The Biosynthesis of Oudenone, a Hypotensive Agent from *Oudemansiella radicata*.

Parsa Famili

A Thesis
in
the Department
of
Chemistry and Biochemistry

Presented in Partial Fulfilment of the Requirements
for the Degree of Master of Science
at Concordia University
Montreal, Quebec
Canada

June 1993

ISBN 0-315-87315-9

Abstract

The biosynthesis of oudenone, a hypotensive agent from *Oudemansiella radicata.*

Parsa Famili

Oudenone (**14**) is a potent inhibitor of catecholamine biosynthesis, and as such it induces hypotension in spontaneously hypertensive rats. The 1,3-cyclopentanedione and tetrahydrofuran ring moieties of **14** are uncommon among fungal metabolites. Therefore, the biosynthetic origin of such structures are of interest. At the present time, the ^{1}H and ^{13}C chemical shifts of **14** have been unambiguously assigned. In addition, it has been shown that oudenone is formed by the condensation of a tetraketide with one succinate unit.

To Mata, Afzal, Mazda, Mamman

and to Deedee

for putting up with me through it all.

Acknowledgements:

First and foremost, I would like to thank Dr. Y. S. Tsantrizos, my supervisor, for constant encouragement, and motivation throughout the project.

I would also like to thank Dr. O. S. Tee, Dr. Paul Joyce and Dr. L. D. Colebrook, my thesis committee, for their wise advice.

I also appreciate the help of my friends and colleagues throughout the project and during the preparation of this thesis.

Table of Contents

Chapter 3: Experimental

List of Schemes

List of Figures

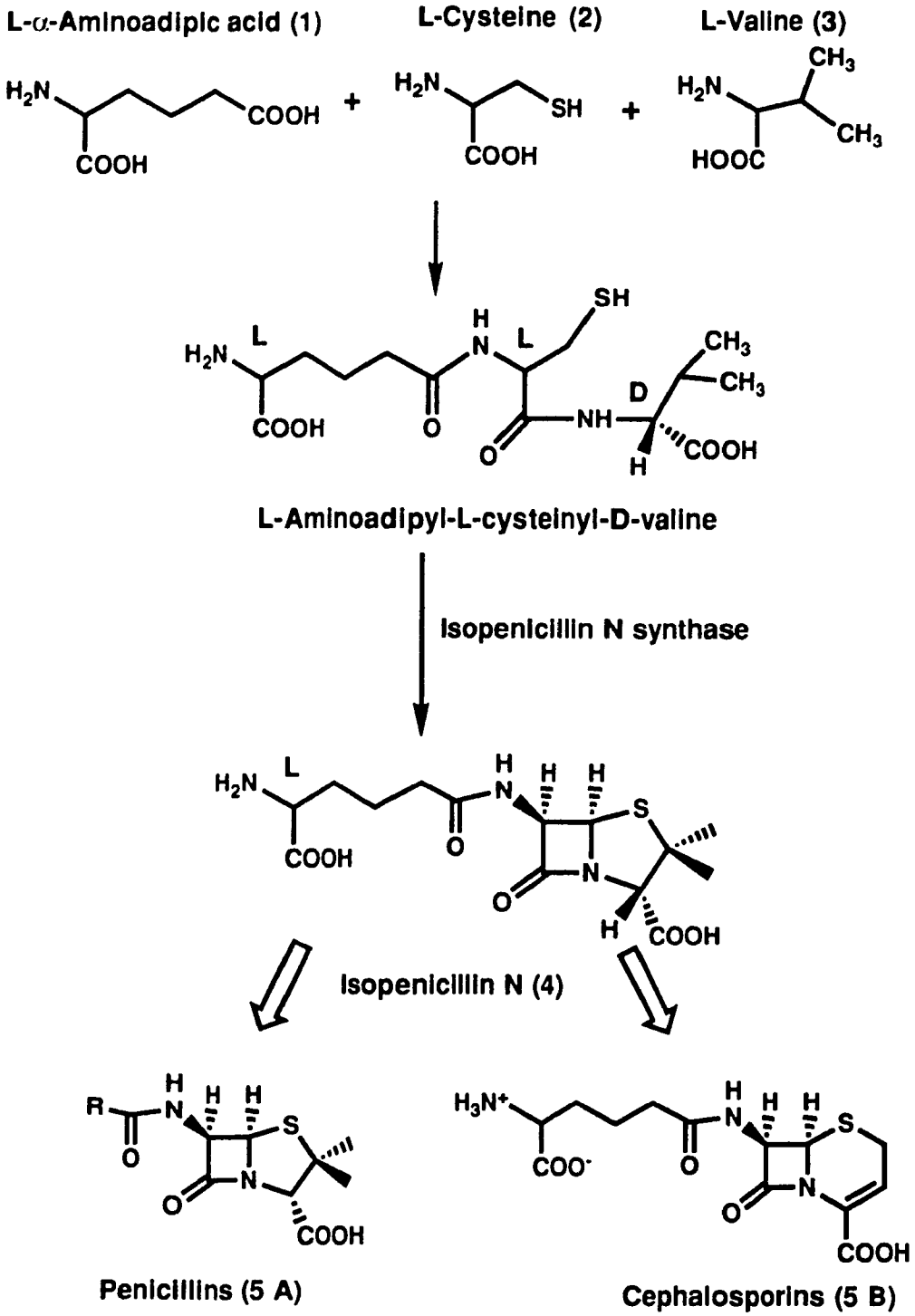
List of Tables

CHAPTER 1 : INTRODUCTION

[1.1] General Introduction

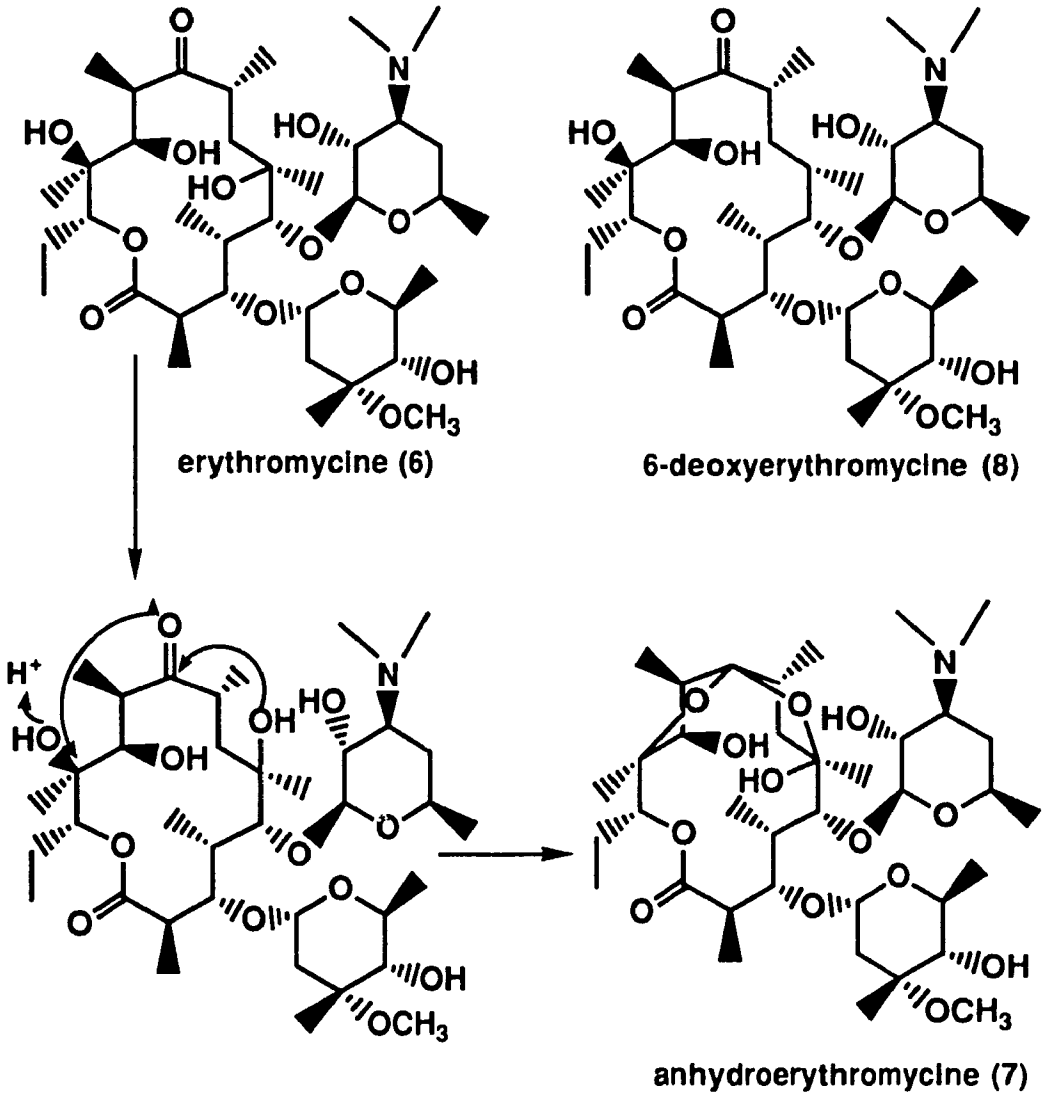
The quest for knowledge is one of the driving forces of humankind. It is the nature of human beings to ask questions. Every question initiates a search for an answer and every answer brings about several new questions. Humankind has always been wondering how nature functions and how it can be used to advance technology and medicine. It is one of our ongoing goals to understand nature at the molecular level, which includes studies on the biosynthesis of secondary metabolites. Such studies provide us with an insight into how nature produces diverse and complex compounds. Secondary metabolites are synthesized naturally from a handful of primary metabolites such as amino acids, acetyl CoA, mevalonic acid, and intermediates of the shikimic acid pathway.[1]

The mechanisms of the biosynthetic pathway can permit us to develop more efficient methods for the total synthesis of bioactive metabolites using biotechnology. There are already a few examples where the same natural machinery has been used in the synthesis of structural analogues. One such example is the work of Baldwin *et al.*[2] who were able to produce various "unnatural" β-lactam antibiotics from false precursors and the purified enzyme isopenicillin N synthase. β-Lactam antibiotics are produced from a tripeptide synthesized from L-amino adipic acid (**1**), L-cysteine (**2**), and L-valine (**3**). This tripeptide undergoes cyclization by isopenicillin N synthase, a ferrous dependent non-heme enzyme, to produce the isopenicillin N (**4**), which is the universal precursor of β-lactam antibiotics (Scheme 1).



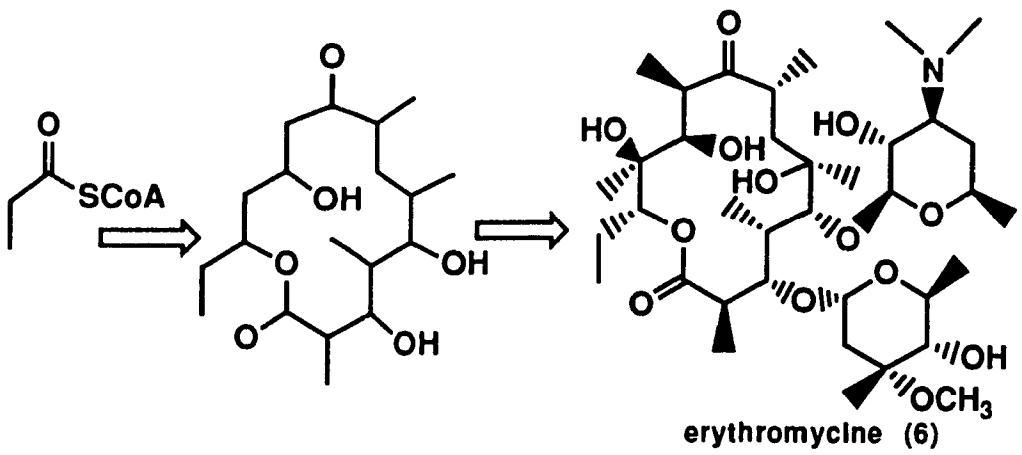
Scheme 1: The biosynthesis of β-lactam antibiotics.

