THE BIOSYNTHESIS OF ANTIBIOTIC F-244

IN *FUSARIUM* SP. ATCC 20788

By

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ABSTRACT

The naturally occurring antibiotic β-lactone F-244, also known as 1233A and L-659,699, is isolated from species of *Fusarium*, *Scopulariopsis* and *Cephalosporium*. This compound has been shown to be a potent specific inhibitor of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase, a key enzyme in the mammalian biosynthesis of cholesterol.

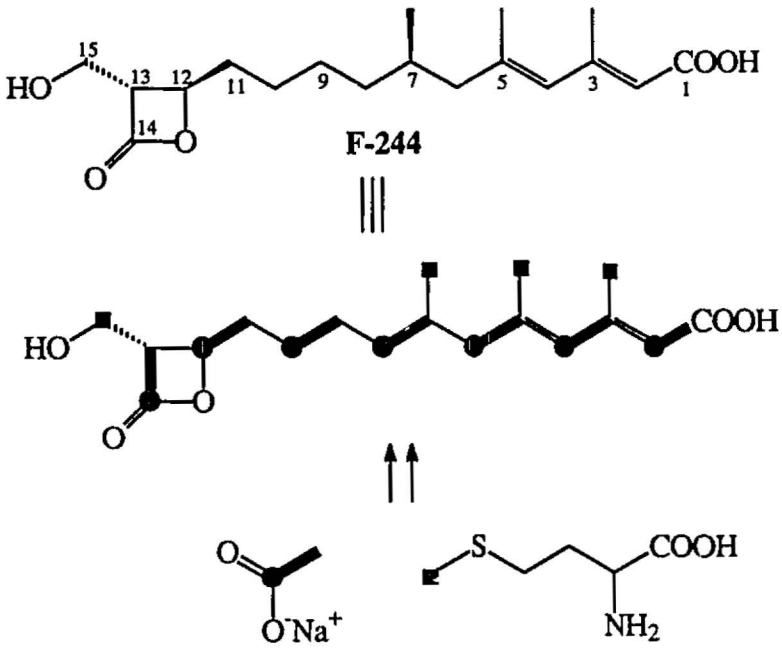
Extensive multidimensional NMR analysis afforded unambiguous assignments of all the proton and carbon-13 resonances of F-244.

The biosynthesis of F-244 in *Fusarium* sp. ATCC 20788 was investigated by incorporation of isotopically labelled precursors such as acetate and methionine into growing cultures. Separation and purification of the resulting labelled F-244 was followed by NMR analysis to determine the location of label.

The results show that F-244 is a polyketide metabolite derived from 7 acetate units arranged in a typical "head-to-tail" fashion; the three methyl groups and the carbon of the hydroxymethyl moiety are derived from L-methionine. The carbon-oxygen bonds at C_{14}=O and C_{12}-O are derived intact from acetate. These results define a probable sequence of events on the polyketide synthase surface, where acetate units are condensed and manipulated to give F-244.

To test this hypothesis, several of the potential intermediates, both as free acids and as N-acetylcysteamine thioesters, with strategically placed deuterium or carbon-13 labels, were synthesized and fed to growing cultures of *Fusarium* sp. ATCC 20788.

The results of feeding experiments using deuterium labelled putative intermediates showed that none of these precursors was incorporated intact. When the carbon-13 labelled intermediates were used, the ^{13}C NMR spectra showed enhancements of the signals that are derived from C-1 of acetate, indicating that the labelled intermediates had been degraded by β-oxidation.



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LIST OF ABBREVIATIONS

ACP	acyl carrier protein
ACQ	acquisition
AT	acetyl transacylase
ATCC	American type culture collection
CI	chemical ionization
CoA	coenzyme A
COSY	correlated spectroscopy
Cys	cysteine
DCC	dicyclohexylcarbodiimide
DH	dehydrogenase
DPPA	diphenylphosphoryl azide
EI	electron impact
ER	enoyl reductase
FAS	fatty acid synthase
FID	flame ionization detector
FT-IR	Fourier transformed infrared spectrometer
GC	gas chromatography
HETCOR	heteronuclear correlated spectroscopy

HMBC	heteronuclear multiple bond correlated spectroscopy
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrum
IC_{50}	inhibitor concentration required to inhibit 50% of the enzyme activity
INADEQUATE	incredible natural abundance double quantum transfer experiment
IR	infrared
KR	keto reductase
KS	keto synthase
LDA	lithium diisopropylamide
LDL	low density lipoprotein
Mp	melting point
MPLC	medium pressure liquid chromatography
MS	mass spectrometry
MT	malonyl transacylase or methyl transferase
NAC	N-acetylcysteamine
nOe	nuclear Overhauser effect
PT	palmitoyl thioesterase

PKS	polyketide synthase
TE	thioesterase
THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	tetramethyl silane
TR	acetyl transfer reaction

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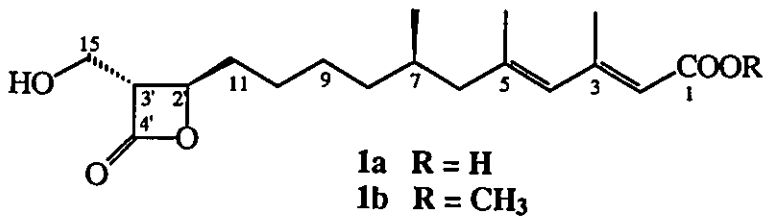
CHAPTER 1

Introduction

1.1. F-244

The natural product antibiotic F-244 (**1a**), a member of a small but growing group of naturally occurring β-lactones, is a novel antibiotic first found over two decades ago during the screening for physiologically active compounds from *Cephalosporium* sp. ACC 1233 by Aldridge's group.[1,2] Subsequently it was also isolated from *Scopulariopsis* sp. by Omura and co-workers,[3,4] and from *Fusarium* sp. by Greenspan's group.[5] The structure and stereochemistry of **1a** have been identified as (*E,E*)-11-[(3'-hydroxymethyl)-4'-oxo-2'-oxetanyl]-3,5,7-trimethyl-2,4-undecadien-oic acid with an absolute configuration of 2'*R*, 3'*R* and 7*R*.[2,6] Recently, some elegant syntheses of F-244 have also been appearing in the literature.[7-10] The structure of β-lactone F-244 is shown in **Figure 1**.



1a R = H
1b R = CH_3

Figure 1. Structure of the Antibiotic F-244

Also known as 1233A and L-659,699, this compound has been shown to be a very selective antimicrobial agent. It is active against *Bacillus subtilis*, *B. cereus*, *Escherischia coli*, *E. cloacae*, *Mycobacterium thermosphactum*, *Citrobacter freundii*, *Penicillium herquei*, and *Piricularia oryzae*, but is inactive against other yeasts, filamentous fungi and bacteria tested.[11,12] On the other hand, a patent indicates that F-244, as well as various derivatives, was active against a broad range of fungal species at unspecified concentrations.[13] Both F-244 and its tetrahydro derivative have also been reported to affect plant growth. Bean and corn plants showed necrotic lesions within 48 hours after treatment with F-244 and tetrahydro-F-244.[12]

While its antimicrobial properties and effect on plant growth could probably provide some interest for researchers involved in microbiology or herbicides, the feature of F-244 that has sparked considerable interest over the last few years is that it is the only known specific inhibitor for the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase, an enzyme involved in an early step in the biosynthesis of cholesterol. Thus, this compound is a potential candidate as an antihypercholesterolemic drug for the treatment of diseases in which the inhibition of cholesterol biosynthesis would be useful, such as atherosclerosis, hyperlipidaemia, and familial hypercholesterolemia.

1.2. Cholesterol Biosynthesis

Cholesterol, the most prominent member of the steroid family, is an important component of many eukaryotic membranes. It is the precursor of two other major classes of steroids: the steroid hormones and the bile acids. Steroid hormones play a key role in the regulation of metabolism. These hormones come in a rich variety, each interacting in a highly specific manner with a receptor protein to effect gene expression in the appropriate target tissue. Bile acids are the primary degradation product of cholesterol. The bile acids are made in the liver, stored in the gall bladder, and secreted into the small intestine. There they aid in the solubilization of lipids, facilitating their digestion by intestinal lipases.[14]

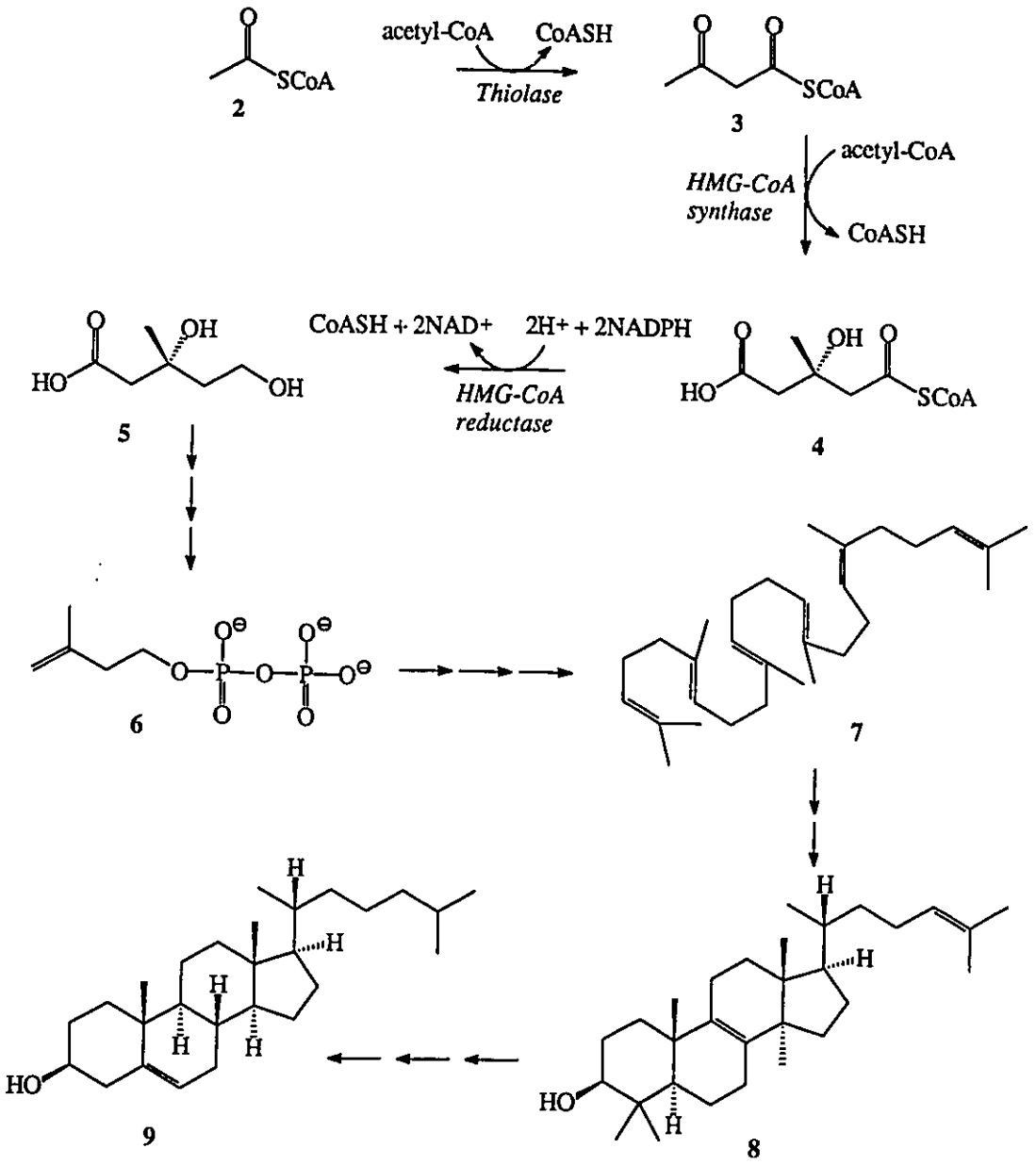
All of these biological roles of the steroids figure prominently in human well-being. Defects in cholesterol metabolism are the major cause of cardiovascular disease. Faulty regulation of cholesterol biosynthesis is one of the factors involved in the pathological process of atherogenesis, the formation of cholesterol- and lipid-rich deposits in arteries and arterioles. These deposits can limit blood flow and cause heart attacks or strokes by depriving the tissue of an adequate supply of oxygen.[15] It is no wonder that steroids are a central concern in medical biochemistry.

Cholesterol is formed from acetyl-CoA in a complex series of reactions, comprising more than 20 individual steps. The sequence of cholesterol biosynthesis (**Scheme 1**), as elucidated by Bloch, Lynen and Cornforth who were rewarded by

Nobel prizes in 1961, begins with condensation in the cytosol of two molecules of acetyl-CoA (**2**), a reaction catalyzed by acetoacetyl-CoA thiolase. The next step requires the enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase. This enzyme catalyzes the condensation of a third acetyl-CoA molecule with acetoacetyl-CoA (**3**) to yield HMG-CoA (**4**). HMG-CoA is then reduced to mevalonic acid (**5**) with HMG-CoA reductase as a catalyst. The formation of mevalonate from HMG-CoA is irreversible. Because the mevalonic acid has no metabolic future except in the formation of terpenes and steroids, this step is often referred to as the committed step in cholesterol biosynthesis.

In the next sequence of reactions three phosphate groups are attached to mevalonate, followed by loss of the carboxyl group and phosphoric acid to yield isopentenyl pyrophosphate (**6**), an activated form of an isoprene unit. Six isopentenyl groups are then assembled, with loss of their pyrophosphate groups, to produce squalene (**7**).

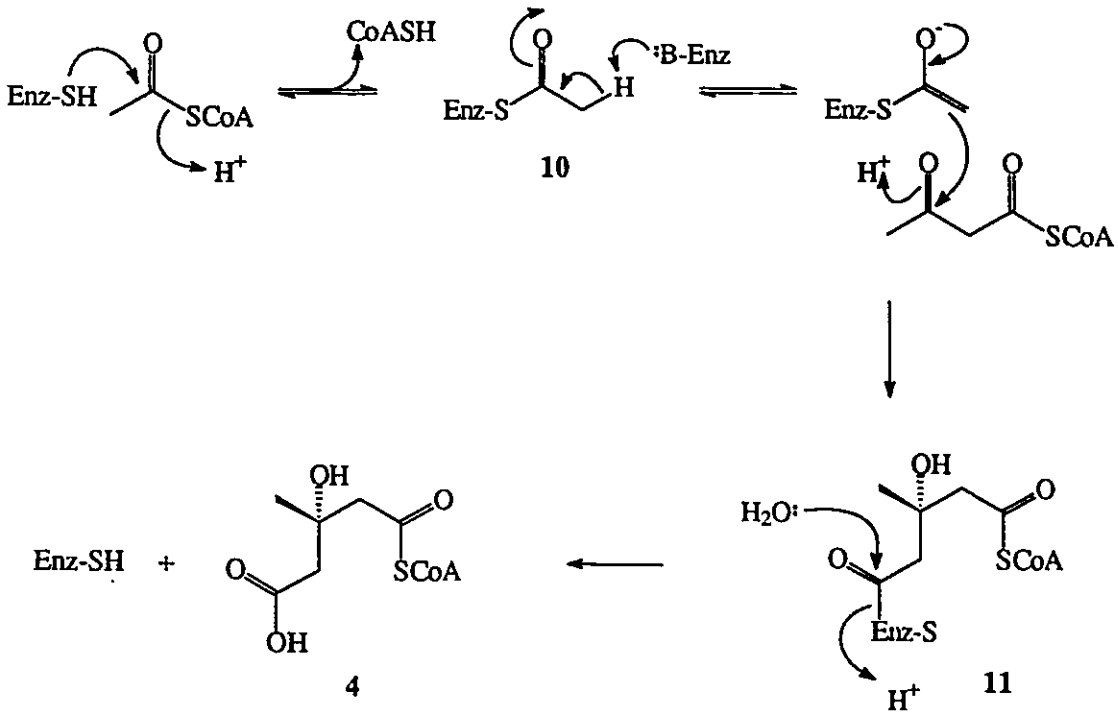
In the next series of reactions, squalene undergoes a series of complex enzymatic reactions in which its linear structure is folded and cyclized to form lanosterol (**8**). The bioconversion of lanosterol into cholesterol (**9**) involves the loss of three methyl groups, the saturation of the side chain double bond and migration of the $\Delta^{8,9}$ double bond to the $\Delta^{5,6}$ position.[15]



Scheme 1. The Biosynthesis of Cholesterol

1.2.1. Mode of Action of HMG-CoA Synthase

The mode of action of HMG-CoA synthase has been shown to involve nucleophilic attack by the thiol group on the side chain of an active site cysteine (Cys) residue, on the carbonyl carbon of acetyl-CoA to form the S-acetyl enzyme intermediate **10** (**Scheme 2**). There have been a number of studies supporting such an intermediate. Middleton and Tubbs[16,17] have demonstrated that stoichiometric release of CoA occurred when equimolar quantities of enzyme were mixed with acetyl-CoA. Miziorko *et al.*[18] have reported similar results and have identified a cysteinyl sulfhydryl as the site of acetylation. Deprotonation of this S-acetyl enzyme species produces an enolate ion, which subsequently undergoes an aldol type condensation with acetoacetyl-CoA to form a 1,5-dithioester of 3-hydroxy-3-methylglutarate (**11**). This intermediate has been trapped and partially characterized by Miziorko *et al.*[19]. Using either acetoacetyl-S[^{3}H]CoA or [^{14}C]acetyl-S-enzyme, they determined that the label from either of these sources could be trapped in the accumulated intermediate with carbon-14 activity appearing in the HMG-CoA moiety. Hydrolysis of the thioester linkage between the enzyme and the condensation product results in the release of **4** and the free enzyme, accounting for the irreversible nature of the overall reaction.

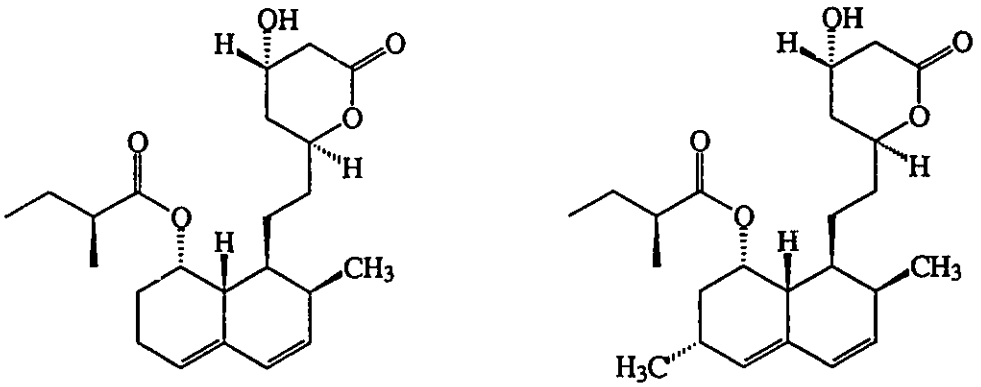


Scheme 2. Mode of Action of HMG-CoA Synthase

1.3. F-244 as a Specific Inhibitor of HMG-CoA Synthase

Epidemiological data revealed the surprising fact that more than half of the people in western industrialized societies have a high level of circulating low density lipoprotein (LDL), particles carrying cholesterol that put them at a high risk of developing atherosclerosis. Brown and Goldstein have demonstrated the mechanism by which the LDL receptors influence cholesterol and atherosclerosis.[20] Since this

evidence was presented, much attention has been directed toward the search for inhibitors of cholesterol biosynthesis as potential antihypercholesterolemic agents. Compactin (ML-236B) (**13**), a fungal metabolite isolated from the strains *Penicillium brevicovactum* and *Penicillium citrinum*[21,22], and mevinolin (monacolin K) (**14**), isolated from *Aspergillus terreus*, and *Monascus* sp.[23,24] (**Figure 2**) were discovered as potent inhibitors of HMG-CoA reductase and were shown to lower plasma cholesterol in humans and dogs.[25,26] Their analogs, pravastatin (CS-514) and simvastatin, have been developed and approved as clinical drugs.[27,28]



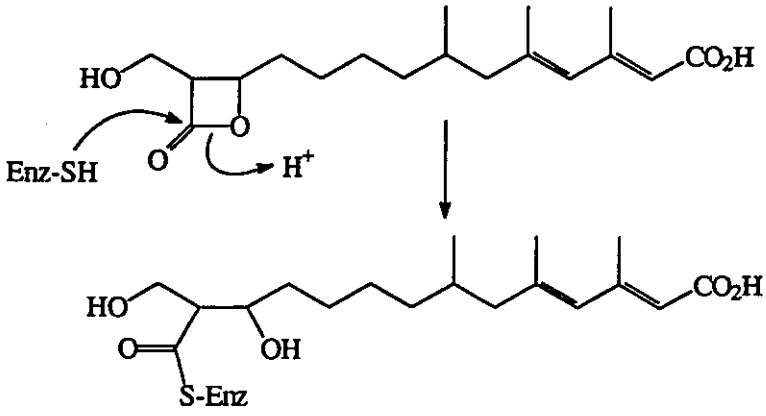
13 **14**

Figure 2. The Structure of Compactin (**13**) and Mevinolin (**14**)

These discoveries were responsible for generating a great deal of interest in finding related compounds and identifying other natural products which act as

inhibitors of cholesterol biosynthesis. During the course of screening for physiologically active metabolites, Omura *et al.*[4] discovered that F-244 is a potent and specific inhibitor of HMG-CoA synthase, with IC_{50} = 0.2 μM. This compound is the only known specific and potent natural inhibitor of HMG-CoA synthase that has ever been reported. Early reports by Greenspan described that the inhibition is reversible upon dilution of the enzyme-inhibitor complex.[30] However, this claim has been disputed by Sunazuka *et al.*[31] and Mayer *et al.*,[32] who claim the inhibition to be irreversible over several hours even after dilution of the enzyme-inhibitor complex. Later, Greenspan *et al.*[33] also reported that indeed inhibition is irreversible *in vitro*, but showed that the inhibition is reversible in culture cells and animals.

A mechanism for the inhibition of HMG-CoA synthase by F-244 has been proposed by Mayer *et al.*[32] (**Scheme 3**). Since the initial step in the catalytic cycle involves nucleophilic attack by an active site cysteine (Cys) residue on the carbonyl group of acetyl-CoA, Mayer proposed that the inactivation of the enzyme is due to the β-lactone functionality. It was suggested that nucleophilic attack by the sulfhydryl group of the active site Cys occurs at the carbonyl carbon of the β-lactone ring. Subsequent ring opening would then proceed to give a covalently bound enzyme-inhibitor thioester complex, effectively inactivating the enzyme.



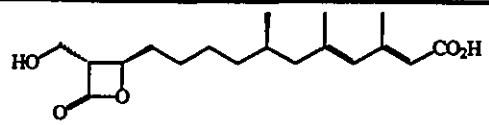
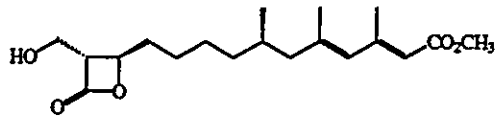
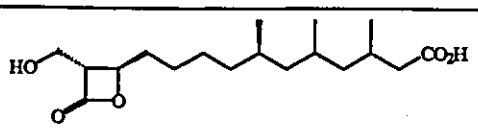
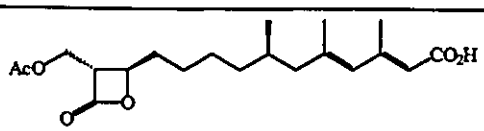
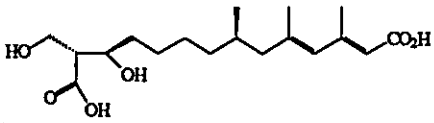
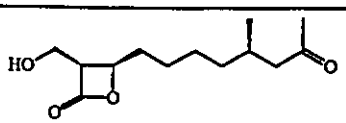
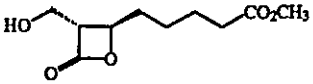
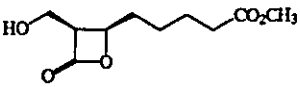
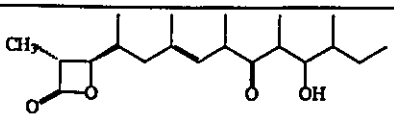
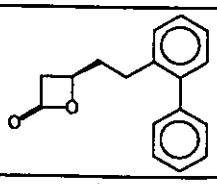
Scheme 3. A Possible Mechanism for the Inhibition of HMG-CoA Synthase by F-244

However, the β-lactone moiety is not the only factor required for inhibition of HMG-CoA synthase. There have been numerous independent studies directed towards determining the component of F-244 necessary for inhibition of HMG-CoA synthase **(Table 1)**.

It has been found that reducing the length of the carbon side chain, e.g., **15,** dramatically reduced the potency of the inhibitor, indicating that the length of the carbon side chain is another factor affecting the inhibitory activity.[5,33] The presence of double bonds is also important. The saturated derivative (**16**) was about 25% as active as the native **1a**.[5,11] It has been found that only the *trans*-β-lactone **17** was active against HMG-CoA synthase, whereas the *cis*-β-lactone **18** showed no inhibitory activity.[31] While the methyl ester derivative (**1b**) exhibited activity comparable to **1a**, demonstrating that the carboxyl group can be modified without significant loss of

activity, the acetyl derivative (**19**) has considerably lower activity, showing that the hydroxymethyl moiety of **1a** is important for potent inhibition, perhaps in spatial interaction with the enzyme.[30,31] Opening the β-lactone ring (**20**) resulted in complete loss of inhibitory activity, which indicates that the lactone ring is essential for potent inhibition of HMG-CoA synthase. Interestingly, ebelactone A (**21**), a metabolite isolated from *Streptomyces* sp. MG7-G1[34], which has a somewhat similar structure to F-244, has no activity against HMG-CoA synthase.[11] On the other hand (±) β-butyrolactone (**23**) was found to inhibit HMG-CoA synthase, although it was very weak compared to **1a**.[35]

Table 1. Inhibitory Activities of Various F-244 Analogs

Compound #	F-244 Analog	IC_{50} (μM)[a]	Reference
1a		0.10[b] 0.34[c] 0.20[c]	5 31 4
1b		0.10[b] 0.27[c]	5 11
16		0.40[b] 0.43[c]	5 11
19		2.0[b] 4.51[c]	5 11
20		no activity[b] no activity[c]	5 11
15		1.6[b] 1.5[b] 115[c]	5 33 33
17		2.08[c]	31
18		>2.31[c]	31
21		no activity[c]	11
22		9.0[b] 12.0[c]	33
23		2000[b]	35

[a] IC_{50} is the concentration of inhibitor required to inhibit 50% of the enzyme activity
[b] enzyme activity measured after 5 minute incubation with the inhibitor
[c] enzyme activity measured without prior incubation with the inhibitor

1.4. Polyketide and Fatty Acid Biosynthesis

Acetic acid is one of the most common "building bricks" used by living organisms for generating complex molecules. Besides being used in fatty acid synthesis, acetic acid – in its activated forms as acetyl CoA and malonyl CoA – is the most important carbon atom source for two large classes of natural substances, the polyketides and the terpenes. Linear Claisen-type condensation leads to β-keto esters, which either by reduction and repeated condensation give fatty acids or by further direct condensation give polyketides. They, in turn, can cyclize to a vast number of aromatics. In a branched condensation, the keto function of acetoacetyl CoA reacts with another acetyl CoA to form β-hydroxy-β-methylglutaryl CoA which is transformed to mevalonic acid and ultimately to the terpenoids and steroids.[36]

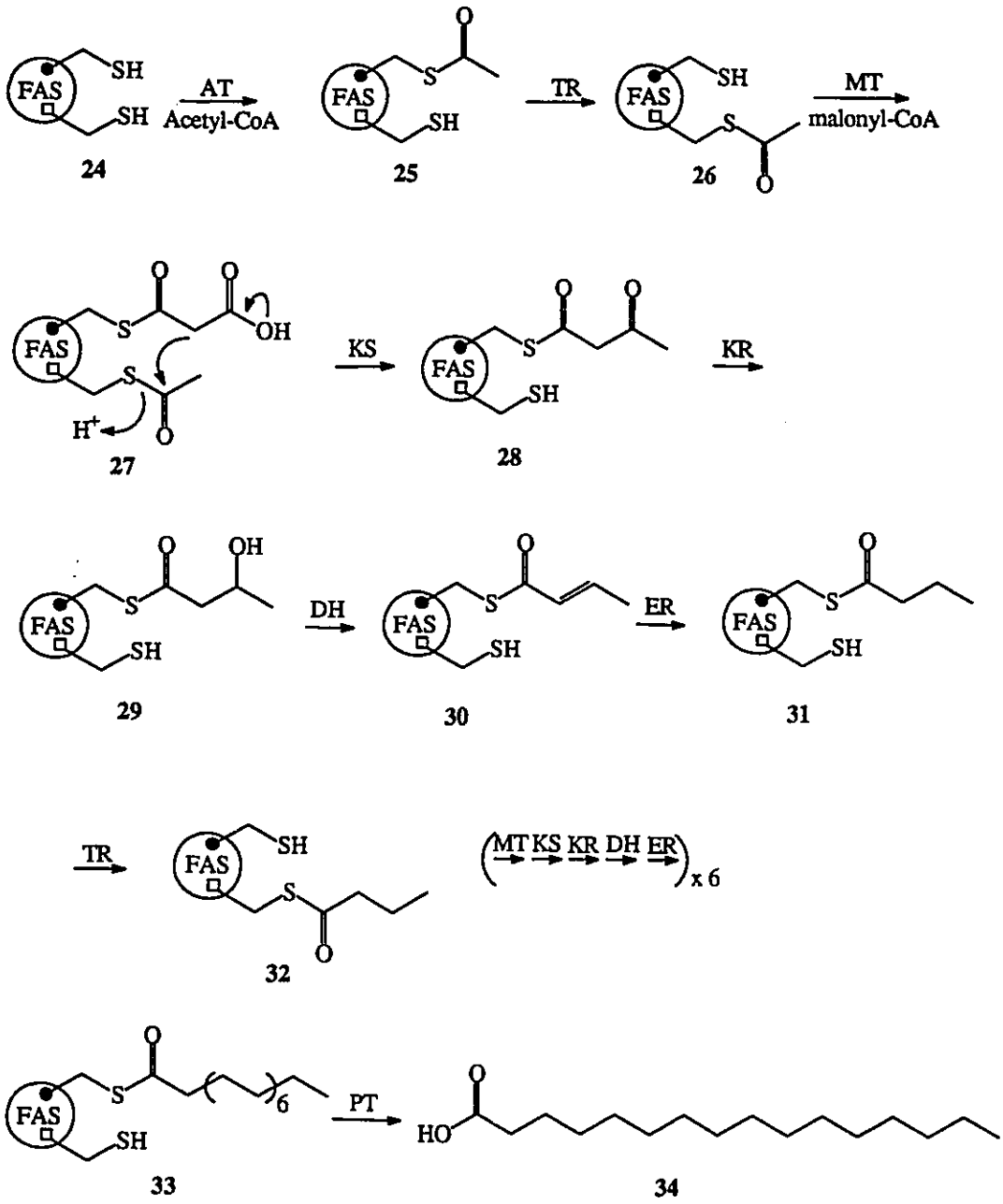
1.4.1. Biosynthesis of Fatty Acids

The ubiquity of many of the common fatty acids, and the vital roles many of them have, puts them into the class of primary metabolites, and it is only the more unusual or uncommon fatty acids that can be considered to be true secondary metabolites.

The construction of fatty acids in all organisms takes place by an essentially universal reaction sequence. They are synthesized on a multifunctional enzyme

complex called fatty acid synthase (FAS), which shows various patterns of both structural and functional variation depending on its origin. Type II FASs, found in most prokaryotes and plants, comprise eight structurally independent and monofunctional enzymes, all of which can be isolated separately. In eukaryotic organisms, all these functionalities are combined with all the catalytic activities required to assemble the fatty acid present in the domains of these multifunctional proteins, termed type I FAS.[37]

Palmitic acid is one of the most common saturated fatty acids produced in animals. Palmitic acid is generated by initiating the fatty acid chain with the acetyl group from acetyl-CoA and adding two carbons at a time from 7 malonyl-CoA molecules. The growing fatty acid chain is covalently bound to the enzyme during the catalytic reactions; it is attached through a thioester linkage to the sulfhydryl group of 4'-phosphopantethoic acid which is covalently linked to a serine residue of the protein.[38] The specific sequence of reactions is shown in **Scheme 4**. The sequence of enzyme activities is as follows: acetyl transacylase (AT), acetyl transfer reaction (TR), malonyl transacylase (MT), β-ketoacyl synthase (KS), β-ketoacyl reductase (KR), dehydratase (DH), and enoyl reductase (ER). In the chicken liver enzyme, the acetyl and malonyl transacylase activities are combined in a single enzyme.[39] When the fatty acid chain of the intermediate is saturated (**30** → **31**), another malonyl residue is transferred to the enzyme (**26** → **27**) and two more carbon atoms are added.



Scheme 4. Reaction Sequence of Fatty acid Biosynthesis.

This cycle continues until a palmitoyl thioester is formed, which is cleaved by palmitoyl thioesterase (PT) to give the free acid.

1.4.2. Biosynthesis of Polyketides

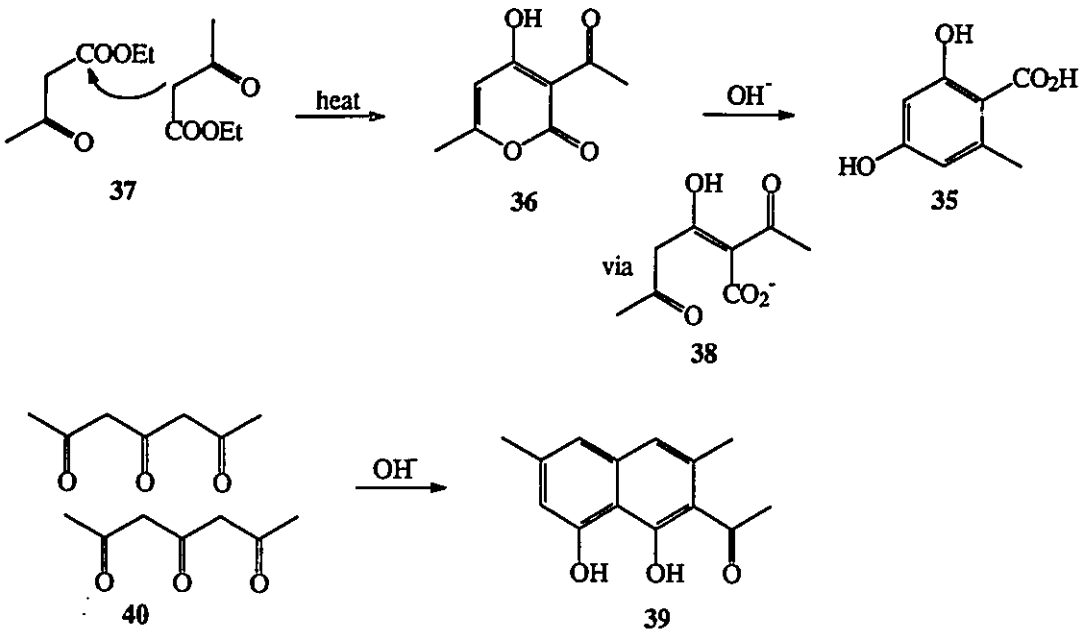
The term polyketide defines a class of molecules produced through the successive condensation of small carboxylic acids to build a carbon chain. The building units are of various kinds: commonly, they are residues of acetate, propionate, and butyrate, but sometimes are more complex. Each unit contributes two carbon atoms to the assembly of the linear chain, of which the β-carbon always carries a keto group. Typically, some of these keto groups are reduced to hydroxyls, or removed, after addition of a unit to the chain. The occurrence of the remaining keto groups at many of the alternate carbon atoms of the chain give rise to the name "polyketide" for this family of compounds.

The second carbon atom donated by each unit to the growing polyketide chain carries different substituents depending on its origin: only hydrogen in the case of acetate residues, a methyl or ethyl group for propionate or butyrate, and other for rarer building units. This side-chain variation, together with the varied fate of each of the keto groups, the possibility of chirality at one or more carbon atoms, and the total length of chain, account for the huge potential diversity built into primary structures of

the molecules by genetic "programming" of the polyketide synthases (PKS) that catalyze chain building.

Polyketides are classified as secondary metabolites, so-called because they occur sporadically, and are probably adaptive only under particular conditions, in contrast to the primary metabolites that provide the structure and energy requirements of all living cells.[40] Their production is usually connected with several external factors, such as replicatory growth, flowering, season, temperature, habitat, length of daylight, etc.[37].

