BIOSYNTHESIS OF β-LACTONES

BIOSYNTHESIS OF EBELACTONE A: ORIGIN OF OXYGEN ATOMS AND MECHANISM OF β-LACTONE RING FORMATION

By

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TITLE: Biosynthesis of β-Lactones: Origin of the Oxygen Atoms and Mechanism of β-Lactone Ring Formation

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ABSTRACT

β-Lactones are natural products containing a four-membered lactone ring and are potent inhibitors of various types of enzymes. The mechanism of β-lactone ring formation was examined *in vivo* in order to identify the genes and proteins that effect cyclization. Incorporation experiments into ebelactone A, a polyketide-derived β-lactone from *Streptomyces aburaviensis*, were conducted. Doubly labelled sodium [1-^{13}C,$^{18}O_2$]propionate prepared from labelled cyanide and water was fed to the cultures of the organism to give labelled ebelactone A. ^{18}O-induced isotopic shifts were observed for all oxygenated carbon atoms of the molecule indicating that these oxygen atoms are all derived from propionate. This result is consistent with formation of the β-lactone ring by nucleophilic attack of the C-3 hydroxyl group of a polyketide precursor onto the C-1 carbonyl carbon, and excludes other putative mechanisms for ring formation. Ebelactone A was chemically converted to its *N*-acetylcysteamine derivative (SNAC). Labelling experiments to test for conversion of this SNAC derivative to ebelactone A *in vivo* showed only a background chemical cyclization reaction.

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LIST OF ABBREVIATIONS

PKS	Polyketide Synthase
TE	Thioesterase
Ph_3P	Triphenylphosphane
DEAD	Diethyl Azodicarboxylate
TMAL	Tandem Mukayama Aldol Lactonization
HTA	Homoserine Transacetylase
KS	Ketosynthase
AT	Acyltransferase
ACP	Acyl Carrier Protein
KR	Ketoreductase
DH	Dehydratase
ER	Enoylreductase
ATP	Adenosine Triphosphate
AMP	Adenosine Monophosphate
NMR	Nuclear Magnetic Resonance
COSY	Correlation Spectroscopy
HSQC	Heteronuclear Single Quantum Coherence
HMBC	Heteronuclear Multiple Bond Correlation
ESI	Electrospray Ionisation
MS	Mass Spectrometry
IR	Infrared

NADPH	Nicotinamide Adenosine Dinucleotide Phosphate
SNAC	*N*-Acetylcysteamine (Derivative)
HPLC	High Pressure Liquid Chromatography
LC-MS	Liquid Chromatography Mass Spectrometry
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
R.T.	Retention Time

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INTRODUCTION

β-Lactones (2-oxetanones) (Fig. 1.1) are compounds that contain a 4-membered lactone ring. They are important structures both as biologically active drugs and as reactive intermediates in synthesis of various structures.[1] A number of naturally occurring β-lactones have been isolated as a result of their potent biological activities.[2] β-Lactones produced by bacteria, plants, fungi and insects are all known[2] and a number of structural analogues of medicinally important lactones have been synthesized.[3] The numbering system used in this thesis is that shown for the β-lactone structure, and corresponds to the convention used in the literature for ebelactone, *i.e.* carbonyl carbon = C-1.

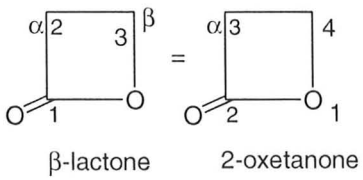
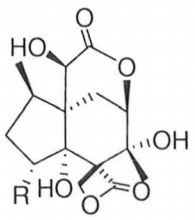


Fig. 1.1: Nomenclature and atom numbering of β-Lactones

1.1 Naturally Occuring β-Lactones

The first naturally occurring β-lactone to be discovered was anistatin (**1**), a potent vegetal poison isolated from the Japanese star anise, *Illicium anistatum*[4] (Fig. 1.2). Although the convulsant activity of the seeds of these plants was known for centuries, the component responsible for the activity was only isolated in 1952, along with neoanistatin (**2**), another convulsive analogue produced by the same plant.[4]

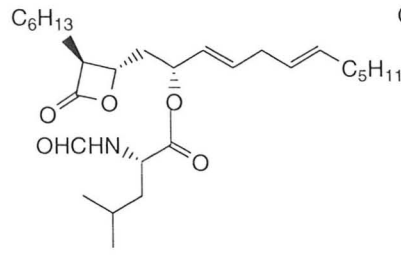


1R=OH: **2** R=H

Fig 1.2: Anistatin and Neoanistatin

Over the last few decades, a large number of β-lactones has emerged as new therapeutic agents for the treatment of various human diseases. Lipstatin (**3**), produced by a terrestrial actinomycete *Streptomyces toxytricini*[5,6] and the synthetic derivative tetrahydrolipstatin (orlistat) (**4**) are inhibitors of pancreatic lipase[7] and are also known to inhibit fatty acid synthesis.[8] Thus, **4** is now approved by the Food and Drug Administration (FDA-U.S.) and used clinically to treat obesity. It is available as Alli (over the counter drug: 60 mg orlistat) and Xenical (prescription drug: 120 mg orlistat).

Esterastin (**5**), isolated from *Streptomyces lavandulae,*[9] valilactone (**6**) produced by *Streptomyces albolongus,*[10] and panclicins A-E (**7a-e**)[11] isolated from *Streptomyces* sp. are structural analogues of **3**, but show less activity as lipase inhibitors (Fig. 1.3).[11]



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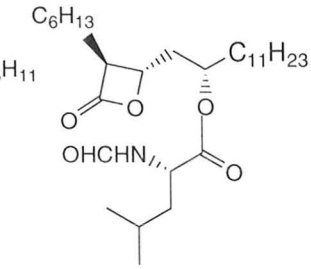

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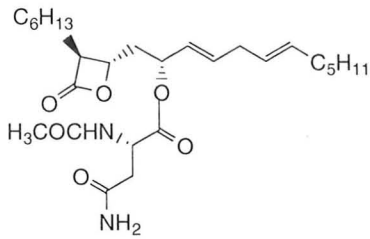

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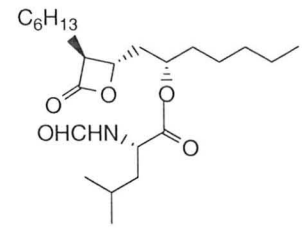

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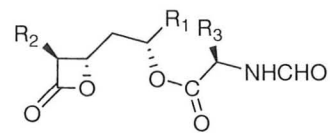


7a R_1=$CH_3(CH_2)_6$: R_2=$(CH_2)_7CH(CH_3)_2$: R_3= CH_3

7b R_1=$CH_3(CH_2)_6$: R_2=$(CH_2)_9CH_3$: R_3= CH_3

7c R_1=$CH_3(CH_2)_6$: R_2=$(CH_2)_7CH(CH_3)_2$: R_3= H

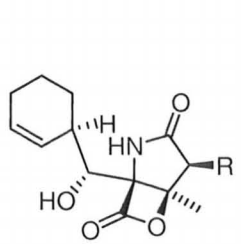
7d R_1=$CH_3(CH_2)_6$: R_2=$(CH_2)_9CH_3$: R_3= H

7e R_1=$CH_3(CH_2)_6$: R_2=$(CH_2)_{11}CH_3$: R_3= H

Fig.1.3: β-Lactone Type Lipase Inhibitors

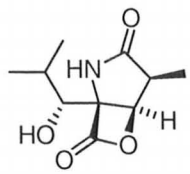
Salinosporamide A (**8**) is a bicyclic β-lactone natural product containing a β-lactone and a γ-lactam which is produced by *Salinospora tropica* (Fig. 1.4).[12] It is a potent inhibitor of 20S proteosomes, and presently is in human clinical trials against multiple myeloma.[13] This compound, along with its deschloro analogue salinosporamide B (**9**)[14] isolated from the same *Streptomyces* sp and omuralide (**10**),[15]

another *Streptomyces* metabolite, show activity against the 20S proteosome with IC_{50} values of 1.3 nM, 13 nM and 49 nM respectively.[16] Recently, a number of salinosporamide type β-lactones have been isolated from a terrestrial *Streptomyces* sp..[17] Named cinnabaramides A-C (**11-13**), these compounds only differ from the salinosporamides in C-2 alkyl side chain and have comparable *in vitro* activity with IC_{50} values in the nanomolar range.[17] A number of structural derivatives of **8** have been chemically synthesised/bio-engineered and tested for activity. These will be discussed in a separate section (Section 1.4).



8 R = CH_2CH_2Cl

9 R = CH_2CH_3



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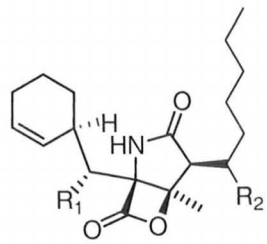


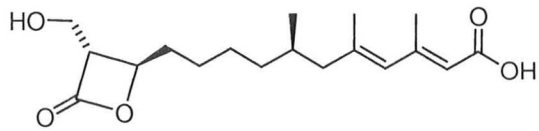
11 R1 = OH: R2 = H

12 R1 = OH: R2 = OH

13 R1 = H: R2 = H

Fig: 1.4: β-Lactone Type Proteosome Inhibitors

Other natural β-lactones studied for their important biological activities include: F244 (**14**), a fungal metabolite isolated from *Fusarium* and *Cephalosporium* sp.[18] which is an inhibitor of 3-hydroxy-3-methylglutaryl Coenzyme A synthase, a key regulatory enzyme in the cholesterol biosynthetic pathway;[19-21] ebelactones A and B (**15, 16**) which show esterase, lipase and cutinase inhibition activity;[22] and vittatalactone (**17**), an aggregation pheromone produced by the striped cucumber beetle, *Acalyma vitatum* (Fig. 1.5).[23]



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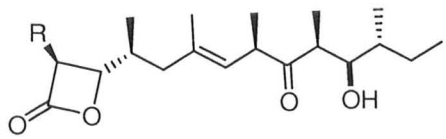

15 R = CH_3: **16** R = CH_2CH_3

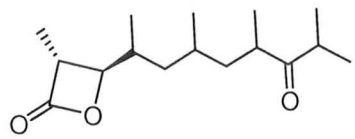

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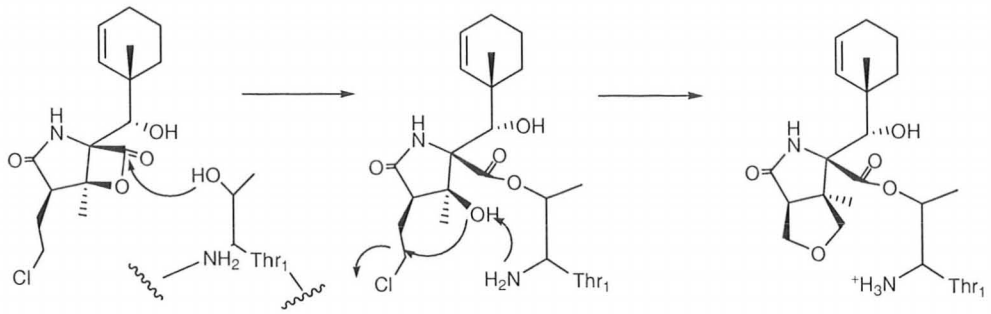
Fig: 1.5: F244, Ebelactone A, B and Vittatalactone

1.2 Mode of Action

Where the mode of action is known, β-lactones act by acylating their target enzymes resulting in ring opening of the lactone moiety. They react with the enzyme active sites by forming reversible/irreversible complexes, and hence may inactivate the target enzyme.[24] In the case of salinosporamide A (Scheme 1.1), the hydroxyl group of a threonine residue in the 20S proteosome forms a covalent ester bond with the lactone carbonyl carbon atom. The β-hydroxy group created by this reaction then attacks the electrophilic C-Cl bond, and forms a tetrahydrofuran ring. The latter reaction prevents hydrolytic attack by water on the ester bond to hydrolyse it, making the binding irreversible, and leading to a higher level of biological activity.[25]



Scheme 1.1: Mode of Action of Salinosporamide A

In the case of lipstatin, it has been shown by biochemical studies that the lactone carbonyl forms an ester linkage with the side chain hydroxyl group of the Ser-152 residue of the lipase, leading to irreversible binding.[26] Covalent ester bond formation is also proposed in the mechanism of action of F244.[27]

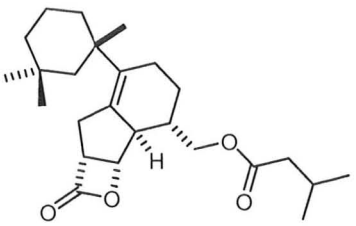
1.3 Biosynthesis of β-Lactones

Biosynthetically, β-lactones show a wide variety of origins. They can apparently be derived from polyketides, amino acids or terpenoids.[28]

1.3.1 Terpenoid Derived β-Lactones

β-Lactones thought to be derived from diterpene, triterpene and sesquiterpene origins are known.[1] However, there is no literature available on studies of their biosynthesis. Nonetheless, these compounds generally posess multiple isoprenoid units. Spongiolactone (**18**), isolated from the mediterranean sponge *Spongionella gracilis*, is a novel β-lactone-diterpene type compound, which contains a highly rearranged terpenoid-like carbon skeleton (Fig. 1.6).[29] Papyriogenin G (**19**) isolated

from the leaves of the plant *Tetrapanax papyriferum* is an oleanane-type triterpene.[30] The sesquiterpene-type β-lactones include guaiagrazielolide (**20**) from *Graziella* sp.[31] and anistatin (**1**) and its analogues neoanistatin (**2**) and the veranistatins A and B (**21 a, b**).[32] Lupeolactone (**22**)[33] from *Antidesma pentandrum* and vibralactones (**23 a-c**)[34] from *Boreostream vibrans* are also likely to be sesquiterpenes. Biological activities and sources of origin of the terpenoid type β-lactones are shown in Table 1.1.



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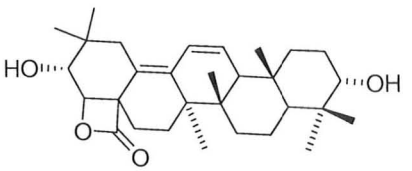

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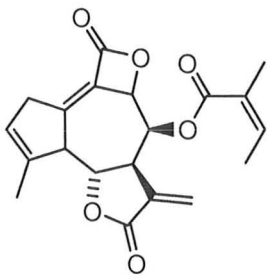

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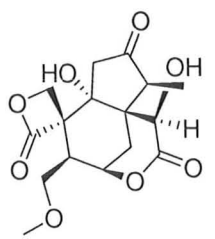

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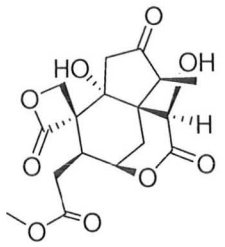
Fig 1.6: Terpenoid Derived β-Lactones



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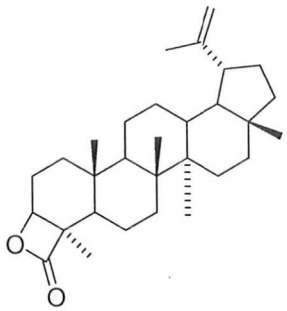

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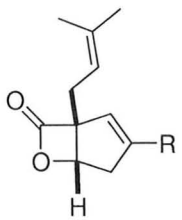
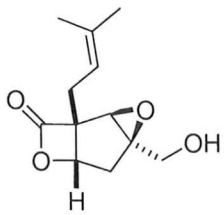


23 a R=CH_2OH

c R= CHO



23 b

Fig 1.7: Terpenoid Derived β-Lactones

Table 1.1: Terpenoid Derived β-Lactones

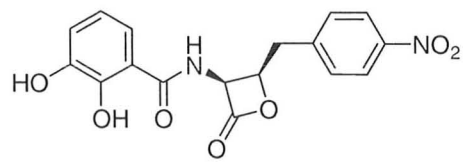
	Structure	Known Activity	Source
Anistatin[4]	1	Convulsant	*Illicium anistatum*
Neoanistatin[4]	2	Convulsant	*Illicium anistatum*
Spongiolactone[29]	18	-	*Spongionella gracilis*
Papiriogenin G[30]	19	-	*Tetrapanax papyriferum*
Guaiagrazielolide[31]	20	-	*Graziella* sp
Veranistatin A, B[32]	21a, 21b	Convulsant	*Illicium verum*
Lupeolactone[33]	22	Lowers serum cholesterol levels	*Antidesma pentandrum* Merr
Vibralactone A-C[34]	23a-23c	Lipase inhibitor	*Boreostereum vibrans*

1.3.2 Amino Acid Derived β-Lactones

The salinosporamides (**8, 9**) are examples of amino acid derived β-lactones. Feeding experiments using stable isotopes have established that part of the lactone ring is derived from the amino acid β-hydroxy-2'-cyclohexylalanine.[35] Omuralide (**10**), on the other hand, has been shown to originate from a valine-derived precursor condensing with a leucine subunit and a cysteine subunit.[36] The cinnabaramides[17] and the *N*-acetylated 3-amino-2-oxetanones SQ 26,517 (**27**)[37], obafluorin (**24**)[38], oxazolomycin (**25**)[39] (Fig. 1.8) curromycins A and B (**28**a, b)[40], lajollamycin (**26**)[41] and belactosin (**29**)[42] also have amino acid based biosynthetic origin (Fig. 1.9). Their origin, structure and biological activities are tabulated in Table 1.2. Recently, in 2009, Manam *et al.* isolated antiprotealide (**30**), a previously known synthetic proteosome inhibitor from the extract of wild type *Salinospora tropica.*[43] Although a number of chemical synthetic routes have been reported for most of these amino acid derived β-lactones, only salinosporamides (**8, 9**)[35], omuralide (**10**)[36] and obafluorin (**24**)[44] have been explored biosynthetically.

Table 1.2: Amino Acid Derived β-Lactones

	Structure	Known activity	Source
Salinosporamide A[12]	8	Proteosome inhibitor	*Salinospora tropica*
Salinosporamide B[14]	9	Proteosome inhibitor	*Salinospora tropica*
Omuralide[15]	10	Proteosome inhibitor	*Streptomyces* sp. OM6519
Cinnabaramides A-C[17]	11-13	Proteosome inhibitor	*Streptomyces* sp.
Obafluorin[38]	24	Antimicrobial activity	*Pseudomonas fluorescens*
Oxazolomycin[39]	25	Antimicrobial, antiviral activity	*Streptomyces* sp.
Lajollamycin[41]	26	Anti microbial	*Streptomyces nodosus*
SQ 26,517[37]	27	Antibiotic	*Bacillus* sp. SC 11,480
Curromycin A, B[40]	28 a, b	-	*Streptomyces hygroscopus*
Belactosin A, C[42]	29 a,b	Proteosome inhibitor	*Streptomyces* sp. UCK14
Antiprotealide[43]	30	Proteosome inhibitor	*Salinospora tropica*



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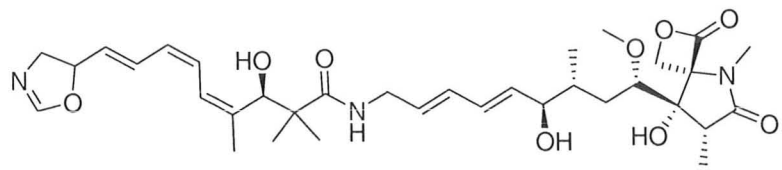

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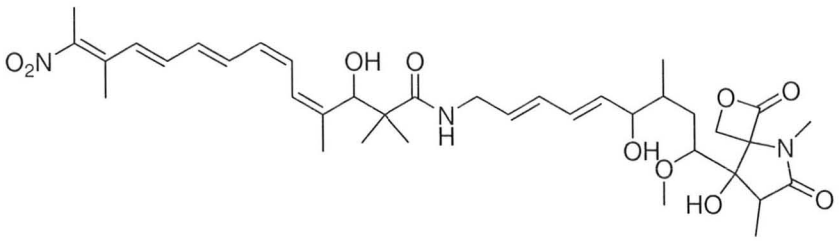
Fig 1.8: Obafluorin and Oxazolomycin



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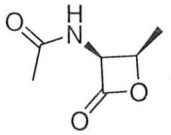

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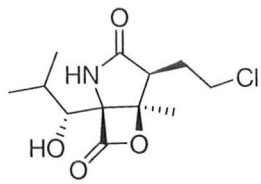

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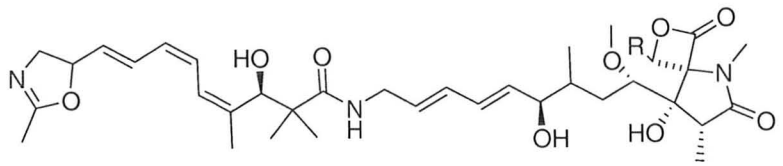
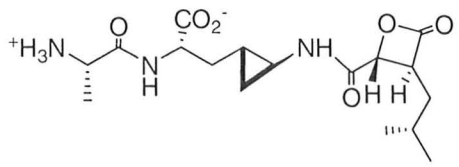


28 a R=CH_2OCH_3: b R=CH_3



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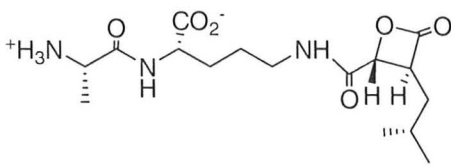


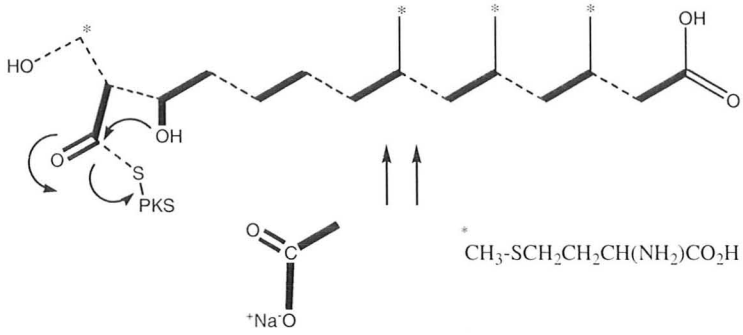
29b

Fig: 1.9: Amino Acid Derived β-Lactones

1.3.3 Polyketide Derived β-Lactones

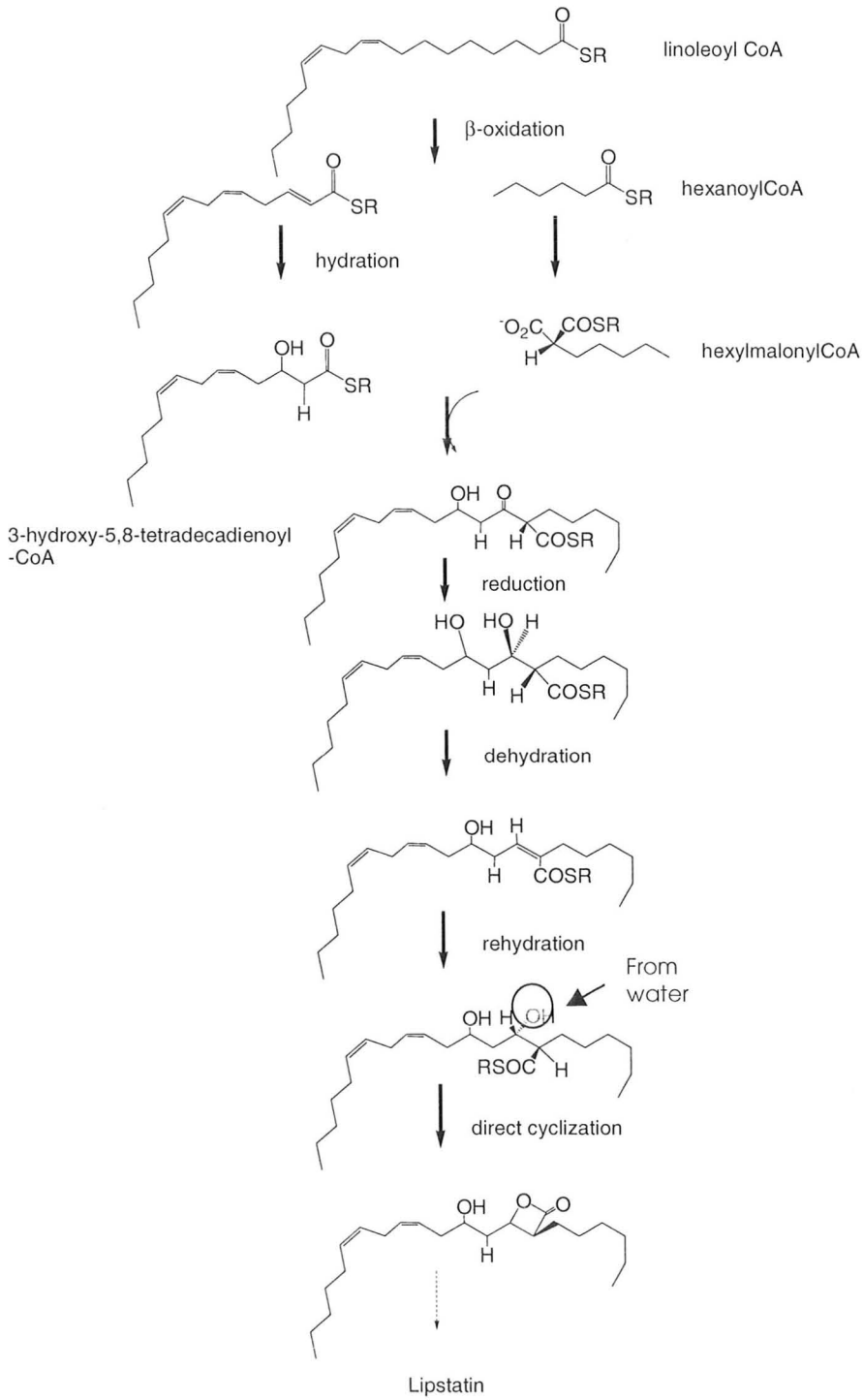
Ebelactones A and B (**15, 16**), F244 (**14**), esterastin (**5**), lipstatin (**3**) and valilactone (**6**) are all examples of β-lactones which show a polyketide type origin. Table 1.3 describes the origin, activity and structures of polyketide derived β-lactones. Polyketides are natural products with 1-3 oxygenation patterns. They are biosynthesised on a large protein known as a polyketide synthase (PKS), using acetate, propionate or butyrate units as precursors among others.[45] These precursors are activated as co-enzyme A thioesters, then loaded onto the PKS enzyme as starter units and extender units, where they are condensed in a "head to tail" manner to form a long, linear polyketide chain.[45] This chain then undergoes chemical modifications to yield several different structures such as aflatoxins, macrolides, chalcones and polyphenols. β-Lactone type polyketides are made in a similar manner and the long polyketide chain cyclizes to give the four-membered lactone ring.

The biosynthesis of F244 (**14**) was studied by several groups. Omura and coworkers investigated the biosynthesis using singly and doubly labelled sodium acetate and methionine and showed that F244 is a heptaketide derived from seven acetate units and four methionines.[46] Saepudin and Harrison investigated the origin of the oxygen and hydrogen atoms of the molecule. The results showed that the carbon-oxygen bonds of the precursor carboxylic acid molecules remained intact (Scheme 1.2), suggesting a thioesterase mediated cyclization mechanism.[47]



Scheme 1.2: Biosynthesis of F244

Lipstatin is biosynthesised by a variation of the fatty acid and polyketide pathways; the work by Eisenreich *et al.* using ^{13}C labelled lipids showed that it is made from C_{14} and C_8 moieties which are products of fatty acid catabolism (Scheme 1.3).[48] The biosynthesis of the lactone ring was studied by Goese *et al.* using deuterium labelling studies and revealed that the hydrogen at the C-3 hydroxyl group of the lactone moiety originates from the water used for fermentation.[49] This result was interpreted in terms of a dehydration-rehydration mechanism that predicts that the ring oxygen atom of the β-lactone would originate from water.



Scheme 1.3: Biosynthesis of Lipstatin

Genetic techniques in the study of biosynthesis have been used extensively to study the PKSs of several compounds including the macrolactone erythromycin (**35**). The structural genes responsible for the biosynthesis of **35** were sequenced, giving insight into the biosynthesis and the lactonization mechanism of erythromycin.[50] A direct cyclization mechanism, catalyzed by the thioesterase (TE) domain of the PKS, which facilitates the cyclization as it cleaves the polyketide from the protein, was suggested for **35** and as well for other macrolactones such as rapamycin, spiramycin and tylosin.[45] However, the mechanism of cyclization has not been studied at the genetic level in the case of β-lactones. This prompted us to explore the biosynthesis and the nature of the PKS in these small ring lactones which display such a wide range of therapeutic properties.