Biosynthetic studies at the enzymatic level have provided significant contributions in the fields of Chemistry and Medicine. For example, the important antibiotic erythromycine A (**6**) is fairly unstable in acidic solution: in an aqueous solution of citrate buffer (pH 2.2) it undergoes 95% inactivation within 2 minutes. Therefore large quantities must be consummed for effective control of infections. The inactivation of erythromycine is believed to occur *via* intermolecular rearrangement, involving the C-6 hydroxyl group, into inactive anhydroerythromycine (**7**) (scheme 2).



Scheme 2: The mechanism of erythromycine deactivation.

Cane *et al.*[3] demonstrated that erythromycine A (**6**) is biosynthesized from 7-propionate units (scheme 3). The enzymes and genes involved in the biosynthesis were later identified by Weber *et al.*[4]

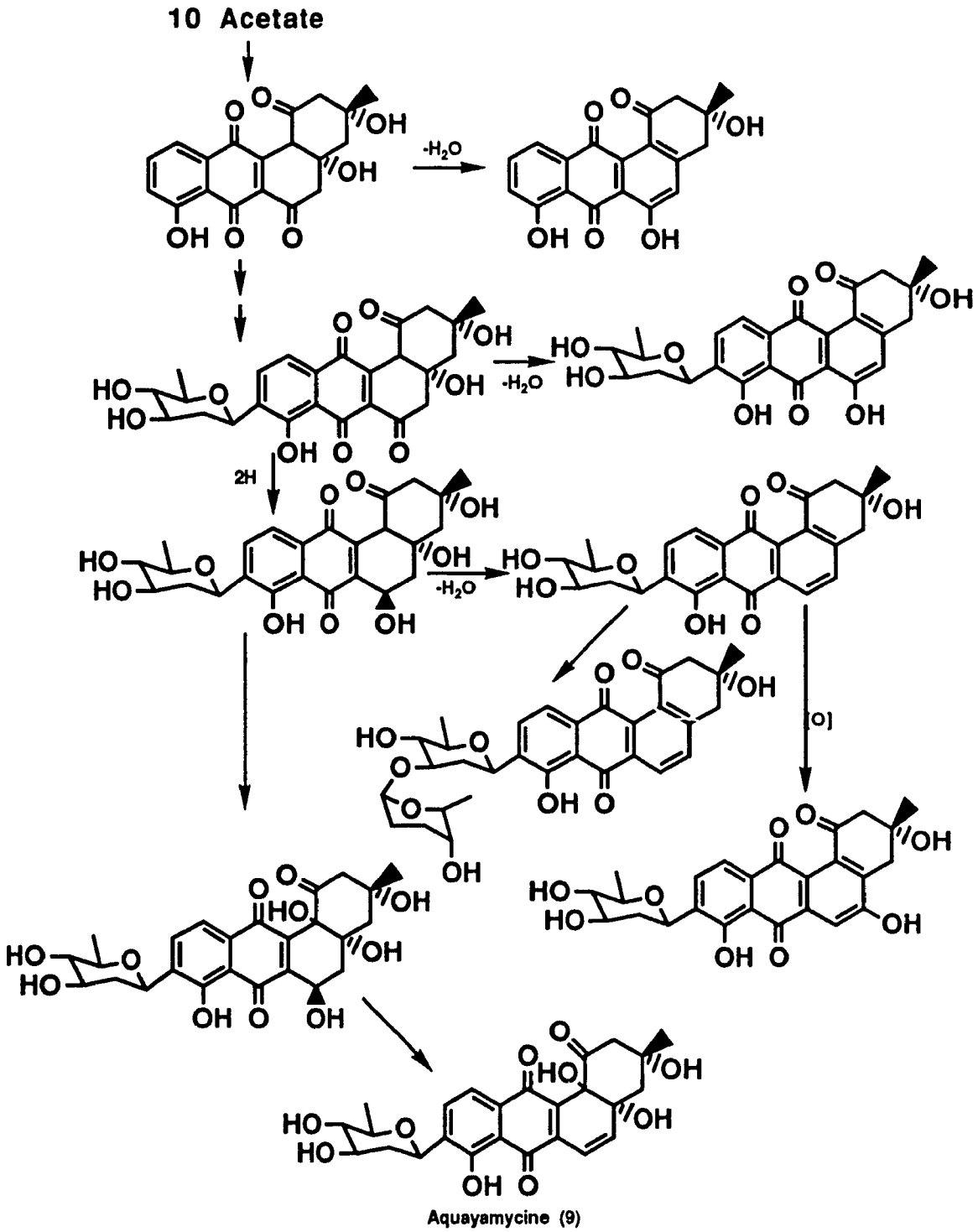


erythromycine (6)

Scheme 3: Biosynthesis of erythromycine A (6).

Site-specific mutation of the erythromycine-producing gene in the bacterium *Saccharopolyspora erythrae* has produced a 6-deoxy derivative of erythromycine (**8**). The 6-deoxy derivative **8**, is much more stable to the acidic pH of the stomach. This example clearly demonstrates the role of biosynthetic studies in the development of novel pharmaceutically important natural products.

Experiments using genetically-altered microorganisms have also been very useful in biosynthetic investigations.[5] These experiments demonstrate the role of mid-stage biosynthetic steps in the formation of natural products. For example, the biosynthetic steps in the formation



Scheme 4: The biosynthesis of aquayamycin (9).

of aquayamycin (**9**), a member of the angucycline group of antibiotics, have recently been investigated using ^{18}O-labelled precursors and identification of the metabolites produced by blocked mutants (Scheme 4).[5,6]

Biosynthetic studies depend to a great extend on advanced NMR techniques and the use of stable isotopes such as ^{13}C, ^{2}H, ^{18}O, ^{15}N. Applications of different NMR techniques can provide evidence for the biosynthetic scheme of a secondary metabolite as well as support for the enzymatic mechanisms involved in its formation.

[1.2] Polyketide Biosynthesis

Several natural products contain repeating acetate units.[7] Experiments using isotope labelling,[8,9] mutant organisms[10,11] and enzyme inhibitors[12,13] support the current belief that polyketide biosynthesis is catalyzed primarily by a multiple enzyme complex analogous to fatty acid synthase.[14] The assembly process is similar to fatty acid biosynthesis, except that reductive steps are bypassed in specific cycles allowing the incorporation of keto, hydroxyl, and olefinic functionalities into the growing polyketide chain.

The biosynthetic precursors for polyacetyl polyketide biosynthesis are acetyl CoA (**10**) and malonyl CoA (**11**). Acetyl CoA is formed from pyruvate in the mitochondrial matrix via the action of the pyruvate dehydrogenase complex, the zwitterion of coenzyme thiamine pyrophosphate and enzyme-bound lipoic acid.[1] Malonyl CoA is synthesized by the carboxylation of acetyl CoA (Figure 1).[15]

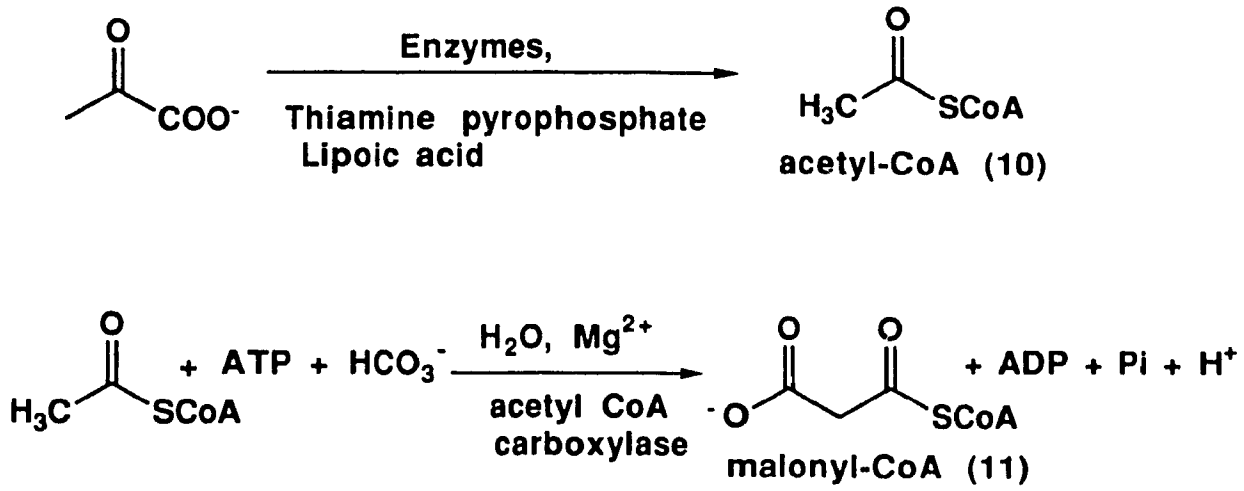


Figure 1: Acetyl CoA and malonyl CoA Synthesis.

Most intermediates of polyacetyl polyketide biosynthesis are thioesters of the acyl carrier protein (ACP) (**12**).[14] ACP, along with six catalytic enzymes, compose the polyacetyl polyketide synthase enzyme complex (**13**). The function of ACP is to act as an anchor for the growing polyketide chain. The chains are linked to the sulfhydryl terminus of the phosphopantetheine group attached to a serine residue of ACP (Figure 2).

The reactions of polyacetyl polyketide synthase are initiated by the transfer of one acetate unit from the thiol group of 4' phosphopantetheine side chain of the coenzyme A to that of ACP (**12**). This reaction is catalyzed by ACP-acyl-transferase, one of the six catalytic enzymes of the polyacetyl polyketide synthase. The acetyl

group is then transferred onto the cysteine residue of the β-keto-acyl-ACP-synthase enzyme.

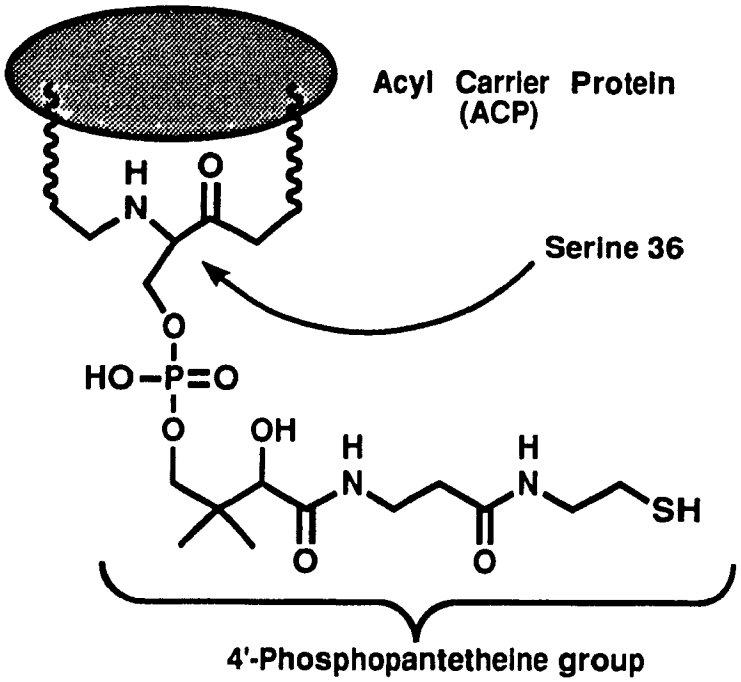


Figure 2: ACP (12) side chain.

The elongation step involves the condensation of a malonyl CoA unit with the enzyme-bound acetyl group catalyzed by the β-ketoacyl-ACP synthase enzyme. The acetoacetyl-ACP which is produced can undergo reduction of the β-carbonyl group to the hydroxyl (catalysed by β-ketoacyl reductase), elimination of the OH group and formation of an unsaturated hydrocarbon chain (catalyzed by enoyl-ACP hydratase), reduction of the double bond to give a saturated hydrocarbon chain

(catalysed by enoyl-ACP reductase) (Figure 3).[1] The chain elongation step can be repeated many times until the predetermined length of the chain is formed, with the necessary functional groups. After the last step, the chain is either cyclized or transferred to coenzyme A and released from the polyacetyl polyketide synthase complex.