As early as 1907, Collie studied the chemistry of polyacetyl (ketomethylenic) compounds and noticed that products identical with, or similar to others already known in nature, could be obtained from these ketomethylenic substances using reactions catalyzed by base.[41] Orselinic acid (**35**) was obtained in this manner from dehydroacetic acid (**36**), which was itself obtained by pyrolysis of acetoacetic ester (**37**). Similarly, the dihydroxynaphthalene **39** was produced from 1,3-diacetyl-acetone (**40**), **Scheme 5**.



Scheme 5.

Collie's idea was not followed up until Birch and Donovan, in 1953, rediscovered and proposed it as a general biogenetic hypothesis.[42] They showed that [1-^{14}C]acetate was incorporated into 6-methyl salicylic acid (**41**) which is produced by many microorganisms, and certain higher plants. The more complex metabolite, griseofulvin (**42**) isolated from *Penicillium griseofulvum* was also studied using [1-^{14}C]acetate. Degradation of **42** gave a labelling pattern consistent with the hypothesis.

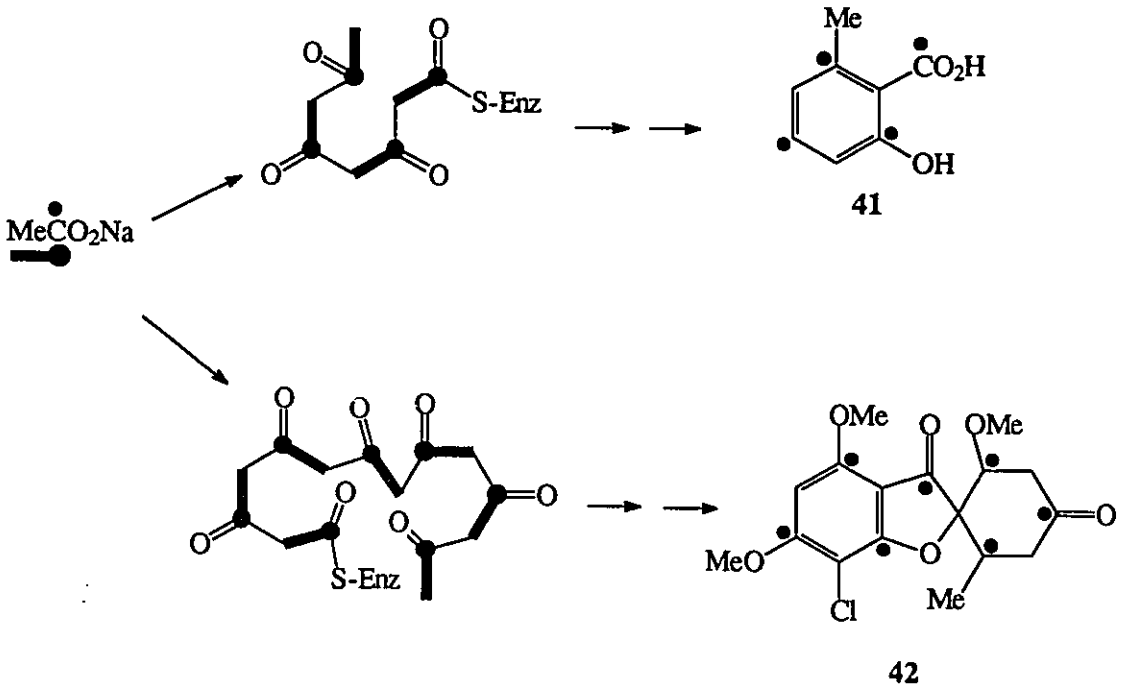


Figure 3. Birch's Observations of Acetate Derived Compounds which Lead to the "Acetate Hypothesis"

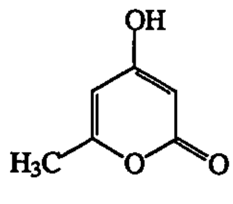
Birch's ^{14}C-labelling pattern of griseofulvin has been confirmed by ^{13}C-labeling and ^{13}C-NMR techniques (*vide infra*), and the methyl groups of the methyl ether moieties are derived from S-adenosyl methionine.[43,44]

One of the most impressive features of the "acetate hypothesis" was its predictive capabilities. Many partial structures of metabolites were known for which ambiguity of total structure existed. Consideration of their probable biogenetic derivation from acetate often led to assignment of a unique total structure.

The diversity of polyketide structures is a direct result of two metabolic processes: the initial formation of a few, basic skeletal types, and the diverse secondary transformations of these structures. Among the smallest are triacetic acid lactone (**43**), a six-carbon chain that cyclizes to form a six-membered lactone ring, and the antibiotic furanomycin (**44**), a linear seven carbon chain that undergoes epoxidation and transamination[45]. The largest known polyketide is brevitoxin B (**45**) with 50 carbon atoms in its chain (**Figure 4**). Many acetate-derived polyketides cyclize to form aromatic rings (e.g., anthracyclines) or lactone rings (e.g., macrolides, polyenes). Many cyclized polyketides undergo glycosidation at one or more sites (e.g., macrolides, anthracyclines), and virtually all are modified during their secondary transformations through hydroxylation, reduction, epoxidation, alkylation, etc.

Many polyketides can also be the source for other polyketide metabolites. One example is **41**. This acid is the source of a variety of metabolites in which hydroxylation and oxidation of the aromatic nucleus and side-chain methyl group occur as major pathways following decarboxylation to m-cresol (**47**).[46]

The [1-^{14}C]acetate labelling in patulin (**46**) showed that no regular polyketide chain is apparent, but the incorporation of radioactive **41** and other related compounds indicate that patulin arises by cleavage of the aromatic ring, **Scheme 6**.[47,48]



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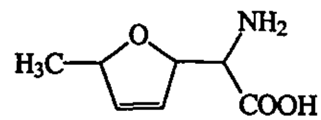

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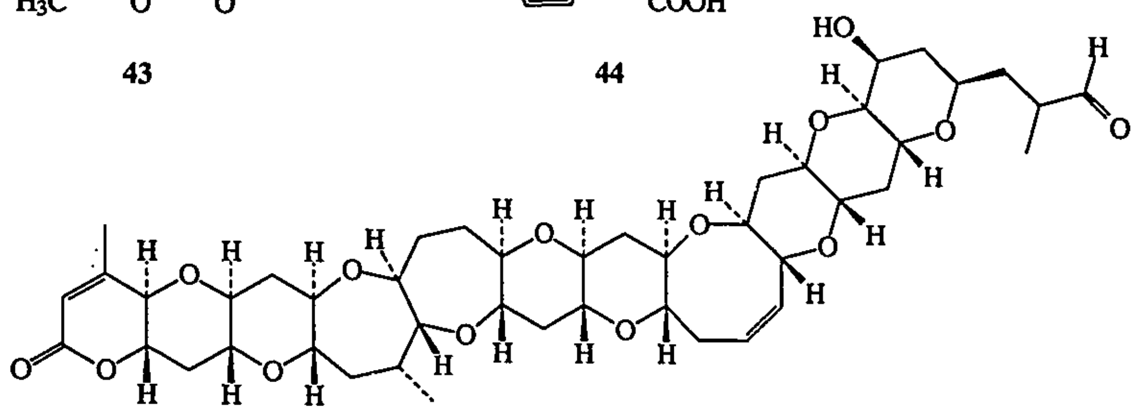

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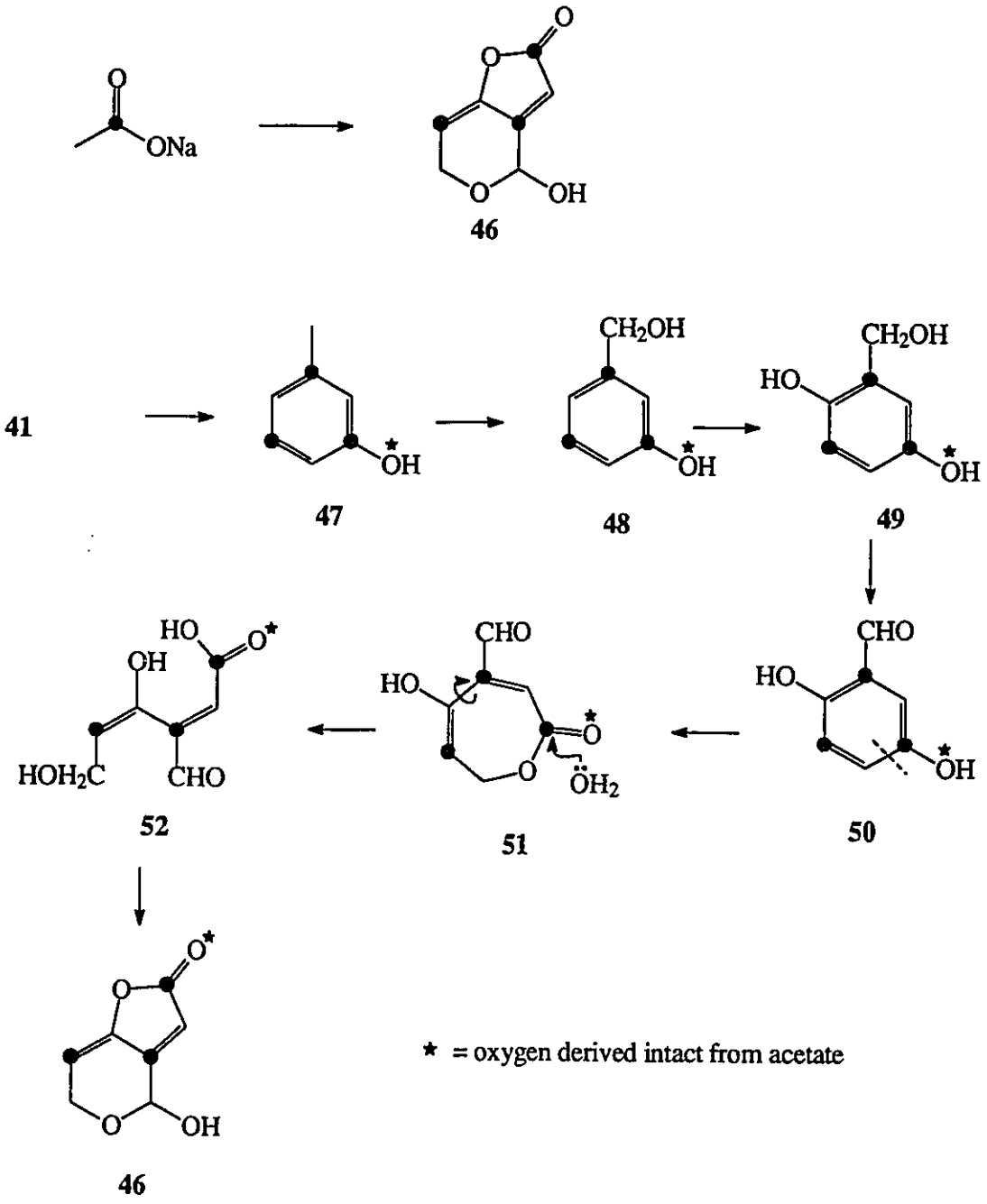
Figure 4. Structure of the Smallest and the Largest Known Polyketides, Triacetic Acid Lactone (**43**), Furanomycin (**44**) and Brevitoxin B (**45**)

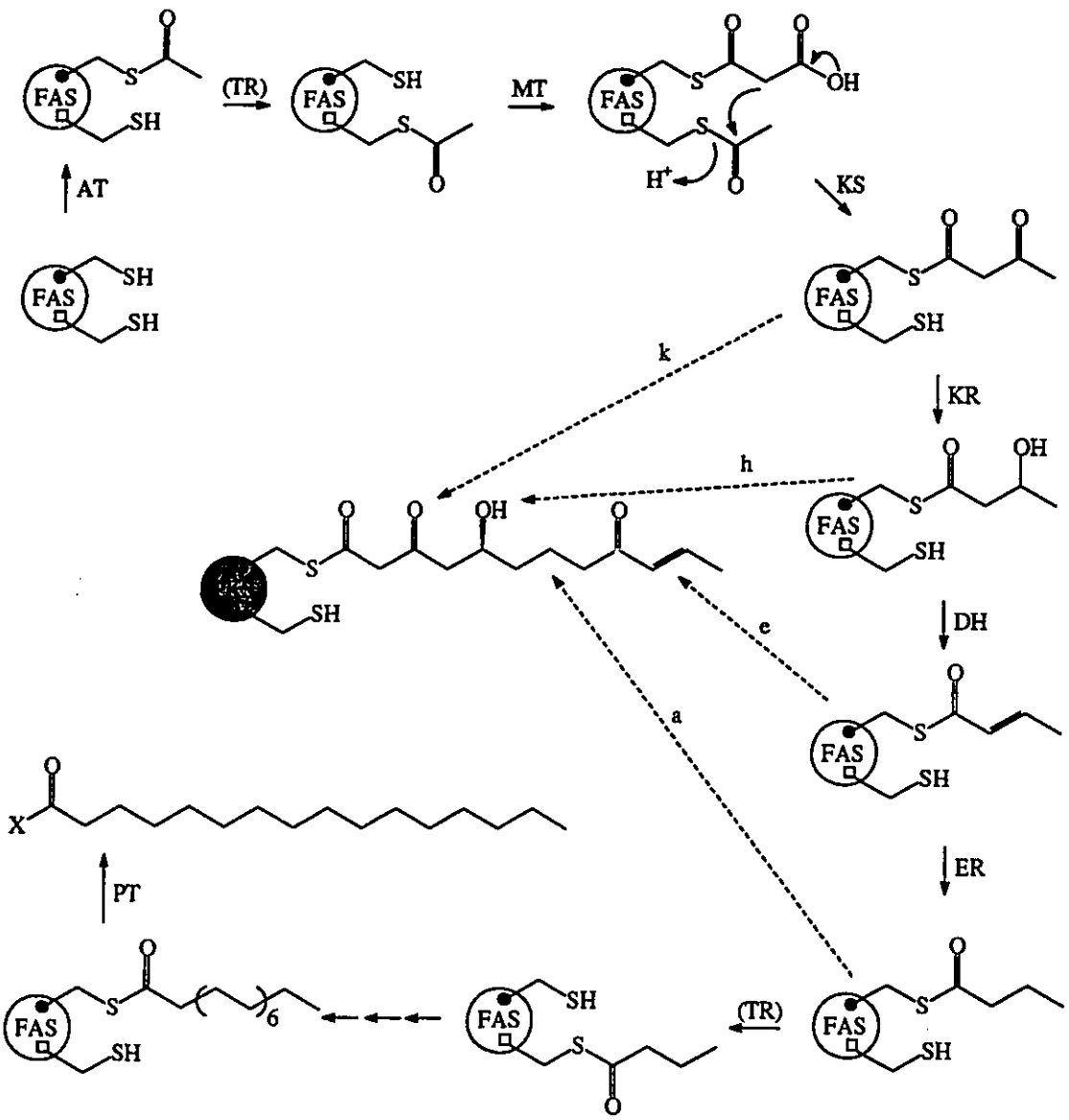


Scheme 6. The Biosynthetic Pathway of Patulin (**46**)

1.4.3. Analogies between Polyketide and Fatty Acid Biosynthesis

The multienzyme polyketide synthases responsible for catalyzing the formation of the carbon backbones of macrolide, polyether, and related antibiotics, predominantly in *Streptomyces* species, have become the subject of intense interest recently.[49-52] The enzymes operating on these pathways are able to assemble the backbone of these complex secondary metabolites, starting typically from activated coenzyme-A derivatives of acetate, propionate, and butyrate. In general, the mode of assembly is believed to involve chemistry which is closely related to that catalyzed by the FAS, except that a regular pattern of chain elongation — reduction — elimination — reduction steps, during the biosynthesis, apparently does not take place. Investigations using molecular biology suggest that the assembly process can bypass certain reductive steps in particular cycles to generate a functionalized carbon skeleton.[53,54] This leads to incorporation of keto, hydroxy, or olefinic functionality in the polyketide chain as it grows.

Scheme 7 shows a schematic representation of fatty acid and polyketide biosynthesis. The circle labelled FAS or PKS represents the fatty acid or polyketide synthase, carrying two thiol groups, one on the β-ketoacyl synthase (condensing enzyme) (□) and the other on the acyl carrier protein (●). The reaction steps are labelled: AT, acetyl transferase; (TR), acetyl transfer reaction, not unambiguously assigned to a specific enzyme component; MT, malonyl transferase; KS, β-ketoacyl



Scheme 7. Schematic Representation of Fatty Acid and Polyketide Biosynthesis

synthase; KR, ketoreductase; DH, dehydrase; ER, enoyl reductase; PT, or TE, palmityl transferase or thioesterase, involved in chain termination to produce palmityl CoA (X = CoA) or free palmitic acid (X = OH) respectively. Arrows k, h, e, and a, represent various possibilities that follow each condensation step to give keto, hydroxyl, or alkyl functionality at specific points in the polyketide product.

In the simplest form outlined above, fatty acid synthesis involves few programmed choices on the part of the FAS; carbon chain length —determined by the number of successive additions of acetyl residues— is the primary one. However, the situation in reality is somewhat more complex. Thus, the synthesis of unsaturated fatty acids in bacteria, depends on a lack of enoyl reduction after the dehydration step at one or two points in chain assembly so that a double bond remains (in mammals, such double bonds are introduced by specific desaturases after chain building is complete, rather than being programmed into chain assembly).[15]

Polyketide biosynthesis may be considered to involve an extension of the limited set of choices made by a FAS, so that PKS needs to be more highly programmed. One example is the biosynthesis of monensin A (**53**)[55-57] in which the PKS makes many choices during chain assembly: a choice of 13 building units in a specific order from acetyl, propionyl, and butyryl residues; a choice of four possibilities for the chemistry after each of the 12 steps in chain building, giving keto, hydroxyl, enoyl, or alkyl functionality at each particular alternating position in the

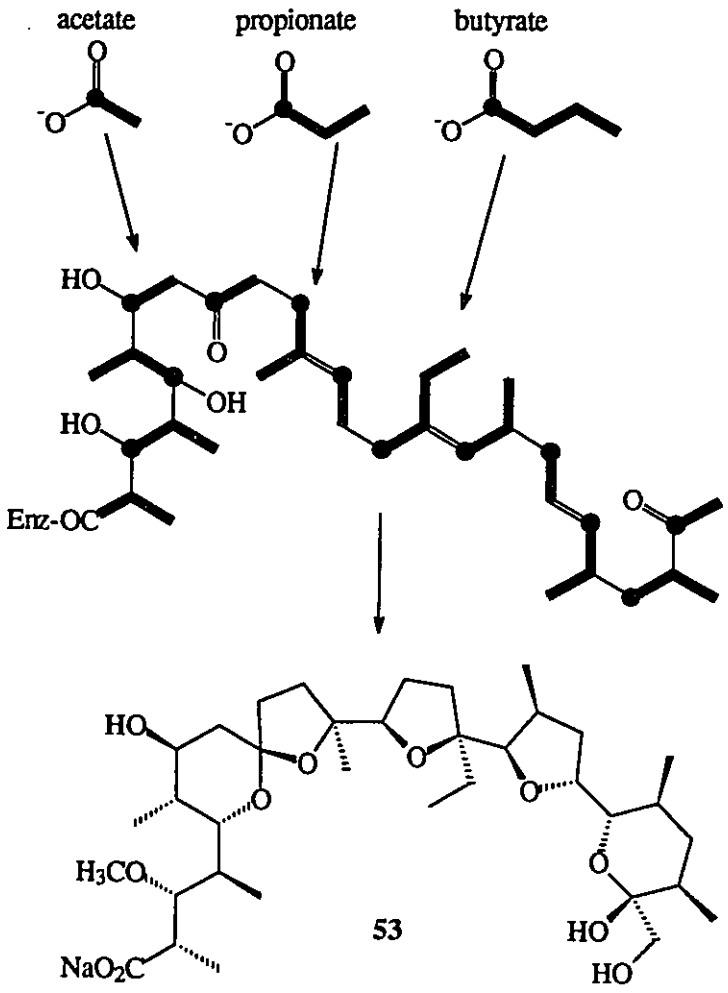


Figure 5. The Biosynthesis of Monensin A

chain; and the correct choice of stereochemistry at 12 positions where R or S configurations of methyl or hydroxyl groups are possible (**Figure 5**). Thus the monensin PKS constructs just one of an enormous number of theoretically possible

carbon chains. Understanding the basis of this programming is the challenge that is believed can now be realistically addressed by the combination of biochemistry and chemistry with the currently developing molecular genetics of polyketide producers.

1.5. The Use of Stable Isotopes in Biosynthetic Studies

The experimental study of biosynthesis has developed largely through the application of tracer studies using compounds isotopically labelled with ^{14}C or ^{3}H. Today, the explosive growth of new techniques in nuclear magnetic resonance spectroscopy has led to the widespread adoption of ^{13}C and ^{2}H as isotopic labels. This rapid, and non-destructive technique for detecting specifically the sites of enrichment in a molecule has contributed to and extended the knowledge of biosynthetic pathways.[58] The complete assignment of ^{1}H and ^{13}C nuclear magnetic resonances of a natural product is usually a prerequisite for ensuing biosynthetic experiments in which stable isotopes are used.

An important advantage in the use of stable isotopes is that studies can be conducted with doubly-labelled precursors such as sodium [1,2-$^{13}C_2$]acetate. [1,2-$^{13}C_2$]Acetate is normally assimilated into a metabolite under conditions where it will be diluted by the organism's own pool of unlabelled acetate. The metabolite will then be formed with almost no likelihood that two labelled acetate units are adjacent to one another. In the NMR spectrum, coupling will now be seen between the signals for ^{13}C

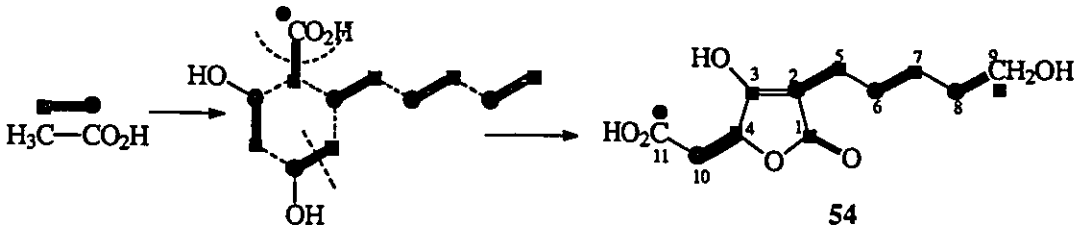
labels of intact acetate units. This approach provides a detailed picture of the bonds broken or formed during the biosynthetic processes of building up the molecule. In multiple label studies, ^{2}H and ^{18}O are often used in conjunction with ^{13}C, such as $CD_3{}^{13}COONa$ or $CH_3{}^{13}C^{18}O_2Na$, providing evidence concerning the integrity of carbon-hydrogen bonds or the origin of carbon-oxygen bonds.

1.5.1. Probing the Origin of the Carbon Skeleton

The first natural abundance ^{13}C NMR spectra were reported by Lauterbur[59] and by Holm[60] in 1957. However, instrumental limitations, combined with the low natural abundance of carbon-13 (1.108 %) and its low sensitivity due to its small magnetogyric ratio, delayed the application of ^{13}C NMR to biosynthetic work until 1970.[61]

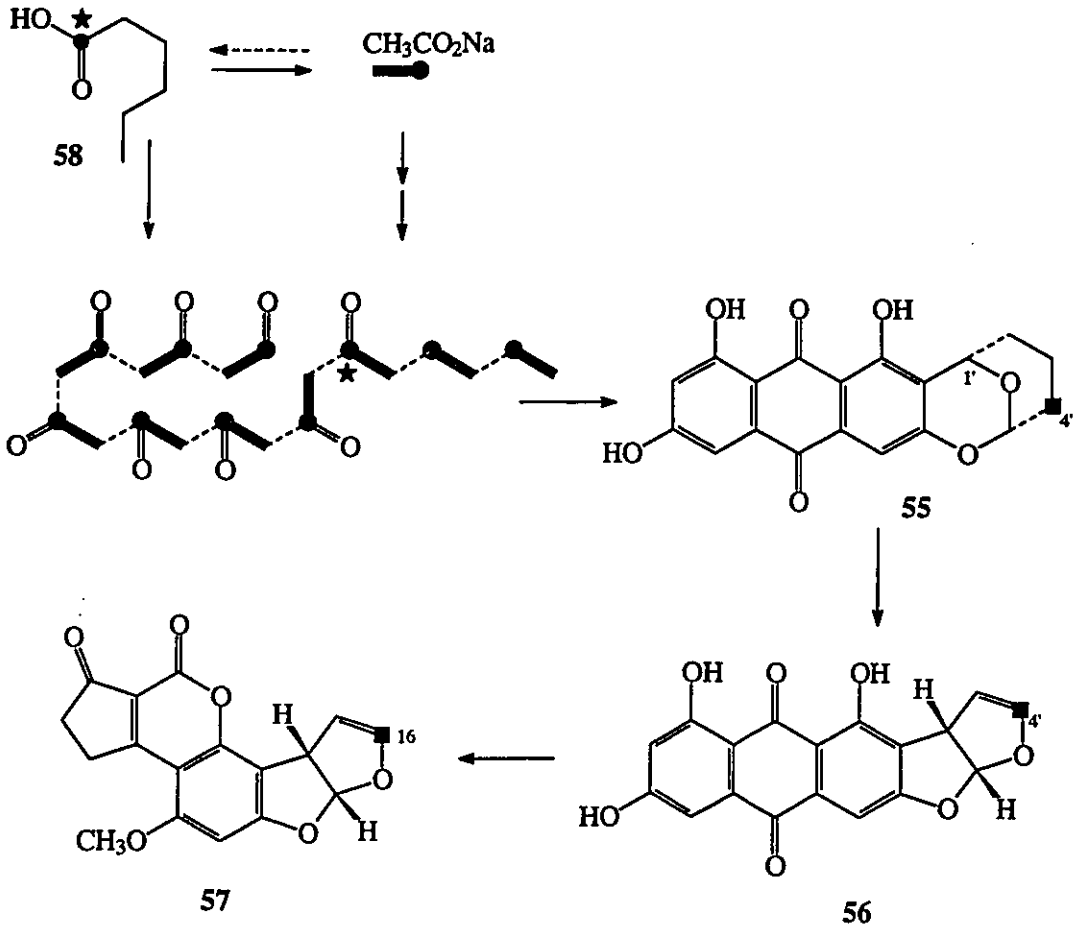
Incorporation of ^{13}C label from a precursor leads to enhanced NMR signals in the metabolite derived from it. Multicolic acid (**54**) biosynthesized from [^{13}C]acetate is one example. Enhanced NMR signals in **54** for C-1, C-3, C-5, C-7, C-9, and C-10 from [2-^{13}C]acetate and complementary results with [1-^{13}C] acetate outline the precursor labelling pattern. When [1,2-$^{13}C_2$]acetate was used as a precursor, the ^{13}C NMR signals showed coupling between C-8 and C-9, C-7 and C-6, C-5 and C-2, C-10

and C-4, but C-1, C-3 and C-11 appeared as singlets. Therefore, it was proposed that the likely biosynthesis of 54 proceeds as shown in **Scheme 8.**[62]



Scheme 8. Proposed Biosynthesis of Multicolic acid (54)

Advanced ^{13}C-labelled polyketide precursors can sometimes be incorporated intact by fungi. In an elegant series of experiments, Townsend and co-workers showed that [4'-^{13}C]averufin (**55**; ■ = ^{13}C) was efficiently transformed by *Aspergillus parasiticus* into [4'-^{13}C]versicolorin A (**56**; ■ = ^{13}C) and into [16-^{13}C]aflatoxin B_1 (**57**; ■ = ^{13}C), as shown in **Scheme 9.**[63,64] Subsequently, these workers also succeeded in obtaining specific incorporation of label from [1-^{13}C]hexanoic acid (**58**; ● =^{13}C) into



★ = ^{13}C Derived intact from hexanoic acid

Scheme 9.

C-1' of averufin (**55**), thereby suggesting that this acid can act as the polyketide starter unit.[65] The ^{13}C NMR spectrum of a sample of averufin that had been derived from these feedings showed a three-fold enhancement of the resonance for C-1' and increases in intensities of peaks of other carbons that had been derived from carboxyl of acetate, corresponding to much smaller enrichment. The latter enrichments are due

to the degradation of **58** to [1-^{13}C]acetate by β-oxidation, a process which is, at least formally, the reverse of fatty acid biosynthesis.

The process of β-oxidation normally occurs during attempts to incorporate fatty acids or related compounds into polyketides, which has become an inherent problem in attempts to incorporate advanced precursors. However, long fatty acids are often incorporated intact. For example [1-^{13}C]octanoate was incorporated intact into fungichromin (**59**) by *Streptomyces cellulosae* with little observable degradation. The remaining carbons of fungichromin were shown to come from acetate and propionate (**Figure 4**).[66] [1-^{13}C]Butyrate was also shown to be efficiently incorporated into ebelactone B (**60**) by *Streptomyces* sp. MG7-G1.[34]

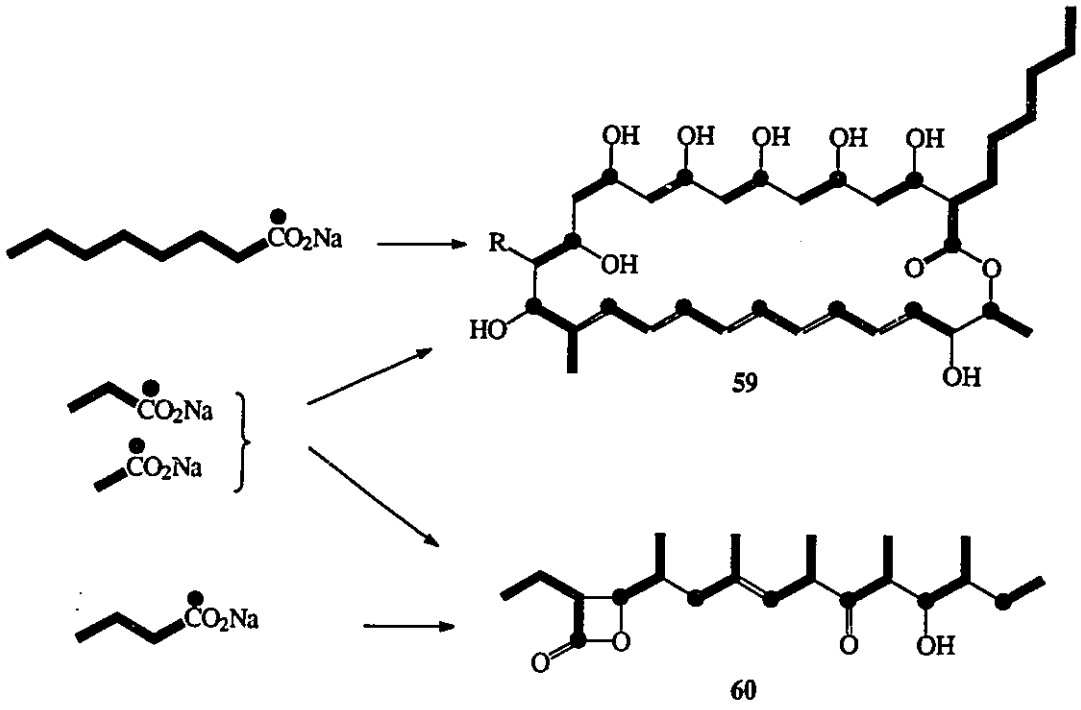


Figure 6. The Biosyntheses of Fungichromin (**59**) and Ebelactone B (**60**)

1.5.2. Probing the Origin of Pendant Methyl Groups

Two sources of pendant methyl groups are commonly found in polyketides, propionate and methionine. The former is generally only utilized in bacteria (e.g., in the biosynthesis of **60** (**Figure 6**), whereas fungi usually use the latter. This has been further confirmed in the following biosynthetic investigations, which illustrate this general phenomenon.

Fusalnipyrone (**61**) isolated from *Fusarium solani* DSM 62416 was formerly thought to be a terpenoid metabolite. The incorporation of six deuterium atoms from

[*methyl*-$^{2}H_3$]methionine into this metabolite has established that **61** is not a terpene but a true polyketide with the pendant methyl groups derived from methionine.[67] This study also suggests that the related metabolite nectriapyrone (**62**) is similarly derived from acetate and methionine (**Figure 7**).

The isolation and structure of cubensic acid (**63**) has been reported from cultures of the fungus *Xylaria cubensis*.[68] A biosynthetic rationale was proposed on the basis of structural similarities to the polyether and macrolide antibiotics, that **63** would be derived from acetate and propionate units. However, it was found that all of the eight pendant methyl groups were enriched after the incorporation of L-[*methyl*-^{13}C]methionine.[69]

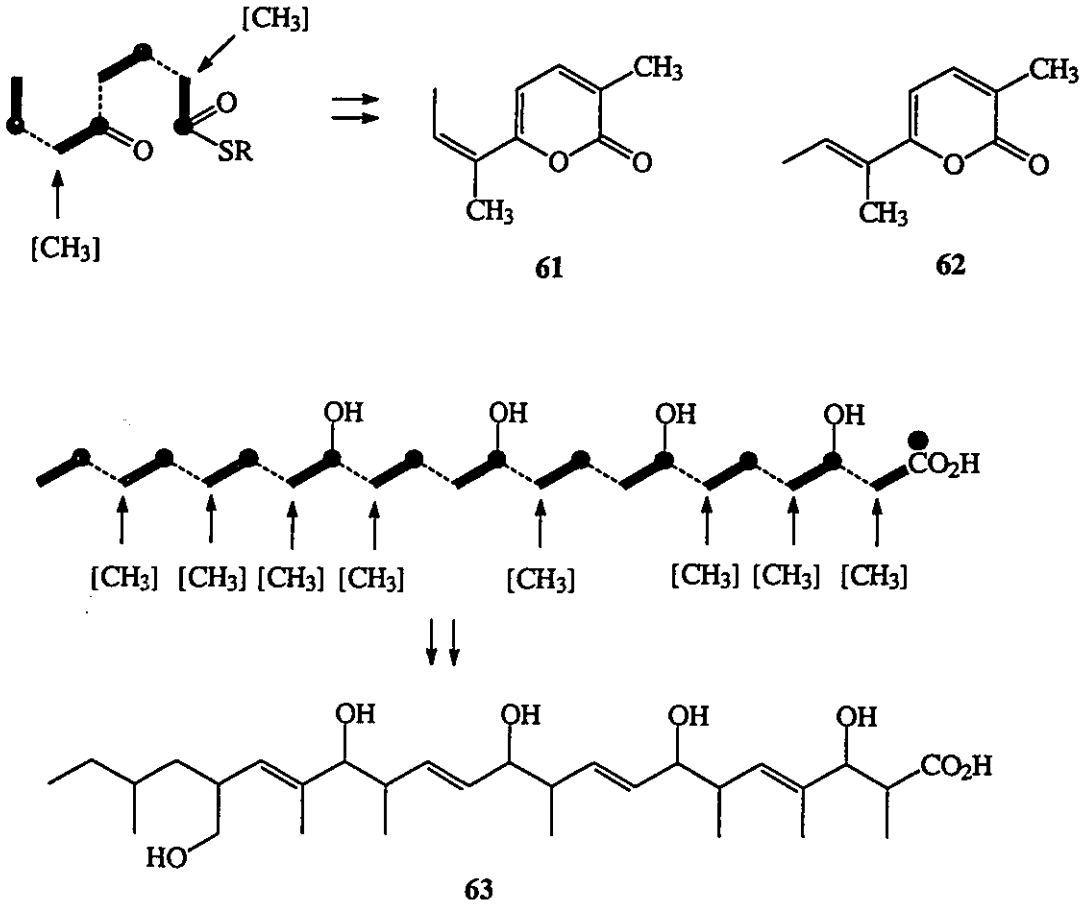


Figure 7. Methionine as the Source of Pendant Methyl Groups in the Biosynthesis of Polyketides Produced by Fungi. [CH_3] represents the methyl group of methionine.