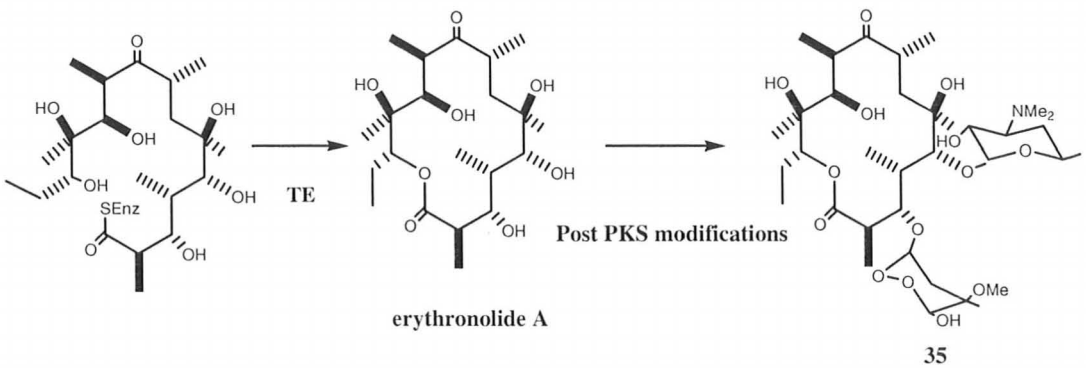


Fig 1.10: Macrolactonization in Erythromycin A

The biosynthetic studies of ebelactones by Umezawa and co-workers and by our group will be discussed in detail in a separate section (1.7.2). Table 1.3 summarizes the polyketide-derived β-lactones.

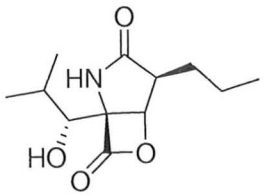
Table 1.3: Polyketide Derived β-Lactones

	Structure	Known Activity	Source
Lipstatin[5, 6]	3	Pancreatic lipase inhibitor	*Streptomyces toxytryicini*
Esterastin[9]	5	Esterase inhibitor	*Streptomyces lavandulae*
Valilactone[10]	6	Esterase inhibitor	*Streptomyces albolongus*
Panclicin A-E[11]	7a-7e	Lipase inhibitor	*Streptomyces* sp
F244[18]	14	3-Hydroxy-3-methylglutaryl coenzyme A synthase inhibitor	*Cephalosporium* sp, *Scopulariopsis* sp, *Fusarium* sp
Ebelactone A, B[22]	15, 16	Esterase, lipase, cutinase inhibitors	*Streptomyces aburaviensis*
Vittalactone[23]	17	Aggregation pheromone	*Acalyma vittatum*

1.4 Synthesized and Bioengineered β-Lactones

Inspired by the biological importance of the β-lactones, many structural analogues of these compounds have been synthesized. Tetrahydrolipstatin (**4**), the anti-obesity drug,[51] PS519 (**31**)[52], the anti-inflammatory lead, and the salinosporamide-omuralide hybrid, antiprotealide (**30**)[53] are a few of the important β-lactones that have been synthesized chemically.

The development of genetic techniques in the 1980s opened the way to bioengineered drugs. Thus, fluorosalinosporamide (**32**) was successfully bioengineered by Moore and co-workers by mutasynthesis using fluoroacetate, and this compound happens to be the most potent proteosome inhibitor tested so far.[54] Antiprotealide (**30**), salinosporamide X1 (**33a**), salinosporamide X2 (**33b**) and salinosporamide J (**34**) are some of the other compounds that have been successfully bioengineered (Fig. 1.11).[55]



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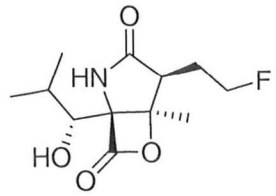

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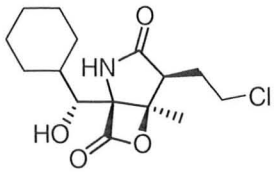
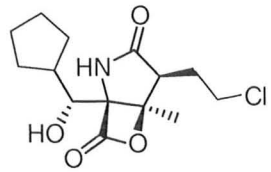


33 a



33b

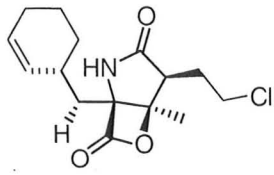


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Fig 1.11: Synthesized and Bioengineered β-Lactones

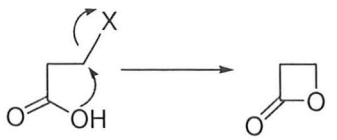
1.5. Chemical Synthesis

Chemically, β-lactones can be synthesised using several different synthetic strategies. Two key processes of relevance to this thesis are:

(a) Alcohol activation

(b) Acid activation

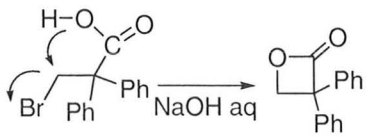
1.5.1 Alcohol Activation



Scheme 1.4: Alcohol Activation Route to β-Lactones

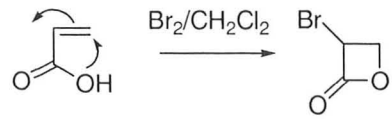
This method involves the activation of an alcohol group at the C-3 position of a β-hydroxy acid and nucleophilic attack of the carboxylate ion on to the C-3 carbon to displace the activated leaving group (Scheme 1.4). This reaction generally results in an inversion of configuration of the carbon atom which bore the leaving group.

The oldest method known to prepare β-lactones is from β-halocarboxylic acid salts (Scheme 1.5). The acid salt is made *in situ* from the acid with a mild base and the cyclization occurs at room temperature.[56]



Scheme 1.5: β-Lactones from β-Haloacid Salts

Halolactonization is also an example of this method. Barnett *et al.* established that α-halo-β-lactones could be prepared from α-β-unsaturated acids using Br_2/I_2 in good yields (Scheme 1.6).[57]



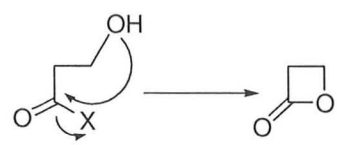
Scheme 1.6: Bromolactonization Route to β-Lactones

Mitsunobu lactonization (PhP_3/DEAD) of β-hydroxy carboxylic acids also generally yields β-lactones, although most commonly carbonyl group activation is observed under normal conditions (see below).[58]

Utilization of selenium reagents for the formation of different heterocycles is well known. However, β-lactones made by this method were not stable and were not isolated. Nicolaou and Lysenko have described a method for internal lactonization of unsaturated carboxylic acids by using phenylselenyl chlorides.[59]

1.5.2 Acid Activation

This method involves the activation of the carboxylate group and subsequent nucleophilic attack of the C-3 hydroxyl group on to the electrophilic carbonyl to displace the leaving group (Scheme 1.7).

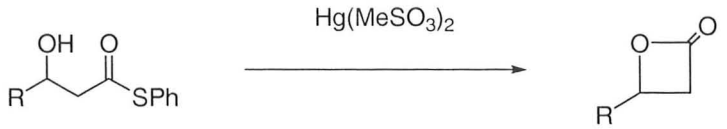


Scheme 1.7: Acid Activation Method to Form β-Lactones

Diassi used the β-hydroxy acid activation method using ethyl chloroformate in pyridine to form a mixed anhydride.[60] This is then attacked by the hydroxyl group

which serves as an internal nucleophile leading to β-lactones with retention of configuration at the β-carbon.

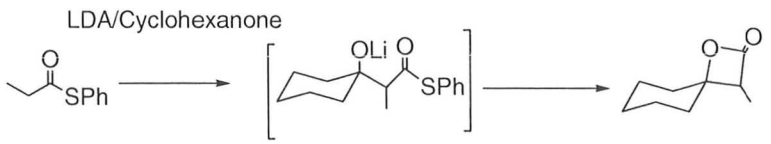
Masamune's reaction for the preparation of β-lactones is also an example of this method (Scheme 1.8).[61] The acid group at the C-1 carbonyl group is activated by conversion to a thiol ester functionality and this is quenched by a thiophilic salt such as mercury methanesulfonate to yield the four-membered lactone.



Scheme 1.8: Masamune Reaction

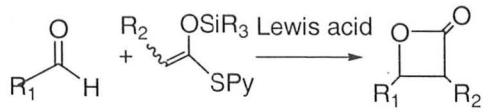
A similar method was used by Adam *et al.* using benzenesulfonyl chloride to activate the β-hydroxyacid.[62]

The Tandem Mukaiyama Aldol Lactonization (TMAL) method was first used by Danheiser[63] and modified by Romo[64] and others. This method involves the addition of thiol ester enolates to carbonyl intermediates and spontaneous cyclization of the resulting alcoholate intermediates, due the activation of the C=O group as a thioester (Scheme 1.9).



Scheme 1.9: Danheiser (TMAL) Method to β-Lactones

Recently, Mitchell and Romo[65] used Lewis acids and substrate control to stereoselectively synthesize both *cis* and *trans* β-lactones using TMAL reactions (Scheme 1.10).



Scheme 1.10: Romo (TMAL) Method

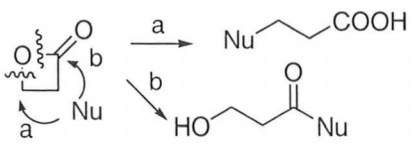
Finally, Jenkins and Harrison[66] developed a method call Tandem Evans type Aldol Lactonization (TEAL) for the preparation of optically active β-lactones. This method was recently modified and tested by Khandker *et al.*.[67]

1.6 Reactivity of β-Lactones

β-Lactones show unusual reactivity compared to larger ring lactones and esters. This reactivity is due to the high strain of the ring system. Among the many reactions that β-lactones undergo, two are of particular importance to the current thesis.

1.6.1 Reactions with Nucleophiles

In the presence of nucleophiles, β-lactones, undergo oxygen-alkyl (a) or oxygen-acyl (b) bond cleavage (a or b in Scheme 1.11). They are therefore ambident electrophiles. Soft nucleophiles typically add to β-lactones at the oxygen-alkyl/C-3 bond (a) with inversion of configuration; neutral or slightly acidic conditions result in C-3 attack. Hard nucleophiles however, cleave the acyl-oxygen/C-1 bond (b) and this reaction is favoured by basic or strongly acidic conditions.[68]



Scheme 1.11: Nucleophilic Ring Opening of β-Lactones

Ring opening via nucleophilic addition at the carbonyl residue affords access to a variety of β-hydroxy-ester or amide adducts depending on the nucleophile, while attack on C-3 can lead to S_N2 reaction to afford access to β-substituted carboxylate derivatives.[69] Nelson *et al.* have used this method successfully in the synthesis of β-peptides via β-amino acids.[70]

Recently, in 2007, Zhang and Romo have used a new strategy to synthesise γ-lactones and 3(2H)-furanones from β-lactones.[71] This involves the acyl C-O nucleophilic ring cleavage at the lactone carbon of bicyclic/tricyclic β-lactones to form δ-hydroxy-α-diazo-β-ketoesters.

1.7 Ebelactones

Ebelactones A and B (**15, 16**) are produced by the terrestrial *Streptomyces* sp. MG7-G1 and were first reported to show inhibitory activity against esterases in 1980.[22]

1.7.1 Bioactivity Studies

Initial biological assay with ebelactone A and B by Umezawa and co-workers showed that these compounds inhibit hog liver esterase activity with IC_{50} values of 0.056 and 0.00035 μg/mL respectively.[21] Over the past three decades, several biological studies have been conducted on these molecules, and these β-lactone-type

compounds have been found to be active against a number of other enzymes. The work by the Japanese group led by Majima showed that ebelactone B is active against rat liver kinases.[72] Ebelactone B is also known to be active against cathepsin A.[73] Earlier work by Koller *et al.* showed that these compounds inhibit cutinase – an enzyme produced by fungal pathogens in order to facilitate penetration into plant bodies.[74] It is also known that ebelactones suppress the activity of aminopeptidases found in cellular membranes of various animal cells.[22] Wright, Harrison and co-workers have recently shown that ebelactone inhibits HTA (homoserine transacetylase).[75]

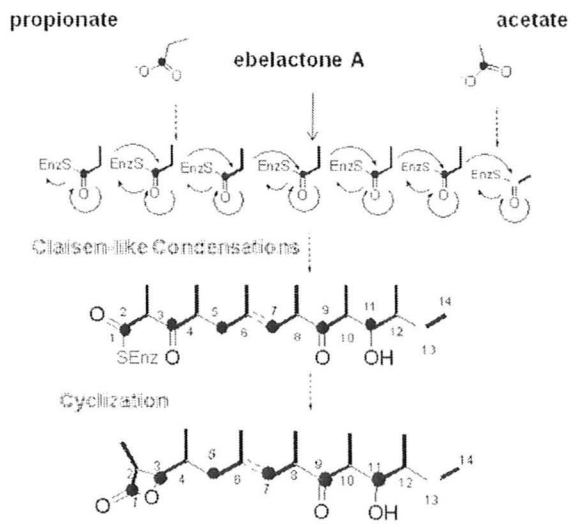
1.7.2 Biosynthesis

Ebelactones A and B consist of a long side chain attached to the β-position of the lactone ring, indicating a polyketide assembly. The side chain includes a methylene, an olefin, a ketone and a hydroxyl functionality, suggesting that it is biosynthesised by a type I/modular polyketide synthase (PKS). We can therefore predict the PKS of ebelactone to consist of seven modules and each module to contain three domains to catalyse one cycle of chain extension: ketosynthase (KS), acyl transferase (AT) and acyl carrier protein (ACP); in addition a variable set of domains, ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER), are responsible for the keto group modifications, where necessary at the appropriate module, in order to furnish the above-mentioned various functionalities.

Although no experimental work exists on this putative PKS, we can expect the first and the fifth modules to consist of ACP, KS, KR, DH and ER domains, in order to catalyze a full set of reductive activities to produce methylene groups at C-13 and

C-5, respectively. The second module will consist of only a KR to reduce the keto group to generate a hydroxyl group at C-11, while module 3 will not have any reducing domains so that the keto group at C-9 remains intact. Module 4 will have a KR and a DH in order to yield the olefin functionality at C-6 /C-7.

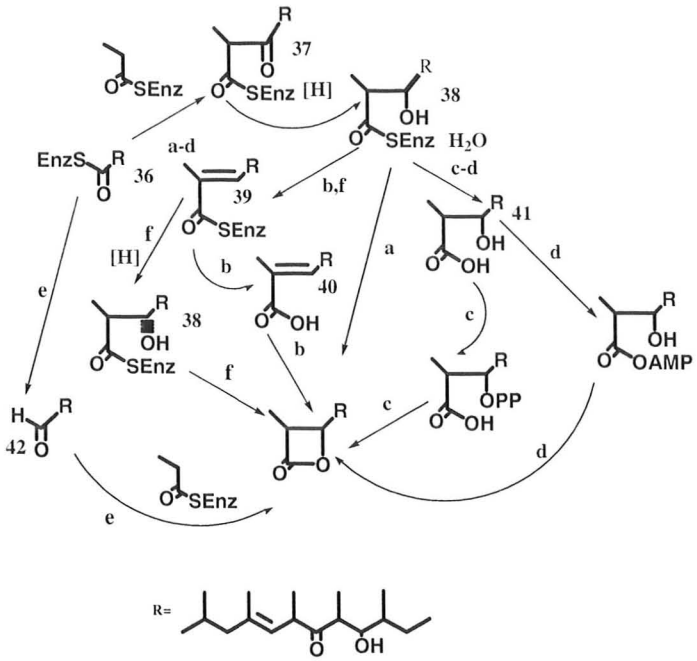
The biosynthesis of ebelactones A and B was studied by Umezawa and co-workers using ^{13}C-labelled sodium acetate, propionate, and butyrate[76], supporting the hypothesis of polyketide origin. These experiments showed that **15** is derived from a "starter" acetate unit and six "extender" units of propionate, joined in the "head-to-tail" arrangement typical of polyketides. Likewise **16** derives from 1 unit of acetate, 5 units of propionate and 1 terminal unit of butyrate[76] (Scheme 1.12).



Scheme 1.12: Biosynthesis of Ebelactone A

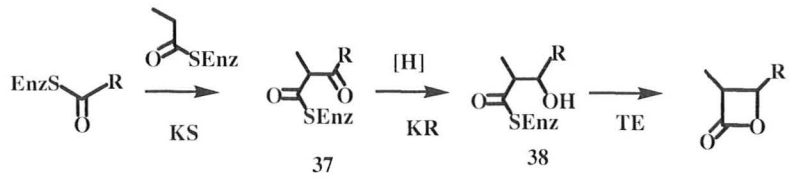
However, the origin of the oxygen atoms in **15** or **16** was not determined, leaving considerable ambiguity in the mechanism of β-lactone ring formation. Several possible mechanisms for the biosynthesis of β-lactones that are feasible based on

biosynthetic and/ or chemical precedents, are shown in Scheme 1.13.[77] In mechanisms a-d and f, the hexaketide intermediate **36** is extended by methylmalonyl CoA on the presumed polyketide synthase (PKS).



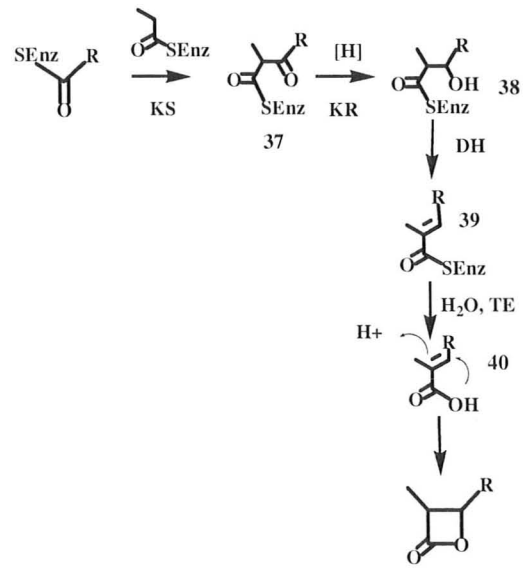
Scheme 1.13: Putative Mechanisms for the Formation of the β-Lactone Ring in Ebelactone A

Next, heptaketide intermediate **37** is reduced to a β-hydroxyacid intermediate (**38**) on the PKS by ketoreductase. In route **a**, intermediate **38** cyclizes directly. This mechanism gives the small ring lactone in a route that is analogous to formation of macrocyclic lactones such as erythromycin *in vivo* (Section 1.3.3),[78] and resembles the chemical lactonization of β-hydroxy-thioesters, (Section 1.5.2). In this route, we also expect to find a thioesterase at the end of the last module which will catalyze the off-loading of the fully grown heptaketide chain (Scheme 1.14).



Scheme 1.14: Mechanism a

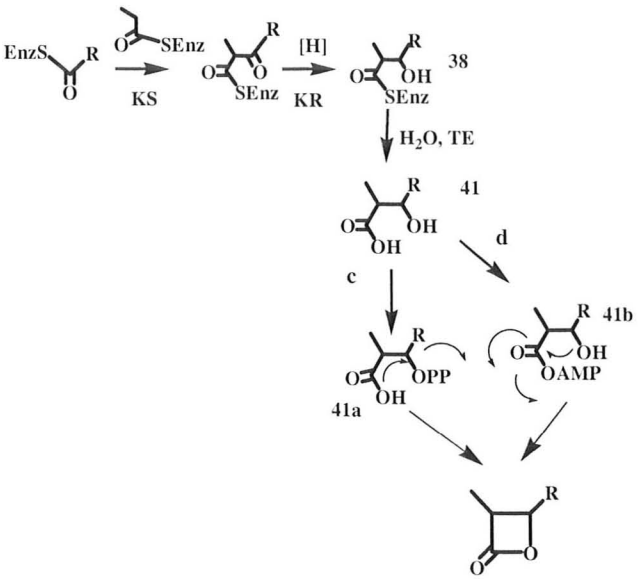
In mechanism **b**, **38** is dehydrated to **39** by the DH domain of PKS, and hydrolyzed from the PKS by a hydrolytic TE. The resulting α, β-unsaturated acid (**40**) could cyclize via protonation at the α-position and nucleophilic attack at C-β by the carboxylate in a process that is reminiscent of the chemical bromo-lactonization of α, β-unsaturated acids (Section 1.51).[57]



Scheme 1.15: Mechanism b

In mechanisms **c** and **d**, intermediate **38** is hydrolyzed to give β-hydroxy acid **41**, then activated by, for example, adenylation by ATP, either at the carboxylate to give **41b** (**d**) or at the hydroxyl group to yield a phosphate group (**41a**) (**c**). This activation facilitates cyclization by mechanisms analogous to the chemical activation of β-hydroxyacids at carboxylate and alcohol groups, respectively (1.5.1).[79] The

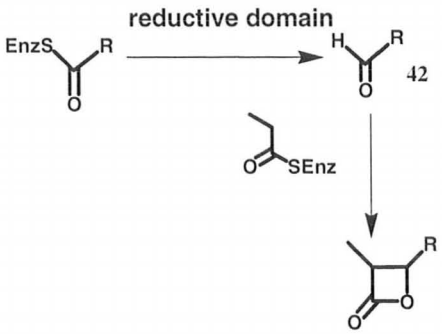
adenylation mechanism **c** resembles the activation of amino acids in the first step of protein synthesis by α-amino acyl tRNA synthetase, while the alkyl phosphate type intermediate in mechanism **d** is reminiscent of the alkyl diphosphates in terpene biosynthesis.



Scheme 1.16: Mechanisms c and d

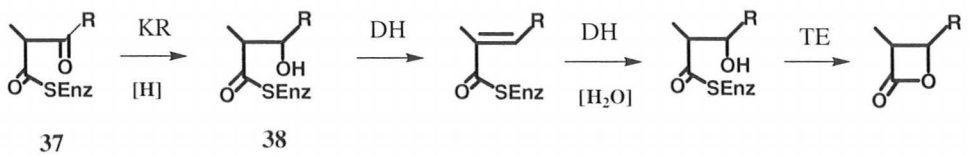
In mechanism **e**, in contrast, the hexaketide precursor is cleaved reductively from the enzyme to furnish aldehyde **42**. Several PKSs are known which contain reductive domains that perform analogous cleavage reactions of acyl groups.[80] Aldehyde **42** can then undergo aldol-type condensation with, *e.g.* propionyl or methylmalonyl CoA, and the resulting aldol adduct can then cyclize directly in a reaction analogous to the chemical Tandem aldol-lactonization reactions developed by Romo and others.[64] Indeed, a TAL-like mechanism has been suggested in the biosynthesis of the salinosporamide β-lactones.[35] In this case, the final module of the

putative PKS will not consist of a terminal TE; instead it will likely contain a "R" domain which will reductively cleave the hexaketide to an aldehyde.



Scheme 1.17: Mechanism e

Finally, in the biosynthesis of lipstatin, yet another mechanism has been proposed based on deuterium labelling experiments (Scheme 1.3). A Claisen-like condensation between two esters derived from fatty acid degradation is followed by reduction of the resulting ketone (*cf* **37** to **38**, Scheme 1.18). The ensuing alcohol then undergoes dehydration-rehydration to effect epimerization, prior to cyclization. An analogous process for ebelactone biosynthesis would correspond to mechanism **f** (Scheme 1.18).



37 **38**

Scheme 1.18: Mechanism f

Mechanisms **a** and **c** require that the lactone ring oxygen atom is derived from the same biosynthetic unit as the carbonyl oxygen, and involve the breaking of the C-β-O bond during biosynthesis. In contrast, in mechanisms **c**, **d** and **e** the C-O bond at

C-β remains intact from the carboxylic acid precursor. Finally, the ring oxygen is predicted to be lost from the precursor in route **f**.

In order to probe the mechanism of β-lactone ring formation, our group used the ^{18}O induced isotopic shift method described originally by Vederas[81,82] to study the origin of the oxygen atoms in F-244 (**14**), which derives from the fungus *Fusarium* sp.[46] The results showed that the β-lactone ring is derived from an intact bond between the β-carbon and the oxygen atom; the carbonyl oxygen atom is derived from an intact C=O bond in the adjacent unit. Thus pathways **a, c** and **f** are excluded as possible mechanisms for lactone formation in this fungal metabolite.[46] However, it is unclear whether this result would still be true in a bacterial β-lactone.

1.8 Objectives of the Study

It is obvious that β-lactones are indeed a very important group of compounds in medicinal chemistry. There are a number of objectives for this study.

1. Biosynthesis β-Lactones

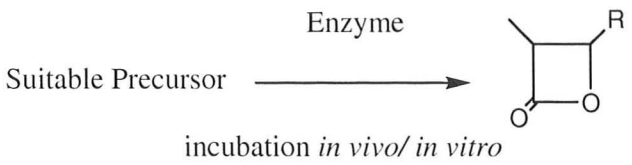
   Understanding the biosynthetic mechanism and thereby identifying the enzyme(s) which catalyze the reaction of lactonization in the small strained ring system. Coding of the genes responsible for this reaction using the *Streptomyces aburaviensis* genomic DNA will give insight into the biosynthetic sequence leading to β-lactone assembly.

2. Production of Non-Natural Natural Products

This information can be used to produce "non-natural natural products" containing the important β-lactone pharmacophore, which can bind to target enzymes covalently, increasing the efficiency of the natural product drugs.

3. Alternative Route to Chemical Synthesis of β-Lactones

Purification of the enzyme which catalyzes the lactonization can lead to a separate route to the preparation of β-lactones, by incubating a suitable precursor with the enzyme under suitable conditions.



Scheme 1.19: Enzymatic Preparation of β-Lactones

Chapter 2

BIOSYNTHESIS OF EBELACTONE A: ORIGIN OF THE OXYGEN ATOMS AND MECHANISM OF β-LACTONE RING FORMATION

2.1 Introduction

This chapter deals with biosynthetic studies on ebelactone A and the results of the isotopic labelling experiments conducted to probe the mechanism of the lactone ring formation.

2.1.1 Application of Isotopic Labelling in Biosynthetic Studies

The use of stable isotopes in the study of biosynthesis became popular at the time of the introduction of NMR spectroscopy.[81] Using this technique, a structural study which could take several years of hard work with chemical degradation would be possible in a matter of a few weeks. The ^{13}C isotope was of particular significance: labelled precursors were incorporated and the natural products were isolated. A ^{13}C NMR spectrum was obtained to determine the sites of isotopic enrichment. Sites where the isotope was incorporated would give rise to signals with enhanced intensities.

Eventually, doubly labelled precursors became available. These would give rise to a pair of ^{13}C-^{13}C coupled doublets in the ^{13}C NMR spectrum. This coupling will still be observed in the labelled natural product, if the relevant carbon-carbon bond remains intact throughout the biosynthetic pathway. Similarly, information can also be obtained using ^{13}C-^{18}O, ^{13}C-^{15}N and ^{13}C-^{2}H labelled precursors. For a ^{13}C-^{18}O labelled precursor, an isotopic shift would be observed in the ^{13}C NMR spectrum, when the relevant bond remains intact.[82]

2.2 Fermentation and Purification of Ebelactone A

Cultures of *Streptomyces* sp. MG7-G1 were purchased from ATCC, and grown on ISP2 agar. The cells were transferred to a liquid medium containing fish meal. Incubation was followed by extraction with ethyl acetate and purification using subsequent chromatography yielded ebelactone A (2 mg/L) (Fig. 2.1) as white crystals. The ESI+ mass spectrum of the compound showed a peak at m/z 339 $[M+H]^+$ consistent with the molecular formula $C_{20}H_{34}O_4$. The proton NMR of the compound showed a doublet at 5.03 ppm corresponding to the olefinic proton at 7-H and a doublet of doublets at 3.86 ppm due to the 3-H lactone proton (Fig. 2.2); these signals are characteristic of the ebelactones. The remainder of the proton NMR spectrum was assigned by comparison to literature data reported by Uotani *et al.*.[76] The carbon-13 NMR spectrum also agreed well with the structure and accounted for two carbonyls, seven methine, two methylene, seven methyl and two olefinic carbons. Assignments of the proton and carbon-NMR resonances are tabulated in Table 2.1.