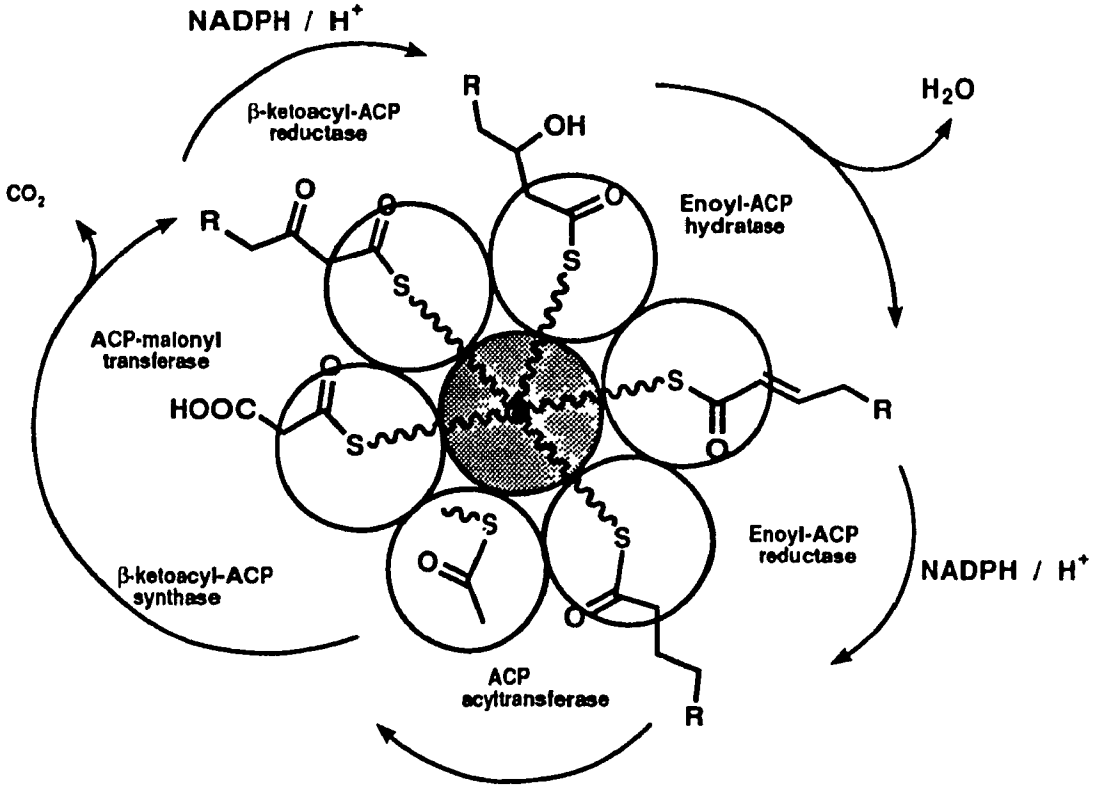


Figure 3: The polyacetyl polyketide synthase complex (13).

[1.3] Oudenone (14)

Oudenone is a fungal metabolite which was first isolated from the liquid culture of *Oudemansiella radicata*.[16] This metabolite inhibits tyrosine hydroxylase and phenylalanine hydroxylase, two key enzymes in the catecholamine biosynthetic pathway.[17] Oudenone inhibits these enzymes by competitively binding to their active site and preventing the binding of their coenzyme tetrahydropterin (Figure 4).[17]

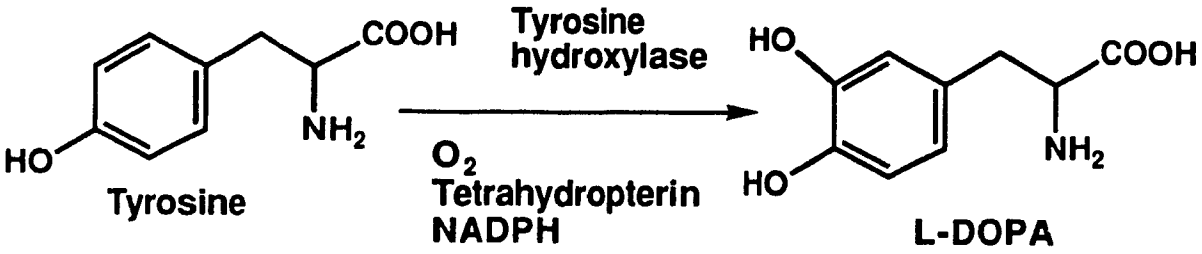
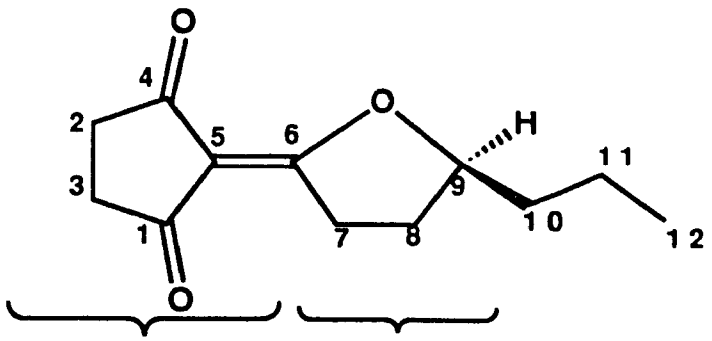


Figure 4: The rate limiting step in the biosynthesis of catecholamines.

The structure of Oudenone (**14 A**) consists of a 1,3-cyclopentanedione and a tetrahydrofuran moieties, which is quite uncommon among fungal metabolites.



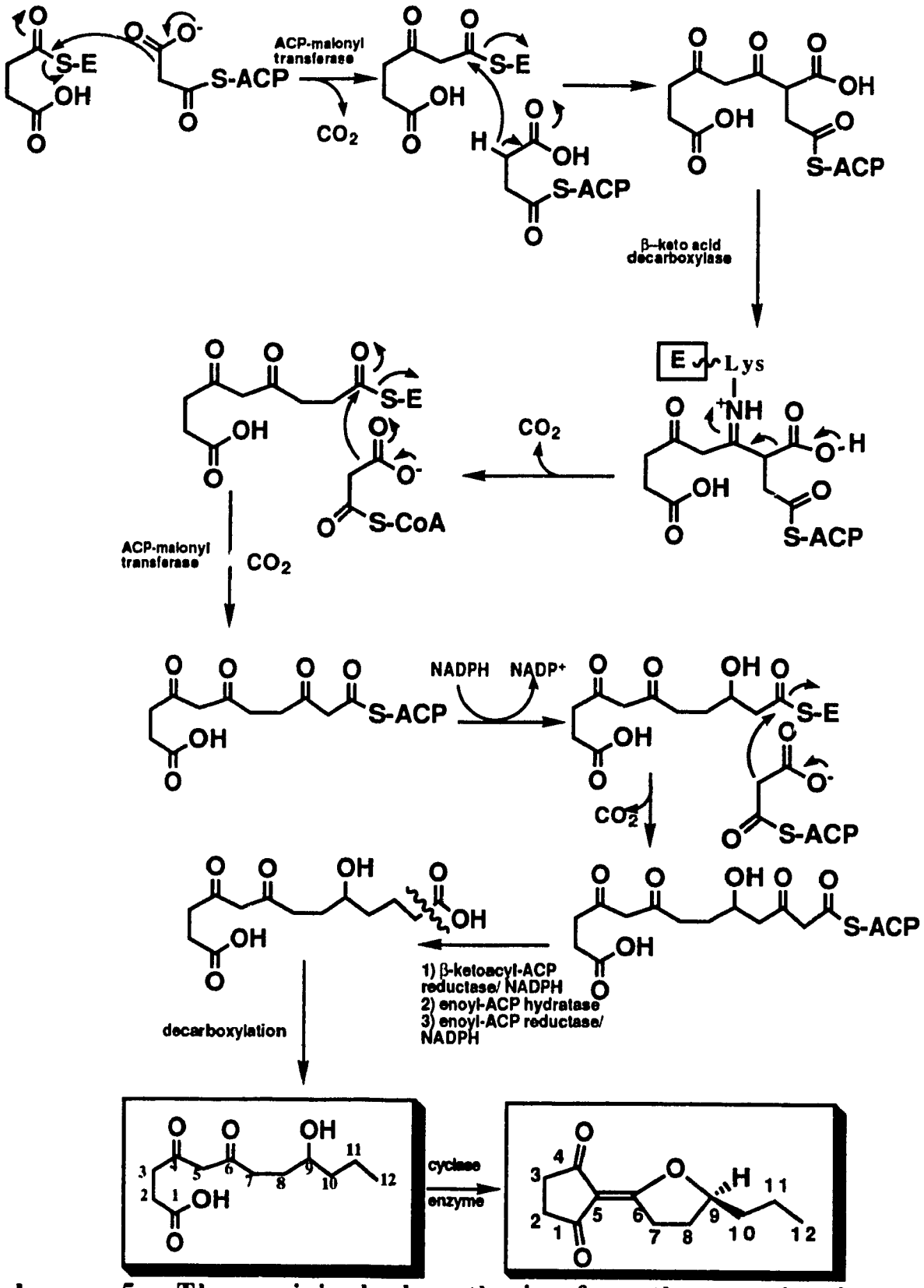
1,3 cyclopentanedione Tetrahydrofuran

Oudenone (14 A)

Based on what is generally known about polyketide biosynthesis, we proposed that oudenone is synthesized by the condensation of three malonyl CoA units with two succinyl CoA units (Scheme 5). The experimental data indicated that oudenone is biosynthesized by the condensation of only one succinate unit and four acetate units, hence a modified proposal will be discussed later (Scheme 7).

[1.4] Objectives

The objectives of this project were to:
(**a**) assign the carbon skeleton of oudenone,
(**b**) determine the production curve of oudenone and,
(**c**) study the biosynthetic pathway of oudenone using ^{13}C-labelled primary precursors.



Scheme 5: The original hypothesis for the production of oudenone.

CHAPTER 2 : RESULTS AND DISCUSSIONS

[2.1] Isolation, purification and NMR assignment of oudenone

Liquid cultures of *O. radicata* grown for 17 days on 500 mL media were extracted with butanol, and the butanol was evaporated under reduced pressure. The remaining syrup was partitioned between ethyl acetate and water (pH 7.0). The pH of the aqueous layer was then adjusted to 3.0 and the oudenone was extracted with ethyl acetate. The crude oudenone was further purified by reversed phase flash column chromatography.[18,19,20] The general isolation scheme of oudenone is illusterated in Scheme 6.