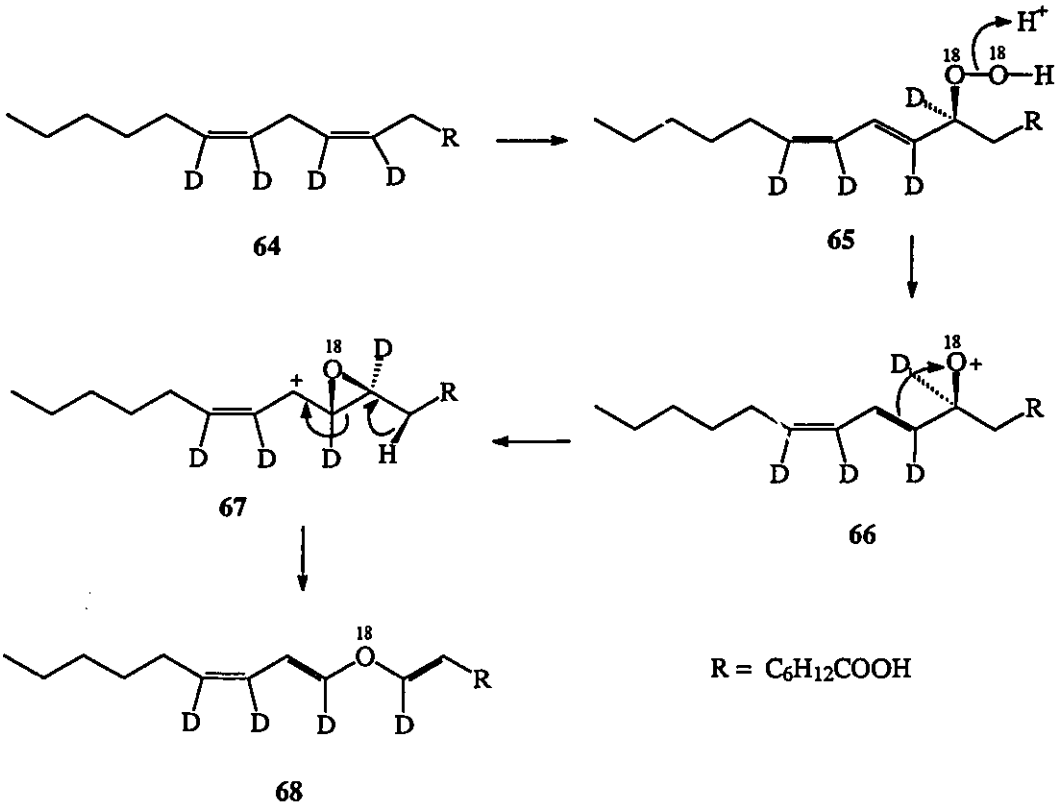
1.5.3. The Use of ^{2}H and ^{18}O Labelled Precursors.

Although most studies in which easily accessible deuterated precursors are used now employ some form of multiple labelling with another isotope, usually ^{13}C, direct ^{2}H NMR is often the most reliable way to determine the actual content of

deuterium at a particular site, and to obtain stereochemical information.[70,71] In the biosynthesis of polyketides, a starter acetate unit usually retains most of the deuterium of [$^2H_3$]acetate. However, extensive loss of deuterium by exchange with the medium often occurs at subsequent units along the chain. Staunton and co-workers reported that such exchange occurs after sodium [$^2H_3$]acetate has been incorporated into mellein by *Aspergillus melleus*, and into alternariol by *Alternaria alternata*.[72]

One of the advantages of ^2H NMR spectroscopy is the ability to observe directly the stereochemistry of hydrogen labels in many cases. If a stereospecifically deuterated precursor is transformed, considerable information about possible biochemical pathways and mechanisms becomes available.

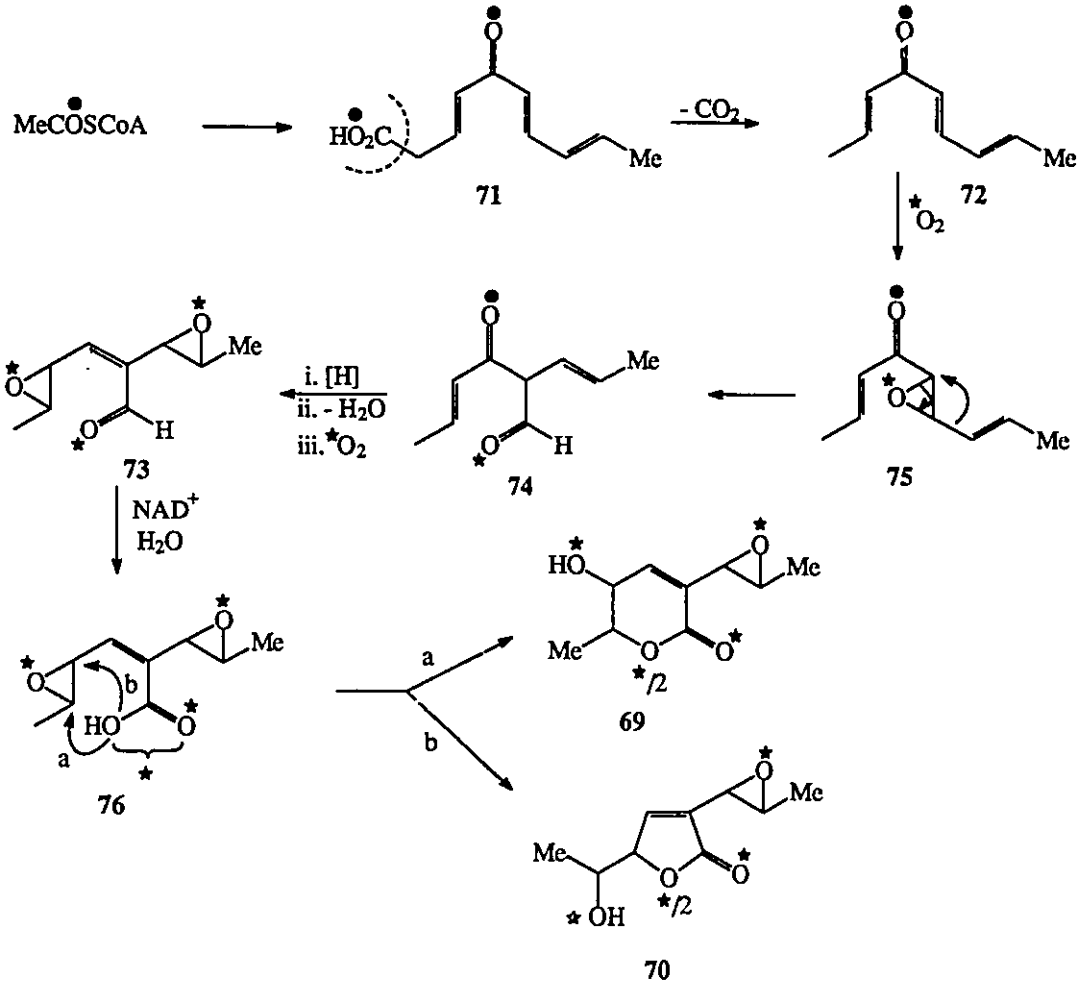
Together with other isotopes, such as ^{13}C or ^{18}O, ^2H provides invaluable information concerning the biochemical assembly of metabolites. For example in the biosynthesis of colneleic acid (**68**), it had previously been suggested[73] that the ether oxygen of this metabolite did not derive from the hydroperoxide oxygen. However, repetition of this experiment in potato, using enzymatically prepared [9-$^{18}O_2$]-(9S)-hydroperoxide **65** from tetradeuterated **64**, has established the origin of the ether oxygen of **68** to be from the hydroperoxide oxygen.[74] When **65** was incubated with potato homogenate to yield **68**, all deuteriums were retained. From these observations, a mechanism was proposed as shown in **Scheme 10**.



Scheme 10.

In probing the mechanism of formation of aspyrone (**69**) and asperlactone (**70**), metabolites produced by *Aspergillus melleus*, Staunton *et al.*[75] used [1-^{13}C, $^{18}O_2$] acetate and $^{18}O_2$ gas as precursors. When [1-^{13}C, $^{18}O_2$] acetate was fed to cultures of *A. melleus*, it was found that no ^{18}O isotope-induced shifts were observed in the ^{13}C NMR spectrum, indicating that no acetate derived oxygen was incorporated into the metabolites. However, when fermentation was carried out in the presence of $^{18}O_2$ gas, isotope shifts were observed at C-5, C-8, and C-9, in the ^{13}C NMR spectrum. In

addition C-2 shows two isotopically shifted signals and C-6 shows one with intensities about half of those observed at C-5, C-8, and C-9. From these results, a pathway was proposed for the biosynthesis of aspyrone and asperlactone as shown in **Scheme 11.**

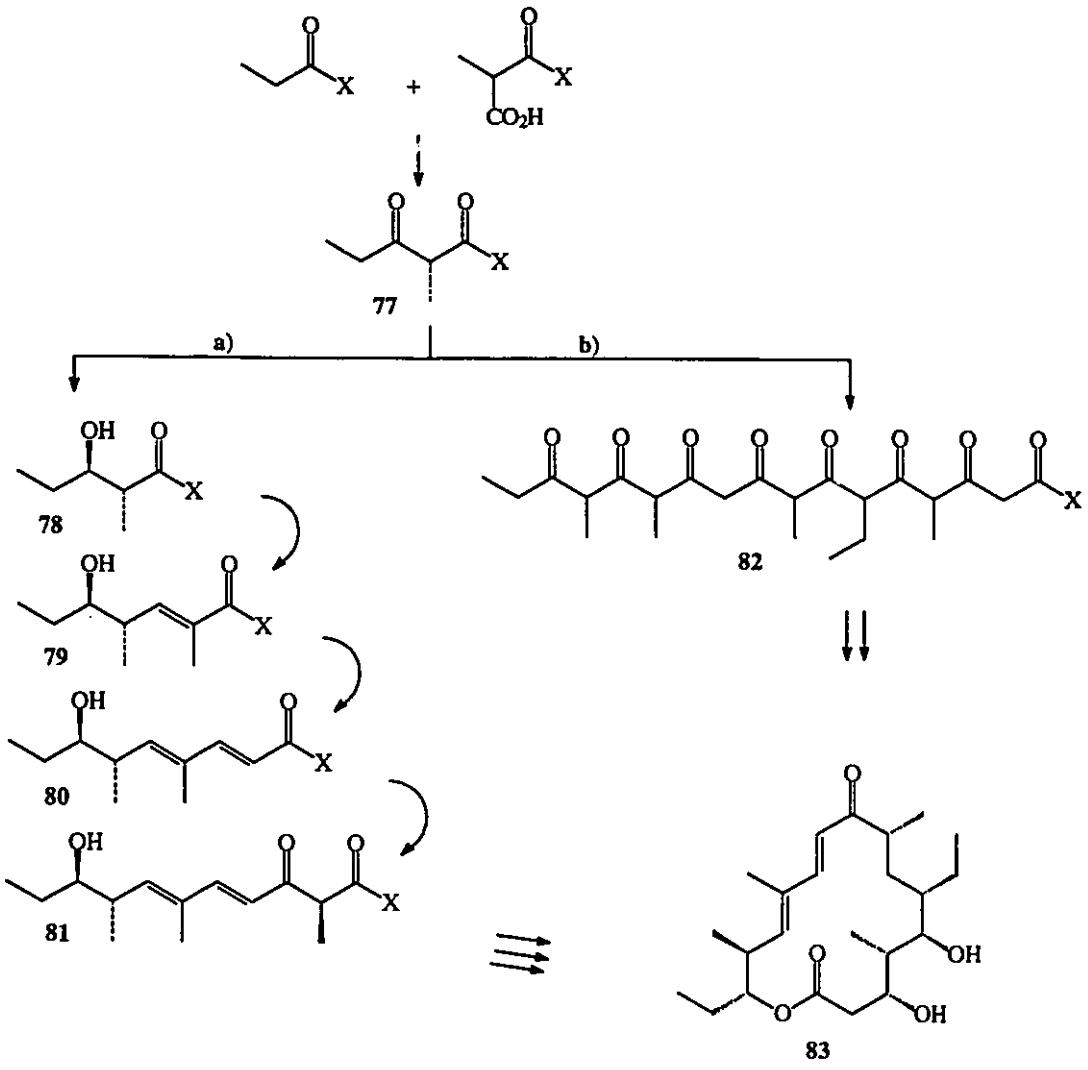


Scheme 11.

1.5.4. The Use of Partially Assembled Precursors

The distinctive feature of a polyketide biosynthetic pathway is found in the initial steps in which small biosynthetic building blocks (usually C_2 or C_3 units) are successively linked to form an extended carbon chain. The product, which has a characteristic linear carbon skeleton and array of functional groups, is occasionally the end product of the pathway. However, it is often elaborated by further biosynthetic reactions to produce a different final metabolite, sometimes with an extensively reorganized carbon skeleton.

There are two concepts that are believed to be operating in the polyketide biosynthetic pathways. Formation of a polyketide as shown in **Scheme 12** (pathway a) is called the "processive" mode, in which the new keto group generated in each cycle is appropriately modified prior to the commencement of the next chain extension cycle.[76] Alternatively, a "non-processive" mode may operate (pathway b), in which a poly-β-keto intermediate is formed by repeated operation of identical chain extension cycles. The array of functional groups would then be produced by subsequent modification of the carbonyl groups. The two concepts for the polyketide biosynthesis, as illustrated for the biosynthesis of tylactone (**83**),[76] can be distinguished if any of the compounds shown in **Scheme 12**, are incorporated intact into **83**.

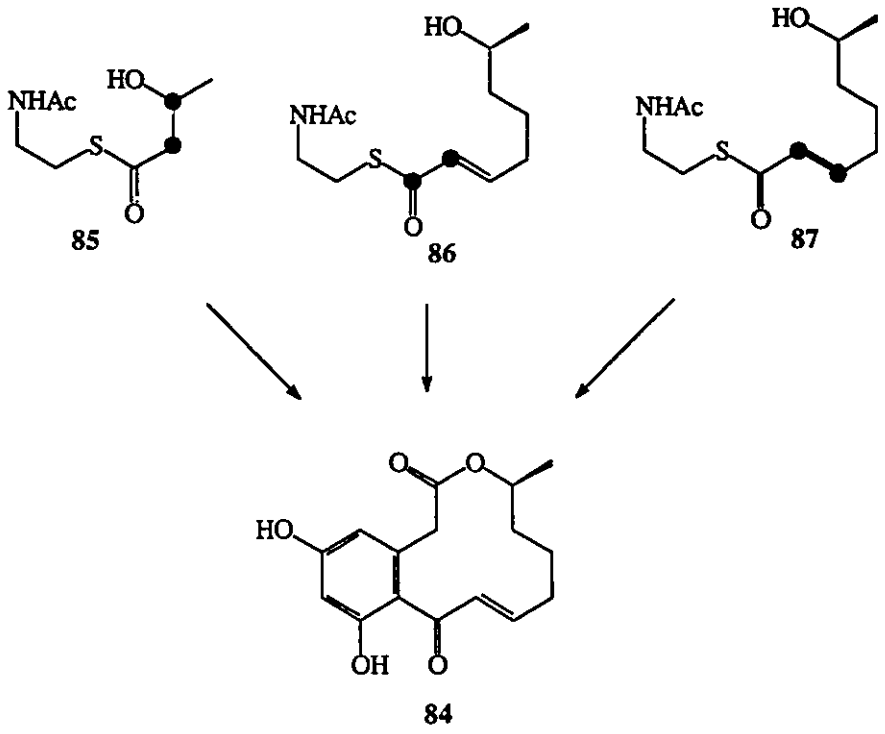


Scheme 12. Two Hypotheses for the Carbon Chain Assembly Mechanism in Tylactone. X indicates attachment of the intermediates to the enzyme, or to N-acetylcysteamine in the putative precursors.

In general it has proved difficult to intercept the PKS and introduce partially assembled fragments. A number of successful cases have been reported both in fungal and bacterial systems,[77] but it is well known throughout the biosynthetic community, as O'Hagan stated,[78] that the reported successes are grossly disproportionate to the failed attempts.

In the tylactone study, success was achieved by feeding intermediates as their N-acetylcysteamine (NAC) thioesters (**77-79**, X = $-SCH_2CH_2NHCOCH_3$). When fed as the corresponding ethyl oxoester, incorporation of label occurred only after prior degradation.[76]

Further support for the "processive" mode comes from the study of dehydrocurvularin (**84**) biosynthesis in *Alternaria cinerariae* ATCC 11784,[79] in which the partially assembled diketide **85** and tetraketide **86** intermediates were successfully incorporated intact into **84** (**Scheme 13**). This success was only achieved, however, after a mutant strain of *A. cinerariae*, deficient in the ability to utilize fatty acids, had been selected and when incubations were carried out in the presence of 4-pentynoic acid, a β-oxidation inhibitor. In the more recent studies,[80] this group was also successful in incorporating the NAC derivative of the tetraketide intermediate **87** into dehydrocurvularin by a wild type *A. cinerariae* in the presence of a β-oxidation inhibitor. Interestingly, attempts to incorporate the triketide intermediate with a large variety of feeding protocols and β-oxidation inhibitors were unsuccessful.



Scheme 13.

1.6. The Purpose of the Current Study

In this study, we wished to address several major questions related to the biosynthesis of F-244. First, what kind of precursors does *Fusarium* sp. ATCC 20788 use to assemble F-244 ? Secondly, how does nature control the formation of F-244 ?

It was believed that F-244, like other polyketide metabolites, is derived from acetate. However, since **1a** has two carboxyl ends, it was difficult to predict the orientation of the acetate units in **1a.**

Further, the origin of the three methyl groups, as well as the hydroxymethyl carbon were of interest. These moieties can be either derived from methionine or from incorporation of propionate instead of acetate into the growing polyketide chain. Thus to probe the origin of these groups, [1-^{13}C]propionate and L-[*methyl*-^{13}C] methionine can be used in incorporation experiments.

Polyketides normally are assembled from acetate units in a "head to tail" fashion, but how does this assembly proceed in the formation of **1a**? How is the β-lactone ring formed? It is interesting to speculate on the mode of formation of the highly strained β-lactone ring.

Chapter 2 describes the growth of *Fusarium* sp. ATCC 20788, production and isolation of F-244 as well as NMR spectroscopic analysis. Chapter 3 then describes the incorporation of simple precursors in order to address the biosynthetic questions above.

The last question we wished to address is the sequence of events in the biochemical transformation from acetate and propionate or from acetate and methionine. When are the double bonds introduced? Along with chain elongation or later after chain elongation? If **1a** is derived from acetate and methionine, when are the methyl groups introduced? To address these questions, some advanced precursors labelled with either ^{2}H or ^{13}C need to be prepared and incorporated into **1a**. The

syntheses of advanced precursors and the results of feeding experiments are described in chapter 4.

At the start of our study, there were no reports of any biosynthetic incorporation experiments into F-244. Recently, a study of the biosynthesis of F-244 in *Scopulariopsis* sp. was reported by Omura and co-workers.[81] Their results are detailed and compared to those in *Fusarium* sp. ATCC 20788.

CHAPTER 2

Production and NMR Assignment of F-244

2.1. General

The study of the biosynthesis of F-244 (**1a**) requires a biological system capable of producing this natural product. Currently, there are three micro-organisms known to produce **1a**. They are *Cephalosporium* sp. ACC 1233, *Fusarium* sp. ATCC 20788, 20789, 20790 and *Scopulariopsis* sp. The use of the micro-organism *Fusarium* sp. ATCC 20788 was found to be convenient in this regard due to the availability of both the organism and information concerning the production of **1a** by *Fusarium* sp. ATCC 20788.[13] Initially, reproduction of this patent literature did not seem to produce F-244 in our hands. However, with a little modification the *Fusarium* cultures were able to produce **1a** in an acceptable yield.

2.2. Cultural Characteristics of *Fusarium* sp. ATCC 20788

The freeze-dried *Fusarium* sp. was grown in petri dishes containing potato sucrose agar which were incubated at 25 °C for 5 - 7 days. After three days of incubation, the mycelia were white and fluffy, gradually changing through pink to

bluish-tan in colour as the culture aged (**Figure 8**). After 7 days, these cultures were stored in a refrigerator at 4 °C as preserved culture sources.

2.3. Production of F-244 (1a)

The preserved source of culture was used to inoculate a liquid growth medium containing tomato paste, dextrose, oat flour, and corn meal. The medium was incubated at 28 °C in an incubator shaker with agitation. When the growth was abundant, usually after 3 - 4 days of incubation, as judged by the change of colour from reddish-white to brown and the liquid becoming more viscous, a portion of this medium (3 mL) was used to inoculate flasks containing a corn meal medium which supports production of **1a**. The corn meal medium was incubated with agitation at 25 °C for 10 days. During this time, the colour of the medium gradually changed, normally after *ca.* 3 days of incubation, from yellow to dark brown (**Figure 9**).

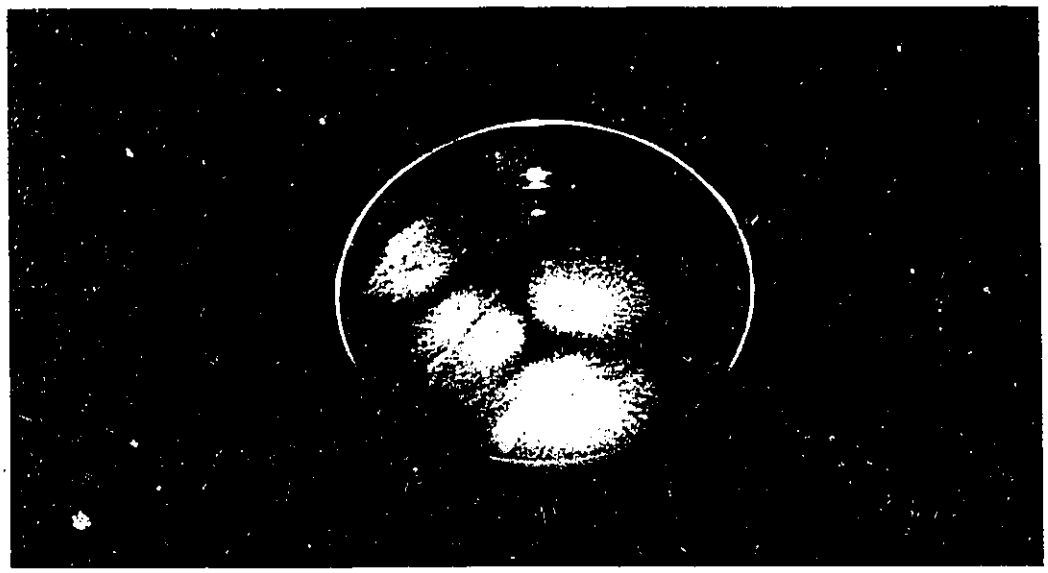


Figure 8. Cultures of *Fusarium* sp. ATCC 20788 Grown on Potato Sucrose Agar

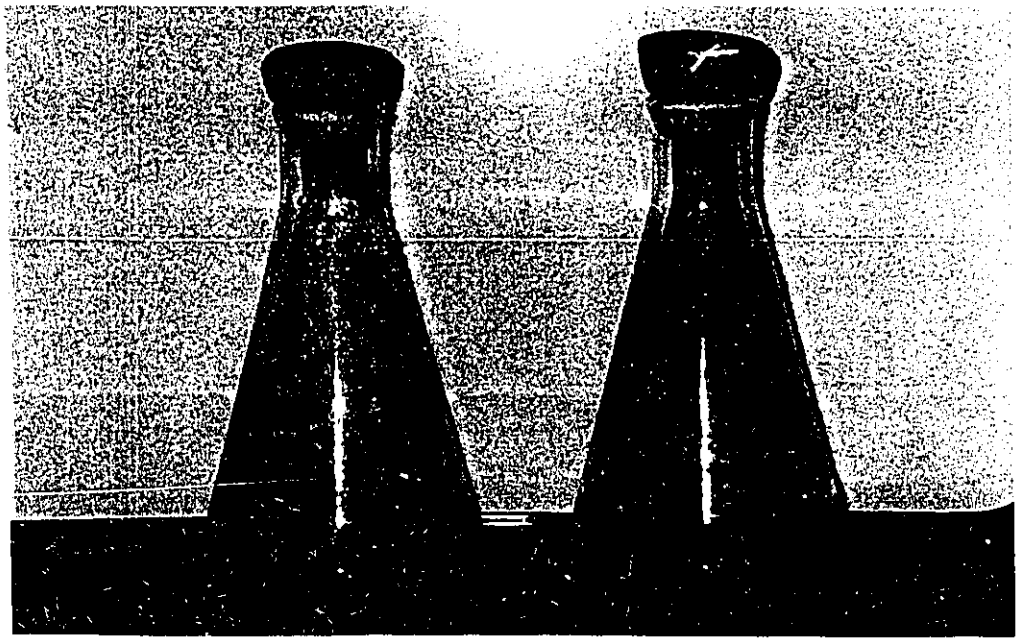


Figure 9. Cultures of *Fusarium* sp. ATCC 20788 Grown in Liquid Medium, Before Incubation (left), After 10 days of Incubation (right)

2.4. Isolation of F-244

The patent literature describes the isolation of **1a** by mixing the fermentation medium with methanol, in a ratio of 1 : 1 by volume. The mixture is then extracted with ethyl acetate or methylene chloride. However, this method proved difficult as the organic solvents are miscible with the aqueous phase in these combinations.

Extraction was better accomplished in our hands by separating the solid cellular material from the liquid medium by centrifugation. The pellet was then extracted with 10% methanol in ethyl acetate, while the supernatant was extracted with chloroform.

F-244 was purified from the combined crude organic extract by column chromatography on silica gel, followed by Sephadex LH-20.

During the course of purification, it was found that converting the free acid **1a** to its methyl ester **1b** by treating the crude extract with diazomethane proved to be advantageous. As **1b** is less polar, it is more readily separable from other unwanted materials on a silica gel chromatographic column, and thus the Sephadex LH-20 step is no longer required. Further, this methyl carbon can serve as an internal standard for carbon-13 NMR when comparing the spectra of labelled samples with that of unlabelled material (see later).

2.5. HPLC Analysis

Initially, following the patent literature, fermentation was performed for 14 days. However, it was not certain that this is the optimum time required for *Fusarium* sp. to produce the maximum amount of F-244. Moreover, from the point of view of studying the biosynthesis, it was necessary to know the best time to start and to stop feeding the precursor, thereby increasing the probability of obtaining a high incorporation.

The analysis of F-244 production was performed using HPLC. Aliquots of the culture medium (5 mL) were removed from the incubation flasks and stirred with 60% CH_3CN in 0.1% aq. H_3PO_4 (10 mL). After filtration, the solution was concentrated to 5 mL and subjected to HPLC analysis using a reverse phase C-18 column, which was eluted isocratically in the same solvent system.[29] The samples of chromatograms and results of the analysis are shown in **Figure 10a** and **10b** respectively. Figure 10a (a) shows the peak due to pure F-244 (7.5 μg/mL). From a series of similar injections, a calibration curve was prepared which was linear from 0 to 20 μg/mL. As shown in Figure 10a (b), F-244 started to appear, within the detection limit of 1 μg/mL, at day 3 after incubation. The concentration of F-244 gradually increased each day to reach a maximum at day 10 (12 μg/mL), then started to decrease. This decrease is perhaps due to F-244, having been released by the microorganism to the environment, being slowly

decomposed in the medium, which becomes noticeably acidic (*p*H 3 - 4) during the course of fermentation.

It appeared that production of **1a** by *Fusarium* sp. is somewhat similar to that of production by *Scopulariopsis* sp., in which case production also reaches a maximum after day 9 of incubation.[29]

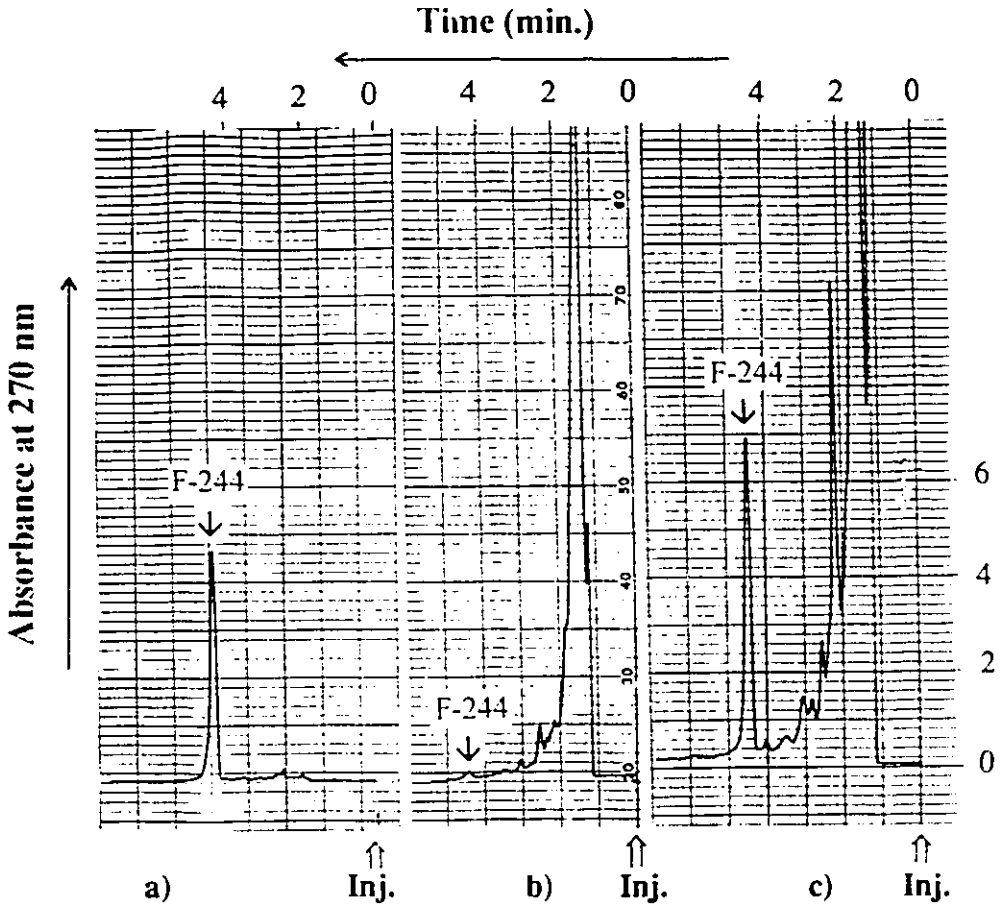


Figure 10a. Sample of Chromatograms from HPLC Analysis of Growing Cultures of *Fusarium* sp. ATCC 20788. a) Standard Purified F-244; b) 3 Days Growth; c) 9 Days Growth

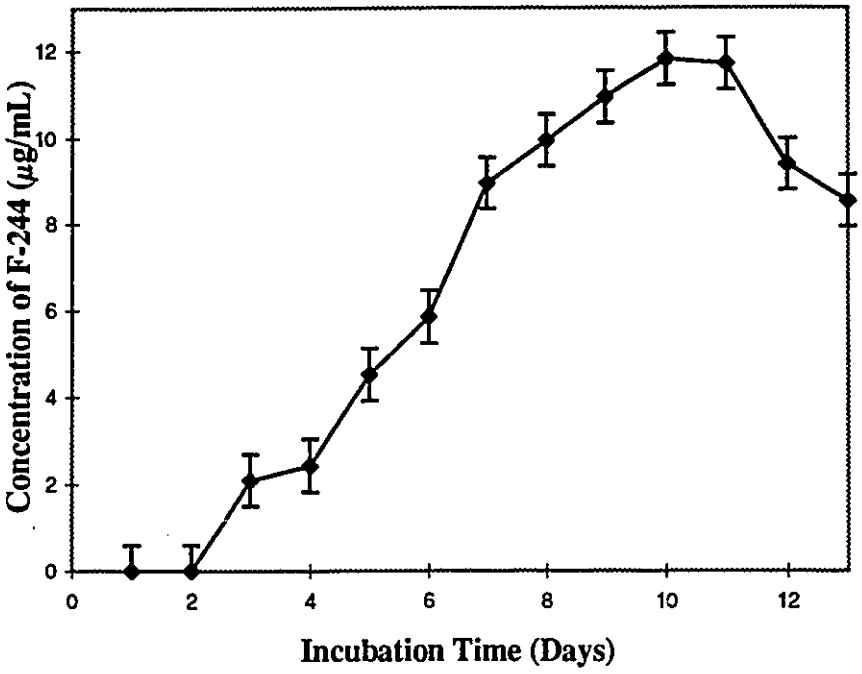


Figure 10b. Production of F-244 (**1a**) by *Fusarium* sp. ATCC 20788. Each data point is the average of 3 determinations. Errors are expressed as the average of the standard deviations of these determinations.

2.6. NMR Assignment of 1a and 1b

The first requirement in using ^{13}C labelling for studying the biosynthesis of a particular compound is to assign the resonances in the natural abundance ^{13}C NMR spectrum of the metabolite. Incorporation of ^{13}C label from a precursor leads to

enhanced NMR signals in the metabolite derived from it. Consequently any mistakes in assignment can lead to false conclusions about the biosynthesis.

The NMR spectra were recorded on a Bruker AC 500 spectrometer at room temperature, and $CDCl_3$ was used as solvent. Various 2-D NMR techniques were used to obtain the complete assignment of **1a** or its more soluble methyl ester **1b**. To simplify the explanation of the assignment, the structure of **1b** was divided into two parts, A and B, as shown in **Figure 11**.

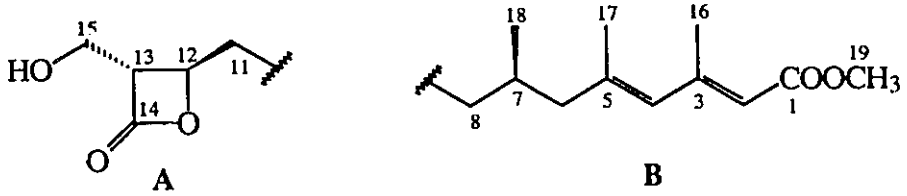


Figure 11. Two Divided Regions of F-244 Methyl Ester

2.6.1. Region A

H-15 and H-15' were easily identified in the proton NMR spectrum as each signal appeared as a doublet of doublets at δ 3.88 (J = 11.6 and 4.2 Hz) and 4.04 (J = 11.6 and 5.0 Hz). The proton - proton COSY spectrum (**Figure 12**) showed correlation between these two protons and a proton multiplet at δ 3.39 (J = 5.0 and 4.2 Hz), attributable to H-13, which in turn is coupled to a multiplet at δ = 4.58 (J = 4.1 Hz) which must therefore be H-12. C-15, C-13, and C-12 were readily assigned at

δ 58.03, 58.61, and 74.91 ppm respectively based on heteronuclear shift correlations to the respective protons (**Figure 14**).

The COSY spectrum (**Figure 12**) also showed correlation between H-12 and two signals at δ 1.90 (J = 4.1 Hz) and 1.76 (J = 4.1 Hz), which were assigned to H-11 and H-11'. Although the signals from H-11 and H-11' were overlapped with other resonances, there was one unique ^{13}C resonance at 33.99 ppm that showed correlation in the heteronuclear shift correlation (HETCOR) spectrum to both δ 1.90 and 1.76 (**Figure 14**). Therefore, the signal at δ 33.99 is C-11. Both H-11 and H-11' also correlated with a multiplet at 1.42 ppm, which therefore corresponds to H-10 and H-10'. These resonances are however convoluted with other signals, and hence the assignments for the H-10 protons are consequently somewhat speculative.

2.6.2. Region B

The three methyl groups were easily identified as they appear as three strong peaks at δ 0.84, 1.79, and 2.23 ppm respectively. The peak at 0.84 ppm appears as a unique doublet attributable to H-18. The corresponding carbon (C-18) was assigned from the HETCOR spectrum. The other two peaks appear as singlets corresponding to H-16 and H-17, but it could not be ascertained which one is which at this point.

The two olefinic protons, H-2 and H-4, appeared at δ 5.66 and 5.70 ppm, however these two protons could not yet be distinguished as they appear as two broadened singlets, and thus no coupling information could be used to help in the assignment. In the shift correlated spectrum (**Figure 16**), these protons correlated to carbons at δ 117.03 and 129.47 ppm, and hence C-2 and C-4.

In the COSY spectrum (**Figure 13**), the methyl doublet H-18 (δ = 0.84), is the best starting place for the assignment of this region. It showed a strong correlation to a signal at δ 1.67 assignable to H-7. H-7 in turn is coupled to two groups of signals which resonate at δ 1.85 and 2.08. These signals, besides being coupled to H-7, exhibited only coupling to each other with a large coupling constant (J = 13.2), indicating that these protons are attached to the same carbon and are adjacent to a quarternary carbon. These signals were assigned to H-6 and H-6'. H-7 was also coupled to protons at δ 1.28 and 1.12. The only possible assignment for these signals is H-8 and H-8'. The corresponding carbons, C-7, C-6, and C-8 were assigned from the HETCOR spectrum (**Figure 15**). Both H-9 and H-9' were found to be overlapped with H-8 and H-10.