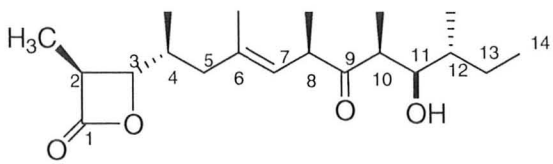


Fig: 2.1: Structure of Ebelactone A

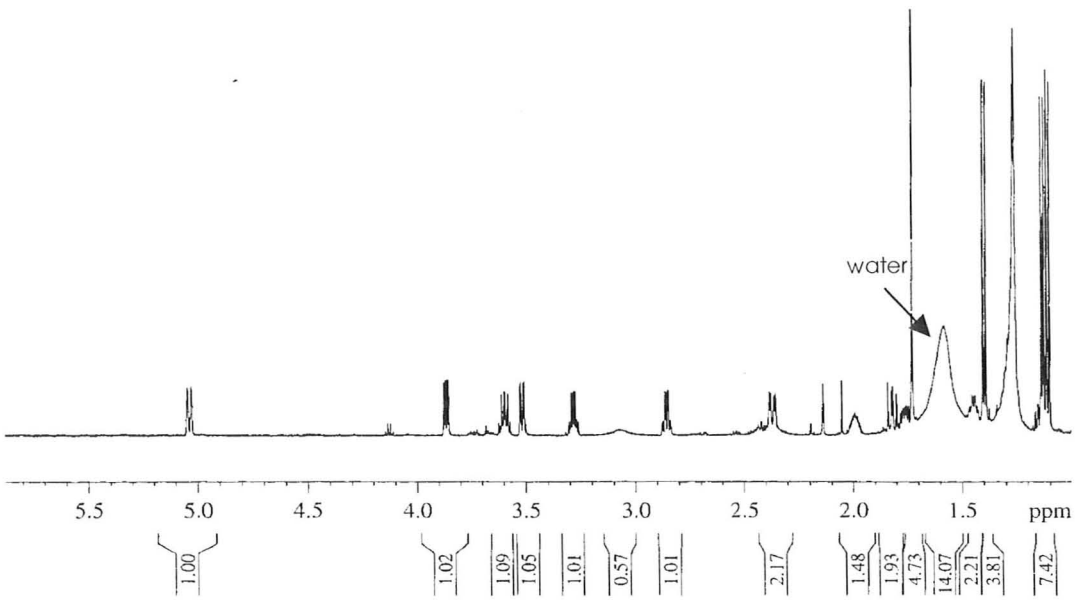


Fig. 2.2 Proton NMR Spectrum of Ebelactone A ($CDCl_3$, 600 MHz)

Table 2.1: Proton and ^{13}C NMR Assignments of Ebelactone A (15)

Assignment	Proton (m) (#H)	Carbon
1	-	171.15
2	3.27 (dq) (H)	48.74
2-CH_3	1.39 (d) (3H)	13.29
3	3.86 (dd) (3H)	83.03
4	2.00 (m) (1H)	35.97
4-CH_3	0.85 (d) (3H)	13.97
5	1.82 (dd) (1H) 2.36 (dd) (1H)	43.39
6	-	135.93
6-CH_3	1.73 (s) (3H)	16.88
7	5.03 (d) (1H)	126.93
8	3.59 (dq) (1H)	45. 77
8-CH_3	1.12 (d) (3H)	16.88
9	-	217.10
10	2.85 (dq) (1H)	45.55
10-CH_3	1.10 (d) (3H)	9.80
11	3.50 (m) (1H)	74.62
11-OH	3.06 (s) (1H)	-
12	1.44 (m) (1H)	37.00
12-CH_3	0.77 (d) (3H)	15.29
13	1.75 (dq) (1H) 1.17 (m) (1H)	25.25
14	0.88 (t) (3H)	11.31

2.3 Ebelactone B

The above mentioned fermentation also yielded ebelactone B in trace quantities (1 mg) (Fig. 2.3); it also is a white solid. The ESI+ mass spectrum of the compound suggested a molecular formula of $C_{21}H_{36}O_4$ with the peak at m/z 353 ($[M+H]^+$). The IR spectrum of the compound exhibited the presence of the hydroxyl (3540 cm^{-1}), β-lactone (1810 cm^{-1}) and carbonyl (1700 cm^{-1}) groups. The proton NMR spectrum of the compound was very similar to that of ebelactone A, and only differed in containing an extra CH_2 signal at 1.86 ppm due to the methylene attached to C-2 and a shifted signal at 1.06 ppm due to the CH_3 of the ethyl group attached to C-2 (Table 2.2). The carbon-13 NMR spectrum also resembled that of ebelactone A, but in addition it contained an extra methylene signal at 21.80 ppm. Thus, based on this analysis, the compound was assigned the structure of ebelactone B, which was also consistent with the reported literature data.[76]

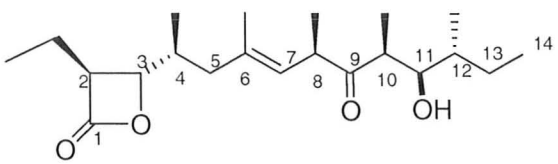


Fig: 2.3: Structure of Ebelactone B

Table 2.2: Proton and ^{13}C NMR Assignments of Ebelactone B (16)

Assignment	Proton (m) (#H)	Carbon
1	-	171.61
2	3.20 (dt) (H)	56.43
2'- H_2	1.86 (d) (2H)	21.80
2'- CH_3	1.06 (t) (3H)	11.31
3	3.92 (dd) (3H)	81.55
4	2.05 (m) (1H)	35.91
4-CH_3	0.86 (d) (3H)	13.12
5	1.87 (dd) (1H) 2.39 (dd) (1H)	43.39
6	-	136.02
6-CH_3	1.73 (s) (3H)	16.85
7	5.04 (d) (1H)	126.90
8	3.59 (dq) (1H)	45. 79
8-CH_3	1.13 (d) (3H)	16.85
9	-	218.11
10	2.85 (dq) (1H)	45.52
10-CH_3	1.10 (d) (3H)	9.83
11	3.51 (m) (1H)	74.95
11-OH	3.08 (s) (1H)	-
12	1.44 (m) (1H)	37.00
12-CH_3	0.78 (d) (3H)	13.29
13	1.78 (dq) (1H) 1.12 (m) (1H)	25.25
14	0.89 (t) (3H)	11.85

2.4 Synthesis of Sodium [1-^{13}C] and [1-^{13}C, $^{18}O_2$]Propionate

The method of Cane *et al.* was used.[83] The reaction of potassium [^{13}C]cyanide with iodoethane in deionised water, followed by distillation and hydrolysis of the resulting propionitrile yielded sodium [1-^{13}C]propionate in 88% yield, as a white powder. Proton NMR spectrum of the compound showed a double triplet at 0.95 ppm and a double quartet at 2.08 ppm corresponding to CH_3 and CH_2 respectively. The ^{13}C NMR spectrum contained an enhanced singlet at 185.03 ppm, a doublet at 30.67 ppm and a singlet at 10.15 ppm, which were assigned to C1, C2 and C3 respectively. The negative ion ESI mass spectrum of the compound showed the molecular ion peak at 74.1 $[M-H]^-$ as expected, confirming the synthesis of the compound.

Likewise, the reaction of potassium [^{13}C]cyanide with iodoethane and $H_2{}^{18}O$ gave similar results yielding [1-^{13}C, $^{18}O_2$] propionate in 82% yield. Proton and carbon-13 NMR spectra of the compound were similar to those of sodium [1-^{13}C] propionate. However, the ESI mass spectrum showed a distribution of isotopes at m/z 77.6, 75.6 and 73.6, with percentage intensities of 100, 75 and 12 respectively, confirming the successful synthesis of ^{18}O labelled propionate. The mass spectra of sodium [1-^{13}C] and [1-^{13}C, $^{18}O_2$] propionates are shown in Fig. 2.4. The data show that the sample contains 75.69% ^{18}O/site. As is common in this synthesis, the enrichment is < 100%, due to the presence of ^{16}O in the starting $H_2{}^{18}O$ and/ or endogenous $H_2{}^{16}O$.

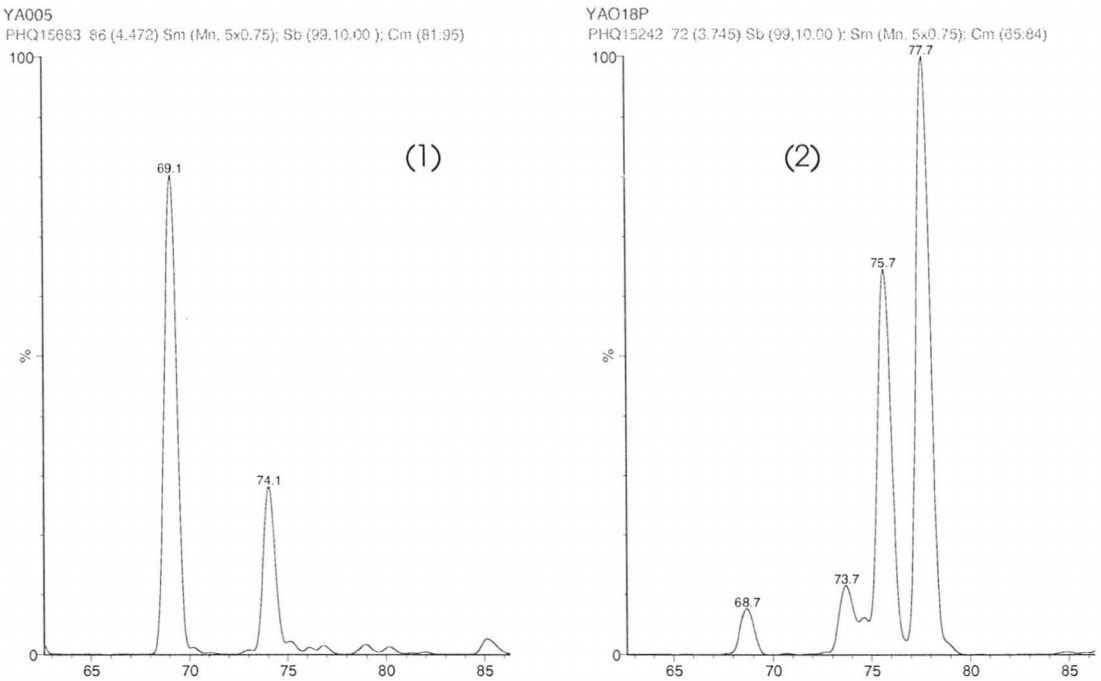
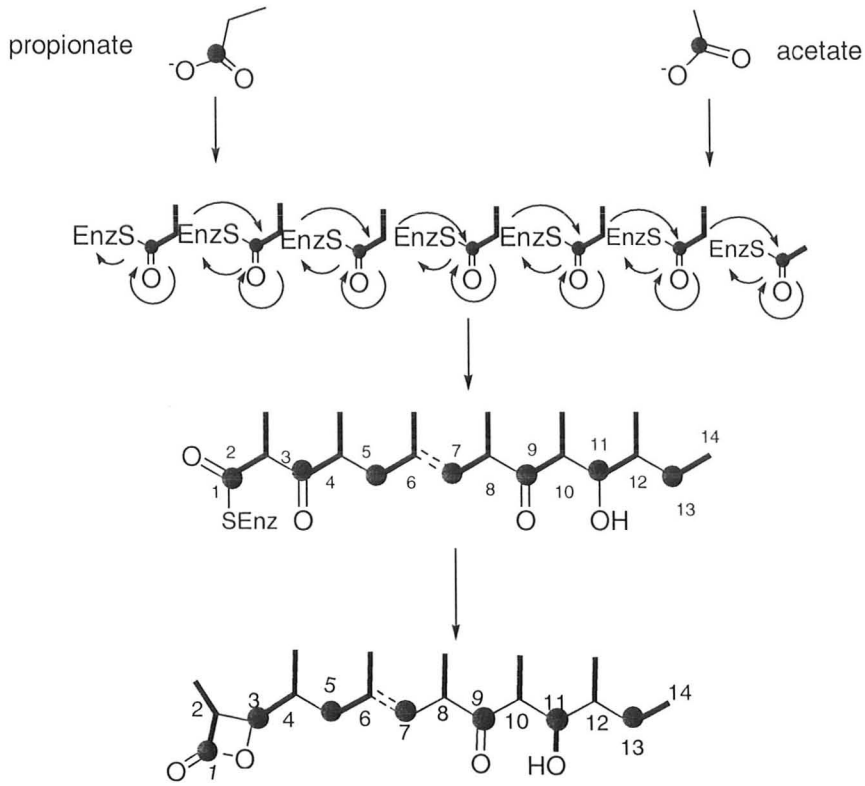


Fig. 2.4: (1) ESI- MS of ^{13}C Labelled Sodium Propionate
(2) ESI- MS of ^{13}C-$^{18}O_2$ Labelled Sodium Propionate

2.5 Feeding Experiments with Sodium [1-^{13}C]Propionate

Cultures of *Streptomyces* sp. MG7-G1 were grown as described previously. Sodium [1-^{13}C]propionate was administered to the cultures containing liquid medium (approximately 10 mg/flask/feeding) at 21 h and 45 h after inoculation. Isolation and purification of ebelactone A was carried out as has been previously reported.

The carbon-13 NMR spectrum of isolated ebelactone A was obtained and compared with that of unlabelled ebelactone A. Enrichment was observed at C-1, C-3, C-5, C-7, C-9 and C-11 (Fig. 2.5) indicating incorporation of propionate groups into ebelactone A as extender units (Scheme 2.1). This result is consistent with the results obtained by Umezawa and co-workers.[84]



Scheme 2.1: Incorporation of Propionates in the Biosynthesis of Ebelactone

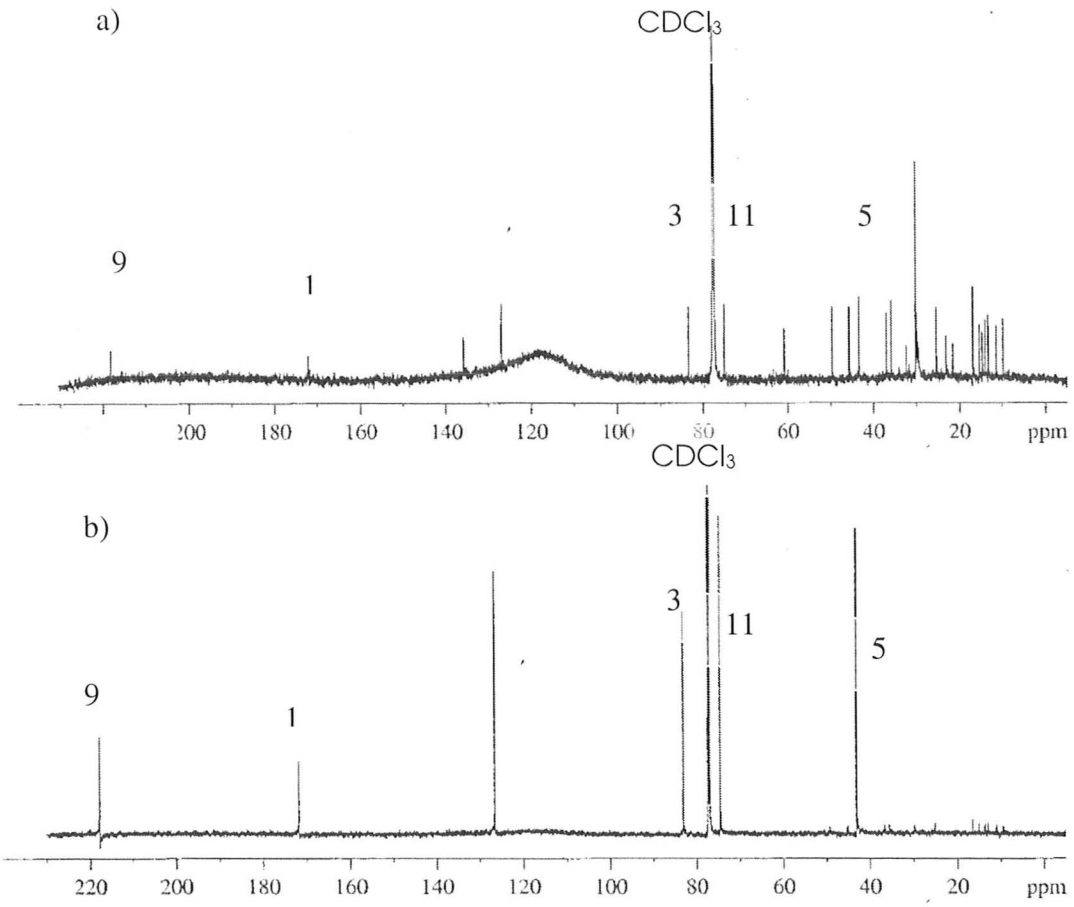


Fig 2.5: ^{13}C NMR in $CDCl_3$ of Ebelactone A (a) Unlabelled (15) and (b) Labelled with Sodium [1-^{13}C]Propionate (15a)

The percentage incorporation levels were determined from the enhancements, and enrichments were approximately 20% for each labelled site well above the required intensity of 1%.

The ESI+ mass spectrum of unlabelled ebelactone A contained the molecular ion peak at m/z 339 and ammonium and sodium adduct peaks at m/z 356 and 361 (Fig 2.6 b) respectively. The mass spectrum of the labelled compound contained similar peaks. In

addition it also contained M+1, M+2, M+3, M+4, M+5, and M+ 6 peaks corresponding to ^{13}C enhanced species with percentage intensities of 100, 100, 100, 98, 66, 30 and 10 respectively (Fig 2.6 a). This result shows that a percentage of molecules were labelled at all six possible sites. Thus, the high level of enrichment shown by ^{13}C-NMR was also demonstrated by the mass spectrum.

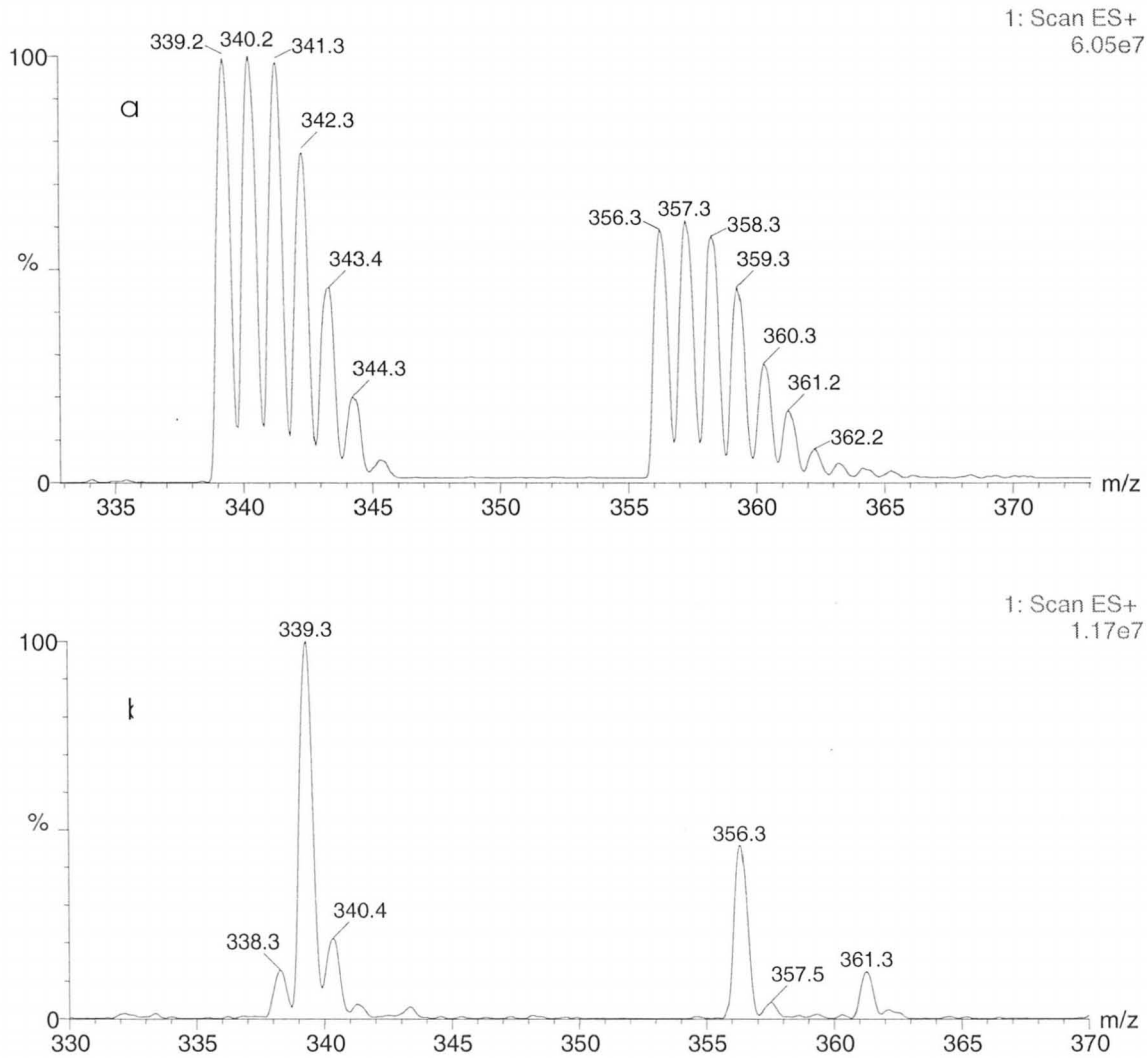


Fig 2.6: ESI+ MS of Ebelactone A (a) Labelled with Sodium [1- ^{13}C]propionate (b) Unlabelled

The results of this feeding experiment clearly indicate that propionate provides the extender units in the biosynthesis of ebelactone A. However, this experiment does not indicate the mechanism of lactone ring formation or the origin of the oxygen atoms of the molecule. This re-examination of the incorporation of propionate was however useful as a control for the oxygen-18 experiment to test the origin of the oxygen atoms of the molecule.

Establishing the origin of the oxygen atoms of ebelactone A is important in the study of the lactonization mechanism. This was tested using the ^{18}O labelled isotopic shift method first described by Vederas.[81,82]

2.6 Feeding Experiments Using Sodium [1- ^{13}C, $^{18}O_2$]Propionate

Addition of sodium [1-^{13}C, $^{18}O_2$]propionate to cultures of *Streptomyces* sp MG7-G1 followed by purification gave labelled ebelactone (**15b**). The ESI mass spectrum of this material showed a distribution of peaks corresponding to molecular ions of unlabelled **15b** ($[M+H]^+$, 339) and labelled **15a** ($[M+H]^+$, 340-346) (Fig. 2.7), along with peaks at m/z 356-363, due to the corresponding $[M+NH_4]^+$ ions.

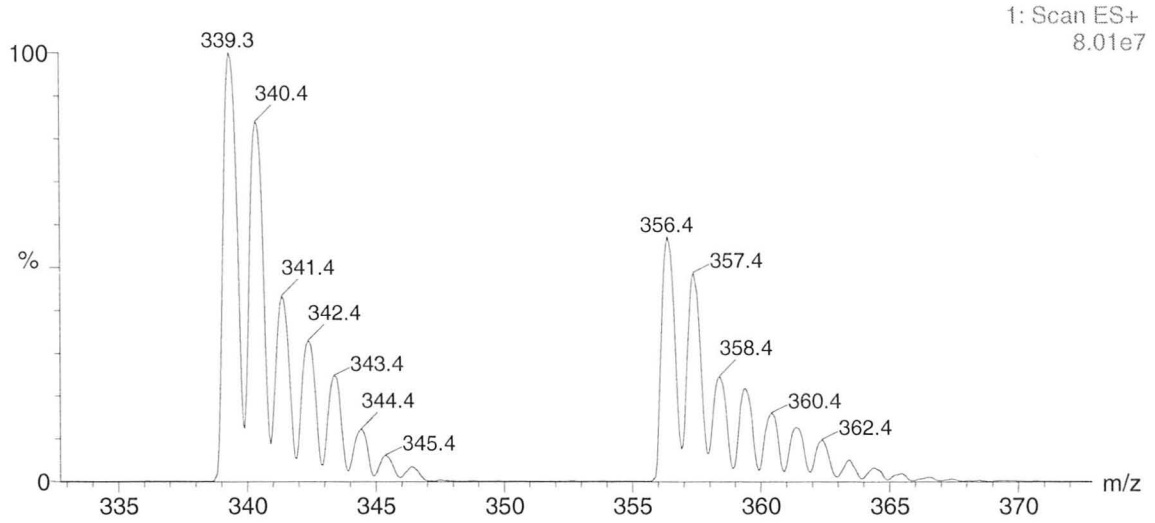


Fig 2.7: ESI+ Mass Spectrum of ^{13}C-and ^{18}O-Labelled Ebelactone A

The ^{13}C NMR spectrum also showed a high level of enrichment (>20%) at all positions derived from C-1 of propionate, similar to the results reported by Uotani *et al.*[76,84] The high resolution ^{13}C NMR spectrum of this sample exhibited isotopically shifted signals induced by the presence of ^{18}O. Single isotopic shifts were observed at all four oxygen-bearing positions, (C-1, C-3, C-7 and C-9) indicating that these carbon atoms were incorporated from propionate with the C-O bonds remaining intact (Fig. 2.8). Isotopic shifts of 0.053 ppm and 0.039 ppm were observed at C-9 and C-1, respectively (Table 2.3), consistent with values expected for C=O double bonds.[81] Similarly, isotopic shifts of 0.029 ppm and 0.024 ppm were observed for C-3 and C-11 respectively; these in turn fall in the range (0.010 - 0.035 ppm) expected for C-O single bonds.

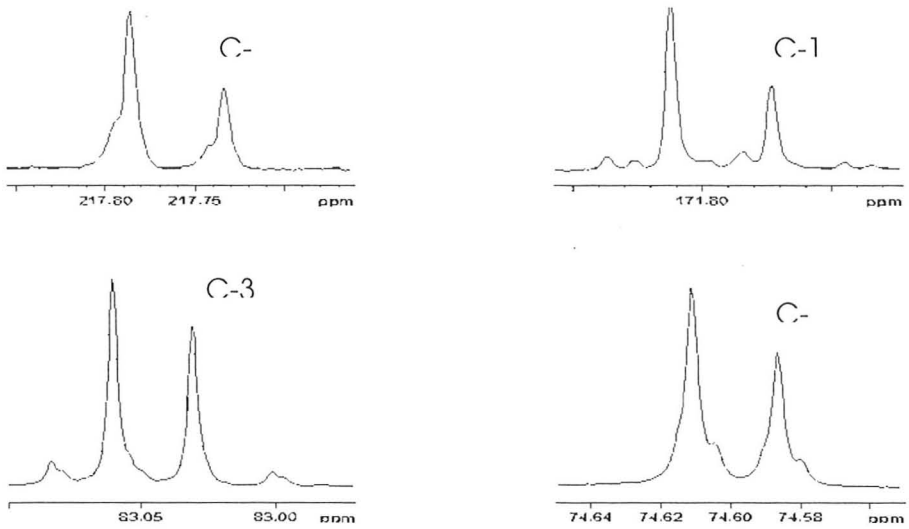


Fig: 2.8 ^{13}C NMR Spectrum of 15b at 700 MHz in $CDCl_3$

(See Section 2.8 for Discussion of Minor Peaks)

Table 2.3 also shows that the level of enrichment in oxygen-18 is similar at all four sites.

Table 2.3: Isotope Shifts and ^{18}O Enrichments Observed for Ebelactone A (15b)

$\delta^{13}C$ (ppm)	C#	$\Delta\delta$ (ppm)	$^{18}O/^{16}O$ Ratio
217.79	9	0.053	0.56
171.81	1	0.039	0.51
83.06	3	0.029	0.69
74.61	11	0.024	0.69

2.7 Discussion

These results show that four carbon-oxygen bonds in ebelactone A remain intact during biosynthesis from propionate (Fig. 2.9).

