancement by Polarization Transfer (DEPT)

2D-Selective DEPT PULSE SEQUENCE

^{1}H: 90^{0}_{x} (P1) — t_1 — 180^{0}_{x} (P2) — t_1 — τ — θy (P4) — τ — decouple

^{13}C: 90^{0}_{x} (P7) 180^{0}_{x}(P6) — 90^{0}_{x} (P5) — 180^{0}_{x} (P6) — FID(t_2)

2D-Selective DEPT PULSE PROGRAM FOR ASPECT 2000 (FP810515).

θy = 30°	θy = 90°	θy = 150°
1 ZE	11 ZE	22 ZE
2 D1S1DO	12 D3S2CW	23 D3S2CW
3 (P1PH1DØ):DP7PH7	13 D1S1DO	24 D1S1DO
4 (P6PH6DØ)	14 (P1PH1DØ):DP7PH7	25 (P1PH1DØ):DP7PH7
5 D2	15 (P6PH6DØ)	26 (P6PH6DØ)
6 (P2PH2D2):DP5PH5	16 D2	27 D2
7 (P3 PH3 D3):D P6 PH6	17 (P2 PH2D2);DP5PH5	28 (P1PH1D2);DP5PH5
8 GO = 2 BB	18 (P4 PH3 D2)DP6PH6	29 (P8 PH3 D2)DP6PH6
9 WR FN30.SER	19 GO = 13 BB	30 GO = 24 BB
10 IF FN30.SER	20 WR FN90.SER	31 WR FN135.SER
	21 IF FN90.SER	32 IF FN135.SER
PH1 = BØ PH5 = AØ		33 IN 1
PH2 = BØ PH6 = AØ		34 DO
PH3 = B1 PH7 = AØ		35 EXIT

DØ ≡ Incremented time delay (1/4 ^1H SW) and set to 5 μs initially.

D_1 ≡ Recycle delay for carbon proton system (1.8s) (t_2).

D_2 ≡ delay for carbon-proton system = $\frac{1}{2} J_{C-H}$ = 0.00388 s for J_{C-H} = 174 Hz. (τ).

Linear combinations of these FID's are used to obtain the CH_3, CH_2 and CH data.

$CH \equiv FID_{\theta 90°}$

$CH_2 \equiv FID_{\theta 30°} - FID_{\theta 150°}$

$CH_3 \equiv FID_{\theta 30°} + FID_{\theta 150°} - FID_{\theta 90°}$

APPENDIX II

Medium A, used in the production of mevinolin, is composed of:

5 g of corn steep liquor.

40 g of tomato paste.

10 g of oat flour.

10 g of dextrose.

10 g of dextrose

10 mL of a trace element solution (composition: 1 g of iron(III) sulphate heptahydrate, 1 g of manganese (II) sulphate tetrahydrate, 25 mg of copper (II) chloride dihydrate, 100 mg of calcium chloride dihydrate, 56 mg of

boric acid, 19 mg of $(NH_4)_6MO_7O_{24}\cdot 4HO$, and 250 mg of zinc sulphate heptahydrate per litre).

in 1 L of triply distilled water at a pH of 6.80.

Medium B, u[redacted] in the production of mevinolin, is composed of:

45g of dextrose.

24 g of peptonised milk.

2.5 mg of yeast extract

2.5 mL of polyethylene glycol P2000.

in 1 L of triply distilled water at pH 7.4.