Assignment of the two carboxyl carbons, C-1 and C-14, of F-244 and its methyl ester (**1a** and **1b**) was easily accomplished by comparing the two ^{13}C spectra. The two carbonyl carbons of **1a** appeared at δ 169.70 and 171.7 ppm. When **1a** was converted to its methyl ester, **1b**, one of the signals (δ 171.7) shifted upfield to δ 167.5

ppm. Therefore this signal was assigned to C-1, and the signal at δ 169.70 is C-14. Similarly, H-19 and C-19 were assigned by comparing the proton and carbon spectra of the two compounds. H-19 appeared as a strong singlet at δ 3.70 in the proton spectrum and at δ 50.85 in the carbon-13 spectrum of **1b**. These resonances were absent in the proton and carbon-13 spectra of **1a**.

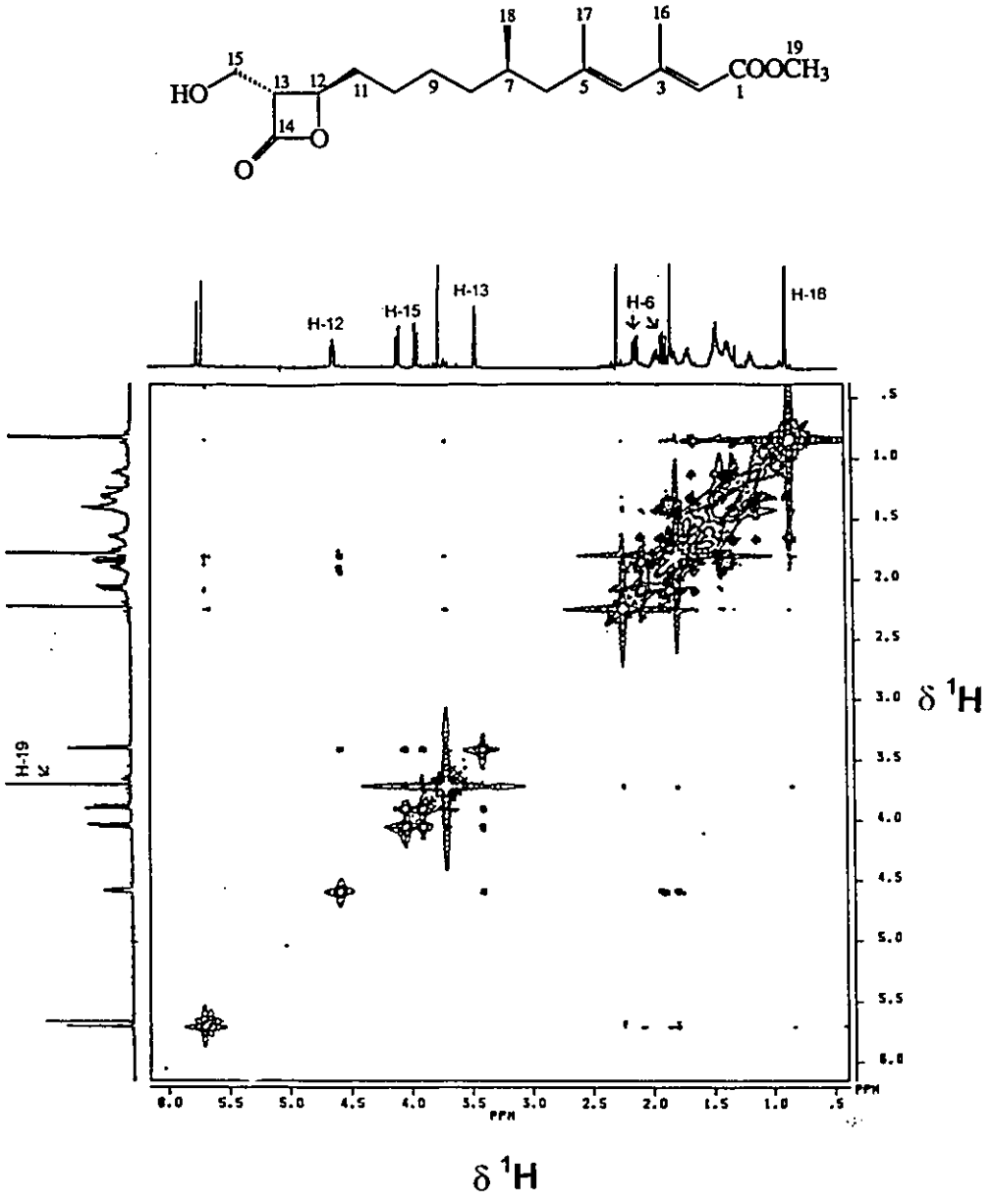


Figure 12. ^{1}H-^{1}H COSY Spectrum of F-244 Methyl Ester (**1b**). The spectrum shown on each axis is the normal 1-D proton NMR spectrum.

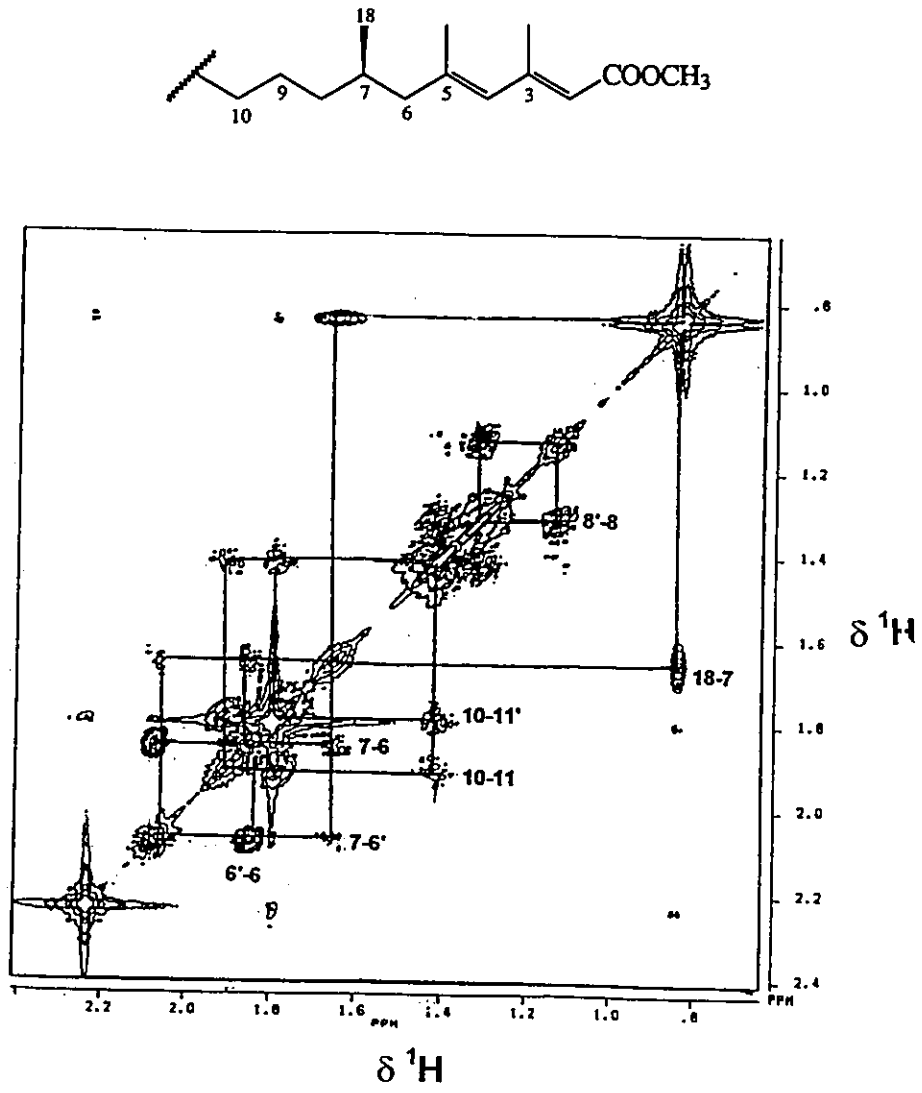


Figure 13. ^{1}H-^{1}H COSY Spectrum of the Aliphatic Region of F-244 Methyl Ester (**1b**). Correlations are represented by A-B, where A represents the proton on vertical axis, and B represents the proton on horizontal axis.

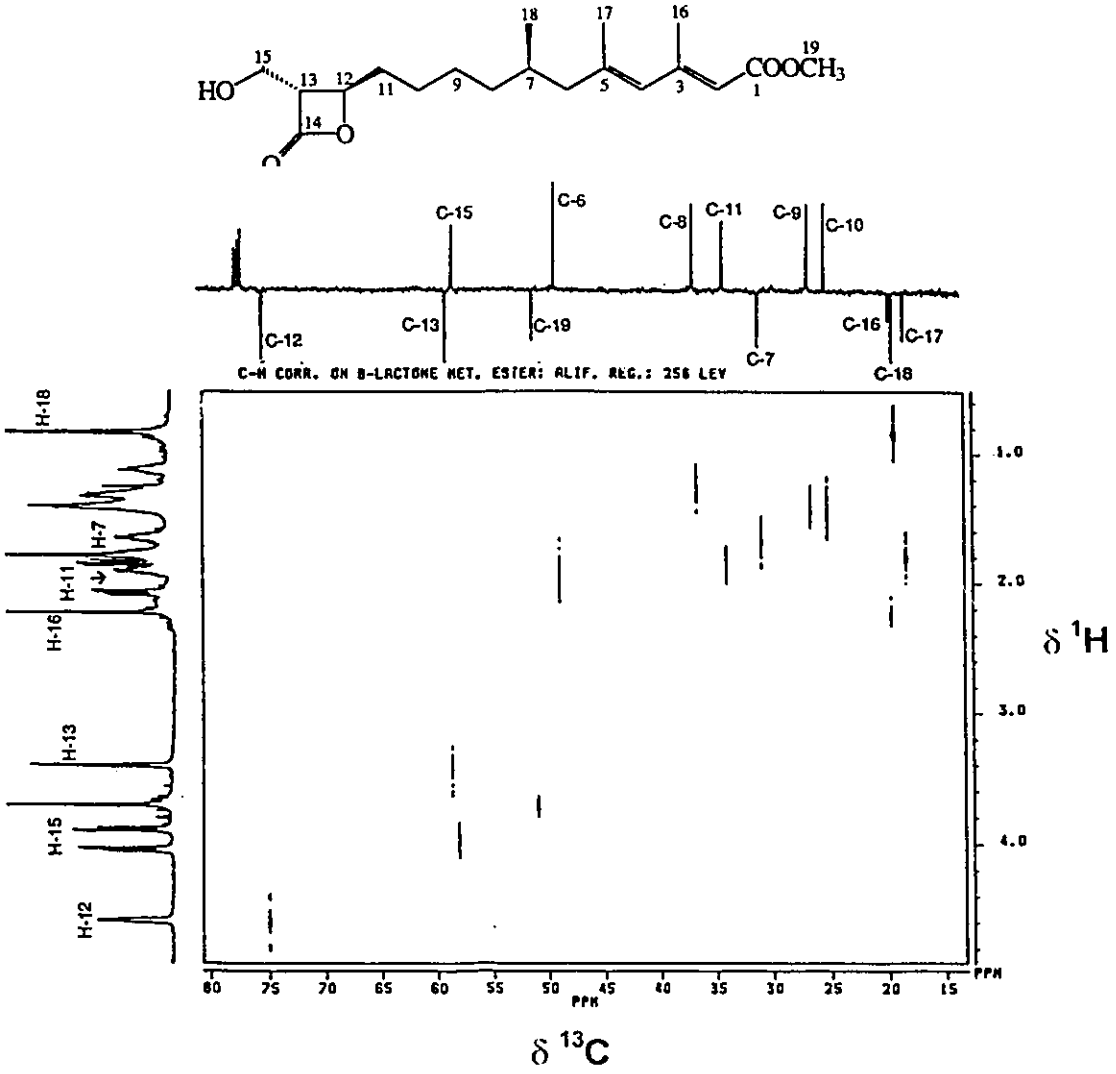


Figure 14. ^{1}H-^{13}C Heteronuclear One-bond Shift Correlation for the Aliphatic Region of F-244 Methyl Ester (**1b**). The spectrum shown on y axis is the normal proton spectrum, and on x axis is the ^{13}C spin-echo spectrum, where negative signals represent CH_3 and CH, positive signals represent CH_2 and CH.

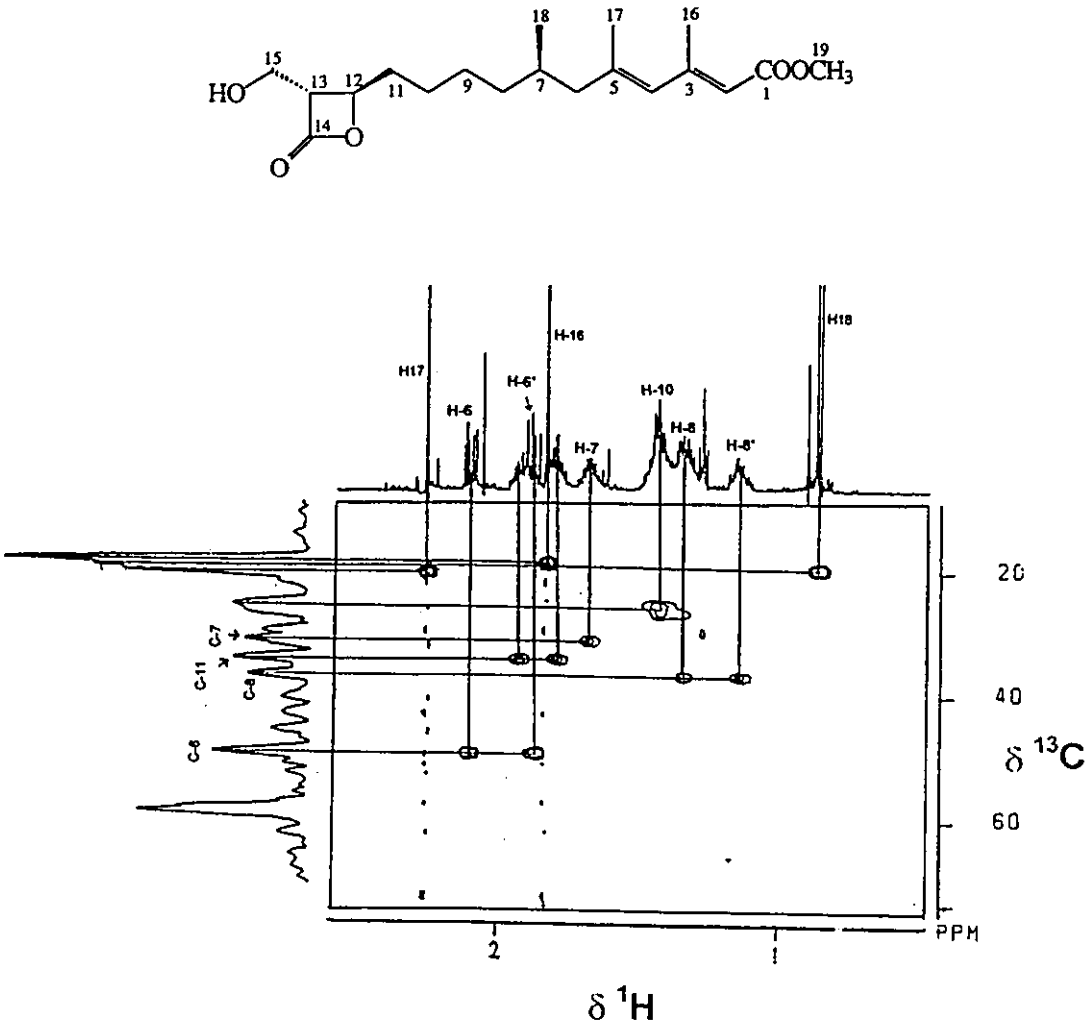


Figure 15. ^{1}H-^{13}C Heteronuclear One-bond Shift correlation Spectrum for the Aliphatic Region of F-244 Methyl Ester (**1b**) Acquired in Inverse Mode. The spectra shown on the x and y axes are 1-D proton and carbon-13 spectra respectively.

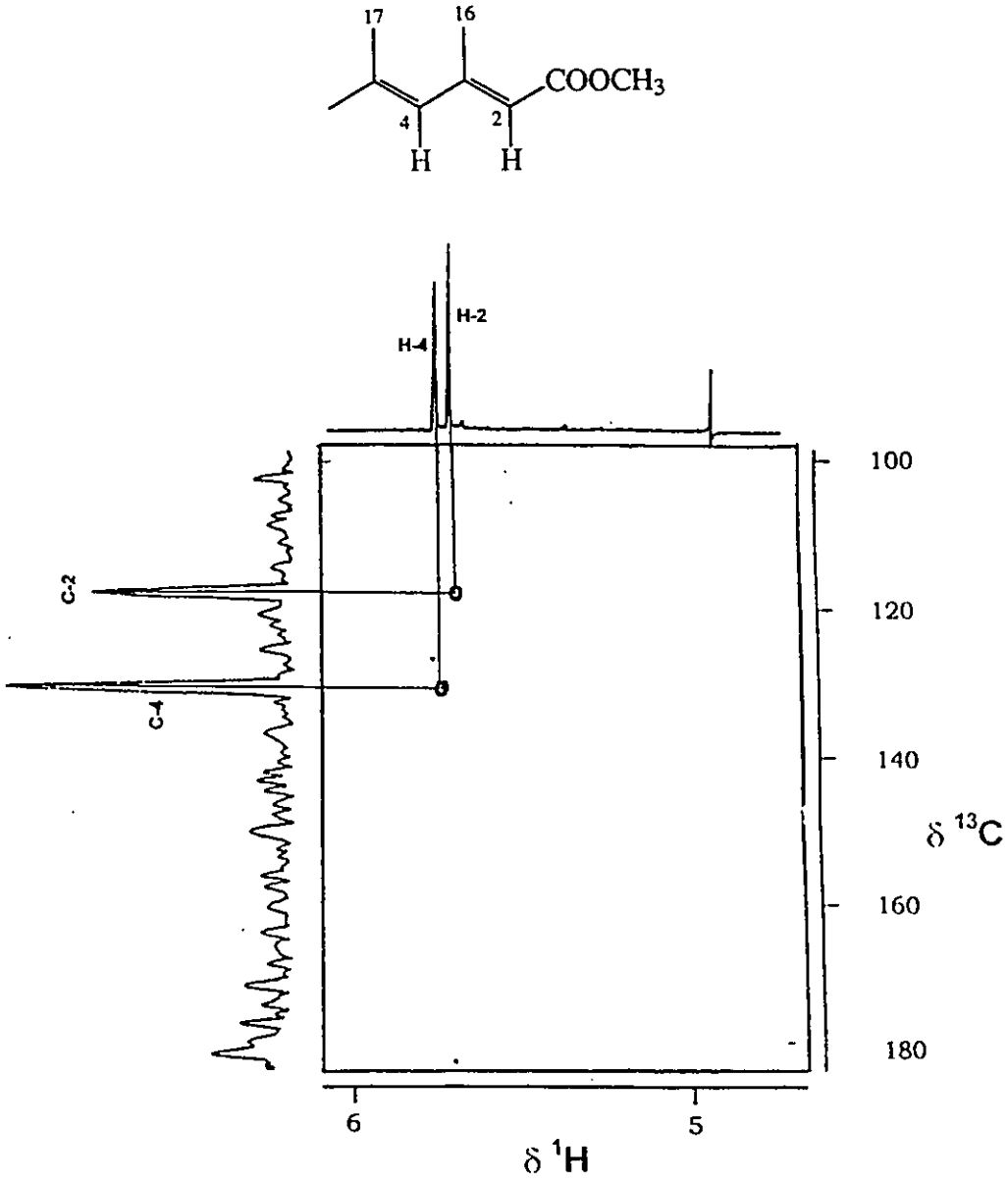
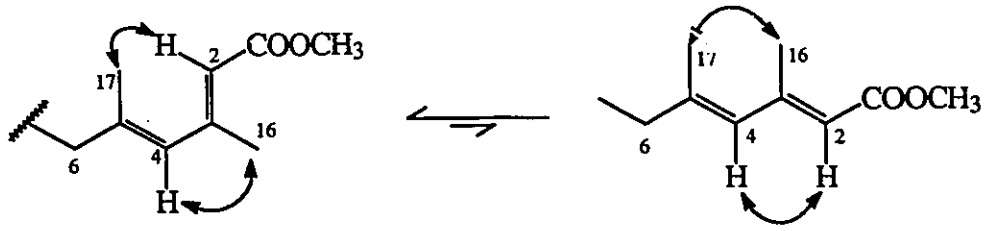


Figure 16. ^{1}H-^{13}C Heteronuclear Shift correlation Spectrum for the Olefinic Region of F-244 Methyl Ester (**1b**) Acquired in Inverse Mode. The spectra shown on the x and y axes are 1-D proton and carbon-13 spectra respectively.

The remaining carbons that were left unassigned by the techniques mentioned above are C-2, C-3, C-4, C-5, C-16, C-17, and the corresponding protons H-2, H-4, H-16 and H-17.

The geometry of the double bonds of **1a** has been determined to be 2*E*-4*E*.[2,6] It was hoped that nuclear Overhauser effects (n.O.e's) could be observed between H-6 and H-4, and between H-4 and H-2, thereby providing the assignment of H-2 and H-4. The result of n.O.e experiments showed that upon irradiation of the signal at δ 5.70, pronounced n.O.e's at H-6, H-6', and the methyl protons at 2.23 ppm, were observed. A n.O.e was also observed at δ 1.79 ppm when the signal at δ 5.66 was irradiated. These results suggest that the signal at 5.70 ppm is H-4 and the signal at 2.23 ppm is methyl protons, H-16. Therefore signals at 5.66 and 1.79 ppm are H-2 and H-17. Corresponding carbons, C-2, C-4, C-16, and C-17, were assigned from the shift correlated spectrum. The fact that n.O.e's were observed between H-4 and H-16 and between H-4 and H-17 suggest that for **1a** and **1b**, the s-*cis* rotamer would be favored over the s-*trans* (**Figure 17**). This perhaps is not surprising, considering that in the s-*cis* rotamer, both methyl groups would have less steric interaction than that in the s-*trans* rotamer. This result was later confirmed by Koseki *et al.*[82] Further evidence for this assignment was obtained from the heteronuclear multiple bond correlation (HMBC) experiment[83] (see below).



s-*cis* s-*trans*

Figure 17. Two Possible Conformations of F-244. NOE experiments showed that the s-*cis* rotamer is favored

In order to assign the two quarternary carbons, C-3 and C-5, an HMBC experiment using an inverse probe was performed. The results showed that H-16 correlated with 3 carbons at δ 117.03, 129.47, and 154.51 ppm. On the other hand, H-17 showed correlation with carbons at δ 48.88, 129.47, 141.12 ppm (**Figure 18**). Since the signal at δ 48.88 and 117.03 are known as C-6 and C-2 respectively, and only C-4 (δ 129.5) showed correlation to both H-16 and H-17. This results show that the signals at δ 154.5 and 141.1 ppm arose from C-3 and C-5 respectively. Thus besides giving direct assignments of C-3 and C-5, this experiment also provides further evidence for the assignments of C-2, C-4, H-16 and H-17, and hence of H-2, H-4, C-16 and C-17.

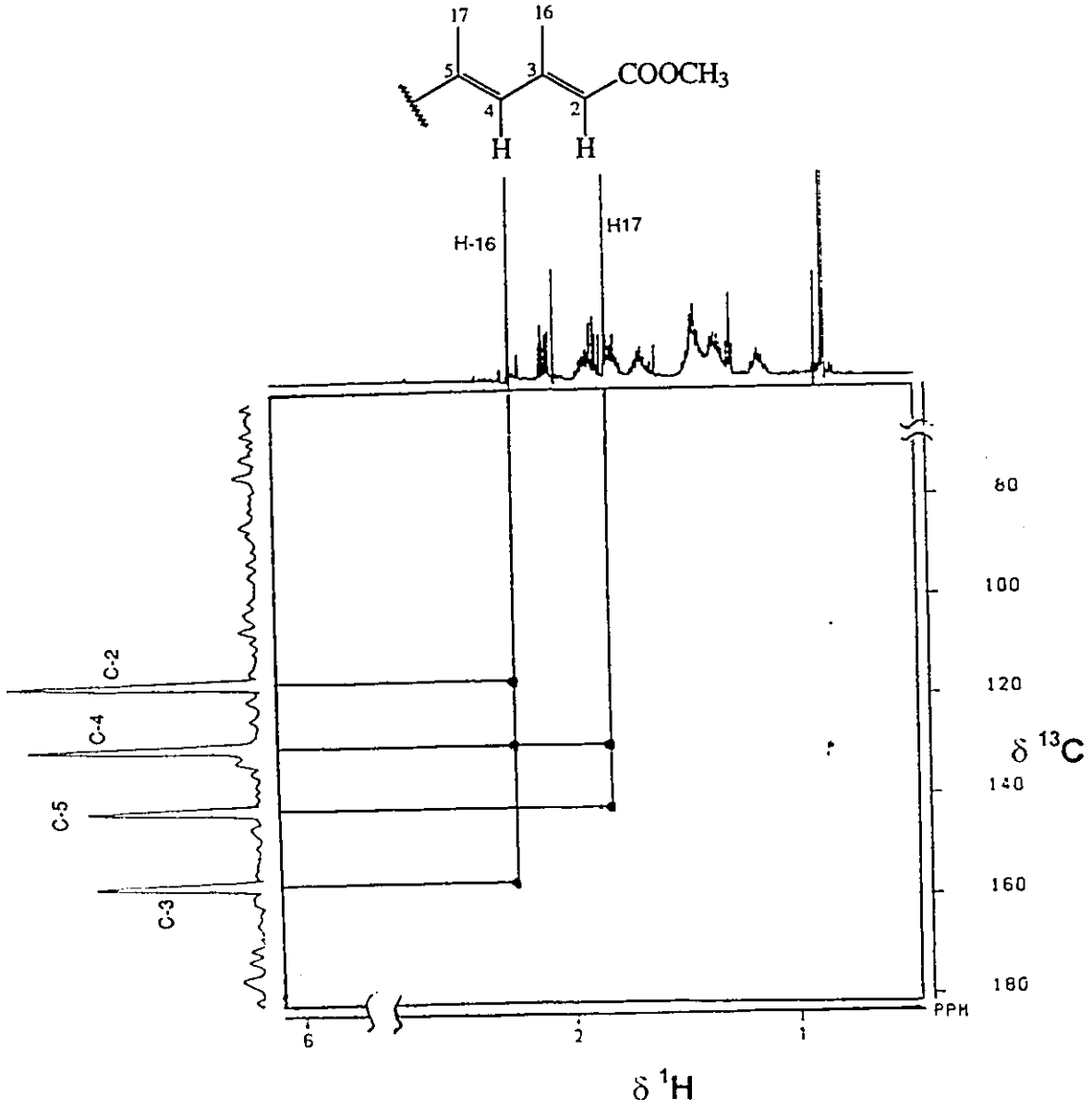


Figure 18. Heteronuclear Multiple Bond Correlation Spectrum for the 'Diene' Region of F-244 Methyl Ester. The spectra shown on the x and y axes are 1-D proton and carbon-13 spectra respectively.

Unequivocal assignments of C-8, C-9, and C-10, were obtained by the double quantum coherence (INADEQUATE) technique.[84] Due to the demand of high concentration of the sample for this technique, the experiment was only possible using **1b** obtained from the incorporation experiments utilizing [1,2-$^{13}C_2$]acetate. Using this technique, the connectivities between enriched pairs of carbon atoms including C-9 and C-10, were observed as expected. Although this experiment does not distinguish the two carbons, feeding experiments involving [1-^{13}C] and [2-^{13}C]acetates confirmed the overall assignment (see Chapter 3). The complete assignment of protons and carbons of **1a** and **1b** is given in **Table 2.**

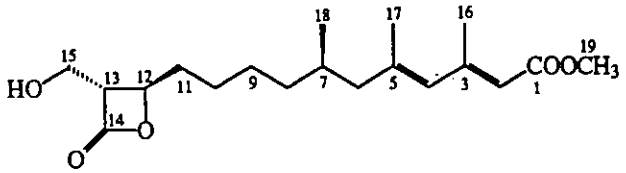
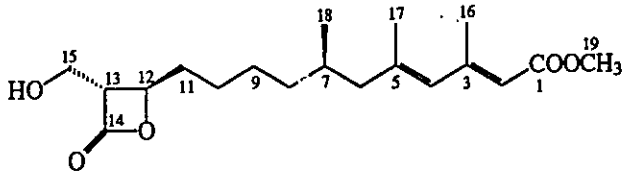


Table 2. NMR Assignment of **1b**

Number	$\delta^{13}C$ (ppm)	$\delta^{1}H$ (ppm)	J (Hz)[a]	Assigned from:
1	167.55	–	–	compared with **1a**
2	117.03	5.66	singlet	HETCOR, nOe & COSY
3	154.51	–	–	HMBC
4	129.47	5.70	singlet	HETCOR, nOe & COSY
5	141.12	–	–	HMBC
6	48.88	2.08	dd, $J_{6\text{-}6'}$= 13.2, $J_{6\text{-}7}$= 8.2	COSY & HETCOR
6'	–	1.85	dd, $J_{6'\text{-}6}$= 13.2, $J_{6'\text{-}7}$= 6.5	COSY & HETCOR
7	30.89	1.66	multiplet	COSY & HETCOR
8	36.57	1.28	multiplet	COSY & HETCOR
8'	–	1.12	multiplet	COSY & HETCOR
9	26.58	1.31	multiplet	COSY & HETCOR
9'	–	1.44	multiplet	COSY & HETCOR
10	25.16	1.42	multiplet	COSY & HETCOR
10'	–	1.42	multiplet	COSY & HETCOR

Continued on the next page.

Table 2 (continued)



Number	$\delta^{13}C$ (ppm)	$\delta^{1}H$ (ppm)	J (Hz)[a]	Assigned from:
11	33.99	1.90	m, $J_{11\text{-}12} = 4.1$	COSY & HETCOR
11'	–	1.76	m, $J_{11'\text{-}12} = 4.1$	COSY & HETCOR
12	74.92	4.58	m, $J_{12\text{-}13} = 4.1$	COSY & HETCOR
13	58.61	3.39	dd, $J_{13\text{-}15} = 5.0$, $J_{13\text{-}15'} = 4.2$	COSY & HETCOR
14	169.70	–	–	compared with **1a**
15	58.03	4.04	dd, $J_{15\text{-}15'} = 11.6$, $J_{15\text{-}13} = 5.0$	COSY & HETCOR
15'	–	3.88	dd, $J_{15'\text{-}15} = 11.6$, $J_{15'\text{-}13} = 4.2$	COSY & HETCOR
16	19.65	2.23	singlet	nOe, HETCOR & HMBC
17	18.36	1.79	singlet	nOe, HETCOR & HMBC
18	19.36	0.84	d, $J_{18\text{-}7} = 6.6$	COSY & HETCOR
19	50.85	3.70	singlet	HETCOR.

[a]Multiplicities and coupling constants (J) determined from the one dimensional ^{1}H spectrum

2.7. Conclusion

The production of F-244 by *Fusarium* sp. ATCC 20788 started after 3 days of incubation and reached a maximum after 10 days of incubation. Methods for subsequent extraction and chromatography were developed to give pure **1a** and **1b**.

Extensive NMR work, using 1-D and 2-D techniques, has established a complete and unambiguous assignment of all of the ^{1}H and ^{13}C resonances for F-244 (**1a**) and F-244 methyl ester (**1b**).

CHAPTER 3

Biosynthetic Studies of F-244 Using Basic Precursors

3.1. The Biogenesis of Carbon Atoms

3.1.1. Preliminary Investigations of Acetate Incorporation

Having fully assigned the ^{13}C NMR signals of F-244 (**1a**), biosynthetic studies can now be performed. The investigation of F-244 biosynthesis in *Fusarium* sp. ATCC 20788 first requires that the origin of the carbon atoms be firmly established. During the course of our study, Omura and co-workers reported the origin of F-244 in *Scopulariopsis* sp.;[81] however these results could not be assumed to apply to *Fusarium* sp. ATCC 20788.

The first incorporation experiment to be performed involved addition of a solution of sodium [1-^{13}C]acetate into the growing *Fusarium* cultures (1g/24 flasks) in 12 portions at 12 h intervals, starting from day 4 until day 9 of incubation. These conditions led to a similar amount of isolated F-244 (30 mg) compared to controls without acetate (35-50 mg/24 flasks). However, no significant enhancements were

noticeable in the ^{13}C NMR spectrum of purified **1a** (for discussion of test of significance, see section 3.1.2). The fact that polyketides are normally derived from acetate prompted us to investigate the culture requirements more closely. It was thought that perhaps this result was due to an inadequate amount of the precursor. In other words, 1 g of acetate was too little to feed to 24 flasks of cultures containing 10 g of corn as a carbon source in each flask. In order to test this assumption, and to determine whether or not **1a** can be produced using a lesser amount of corn, a series of cultures with different amounts of corn were prepared. Unlabelled sodium acetate at different concentrations was added to each culture using the same method as described above. The results showed that without addition of acetate, production of **1a** declines approximately proportionally as the amount of corn decreases (**Figure 19**). Addition of acetate to the cultures containing 2g and 4 g of corn marginally increases the production of **1a**, while addition of acetate to the rest of the cultures either does not affect or slightly decreases production of **1a** (**Figure 20**). Perhaps addition of acetate compensates for the lack of carbon source and other nutrients in cultures containing only 2 g and 4 g of corn.

With these results in hand, a level of 4 g of corn per culture flask was selected for use in all subsequent experiments.

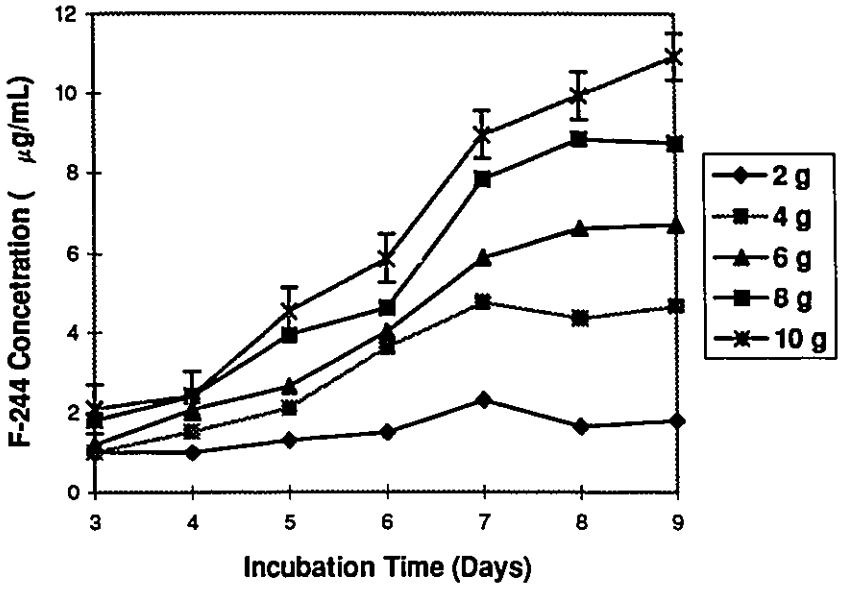


Figure 19. Production of **1a** as a Function of Corn Content (g/flask).Errors are based on production curve (section 2.5) and apply to all data points.