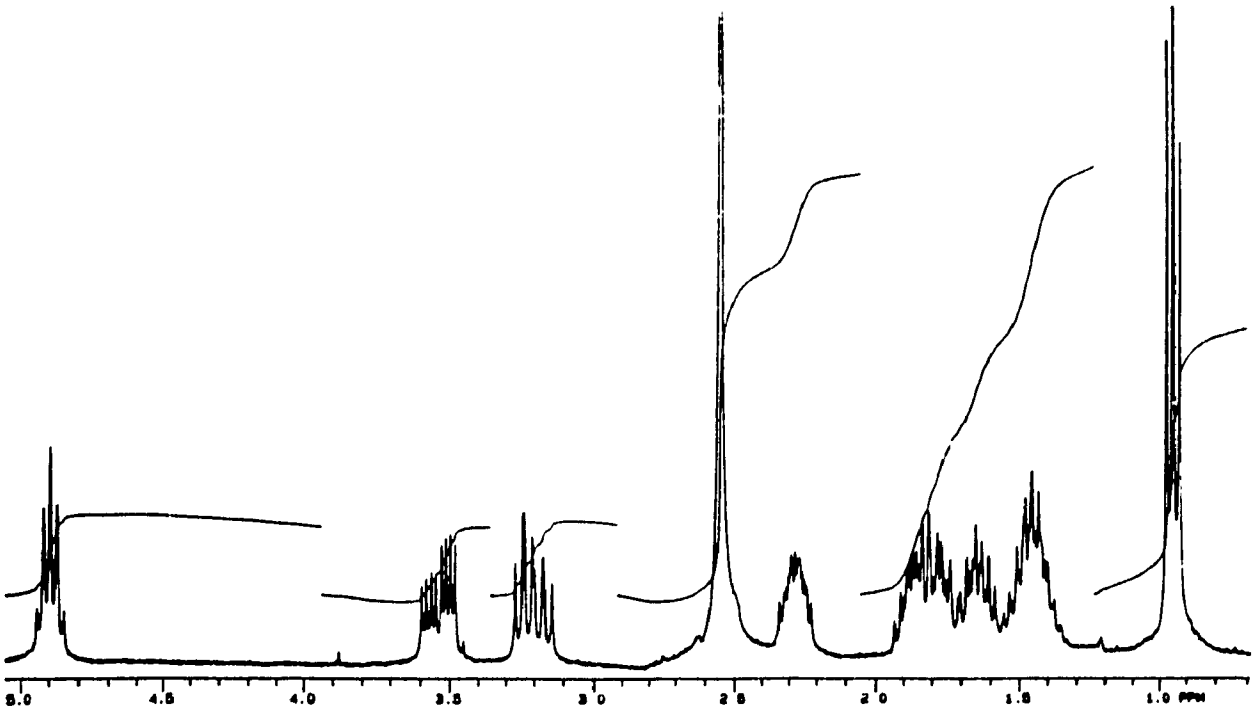
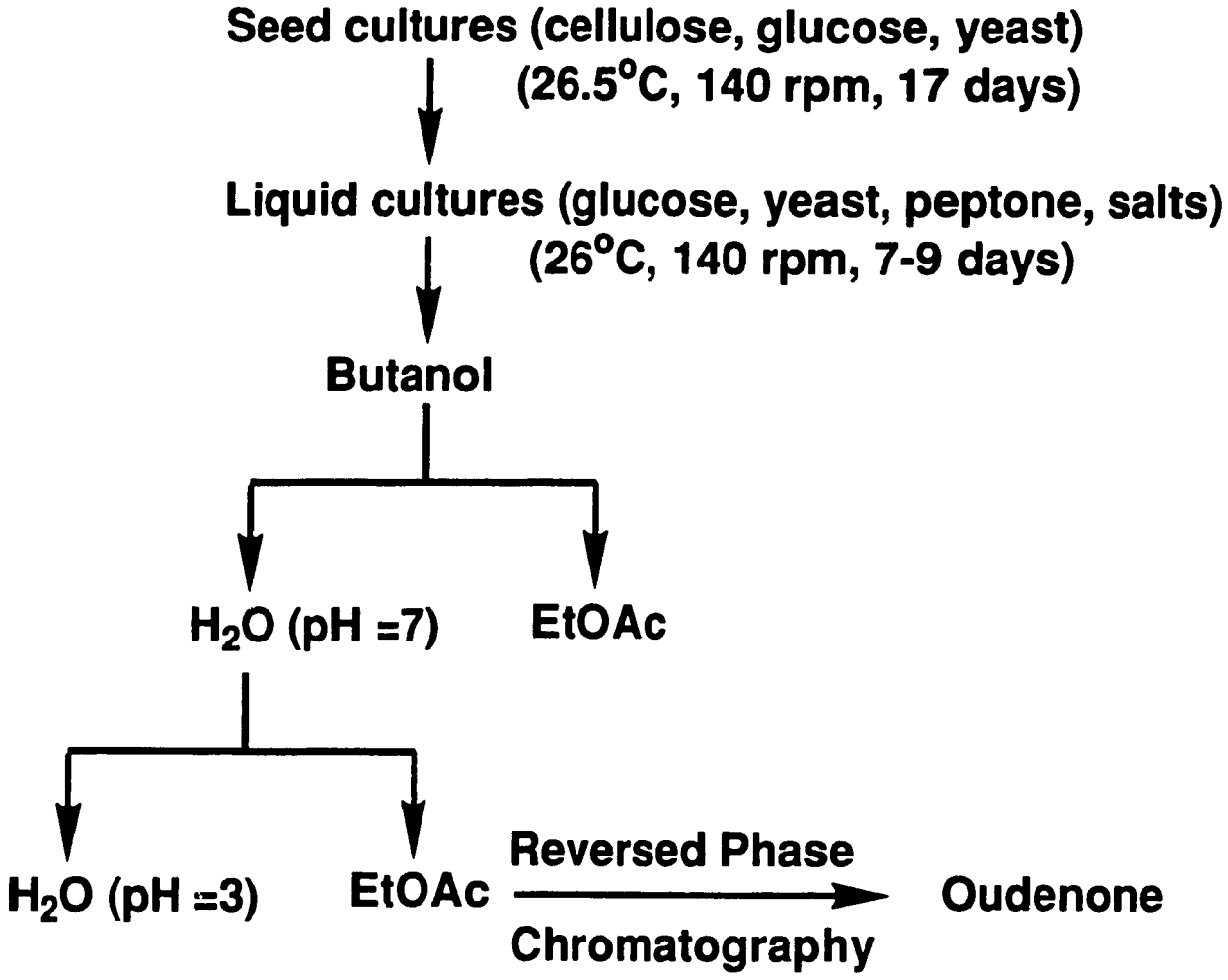


Figure 5: Proton spectra of oudenone (14 A) in $CDCl_3$.



Scheme 6: Isolation of oudenone from *Oudemansiella radicata*.

Table 1: NMR data of oudenone (14 A).

	^{1}H δ (ppm)	COSY	^{13}C δ (ppm)	HETCOR
C-1			200.7	
C-2	2.55(s)		34.4	H2
C-3	2.55(s)		34.8	H3
C-4			204.3	
C-5			109.1	
C-6			184.4	
C-7	3.2-3.5(m)	H8	33.6	H7
C-8	1.7-2.3(m)	H7,H9	26.7	H8
C-9	4.89(q)	H8,H10	90.3	H9
C-10	1.6-1.9(m)	H9,H11	36.6	H10
C-11	1.45(m)	H10,H12	18.5	H11
C-12	0.95(t)	H11	13.7	H12

The ^{1}H NMR (Figure 5, Table 1), similar to that described by Ohno *et al.*[21] could be only partially assigned. The triplet of 3H at δ 0.9 was assigned to the methyl group (C-12). The singlet of 4H at δ 2.5 arose from 2H2 and 2H3, and the multiplet at δ 4.9 was assigned to H9.

A COSY NMR data (Figure 6), was used to unambiguously assign the remaining proton signals. The NMR spectrum illustrated that the H12 protons at δ 0.95 were coupled to the H11 at δ 1.45. The protons of C-11 were coupled to the two non-equivalent H10 protons at δ 1.6 and 1.9. The protons of C-10 were coupled to H9 at δ 4.89. The proton of C-9 was coupled to the two non-equivalent protons of C-8 at δ 1.6 and 1.9. The protons of C-8 were coupled to two non-equivalent H7 protons at δ 3.2 and 3.5. All the data obtained from the COSY experiment are shown in Table 1.

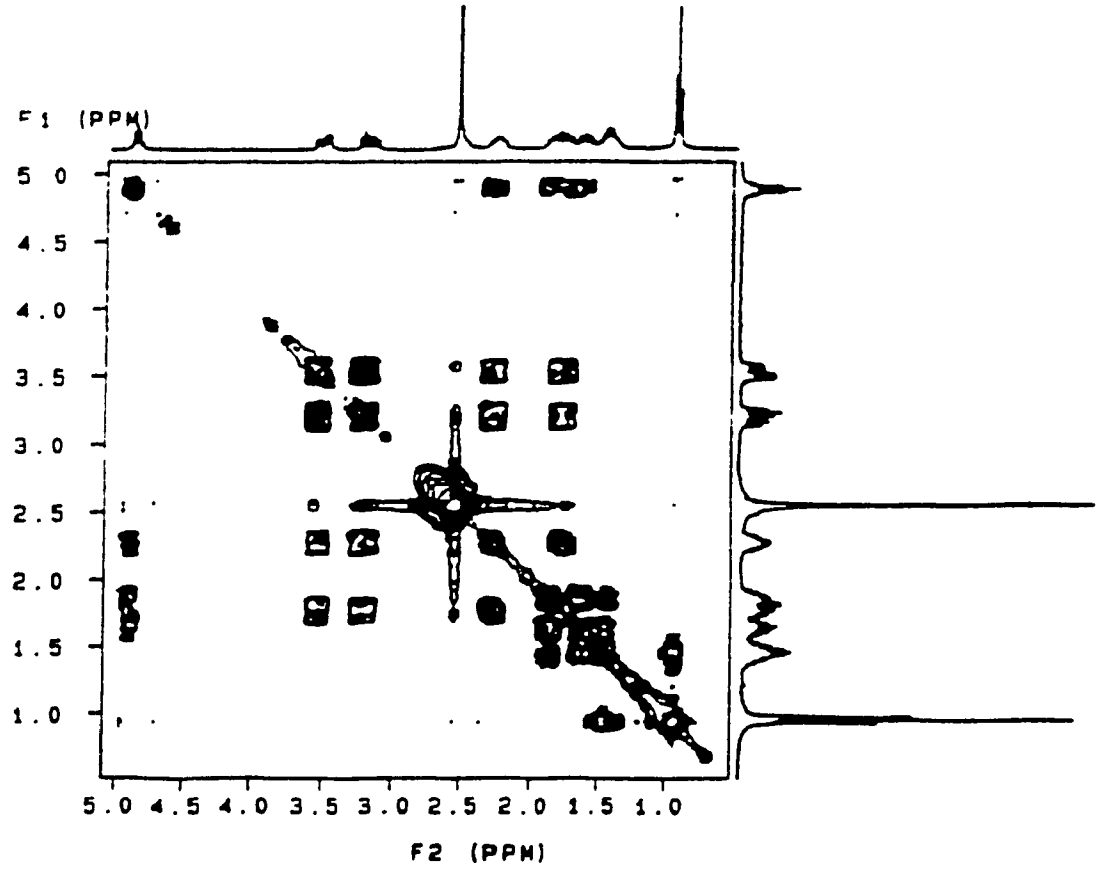


Figure 6: The COSY NMR spectra of oudenone in $CDCl_3$.

Despite the previous chemical synthesis of oudenone,[21,22,23] the ^{13}C NMR spectrum had not been assigned. For the purpose of our experiments it was absolutely essential to assign all chemical shifts of oudenone unambiguously. In the ^{13}C NMR spectrum of oudenone (Figure 7), the signals at δ 200 and 204 ppm were assigned to the carbonyls of C-1, and C-4. The signal at δ 185 corresponded to C-6 (a quaternary carbon adjacent to an oxygen) and the signal at δ 109 was assigned to the olefinic quaternary carbon C-5. A HETCOR NMR was used to assign the chemical shifts of the remaining carbon signals.