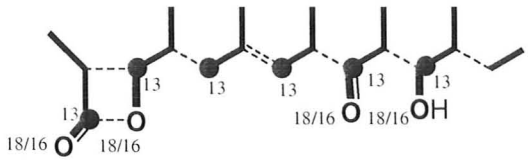
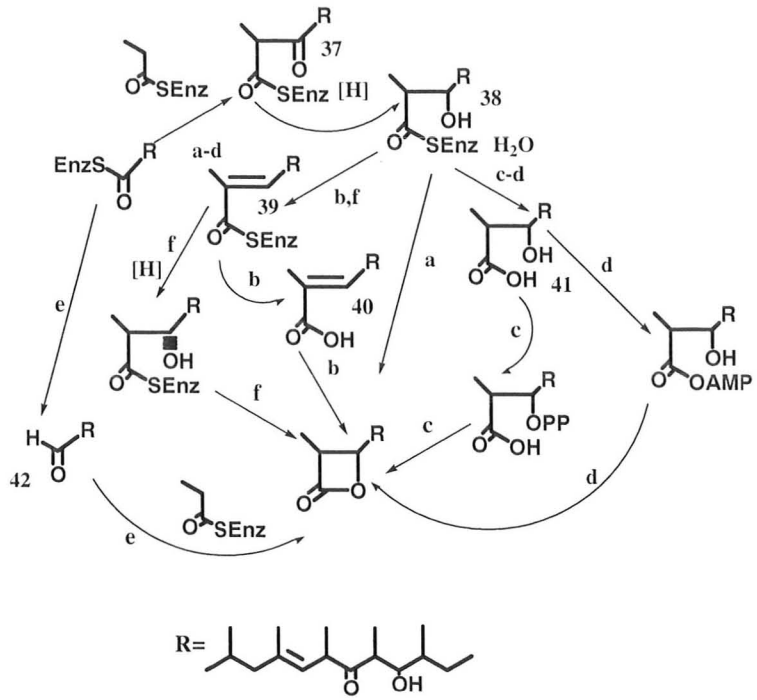


Fig 2.9: Ebelactone A Showing Intact Incorporation of ^{13}C-^{18}O Bonds from Propionate

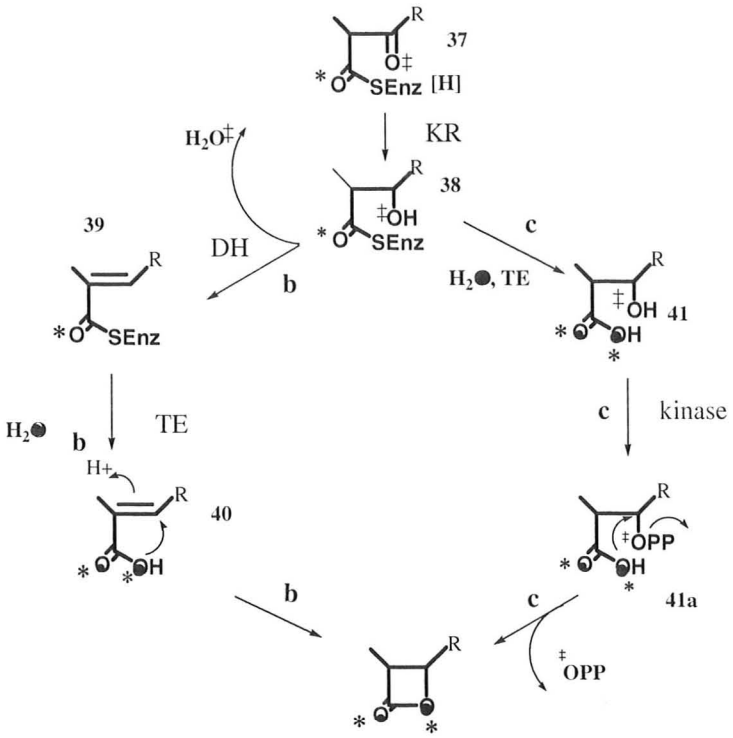
According to Scheme 2.2, the intact C-O bond between C-3 and the ring oxygen supports mechanisms **a**, **d** and **e**, while mechanisms **b**, **c** and **f** require a different origin. Thus pathways **b**, **c** (Scheme 2.3) and **f** can be excluded as possible mechanisms for the formation of the β-lactone moiety. In these mechanisms, the C-O bond at C3 is cleaved during biosynthesis.



Scheme 2.2: Putative Mechanisms for the Formation of the β-Lactone Ring in Ebelactone A

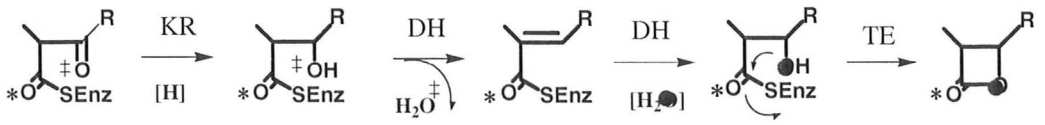
In mechanism **b**, the intermediate **37** is reduced to **38,** then dehydrated to give intermediate **39**. In this step the ^{13}C-^{18}O bond at C-3 is cleaved and the ^{18}O is lost from the pathway. Although the ring oxygen is labelled with ^{18}O in this mechanism, it is the C(=O)-O bond that is intact, rather than the C-3-O bond (scheme 2.3).

Similarly, in mechanism **c**, intermediate **38** is directly cleaved as a hydroxy acid (**41**) by hydrolytic TE. This is then phosphorylated to give **41a**, which cyclizes by a mechanism similar to alcohol activation, in which the ^{13}C-3-^{18}O bond is cleaved as the phosphate group is lost from the molecule (Scheme 2.3).



Scheme 2.3: Mechanisms b and c

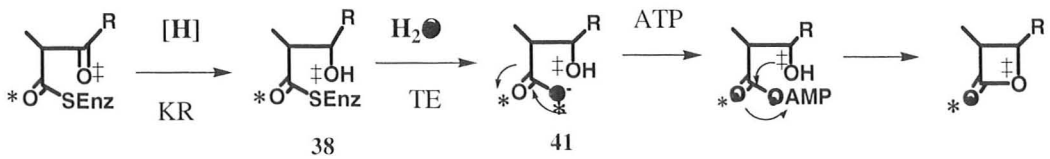
Likewise, oxygen exchange as proposed for lipstatin in mechanism **f** does not occur in biosynthesis of **15b**.[48] This again requires a dehydration-rehydration mechanism which implies the breaking of the ^{13}C-^{18}O-3 bond (Section 1.3.3). This is not consistent with the observed origin of oxygen at C-3 (Scheme 2.4).



Scheme 2.4: Mechanism f

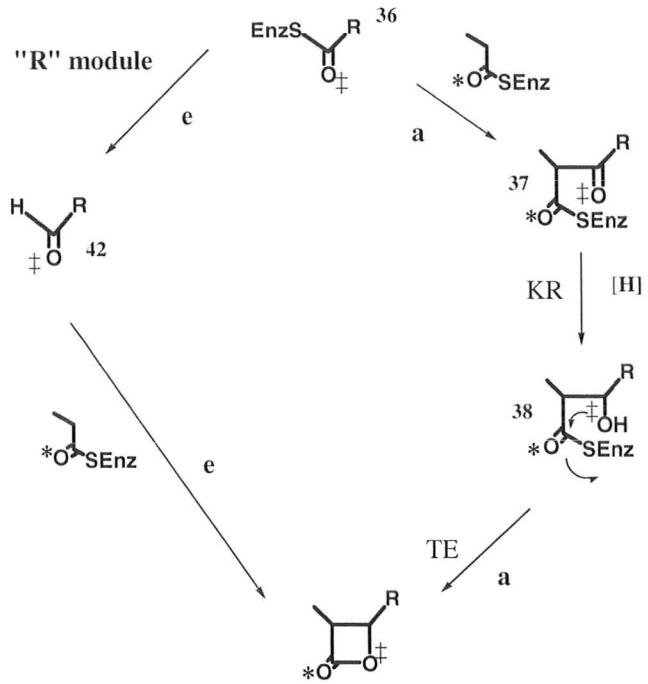
The remaining mechanisms (**a, d** and **e**) are all consistent with the qualitative results obtained, since each atom has the same biosynthetic origin in all cases. This suggests that the ring formation takes place by attack of a hydroxyl group at C-3 of the polyketide onto the terminal carbonyl group but not by the attack of the carboxylate onto the C-3 carbon of the polyketide.

Further, we observed an essentially equal level of oxygen-18 enrichment at C-1, when compared to C-3, C-9 and C-11 (Table 2.3). This result indicates that a free carboxylic acid (e.g. **41**) cannot be involved in the biosynthetic pathway, because hydrolysis of an intermediate acyl-enzyme species (e.g. **38**) would require incorporation of unlabelled water (Scheme 2.5). Cyclization of the ensuing acid would then result in loss of one-half of the labelled oxygen atoms at the C=O group. Hence, pathway **d** can also be excluded.



Scheme 2.5: Mechanism d

Therefore, it can be concluded that the lactone ring of ebelactone is either formed by direct displacement of the acyl intermediate while still attached to the PKS, thereby releasing the cyclized product (mechanism **a**) (Scheme. 2.6), or through a route similar to the tandem aldol lactonization (TAL) (**e**) proposed in the biosynthesis of salinosporamides.[35] This result is in agreement with previous findings in our group on the biosynthesis of F244,[46] suggesting that both fungal and bacterial β-lactones are made by similar mechanisms.



Scheme 2.6: Mechanisms a and e

2.8 Multiple Incorporation

In addition to the large peaks shown in Fig. 2.8, lower intensity peaks were observed at many of the ^{13}C labelled positions in **15b**. These peaks were consistent with incorporation of multiple labelled propionate units at adjacent sites due to the high level of incorporation, giving rise to long-range ^{13}C-^{13}C couplings. Thus, two-bond couplings between C-11 and C-9, C-9 and C-7, C-7 and C-5 and C-5 and C-3 account for all the observed minor peaks at these positions. In addition, a two-bond ^{13}C-^{13}C coupling of 9.3 Hz ($^{2}J_{c\text{-}c}$) was observed between the C-1 and C-3 positions (Fig. 2.10). This unusually large coupling constant is fully consistent with literature values of $^{2}J_{13C\text{-}13C}$ for cyclobutanone,[85] and can be explained by the two separate 2-bond coupling paths across

the lactone ring. Fig. 2.10 shows all the possible isotope labelling patterns for the lactone ring of **15b**, and NMR signals assigned to each.

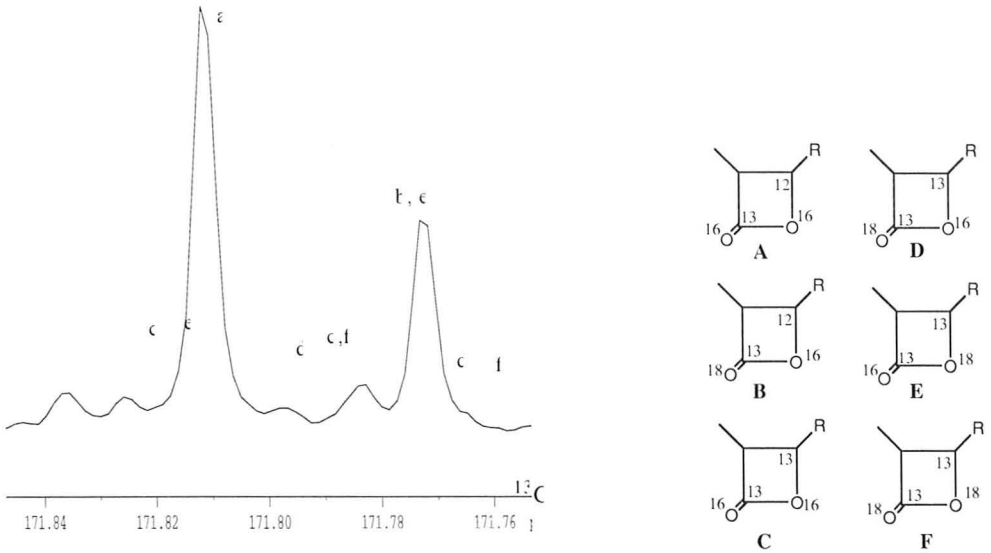


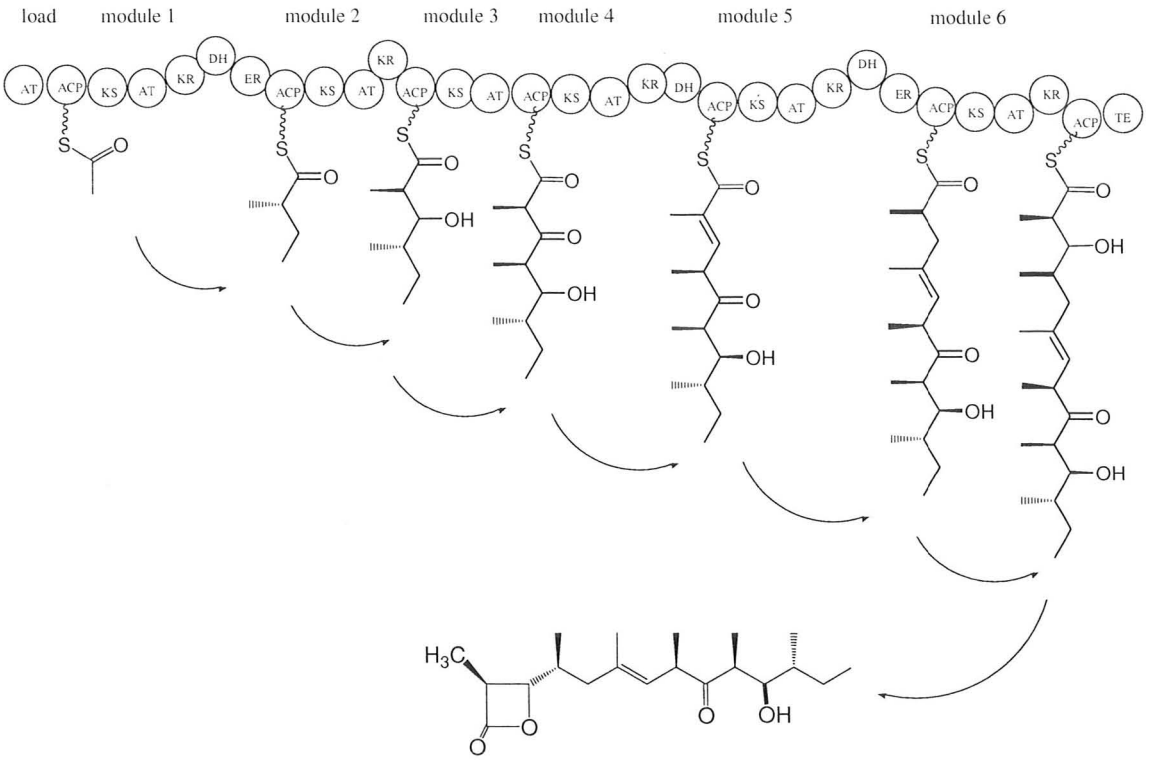
Fig 2.10: Possible isotope labelling patterns of the β-lactone ring in ebelactone A. Isotopomers A, B give rise to the peak and the isotope shift at C-1 in the ^{13}C NMR spectrum (a, b respectively), while isotopomers C-F give coupled signals, labelled c-f respectively on the spectrum.

2.9 Conclusion

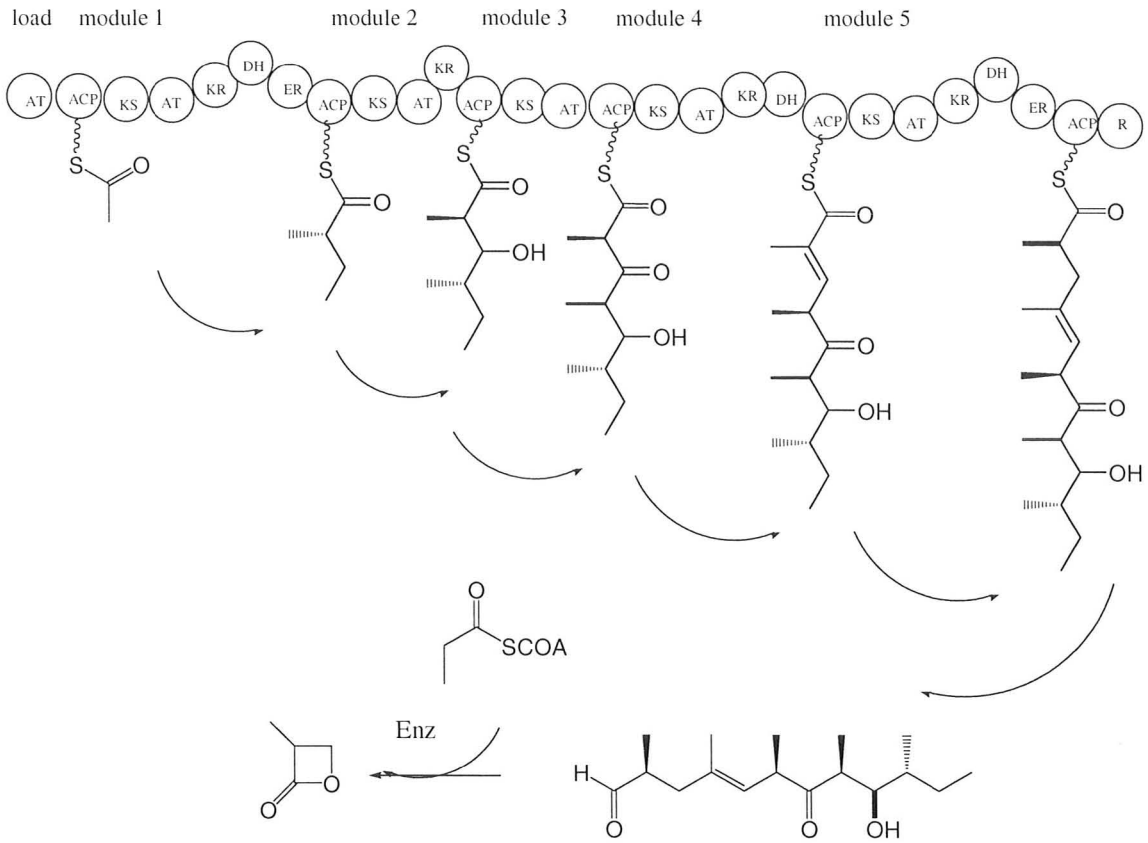
From a wide range of mechanisms, only **a** and **e** are consistent with the ^{18}O labelling data presented here. Since mechanisms **a** and **e** are indistinguishable through isotope labelling, it is clear that in order to further understand the assembly of the β-lactone ring, exploration of the protein(s) which catalyze ring formation is needed.

According to mechanism **a**, TE, which cyclises the four membered lactone while cleaving it from the PKS is involved in the final step of the biosynthesis. Thus, the last

dependent "R" domain, which is responsible for the reductive cleavage of the hexaketide thioester to the corresponding aldehyde, will be present.[86] Schematic representations of the gene clusters for mechanisms **a** and **e** are shown in Schemes 2.7 and 2.8.



Scheme 2.7: Proposed Domain Organization of Ebelactone A PKS ; Mechanism a



Scheme 2.8: Proposed Domain Organization of Ebelactone A PKS; Mechanism e

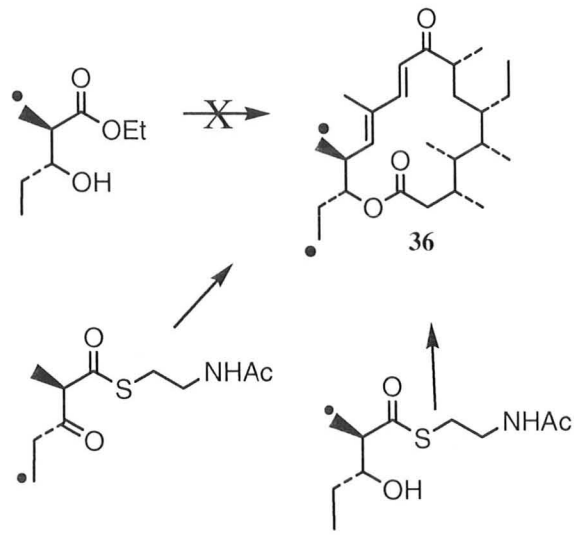
Experiments involving other labelled precursors involved in advanced stages of the biosynthesis to test TE involvement and identification of the protein(s) that catalyze lactonization with a view to understanding the nature of ring formation will be discussed in Chapter 3.

Chapter 3

STUDIES ON THE CYCLIZATION MECHANISM OF β-LACTONES USING ADVANCED PRECURSORS

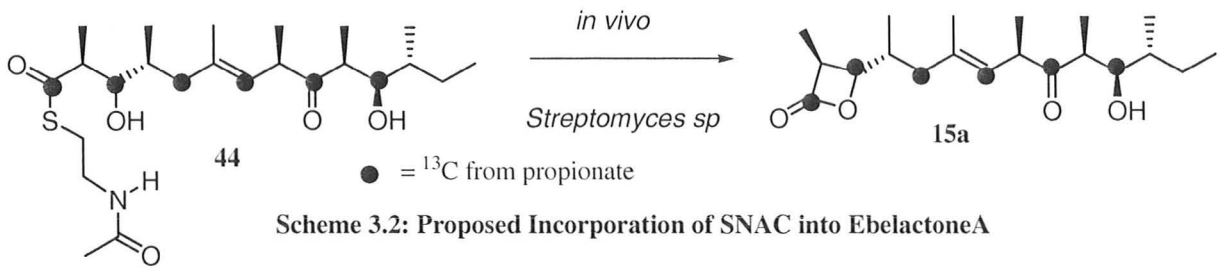
Labelling experiments using sodium [1-^{13}C,$^{18}O_2$]propionate supported β-lactone ring formation by attack of a β-hydroxy-group onto the carbonyl moiety of an acyclic precursor, and excluded other putative processes. To further explore the mechanism, we planned to use labelled precursors which could be involved in later stages of the biosynthesis.

Use of labelled precursors other than acetates and propionates has often not been successful due to the permeability of these precursors through cell membranes.[87] Experiments with advanced precursors such as carboxylic acid and acyl esters showed no incorporation and in some cases some of these substrates were shown to degrade before incorporation.[88] However *N*-acetylcysteamine (SNAC) type thioesters have been successfully incorporated into many polyketide intermediates, such as tylactone (**48**)[88] (Scheme 3.1) and erythromycin A.[89]



Scheme 3.1: Incorporation of SNAC Precursors in to Tylactone (48)

To further test the mechanism of direct cyclization in β-lactones and to show the mediation of this reaction by a TE, we decided to use a SNAC derivative of ebelactone. Experiments with dissected thioester intermediates on macrolides such as erythromycin have been successfully conducted to show that TE, which is present at the end of the PKS cleaves the fully grown polyketide chain from the protein and catalyzes the cyclization to form the large ring lactone.[90] Genetic studies on the erythromycin PKS gene, where the TE domain was moved, for example, to the end of the fourth or fifth module resulted in a tetraketide or pentaketide lactone respectively, confirming the involvement of TE in the cyclization.[91,92] Based on these results, we anticipated the incorporation of the SNAC derivative **44** into the cultures of *Streptomyces* sp. MG7-G1 to give ebelactone as the cyclized product (Scheme 3.2). We also expect to find a TE domain at the end of the ebelactone PKS during ongoing genetic studies.

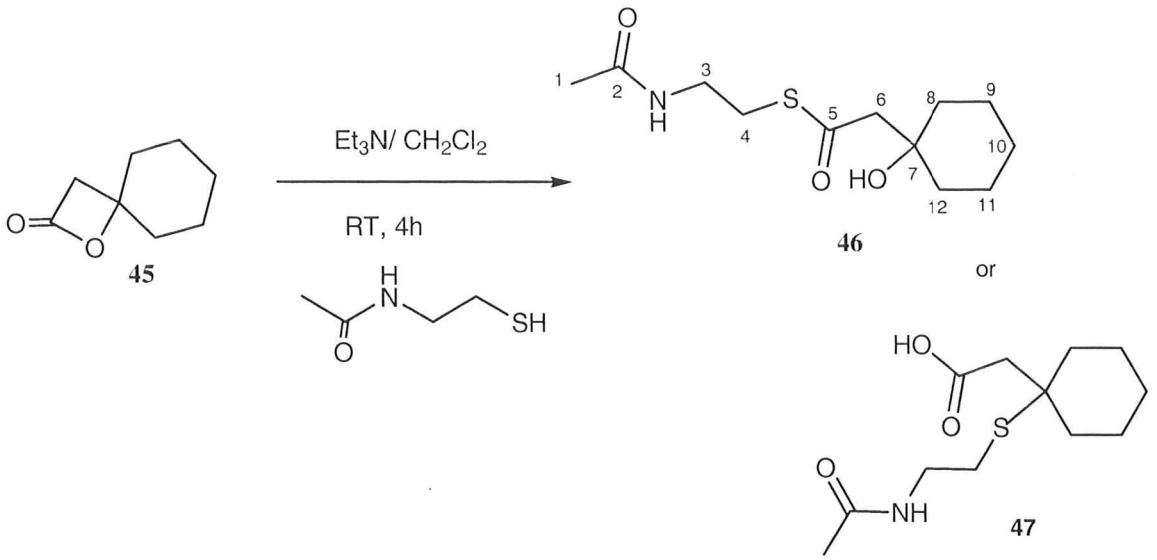


Scheme 3.2: Proposed Incorporation of SNAC into EbelactoneA

3.1 Synthesis of SNAC Derivatives

We envisioned that the preparation of the intermediate **44** could be possible by a ring opening reaction of ebelactone with an appropriate nucleophile. Use of a similar strategy was adapted by Corey *et al.* in the preparation lactacystin:[93] the β-lactone omuralide was synthesised and then the lactone ring was opened at the oxygen-acyl bond using *N*-acetylcysteine as nucleophile. This reaction was first tested on a model compound prepared in our laboratory, 4-spirocyclohexyloxetan-2-one (**45**) (Scheme 3.3).

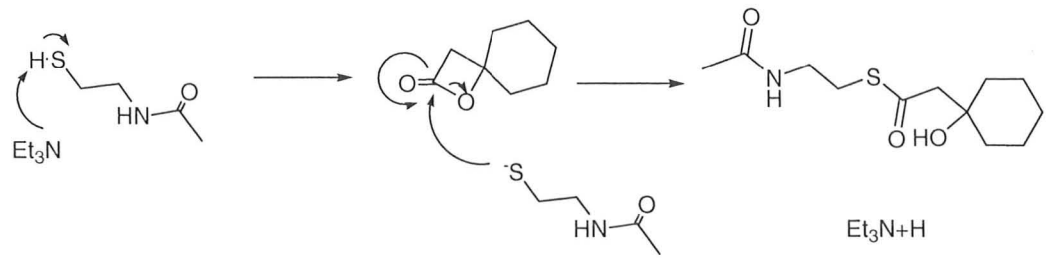
3.2 Preparation of 4-Spirocyclohexyloxetan-2-one-SNAC derivative



Scheme 3.3: Preparation of 46

Reaction of 4-spirocycyclohexyloxetan-2-one (**45**) with *N*-acetylcysteamine and triethylamine (Et_3N) in dichloromethane at rt for 4 h gave a product in 35% yield after purification by column chromatography using methanol and chloroform.

Thiols are soft nucleophiles. Therefore, we can expect them to react at soft electrophilic sites better than would hard nucleophiles. β-Lactones have two electrophilic sites. The lactone carbonyl carbon is highly selective, due to the charged resonance structure. The β-carbon bonded to oxygen behaves as a soft electrophile (Section 1.6.1). Therefore, we can expect the soft thiol to attack the β-carbon as soft nucleophiles prefer to react with soft electrophiles due to similar energies of the relevent bonding orbitals, giving **47**. However, in this case we desired the thiol to attack the carbonyl carbon leading to the product **46**. Therefore, it was important to establish the site of nucleophilic attack.



Scheme 3.4: Mechanism of Reaction of SNAC with 4-Spirocyclohexyloxetan-2-one

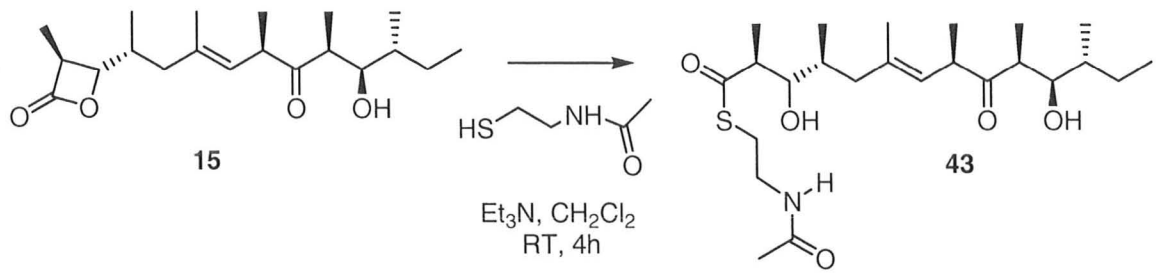
The high resolution ESI+ mass spectrum of the product showed a peak at m/z 260 ($[M+H]^+$) consistent with the molecular formula of $C_{12}H_{21}O_3NS$. The IR spectrum of the compound showed an OH stretch at 3294 cm^{-1} and a thioester/carbonyl band at 1653 cm^{-1} indicating the successful opening of the lactone ring. The proton NMR spectrum showed a broad singlet at 5.91 ppm due to the NH proton, and sharp singlets at 2.74 (2H) ppm and 1.96 (3H) ppm corresponding to the methylene protons at the C6 position and the methyl group respectively (See Scheme 3.3 for numbering). The double triplet at 3.43 (2H) ppm and the triplet at 3.03 (2H) ppm were due to the CH_2-CH_2 group of the cysteamine residue. The rest of the signals, which were unresolved at 1.74-1.24 ppm (11H) were due to the cyclohexyl ring of the compound and due to the hydroxyl proton. The ^{13}C NMR spectrum of the compound contained a peak at 200.12 ppm corresponding to the newly formed thioester bond and a signal due to the oxygenated carbon (C7) at 71.52 ppm. The rest of the peaks in the spectrum were all consistent with the structure **46**; thus the compound was assigned as the desired 4-spirocyclohexyloxetan-2-one-SNAC derivative. Table 3.1 shows the ^1H and ^{13}C NMR assignments of **46**.