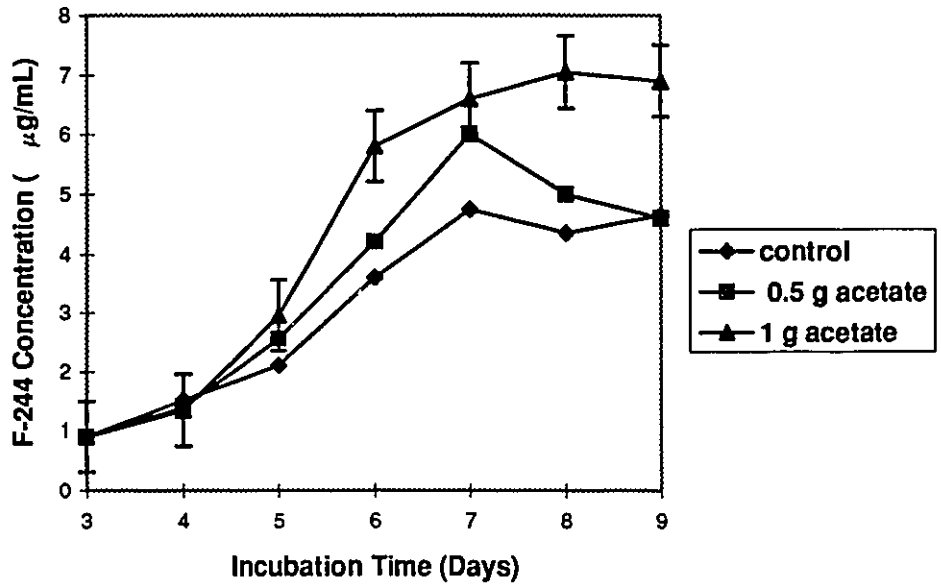


Figure 20. Addition of Sodium Acetate to a *Fusa...m* Culture Containing 4 g of Corn

3.1.2. Incorporation of [1-^{13}C]Acetate

Addition of sodium [1-^{13}C]acetate to the growing *Fusarium* cultures gave **1a** which was subsequently converted to its methyl ester **1b** (8 mg). The ^{13}C NMR spectra of this sample as well as an unlabelled sample (5 mg) were then acquired using identical parameters. The extent of isotopic labelling at each position in F-244 due to the addition of the labelled acetate was determined by comparing peak heights for the labelled and unlabelled methyl ester derivative of F-244 (**1b**), after each peak height was standardized relative to the height of the signal from the methyl ester group (C-19) (**Figure 21a** and **b**). The resulting signal enhancements were converted to % incorporations based on the 1.1% natural abundance of ^{13}C in unlabelled F-244 using the formula:

$$\% \text{ Incorporation} = \frac{\text{Peak height (labelled)} - \text{Peak height (unlabelled)}}{\text{Peak height (unlabelled)}} \times 1.1\%$$

The errors associated with peak height measurement in the ^{13}C spectrum of F-244 can be derived from a series of spectra of different unlabelled samples. Since a spectrum of unlabelled F-244 was acquired each time an incorporation experiment was performed, these data provide a reliable estimate of this type of error. The errors generated by these measurements are shown in **Table 3.**

Table 3. Carbon-13 NMR Spectral Peak Heights, Mean Peak Heights, and Standard Deviation of Peak Heights from 3 Different Spectra of Unlabelled F-244 Methyl Ester

Carbon #	Peak Height Spec. 1	Peak Height Spec. 2	Peak Height Spec. 3	Mean	Standard. Deviation.
1	1.25	1.15	1.22	1.21	0.05
2	1.30	1.30	1.35	1.32	0.03
3	1.15	1.32	1.20	1.22	0.09
4	1.42	1.50	1.44	1.45	0.04
5	1.80	1.60	1.85	1.75	0.13
6	1.65	1.50	1.60	1.58	0.08
7	1.85	1.82	2.03	1.90	0.11
8	2.05	2.00	2.00	2.02	0.03
9	1.85	1.90	1.80	1.85	0.05
10	2.00	1.85	2.05	1.97	0.10
11	1.85	1.90	1.83	1.86	0.04
12	1.32	1.38	1.20	1.30	0.09
13	1.85	1.90	1.90	1.88	0.03
14	0.60	0.79	0.52	0.64	0.14
15	1.40	1.50	1.40	1.43	0.06
16	1.30	1.30	1.30	1.30	0.00
17	1.70	1.70	1.68	1.69	0.01
18	2.05	1.96	2.08	2.03	0.06
19	1.00	1.00	1.00	1.00	0.00

From these results, the largest value of the standard deviation of a peak height was shown to be 0.14. Because % incorporation was determined by comparing each peak height in a labelled F-244 spectrum with the corresponding peak height in the unlabelled F-244 spectrum, the standard deviation in any peak height can be used to calculate the error in % incorporation. Thus, if the value of 0.14 is chosen as the overall measurement error, this would give a calculated error in % incorporation of ±0.21%. This value was chosen as the criterion for a reliable incorporation of carbon-

13 at any site. Values of % incorporation of 0.2 or below require more rigorous analysis to determine their significance.

The ^{13}C NMR spectrum showed that **1b** was enriched at 7 alternating carbons throughout the chain (C-2, C-4, C-6, C-8, C-10, C-12, and C-14) (**Figure 21b**). These signals were enhanced *ca.* 3 fold corresponding to 2.2% incorporation, while the remaining carbon signals gave calculated % incorporations of -0.2 to +0.2 (**Table 4**). Therefore any incorporation of ^{13}C at these sites (e.g., through the citric acid cycle)[15] is small and falls within the calculated errors of the experiment.

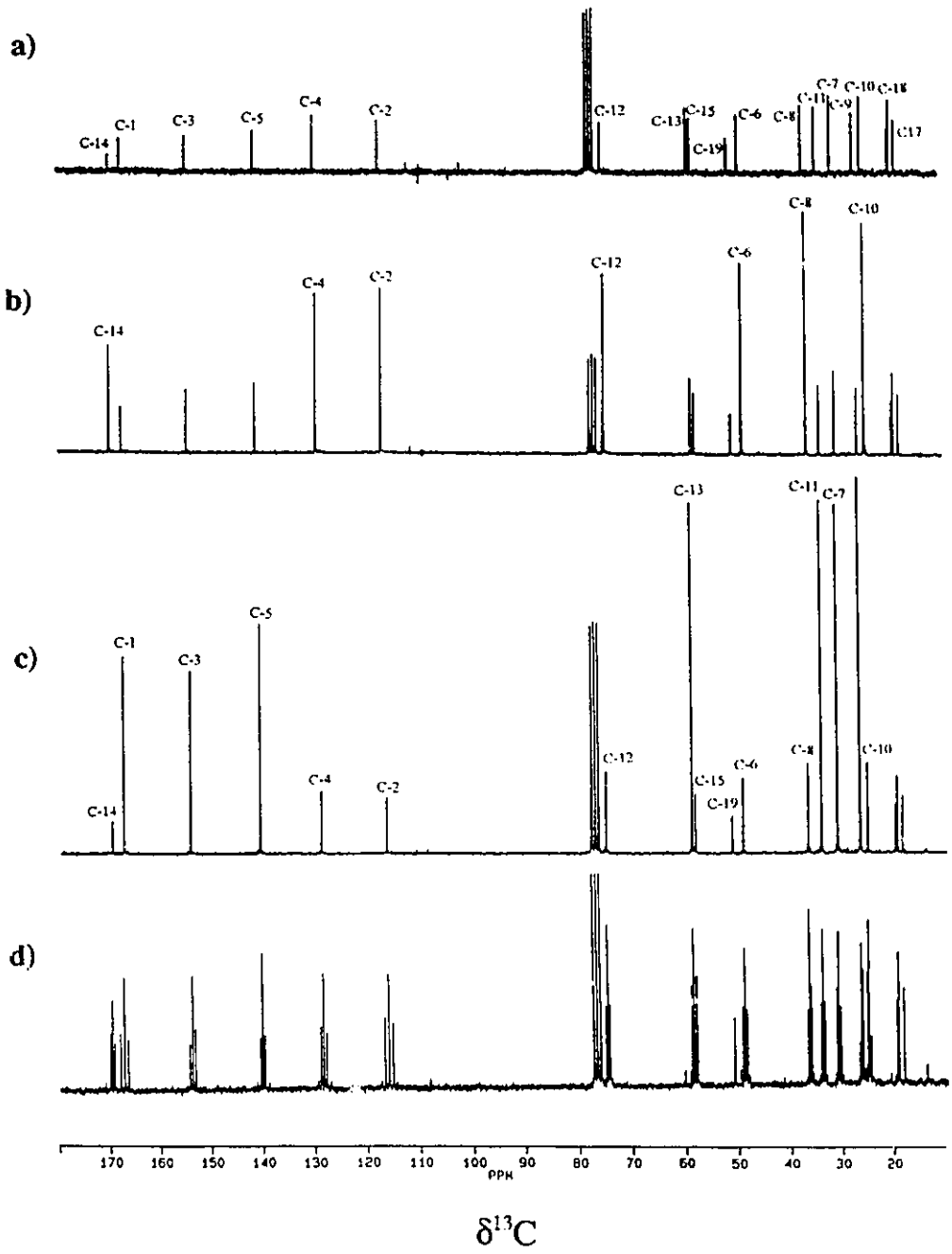


Figure 21. ^{13}C NMR Spectra of F-244 Methyl Ester, a) Unlabelled; and Labelled with b) Sodium [1-^{13}C]Acetate; c) Sodium [2-^{13}C]Acetate; d) Sodium [1,2-$^{13}C_2$]Acetate.

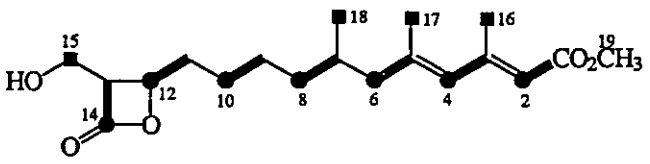


Table 4. Isotopic Incorporations from Singly-labelled Precursors into F-244 and Coupling Constants of F-244 Derived from [1,2-$^{13}C_2$]Acetate .

Carbon	$\delta^{13}C$	% Incorporation[a] from			[1,2-$^{13}C_2$] acetate
		[1-^{13}C]acetate	[2-^{13}C]acetate	[*methyl*-^{13}C] methionine	$J_{C\text{-}C}$ (Hz)
1	167.5	0.1	4.9	0.1	76
2	117.0	1.8	0	0.0	76
3	154.5	0.2	4.0	-0.1	53
4	129.5	1.5	0	-0.2	53
5	141.1	0.1	3.9	-0.3	42
6	48.9	1.8	0.2	-0.2	42
7	30.9	-0.1	4.1	-0.2	35
8	36.6	1.9	0.2	0.0	35
9	26.6	-0.1	5.5	0.0	35
10	25.2	1.7	0.1	0.0	35
11	34.0	-0.2	4.5	0.0	39
12	74.9	2.2	0.2	0.3	39
13	58.6	0	4.7	-0.2	41
14	169.7	4.4	0.2	0.0	41
15	58.0	-0.1	0	18.8	*b*
16	19.6	0	0	19.5	*b*
17	18.4	-0.1	0	19.1	*b*
18	19.4	-0.1	0	15.5	*b*
19[c]	50.8	0	0	0	*b*

[a]Incorporation expressed after subtraction of the peak height for unlabelled **1a**.
[b]No coupling observed.
[c]Unlabelled internal standard

3.1.3. Incorporation of Other Carbon-13 Labelled Basic Precursors

A similar incorporation experiment using sodium [2-^{13}C]acetate gave **1a** which was labelled at the complementary positions C-1, C-3, C-5, C-7, C-9, C-11, and C-13.

Incorporation of sodium [1,2-$^{13}C_2$]acetate gives rise to doublet signals superimposed on natural abundance ^{13}C singlets from unlabelled **1b**, indicating that each of the acetate units was incorporated with the carbon-carbon bond intact. The connectivity between enriched pairs of carbon atoms was confirmed by a two-dimensional INADEQUATE experiment as shown in **Figure 22**. The key feature of the ^{13}C NMR spectrum of this material was the fact that each of the absorptions for the carbon atoms in the main skeleton contained ^{13}C satellites confirming the polyketide origin of the skeleton and establishing the incorporation of the seven intact double-labelled acetate units. In principle, one can identify the individual acetate units by pairing the coupling constants given by the satellite separations provided that the *J* values for each unit are significantly different. For **1b**, this is not the case since the methylene carbons at 25.2, 26.6, 30.9, and 36.6 all exhibited satellites separated by 35 Hz. In a two-dimensional INADEQUATE experiment, however, coupled spins are identified by the double quantum coherence which is equal to the sum of the chemical shift of the two carbon sites measured with respect to the transmitter frequency.[84,85] INADEQUATE is an excellent correlation technique for tracing the backbone of a molecule, but relatively large samples are required because of the low probability of

occurrence of contiguous ^{13}C nuclei (*ca.* $(0.01)^2$ in a sample with natural abundance ^{13}C). In an enriched compound obtained from experiments utilizing doubly labelled acetate, however, the double quantum coherence of the pairs of carbons arising by incorporation of intact acetate units is much more readily determined even at low incorporation levels, and thus, smaller samples are required to identify these coupled pairs.

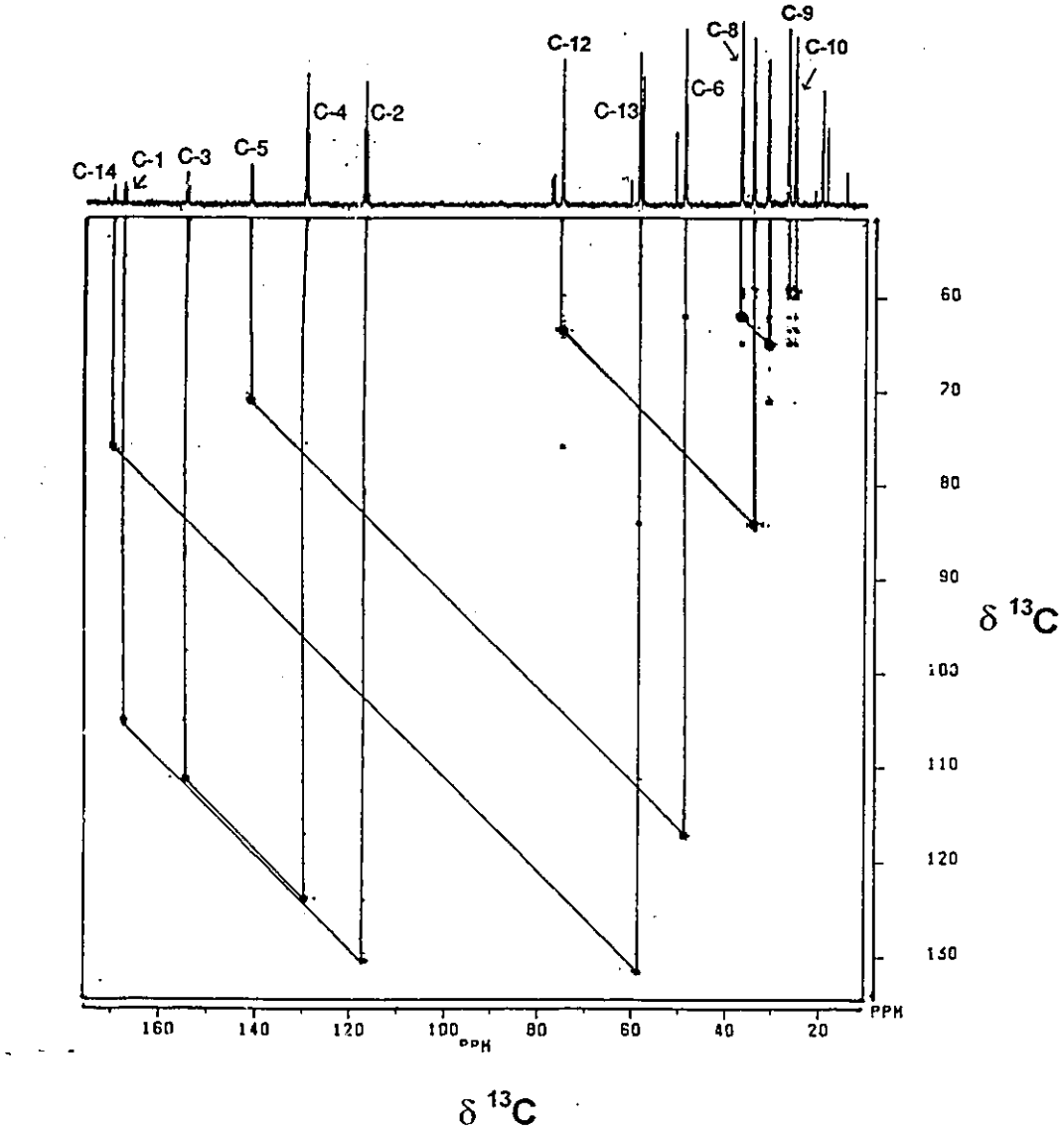


Figure 22. 2-Dimensional INADEQUATE Spectrum of F-244 Derived from [1,2-$^{13}C_2$]Acetate, Showing the Connectivity of Enriched Carbon Atoms.

In order to determine the biosynthetic source of the four remaining carbons in F-244 (i.e. C-15 to C-18), an incorporation experiment using sodium [1-^{13}C]propionate was performed. However, the carbon-13 NMR spectrum of the purified **1b** showed no significant enhancement (within ±0.2 % error) of all carbon signals of **1b**, indicating that propionate was not incorporated into F-244. In contrast, addition of L-[*methyl*-^{13}C]methionine to the *Fusarium* cultures gave large incorporations at the four remaining carbon atoms, C-15, C-16, C-17, and C-18 (**Figure 23**). The extent of methionine incorporations were of the order of 15 to 20%, whereas those of labelled acetates were only of the order of 2 to 5% (Table 3).

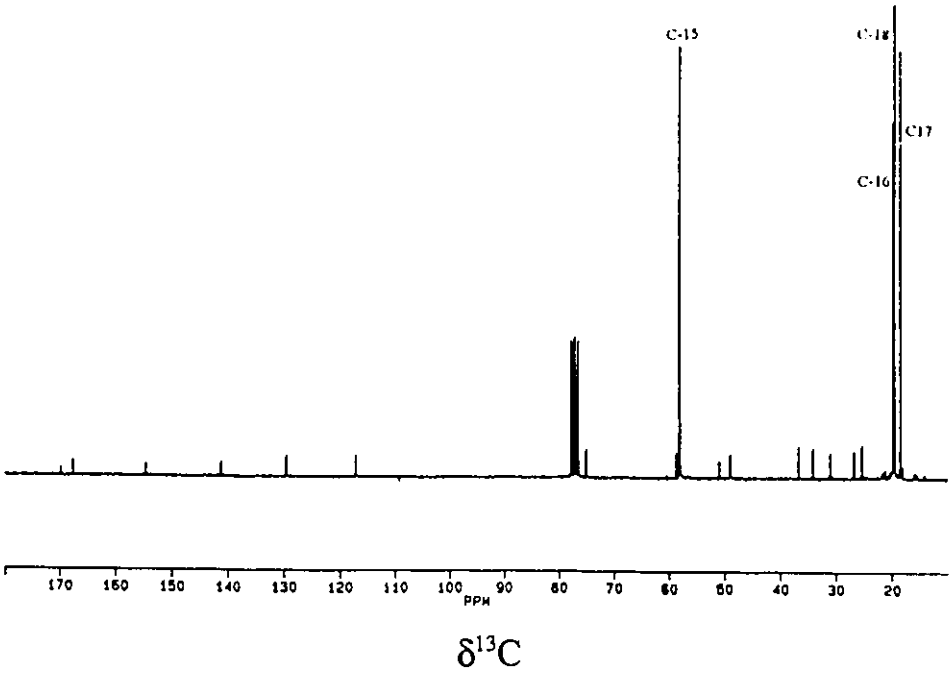


Figure 23. The ^{13}C NMR Spectrum of 1b Derived from Incorporation of L-[*Methyl*-^{13}C] Methionine.

The results from carbon-13 labelled acetate incorporation experiments clearly show that F-244 in *Fusarium* sp. ATCC 20788 is derived from coupling of seven acetate units, arranged in the "head-to-tail" sequence typical of the polyketide metabolites. Further, the arrangement of acetate units is identical to that observed by Omura in *Scopulariopsis* sp.,[81] indicating strong similarities in the biosynthesis of **1a** in the two microorganisms. The carbon atoms of the three methyl groups, as well as C-15 are derived from methionine in both organisms, rather than through incorporation of propionate into the extending polyketide chain. This result is in agreement with a variety of studies which have shown that pendant methyl groups in fungi are derived from methionine rather than propionate. (**Figure 24**).

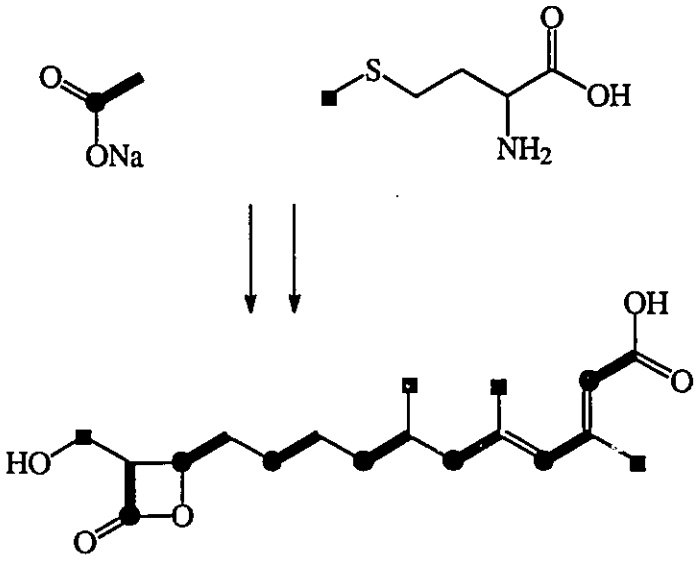


Figure 24. Biosynthesis of F-244: Origin of the Carbon Atoms

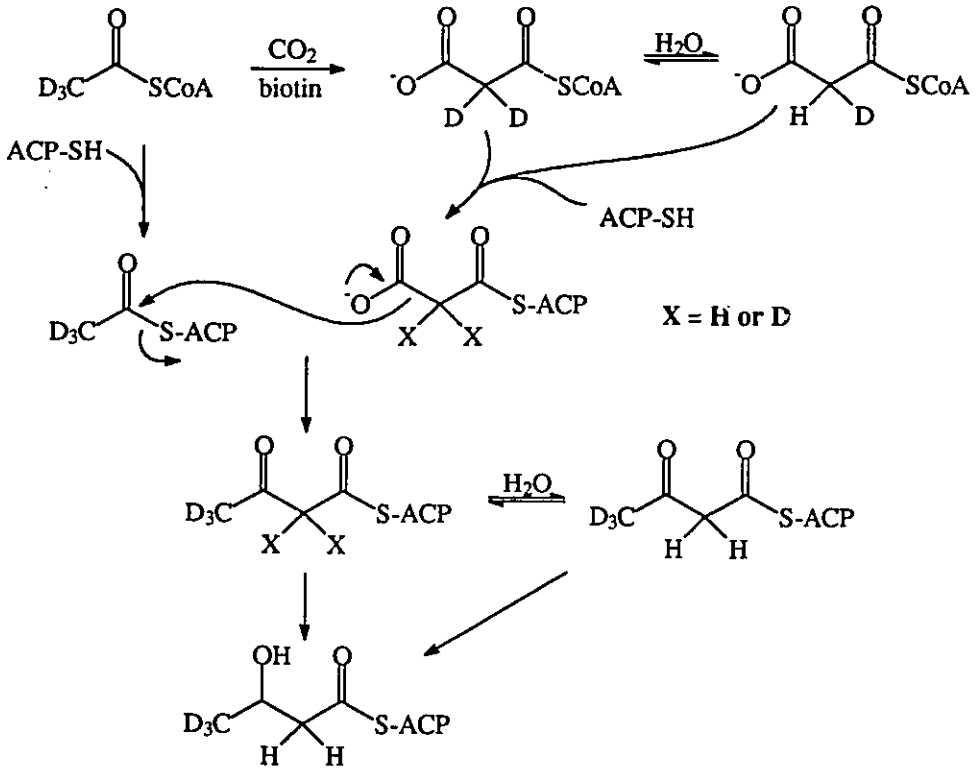
3.2. The Biogenesis of the Hydrogen and Oxygen Atoms of F-244

With the biogenesis of the carbon atoms established, the biosynthetic origin of the hydrogen atoms of F-244 was investigated next. Incorporation experiments using sodium [1-^{13}C,^{2}H$_3$]acetate gave **1a** that was enriched with carbon-13 at all the positions derived from C-1 of acetate as expected. However, none of the signals exhibited a β-deuterium isotope shift. Further, deuterium NMR analysis showed no deuterium signal in the deuterium NMR spectrum after 18 hours of acquisition time, indicating that the level of labelling was less than 0.1% incorporation (for the discussion of detection limit, see section 4.3).

The fact that incorporation of carbon-13, but not deuterium, was observed when sodium [1-^{13}C,^{2}H$_3$]acetate was administered to the cells strongly suggests that "washout" occurred during the biosynthetic process. Washout processes are common in polyketide biosynthetic studies and normally occur during chain elongation. Thus, when C-2 of acetyl-CoA is converted to C-2 of malonyl-CoA then to a β-ketoacyl enzyme intermediate, the two protons in malonate as well as in acetoacetate are sufficiently acidic to undergo an exchange process with the aqueous environment (**Scheme 14**).

Additional experiments in our laboratory, performed by Dr. Louise Edwards, showed that addition of [*methyl*-^{13}C,^{2}H$_3$]methionine to the cultures of *Fusarium* sp. ATCC 20788 gave **1a**, which exhibited three doublets in the deuterium NMR

spectrum corresponding in chemical shift to the three methyl groups of F-244. As well, signals at deuterium chemical shifts equivalent to those for the diastereotopic protons, H-15 and H-15' were observed. This result demonstrates intact incorporation of the ^{13}C-^{2}H bonds. This observation is consistent with Omura's description of incorporation of tritium from L-[^{3}H]methionine into F-244.[81]



Scheme 14. The Process of Chain Elongation in Polyketide Biosynthesis Can Result in Loss of Deuterium Label from [CD_3]Acetate.

The next experiment aimed to determine the biosynthetic origin of the oxygen atoms in F-244. This experiment would also address questions about the mechanism of

formation of the β-lactone moiety. According to the Birch hypothesis, oxygen atoms on carbons derived from C-1 of acetate should originate from acetate, since the carbon-oxygen bond remains intact during the condensation of acetate and malonate to give the β-ketoester, and in the subsequent reduction step to the β-hydroxyester.[43] However, in the case of F-244, the oxygen atom in the β-lactone ring could originate from one of two possible acetate units.

Labelling experiments with both isotopes of oxygen, ^{17}O and ^{18}O, are documented in the literature.[58,86] Oxygen-17 has a spin of 5/2, and thus can be directly detected by NMR. Despite this advantage, ^{17}O has been used relatively little as a biosynthetic tracer. The reason for this neglect is the difficulty of acquiring good quality ^{17}O NMR spectra. The nucleus is quadrupolar and thus relaxes very rapidly, resulting in broad signals.[87] Oxygen-18 has no spin, but can be detected by indirect observation through an isotope shift effect on the attached carbon-13 signal.[88,89] The magnitude of these induced isotope shifts are in the range of 0.01 to 0.05 ppm, depending on the nature of the bond. Isotope shifts of $^{13}C=^{18}O$ double bonds are larger that those of $^{13}C-^{18}O$ single bonds.[90] This isotope shift effect permits bond labelling. Thus, incorporation of [1-^{13}C,$^{18}O_2$]acetate into a natural product normally gives a detectable isotope shift if at least one of the carbon-oxygen bonds remains intact.

In order to determine the origin of the oxygen atoms and the mode of cyclization of the β-lactone ring in F-244, a biosynthetic incorporation experiment was

performed using sodium [1-^{13}C,$^{18}O_2$]acetate. This technique has been used to demonstrate that a variety of six-membered or larger lactone rings are formed with retention of the alkyl carbon-oxygen bond. Examples include Brefeldin A3 from *Penicillium brefeldianum*[90], erythromycin from *Streptomyces erythraeus*, nargenicin from *Nocardia argentinensis*, and avermectin from *Streptomyces avermitilis*.[60] In each case, the results are consistent with formation of the lactone being the last step on the PKS enzyme. Intramolecular nucleophilic attack by an OH group onto the carbon atom of the acyl-S-PKS thioester occurs, releasing the cyclized product and regenerating the enzyme. There are, however, no reported examples of the use of the ^{18}O-labelling technique to study the biosynthesis of β-lactones. Due to the ring strain inherent in the β-lactone, it was unclear whether ring formation could occur on the polyketide synthase, or whether a separate enzyme was required to effect the lactonization in F-244.

Addition of [1-^{13}C,$^{18}O_2$]acetate to the growing cultures of *Fusarium* sp. gave F-244 that was enriched with carbon-13 at each of the positions that had been labelled by [1-^{13}C]acetate. High resolution carbon-13 NMR showed that two signals (C-12 and C-14) exhibited isotopically shifted signals induced by the presence of oxygen-18 (**Figure 25**). The magnitude of the shift is 0.037 ppm for C-14, which is consistent with that expected for a signal due to the presence of ^{18}O attached to ^{13}C via a double bond. There was no apparent signal due to the presence of carbon-13 attached to either oxygen-18 within the ring (i.e., $^{16}O=^{13}C-^{18}O$), or to two oxygen-18 atoms (i.e.,

$^{18}O=^{13}C-^{18}O$). At C-12, a small but significant single isotopically shifted signal was observed. The magnitude of the shift ($\Delta\delta$ 0.031 ppm) is rather large compared to many $^{13}C-^{18}O$ single bonds,[92] perhaps as a result of the ring strain inherent in the β-lactone moiety. In addition to these isotopically-shifted signals, smaller signals were also noticeable at δ 74.84, 74.94, and 169.81. These signals probably arose from long (2-bond) range carbon-carbon coupling resulting from multiple intramolecular precursor incorporation. This phenomenon has been observed in sterigmatocystin from *Aspergillus versicolor*.[90] No detectable induced isotope shift was observed at either C-1 or C-15 as expected, since these carbon atoms do not derive from C-1 of acetate.

These observations are consistent with formation of the β-lactone ring by attack of the hydroxyl group at C-12 of the nascent polyketide onto C-14 (**Figure 26**, pathway **a** or **b**), but not with nucleophilic attack by a C-14 carboxylate onto C-12 (pathway **c**). Perhaps the most likely pathway is pathway **a** which involves the formation of the β-lactone ring by direct displacement of the acyl intermediate while still attached to the polyketide synthase (PKS), thereby releasing the cyclized product. In contrast to pathways **b** and **c**, pathway **a** does not require a unique β-lactone cyclizing enzyme.

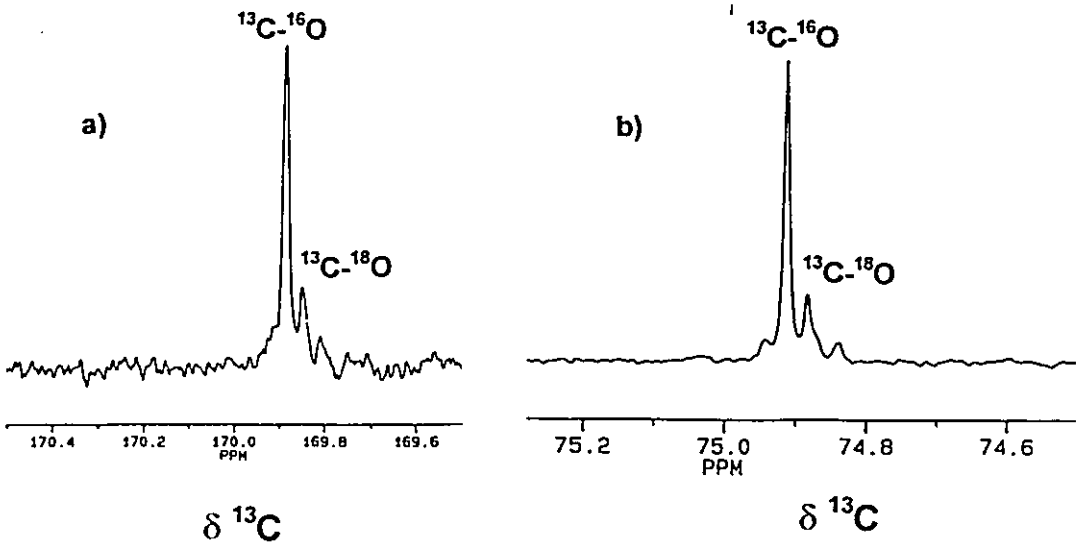


Figure 25. Signals for the ^{13}C–^{18}O Labelled Carbons of **1b**. The resonances due to ^{13}C–^{18}O labelled carbons are on the right hand side of each set of signals, a) the resonance of C-14 and b) the resonance of C-12.

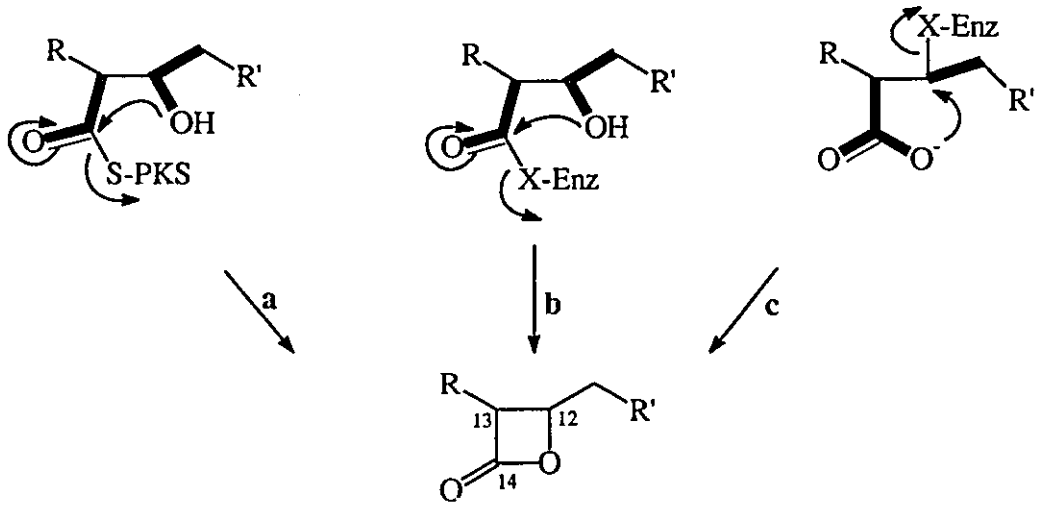


Figure 26. Possible Mechanisms for β-Lactone Ring Formation in F-244

3.3. Conclusion

The biosynthesis of F-244 in *Fusarium* sp. ATCC 20788 proceeds by the normal polyketide pathway, with seven acetate units condensing in a "head to tail" fashion. The pendant hydroxymethyl and three methyl moieties of F-244 are derived from methionine. A labelling experiment with sodium [1-^{13}C,$^{18}O_2$]acetate shows that the β-lactone ring is formed with retention of the C-12—O bond, suggesting nucleophilic attack of a hydroxyl group at C-12 of the putative precursor, possibly still attached to the polyketide synthase, onto an activated carboxylic acid derivative at C-14. Failure to observe an induced isotope shift at C-1 or C-15 is consistent with the oxygen atoms at these sites being derived from oxygen gas in a late stage oxidation.[91]

The results of feeding experiments using [1-^{13}C]acetate and L-[methyl-^{13}C] methionine show that there are strong similarities in the biosynthesis of F-244 between *Fusarium* sp. and *Scopulariopsis* sp.. However, since Omura's group only used ^{13}C-labelled precursors, their study did not reveal any information about the mechanism of formation of β-lactone moiety of F-244 by *Scopulariopsis* sp.; indeed, the ^{18}O-induced isotope shift method, which has been applied extensively to larger ring lactones, has not been applied to β-lactones to our knowledge. Therefore, our investigation provides new information about the mechanism of β-lactone formation in F-244, which may be of general applicability.