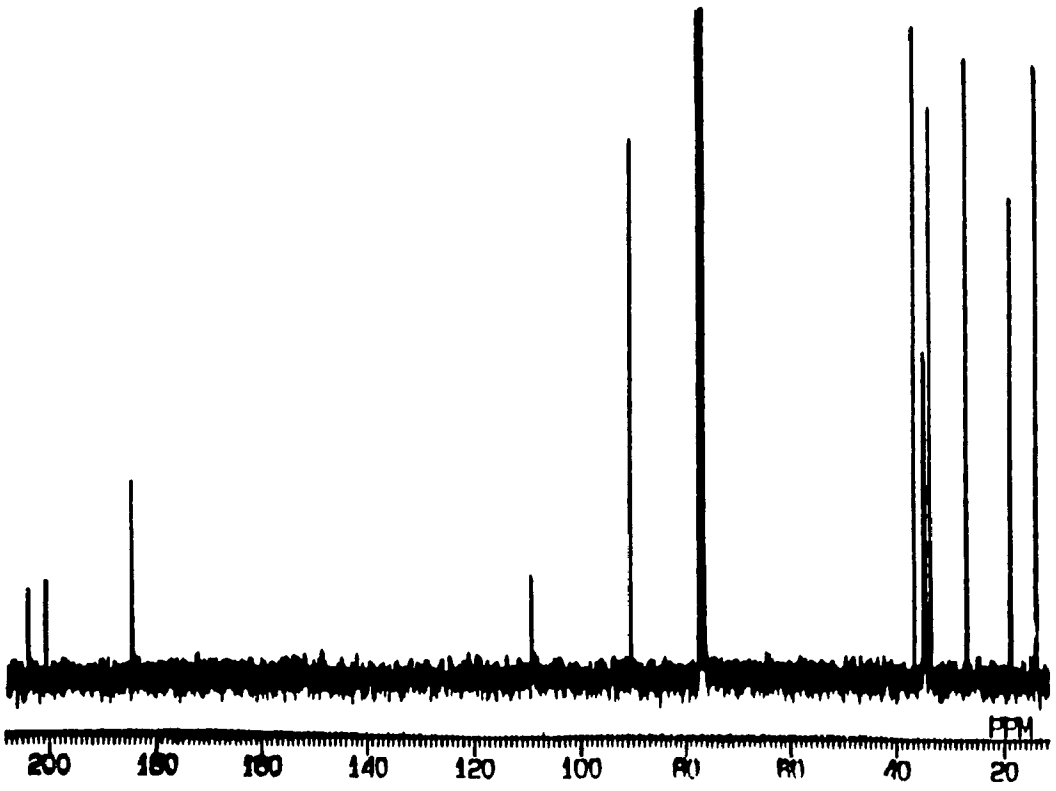


Figure 7: ^{13}C spectrum of oudenone in $CDCl_3$.

The HETCOR NMR spectrum of oudenone (Figure 8) demonstrated that the carbon at δ 13.7 was coupled to the triplet at δ 0.9 , therefore it was assigned to C-12. The carbon at δ 18.5 was assigned to C-11 since it was coupled to the multiplet at δ 1.44. The carbon at δ 36.6 was coupled to two multiplets at δ 1.6 and at δ 1.9, hence it was identified as C-10. The assignment data obtained from the HETCOR experiment is shown in Table 1. It was not possible to differentiate the signals at δ 34.4 and 34.8 (belonging to C-2 and C-3) since they were both coupled to the singlet at 2.55 ppm.

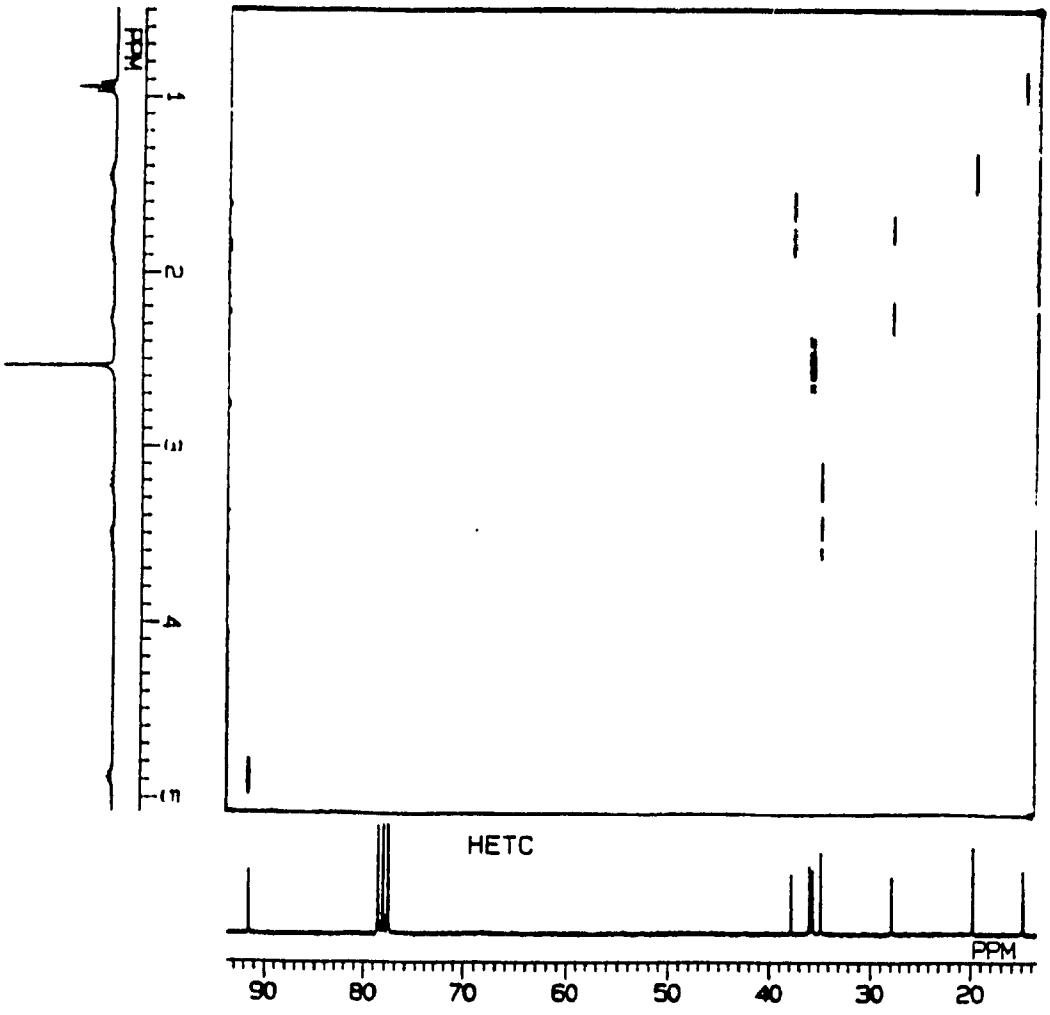


Figure 8: The HETCOR NMR spectra of oudenone in $CDCl_3$.

[2.2] Production curve of oudenone

The precise timing for the administration of labelled precursors is critical in achieving intact incorporation of the ^{13}C-labelled precursors.[8,12] In order to achieve incorporation of the ^{13}C-labelled precursors, it was necessary to determine the production curve of oudenone (Figure 9). For maximum incorporation, it was important to feed the precursors soon after the production of oudenone was initiated. Therefore the production of oudenone with respect to period of culture growth had to be determined (Figure 9). Early, or late feeding, could result in the random incorporation of the precursors along other biosynthetic or metabolic pathways such as Krebs cycle[24] and β-oxidative pathway.

The oudenone production curve was obtained by monitoring the UV of oudenone present in the liquid cultures. A sample was withdrawn from an actively fermenting culture, filtered and diluted with water. Two aliquots of the diluted solution were transferred into each of a phosphate buffer (pH 7.0) and 0.1 N HCl solution. The production of oudenone was estimated from the difference in the UV absorbance of the crude extract in buffer and that obtained in acid at 246 nm (Figure 10).[16]

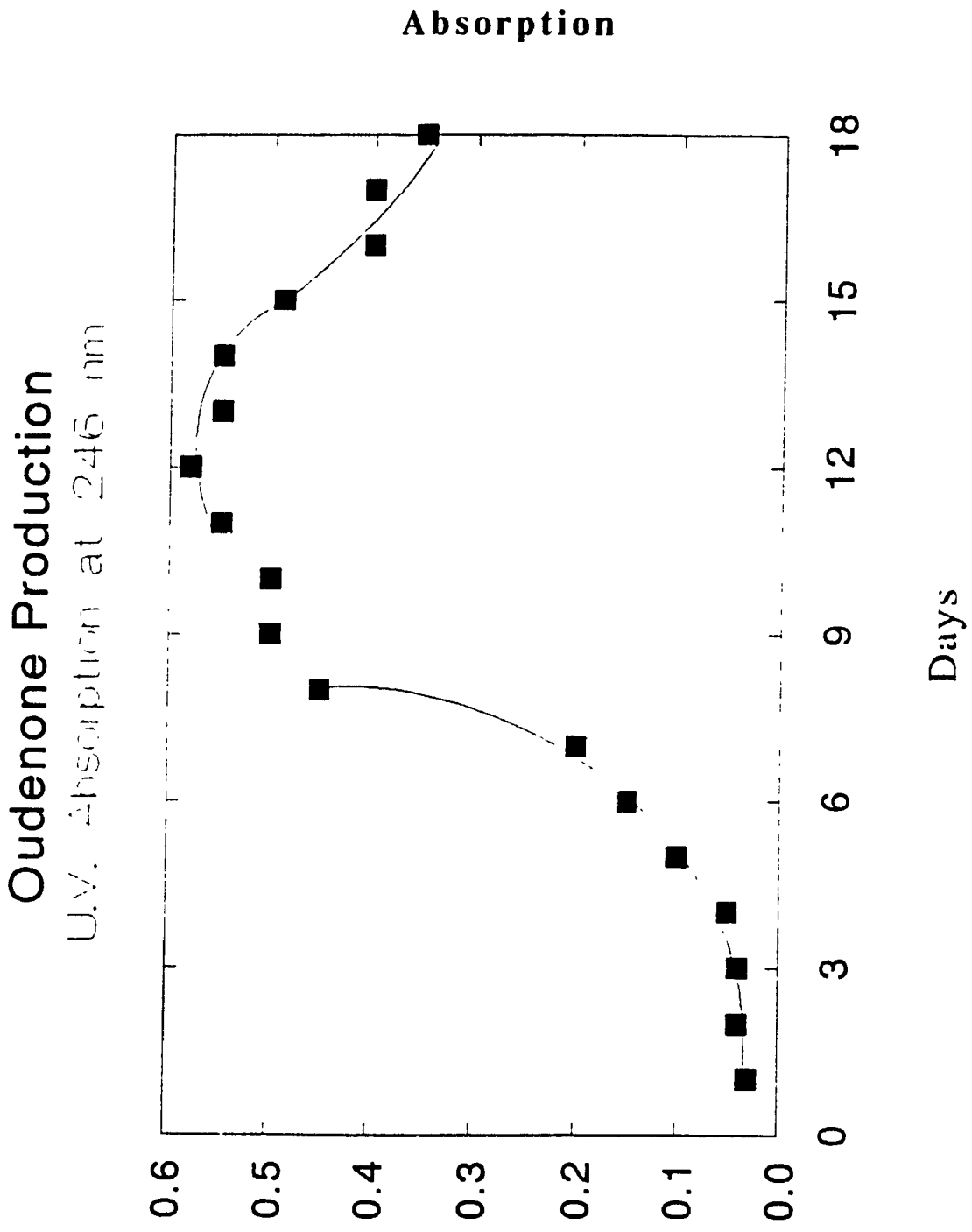


Figure 9: The production curve of oudenone.