Table 3.1: Proton and ^{13}C NMR Assignments of 46

Assignment	Proton	Carbon
1	1.96 (s, 3H)	23.38
2	-	170.95
NH	5.91 (s, 1H)	-
3	3.43 (dt, J = 6.0, 6.2 Hz, 2H)	39.50
4	3.03 (t, J = 6.0 Hz, 2H)	37.56
5	-	200.12
6	2.74 (s, 2H)	54.68
7	1.74 (s, 1H, OH)	71.52
8		28.99
9		25.61
10	1.24-1.64 (m, 10H)	22.12
11		25.61
12		28.99

3.3 Preparation of Ebelactone SNAC Derivative

Following the successful conversion of **45** to **46**, we used the same conditions on ebelactone A to access the precursor **43** (Scheme 3.5).

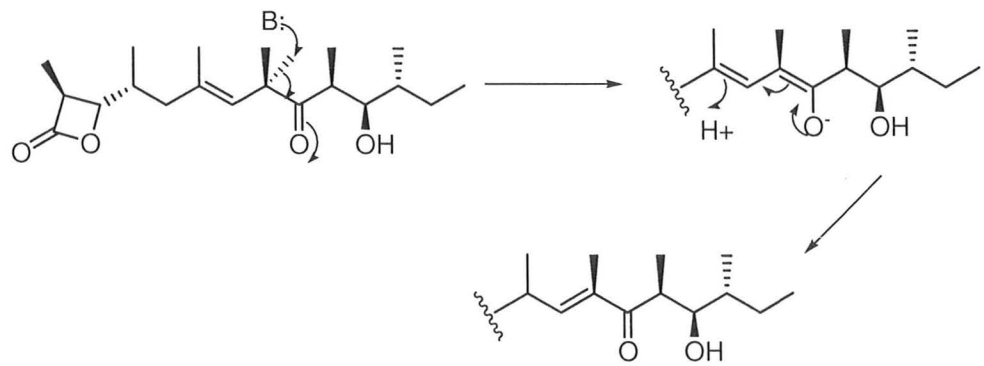


Scheme 3.5: Preparation of 43

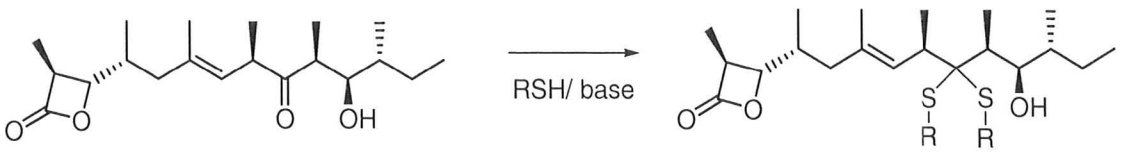
Ebelactone A (4.2 mg) was used under similar conditions as described above, to yield 50% of a product as colourless crystals. The mechanism of this reaction can be expected to proceed via a similar route to that shown in Scheme 3.4. However, the long side chain substituent at C3 with several functionalities is potentially susceptible to other reactions under these reaction conditions. For example:

The olefin functionality at C6-C7 can isomerise to the C7-C8 isomer, which is now an α, β unsaturated system (Scheme 3.6).

The ketone functionality at C9 can react with the nucleophile yielding a dithiane (Scheme 3.7).



Scheme 3.6: Tautomeric Equibriation of Unsaturated Ketone α, γ to α, β Unsaturated Isomer



Scheme 3.7 : Dithiane Formation

However, the analysis of the product confirms that these functionalities are untouched and unreactive under these conditions. The ESI+ mass spectrum showed a peak at m/z 458 ($[M+H]^+$) consistent with the formula of $C_{24}H_{43}O_5SN$ (Fig. 3.1). The IR spectrum showed a broad band at 3323 cm^{-1} corresponding to OH groups and absorptions at 1660 and 1690 cm^{-1} corresponding to the ketone and thioester functionalities.

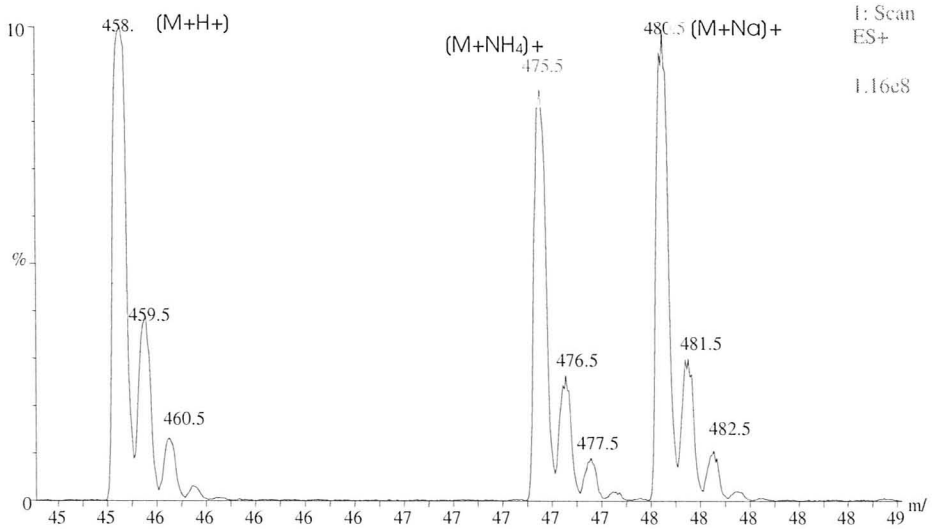
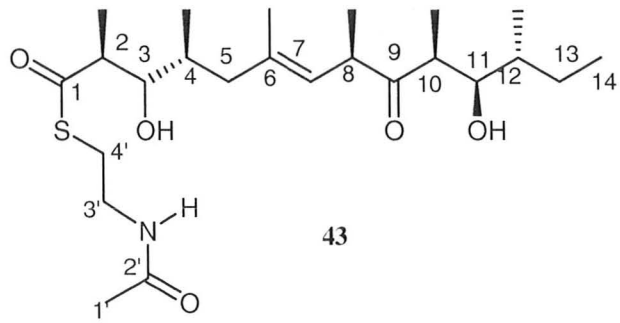


Fig: 3.1 ESI+ MS of 43

The proton NMR spectrum of the product showed a broad singlet at 5.78 ppm due to NH, and a doublet at 5.00 ppm, essentially unchanged from its position in ebelactone, thus confirming the presence of the olefin group. The assignment of the rest of the proton spectrum was complicated and was done with the help of 2D spectra (COSY, HMBC and HSQC; Fig. 3.5, 3.6, 3.7 respectively). The side chain resonances of the product were similar to those of ebelactone A, and the cysteamine side chain resonances resembled those of the NAC-SH thiol starting material, but the ring resonances showed some changes as we anticipated. The NH proton at 5.78 ppm showed a correlation to the signal at 3.45 ppm on COSY experiments. Therefore the signal at 3.45 ppm (t) was assigned as 3'-H. In addition, the 3.45 ppm signal also showed correlation to the triplet at 2.95 ppm in the COSY which was then assigned to 4'-H.



43

Fig: 3.2: Ebelactone SNAC Derivative

The ^{13}C NMR spectrum of the compound contained a peak at 204.24 ppm corresponding to the newly formed thioester bond and the peaks at 78.07 and 74.01 ppm were due to hydroxyl substitution at C3 and C11 respectively. The peak at 78.07 ppm showed correlation to the proton signal at 3.42 ppm in the HSQC spectrum and therefore the latter was assigned as 3-H. The HMBC spectrum of the compound displayed

correlation between the carbon signals at 125.21 (C7) and 136.49 (C6) to the proton doublet at 2.36 ppm, which enabled the assignment of 5_B-H. The 5_B-H proton signal showed COSY correlation to the peaks at 3.42 (3-H) and 2.54 ppm, which was assigned to 4-H accordingly. The 4-H signal showed HMBC correlation to 78.07 (C3) peak, which in turn was correlated to the proton signals at 1.28 ppm (2-CH_3) and at 0.86 ppm (4-CH_3). The rest of the peaks in the proton NMR spectrum of **39** were assigned in comparison to those of ebelactone A and the carbon-13 NMR peaks were assigned using HSQC and HMBC correlations. The proton and ^{13}C assignments of **43** are shown in Table 3.2.

Table 3.2: Proton and ^{13}C NMR Assignments of 43

Assignment	Proton	Carbon
1	-	204.24
2	2.93 (dq, *J* 7.5, 4.0 Hz, 1H)	50.32
2-CH_3	1.28 (d, *J* 7.5 Hz, 3H)	14.34
3	3.42 (dd, *J* 8.0, 4.0 Hz, 1H)	78.07
4	2.54 (d, *J* 8.0 Hz, 1H)	40.75
4-CH_3	0.86 (d, *J* 6.8 Hz, 3H)	10.38
5_A 5_B	1.76 (dd, *J* 13.3, 10 Hz, 1H) 2.36 (d, *J* 10.0 Hz, 1H)	41.92
6	-	136.49
6-CH_3	1.72 (s, 3H)	16.08
7	5.00 (d, *J* 10.0 Hz, 1H)	125.21

8	3.56 (dq, *J* 8.2, 6.7 Hz, 1H)	45.01
8-CH_3	1.12 (d, *J* 6.7 Hz, 3H)	15.81
9	-	217.38
10	2.85 (dq, *J* 1.8, 7.2 Hz, 1H)	45.01
10-CH_3	1.09 (d, *J* 7.2 Hz, 3H)	8.89
11	3.44 (m, 1H)	74.01
11-OH	3.13 (s, 1H)	-
12	1.44 (m, 1H)	33.99
12-CH_3	0.78 (d, *J* 6.8 Hz, 3H)	15.50
13_A 13_B	1.74 (dq, *J* 7.6, 2.6 Hz, 2H) 1.25 (m, 1H)	24.46
14	0.88 (t, *J* 7.6 Hz, 3H)	13.64
1'	1.96 (s, 3H)	22.75
2'	-	169.87
NH	5.78 (s, 1H)	-
3'	3.45 (t, *J* 6.2 Hz, 2H)	36.05
4'	2.95 (t, *J* 6.2 Hz, 2H)	28.83

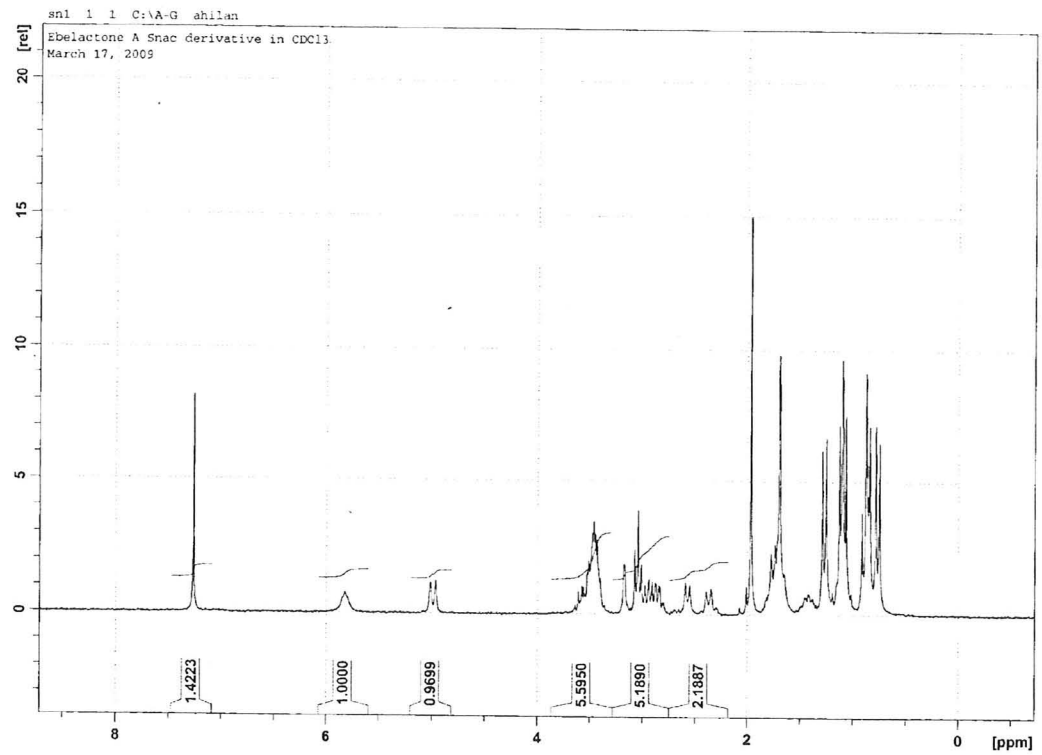


Fig. 3.3: Proton NMR Spectrum of 43

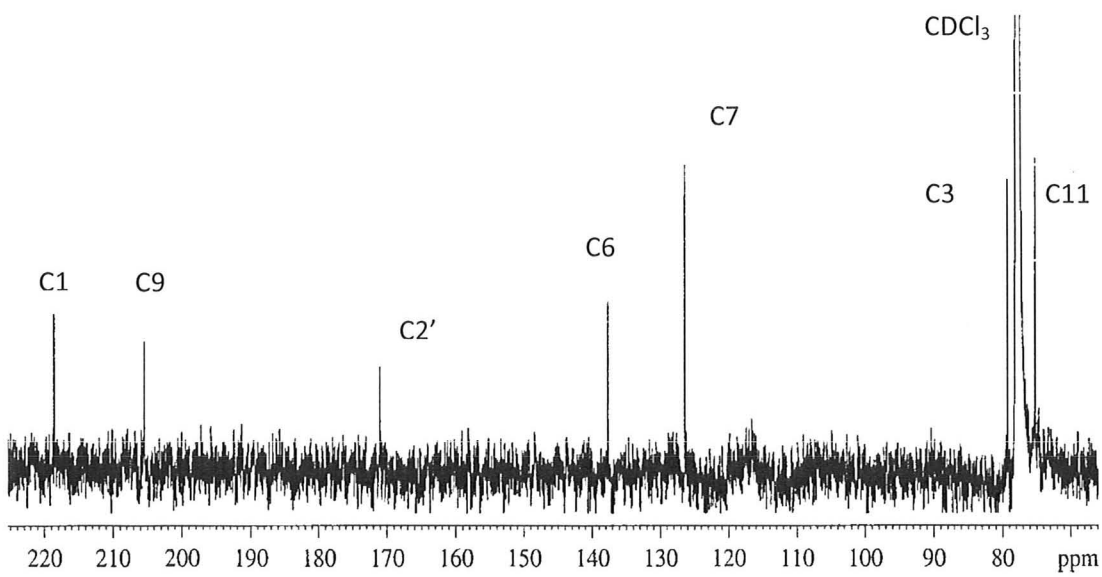


Fig 3.4: Part of ^{13}C Spectrum of 43 in $CDCl_3$ at 150 MHz

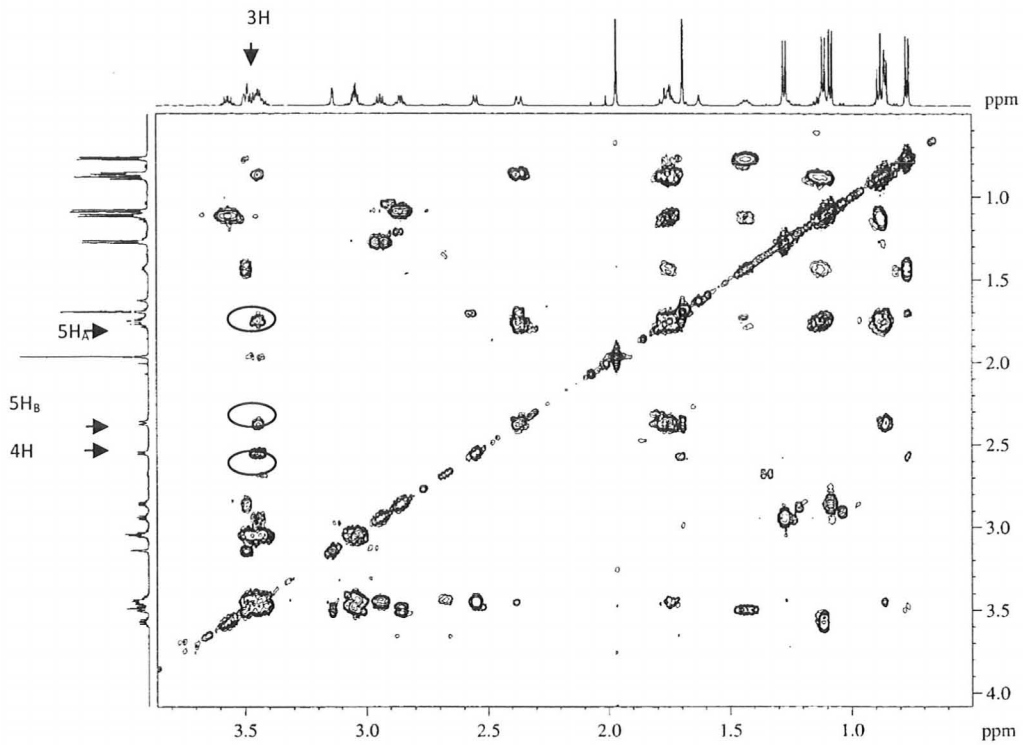


Fig: 3.5: ^{1}H-^{1}H COSY Spectrum of 43 Showing Correlations to 3H from 4H and 5H

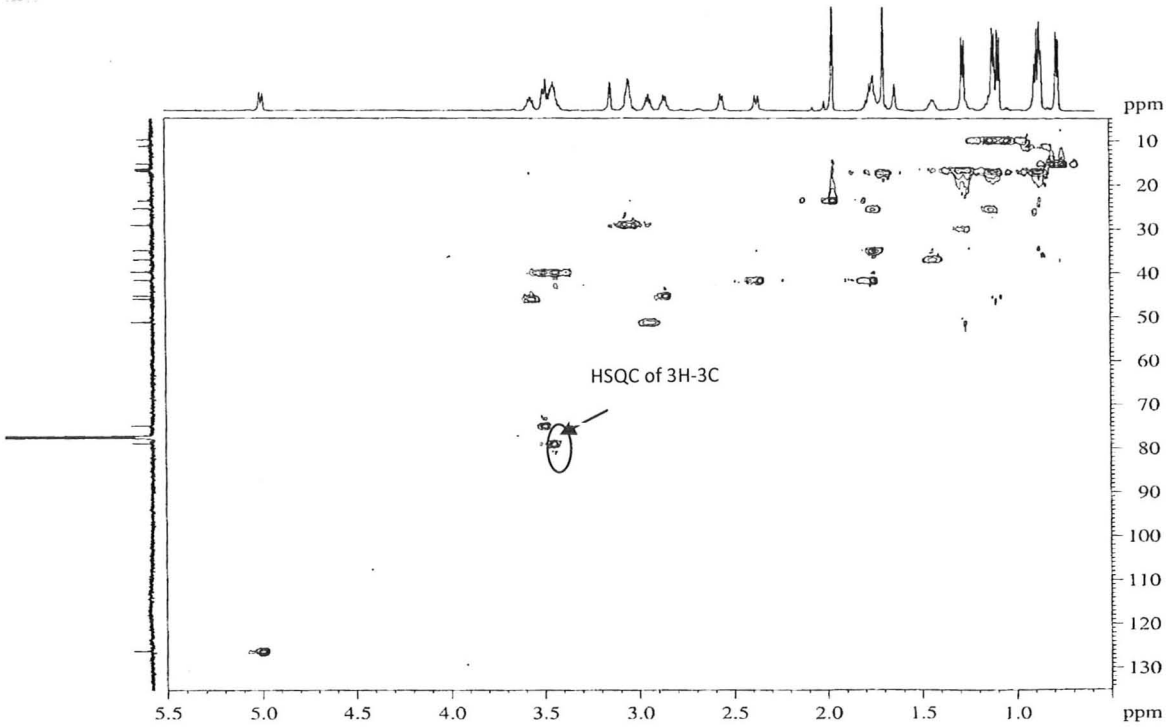


Fig 3.6: HSQC Spectrum of 43

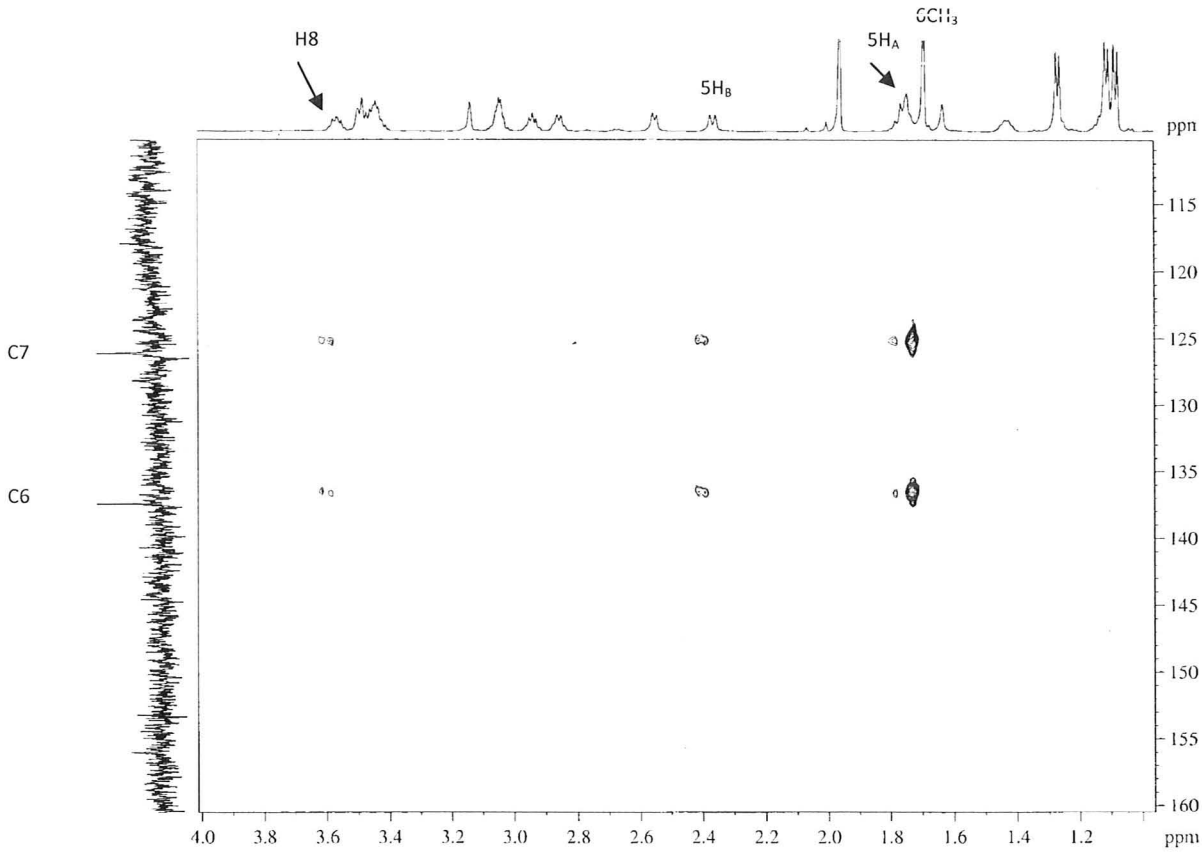
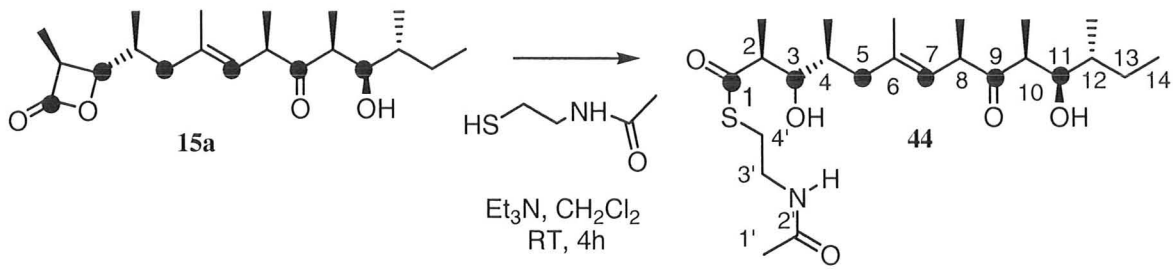


Fig. 3.7: Expanded HMBC Spectrum of 43 Showing Correlations Between C6, C7 to 5_AH, 5_BH, 8H and $6CH_3$

The low percentage yield obtained was due the oxidation of the starting material, *N*-acetylcysteamine to the corresponding disulphide during reaction. The disulphide also caused difficulty during separation of the product. This problem was overcome by stirring the reactants under a constant flow of nitrogen, although this seemed to dry the solvent and re-addition of dichloromethane was required. An excess of N-acetyl cysteamine was also used. The yield obtained was thereby increased to approx. 65%.

3.4 Preparation of Ebelactone A SNAC Derivative Labelled with ^{13}C



Scheme 3.8: Preparation of 44

Following the successful synthesis of the required product in an acceptable yield, the next stage was the preparation of the labelled SNAC derivative **44** (Scheme 3.8). Thus 12 mg of ^{13}C labelled ebelactone A prepared from propionate (Chapter 2) was used under similar conditions to those described above, to give 9.5 mg of labelled product (63% yield). The proton NMR spectrum of the compound was similar to that of **43**. The ^{13}C NMR spectrum showed enrichments at C1, C3, C5, C7, C9 and C11 positions as anticipated (Fig 3.9). The ESI+ MS of **44** contained peaks at m/z 458-463 ($[M+H]^+$-$[M+H+6]^+$) confirming the successful opening of the ring in labelled ebelactone A. The ESI+ MS of the product is shown in Fig. 3.8.

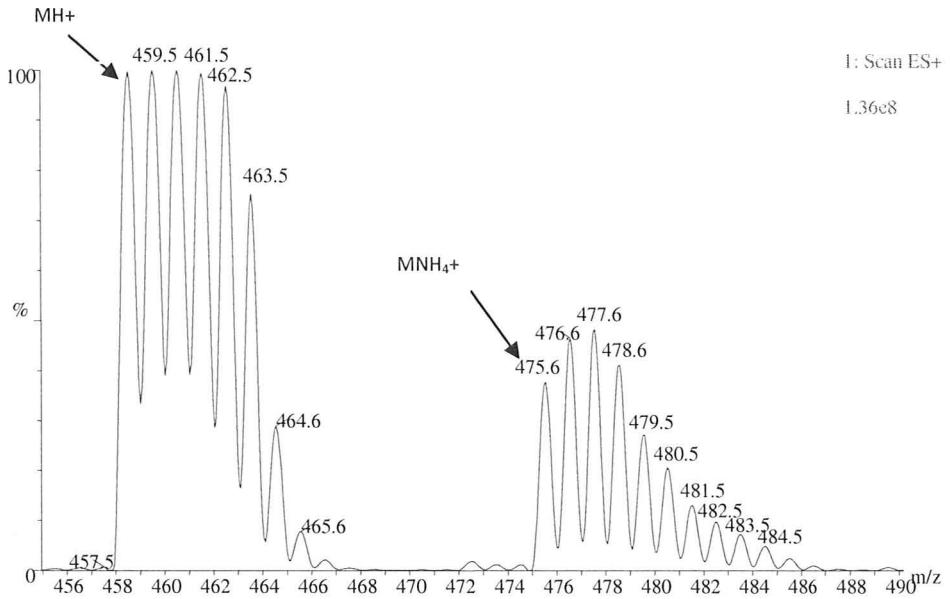


Fig: 3.8 ESI+ MS of 44

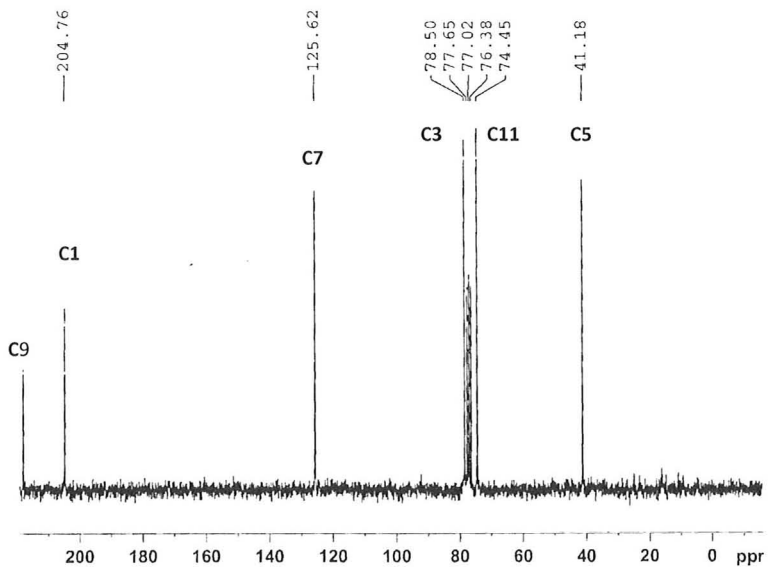


Fig: 3.9 ^{13}C NMR Spectrum of 44 (50 MHz, $CDCl_3$) Showing Enriched Carbon Signals C1, C3, C5, C7, C9, C11

Following the successful preparation of the labelled SNAC derivative of **15a**, we turned to the incorporation experiments to track cyclization. HPLC-MS was used to monitor conversion of **44** to labelled ebelactone A (**15a).** The prepared SNAC derivative (**44**) had a retention time of 3.1 min whereas ebelactone A (**15a**) had a retention time of 5.2 min, and thus these compounds were readily separable on a C_{18} column (Fig. 3.10).