CHAPTER 4

Biosynthetic Studies of F-244 Using Advanced Precursors

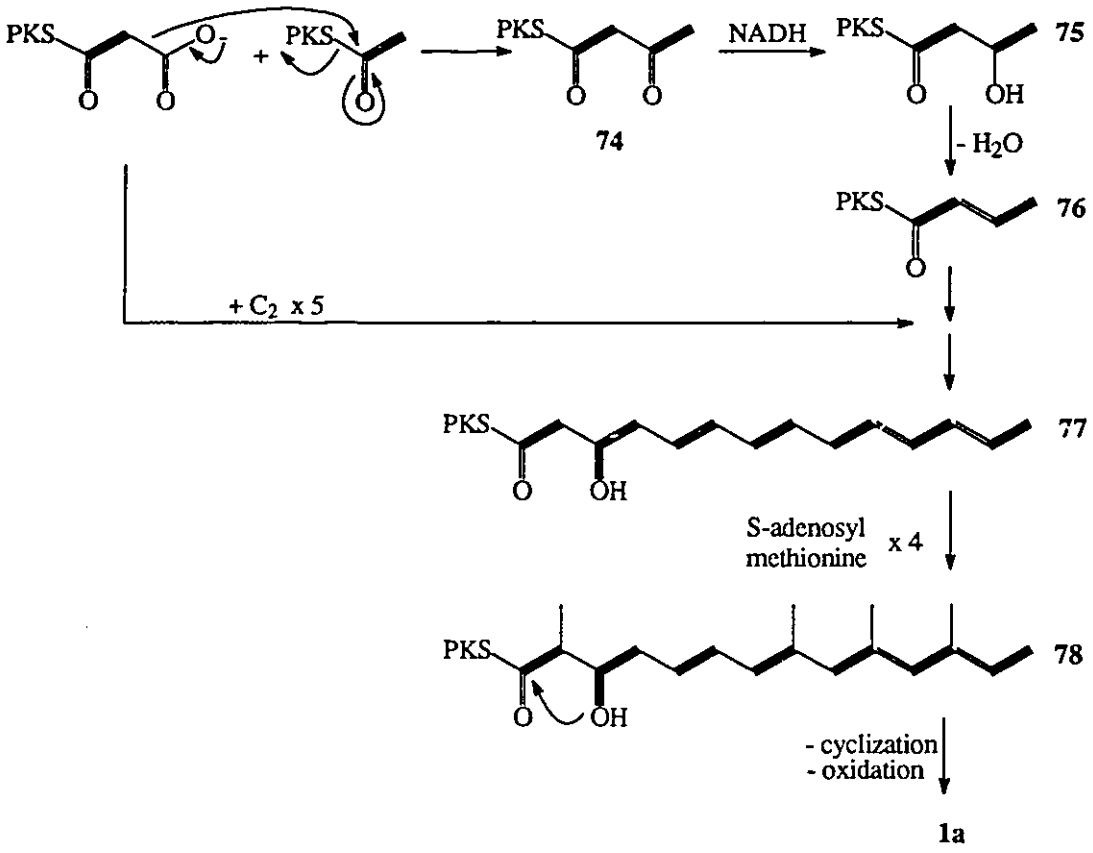
4.1. Hypotheses for the Biosynthetic Pathway

The close parallel between fatty acid biosynthesis and the chain building steps of polyketide biosynthesis has long been recognized, but efforts to provide experimental proof by isolating cell free systems that produce complex polyketide synthase enzymes responsible for formation of polyketides have proved elusive. Presently, the only polyketide synthase proteins that have been characterized construct relatively simple aromatic compounds such as orsellinic acid,[92] and 6-methylsalicylic acid.[93] In addition, classical methods of biosynthetic study using intact organisms and labelled precursors have been frustrated by at least one of two factors: 1) labelled precursors are rapidly degraded by efficient β-oxidation to acetate or propionate, which may then undergo *de novo* incorporation, 2) intermediates need to become bound to the enzyme system by covalent bonds rather than by relatively simple non-covalent molecular interactions. A number of groups, working on various metabolites, have shown that this barrier can sometimes be overcome by administering the

proposed acyl intermediates as thioesters of N-acetyl cysteamine.[76,79,94,95] How this incorporation is achieved is still unclear, but it is likely this small residue can effectively mimic the thiol terminus of the pantotheine group which is present in coenzyme A and the acyl carrier protein (ACP) component of the PKS, thus allowing the added labelled intermediate to be elaborated.

If the formation of the backbone of F-244 resembles the biosynthesis of fatty acids, as is currently believed, this process would involve assembly of two-carbon precursors via enzyme-bound, thioester intermediates to give a four-carbon chain. There are several plausible sequences that can be proposed from here.

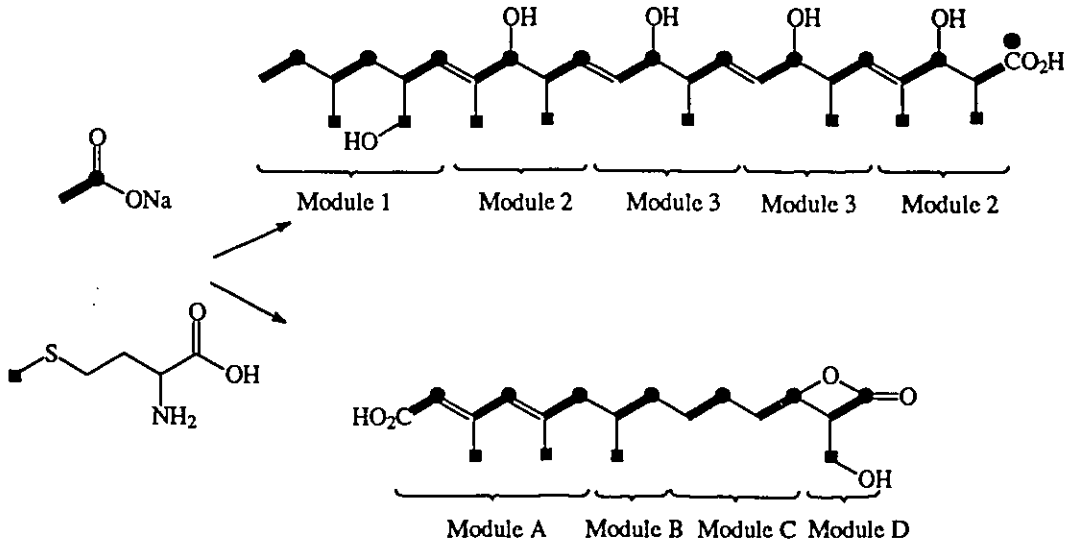
The first mechanism (a) would involve successive condensation of acetate units until all seven acetates are assembled to form a long β-hydroxy carboxylic acid intermediate. Four methyl groups are then introduced, followed by cyclization and oxidation to form **1a**. Thus, acetate is first condensed with malonate to give β-ketoacyl residue **74** with concomitant decarboxylation. Reduction of the keto group gives the corresponding β-hydroxyacyl analog **75**, which is then dehydrated to the enoyl derivative **76** before its reaction with the next chain-extending, two-carbon unit (malonate). This process is repeated with addition of malonate units until 7 acetate units are assembled and ready for the next conversion (**Scheme 15**). It is generally accepted that formation of hydroxy, olefinic, or other functionalities are adjusted as the chain grows.[38,56]



Scheme 15. Hypothesis (a) for Assembly Process of F-244

The second mechanism (b) involves the introduction of methyl groups from S-adenosylmethionine (SAM) during the chain growth. In this mechanism, the methyl group is added immediately after a two-carbon unit is added as needed. Thus, three methyl groups are added during the first three cycles, and one in the seventh cycle. Such a mechanism has been proposed by O'Hagan *et al.* for the biosynthesis of cubensic acid in *Xylaria cubencis*.[71] Based on the repeating sequence of units cubensic

acid, O'Hagan proposed a modular enzymatic sequence, modules 1-3, that may be responsible for the biosynthesis of cubensic acid. Assuming that O'Hagan's hypothesis is correct, a similar series of enzymatic modules may operate for the biosynthesis of **1a**, as can be seen in **Scheme 16**.



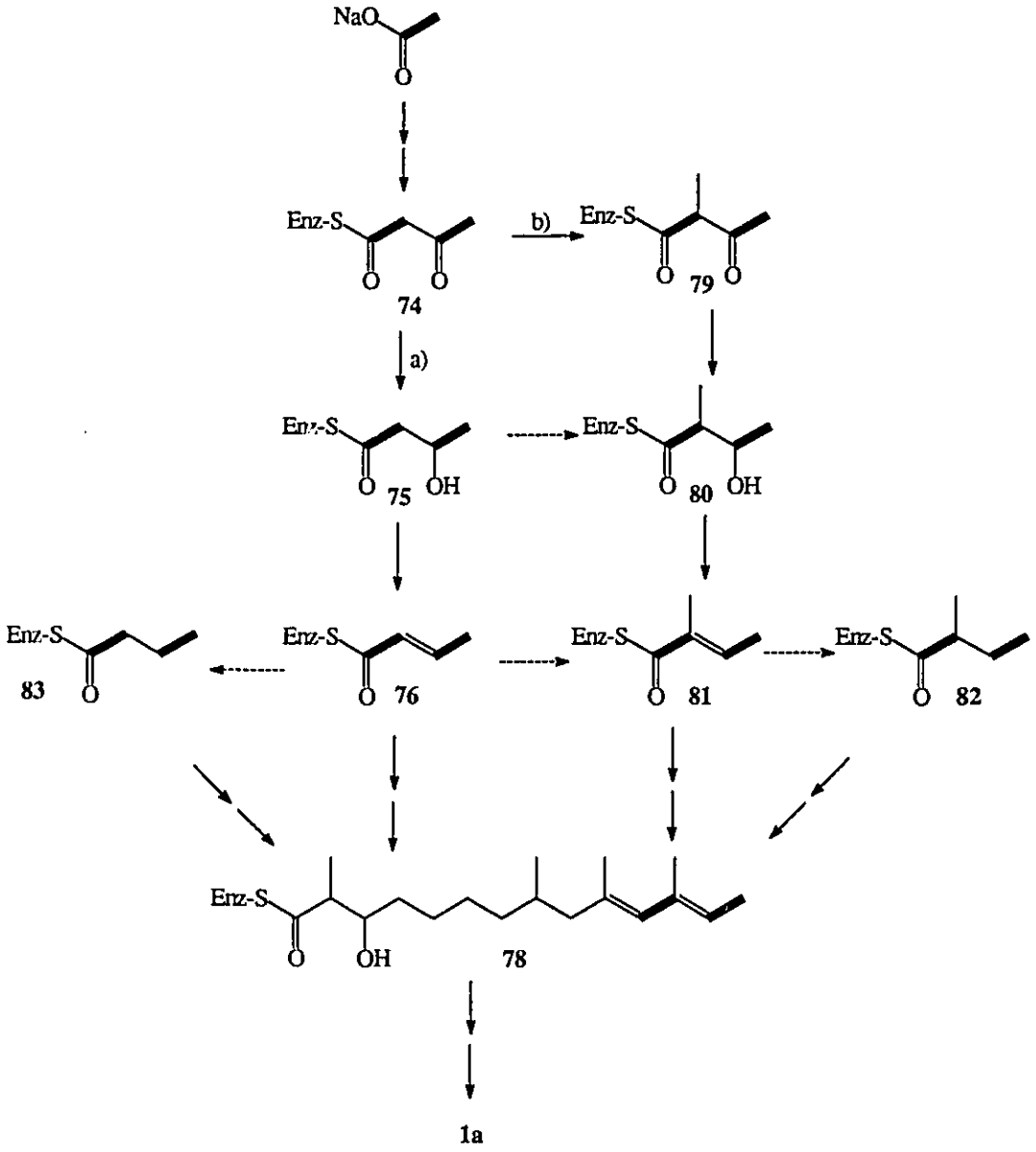
Module 1 KS,MT, KR, DH, ER, KS, MT, KR, DH, ER.
Module 2 KS, MT, KR, DH, KS, MT, KR.
Module 3 KS, MT, KR, DH, KS, KR.

Module A KS, MT, KR, DH, KS, MT, KR, DH
Module B KS, MT, KR, DH, ER.
Module C KS, KR, DH, ER, KS, KR, DH, ER.
Module D KS, MT, KR.

Scheme 16. Proposed modular assembly of cubensic acid and F-244. KS = β-ketoacyl synthase, MT = methyl transferase, KR = β-keto reductase, DH = dehydratase, ER = enoyl reductase

Other mechanisms which are intermediate in between the two mechanisms (a) and (b) above may also be considered. Inevitably, as more two-carbon units are added

to the growing chain, more alternative pathways can be proposed, as can be seen in **Scheme 17.**



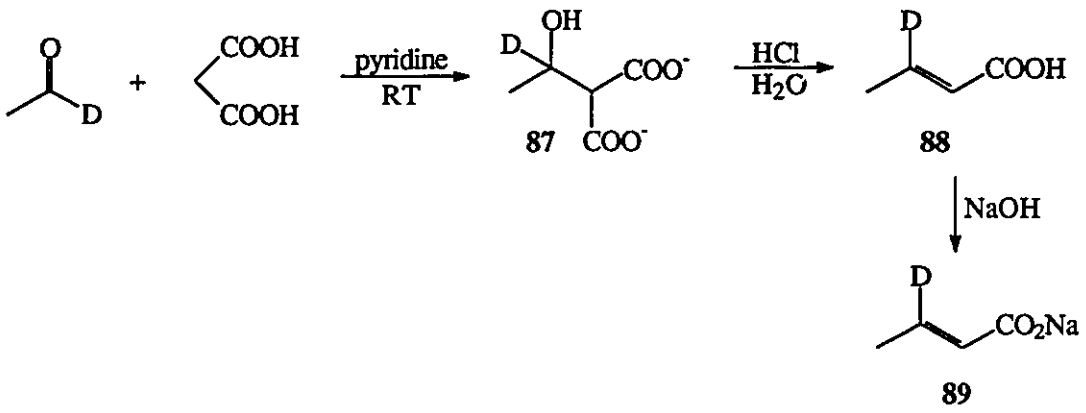
Scheme 17. Possible Alternative Mechanisms for Formation of F-244

4.2. Synthesis of Advanced Labelled Precursors

In order to test the hypotheses discussed above, several of the potential intermediates, both as free acids and as N-acetylcysteamine thioesters, with strategically placed deuterium or carbon-13 labels, were synthesized or purchased if commercially available. The deuterium-labelled precursors that were synthesized would not be degraded to labelled acetate as a consequence of fatty acid degradation (β-oxidation) pathway because the deuterium is placed at a site which would ultimately be converted to the carboxyl group of acetate rather than to the methyl group.

4.2.1. Sodium [3-^{2}H]Crotonate

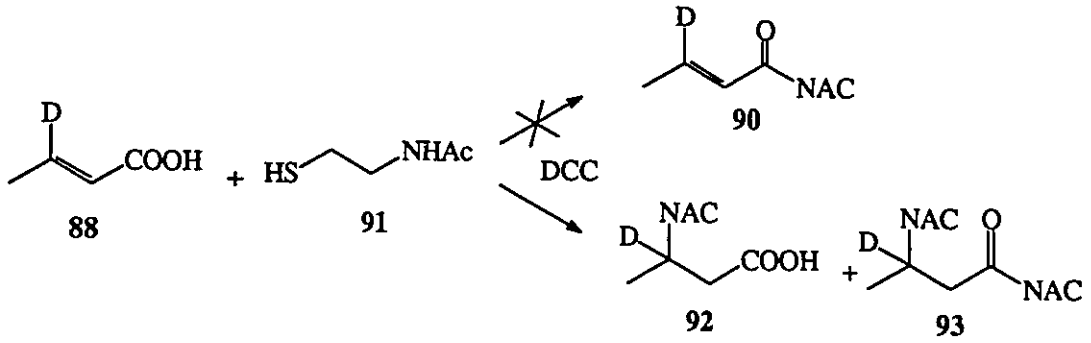
The deuterium-labelled crotonate **89** was prepared following the method developed by Allen and Allan for synthesis of sorbic acid.[96] Thus **89** was readily synthesized from [1-^{2}H]acetaldehyde with malonic acid in pyridine (**Scheme 18**). The [1-^{2}H]acetaldehyde was added to a solution of malonic acid in pyridine and stirred. Addition of a dilute solution of HCl caused decarboxylation of intermediate **87** to produce the acid **88**. The acid was then converted to its sodium salt with addition of one equivalent of aqueous NaOH and the resulting aqueous solution was lyophilized, giving **89** in 64% yield.



Scheme 18.

4.2.2. S-[3-^{2}H]Crotonyl,N-Acetylcysteamine (90)

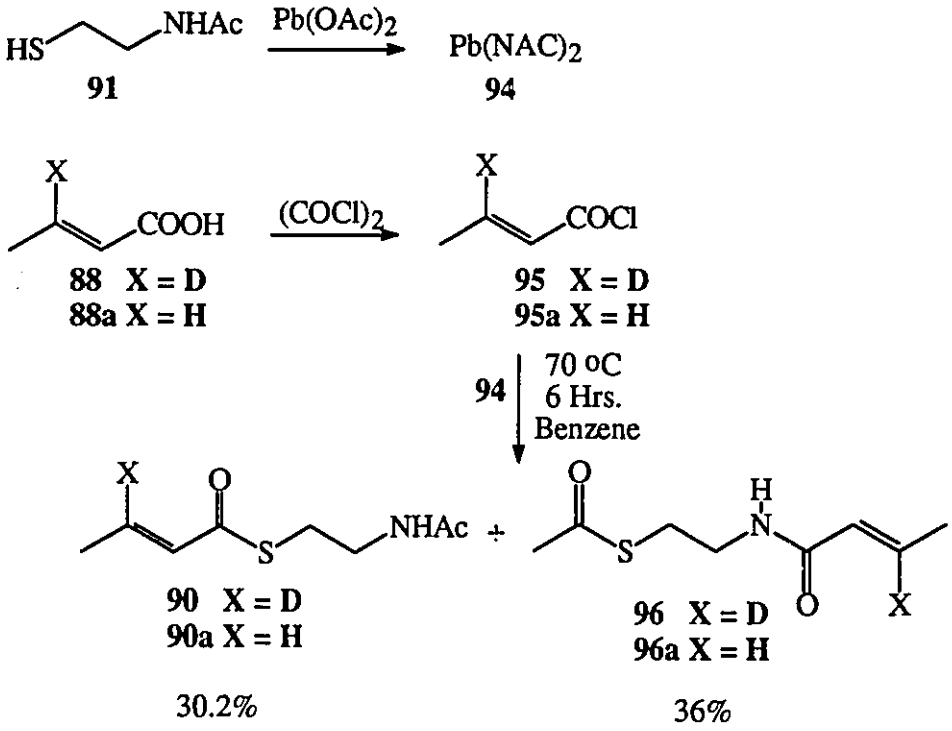
The initial approach to the synthesis of S-[3-^{2}H]crotonyl,N-acetylcysteamine (**90**) was to mix **88** and N-acetylcysteamine (NAC) in the presence of the coupling reagent dicyclohexyl carbodiimide (DCC) at room temperature. N-acetyl-cysteamine (**91**) was itself generated by selective hydrolysis of N,S-diacetylcysteamine.[97] However, the result was not as expected, instead of **90**, compounds **92** and **93** were produced. The structures were substantiated by NMR, IR and MS. Apparently, instead of the desired coupling reaction, Michael type addition to the existing conjugated double bond in **88** occurred (**Scheme 19**). Attempts to prevent this addition reaction by lowering the reaction temperature to 0 °C or replacing DCC with diphenylphosphoryl azide (DPPA) failed to give the desired product.



Scheme 19.

To prevent this Michael type addition, **88** was converted to acyl chloride **95** by addition of oxalyl chloride, and **91** was converted to its lead salt **94** by reaction with lead acetate.[99] These conversions were intended to increase the activity of the carboxyl group of the crotonate, while decreasing the activity of the sulfhydryl group of N-acetylcysteamine. Stirring the acyl chloride **95** with the lead salt **94** at 70 °C in benzene gave two major products, **90** and **96**, which were separable by column chromatography, in a ratio of 5 : 6. The proton NMR spectrum of **96** is very similar in many respects to that of **90**. However, the chemical shifts of the acetyl group and the proton on the double bond are slightly different indicating that the acetyl and crotonyl groups of **90** and **96** have different positions. This observation deserved further analysis. Therefore, nOe experiments using unlabelled materials **90a** and **96a** were performed. Upon irradiation of the N-H proton of **90a**, nOe's were observed at 1.96 ppm (CH_3CO), 3.0 and 3.5 ppm (CH_2S and CH_2N), while irradiation of the N-H

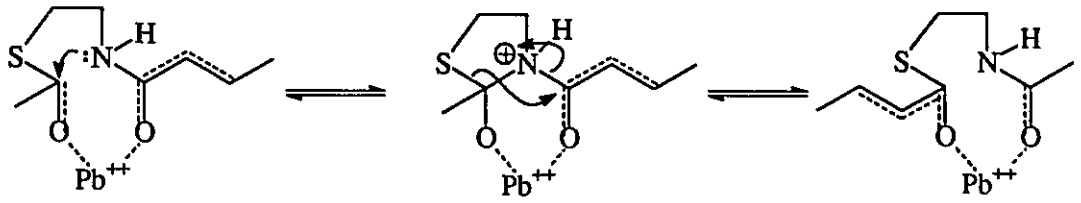
proton of **96a** gave nOe's at 5.8 and 6.8 ppm (CH=CH) as well as at δ 3.0 and 3.5 (CH_2S and CH_2N). These results show that **96a** is the isomer of **90a** in which the acetyl and crotonyl positions have been switched. Thus the structure of **96a** is S-acetyl,N-crotonylcysteamine (**Scheme 20**).



Scheme 20.

It was unclear how the acetyl group and crotonyl group could switch their position. Perhaps this is due to the presence of the lead salt which might effect the migration of these groups, as shown in **Scheme 21**. Efforts to reduce this migration by

varying the reaction time or reducing the reaction temperature only resulted in a lower yield of **90** with an unchanged the ratio of the two compounds.

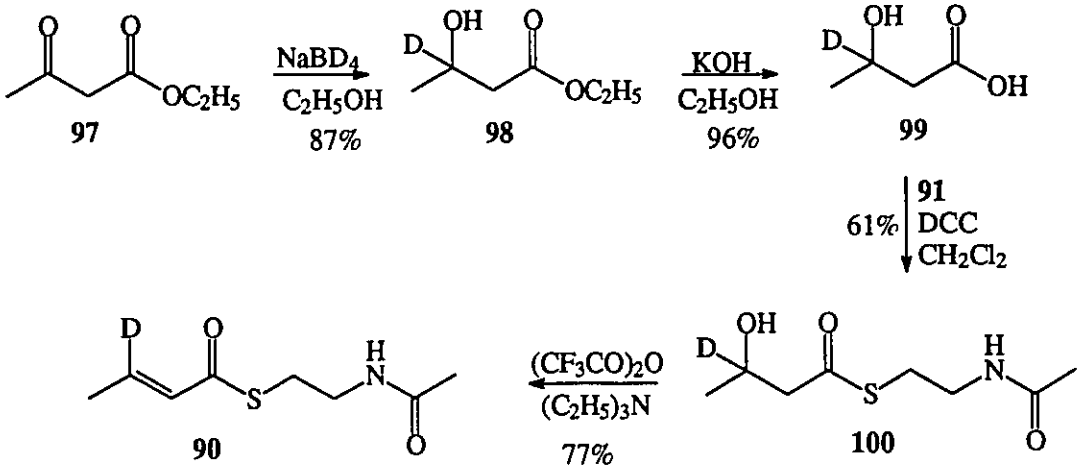


Scheme 21.

Because of the poor yield of the final product **90** (the overall yield from acetaldehyde was only 19%), this method was abandoned. Jacobs *et al.*[98] prepared a labelled hydroxy acid analog **100** using ethyl acetoacetate (**97**) as a starting material. It appears that this method is quite feasible for the preparation of **90.** Thus, **97** was reduced with sodium borodeuteride to give ethyl 3-hydroxy-[3-^{2}H]butyrate (**98**) in 87% yield. Hydrolysis of **98** with KOH/ethanol gave the hydroxy acid **99** in 96% yield. Reaction of the acid **99** with **91** in the presence of DCC in dichloromethane afforded the hydroxy acid analog **100** in 61% yield.

Sheehan and Beck[99] prepared **90** by heating **100** at 150 °C. However, this method could not be reproduced in our laboratory. Heating of **100** for prolonged times only resulted in decomposition, with no noticeable formation of **90**. Reaction of **100**

with *p*-toluenesulfonyl chloride in triethylamine also failed to give the desired product. However, upon addition of trifluoroacetic anhydride to **100**, followed by treatment with an excess of triethylamine, reaction proceeded smoothly giving **90** in 77% yield (**Scheme 22**). Extensive purification allowed isolation of the pure product, which gave satisfactory spectral data to confirm the structure. However, the physical property was found to be different to that reported by Sheehan and Beck.[101] We found that **90** was a solid, whereas Sheehan and Beck reported that **90** was liquid. Perhaps this is due to the difference in purity of the compound.

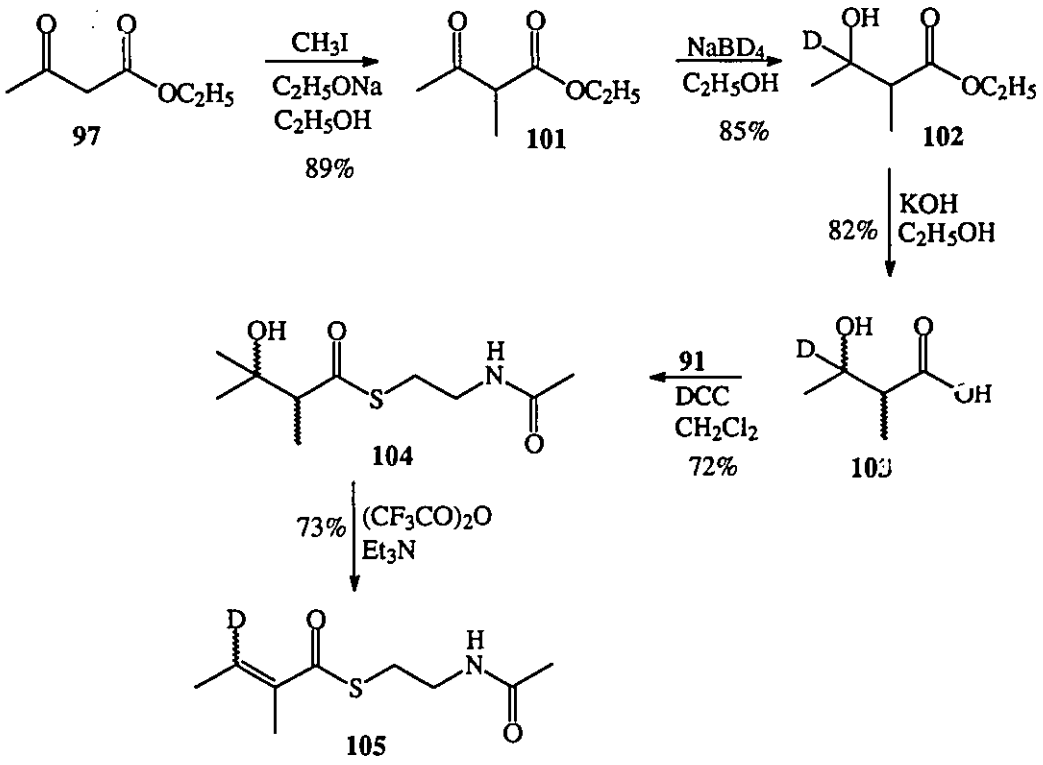


Scheme 22.

4.2.3. [3-^{2}H]2-Methylcrotonyl-NAC Thioester (105)

[3-^{2}H]2-Methylcrotonyl-NAC (**105**) was prepared as shown in **Scheme 23**. The starting material **97** was treated with sodium ethoxide in ethanol followed by

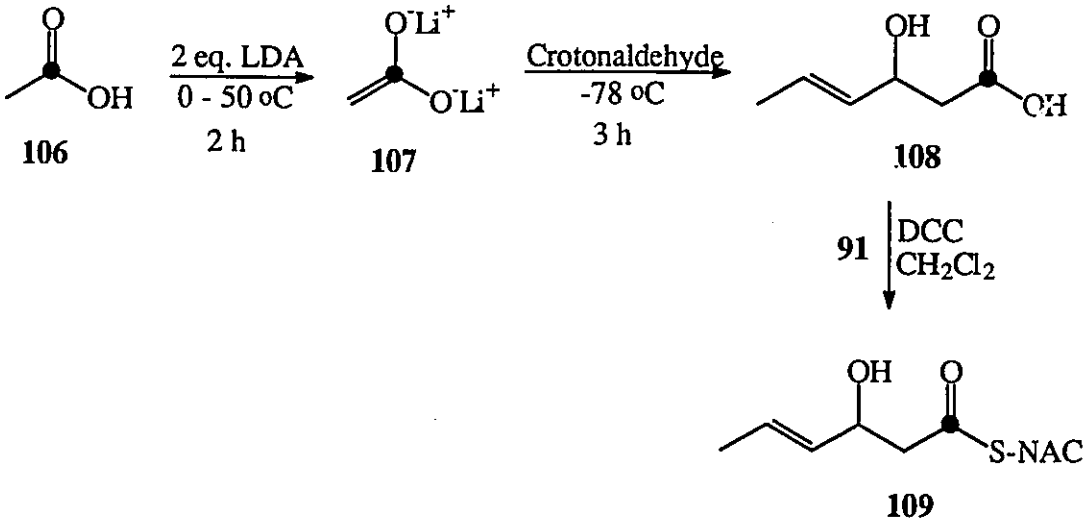
addition of methyl iodide.[100] Upon heating, **101** was formed, which was easily separated from the solvent and the resulting solid sodium iodide by filtration and distillation. Compound **101** reacts readily with sodium borodeuteride to afford **102** as a racemic mixture of two diastereomers. Hydrolysis of **102** gave the acid **103** in 82% yield. Reaction of the acid **103** with **91** in the presence of DCC furnished the thioester **104**. Reaction with trifluoroacetic acid and triethylamine afforded **105** in 73% yield as a mixture of *cis* and *trans* isomers. No effort was made to separate this mixture.



Scheme 23.

4.2.4. [1-^{13}C]3-Hydroxy-4-hexenoyl-NAC Thioester (109)

[1-^{13}C]3-Hydroxy-4-hexenoyl-NAC thioester (**109**) was synthesized from [1-^{13}C]acetic acid (**106**), following the method developed by Adam *et al.*[101] for α-alkylating aliphatic carboxylic acids by treating an α-lithio carboxylate salt with an aldehyde. The acid **106** was obtained from the reaction of sodium [1-^{13}C]acetate and *p*-toluenesulfonic acid. Treatment of **106** with 2 equivalents of LDA at 0 - 50 °C followed by addition of crotonaldehyde at -78 °C gave the hydroxy acid **108** in 67% yield. Reaction of **108** with **91** in the presence of DCC furnished **109** as a clear oil in 61% yield.

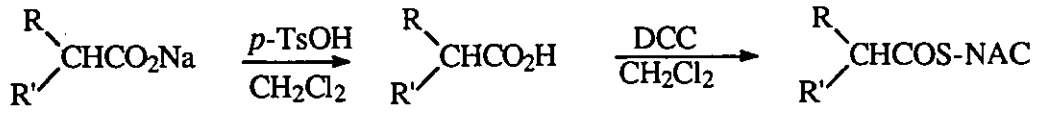


106 **107** **108** **109**

Scheme 24.

4.2.5. S-[1-^{13}C]Acetyl-NAC (110), S-[1-^{13}C]Butyryl-NAC (111), and 2-Methyl [1-^{13}C] Butyryl-NAC Thioester (112)

The three compounds **110**, **111**, and **112** were prepared in a similar manner, starting from the commercially available sodium salts (**Scheme 25**). Thus the sodium salts were converted to the corresponding acids by stirring with *p*-toluenesulfonic acid in dichloromethane. The resulting acids were then reacted with **91** and DCC to afford **110**, **111**, and **112**.



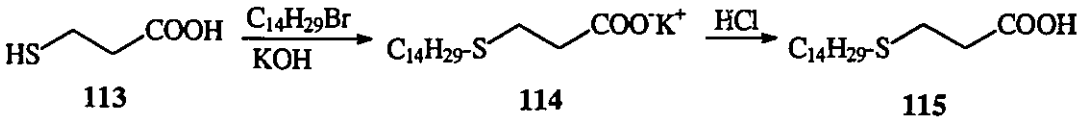
110 R = R' = H
111 R = C_2H_5, R' = H
112 R = C_2H_5, R' = CH_3

Scheme 25.

4.2.6. 3-Tetradecylthiopropionic Acid (115)

3-Tetradecylthiopropionic acid (**115**) was synthesized from 3-mercaptopropionic acid (**113**) and tetradecyl bromide using the method developed by Spydevold and Bremer[102] (**Scheme 26**). Thus **113** was converted to its potassium salt by addition of 2.5 equivalents of KOH. Heating the salt with tetradecyl bromide

followed by addition of concentrated HCl afforded **115** as white silvery crystals in 92% yield.



113 **114** **115**

Scheme 26.

4.3. Feeding experiments

With the labelled putative precursors in hand, incorporation experiments can now be performed. All of the thioester derivatives except [1-^{13}C]acetyl-NAC (**110**) have poor solubility in water; therefore 4 - 5% ethanol in water was used to prepare the corresponding solutions. Experiments were monitored using HPLC analysis of F-244 production and compared with controls which were performed simultaneously.

The first attempt to incorporate a partially assembled precursor was to incorporate crotonate **89** into F-244 using the same protocol that was used in feedings with labelled acetates. The isolated F-244 was methylated with diazomethane to give compound **1b** (6 mg). However, no deuterium signal from the F-244 was observed in the ^{2}H NMR spectrum of this material after 18 hours of acquisition time.

The failure to observe any deuterium signal from this experiment prompted us to determine the detection limit of deuterium on the Bruker AM-500 spectrometer. The detection limit was calculated based on the natural abundance deuterium signal in chloroform (**Appendix 1**). Assuming that the minimum acceptable S/N ratio to detect a peak is 3 : 1, it was estimated that for a sample of F-244 in 0.75 mL $CHCl_3$, the detection limit of the spectrometer is 22 nmol of deuterium in 18 hours of acquisition, which corresponds to 0.12% of deuterium incorporation in 6 mg of F-244. It is therefore defined from here on, that no incorporation or no observable deuterium in

the ^2H NMR spectrum means incorporation is less than 0.12% for a 6 mg sample of F-244.

Thus, the extent of deuterium incorporation of crotonate **89** is less than 0.12%. The lack of any observable deuterium at the C-2 position of **1b** indicates that either (a) the labelled crotonate cannot cross the cell wall; or (b) it is not accessible to the F-244 PKS *in vivo*; or (c) it was efficiently degraded by β-oxidation before entering the F-244 PKS; or (d) it is not a true biosynthetic precursor.

Another approach was thus sought using the labelled putative intermediates as thiol ester N-acetylcysteamine derivatives **90** and **100**. However, when crotonate **90** and hydroxybutyrate (**100**) were administered to the culture of *Fusarium* sp. in two separate experiments, neither of the isolated F-244 samples exhibited any observable signal in the ^2H NMR spectra, suggesting that neither of these labelled materials was incorporated intact into F-244. Since there have been a number of examples of successful experiments utilizing derivatives of N-acetylcysteamine which suggest that the precursor should be able to enter the cell, this failure is most likely interpreted in one of two ways: either the crotonate and the hydroxybutyrate could not survive in the cell due to β-oxidation, or else these units are not intermediates in the assembly process.

Because the crotonate and the hydroxybutyrate were designed in such a way that if any degradation occurs the deuterium would not be retained, it becomes

difficult to determine if any incorporation had actually taken place. Therefore carbon-13 labelled compounds were prepared to distinguish the possibilities. To ensure that the NAC derivative of the presumed intermediates can indeed be delivered into the *Fusarium* cells more readily than the corresponding salts, S-[1-^{13}C]acetyl-NAC (**110**) was tested in feeding experiments as a comparison to the feeding with sodium [1-^{13}C]acetate. Unfortunately, for no apparent reason, HPLC analysis showed that the *Fusarium* stopped producing F-244 after 2 days of addition of **110**. The amount of F-244 slowly decreased in the following days, and after 6 days subsequent addition of this material, the HPLC signals due to F-244 had completely disappeared. It was unclear whether this was caused by traces of impurities in the precursor which were not detected by NMR or HPLC analysis, or by other factors.

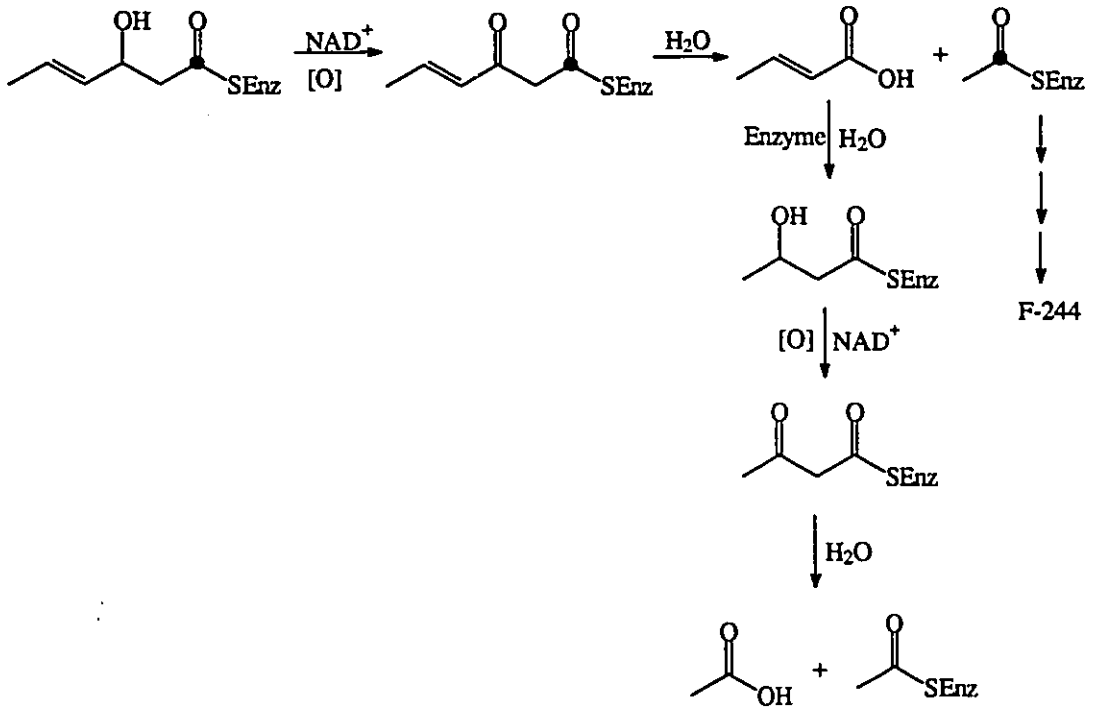
The next experiment used a potential triketide intermediate, [1-^{13}C]3-hydroxy hexenoyl-NAC. Thus, [1-^{13}C]3-hydroxyhexenoyl-NAC (**109**) was fed to the growing *Fusarium* cultures in the usual way and the isolated F-244 was converted to compound **1b** (8 mg). The ^{13}C NMR spectrum showed enrichments at all of the carbon atoms that are derived from C-1 acetate with no apparent intact incorporation of the labelled triketide unit (**Table 5**). This result confirms that indeed [1-^{13}C]3-hydroxyhexenoate had been successfully delivered into the cell, but suffered from β-oxidation, resulting in complete degradation to acetate as illustrated in **Scheme 27**.