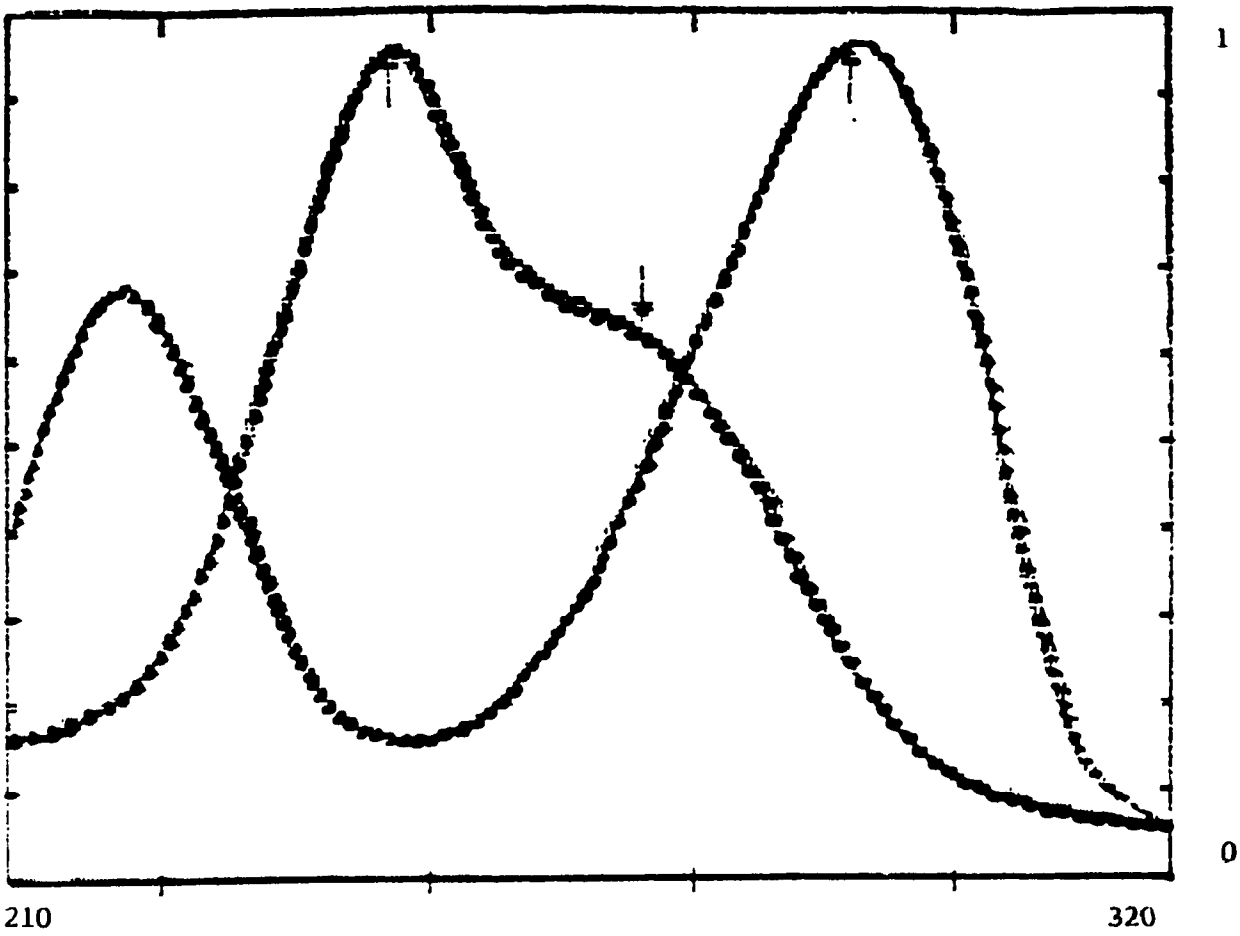
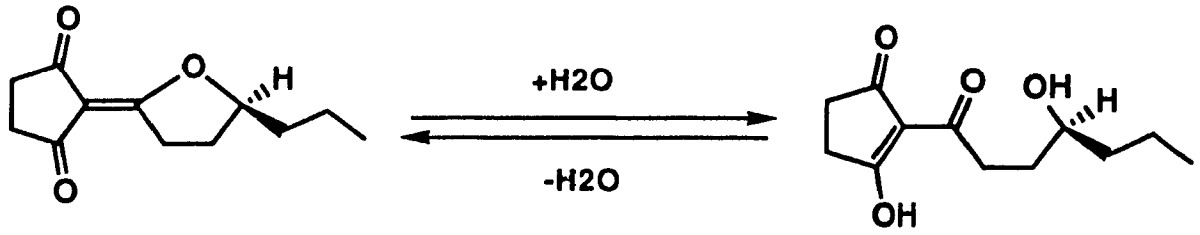


Figure 10: The ultraviolet spectrum of oudenone in neutral (phosphate buffer, pH 7) and acidic solution (0.1 N HCl).

The production curve of oudenone was obtained using the fact that oudenone could undergo a dynamic change to yield the open chain of oudenone (β-trione) by the addition of water (Figure 11).[17] Oudenone has a maximum UV absorbance at 246 nm in buffer (pH 7.0) which shifts to 284 nm in acid because of this structural change. Therefore, by subtracting the background UV absorbance at 246 nm obtained in acid

from that of oudenone plus background (obtained in pH 7), the amount of oudenone produced could be estimated.



(14 A) (14 B)

Figure 11: Equilibrium between the open (14 B) and closed (14 A) form of oudenone.

Oudenone production was usually first observed on days 5-7 and the production reached its maximum on days 7-11.

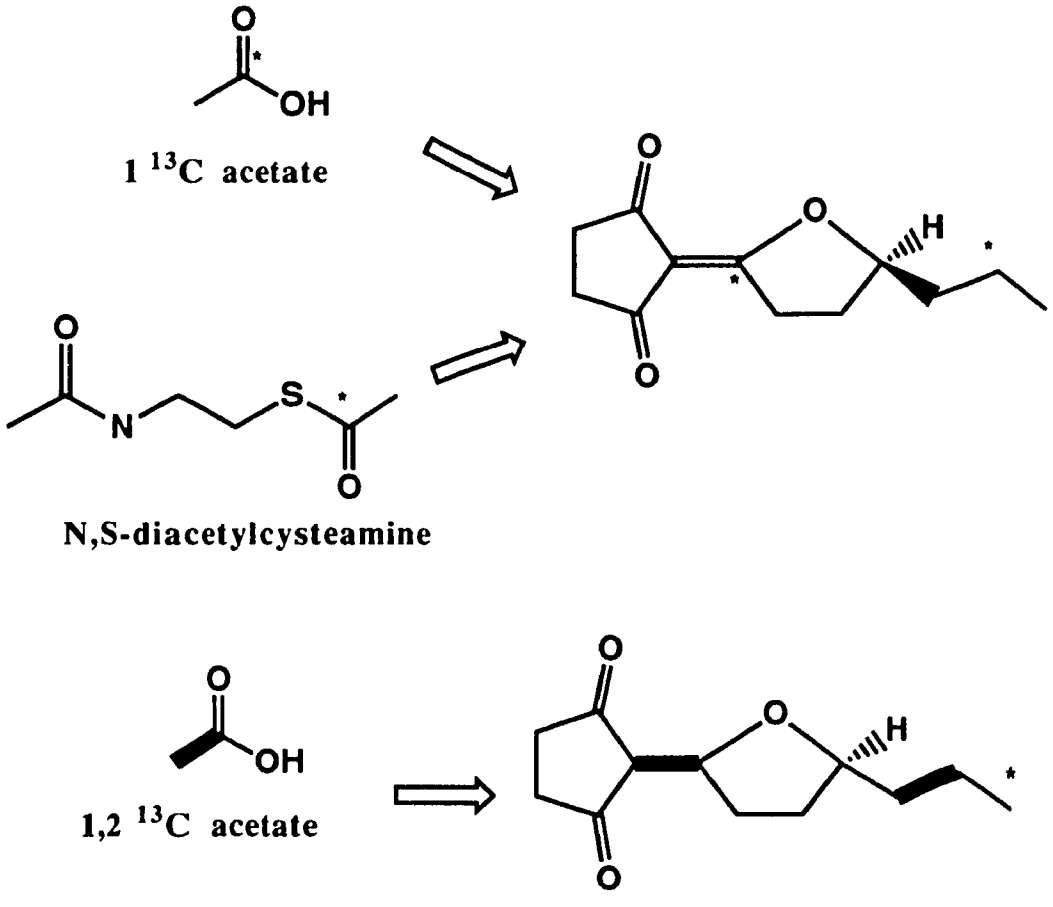
[2.3] Incorporation studies of simple precursors

[2.3.1] [1-^{13}C] Sodium Acetate

After studying the production curve of oudenone and characterizing the key chemical shifts of the NMR data, we were able to study the incorporation of labelled precursors. Since our initial proposal assumes the involvement of acetate, ^{13}C-sodium acetate was used in the first

feeding experiments. The maximum amount of acetate which did not adversely affect the production of oudenone using sodium acetate was established to be 100 mg per 500 mL flask fed in 2 doses. This amount was added to the liquid cultures on days 5-7 and the cultures were harvested after 48 hours.

Based on the proposed biosynthetic pathway (scheme 3) it was expected that feeding [1-^{13}C] acetate to the liquid cultures of *O. radicata* would result in signal enhancements at C-6 and C-11. Intact incorporation of acetate could then be demonstrated by feeding [1,2-^{13}C] acetate and observing J-coupling between C-5 and C-6, C-10 and C-11 (scheme 7).



Scheme 7: Proposed sites of acetate and N,S-diacetylcysteamine incorporation.

[1-^{13}C] Sodium acetate, dissolved in distilled water, was added to the 500 mL liquid culture of *O. radicata*, in two equal portions of 50 mg each. The data obtained from this experiment are shown in Table 2, columns 1 and 2. Incorporation was observed in C-11, C-9, C-7, and C-5. Random incorporation was also observed in experiments when the production of oudenone was very slow. The incorporation ratio was obtained by dividing the peak intensity ratio in labelled oudenone by the peak intensity ratio in unlabelled oudenone. The intensity ratio is defined as the intensity of the desired carbon divided by the intensity of a reference signal (C-12).

In order to obtain further support for our results, [1-^{13}C] labelled N-S-diacetylcysteamine was synthesized. The N,S-diacetylcysteamine derivatives are believed to mimic the side chain of acetyl CoA (Figure 2) and have been used before to enhance intact incorporation of polyketide precursors.[8, 12,25]

[2.3.2] [1-^{13}C] Thioester of N,S-diacetylcysteamine

^{13}C-Labelled N,S-diacetylcysteamine was synthesized *via* the reaction of [1-^{13}C] acetyl chloride with the thallium salt of N-acetylcysteamine (Scheme 8). The ^{1}H NMR of the ^{13}C labelled N,S-diacetylcysteamine showed the expected ^{13}C-^{1}H coupling between the carbonyl and the protons on the adjacent methyl group, therefore transforming the singlet at δ 2.38 in the unlabelled ^{1}H NMR to a doublet (Figure 12). The triplet (CH_2S) at δ 2.9 became a multiplet due to the ^{1}H-^{13}C coupling. The ^{13}C NMR of N,S-diacetylcysteamine illustrated an intense peak at δ 196 due to labelled thioester and ^{13}C-^{13}C coupling of

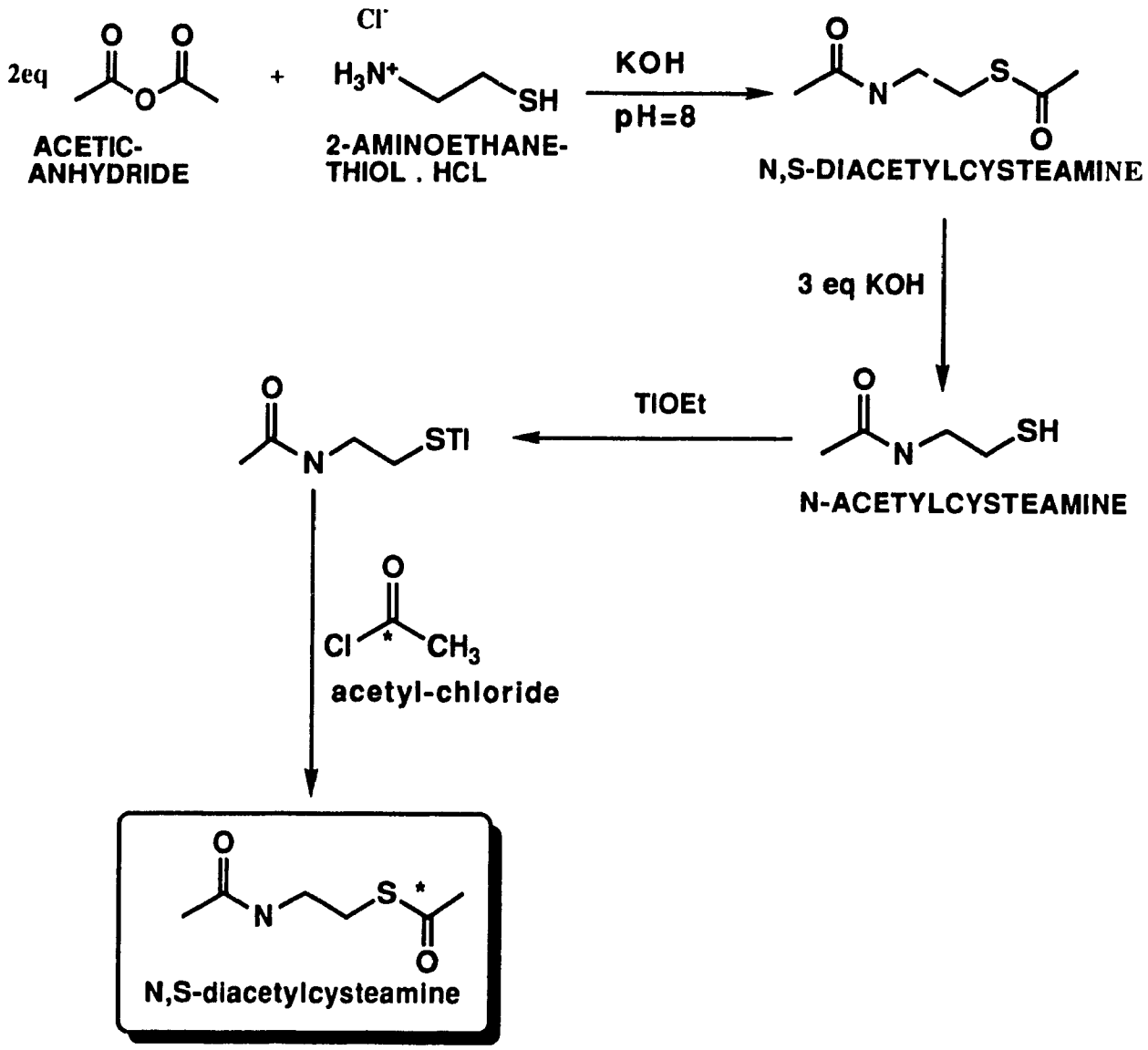
Table 2: Feeding experiments using [1-^{13}C] sodium acetate and ^{13}C labelled N,S-diacetylcysteamine.