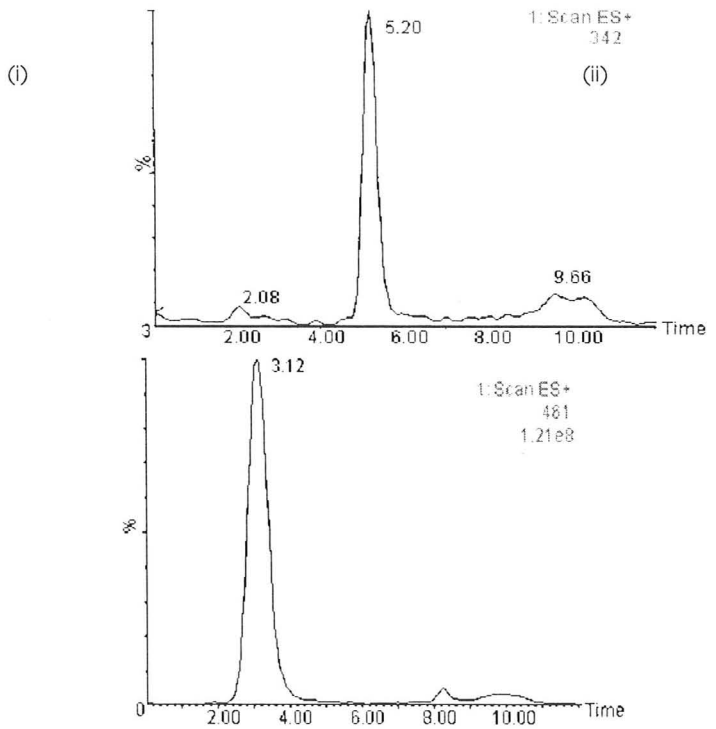


Fig: 3.10 HPLC Chromatograms of Labelled (i) Ebelactone A (15a) (ii) SNAC Derivative (44)

Further, the mass spectra of **44** and **15a** were completely distinguishable, as **44** had molecular ion peaks ranging from 458 to 464 (m/z) whereas **15a** contained peaks from 339 to 345 (Fig. 3.11).

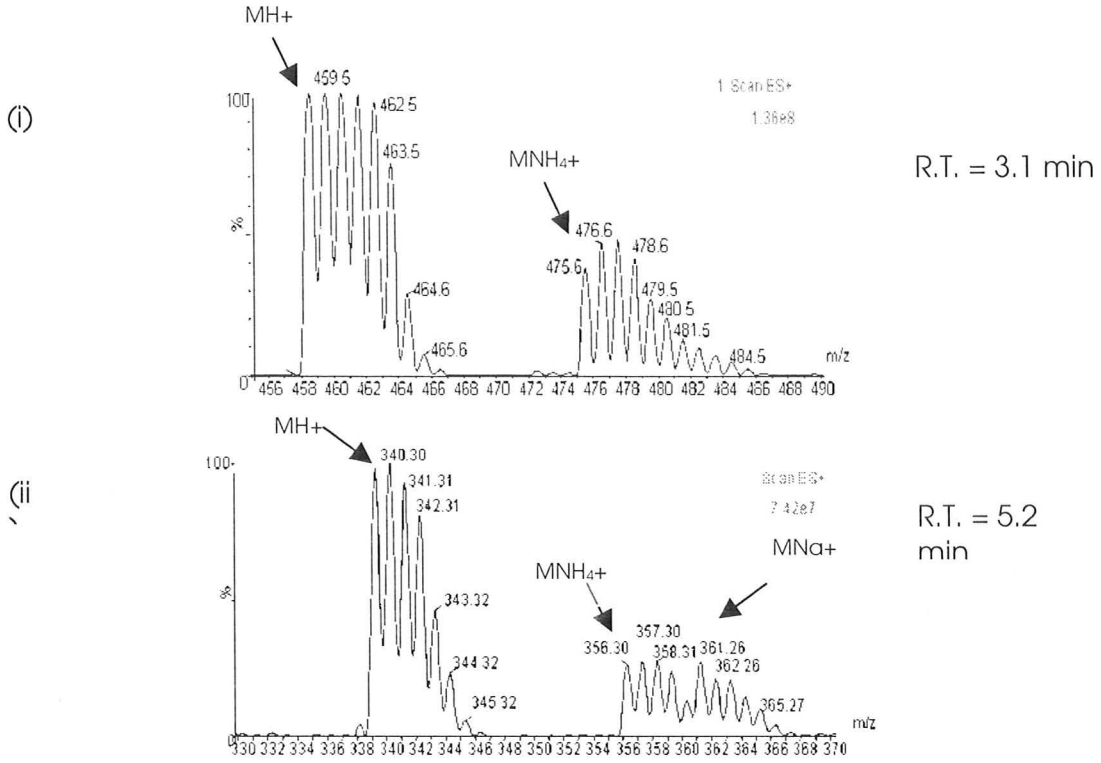


Fig: 3.11: HPLC ESI+ Mass Spectrum of (i) 44 at R.T= 3.1 min (ii) 15a at R.T= 5.2 min

3.6 Experiments with Unlabelled SNAC to Check Stability in Growth Medium

The first experiment conducted was to check the stability of the thioester in aqueous medium. Thioesters are relatively reactive compounds due to the poor overlap of orbitals in C-S bonds as a result of the incompatible size of the relevant orbitals, and therefore thiolates are good leaving groups. In aqueous medium at basic pH, thioesters can be easily hydrolysed by hydroxyl ions to β-hydroxy acids. Unlabelled SNAC derivative **43** was added to sterile media. Aliquots were taken at different times, extracted with ethyl acetate, and screened for the presence of SNAC derivative (m/z 458) and ebelactone A (m/z 339) using LC-MS. After 1 h and 3 h of addition, **43** was still present

in the sample, although **15** was not present at detectable levels. However, after 24 h, **15** was detected in very small quantities (Fig. 3.12).

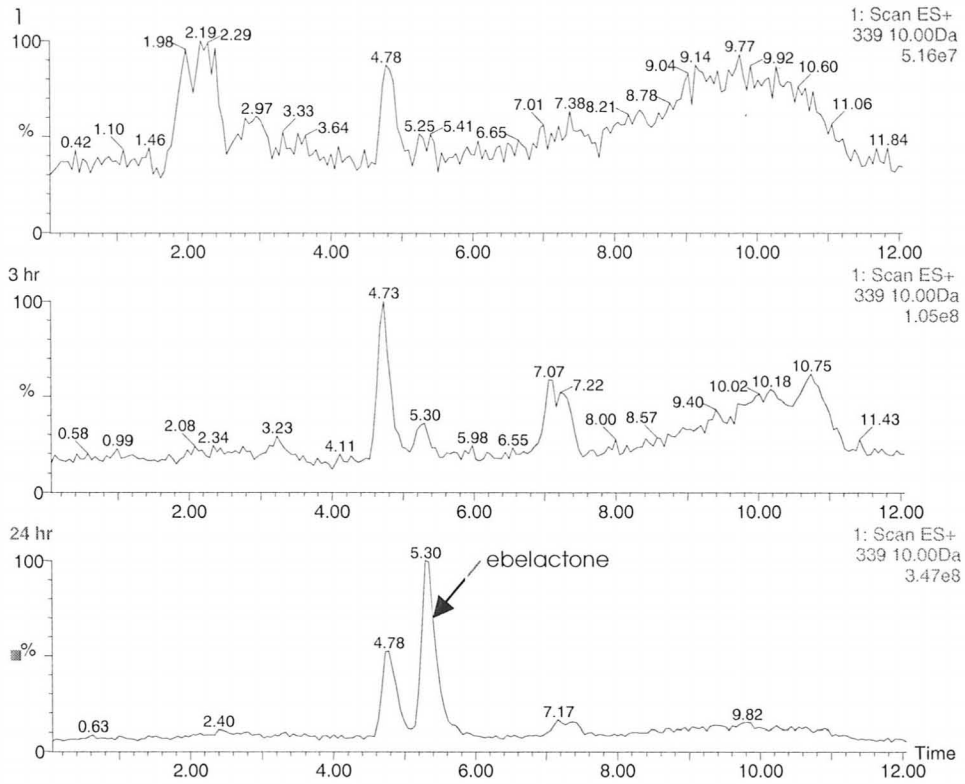


Fig: 3.12 LC-MS Analysis of Media at m/z 339 (After Different Times of Incubation with Precursor)

3.7 Experiment with Labelled SNAC for Incorporation into Whole Cells

Cultures of *S. aburaviensis* were tested for incorporation of precursor by adding labelled derivative **44** at 12 h after inoculation. The LC-MS analysis revealed chemical cyclization in addition to the natural production of unlabelled ebelactone A (Fig. 3.13). The experiment (i) had living cells in culture medium to which, labelled **44** was added. Controls (ii) and (iii) had living cells only (no **44**) and sterile media and labelled **44,** respectively. After incubation for another 12 h the cultures were scanned for the presence

of labelled lactone at m/z 342. Fig. 3.13 shows that no labelled lactone was present in control (ii) but it was present in (i) and (iii).

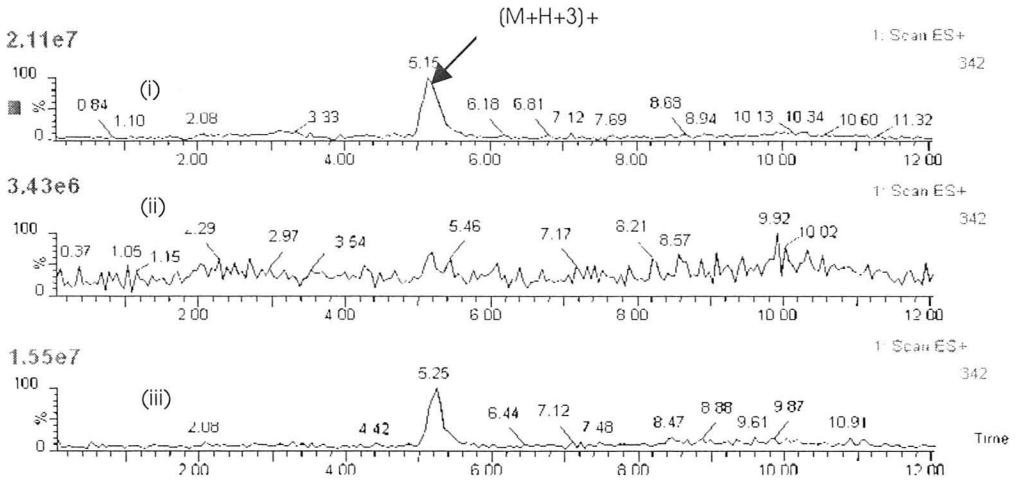


Fig: 3.13 LC-MS Analysis of Cultures at m/z 342 (Labelled Ebelactone MH+)

(i) Media+ cells + labelled SNAC @ 27°C for 24 h
(ii) Media + cells only incubated @ 27°C for 24 h CONTROL
(iii) Sterile media + labelled SNAC @ 27°C for 24 h CONTROL

The ESI+ mass spectra of these samples showed that (i) contained a mixture of labelled and unlabelled lactones as expected. This was due to the natural production of ebelactone by the organism in addition to the cyclization induced by the addition of labelled SNAC precursor. Sample (ii) contained only unlabelled ebelactone produced by the organism naturally, and no labelled lactone was detected as there were no precursors added to this sample.

Surprisingly, sample (iii) showed cyclization even in the absence of any living cells. This result therefore should be due to some chemical cyclization of the added SNAC precursor (**44**).

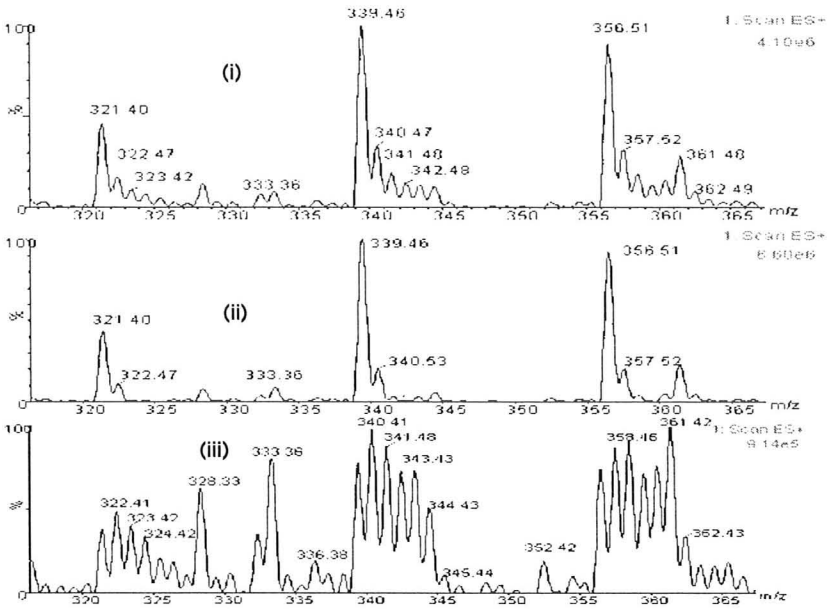
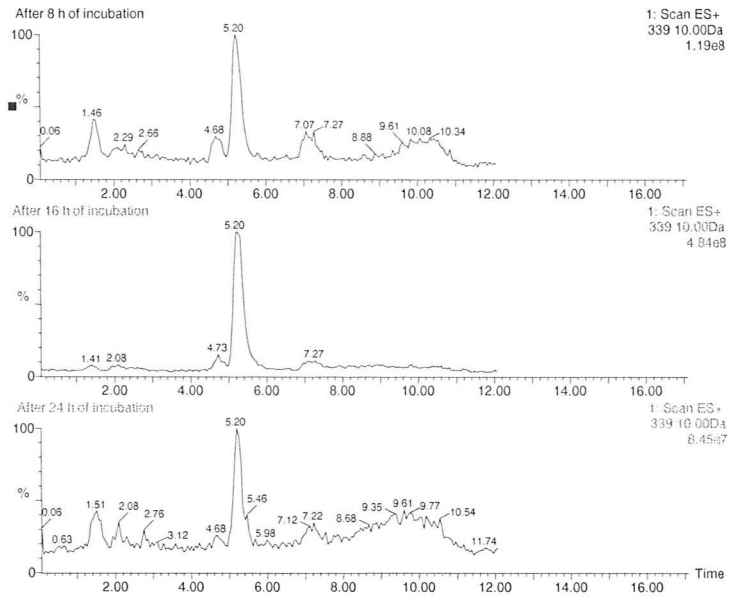


Fig: 3.14 ESI+ MS of Chromatograms Shown in Fig. 3.14.
The M.S. of the Ebelactone Peak at 5.15-5.25 mins are Shown

3.8 Experiments with Labelled SNAC with Time

The next experiment focused on the incubation time and rate of the chemical cyclization. The samples, which comprised cells in media with the addition of labelled precursor, along with two controls, (i) supernatant only (no cells) with labelled SNAC and (ii) cells in media (no SNAC), were incubated for 8, 16 and 24 h intervals. The results (Fig. 3.15) showed that the chemical cyclization could be detected after 8 h of addition of the SNAC and the amount of the cyclized lactone increased with time of incubation, as determined from peak intensities.

a-i



a-ii

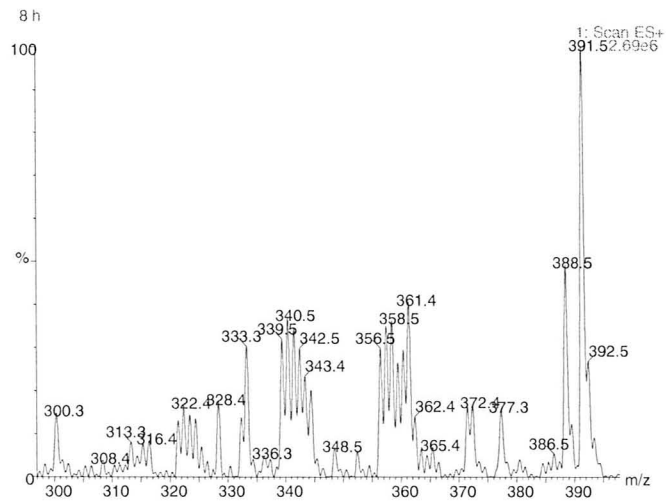
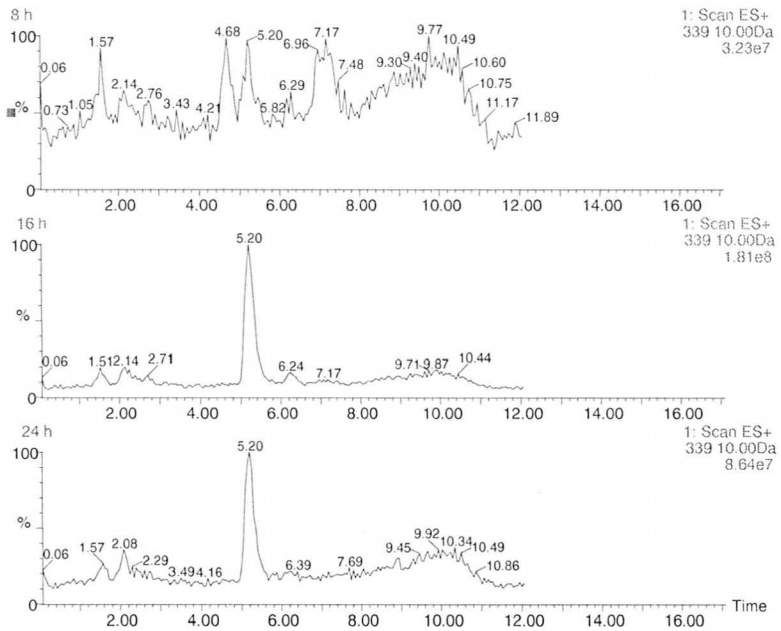


Fig: 3.15 (a-i) HPLC-MS Trace of Supernatant and SNAC with Time (8, 16, 24 h) at m/z 339 (a-ii) LC-MS of a at R.T = 5.20 min. (8 h)

b-i



b-ii

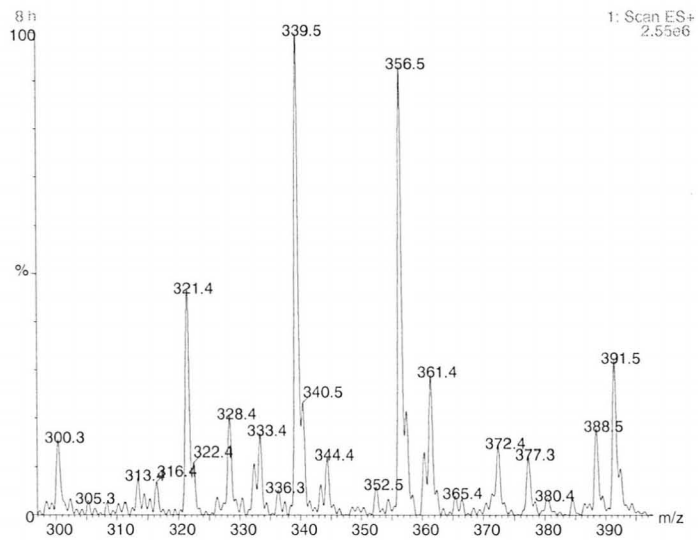
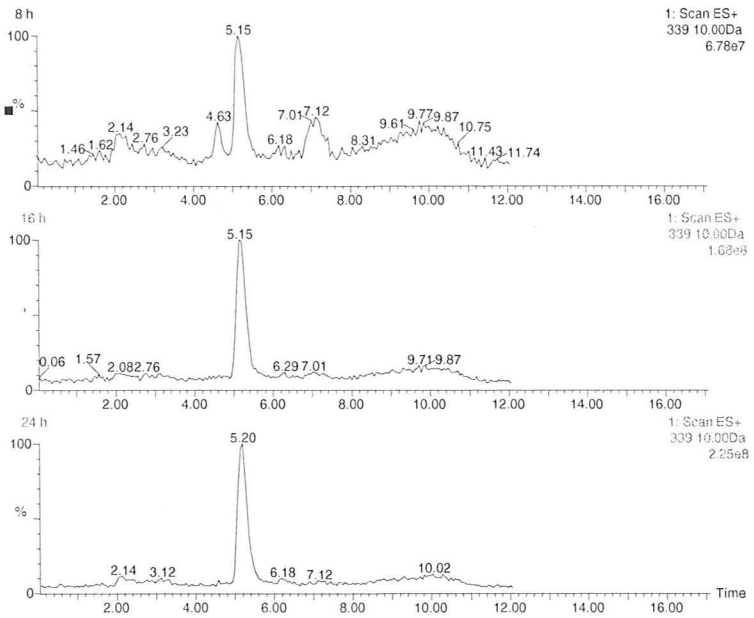


Fig. 3.15 (b-i) HPLC-MS Traces of Cells without SNAC with Time (8, 16, 24 h) (b-ii) LC-MS of b at R.T= 5.20 min. (8 h)

c-i



c-ii

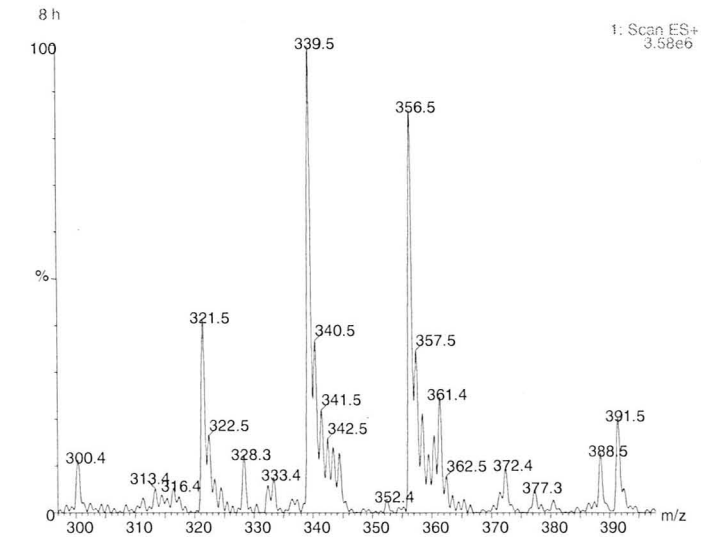


Fig. 3.15 (c-i) HPLC Traces of Cells in Media with SNAC with Time (8, 16, 24 h) (c-ii) LC-MS of c at R.T. 5.20 min. (8 h)

3.9 Experiments to Track Chemical Cyclization in Water/Calcium Carbonate

Since chemical cyclization to give ebelactone from its SNAC derivative was confirmed by the above-mentioned experiments, we wanted to identify the component in

the media which was responsible for this reaction. The basic pH needed for the growth of the organism was due to calcium carbonate and we decided to test the involvement of this component in cyclization. Fig. 3.16 shows the result of the experiment showing the involvement of calcium carbonate. The results show that there is no chemical cyclization detectable in deionised water only. However, the addition of calcium carbonate induced cyclization. After reaction, the mixture was extracted and HPLC-MS was used for analysis at m/z 339 (unlabelled lactone).

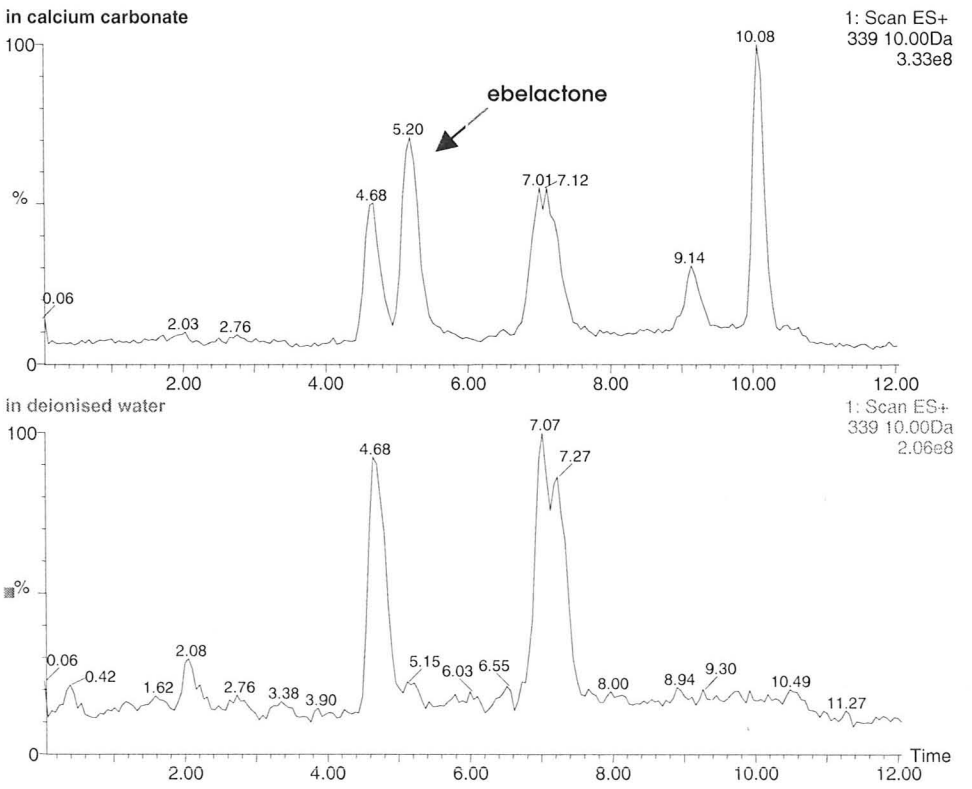


Fig. 3.16 Stability of SNAC Derivative in (a) Calcium Carbonate (b) Water

3.10 Experiments with Cell-Free Extracts

As calcium carbonate and the basic pH of the medium seemed to be causing the background cyclization, we decided to opt for cell-free experiments. HEPES[94] buffer (pH 7.5) was used and the extracts were prepared from *S. aburaviensis* cultures in a bead beater at 4°C. Aliquots of these extracts were incubated with **44** for 1 h at room temperature. But, the results (Fig. 3.17) showed chemical cyclization even under these conditions.