Table 5. Percent Incorporation of Carbon-13 from the Incorporation of [1-^{13}C]3-Hydroxyhexenoyl-NAC (**109**) into F-244

Carbon #	Chem. Shift	% ^{13}C enhancement [a]
1	167.57	-0.21
2	117.02	0.24
3	154.50	0.00
4	129.45	0.40
5	141.12	-0.09
6	48.86	0.39
7	30.88	-0.14
8	36.57	0.39
9	26.58	-0.10
10	25.15	0.40
11	33.97	-0.04
12	74.97	0.28
13	58.63	0.13
14	169.85	0.14
15	57.94	0.12
16	19.63	-0.14
17	18.34	-0.05
18	19.33	0.14
19	50.83	0.00

[a] Incorporation expressed after subtraction of the peak height for unlabelled **1b**.

In an attempt to suppress β-oxidation, the β-oxidation inhibitor 3-tetradecyl thiopropanoic acid (**115**) was used. This compound is known to inhibit β-oxidation in rat liver.[103,104] Using this compound as a β-oxidation inhibitor, Vederas and co-workers successfully incorporated the NAC derivative of presumed chain elongation tetraketide intermediates, with an amazing 70% incorporation into the framework of



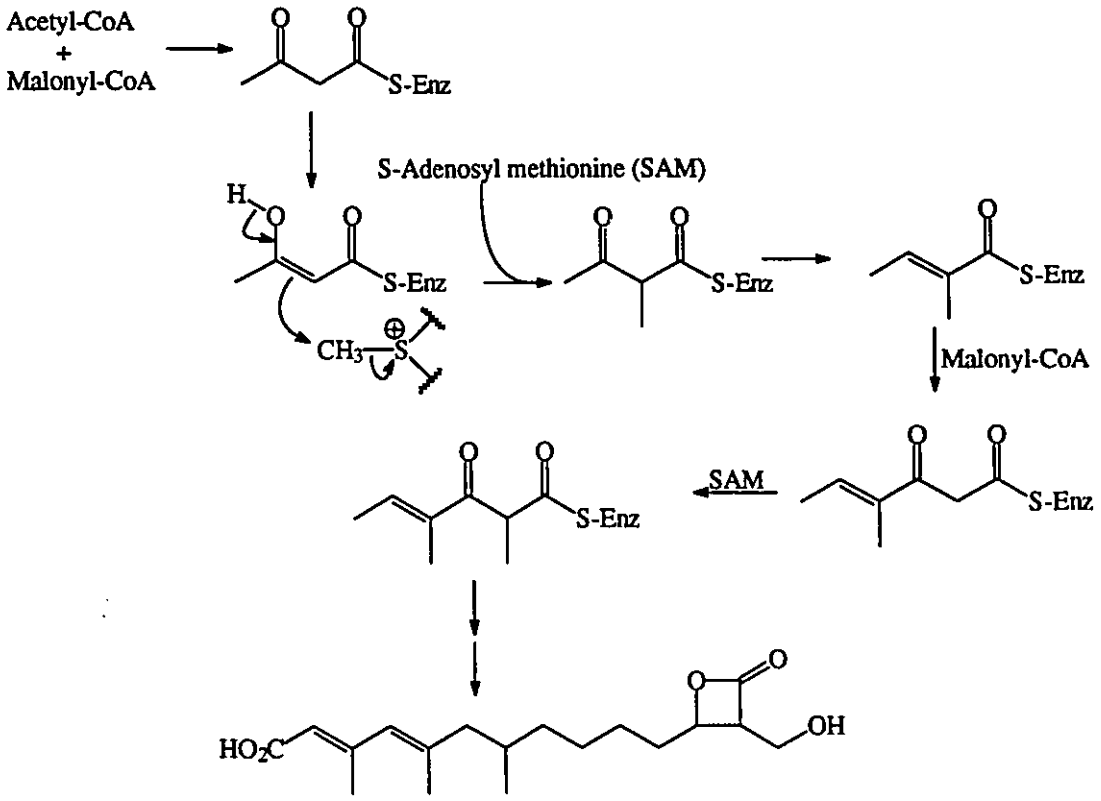
Scheme 27.

dehydrocurvularin.[80] Accordingly, following the method described in this literature, the inhibitor was administered together with crotonyl-NAC **90** to the growing *Fusarium* cultures. Subsequent work up in the usual way afforded **1b** . The sample of **1b** from this experiment, however, showed no observable deuterium in the ^{2}H NMR spectrum after 18 hours of acquisition time. Instead, it was found that some of the precursor was recovered unchanged. The yield of F-244 isolated from this experiment was low (3 mg from 12 culture flasks), indicating that the *Fusarium* sp. was substantially affected by the addition of this inhibitor.

The fact that many attempts to incorporate the presumed diketide and triketide intermediates met with failure suggests that these compounds may not be the intermediates in the biochemical assembly of F-244. While the use of doubly-labelled precursors can be considered for this experiment, the high cost associated with preparation of such compounds has to date prevented us from performing such an experiment.

The next experiment was designed to test whether mechanism **b** was operating. It has been documented in the literature, mainly in aromatic polyketides, that C-methylation occurs before release of the completed polyketide from the synthase complex. Evidence accumulated from biosynthetic studies of gliorosein,[105] barnol,[106] and atranorin,[107] indicates that C-methylation takes place before aromatization of the intermediate, when the intermediate is still in the form of a polyketone.

Although it is unlikely that the chain assembly process of F-244 proceeds through a polyketone intermediate like those in aromatic polyketides, the evidence presented above provides a hint that methylation in chain assembly of F-244 may occur during chain elongation, while the intermediates still possess a diketone moiety as illustrated in **Scheme 28.**



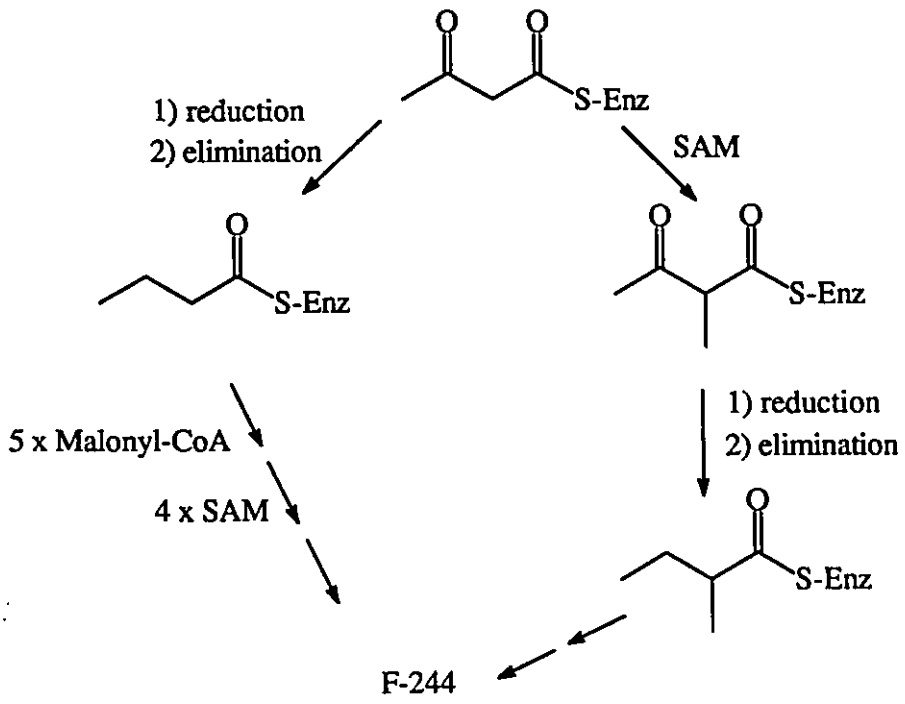
1a

Scheme 28.

In order to test this hypothesis, [3-^{2}H]2-methylcrotonyl-NAC thioester (**105**) was used as a precursor. This experiment also failed to give any detectable deuterium in the ^{2}H NMR spectrum of the resulting F-244 after 18 hours of acquisition. The next experiment involved addition of **105** together with the inhibitor 3-tetradecylthio propionic acid (**113**). Upon monitoring this experiment by HPLC, however, it was revealed that *Fusarium* no longer produced F-244 after a period of 2 days of addition

of these compounds. Continuing addition of this combination for a further 3 days resulted in complete loss of F-244. It was at first thought that this loss of F-244 might be due to overloading the inhibitor, but a similar result was obtained even when the amount of inhibitor was reduced to 25% of that used in the first case. Thus, this experiment proved that an inhibitor that is effective for one type of organism may not always work in another organism.

After these attempts to probe mechanisms **a** and **b** failed, another mechanism was considered. This hypothesis is based on the structural features of F-244. Since F-244 has a linear carbon chain backbone similar to fatty acids, it is possible that F-244 is built in a manner similar to the biosynthesis of fatty acids. In this hypothesis, all the double bonds that are formed after elimination of water will be subsequently reduced to single bonds. There are still 2 possible C-methylations in this mechanism, as in mechanisms **a** and **b**; in either case the double bond is subsequently reduced. The downside of this mechanism is that the double bonds will have to be reintroduced once the chain has been completed, but that is justifiable since oxidations will still have to be performed to form the final product. To test this hypothesis both [1-^{13}C]butyrate and [1-^{13}C]2-methylbutyrate were used as precursors. One might argue that if this is the case, crotonate or 2-methylcrotonate can still be incorporated into F-244. This can be rationalized from substrate specificity. Thus if crotonate or 2-methylcrotonate is not the substrate for the enzyme, it will not be taken up by the enzyme.



Scheme 29.

The results of feeding experiments using sodium [1-^{13}C]butyrate (**116**), [1-^{13}C] butyryl-NAC (**111**), and 2-methyl-[1-^{13}C]butyryl-NAC (**112**) showed strikingly that both butyrate and butyryl thioester had been metabolized and completely degraded to acetate, without any noticeable intact incorporation. It is interesting to note (within ± 0.2% error) that the extent of incorporation of sodium butyrate (as acetate units) is larger than that of the corresponding NAC thioester.

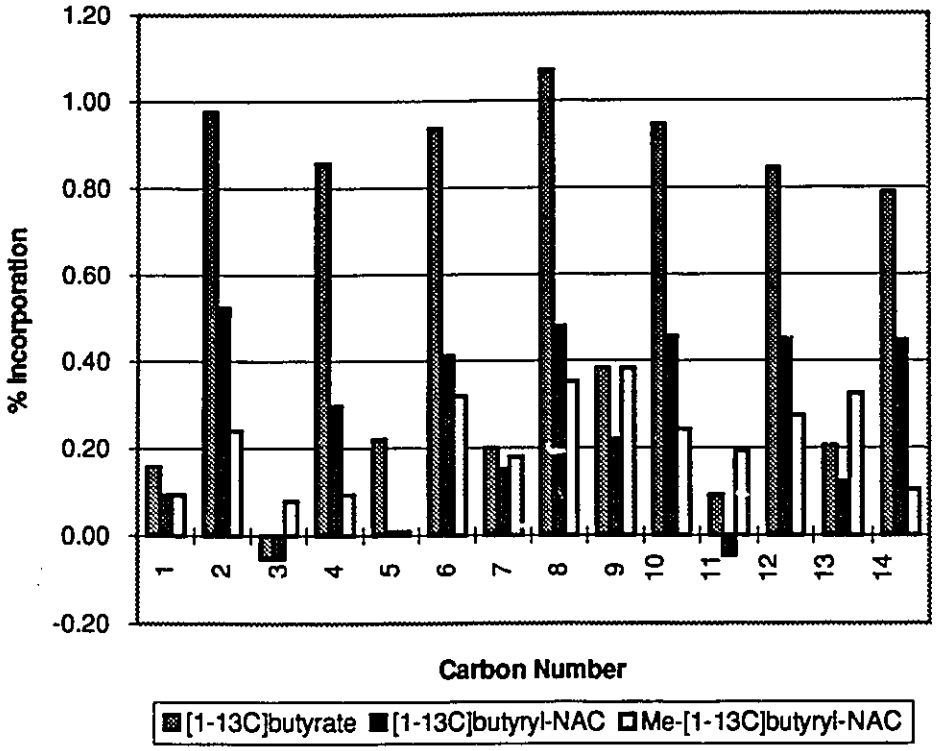


Figure 27. Percent Incorporation of Carbon-13 from Incorporation Experiments Using Sodium [1-^{13}C]Butyrate, [1-^{13}C]Butyryl-NAC, and 2-Methyl [1-^{13}C]Butyryl-NAC

The failure to observe any intact incorporation of any labelled compounds that were used is difficult to predict up to this point. In the case of deuterium labelled compounds, it certainly is not the "washout" process since the deuterium at that position is not exchangeable if any of these compounds is incorporated intact into F-244. The results of acetate feeding experiments clearly indicate that two acetate units must first be combined to produce the four-carbon unit, acetoacetate. In any case, the

next transformation must proceed through the same compounds that were used in this study. Although β-oxidation is usually the culprit of many incorporation experiments, as has been shown in this study, another possibility may also exist. It is possible that the whole process of F-244 assembly occurs rapidly on one multiple enzyme complex, without release, or acceptance of any intermediate externally. This phenomenon has been observed in the biosynthesis of 6-methylsalicylic acid.[108-110] Thus all attempts to probe the biosynthetic sequence by normal feeding experiments with possible oligoketide intermediates failed.

4.4. Conclusions

Based on the repeating sequence of the acetate labelling pattern, three distinct possible mechanisms of biosynthetic assembly of F-244 have been proposed, and several presumed intermediates labelled with either deuterium or carbon-13 have been synthesized by novel or literature procedures. Notably, an efficient method for conversion of the hydroxy acid of NAC derivatives to crotonyl-NAC derivatives has been developed.

Incorporation experiments to prove those three hypotheses using labelled putative intermediates showed that none of the labelled intermediates, within the limits of detection, was incorporated intact. The labelling pattern of carbon-13 of F-244

derived from incorporation of all carbon-13 labelled intermediates clearly indicates that these intermediates had been metabolized and degraded to acetate by β-oxidation. The use of the β-oxidation inhibitor 3-tetradecylthiopropionic acid which has been shown to be a very effective inhibitor in *A. cinerariae* failed to prevent degradation of intermediates in *Fusarium* sp. ATCC 20788.

4.5. Suggestions for Future Work

This work has shown that all attempts to incorporate labelled advanced intermediates were plagued by rapid degradation of the precursors by efficient β-oxidation to acetates. Thus, in order to achieve successful incorporation experiments, this β-oxidation process must be prevented. Currently, there are several known β-oxidation inhibitors documented in the literature, such as hypoglycin,[111-113] pentenoic acid,[114] 3-octylthiopropionic acid,[102] ethyl 3-hydroxy-4-pentynoate,[80] etc. It might be of interest to explore the efficacy of these inhibitors in *Fusarium* sp. ATCC 20788.

For isotopic labelling studies, the use of radioactive compounds should be considered for a preliminary test in order to obtain more sensitive detection of the isotope. Tritium would be the best candidate for this purpose since the methods for synthesis of the required tritium labelled compounds will basically be the same as those developed in this work.

The use of doubly labelled compounds should be strongly considered in order to detect intact incorporation of the putative precursor. If the two labels e.g. ^{13}C-^{13}C are placed at either end of the cleavable bond, any appearance of two doublet signals in the carbon-13 NMR spectrum would confirm that the precursor is incorporated intact.

Finally, genetic studies could be undertaken to determine the sequence of events leading to F-244.

CHAPTER 5

Experimental

5.1. Materials

All reactions requiring non-aqueous conditions were performed in flame-dried glassware under a positive pressure of nitrogen. Commercial grade reagents and solvents were used without further purification except as indicated below. Crotonaldehyde was distilled under reduced pressure. Dichloromethane and diisopropylamine were distilled from calcium hydride. Tetrahydrofuran was distilled from benzophenone and potassium. Water was obtained from a Millipore purification system. All organic layers obtained from extractions were dried over anhydrous sodium sulfate or magnesium sulfate. Flash column chromatography was performed on silica gel 60 (230 - 400 mesh ASTM, E. Merck). All reactions were followed by thin layer chromatography (TLC) using either UV fluorescence, iodine staining, or *p*-anisaldehyde solution for visualization. Commercial TLC plates were Merck 60 F-254 (silica) or Merck RP-8 F-254S (reverse phase). Medium pressure liquid

chromatography (MPLC) was performed using Merck type Lobar pre-packed columns (LiChroprep Si60 20-63 μm).

Sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate, sodium [1,2-$^{13}C_2$]acetate, sodium [1-^{13}C]propionate, and L-[*methyl*-^{13}C] methionine were purchased from MSD Isotopes (Dorval, Quebec). Sodium [1-^{13}C]butyrate, and sodium [1-^{13}C]2-methyl butyrate were purchased from C/D/N Isotopes (Vaudreuil, Quebec). Sodium [1-^{13}C,$^{18}O_2$]acetate was purchased from Cambridge Isotope laboratories (Woburn, MA).

High performance liquid chromatography (HPLC) was performed using a Beckman system fitted with an ultrasphere ODS column (4.6 x 150 mm). The operation conditions were as follows: volume of sample, 10 μL; solvent, 60% CH_3CN in 0.1% aq. H_3PO_4 ; flow rate, 1.0 mL/minute; detection, UV set at 270 nm. Gas chromatography (GC) was performed on a Hewlett-Packard 5890A gas chromatograph fitted with an HP-1 methyl silicone gum capillary column (9 m), with helium as the carrier gas. Compounds were detected using a flame ionization detector (FID). Melting points (Mp) were determined on a Gallenkamp melting point apparatus using open capillary tubes and are uncorrected. Infrared (IR) spectra were determined on a BIO-RAD FTS-40 FT-IR spectrometer. Mass spectra (MS) were recorded at an ionizing voltage of 70 eV on a VG ZAB-E, run at 200 °C source temperature, using either electron impact (EI) or chemical ionization (CI). High resolution mass spectra were run at 3000 - 4000 resolution.

Nuclear magnetic resonance (NMR) spectra were recorded on either a Bruker AM-200 or a Bruker AC-500 spectrometer equipped with a 5 mm dual frequency ^{1}H-^{13}C probe. Sample temperature was maintained at 27 °C by a Bruker Eurotherm B-VT 1000 variable temperature unit. All 2-D experiments were performed on the Bruker AC-500 instrument. $CDCl_3$ and D_2O were used as solvents, and tetramethylsilane (TMS) was used as an internal standard. The chemical shifts are reported in δ values (ppm), followed in brackets by the multiplicity symbol (s = singlet, d = doublet, dd = doublet of doublets, ddd = doublet of doublet of doublets, t = triplet, dt = doublet of triplets, q = quartet, dq = doublet of quartets, m = multiplet, and br = broad), the coupling constant where appropriate, the number of protons, and the type of group.

Proton COSY 2-D NMR spectra were recorded in the absolute value mode using either of the pulse sequences 90° - t_1 - 90° - ACQ. or 90° - t_1 - 45° - ACQ. Spectra were acquired in 16 scans for each of the 128 FID's that contained 1K data points. A 1.0 s relaxation delay was employed between acquisitions. The data were zero filled to 2K in F1 and subjected to Fourier transformation using Gaussian line shaping in both dimensions. The transformed data were then symmetrized.

The ^{13}C-^{1}H 2-D heteronuclear shift correlation spectra were acquired using the standard pulse sequence incorporating the BIRD pulse during the evolution period for ^{1}H - ^{1}H decoupling in F1. The spectra in F2 were recorded over a spectral width of 8474 Hz in 2K data points. The 108 FID's in F1 were obtained over a ^{1}H spectral

width of 1101 Hz. Each FID was acquired in 256 scans. The relaxation delay was 1 s, and J_{C-H} was selected at 130 Hz. The data were zero filled to 4K in F2 and to 512W in F1 and subjected to Fourier transformation using exponential multiplication.

Inverse detected ^{1}H -^{13}C 2-D heteronuclear shift correlation spectra were acquired in the phase-sensitive mode using a 5 mm broadband inverse probe. The FID's in the F2 (^{1}H) dimension were recorded over a spectral width of 2824 Hz in 1K data points. The 256 FID's in the F1 (^{13}C) dimension were obtained over a 10869 Hz spectral width. Each FID was acquired in 8 scans. The fixed delays during the pulse sequence were a 1.0 s relaxation delay, a 0.003 s delay for the BIRD pulse, a 0.3 s delay between the BIRD pulse and the inverse pulse sequence, and a polarization transfer delay of 0.0001 s. The 90° ^{1}H pulse was 9.2 μs while the ^{13}C 90° pulse was 10.1 μs. The data were processed using a sine-bell squared window function shifted by $\pi/2$ in both dimensions.

Inverse detected ^{1}H-^{13}C 2-D chemical shift correlation spectra through two and three-bond coupling interaction were acquired in the absolute value mode using a 5 mm broadband inverse probe. The FID's in the F2 (^{1}H) dimension were recorded over 3268 Hz spectral width in 2K data points. The 512 FID's in F1 (^{13}C) dimension were obtained over 18519 Hz spectral width. Each FID was acquired in 64 scans. The fixed delays during the pulse sequence were a 1.0 s relaxation delay, a 0.003 s delay for the low pass J-filter and 0.05 s delay to allow evolution of the long-range coupling. The

90° ^{1}H pulse was 9.4 μs while the 90° ^{13}Cpulse was also 9.4 μs. The data were process using a sine-bell squared window function shifted by $\pi/2$ in both dimensions.

Proton-proton nOe difference spectra were acquired by subtraction of a control FID from an on-resonance FID. The decoupler in the control FID irradiated a position in the spectrum where there were no proton signals. The on-resonance FID was obtained with the signal of interest being selectively saturated. In both cases the same decoupler power and duration of saturation (5.0 s) were used. This saturation period also served as a relaxation delay for both the control and on-resonance FID's. The decoupler was gated off during acquisition. Eight scans were acquired for both the control and on-resonance FID's. This cycle of alternate acquisition of control and on-resonance FID's was repeated 4 times for a total of 32 scans for the final difference FID. A 90° ^{1}H pulse width of 18.6 μs was used. The difference FID was processed using exponential multiplication (line broadening: 4.0 Hz) and zero-filled to 32 K before Fourier transformation. The sample was not degassed.

The 2-D INADEQUATE spectrum was recorded over a spectral width of 5208 Hz in 1K data points. 128 Experiments were performed, accumulating 256 scans per experiment. The value of $J_{C\text{-}C}$ selected was 80 Hz and the relaxation delay was set to zero. The data were zero-filled to 2K in F1 and were processed using exponential multiplication in the F1 dimension. The transformed data were then symmetrized.

Isotopic incorporations into F-244 were determined by comparing the peak heights for each ^{13}C NMR signal in the labelled F-244 methyl ester samples (**1b**) to those of unlabelled **1b**. Each ^{13}C NMR spectrum was acquired with 1 s relaxation delay. The spectra were acquired over a 20833 Hz spectral width in 16K data points. The FID's were processed using exponential multiplication and zero filled to 32K before Fourier transformation.

5.2. Preparation of Potato Sucrose Agar Slants

Diced potatoes (200 g) were boiled in water (0.5 L) for 10 minutes, then filtered through cheesecloth. Water was added to the filtrate to 1.0 L. Agar (20 g) and sucrose (20 g) were added, and the solution was sterilized at 121 °C for 20 min before pouring into Petri dishes. Freeze-dried specimens of *Fusarium* sp. ATCC 20788 were soaked in sterile water (1 mL) for 5 min, and transferred to 10 potato sucrose agar slants. The slants were incubated at 25 °C for 5 days. The cultures were then stored at 4 °C.

5.3. Production of F-244

Mycelia from the preserved culture of *Fusarium* sp. were suspended in water (3 mL), and the suspension was transferred to a 500 mL Erlenmeyer flask containing liquid growth medium (100 mL), prepared from corn meal (0.5 g, Primo brand), tomato paste

(4 g, Primo brand), oat flour (1 g), dextrose (1 g), and water (100 mL), which had been autoclaved at 121 °C for 20 minutes. The flask was incubated in an incubator-shaker at 28 °C and 212 rpm for 3 days.

Twenty-four 500 mL Erlenmeyer flasks containing 200 mL of corn meal medium were prepared. The medium contained corn meal (4 g), water (175 mL), and a trace element solution (25 mL) containing $MgSO_4.7H_2O$ (0.1 g/L), sodium tartrate (0.1 g/L), $FeSO_4.7H_2O$ (0.01 g/L) and $ZnSO_4$ (0.01 g/L). The flasks were autoclaved at 121 °C for 20 min and then inoculated with 3 mL of the growth medium for each flask. These flasks were then incubated in the incubator-shaker for 10 days at 25 °C and 220 rpm.

5.4. Incorporation Experiments

5.4.1. Sodium [1-^{13}C]Acetate, Sodium [2-^{13}C]Acetate, Sodium [1,2-$^{13}C_2$]Acetate, Sodium [1-^{13}C,$^{2}H_3$]Acetate, and Sodium [1-^{13}C]Propionate

Each of the sodium salts (1 g) was dissolved in sterile water (25 mL), and added batchwise using a disposable plastic syringe, in equal portions, to each of twenty four 500 mL shake flask fermentation cultures of *Fusarium* sp. ATCC 20788, starting from day 4 until day 9 of incubation, at 10 - 14 h intervals. After 10 days of growth, F-244 was isolated by extraction of the whole cultures which will be described later in this chapter.

5.4.2. Sodium [1-^{13}C,$^{18}O_2$]Acetate

Sodium [1-^{13}C,$^{18}O_2$]Acetate (0.5 g) was dissolved in sterile water (25 mL), and added batchwise using a disposable plastic syringe, in equal portions, to each of twelve 500 mL shake flask fermentation cultures of *Fusarium* sp. ATCC 20788, starting from day 4 until day 9 of incubation, at 10 - 14 h intervals. F-244 was isolated after 1 day further incubation.

5.4.4. L-[*methyl*-^{13}C]Methionine

L-[*methyl*-^{13}C]Methionine (1 g) was dissolved in sterile water (50 mL), and then administered in equal batches to each of twenty four fermentation flasks during 6 days, starting when the cultures had begun to turn brown (day 4 - 9). The cultures were harvested after 1 additional day of incubation.

5.4.5. Labelled Putative Intermediates (89, 90, 100, 105, 109, 110, 111, 112 and 116)

All labelled compounds (300 mg) except for **89, 110** and **116**, were dissolved in ethanol (2 mL) then diluted with sterile water (23 mL). Compounds **89, 110,** and **116** were dissolved in water (25 mL). Each of the solutions was administered in equal batches to each of six shake fermentation flasks in a period of 6 days every 10 - 14 h

starting on day 4 of incubation. After 1 additional day of incubation, F-244 was isolated.

5.5. Isolation of F-244 (1a)

The corn meal medium was collected and centrifuged (12000 rpm) for 10 min to obtain about 2.5 L of supernatant fluid. The supernatant was extracted with chloroform (3 x 400 mL). After allowing the emulsion to settle for 10 min, the organic phase was filtered through phase separator paper (Whatman 1PS), and the filtrate was collected. The pellet from the centrifugation step was stirred with 5% methanol in ethyl acetate (300 mL) until the liquid became bright orange. The liquid phase was separated and collected, and the solid phase was repeatedly stirred with the same solvent until the liquid phase no longer turned orange. The extracts from the supernatant and pellet were combined and concentrated on the rotary evaporator to afford *ca.* 3.5 g of brown crude extract. The crude extract was loaded onto a chromatography column of silica gel (5 cm diameter), which was then eluted with step gradients of ethyl acetate in hexane (30%, 40%, 50%, and 60%) containing 0.1% of acetic acid. Fractions were collected every 50 mL and TLC was used to detect which fractions contained the F-244 (**1a**). The fractions containing **1a** were combined and concentrated on the rotary evaporator, then reapplied to a smaller flash chromatography column, and eluted with chloroform : methanol : acetic acid (97 : 3 : 0.1). The polarity of the solvent was gradually increased to 4%, 6%, 8%, and 10% of methanol

while maintaining 0.1% acetic acid and the desired fractions were collected and weighed. Finally, the impure **1a** was loaded on a column of Sephadex LH-20 (2 cm diameter containing 10 g of Sephadex LH-20) which was washed with methylene chloride : hexane : methanol (10:10:1). The desired product was then eluted from the column with methanol, giving pure **1a** (50 mg) as pinkish white needles.

Mp. : 75 - 76 °C (lit.[2] mp 76 - 77 °C)

^{1}H NMR ($CDCl_3$, 500 MHz) δ: 0.84 (d, 3 H); 1.14 (m, 1 H); 1.24-1.44 (m, 4 H); 1.66 (m, 1 H); 1.75-1.81 (m + s, 4 H); 1.86 (dd, 1 H); 1.90 (m, 1 H); 2.08 (dd, 1 H); 2.23 (s, 3H); 3.40 (m, 1 H); 3.88 (dd, 1 H); 4.04 (dd, 1 H); 4.58 (m, 1 H), 5.68 (s, 1 H); 5.72 (s, 1H). The assignments for each resonance are given in Chapter 3.

^{13}C NMR ($CDCl_3$, 125 MHz) δ: 18.48; 19.35; 19.95; 25.15; 26.58; 30.91; 33.96; 36,56; 48.95; 57.89; 58.61; 75.04; 116.76; 129.49; 142.06; 157.02; 169.75; 171.71.

FTIR ($CHCl_3$,) ν: 3510 (OH); 3070; 2930; 2850; 1820 cm^{-1} (β-lactone C=O); 1785; 1685; 1670; 1620; 1460; 1425; 1391; 1372; 1315; 1291; 1249; 1210 cm^{-1}.

MS: (CI, m/z): 342 ($[M + NH_4]^+$, 100); 325 (M^++ 1, 35); 307 (78); 280 (8); 263 (28); 217 (10); 142 (5).

5.6. Preparation of F-244 Methyl Ester (1b)

To a stirred solution of crude extract (3 g) in anhydrous diethyl ether (5 mL) at room temperature was added a solution of diazomethane in diethyl ether dropwise, until no more bubbles of nitrogen were released. The excess diazomethane was removed by bubbling nitrogen through the reaction mixture, and the solution was concentrated under reduced pressure. The desired product was purified by flash column chromatography on silica gel, eluted with ethyl acetate in hexane (40%, 50%, and 60%) to yield **1b** (30 mg) as a yellow oil.

^{1}H NMR ($CDCl_3$, 500 MHz) δ: 0.84 (d, 3 H); 1.13 (m, 1 H); 1.25-1.46 (m, 4 H); 1.66 (m, 1 H); 1.75-1.92 (m + s, 6 H); 2.08 (dd, 1 H); 2.23 (s, 3H); 3.40 (q, 1 H); 3.70 (s, 3 H); 3.88 (dd, 1 H); 4.04 (dd, 1 H); 4.58 (m, 1 H), 5.66 (s, 1 H); 5.70 (s, 1H).

^{13}C NMR ($CDCl_3$, 125 MHz) δ: 18.32; 19.29; 19.64; 25.13; 26.57; 30.81; 33.94; 36.53; 48.86; 50.86; 57.82; 58.86; 74.97; 116.93; 129.41; 141.20; 154.56; 167.58; and 170.00.

MS (CI, m/z): 356 [$(M + NH_4)^+$, 65]; 339 ($M^+ + 1$, 100); 324 (16); 307 (29); 277 (6); 139 (68).

5.7. Preparation of [3-^{2}H]Crotonic Acid (88)

A modification of the method of Allen and Allan was used.[98] Malonic acid (2.3 g, 22.1 mmol) was dissolved in pyridine (10 mL) at 20 °C. [1-^{2}H]Acetaldehyde (1 g,

22.2 mmol) was added to the solution and a condenser was attached to the flask. The mixture was stirred overnight at 20 °C then warmed to 50 °C for 2 h. After the mixture was cooled to room temperature, dilute HCl was added dropwise until the *p*H of the solution was ≤ 3. The mixture was extracted with chloroform (3 x 15 mL). The combined extract was washed with saturated $CuSO_4$ (5 mL) to remove the remaining pyridine. The organic phase was separated, dried over anhydrous sodium sulfate, and concentrated under reduced pressure, giving 2.13 g (64.7%) of **88** as a white solid.

Mp. : 71 -72 °C

^{1}H NMR ($CDCl_3$, 200 MHz) δ: 1.87 (s, 3H, CH_3-); 5.85 (s, 1 H, CH-).

^{13}C NMR ($CDCl_3$, 50 MHz) δ: 18.08 (C-4); 122.20 (C-2); 172 (C-1).

FTIR (KBr pellet) ν: 2950; 2653; 1622; 1450; 1380; 1340; 1245; 970 cm^{-1}.

5.8. Preparation of N,S-Diacetylcysteamine

The procedure of Gerstein and Jencks was used.[115] To a three-necked 250 mL round-bottomed flask equipped with a *p*H electrode and two addition funnels was added 2-mercaptoethylamine hydrochloride (3.52 g, 0.031 mol) in water (10 mL) at 0 - 3 °C. One addition funnel was charged with acetic anhydride (9.06 mL, 0.093 mol) and the other with 8 M KOH (*ca.* 50 mL). After the contents of the flask had been cooled and the *p*H had been adjusted to 8.0 (by addition of a portion of the KOH

solution), acetic anhydride was added dropwise with vigorous stirring along with sufficient KOH solution to maintain the *p*H at 8.0. When all of the acetic anhydride had been added, the *p*H of the reaction mixture was brought to 7.0 by addition of 2 N HCl, and the mixture was stirred for 1 h at room temperature. The mixture was saturated with NaCl, extracted with ether (5 x 25 mL) and the extract was dried over anhydrous sodium sulfate. After evaporation of solvent, the product was distilled at 140 - 142 °C (0.85 mm) giving 4.74 g (95%) of a clear, colorless liquid, which GC analysis showed to comprise only one compound.

^{1}H NMR ($CDCl_3$, 200 MHz) δ:1.82 (s, 3H, CH_3CON-); 2.17 (s, 3H, CH_3COS-); 2.82 (t, J = 6.5 Hz, 2H, CH_2S-), 3.22 (m, J = 6.6 Hz, 2H, CH_2N-), 8.70 (br s, 1H, N*H*).

^{13}C NMR ($CDCl_3$, 50 MHz) δ: 22.99; 28.63; 30.48; 39.37; 170.35 (CON-); 196.06 (COS-).

FTIR (thin film) ν: 3290 (N-H); 3082 (C-H); 2934 (C-H); 1693 (C=O); 1656 (C=O); 1555; 1336; 1374; 1359; 1292; 1135; 958 cm^{-1}.

5.9. Preparation of Lead N-Acetyl Cysteamine (94)

The procedure of Kass and Brock was used.[99] A 250 mL round-bottomed flask was charged with a solution of N,S-diacetyl cysteamine (1 g, 6.2 mmol) in water (30 mL). After the solution had been chilled to 10 °C, solid KOH (1.12 g, 20 mmol) was

added and the resulting cloudy mixture was stirred at room temperature under a slow stream of nitrogen for 45 min. During this time the reaction mixture became clear. The *p*H was adjusted to 7.0 by addition of 5N HCl, and the solution was saturated with NaCl. The mixture was extracted with diethyl ether (6 x 15 mL) and the organic phases were combined and concentrated under reduced pressure. The residue was then added dropwise to a flask containing a solution of lead (II) acetate (1.63 g, 5 mmol) in water (8 mL). A heavy yellow precipitate formed immediately. The reaction mixture was stirred in an ice bath for 30 min. The yellow precipitate was collected by vacuum filtration, washed with a large amount of water, and dried under vacuum to afford 2.42g (88%) of **94** as a yellow powder. The compound was insoluble in either water or organic solvents, therefore no further analysis was performed.