		experiment 1	experiment 2	experiment 3	experiment 4
δ (ppm)		I.R*	I.R*	I.R*	I.R*
13.87	(C-12)	1	1	1	1
18.72	(C-11)	2.72	1.50	1.81	5.42
26.91	(C-8)	1.45	1.13	1.43	1.04
33.79	(C-7)	1.67	0.84	1.52	1.51
34.67	(C-2)	1.10	1.05	1.69	2.17
35.00	(C-3)	1.09	1.83	2.43	2.51
36.81	(C-10)	1.48	0.92	1.41	0.70
90.53	(C-9)	1.75	1.23	1.31	2.13
109.34	(C-5)	2.63	1.00	1.37	1.88
184.68	(C-6)	1.48	0.80	1.51	0.97
200.90	(C-1)	1.20	1.93	2.47	2.46
204.28	(C-4)	1.21	2.27	3.07	2.78

* Incorporation ratio = $\frac{\text{peak height ratio Cn/C12 in labelled oudenone}}{\text{peak height ratio Cn/C12 in unlabelled oudenone}}$

adjacent carbons (Figure 13). In order to confirm the ^{1}H-^{13}C coupling, a HETCOR NMR was performed (Figure 14).

Labelled N,S-diacetylcysteamine dissolved in 99% EtOH was fed in two equal portions to the liquid cultures of *O. radicata*. Once again, the ^{13}C NMR spectrum of the isolated oudenone demonstrated an incorporation at C-11, C-9, C-7 and C-5.



Scheme 8: The synthesis scheme for ^{13}C-labelled N,S-diacetylcysteamine.

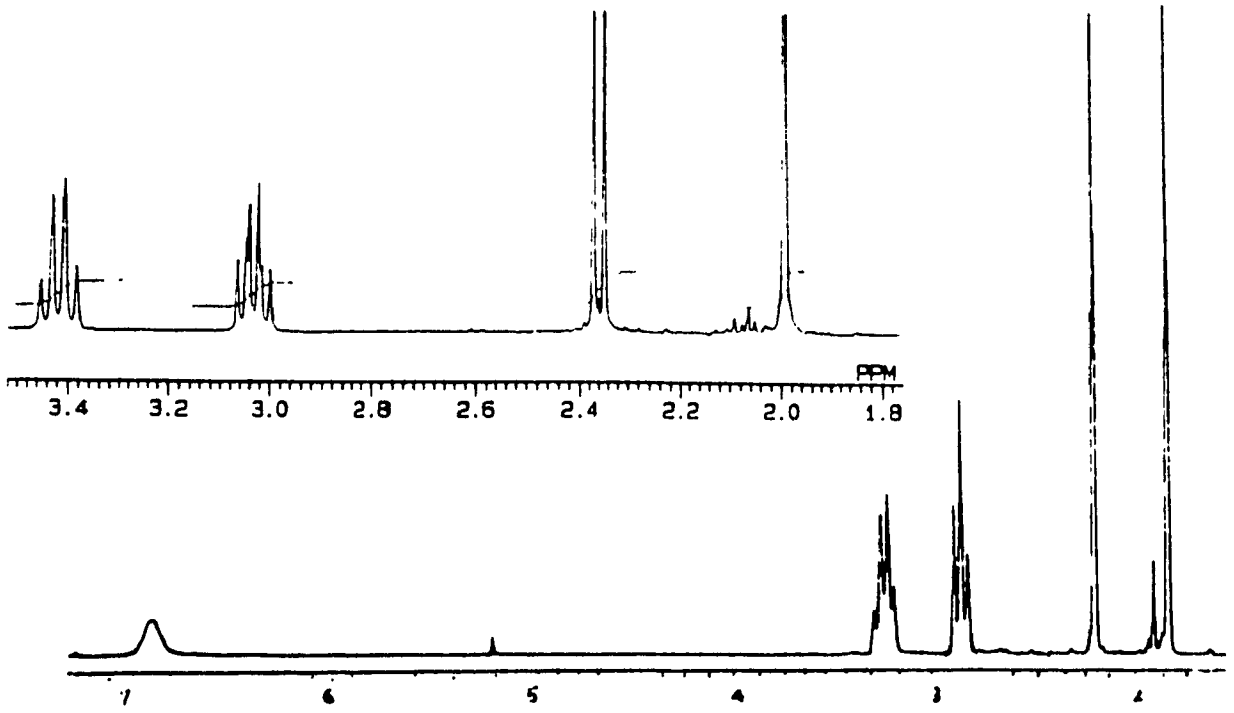


Figure 12: ^{1}H NMR spectra of normal and labelled N,S-diacetylcysteamine.

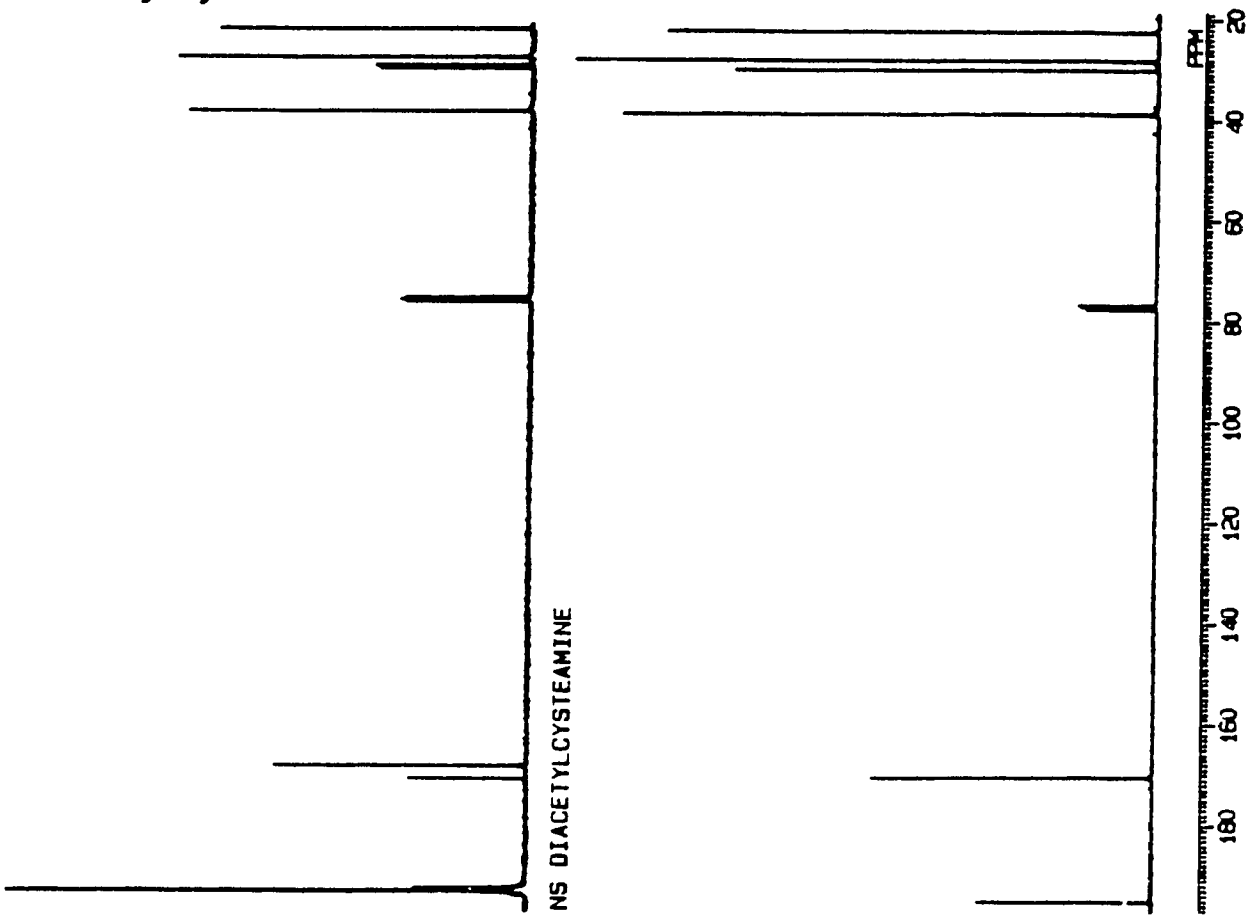


Figure 13: ^{13}C NMR spectra of normal and labelled N,S-diacetylcysteamine.