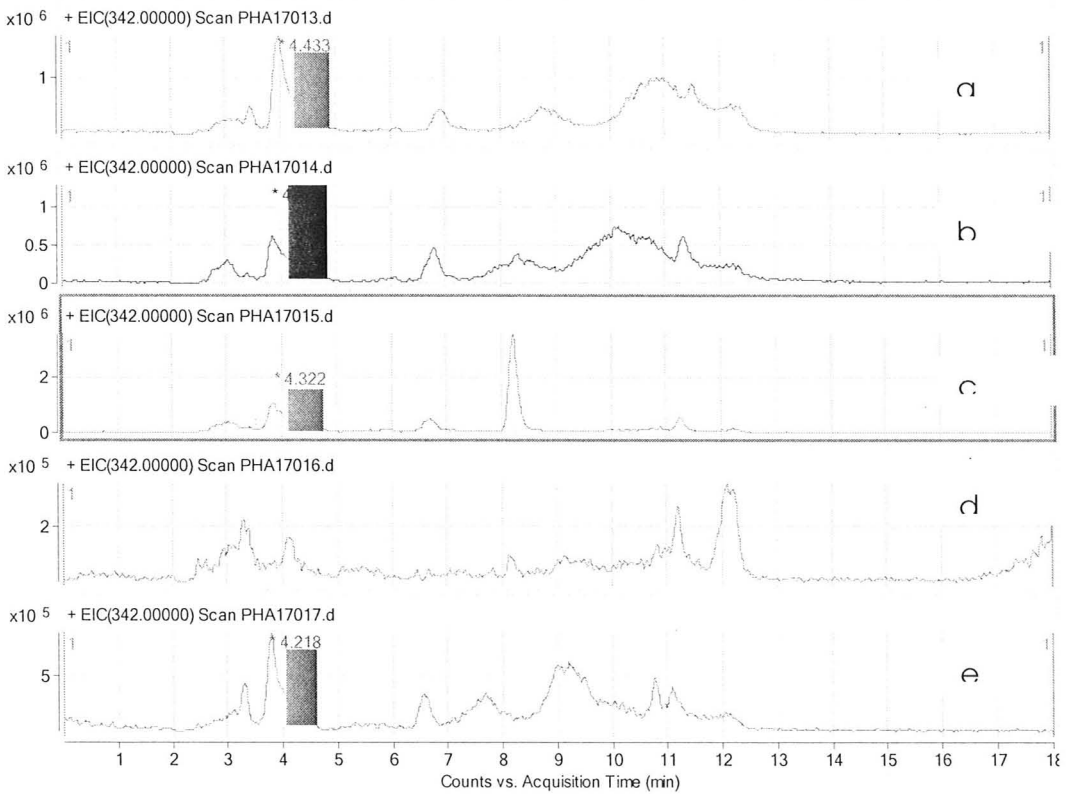
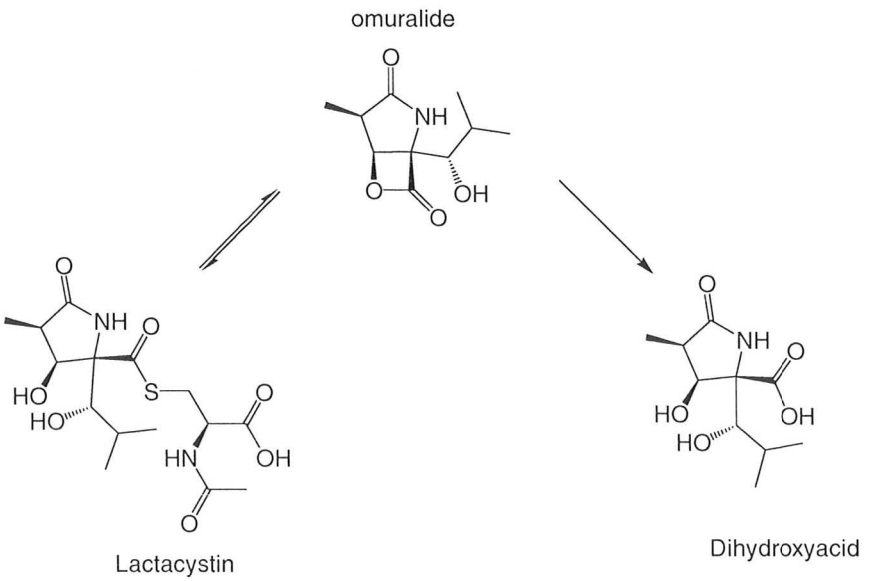


Fig. 3.17: HPLC Chromatograms of (a) Cell-Free Extract and Labelled SNAC (b) HEPES Buffer with Labelled SNAC (c) Media Pellet (no cells) in Buffer and Labelled SNAC (d) Cell-Free Extract with no SNAC (e) Uncentrifuged Cell-Free Extract and Labelled SNAC

Experiment (a) contained cell free extract with labelled SNAC while control 1 (b) had HEPES buffer with labelled SNAC, control 2 (c) had media pellet (no cells) in buffer and labelled SNAC, control 3 (d) cell free extract with no SNAC and control 4 (e) had uncentrifuged cell free extract and labelled SNAC. After incubation a, b, c and e contained labelled lactone due to chemical cyclization.

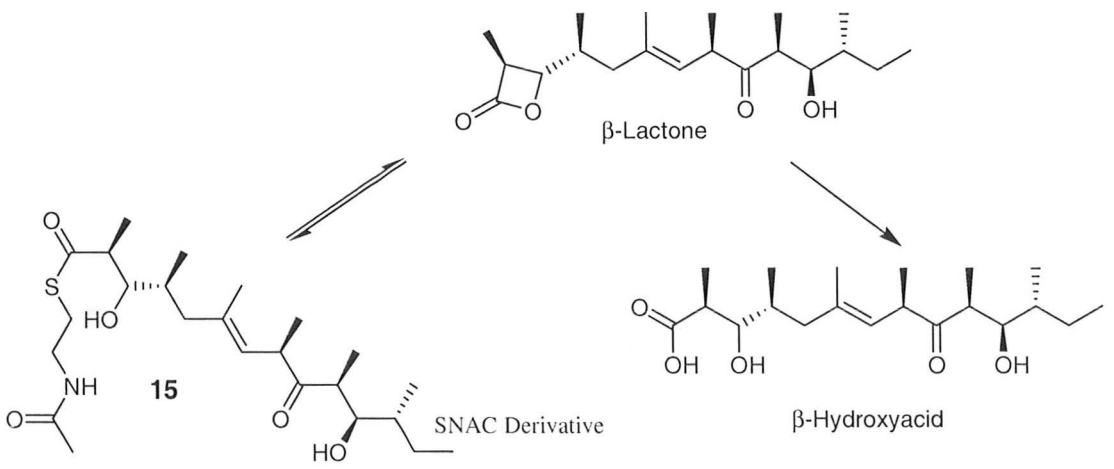
The decomposition of the SNAC derivative in the medium is not surprising as the pH was measured to be 7.5. However, we did not expect to see the thioester cyclize to give the four membered ring lactone so easily. In fact, the stability of the β-lactone in water is surprising. We decided to search the literature for reports on similar experiments. The work by Dick *et al.* on the well known proteosome inhibitor omuralide and its SNAC product lactacystin, which were isolated from *Streptomyces* sp explains the mechanism of hydrolysis of this fused bicyclic β-lactone.[95]



Scheme: 3.9 Mechanism of Hydrolysis of Lactacystin

Scheme 3.9 shows the mechanism by which lactacystin cyclizes to β-lactone (omuralide) before dissociating to its hydroxy acid. The study was conducted at pH 8.0 in HEPES buffer at 37°C. According to this study, in the incorporation experiments in ebelactone A, which were conducted at pH 7.5 (for both cell free and whole cell experiments), we can expect to see a small amount of both lactone and free acid in our samples due to hydrolysis of the SNAC derivative.

A similar mechanism could be proposed for the hydrolysis of **15** (Scheme 3.10). This explains the background cyclization encountered during the incorporation experiments.



Scheme: 3.10 Proposed Mechanism of Hydrolysis of Ebelactone SNAC Derivative

Conclusions on the incorporation experiments along with future work are described in Chapter 4.

Chapter 4

CONCLUSIONS AND FUTURE WORK

4.1 Conclusions from ^{18}O Labelling Experiments

The results of the labelling experiments using sodium [1-^{13}C, $^{18}O_2$] propionate confirmed that the lactonization in ebelactone A proceeds via the attack of the C3 oxygen onto a carbonyl derivative at C1 and excludes all other putative mechanisms for ring formation. The results further pinpoint that either a direct cyclization mechanism through mediation of TE as in macrolactones or a novel off-loading mechanism similar to the TMAL method as proposed in the biosynthesis of salinosporamides could take place.

4.2 Conclusions from SNAC Labelling Experiments

Experiments conducted so far, with labelled SNAC derivatives have failed to establish anything regarding the biosynthetic ring formation. This is due to the background cyclization of the precursor under control conditions, in both microbiological media and the buffer used for the cell-free experiments. These results, however have led to some conclusions regarding the nature of the chemical cyclization. Chemical cyclization is induced by basic pH in aqueous medium, as an intermediate in the hydrolysis process.

4.3 Future Work

(1) Experiments on different β-lactones for chemical cyclization at different pHs and in different buffers. These experiments are proposed to be carried out with different synthetic lactones, to obtain a suitable pH range that does not allow spontaneous lactonization, and this suitable pH range can be used for the cell-

free experiments involving incorporation of SNAC **44** into ebelactone A. This will show whether the ebelactone SNAC derivative is biologically cyclized to give the β-lactone.

(2) Genetic experiments to identify the gene cluster which encodes the lactone formation. Isolation of genomic DNA of *S. aburaviensis* and PCR experiments to find the TE of the putative ebelactone polyketide synthase are already underway. These results will be used to clone the genes responsible for lactonization using reverse genetics.

(3) Synthesis of β-lactones from their respective SNAC derivatives in aqueous medium at basic pH. A range of SNAC derivatives will be synthesized and tested using the new reaction conditions. Different conditions, such as higher temperatures and reaction times and different pH will be tested. These results will be helpful in determining the reaction rate and yield of this reaction.

(4) Studies on biosynthesis of β-lactones of different biosynthetic origins. There are a number of biologically important lactones of terpenoid, amino acid and mixed origins. Biosynthetic and genetic studies on these molecules leading to the identification of gene clusters could be useful in treatment of different diseases of human, plant and animal species.

Chapter 5

EXPERIMENTAL

5.1 Materials and Methods

ISP2 agar (Difco), fish meal (Sigma or Rothsay), $CaCO_3$ (Aldrich), glycerol (Caledon Laboratories) and pure water were used to prepare all media.

EMD silica gel 60 was used for normal phase flash chromatography columns and a prepacked Merck Lobar RP-18 column (240-10 mm) for reverse phase chromatography. Normal phase TLC was performed on Machery Nagel alugram sil G/uv $_{254}$ plates, while reverse phase TLC used Merck silica gel RP-18 F_{254} plates. Proton and ^{13}C NMR spectra were recorded on Bruker AV-200 (200 MHz, 50 MHz), AV-600 (600 MHz, 150 MHz) and AV-700 (700 MHz, 350 MHz) spectrometers. 2D spectra were recorded on the Bruker AV 600 spectrometer. IR spectra were recorded on a Nicolet 600 spectrometer. Mass spectra were recorded on a Quattro Ultima Micromass spectrometer.

5.2 Growth of *Streptomyces* sp. MG7-G1[21]

Cultures of *Streptomyces* sp MG7-G1 from American Type Culture Collection (ATCC 31860) were grown in Petri dishes at 28 °C for 5 days on ISP medium 2 agar plates (36.6 g in 1 L pure water).

The resulting spores were suspended in sterile water and transferred to liquid growth medium containing 3% glycerol, 2% fish meal and 0.2% $CaCO_3$ in 100 mL pure water in a 500 mL Erlenmeyer flask which had been autoclaved at 121 °C for 20 min. The flask was incubated

in a rotary shaker-incubator at 28 °C and 180 rpm for 2 days. Aliquots of the resulting culture (2 mL) were inoculated into twenty two 500 mL Erlenmeyer flasks containing the same sterile liquid medium as described above. The flasks were incubated in the rotary shaker-incubator at 28 °C and 180 rpm for 2 days.

5.3 Extraction and Isolation of Ebelactones A and B

Ethyl acetate (100 mL) was added into each culture flask and the mixture was shaken on the shaker-incubator at 180 rpm for 1 h. The resulting liquid was centrifuged at 4000 rpm at 4 °C for 10 minutes. The ethyl acetate layer was removed, and the remaining aqueous layer was then extracted twice with equal volumes of ethyl acetate (4.4 L of ethyl acetate in total). The combined organic layers were concentrated to give approximately 3 g of crude extract. Flash chromatography with 5:5:1 hexanes:chloroform:ethyl acetate gave fractions containing **15**, which were combined and concentrated. The residue was then filtered using a RP-18 SPE cartridge (E. Merck, adsorbex 400 mg) with 50:50 methanol-water as eluent. The filtrate was concentrated by azeotropic distillation with ethanol, and then subjected to reverse phase chromatography with a step gradient elution from 50:50 to 70:30 methanol-water to give fractions containing pure ebelactone A (**15**) and B (**16**) (Fig. 5.1). Yield: 4 mg of white solid, **15**; 1 mg of **16**.

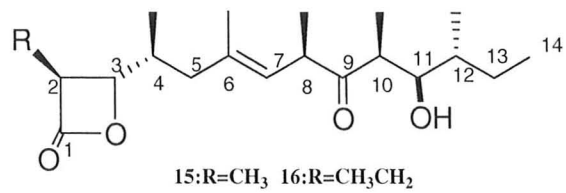
5.4 Ebelactone A (Unlabelled)

^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.39 (d, *J* 7.5 Hz, 3H, 2-CH_3), 3.27 (dq, *J* 4.0, 7.4 Hz, 1H, 2-H), 3.86 (dd, *J* 8.0 Hz, 4.0 Hz, 1H, 3-H), 2.00 (m, 1H, 4-H), 0.85 (d, *J* 6.8 Hz, 3H, 4-CH_3), 1.82 (dd, *J* 13.3, 10.0 Hz, 1H, 5-H_2), 2.36 (dd, *J* 13.3, 3.8 Hz, 1H, 5-H_1), 1.73 (s, 3H, 6-CH_3), 5.03 (d, *J*

10.0 Hz, 1H, 7-H), 3.59 (dq, *J* 10.0, 6.7 Hz, 1H, 8-H), 1.12 (d, *J* 6.7 Hz, 3H, 8-CH_3), 2.85 (dq, *J* 1.8, 7.2 Hz, 1H, 10-H), 1.10 (d, *J* 7.2 Hz, 3H, 10-CH_3), 3.50 (m, 1H, 11-H), 3.06 (bs, 1H, 11-OH), 1.44 (m, 1H, 12-H), 0.77 (d, *J* 6.8 Hz, 3H, 12- CH_3), 1.75 (dq, *J* 7.6, 2.6 Hz, 2H, 13-H_2),1.17 (m, 1H, 13-H_1), 0.88 (t, *J* 7.6 Hz, 3H, 14-CH_3).

^{13}C NMR ($CDCl_3$, 150 MHz) δ 171.15 (C-1), 48.74 (C-2), 83.03 (C-3), 35.97 (C-4), 43.39 (C-5), 135.93 (C-6), 126.93 (C-7), 45.77 (C-8), 217.10 (C-9), 45.55 (C-10), 74.62 (C-11), 37.00 (C-12), 25.25 (C-13), 11.31 (C-14), 13.29 (CH_3-2), 13.97 (CH_3-4), 16.88 (CH_3-6), 16.88 (CH_3-8), 9.80 (CH_3-10), 15.29 (CH_3-12).

MS (ESI +ve) m/z 339.3 $[M+H]^+$, 340.4 $(M+H+1)^+$



15:R=CH_3 16:R=CH_3CH_2

Fig 5.1: Ebelactones A and B

5.5 Ebelactone B

^1H NMR ($CDCl_3$, 600 MHz) δ 1.86 (d, *J* 7.5 Hz, 2H, 1'-H_2), 1.06 (t, *J* 7.5 Hz, 3H, 2'-H_3), 3.20 (dt, *J* 4.0, 7.5 Hz, 1H, 2-H), 3.92 (dd, *J* 8.0 Hz, 4.0 Hz, 1H, 3-H), 2.05 (m, 1H, 4-H), 0.86 (d, *J* 6.8 Hz, 3H, 4-CH_3), 1.87 (dd, *J* 13.3, 10.0 Hz, 1H, 5-H_2), 2.39 (dd, *J* 13.3, 3.8 Hz, 1H, 5-H_1), 1.73 (s, 3H, 6-CH_3), 5.04 (d, *J* 10.0 Hz, 1H, 7-H), 3.59 (dq, *J* 10.0, 6.7 Hz, 1H, 8-H), 1.13 (d, *J* 6.7 Hz, 3H, 8-CH_3), 2.85 (dq, *J* 1.8 , 7.2 Hz, 1H, 10-H), 1.10 (d, *J* 7.3 Hz, 3H, 10-CH_3), 3.51 (m,

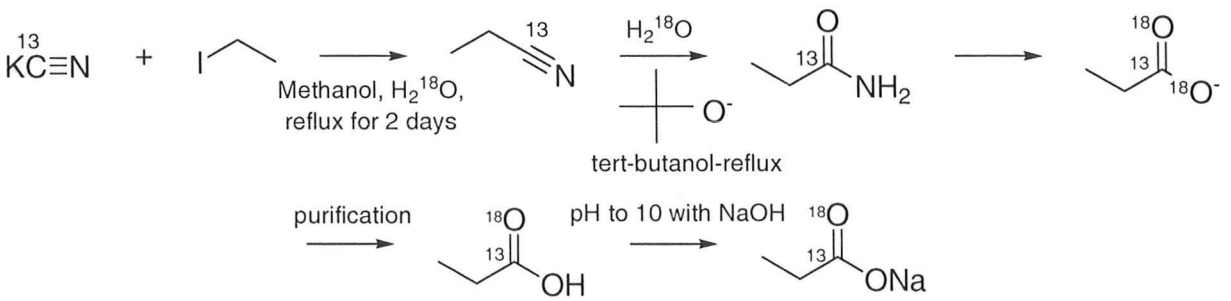
1H 11-H), 3.08 (bs, 1H, 11-OH), 1.44 (m, 1H, 12-H), 0.78 (d, *J* 6.8 Hz, 3H, 12- CH_3), 1.78 (dq, *J* 7.6, 2.6 Hz, 2H, 13-H_2), 1.12 (m, 1H, 13-H_1), 0.89 (t, *J* 7.6 Hz, 3H, 14-CH_3).

^{13}C NMR ($CDCl_3$, 150 MHz) δ 171.61 (C-1), 56.43 (C-2), 81.55 (C-3), 35.91 (C-4), 43.39 (C-5), 136.02 (C-6), 126.90 (C-7), 45.79 (C-8), 218.11 (C-9), 45.52 (C-10), 74.95 (C-11), 37.00 (C-12), 25.25 (C-13), 11.85 (C-14), 11.31 (CH_3-2'), 21.80 (CH_2-2'), 13.12 (CH_3-4), 16.85 (CH_3-6), 16.85 (CH_3-8), 9.83 (CH_3-10), 13.29 (CH_3-12).

MS m/z 353.5 (28%) $[M+H]^+$, 370.5 (100%) $[M+NH_4]^+$, 375.5 (65%) $[M+Na]^+$, 391.4 (30%) $[M+K]^+$.

IR ($CHCl_3$, cm^{-1}), 3512.8 (OH), 1824.4 (C1, lactone-carbonyl), 1700.5 (C9).

5.6 Preparation of Sodium [1-^{13}C, $^{18}O_2$]Propionate



Scheme 5.1: Preparation of Sodium [1-^{13}C, $^{18}O_2$]Propionate

Ethyl iodide (1.58 g, 0.9 mL, 10.1 mmol), potassium [^{13}C]cyanide (>99%^{13}C, 661 mg, 10.0 mmol), [^{18}O] water (95% ^{18}O, 0.1 g) and anhydrous methanol (1.6 mL) were heated to

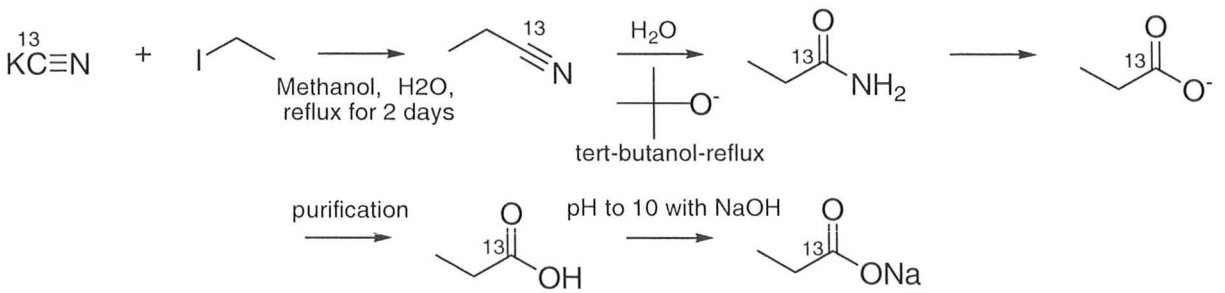
reflux for 5 hours and then at 80 °C for 40 hours. The mixture was distilled and the distillate was treated with $H_2{}^{18}O$ (0.4 g, 20 mmol) and a solution of potassium tert-butoxide in tertiary butanol, (0.85 M, 11.5 mL). The mixture was heated at reflux for 48 hours, then cooled and concentrated *in vacuo*. The residue was then treated with H_2O (5 mL) and cation exchange resin (Dowex 50W-X1, 2.5 g). The mixture was stirred for 10 minutes and filtered; the filtrate was adjusted to pH 10.0 with 5 M NaOH and evaporated *in vacuo* to afford 820 mg (8.2 mmol, 82%) of sodium [1-^{13}C, $^{18}O_2$]propionate as a white solid.

^{1}H NMR (D_2O, 200 MHz) δ 2.08 (d, ^2J (^{13}C-^{1}H) 6.1 Hz, q, 7.7 Hz, 2H, CH_2), 0.94 (d, ^3J (^{13}C-^{1}H) 5.1 Hz, t, 7.7 Hz, 3H, CH_3).

^{13}C NMR (D_2O, 50 MHz) δ 184.97 (enhanced), 30.65 (d, ^1J (^{13}C-^{13}C) 51.1 Hz), 10.12 (s).

MS (ESI –ve) m/z 77.6 [M-H]$^-$, $^{18}O_2$, (100%), 75.6 [M-H]$^-$, $^{18}O^{16}O$, (75%), 73.6 [M-H]$^-$,$^{16}O_2$ (12%).

5.7 Preparation of Sodium [1-^{13}C]Propionate



Scheme 5.2: Preparation of Sodium [1-^{13}C]Propionate

Sodium [1-^{13}C]propionate was prepared using the same procedure as described above using nano pure water instead of $H_2{}^{18}O$ to afford 840 mg (8.7 mmol, 86%) of sodium [1-^{13}C] propionate as a white solid.

^{1}H NMR, (D_2O, 200 MHz), δ 2.08 (d, ^2J (^{13}C-^{1}H) 6.1 Hz, t, 7.7 Hz, 2H, CH_2), δ 0.95 (d, ^3J (^{13}C-^{1}H) 5.1 Hz, q, 7.7 Hz, 3H, CH_3).

^{13}C NMR, (D_2O, 50 MHz), δ 185.03 (enhanced), 30.67 (d, ^1J (^{13}C-^{13}C) 51.0 Hz), 10.15 (d, ^2J (^{13}C-^{13}C) 2.5 Hz).

MS (ESI –ve) m/z 73.9 $[M-H]^-$.

5.8 Addition of Labelled Sodium [1-^{13}C, $^{18}O_2$]Propionate

Unlabelled sodium propionate (Fisher Laboratories, 250 mg) sodium [1-^{13}C,$^{18}O_2$] propionate (150 mg) and sodium [1-^{13}C]propionate (100 mg) were dissolved in sterile water (8.8 mL) and added to the 22 flasks at 21 hours and at 45 hours after inoculation (100 μL per flask per feeding). Ebelactone A was isolated as above.

5.9 Ebelactone A (Labelled with Sodium [1-^{13}C, $^{18}O_2$]Propionate)

^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.39 (d, *J* 7.5 Hz, 3H, 2-CH_3), 3.27 (dq, *J* 4.0, 7.5 Hz, 1H, 2-H), 3.86 (dd, *J* 8.0 Hz, 4.0 Hz, 1H, 3-H), 2.00 (m, 1H, 4-H), 0.85 (d, *J* 6.8 Hz, 3H, 4-CH_3), 1.82 (dd, *J* 13.3, 10.0 Hz, 1H, 5-H_2), 2.36 (dd, *J* 13.3, 3.8 Hz, 1H, 5-H_1), 1.73 (s, 3H, 6-CH_3), 5.03 (d, *J* 10.0 Hz, 1H, 7-H), 3.59 (dq, *J* 8.2, 6.7 Hz, 1H, 8-H), 1.12 (d, *J* 6.7 Hz, 3H, 8-CH_3), 2.85 (dq, *J* 1.8, 7.2 Hz, 1H, 10-H), 1.10 (d, *J* 7.2 Hz, 3H, 10-CH_3), 3.50 (m, 1H 11-H), 3.06 (bs, 1H, 11-OH)

1.44 (m, 1H, 12-H), 0.77 (d, *J* 6.8 Hz, 3H, 12- CH_3), 1.75 (dq, *J* 7.6, 2.6 Hz, 2H, 13-H_2), 1.17 (m, 1H, 13-H_1), 0.88 (t, *J* 7.6 Hz, 3H, 14-CH_3).

^{13}C NMR ($CDCl_3$, 150 MHz) 171.15 (enhanced C-1, $\Delta\delta$ 0.039 ppm), 48.74 (C-2), 83.03 (enhanced C-3, $\Delta\delta$ 0.029 ppm), 35.97 (C-4), 43.39 (enhanced C-5), 135.93 (C-6), 126.93 (enhanced C-7), 45.77 (C-8), 217.10 (enhanced C-9, $\Delta\delta$ 0.053 ppm), 45.55 (C-10), 74.62 (enhanced C-11, $\Delta\delta$ 0.029 ppm), 37.00 (C-12), 25.25 (C-13), 11.31 (C-14), 13.29 (CH_3-2), 13.97 (CH_3-4), 16.88 (CH_3-6), 16.88 (CH_3-8), 9.80 (CH_3-10), 15.29 (CH_3-12).

MS (ESI +ve) m/z 339.3 (100%), 340.4 (85%), 341.4 (45%), 342.4 (35%), 343.4 (28%), 344.4 (12%), 346.0 (3%).

5.10 Addition of Labelled Sodium [1-^{13}C]Propionate

Sodium [1-^{13}C]propionate (400 mg) (see above) in 8.8 mL dI water was added to cultures at 21 h and at 45 h after inoculation. Ebelactone was isolated as above.

5.11 Ebelactone A (Labelled with Sodium [1-^{13}C]Propionate)

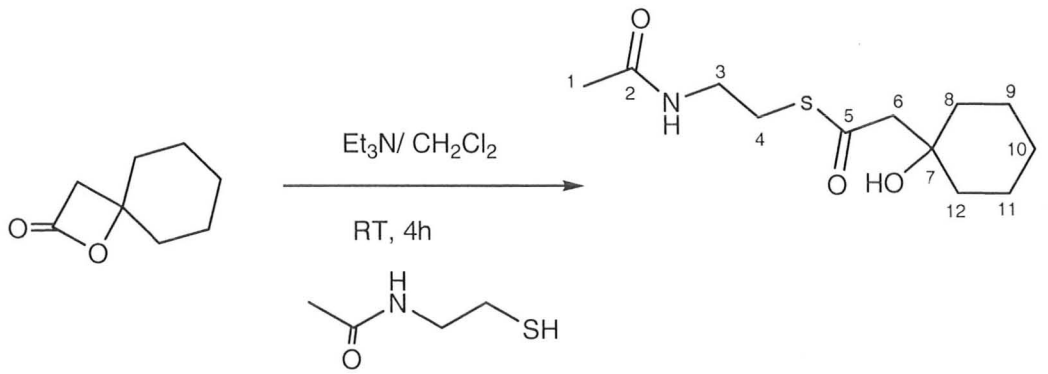
^1H NMR ($CDCl_3$, 600 MHz) δ 1.39 (d, *J* 7.5 Hz, 3H, 2-CH_3), 3.27 (dq, *J* 4.0, 7.5 Hz, 1H, 2-H), 3.86 (dd, *J* 8.0 Hz, 4.0 Hz, 1H, 3-H), 2.00 (m, 1H, 4-H), 0.86 (d, *J* 6.8 Hz, 3H, 4-CH_3), 1.82 (dd, *J* 13.3, 10.0 Hz, 1H, 5-H_2), 2.36 (dd, *J* 13.3, 3.8 Hz, 1H, 5-H_1), 1.73 (s, 3H, 6-CH_3), 5.03 (d, *J* 10.0 Hz, 1H, 7-H), 3.59 (dq, *J* 10.0, 6.7 Hz, 1H, 8-H), 1.12 (d, *J* 6.7 Hz, 3H, 8-CH_3), 2.85 (dq, *J* 1.8 , 7.2 Hz, 1H, 10-H), 1.10 (d, *J* 7.2 Hz, 3H, 10-CH_3), 3.50 (m, 1H 11-H), 3.06 (bs, 1H, 11-

OH), 1.44 (m, 1H, 12-H), 0.77 (d, *J* 6.8 Hz, 3H, 12- CH_3), 1.75 (dq, *J* 7.6, 2.6 Hz, 2H, 13-H_2), 1.12 (m, 1H, 13-H_1), 0.88 (t, *J* 7.6 Hz, 3H, 14-CH_3).

^{13}C NMR ($CDCl_3$, 150 MHz) δ 171.15 (enhanced C-1), 48.74 (C-2), 83.03 (enhanced, C-3), 35.97 (C-4), 43.39 (enhanced C-5), 135.93 (C-6), 126.93 (enhanced C-7), 45.77 (C-8), 217.10 (enhanced C-9), 45.55 (C-10), 74.62 (enhanced C-11), 37.00 (C-12), 25.25 (C-13), 11.31 (C-14), 13.29 (CH_3-2), 13.97 (CH_3-4), 16.88 (CH_3-6), 16.88 (CH_3-8), 9.8 (CH_3-10), 15.29 (CH_3-12).