5.10. Preparation of S-[3-^{2}H]Crotonyl-NAC (90) and S-Acetyl,N-[3-^{2}H]Crotonyl Cysteamine (96) (Lead(NAC)$_2$ Method)

The method of Kass and Brock was followed.[99] Freshly distilled oxalyl chloride (5 mL) was added to a 50 mL round-bottomed flask containing [3-^{2}H]crotonic acid (272 mg, 3.12 mmol) with stirring, and the solution was heated at 50 °C for 30 min. The excess of oxalyl chloride was evaporated under a gentle stream of nitrogen. 1 mL of benzene was added, and then removed under a gentle stream of nitrogen. The benzene wash was repeated once. The crotonyl chloride was then dissolved in benzene

(5 mL). Lead-NAC (692 mg, 1.56 mmol) was added and the suspension was heated to reflux for 2 h. During this time the color of the suspension gradually changed from yellow to white as lead chloride formed. The reaction mixture was diluted with benzene, filtered, washed with 5% $NaHCO_3$ and 5% NaCl, and dried over magnesium sulfate. After evaporation of solvent, the mixture was loaded onto a chromatography column (silica gel) and eluted with a mixture of hexane : ethyl acetate : methanol (50 : 50 : 0, 50 : 50 : 1, 50 : 50 : 3, and 50 : 50 : 5) to give pure S-[3-^{2}H]crotonyl NAC as a white waxy solid (194 mg, 33 %) and S-acetyl, N-[3-^{2}H]crotonyl cysteamine as a white solid (212 mg, 36%).

S-[3-^{2}H]Crotonyl-NAC (90)

Mp : 53 - 54 °C

^{1}H NMR ($CDCl_3$, 200 MHz) δ: 1.85 (s, 3H, C*H*$_3$-CD=); 1.92 (s, 3H, C*H*$_3$CO-); 3.05 (t, *J* = 6.2, 2H, C*H*$_2$-S); 3.43 (q, *J* = 6.2, 2H, C*H*$_2$-N); 6.08 (br, 1H, N*H*); and 6.12 (s, 1H, C*H*=C).

^{13}C NMR ($CDCl_3$, 50 MHz) δ: 17.89; 23.01; 28.01; 39.60; 129.73; 170.40; and 190.06.

FTIR (KBr pellet) ν: 3252 (N-H); 3071; 2944 (C-H); 2864 (C-H); 1661 (C=O); 1567 (C=O); 1435; 1371; 1300; 1204; 1161; 1122; 1092; 1034; 1000; 888; 851; 764; 628; 609; 559; 481; 438 cm^{-1}.

MS (EI, m/z) : 189 (M^++1, 12); 129 (13); 118 (12); 86 (10); 70 (100); 60 (8); 43 (33).

HRMS: 189 (M^++1): $C_8H_{13}{}^2HO_2NS$, calcd. 189.0795, found: 189.0808.

S-Acetyl-N-[3-^{2}H]Crotonyl Cysteamine (96)

Mp : 68 °C

^{1}H NMR ($CDCl_3$, 200 MHz) δ: 1.82 (s, 3H, C*H*$_3$-CD=); 2.33 (s, 3H, C*H*$_3$CO-); 3.02 (t, *J* = 6.2, 2H, C*H*$_2$-S); 3.48 (q, *J* = 6.2, 2H, C*H*$_2$N); 5.73 (s, 1H, C*H*=C); 5.78 (br, 1H, N*H*).

^{13}C NMR ($CDCl_3$, 50 MHz) δ: 17.57; 28.63; 30.44; 39.22; 124.75; 166.17; and 196.09.

FTIR (KBr pellet) ν: 3268 (N-H); 3077; 2968; 2916 (C-H); 1695; 1627; 1557; 1439; 1345; 1281; 1225; 1133; 1105; 969; 943; 908; 837; 743; 673; 628; 513 cm^{-1}.

MS (EI, m/z): 189 (M^+ + 1, 8); 146 (20); 113 (6); 99 (9); 87 (10); 70 (100); 43 (29)

HRMS : 189 (M^+ + 1), $C_8H_{13}{}^2HO_2NS$, calcd. 189.0808, found 189.0796.

5.11. Preparation of Ethyl [3-^{2}H]3-Hydroxybutyrate (98)

The method of Jacobs *et al.* was used.[100] A 50 mL round bottomed flask was charged with a solution of freshly distilled ethyl acetoacetate (6.21 g, 47.7 mmol) in ethanol (25 mL). After the solution had been cooled to 0°C in an ice bath, $NaBD_4$ (1 g, 23.9 mmol) was added slowly and the mixture was stirred for 2 h at room

temperature. The reaction mixture was concentrated on a rotary evaporator to *ca.* 5 mL, and diluted with water (25 mL). The solution was then acidified to *p*H=5 with HCl, saturated with NaCl and extracted with chloroform (5 x 10 mL). The extracts were combined, dried over anhydrous sodium sulfate and concentrated on a rotary evaporator giving **98** (5.53 g, 87%) as a clear liquid.

^{1}H NMR ($CDCl_3$, 200 MHz) δ: 1.24 (m, 6 H, 2 CH_3-); 2.47 (s, 2 H, CH_2-); 3.43 (s, 1 H, -OH); 4.20 (q, J = 8 Hz, 2 H, -CH_2-O).

^{13}C NMR ($CDCl_3$, 50 MHz) δ: 22.24; 23.16; 42.64; 51.26; 63.86 (t, weak, *C*DOH); 172.66 (C=O).

5.12. Preparation of [3-^{2}H]3-Hydroxybutyric Acid (99)

A 50 mL round-bottomed flask was charged with a solution of ethyl [3-^{2}H]3-hydroxybutyrate (2 g, 15.0 mmol) in ethanol (5 mL). KOH solution (1.26 g, 22.5 mmol) in ethanol (10 mL) was added and a condenser was attached to the flask. The mixture was stirred and heated to 70 °C for 2 h. After the mixture was cooled to room temperature, the solution was concentrated on a rotary evaporator to about 5 mL, then poured into a flask containing 1 N HCl (20 mL). The solution was saturated with NaCl, loaded to a continuous extraction apparatus and extracted with ether overnight. The extract was dried over anhydrous sodium sulfate and concentrated on a rotary evaporator to afford **99** (1.51 g, 96%) as a clear liquid.

^{1}H NMR ($CDCl_3$, 200 MHz) δ: 1.28 (s, 3 H, CH_3); 2.52 (s, 2 H, CH_2); 7.79 (br. s, 2H, OH).

^{13}C NMR ($CDCl_3$, 50 MHz) δ: 22.30; 42.64; 64.40 (t, weak, CDOH); 176.26 (C=O).

5.13. Preparation of [3-^{2}H]3-Hydroxybutyryl NAC Thioester (100)

The method of Jacobs *et al.* was used.[100] To a stirred mixture of [3-^{2}H]3-hydroxybutyric acid (1.678 g, 16.12 mmol) and a freshly prepared solution of N-acetyl cysteamine (2.242 g, 18.81 mmol) in dichloromethane (15 mL) was added DCC (3.31 g, 16.12 mmol) with ice cooling under a slow stream of nitrogen. After stirring at room temperature overnight, the resulting white precipitate was filtered and washed with dichloromethane. The combined filtrates were evaporated to dryness, and the product was then purified by flash column chromatography with 5% methanol in chloroform as eluent. Yield 2.02 g (61%).

^{1}H NMR ($CDCl_3$, 200 MHz) δ : 1.24 (s, 3 H, CH_3); 1.98 (s, 3 H, CH_3CO); 2.73 (s, 2 H, CH_2CO); 3.04 (t, J = 6.3, 2 H, CH_2S-); 3.42 (q, J = 6.3, 2 H, CH_2N); 6.46 (br s, 1 H, NH).

^{13}C NMR ($CDCl_3$, 50 MHz): 22.51(CH_3-CO) ; 22.88 (CH_3-CD-); 28.54; 39.13; 52.32 (CH_2-COS); 64.57 (t, weak, CDOH); 171.13 (CON); 198.99 (COS).

FTIR (thin film) ν: 3299 (O-H and N-H); 3090; 2972; 2933; 2873; 1685; 1658; 1555; 1439; 1375; 1293; 1237; 1134; 1128; 1032; 936; 752 cm^{-1}.

5.14. Preparation of [3-^{2}H]Crotonyl-NAC (90)

Trifluoroacetic anhydride (1.78 g, 8.5 mmol) was added dropwise to a solution of [3-^{2}H]3-hydroxybutyryl-NAC (0.82 g, 4 mmol) in dichloromethane (5 mL). The reaction mixture was stirred for 15 min at room temperature followed by addition of excess triethyl amine. The reaction mixture was stirred for 30 - 45 min at room temperature until no more fumes were released. The mixture was concentrated and subjected to flash column chromatography and eluted with hexane : ethyl acetate : methanol (50 : 50 : 5). Yield 0.5771 g (77%).

Mp : 53 - 54 °C

^{1}H NMR ($CDCl_3$, 200 MHz): 1.85 (s, 3 H, CH_3-CD=); 1.92 (s, 3 H, CH_3CO-); 3.05 (t, J = 6.2, 2 H, CH_2-S); 3.43 (q, J = 6.2, 2 H, CH_2-N); 6.08 (br, 1 H, NH); and 6.12 (s, 1 H, CH=C).

^{13}C NMR ($CDCl_3$, 50 MHz): 17.89; 23.01; 28.01; 39.60; 129.73; 170.40; and 190.06.

FTIR (KBr pellet) ν: 3252 (N-H); 3071; 2944 (C-H); 2864 (C-H); 1661 (C=O); 1567 (C=O); 1435; 1371; 1300; 1204; 1161; 1122; 1092; 1034; 1000; 888; 851; 764; 628; 609; 559; 481; 438 cm^{-1}.

MS (EI, m/z): 189 (M^++1, 12); 129 (13); 118 (12); 86 (10); 70 (100); 60 (8); 43 (33).

HRMS: 189 (M^++1): $C_8H_{13}{}^2HO_2NS$, calcd. 189.0795, found: 189.0808.

5.15. Synthesis of S-[1-^{13}C]Acetyl-N-Acetyl Cysteamine (110).

Sodium [1-^{13}C]acetate (0.51 g, 6.2 mmol) was added to a 50 mL round-bottomed flask containing a solution of *p*-toluenesulfonic acid (1.3 g, 6.2 mmol) in dichloromethane (25 mL). The flask was capped with a rubber stopper and the mixture was stirred overnight. The mixture was filtered, the residue was washed with dichloromethane (3 x 5 mL), and the filtrates were combined and transferred to a flask containing N-acetyl cysteamine (0.767 g, 6.68 mmol). DCC (1.28 g, 6.2 mmol) was added to the reaction mixture, which was then stirred for 4 h at 0 °C. The resulting white precipitate was filtered and washed with dichloromethane (3 x 5 mL). The combined filtrate was then concentrated on a rotary evaporator and purified by flash column chromatography eluting with 30% ethyl acetate in hexane containing 0.1% acetic acid to give 0.8981 g (89%) of pure S-[1-^{13}C]acetyl-NAC as a clear oil.

^{1}H NMR ($CDCl_3$, 200 MHz) δ: 1.98 (s, 3 H, CH_3CON-); 2.35 (d, $^2J_{C\text{-}H}$ = 6.8 Hz, 3 H, CH_3COS-); 2.82 (t, J = 6.5 Hz, 2 H, CH_2S-), 3.22 (m, J = 6.6 Hz, 2 H, CH_2N-), 8.70 (broad s, 1 H, NH).

^{13}C NMR ($CDCl_3$, 50 MHz) δ: 22.86; 28.52; 30.06 (d, $J_{C\text{-}C}$ = 42, *C*-^{13}CO); 39.37; 170.35 (*C*ON-); 195.83 (enhanced, ^{13}*C*OS-).

FTIR (KBr pellet) ν: 3287 (N-H); 3078; 2993 (C-H); 2870; 1655 (C=O); 1551; 1435; 1373; 1359; 1289; 1238; 1198; 1102; 1041; 995; 948; 759; 681; 620; 603; 474; 417 cm^{-1}.

MS (CI, m/z): 180 ($M + NH_4^+$, 69); 163 ($M^+ + 1$, 100); 137 (31); 120 (29); 102 (10); 86 (9); 77 (11); 52 (42); 43 (18).

HRMS: 163 ($M^+ + 1$), $C_5{}^{13}CH_{12}NO_2S$, calcd. 163.0622, found 163.0621.

5.16. Synthesis of [1-^{13}C]3-Hydroxy-4-hexenoic Acid (108)

The procedure of Adam *et al.*[103] was used, with the following exception. Sodium acetate (0.918 g, 11.2 mmol) was added to a solution of *p*-toluene sulfonic acid (2.32 g, 11.2 mmol) in dichloromethane (10 mL) and the mixture was stirred overnight. The mixture was filtered and the residue was washed with dichloromethane (3 x 5 mL). The filtrates were combined, dichloromethane was removed by fractional distillation at 70 °C and [1-^{13}C]acetic acid was purified by a short path distillation at 116 °C.

To a dry 50 mL round bottomed flask equipped with a magnetic stir bar and a rubber septum, was added via syringe diisopropylamine (1.82 mL, 13 mmol) and dry

THF (8.0 mL), and the solution was cooled to 0 °C in an ice bath under an atmosphere of nitrogen. n-Butyl lithium (9 mL, 12.4 mmol) was introduced in a rapid, dropwise manner via syringe and the reaction mixture was stirred for 40 min. [1-^{13}C]Acetic acid (0.32 mL, 5.6 mmol) was added to the solution at 0 °C, and a condenser was then added to the reaction flask and the mixture was heated at 50 °C for 2 h. The reaction mixture was cooled to -78 °C prior to the addition of crotonaldehyde (0.36 mL, 4.3 mmol), and stirred at -78 °C for 2 h. The flask was raised slightly above the ice bath for 1 h to raise the temperature to 0 °C, and stirred for 30 min at room temperature. The reaction mixture was quenched with aqueous sodium hydrogen sulfate (1 M, 75 mL). The mixture was concentrated on a rotary evaporator to about 25 mL, loaded onto a continuous extraction apparatus, and extracted with diethyl ether for 16 h. The extract was dried over sodium sulfate, concentrated on a rotary evaporator and pumped to a constant weight to give 0.884 g (67%) of [1-^{13}C]3-hydroxy-4-hexenoic acid as a pale yellow oil.

^{1}H NMR ($CDCl_3$, 200 MHz): 1.71 (d, J = 6.4, 3 H, H-6); 2.57 (d, J = 5.9, 2 H, H-2); 4.52 (dd, J = 6.5 and 5.9, 1 H, H-3); 5.53 (dd, J = 15.3 and 6.4, 1 H, H-4); 5.73 (dq, J = 15.3 and 6.4, 1 H, H-5); 6.20 (br s, 2 H, OH and COOH).

^{13}C NMR ($CDCl_3$, 50 MHz): 17.57 (C-6); 41.32 (d, $J_{C\text{-}C}$ = 54.5, C-2); 68.87 (C-3); 127.89 (C-4 or C-5); 131.38 (C-4 or C-5); 176.46 (enhanced, C-1).

5.17. Synthesis of [1-^{13}C]3-Hydroxy-4-hexenoyl-NAC Thioester (109)

[1-^{13}C]3-Hydroxy-4-hexenoic acid (800 mg, 6.1 mmol) was added to a solution of freshly prepared N-acetyl cysteamine (946 mg, 7.94 mmol) in dichloromethane (15 mL) under a slow stream of nitrogen and cooled to 0°C. DCC (1.26 g, 6.1 mmol) was added to the reaction mixture. After stirring at room temperature overnight, the resulting white precipitate was filtered and washed with dichloromethane (3 x 10 mL). The combined filtrate was evaporated on a rotary evaporator to dryness. Flash column chromatography on silica and 50% ethyl acetate in hexane containing 0.1% acetic acid as solvent, afforded 0.862 g (61%) of [1-^{13}C]3-hydroxy-4-hexenoyl-NAC as a clear oil, which solidified to a waxy white solid upon cooling.

Mp : 40 - 41 °C

^{1}H NMR ($CDCl_3$, 200 MHz) δ: 1.71 (d, J = 7.2, 3 H, CH_3-CH); 1.97 (s, 3 H, CH_3-CO); 2.79 (m, 2 H, CH_2-^{13}CO); 3.04 (dt, $J_{H\text{-}H}$ = 6.9, $^{2}J_{H\text{-}C}$ = 4.9, 2 H, CH_2-S); 3.44 (q, J = 6.8, 2 H, CH_2-N), 5.48 (dd, J = 15.2, 6.6, 1 H, CH-COH); 5.75 (dq, J = 15.2, 6.4, 1 H, CH-CH_3); 5.90 (br s, 1 H, NH).

^{13}C NMR ($CDCl_3$, 50 MHz) δ: 17.63; 23.18; 28.80; 39.32; 50.53 (d, $J_{C\text{-}C}$ = 45.5); 69.55; 127.89; 131.56; 171.0 (CON); 198.73 (enhanced, COS).

FTIR (KBr pellet) ν: 3285 (OH and NH); 2965 (C-H); 2856 (C-H); 1649 (C=O); 1555; 1435; 1375; 1299; 1236; 1199; 1121; 1086; 1039; 965; 862; 753; 602 cm^{-1}.

MS (EI, m/z): 233 (M^++1, 20); 215 (40); 162 (5); 119 (100); 96 (38); 86 (12); 71 (65); 60 (82).

HRMS: 233 (M^++1), $C_9{}^{13}CH_{18}O_3NS$, calcd. 233.1041, found 233.1041.

5.18. Synthesis of Ethyl 2-Methylacetoacetate (101)

A modification of the method of Marvel and Hager was used.[102] Metallic sodium (2.87 g, 0.125 mol) was added gradually to a flask containing anhydrous ethanol (60 mL). After all the sodium had dissolved, ethyl acetoacetate (16.25 g, 0.125 mol) was added. The solution was heated to gentle boiling for 30 min and the solution was cooled to room temperature prior to the addition of CH_3I (23.1 g, 0.625 mol). A condenser was attached to the reaction flask and the mixture was heated to reflux until the solution was neutral to moist litmus paper (10 h). The ethanol was removed by distillation leaving solid sodium iodide and the product. Sodium iodide was filtered off, and the brown solution was distilled at 177 °C to afford 16 g (89 %) of ethyl 2-methyl acetoacetate.

^{1}H NMR ($CDCl_3$, 200 MHz) δ : 1.30 (t, J = 6.7 Hz, 3 H, CH_3CH_2O); 1.35 (d, J = 7.8 Hz, 3 H, CH_3CH); 2.26 (s, 3 H, CH_3CO); 3.52 (q, J = 7.9 Hz, 1 H, CH_3CH); 4.20 (q, J = 6.7 Hz, 2 H, CH_3CH_2O).

^{13}C NMR ($CDCl_3$, 50 MHz); δ: 12.57; 13.93; 28.28; 53.48; 61.23; 170.43; and 203.61.

5.19. Synthesis of Ethyl [3-^{2}H]3-Hydroxy-2-methylbutyrate (102)

$NaBD_4$ (1 g, 23 mmol) was added to a solution of ethyl 2-methyl acetoacetate (10 g, 69 mmol) in ethanol (30 mL) at 0 °C. After stirring for 2 h at room temperature, the mixture was concentrated on a rotary evaporator to about 15 mL, and diluted with water (30 mL). The *p*H was adjusted to 5 by addition of HCl, and the solution was saturated with NaCl and extracted with chloroform (5 x 20 mL). The organic extracts were combined, dried over anhydrous sodium sulfate and concentrated on a rotary evaporator, giving 8.7 g of ethyl [3-^{2}H]3-hydroxy-2-methyl butyrate (85%) as a clear oil (mixture of 2 diastereomers).

^1H NMR ($CDCl_3$, 200 MHz) δ : 1.25 (m, 9H, -CH_3); 2.42 and 2.48 (2 q, J = 7.2 Hz, 1 H, C*H*-COO-); 2.83 and 2.95 (2 br s, 1 H, OH), 4.19 (q, J = 6.7, CH_3-CH_2-O).

^{13}C NMR ($CDCl_3$, 50 MHz) δ : 11.08 (*C*H_3-CH_2-O); 13.87 and 14.08 (CH_3-); 19.78 and 20.56 (CH_3-); 45.57 and 46.88 (-*C*H-COO); 60.48 (-CH_2-O); 67.95 and 69.28 (2 weak t, CDOH); 175.85 and 176.02 (C=O).

5.20. Synthesis of [3-^{2}H]3-Hydroxy-2-methylbutyric Acid (103)

KOH (3.4 g, 60 mmol) in ethanol (10 mL) was added to a flask containing ethyl [3-^{2}H]3-hydroxy-2-methylbutyrate (6 g, 41 mmol). The mixture was stirred at 70 °C for 2 h. After cooling, the solution was poured into a flask containing 1 M HCl (75 mL) and saturated with NaCl. The mixture was charged into a continuous extraction apparatus and extracted with diethyl ether overnight. The extract was dried over anhydrous sodium sulfate and concentrated in vacuo to give 4.0 g of [3-^{2}H]3-hydroxy-2-methylbutyric acid (82%).

^{1}H NMR ($CDCl_3$, 200 MHz) δ : 1.22 (m, 6 H, CH_3-); 2.58 (m, 1 H, CH_3-C*H*-); 6.30 (br s, -OH).

^{13}C NMR ($CDCl_3$, 50 MHz) δ : 10.77 and 12.67 (CH_3-); 20.44 and 22.50 (CH3-); 45.14 and 46.73 (-*C*H-COO); 68.07 and 69.00 (2 weak t, -CDOH); 180.17 and 180.31 (C=O).

5.21. Synthesis of [3-^{2}H]3-Hydroxy-2-methylbutyryl-NAC Thioester (104)

Freshly prepared N-acetyl cysteamine (3.36 g, 28.2 mmol) was added to a solution of [3-^{2}H]3-hydroxy,2-methylbutyric acid (2.8 g, 23.5 mmol) in dichloro-methane (50 mL) under a slow stream of nitrogen. DCC (4.85 g, 23.5 mmol) was added to the solution at 0 °C, and the mixture was stirred overnight (16 h). The

resulting precipitate was filtered and washed with dichloromethane (3 x 10 mL). The filtrates were combined and concentrated in *vacuo*. Flash column chromatography eluted with 50% ethyl acetate in hexane, afforded [3-^{2}H]3-hydroxy-2-methylbutyryl-NAC (3.73 g, 72%) as a clear oil.

^{1}H NMR ($CDCl_3$, 200 MHz) δ: 1.21 (m; 6 H, C*H*$_3$-CDOH-and C*H*$_3$-CH) 1.94 (s, C*H*$_3$CO-); 2.15 (br s, 1 H, OH); 2.70 (m, 1 H, CH$_3$-C*H*-); 3.05 (t, *J* = 6.2, 2H, C*H*$_2$-S); 3.43 (q, *J* = 6.2, 2H, C*H*$_2$-N); 6.70 (br s, 1 H, N*H*).

^{13}C NMR ($CDCl_3$, 50 MHz) δ : 12.66 and 13.61 (CH_3-); 16.52 and 17.26 (CH_3-); 22.94 (*C*H$_3$-CO); 28.50; 39.13; 52.17 and 52.29 (*C*H-COO-); 170.48 (CH$_3$-*C*O); 200.02 (CH-*C*OS). Carbon attached to deuterium (CD-OH) was lost in the noise.

5.22. Synthesis of [3-^{2}H]2-Methylcrotonyl-NAC Thioester (105)

Trifluoroacetic anhydride (2.41 g, 11.5 mmol) was added dropwise to a solution of **104** (1.20 g, 5.4 mmol) in dichloromethane (10 mL). The reaction mixture was stirred for 15 min at room temperature followed by addition of excess triethyl amine (10 mL). The reaction mixture was stirred for 30 - 45 min at room temperature, then heated to 60 °C for 30 min. The mixture was concentrated and subjected to flash column chromatography and eluted with hexane : ethyl acetate : methanol (50 : 50 : 5). Yield 0.823 g (75%).

^{1}H NMR ($CDCl_3$, 200 MHz) δ : 1.22 (s, 3 H, CH_3-C-CO); 1.39 (s, 3 H, CH_3-CD); 3.04 (t, 2 H, J = 6.2, CH_2S); 3.43 (q, 2 H, J = 6.1, CH_2N); 6.21 (br s, 1 H, N*H*).

^{13}C NMR ($CDCl_3$, 50 MHz) δ : 12.37; 14.04; 28.17; 39.40; 136.60; 170.30 (*C*ON); 193.61 (*C*OS).

FTIR (thin film) ν: 3291 (N-H); 3087; 2979 (C-H); 2935 (C-H); 2878; 1723; 1658 (C=O); 1551 (C=O); 1440; 1375; 1291; 1261; 1200; 1162; 1112; 1008; 968; 858; 660; 450 cm^{-1}.

MS (EI, m/z); 203 (M^+ +1, 4); 143 (12); 119 (12); 102 (8); 84 (100); 72 (10); 56 (85); 43 (90).

HRMS : 203 (M^+ +1), $C_9H_{15}DNO_2S$, calcd. 203.0964, found 203.0969.

5.23. Synthesis of [1-^{13}C]Butyryl-NAC Thioester (111)

Sodium [1-^{13}C]butyrate (0.7 g, 6.3 mmol) was added to a solution of *p*-toluene sulfonic acid (1.20 g, 6.3 mmol) in dichloromethane (15 mL). The mixture was stirred for 6 h at room temperature. The solid was filtered, and washed with dichloromethane (3 x 5 mL). The filtrates were combined, dichloromethane was removed by fractional distillation, and a short path distillation at 162 °C gave 0.51 g of [1-^{13}C]butyric acid. Freshly prepared N-acetyl cysteamine (0.82 g, 6.88 mmol) was added to the flask containing [1-^{13}C]butyric acid (0.51 g, 5.73 mmol) in dichloromethane (20 mL) under a slow stream of nitrogen. DCC (1.18 g, 5.73 mmol) was added to the reaction mixture at 0

°C, and the mixture was stirred overnight. The resulting precipitate was filtered and washed with dichloromethane (3 x 10 mL). The filtrates were combined and concentrated *in vacuo*. Flash column chromatography gave pure [1-^{13}C]butyryl-NAC thioester (0.63 g, 58%) as a clear oil which solidified upon cooling.

Mp. : 26 - 27 °C

^{1}H NMR ($CDCl_3$, 200 MHz) δ : = 0.96 (t, J = 7.3, 3 H, CH_3-CH); 1.69 (d of sextet, $J_{H\text{-}H}$ = 7.3, $^{2}J_{H\text{-}C}$ = 4.3, -CH_2-CH_3); 1.99 (s, 3 H, CH_3-CO); 2.54 (dt, $J_{H\text{-}H}$ = 7.4, $J_{C\text{-}H}$ = 5.8, 2 H, CH_2-^{13}CO); 3.03 (dt, $J_{H\text{-}H}$ = 6.3, $^{2}J_{C\text{-}H}$ = 4.6, 2 H, -CH_2-S); 3.42 (q, J = 6.3, 2 H, CH_2N); 6.15 (broad s, 1 H, NH).

^{13}C NMR ($CDCl_3$, 50 MHz) δ : = 13.28; 19.04; 23.00; 28.22; 39.51; 45.31 (d, $J_{C\text{-}C}$ = 44.5, CH_2-CO); 170.34 (CO-N); 199.83 (enhanced, CO-S).

FTIR (KBr pellet) ν: 3293 (N-H); 3079; 2966 (C-H); 2935; 2876; 1654 (C=O); 1553 (C=O); 1461; 1374; 1291; 1238; 1198; 1111; 1043; 990; 971; 886; 754; 693; 601; 418 cm^{-1}.

MS (EI, m/z): 191 (M^+ + 1, 11); 161 (4); 131 (12); 119 (95); 102 (15); 86 (17); 72 (88); 60 (95); 43 (100).

HRMS : 191 (M^+ + 1), $C_7{}^{13}CH_{16}NO_2S$, calcd. 191.0935, found 191.0941.

5.24. Synthesis of [1-^{13}C]2-Methylbutyryl-NAC Thioester (112)

Sodium 2-methyl [1-^{13}C]butyrate (0.7 g, 5.6 mmol) was added to a solution of *p*-toluene sulfonic acid (1.118 g, 6.16 mmol) in dichloromethane (15 mL). The mixture was stirred for 6 h at room temperature. The solid was filtered, and washed with dichloromethane (3 x 5 mL). The filtrates were combined, dichloromethane was removed by distillation at 60 °C, and a short path distillation under reduced pressure (50 mm Hg) at 100 °C gave 0.49 g (85%) of [1-^{13}C]2-methylbutyric acid. Freshly prepared N-acetyl cysteamine (0.68 g, 5.71 mmol) was added to the flask containing [1-^{13}C]2-methylbutyric acid (0.49 g, 4.76 mmol) in dichloromethane (20 mL) under a slow stream of nitrogen. DCC (0.98 g, 4.76 mmol) was added to the reaction mixture at 0 °C, and the mixture was stirred overnight. The resulting precipitate was filtered and washed with dichloromethane (3 x 10 mL). The filtrates were combined and concentrated *in vacuo*. Flash column chromatography gave pure 2-methyl [1-^{13}C]butyryl-NAC thioester (0.57 g, 58%) as a clear oil.

^{1}H NMR ($CDCl_3$, 200 MHz) δ : 0.92 (t, J = 7.4, CH_3-CH_2); 1.17 (dd, $J_{H\text{-}H}$ = 6.9, $^{2}J_{C\text{-}H}$ = 5.4, 3 H, CH_3-CH); 1.73 - 1.47 (m, ABX system, 2 H, CH_2-CH); 1.97 (s, 3 H, CH_3-CO); 2.60 (m, 1 H, CH-CO); 3.03 (dt, $J_{H\text{-}H}$ = 6.2, $^{2}J_{C\text{-}H}$ = 4.4, 2 H, CH_2-S); 3.48 (q, J = 6.2, 2 H, CH_2-N); 6.10 (broad s, 1 H, NH).

^{13}C NMR ($CDCl_3$, 50 MHz) δ : 11.49; 17.12; 23.10; 27.08; 28.04; 39.78; 49.66 (d, $J_{C\text{-}C}$ = 44.6, *C*H-CO); 170.33 (CO-N); 204.45 (enhanced, CO-S).

FTIR (thin film) ν: 3286 (N-H); 3080; 2969 (C-H); 2934; 2877; 1653 (C=O); 1554 (C=O); 1459; 1440; 1374; 1291; 1239; 1199; 1162; 1106; 968; 938; 830; 809; 755; 446 cm^{-1}.

MS (EI, m/z): 205 (M^+ + 1, 75); 145 (9); 120 (40); 102 (13); 86 (45); 72 (11); 57 (100); 43 (32).

HRMS: 205 (M^+ + 1), $C_8{}^{13}CH_{18}O_2NS$, calcd. 205.1092 found. 205.1094.

5.25. Synthesis of S-Tetradecyl-3-Mercaptopropionic acid (115)

The method of Spydevold and Bremer[104] was used. Mercaptopropionic acid (6.09 g, 57.4 mmol) and KOH (7.72 g, 137.5 mmol) were dissolved in methanol (75 mL). Tetradecyl bromide (10.6 g, 38.2 mmol) was added gradually to the solution at room temperature with stirring. The stirring was continued overnight. Water (150 mL) was added and the mixture was heated until a slightly turbid solution was obtained. The potassium salt was precipitated upon cooling to room temperature and was isolated by filtration. The precipitate was recrystallized once from hot water (200 mL). The crystals were redissolved in hot water and the free acid was precipitated by addition of concentrated HCl (5 mL). The precipitate was then filtered and recrystallized twice from hot acetone : water (150 mL, 9 : 1), giving 10.6 g (92%) of **115** as white silvery crystals.

^{1}H NMR ($CDCl_3$, 200 MHz) δ :0.88 (t, J = 6.7, 3 H, CH_3); 1.26 (m, 22 H); 1.58 (dt, J = 7.1, 2 H, CH_2-CH_2-S); 2.53 (t, J = 7.1, 2 H, CH_2-COOH); 2.63 (t, J = 7.2, 2 H, CH_2-CH_2-S); 2,76 (t, J = 6.9, 2 H, CH_2-S-CH_2); 8.44 (broad s, 1H, COO*H*)

^{13}C NMR ($CDCl_3$, 50 MHz) δ : 14.09; 22.67; 26.76; 28.88; 29.23; 29.34; 29.53 (signals are overlapped); 29.60; 29.65; 31.90; 32.16; 34.90; 177.79.

FTIR (KBr pellet) ν: 3126 (OH); 2919 (C-H); 2848 (C-H); 2590; 2526; 1686 (C=O); 1463; 1428; 1409; 1371; 1337; 1267; 1234; 1189; 1160; 1080; 917; 722; 659; 491 cm^{-1}.

MS (EI, m/z): 302 (M^+, 19); 229 (100); 161 (5); 106 (23); 89 (18); 55 (22).

HRMS: 302 (M^+), $C_{17}H_{34}O_2S$, calcd.302.2279, found 302.2278.

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Appendix 1

Calculation of Detection Limit of Deuterium in Deuterium NMR on the Bruker AM 500 Spectrometer

$CHCl_3$ (MW=119.38, d=1.47) contains 0.015% $CDCl_3$, therefore

$$[D] = \frac{0.015}{100} \times [CHCl_3]$$

$$[CHCl_3] = \frac{1.47g}{1ml} \times \frac{1mol}{119.38g} = 0.01231 \, mol/ml = 12.31M$$

$$[D] = \frac{0.015}{100} \times 12.31M = 1.85 \times 10^{-3} M$$

Where [X] is the concentration of component X.

For 6 mg of F-244 (MW= 324) in 0.75ml of $CHCl_3$, the concentration of F-244 is,

$$[F-244] = \frac{6\,mg \times \frac{1\,mmol}{324mg}}{0.75\,ml} = 2.47 \times 10^{-2} \, M$$

For a deuterium content of y% in a given site of F-244,

$$[D] \text{ in } F-244 = \frac{y}{100} \times 2.47 \times 10^{-2} \, M = 2.47 \times 10^{-4} \, y \, M$$

For a sample of $CHCl_3$, a deuterium NMR spectrum was acquired on the AM-500 spectrometer. The S/N ratio increased as the square root of the number of scans as

expected. After 10,500 scans were acquired at 1.72 sec/scan (total acquisition time 5 h), the S/N ratio was found to be 97 : 1.

Assuming that minimum acceptable S/N ratio is 3 : 1, then the number of scan required is:

$$S/N \propto \sqrt{NS}$$

$$NS = \left(\frac{\sqrt{10500}}{97} \times 3\right)^2$$

$$NS = 10$$

Thus, the time required to detect 1.85×10^{-3} M of deuterium is:

$$T = 10 \times 1.72 \text{ sec.} = 17.2 \text{ sec.}$$

Since [D] in F-244 is $2.47 \times 10^{-4} y\, M$, where y is deuterium content (%), therefore :

$$y = \frac{1.85 \times 10^{-3}}{2.47 \times 10^{-4}}$$

y = 7.5 for 6 mg of F - 244

Thus, to detect a peak of deuterium in F-244 in 17.2 second (0.005 h) requires 7.5% of incorporation.

If reducing the amount of deuterium by one half requires 4 times more scans, then the limit of detection in terms of % incorporation of deuterium is:

Amount of F-244 (mg)	% incorporation	Time (s).	Time (h)
6	7.5	17.2	0.005
6	3.75	68.8	0.019
6	2	241.9	0.067
6	1	967.5	0.269
6	0.5	3870.0	1.075
6	0.2	24187.5	6.719
6	0.12	67187.5	18.663

Conclusion:

For 18 hours of acquisition, the minimum limit of deuterium detection is :

$$\frac{2.47 \times 10^{-4}\ \text{mmol}}{1\ \text{ml}} \times 0.75\ \text{ml} \times 0.12 = 2.2 \times 10^{-5}\ \text{mmol} = 22\ \text{nmol}$$

Therefore, the limit of detection of incorporation of deuterium is 0.12 % for 6 mg F-244. As the quantity of F-244 doubles or halves, the limit of detection of incorporation is reduced or increased by a factor of 4, respectively.