MS (ESI +ve) m/z 339.3(100%) $[M+H]^+$, 340.3 (100%) $[M+H+1]^+$, 341.2 (100%) $[M+H+2]^+$, 342.3 (98%) $[M+H+3]^+$, 343.3 (66%) $[M+H+4]^+$, 344.3 (30%) $[M+H+5]^+$, 345.3 (10%) $[M+H+5]^+$.

5.12 Preparation of 4-Spirocyclohexyloxetan-2-one-SNAC Derivative



Scheme 5.3: Preparation of 4-Spirocyclohexyloxetan-2-one-SNAC Derivative

The method used by Corey *et al.*[93] for opening the ring in omuralide with cysteine was modified. A solution of 4-spirocyclohexyloxetan-2-one (20 mg, 0.14 mmol), *N*-acetylcysteamine (47 mg, 0.40 mmol) and Et_3N (7.5 mg) in CH_2Cl_2 (0.4 mL) was stirred at room temperature for 4 h and the contents were evaporated under vacuum and chromatographed on a flash column with 2% - 5% methanol in chloroform as eluent to yield 12 mg (0.043 mmol, 34%) of product as a colourless liquid.

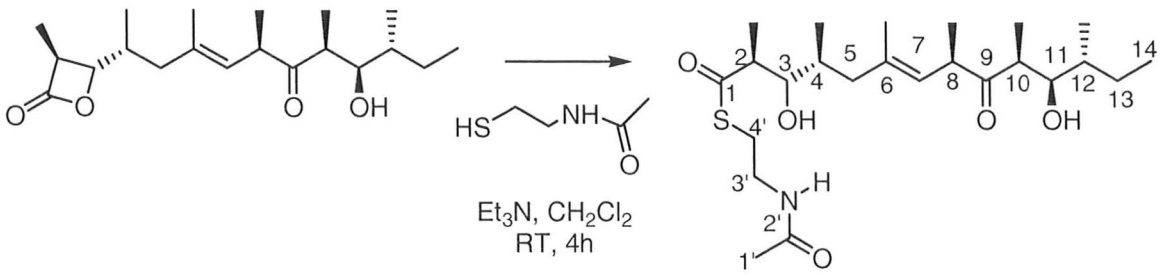
^{1}H NMR, ($CDCl_3$, 600 MHz), δ 5.91 (s, 1H, N-H), 3.43 (dt, *J* 6.2, 6.0 Hz, 2H, 3-H), 3.03 (t, *J* 6.0 Hz, 2H, 4-H), 2.74 (s, 2H, 6-H), 1.24-1.64 (m, 10H, cyclohexane), 1.96 (s, 3H, CH_3).

^{13}C NMR, ($CDCl_3$, 150 MHz), δ 23.38 (C-1), 170.95 (C-2), 39.50 (C-3), 37.56 (C-4), 200.12 (C-5), 54.68 (C-6), 71.52 (C-7), 28.99 (C-8, C-12), 25.61 (C-9, C-11), 22.12 (C-10).

MS (ESI +ve) m/z 260.1 (100%) $[M+H]^+$, 282 (47%) $[M+Na]^+$.

IR cm^{-1} ($CHCl_3$), 3294 (OH), 1653 (carbonyl).

5.13 Preparation of Ebelactone-SNAC Derivative



Scheme 5.4: Preparation of Ebelactone-SNAC Derivative

A mixture of of ebelactone A (4.0 mg, 0.017 mmol), *N*-acetylcysteamine (4.2 mg, 0.035 mmol), and Et_3N (2.6 mg) in CH_2Cl_2 (0.05 mL) was stirred at room temperature for 4 h. The mixture was evaporated under vacuum and subjected to chromatography on a silica column with 2%-4% methanol in chloroform as eluent to yield 3.5 mg (0.006 mmol, 40%) of product as a colourless liquid.

^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.28 (d, *J* 7.5 Hz, 3H, 2-CH_3), 2.93 (dq, 4.0, 7.5 Hz, 1H, 2-H), 3.42 (dd, *J* 8.0 Hz, 4.0 Hz, 1H, 3-H), 2.54 (d, *J* 8.0 Hz, 1H, 4-H), 0.86 (d, *J* 6.8 Hz, 3H, 4-CH_3), 1.76 (dd, *J* 13.3, 10.0 Hz, 1H, 5-H_A), 2.36 (d, *J* 10.0 Hz, 1H, 5-H_B), 1.72 (s, 3H, 6-CH_3), 5.00 (d, *J* 10.0 Hz, 1H, 7-H), 3.56 (dq, *J* 8.2, 6.7 Hz, 1H, 8-H), 1.12 (d, *J* 6.7 Hz, 3H, 8-CH_3), 2.85 (dq, *J* 1.8 , 7.2 Hz, 1H, 10-H), 1.09 (d, *J* 7.2 Hz, 3H, 10-CH_3), 3.44 (m, 1H, 11-H), 3.13 (s, 1H, 11-OH), 1.44 (m, 1H, 12-H), 0.78 (d, *J* 6.8 Hz, 3H, 12- CH_3), 1.74 (dq, *J* 7.6, 2.6 Hz, 1H, 13-H_A), 1.25 (m,

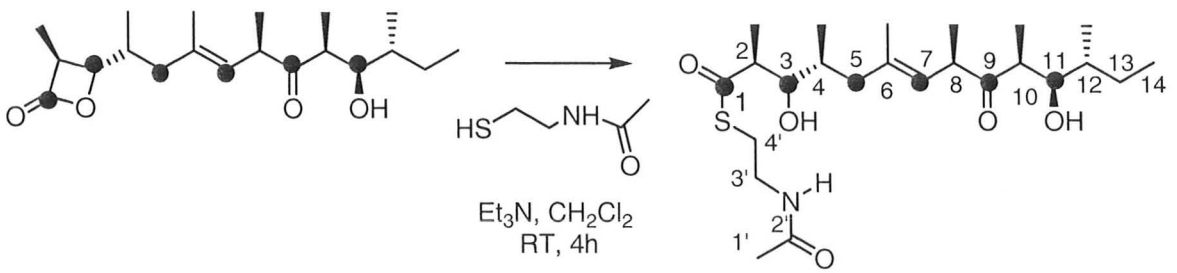
1H, 13-H_B), 0.88 (t, *J* 7.6 Hz, 3H, 14-CH_3), 1.96 (s, 3H, 1'-CH_3), 3.45 (t, *J* 6.2 Hz, 2H, C-3'), 2.95 (t, *J* 6.2 Hz, 2H, C-4'), 5.78 (s, 1H, NH).

^{13}C NMR ($CDCl_3$, 150 MHz) δ 204.24 (C-1), 50.32 (C-2), 78.07 (C-3), 40.75 (C-4), 41.92 (C-5), 136.49 (C-6), 125.21 (C-7), 45.01 (C-8), 217.38 (C-9), 45.01 (C-10), 74.01 (C-11), 33.99 (C-12), 24.46 (C-13), 13.64 (C-14), 14.34 (CH_3-2), 10.38 (CH_3-4), 16.08 (CH_3-6), 15.81 (CH_3-8), 8.89 (CH_3-10), 15.50 (CH_3-12), 169.87 (C2'), 36.05 (C3'), 28.83 (C4'), 22.75 (CH_3-1').

MS m/z 458.5 $[M+H]^+$ (100%), 475.5 $[M+NH_4]^+$ (42%), 480.5 $[M+Na]^+$ (22%).

IR cm^{-1} ($CHCl_3$), 3323 (OH), 1690, 1660 (carbonyl).

5.14 Preparation of Ebelactone-SNAC Derivative Labelled with ^{13}C



Scheme 5.5: Preparation of Ebelactone-SNAC Derivative Labelled with ^{13}C

A mixture of of ebelactone A (labelled, 12.0 mg, 0.03 mmol), *N*-acetylcysteamine (9.5 mg, 0.08 mmol) and Et_3N (7.5 mg) in CH_2Cl_2 (0.2 mL) was stirred at room temperature for 4 h. The mixture was evaporated under vacuum and subjected to chromatography on a silica column

with 2%-4% methanol in chloroform as eluent to yield 9.5 mg (0.02 mmol, 63%) of product as a colourless liquid.

^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.28 (d, *J* 7.5 Hz, 3H, 2-CH_3), 2.93 (dq, *J* 4.0, 7.0, Hz, 1H, 2-H), 3.42 (dd, *J* 8.0 Hz, 4.0 Hz, 1H, 3-H), 2.54 (d, *J* 8.0 Hz, 1H, 4-H), 0.89 (d, *J* 6.8 Hz, 3H, 4-CH_3), 1.79 (dd, *J* 13.3, 10.0 Hz, 1H, 5-H_A), 2.38 (dd, *J* 13.3, 3.8 Hz, 1H, 5-H_B), 1.72 (s, 3H, 6-CH_3), 5.00 (d, *J* 10.0 Hz, 1H, 7-H), 3.58 (dq, *J* 8.2, 6.7 Hz, 1H, 8-H), 1.12 (d, *J*, 6.7 Hz, 3H, 8-CH_3), 2.86 (dq, *J* 1.8 , 7.2 Hz, 1H, 10-H), 1.09 (d, *J* 7.2 Hz, 3H, 10-CH_3), 3.49 (m, 1H, 11-H), 3.15 (s, 1H, 11-OH), 1.45 (m, 1H, 12-H), 0.77 (d, *J* 6.8 Hz, 3H, 12- CH_3), 1.76 (dq, *J* 7.6, 2.6 Hz, 2H, 13-H_A), 1.19 (m, 1H, 13-H_B), 0.87 (t, *J* 7.6 Hz, 3H, 14-CH_3), 1.98 (s, 3H, 1'-CH_3), 3.45 (t, *J* 6.2 Hz, 2H, 3'-H_2), 2.95 (t, *J* 6.2 Hz, 2H , 4'-H_2), 5.78 (s, 1H, NH).

^{13}C NMR ($CDCl_3$, 150 MHz) δ 204.24 (C-1, enhanced), 50.32 (C-2), 78.07 (C-3, enhanced), 40.75 (C-4), 41.92 (C-5, enhanced), 136.49 (C-6), 125.21 (C-7, enhanced), 45.01 (C-8), 217.38 (C-9, enhanced), 45.01 (C-10), 74.01 (C-11, enhanced), 33.99 (C-12), 24.46 (C-13), 13.64 (C-14), 14.34 (CH_3-2), 10.38 (CH_3-4), 16.08 (CH_3-6), 15.81 (CH_3-8), 8.89 (CH_3-10), 15.50 (CH_3-12), 169.87 (C-2'), 36.05 (C-3'), 28.83 (C-4'), 22.75 (CH_3-1').

MS m/z 458.5 $[M+H]^+$, 459.5 $[M+H+1]^+$, 460.5 $[M+H+2]^+$, 461.5 $[M+H+3]^+$, 462.5 $[M+H+4]^+$, 463.7 $[M+H+5]^+$.

Incorporation Experiments with SNAC Derivative

5.15 General Procedure

S. aburaviensis was grown in a 500 mL Erlenmeyer flask for 24 h after inoculation and 1 mL aliquots were transferred to sterile 20 mL culture tubes (pyrex) under sterile conditions. Labelled/unlabelled SNAC derivative (100 μg in 10 μL DMSO) was added to the culture tubes containing the cells. These were incubated at 26 ^{o}C for 24 h and then extracted with an equal volume of ethyl acetate. The analysis was performed by HPLC-MS on a Waters 2695 Micromass Ultima controlled by Masslynx software. A Luna C-18 reverse phase column was used with 75:25 - 95:5 gradient elution of acetonitrile: 10 mM ammonium acetate. Controls for these experiments were carried out using 1 mL of sterile media instead of the inoculum.

5.15.1. Experiment with Unlabelled SNAC to Check Stability in Growth Medium

This experiment was carried out using the general procedure by adding unlabelled SNAC (100 μg) to 5 mL of sterile media. Aliquots (1 mL) were taken and extracted with an equal volume of ethyl acetate at 1 h, 3 h and 24 h, and screened for the presence of the SNAC derivative and ebelactone by LC-MS.

5.15.2. Experiment with Labelled SNAC for Incorporation

This experiment was carried out using the general procedure by adding labelled SNAC to:

(i) Cells in media

(ii) Living cells only (no SNAC)

(iii) Sterile media

After 24 h, samples were extracted as above. Extracts were screened for the presence of labelled ebelactone using LC-MS.

5.15.3. Experiment with Labelled SNAC with Time

Experiment was carried out using the general procedure by adding labelled SNAC to:

(i) Supernatant only (prepared from cells in media by centrifugation at 4000 r.p.m for 1 min)

(ii) Cells in media (no SNAC)

(iii) Cells in media

Aliquots were taken at 8 h, 16 h and 24 h intervals and analyzed as described above.

5.15.4. Experiment for Cyclization in Water/Calcium Carbonate

A similar procedure to that above was followed and labelled SNAC was added to:

(a) Calcium carbonate in pure, deionized water

(b) Pure deionized water

5.15.5. Experiments with Cell Free Extracts

Cells were grown for two days in a similar way to that described above. They were centrifuged at 4000 rpm for 10 minutes at 4 ^{o}C. The supernatant was decanted off and the pellets were stored on ice. They were resuspended in 50mM HEPES (50 mmol, pH 7.5) and sterile glycerol was added to 5% concentration. Cells were then lysed in a bead beater for 10 min., then the suspension was centrifuged and the supernatant was carefully removed. 1 mL of supernatant was

then incubated with labelled SNAC (100 μg) (a) for 1 h at room temperature with the following controls;

Control 1 (b) HEPES buffer with labelled SNAC

Control 2 (c) media pellet (no cells) in buffer and labelled SNAC

Control 3 (d) cell free extract with no SNAC

Control 4 (e) uncentrifuged cell free extract and labelled SNAC

Chapter 6

REFERENCES

1) Pommier, A.; Pons, J. M. *Synthesis,* **1993**, 441-459.

2) Pommier, A.; Pons, J. M. *Synthesis,* **1995**, 729-744.

3) Reddy, L. R.; Fournier, J-F.; Reddy, B. V. S.; Corey, E. J. *Org. Lett.* **2005**, *7*, 2699-2701.

4) Lane, J. F.; Koch, W. T.; Leeds, N. S.; Gorin, G. *J. Am. Chem. Soc.* **1952**, *74*, 3211-3214.

5) Weibel, E. K.; Hadvary, P.; Hochuli, E.; Kupfer, E.; Lengsfield, H. *J. Antibiot.* **1987**, *40*, 1081-1085.

6) Hochuli, E.; Kupfer, E.; Maurer, R.; Meister, W.; Mercadal, Y.; Schimidt, K. *J. Antibiot.* **1987**, 40, 1086-1091.

7) Anderson, J. W. *Exp. Opin. Pharmacother.* **2007**, *8*, 1733-1742.

8) Kridel, S. J.; Axelrod, F.; Rozentkrantz, N.; Smith, J. W. *Cancer Res.* **2004**, *64*, 2070-2075.

9) Umezawa, H.; Aoyagi, T.; Hazato, T.; Uotani, K.; Kojima, F.; Hamada, M.; Takeuchi, T. *J. Antibiot.* **1978**, *31*, 639-641.

10) Kitahara, M.; Asano, M.; Naganawa, H.; Maeda, K.; Hamada, M.; Aoyagi, T.; Umezawa, H.; Iitaka, Y.; Nakamura, H. *J. Antibiot.* **1987**, *40*, 1647-1650.

11) Mutoh, M.; Nakada, N.; Shoko, M.; Shoichi, O.; Kiyoshi, Y.; Junko, W.; Mikio, A. *J. Antibiot.* **1994**, *47*, 1369-1375.

12) Feling, R. H.; Buchanan, G. O.; Mincer, T. J.; Kauffman, C. A.; Jensen, P. R.; Fenical, W. *Angew. Chem. Int. Ed.* **2003**, *115*, 369-371.

13) Moore, B. S.; Eustaquio, A. S.; McGlinchey, R. P. *Curr. Opin. Chem. Biol.* **2008**, *12*, 434-440.

14) Williams, P. G.; Buchanan, G. O.; Feling, R. H.; Kauffman, C. A.; Jensen, P. R.; Fenical, W. *J. Org. Chem.* **2005**, *70*, 6196-6203.

15) Corey, E. J.; Li, W-J. Z. *Chem. Pharm. Bull.* **1999**, *47*, 1-10.

16) Macherla, V. R.; Mitchell, S. S.; Manam, R. R.; Reed, K. A.; Chao, T. H.; Nicholson, B.; Deyanat-Yazdia, G.; Mai, B.; Jensen P. R.; Fenical, W. *J. Med. Chem.* **2005**, *48*, 3684-3687.

17) Stadler, M.; Bitzer, J.; Mayer-Bartschmid, A.; Muller, H.; Benet-Buchholz, J.; Gantner, F.; Tichy, H-F.; Reinemer, P.; Baco, K. B. *J. Nat. Prod.* **2007**, *70*, 246-252.

18) Aldridge, D. C.; Giles, D.; Turner, W. B. *J. Chem. Soc., Chem. Commun.* **1970**, *11*, 639-640.

19) Tomoda, H.; Kumagai, H.; Takahashi, Y.; Tanaka, Y.; Iwai, Y.; Omura, S. *J. Antibiot.* **1988**, *41*, 247-249.

20) Tomoda, H.; Kumagai, H.; Tanaka, H.; Omura, S. *Biochim. Biophys. Acta.* **1987**, *922*, 351-356.

21) Kumagai, H.; Tomoda, H.; Omura, S. *J. Antibiot.* **1990**, *43*, 397-402.

22) Umezawa, H.; Aoyagi, T.; Uotani, K.; Hamada, M.; Takeuchi, T.; Takahashi, S. *J. Antibiot.* **1980,** *12,* 1594-1596.

23) Morris, B. D.; Smyth, R. R.; Foster, S. P.; Hoffmann, M. P.; Roelofs, W. L.; Franke, S.; Franke, W. *J. Nat. Prod.* **2005**, *68*, 26-30.

24) Groll, M.; Balskus, E. P.; Jacobsen, E. N. *J. Am. Chem. Soc.* **2008**, *130*, 14981-14983.

25) Groll, M.; Huber, R.; Potts, B. C. M. *J. Am. Chem. Soc.* **2006**, *128*, 5136-1541.

26) Luthi-peng. Q.; Marki, H. P.; Hadvary, P. *FEBS. Lett.* **1992**, *299*, 111-115.

27) Mizioko, H. M.; Lane, M. D. *J. Biol. Chem.* **1977**, *252*, 1414-1420.

28) Lowe, C.; Vederas, J. C. *Org. Prep. Proc.* **1995**, *27*, 305-346.

29) Mayol, L.; Piccialli, V.; Sica, D. *Tetrahedron Lett.* **1987**, *28*, 3601-3604.

30) Ogihara, Y.; Asada, M. *J. Chem. Soc. Chem. Commun.* **1978**, *8*, 364-365.

31) Bohlmann, F.; Paul, A.H.K. *Tetrahedron Lett.* **1984**, *25*, 1697-1700.

32) Okuyama, E.; Nakamura, T.; Yamazaki, M.; *Chem. Pharm. Bull.* **1993**, *41*, 1670-1671.

33) Kikuchi, H.; Tensho, A.; Shimizu, I.; Shiokawa, H.; Kuno, A.; Yamada, S.; Fujiwara, T.; Tomita, K. *Chem.. Lett.* **1983**, 603-606.

34) Liu, D-Z.; Wang, F.; Liao, T.G.; Tang, J-G.; Steglich, W.; Zhu, H. J.; Liu, J-K. *Org Lett.* **2006**, *8*, 5749-5752.

35) Beer, L. L.; Moore, B. S. *Org. Lett.* **2007**, *9*, 845-848.

36) Takahashi. S.; Uchida, K.; Nakagawa, A.; Miyake, Y.; Kainosho, M.; Matsuzaki, K.; Omura, S. J. *Antibiot.* **1995**. *48*, 1015-1020.

37) Wells, J. S.; Hunter, J. C.; Astle, G. L.; Sherwood, J. C.; Ricca, C. M.; Trejo, W. H.; Bonner, D. P.; Sykes, R. B. *J. Antibiot.* **1992**, *35*, 814-821.

38) Wells, J. S.; Trejo, W. H.; Principe, P. A.; Sykes, R. B. *J. Antibiot.* **1984**, *37*, 802-803.

39) Mori, T.; Takahashi, K.; Kashiwabara, M.; Uemura, D.; Katayama, C.; Iwadare, S.; Shizuri, Y.; Mitomo, R.; Nakano, F.; Matsuzaki, A. *Tetrahedron Lett.* **1985**, *26*, 1073-1076.

40) Ogura, M.; Nakayama, H.; Furihata, K.; Shimazu, A.; Seto, H.; Otake, N. *J. Antibiot* , **1985**, *38*, 669-673.

41) Manam, R. R.; Teisan, S.; White, D. J.; Nicholson, B. C. M. *J. Nat. Prod.* **2005**, *68*, 240-243.

42) Asai, A.; Hasegawa, A.; Ochiai, K.; Yamashita, Y.; Mizukami, T. *J. Antibiot.* **2000**, *53*, 81-83.

43) Manam, R. R.; Macherla, V. R.; Tsueng, G.; Dring, C. W.; Weiss, J.; Neuteboom, S. T. C.; Lam, K. S.; Mosca, D. A.; Lloyd, G. K.; Potts, B. C. M. *J. Nat. Prod.* **2009**, *72*, 295-297.

44) Herbert, R. B.; Knags, A. R. *Tetrahedron Lett.* **1990,** 7517-7520.

45) Staunton, J.; Weissmann, K. J. *Nat. Prod. Rep.* **2001**, *18*, 380-416.

46) Kumagai, H.; Tomoda, H.; Omura, S. *J. Antibiot.* **1990**, *43*, 397-402.

47) Saepudin, E.; Harrison, P.; *Can. J. Chem.* **1995**, *73*, 1-5.

48) Eisenreich, W.; Kupfer, E.; Stohler, P.; Weber, W.; Bacher, A. *J. Med. Chem.* **2003**, *46*, 4209-4212.

49) Goese, M.; Eisenreich, W.; Kupfer, E.; Weber, W.; Bacher, A. *J. Biol. Chem.* **2000**, *275*, 21192-21196.

50) Bevitt, D. J.; Cortes, J.; Haydock, S. F; Leadlay, P.F. *Eur. J. Biochem.* **1992**, *204*, 39-49.

51) Richardson, R. D.; Ma, G.; Oyola, Y.; Zancanella, M.; Knowles, L. M.; Cieplak, P.; Romo, D. *J. Med. Chem.* **2008**, *51*, 5285-5296.

52) Elliot, P. J.; Zollner, T. M.; Boehncke, W. H. *J. Mol. Med.* **2003**, *81*, 235-245.

53) Reddy, L. R.; Fournier, J. F.; Reddy, B. V. S.; Corey, E. J. *J. Am. Chem. Soc.* **2005**, *127*, 8974-8976.

54) Eustaquio, A. S.; Moore, B. S.*Angew. Chem. Int. Ed.* **2008**, *47*, 3936-3938.

55) McGlinchey, R. P.; Nett, M.; Eustaquio, A. S.; Asolkar, R. N.; Fenical, W.; Moore, B. S. *J. Am. Chem. Soc.* **2008**, *130*, 7822-7823.

56) Zaugg, H. E. *J. Am. Chem. Soc.* **1950**, *72*, 2998-3001.

57) Barnett, W. E.; McKenna, J. C. *J. Chem. Soc., Chem. Commun.* **1971**, *11*, 551-551.

58) Mulzer, J.; Bruntrup, G.; Chucholowski, A. *Angew. Chem.* **1979**, *91*, 654-655.

59) Nicolaou, K. C.; Lysenko, Z. *J. Am. Chem. Soc.* **1977**, *99*, 3185-3187.

60) Diassi, P. A.; Dylion, C. M. *J. Am. Chem. Soc.* **1958**, *80*, 3746-3748.

61) Masamune, S.; Hayase, Y.; Wan, K. C.; Sobsczak, R. L. *J. Am. Chem. Soc.* **1976**, *98*, 7874-7874.

62) Adam, W.; Albert, R.; Grau, N. D.; Hasemann, L.; Nestler, B.; Peters, E. M.; Peters, K.; Prechtl, F.; Von Schnering, H. G. *J. Org. Chem.***1991**, *56*, 5778-5781.

63) Danheiser, R. L.; Nowick, J. S. *J. Org. Chem.***1991**, *56*, 1176-1185.

64) Cho, S. W.; Romo, D. *Org. Lett.* **2007**, *9*, 1537-1540.

65) Mitchell, T. A.; Romo, D. *J. Org. Chem.* **2007**, *72*, 9053-9059.

66) Jenkins, S. I. Dept. of Chemistry, McMaster University, **2003**, PhD. Thesis.

67) Khandker, J. A. Dept. of Chemistry, McMaster University, **2008**, M.Sc. Thesis.

68) Ramer, S. E.; Moore, R. N.; Vederas, J. C. *Can. J. Chem. Soc.* **1986**, *64*, 706-713.

69) Arnold, L. D.; Kalantar, T. H.; Vederas, J. C. *J. Am. Chem. Soc.* **1985**, *107*, 7105-7109.

70) Nelson, S. G.; Spencer, K. L.; Cheung, W. L.; Mamie, S. J. *Tetrahedron.* **2002**, *58*, 7081-7091.

71) Zhang, W.; Romo, D. *J. Org. Chem.* **2007**, *72*, 8939-8942.

72) Majima, M.; Kuribayashi, Y.; Ikeda, Y.; Adachi, K.; Kato, H.; Katori, M, Aoyagi, T. *Jpn. J. Pharm.* **1994**, *65*, 79-82.

73) Ostrawska, H.; Dabrowska, M.; Osada, J.; Manthur, M. *Ann. Acad.Biol.* **2003**, 150-153.

74) Koller, W.; Trail, F.; Parker, D. M. *J. Antibiot.* **1990**, *48*, 734-735.

75) Wright, G; Harrison, P. Unpublished Results.

76) Uotani, K.; Naganawa, H.; Aoyagi, T.; Umezawa, H. *J. Antibiot.* **1982**, *35*, 1670-1674.

77) Ahilan, Y.; Harrison, P. Unpublished Results.

78) O'Hagan, D. *Nat. Prod. Rep.* **1986,** *6,* 205-219.

79) Parenty, A.; Moreau, X.; Campagne, J. M. *Chem. Rev.* **2006,** *106,* 911-939.

80) Bergmann, S.; Schumann, J.; Scheltach, K.; Lange, C.; Brakhage, A. A.; Hertweck, C. *Nat. Chem. Biol.* **2007,** *3,* 213-217.

81) Vederas, J. C. *Nat. Prod. Rep.* **1987**, *4*, 277-377.

82) Vedaras, J. C.; Nakashima, T. T. *J. Chem. Soc. Chem. Commun.* **1980**, 183-185.

83) Cane, D. E.; Yang, C. C. *J. Am. Chem. Soc.* **1984,** *106*, 784-787.

84) Uotani, K.; Naganawa, H.; Kondo, S.; Aoyagi, T.; Umezawa, H. *J. Antibiot.* **1982**, *35*, 1495-1499.

85) Jokisaari, J. *Org. Mag. Res.* **1978**, *11*, 157-159.

86) Bailey, A. M; Cox, R. J.; Harley, K.; Lazarus, C. M.; Simpson, T. J.; Skellam, E. *Chem.Commun.* **2007**, 4053-4055.

87) Harrison, P. H.; Noguchi, H.; Vederas, J. C. *J. Am. Chem. Soc.* **1986**, *108*, 3833-3834.

88) Yue, S.; Duncan, J. S.; Yamamoto, Y.; Hutchinson, Y. R. *J. Am. Chem. Soc.* **1987**, *109*, 1253-1255.

89) Cane, D. E, Yang, C. *J. Am. Chem. Soc.* **1987**, *109*, 1255-1257.

90) Cortes, J.; Weismann, K. E. H.; Roberts, G. A.; Brown, M. J. B.; Staunton, J.; Leadley, P. F., *Science*, **1995**, *268*, 1487-1489.

91) Kao, C. M.; Luo, G.; Katz, I.; Cane, D. E.; Khosla, C. *J. Am. Chem. Soc.* **1995**, *117*, 9105-9106.

92) Bohm, I.; Holzbaur, I. E.; Hanefeld, U.; Cortes, J.; Staunton, J.; Leadley, P. F. *Chem Biol.*, **1998**, *5*, 407-412.

93) Corey, E. J.; Li, W.; Reichard, G. A. *J. Am. Chem. Soc.* **1998**, *120*, 2330-2336.

94) Sambrook, J.; Russel, D. W. *Molecular Cloning*. Cold Spring Harbor Laboratory Press. **2001**, 3rd Ed, A1.5.

95) Dick, C. R.; Cruikshank, A. A.; Grenier, I.; Melandri, F. D.; Nunes, S. L.; Stein, R. L. *J. Biol. Chem.* **1996**, *271*, 7273-7276.