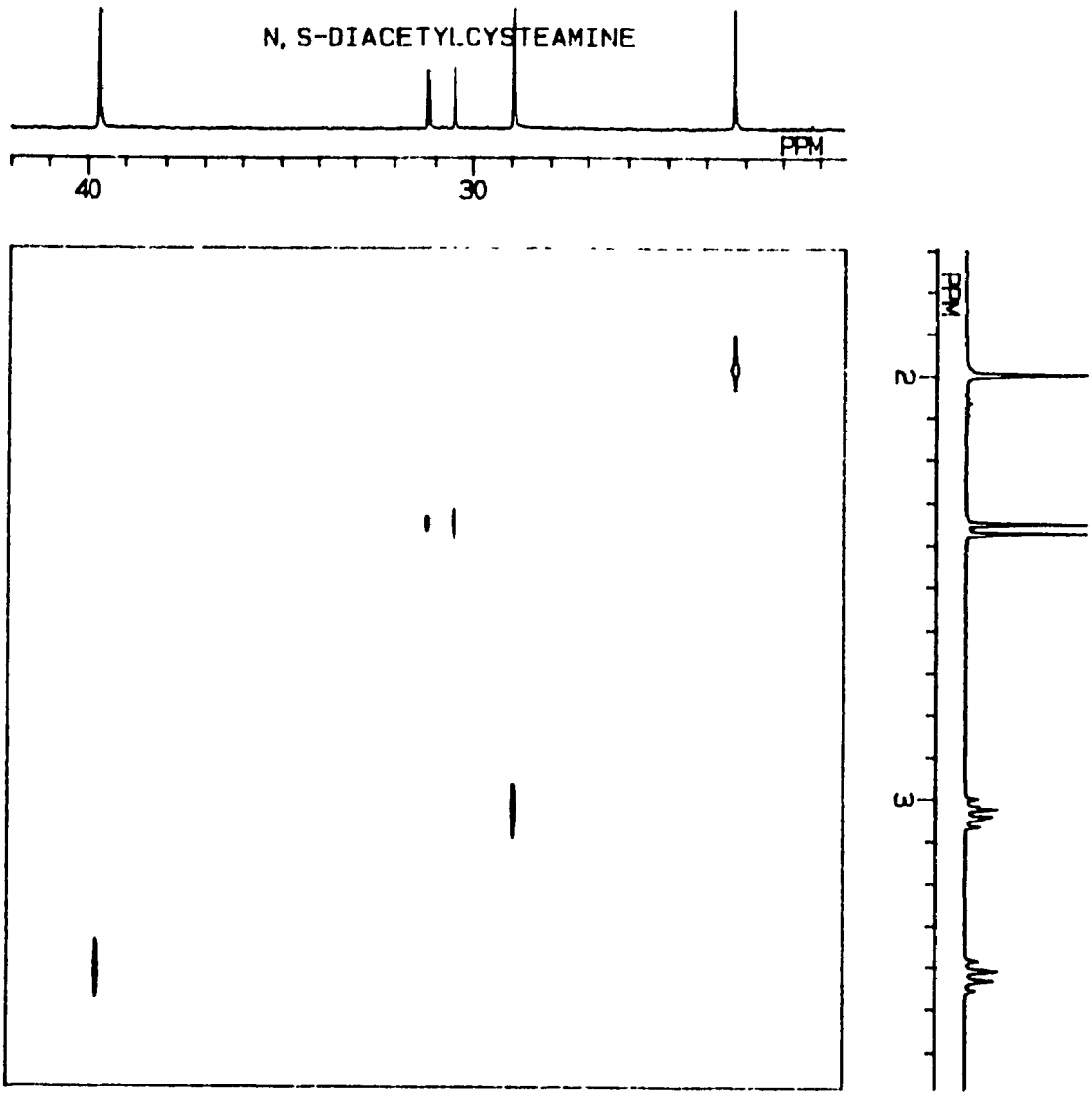


Figure 14: HETCOR NMR spectrum of labelled N,S-diacetylcysteamine.

Columns 3 and 4 of Table 2 illustrate the complete data obtained from the N,S-diacetylcysteamine feeding experiments. As observed, there was some random incorporation in C-1, C-2, C-3 and C-4. Some random incorporation will always occur since acetate is a good substrate for the Krebs cycle (Figure 15). Random incorporation in C-2 and C-3 is discussed in detail in the butyrate feeding experiments (section [2.4], scheme 11).

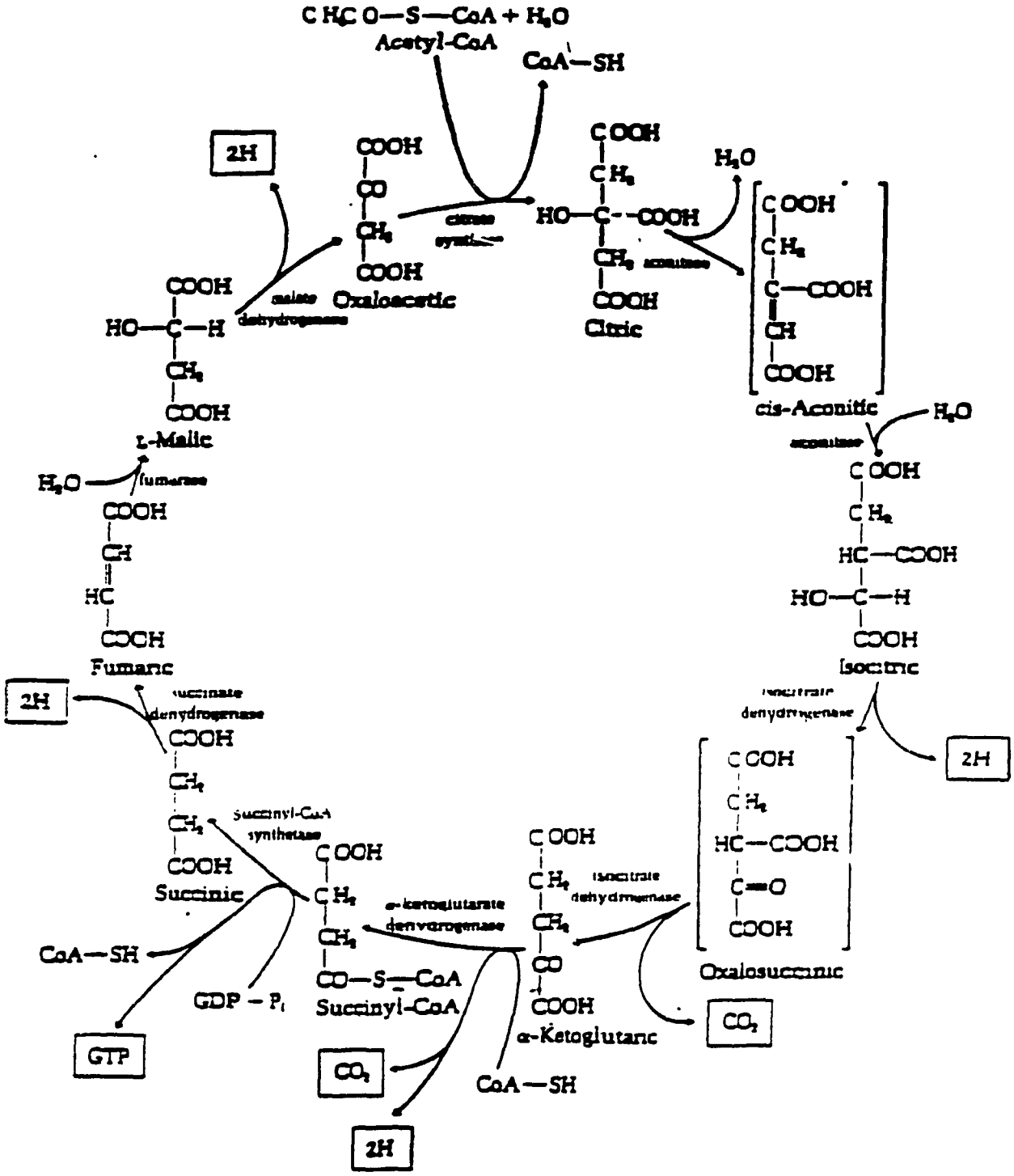


Figure 15, The citric acid (Krebs) cycle[14]

The ^{13}C incorporation pattern observed (Table 2) indicated that there may be four carbonyl carbons derived from acetate units. In order to confirm this hypothesis [1,2-$^{13}C_2$] sodium acetate was fed.

[2.3.3] [1,2-$^{13}C_2$] Sodium Acetate.

[1,2-$^{13}C_2$] Sodium acetate dissolved in distilled water was fed to liquid cultures of *O. radicata*, in two equal portions. The ^{13}C NMR a portion of which is shown in Figure 16, demonstrated that oudenone was made of four acetate units condensed in a head to tail fashion. As observed, the carbon peaks are triplets. The triplets are in fact a doublet from the adjacent ^{13}C-^{13}C coupling and a singlet from the uncoupled ^{13}C incorporation. Table 3 illustrates the experimental results from the doubly labelled sodium acetate feeding.

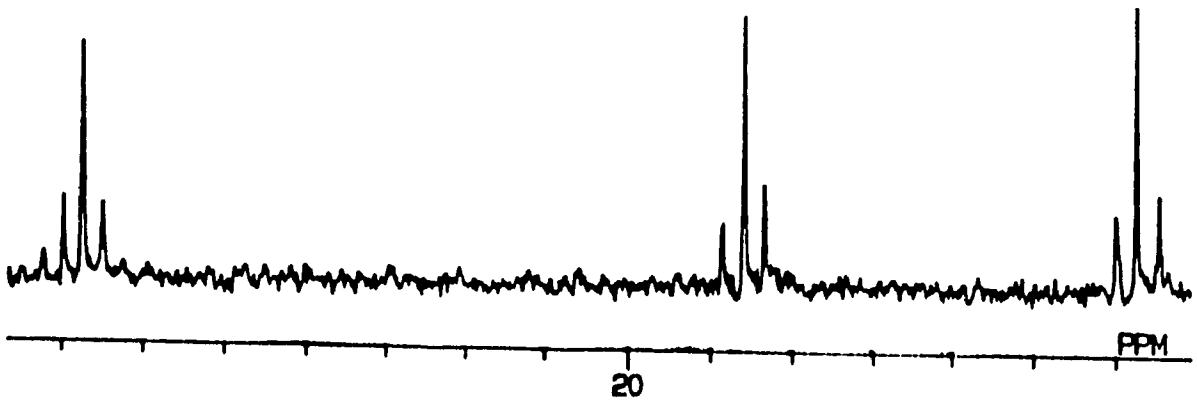


Figure 16: Partial ^{13}C NMR of oudenone after incorporation of [1,2- ^{13}C] acetate.

Table 3: The experimental results from feeding doubly labelled acetate.

	[1,2-^{13}C] acetate	
δ (ppm)	J (Hz)	%E incorporation[20]
13.87 (C12)	35.2	0.75
18.72 (C11)	35.3	0.75
36.81 (C10)	38.6	0.74
90.53 (C9)	38.7	0.74
26.91 (C8)	31.9	0.70
33.79 (C7)	32.0	0.70
184.68 (C6)	76.0	0.54
109.34 (C5)	76.0	0.54
204.28 (C4)	37.5	0.67
35.00 (C3)	37.5	0.67
34.67 (C2)	36.3	0.65
200.90 (C1)	36.4	0.64

$$\text{eq 1:} \quad \%E = \frac{1.1(I_{d1} + I_m)}{I_C - f\,(I_{d1} + I_m)}$$

I_m = total intensity of multiplet (the two furthest peaks) due to coupling with two adjacent carbons.

I_{d1}= total intensity of a pair of doublet resonances due to ^{13}C-^{13}C coupling as satellites of a singlet resonance I_C.

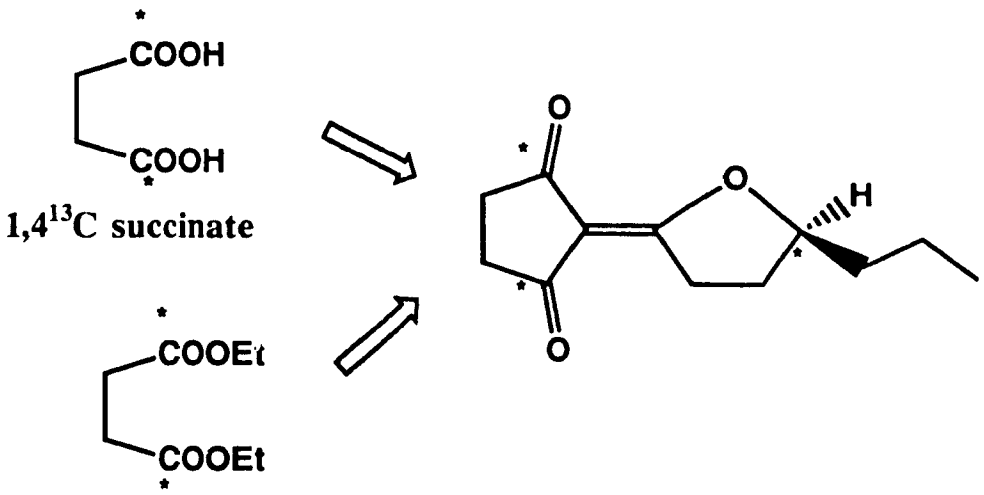
f= the ratio of singly and doubly labelled acetate (1/99)

As seen in Table 3, coupling constants of C-12 and C-11, C-10 and C-9, C-8 and C-7, C-6 and C-5 were identical, indicating an intact incorporation of [1,2- ^{13}C] acetate. The random incorporation in C-1 and C-2, C-3 and C-4 can be explained by the fact that acetate is a good substrate for the Krebs cycle.[19] The percent incorporation was calculated to be between 0.5 and 0.8 per site.[26] The percent enrichment was calculated by equation 1, where "Im" is the sum of intensities of the two furthest peaks due to coupling with two adjacent carbons (^{13}C-^{13}C-^{13}C), "Id1" is the sum of the intensities of the doublet satellite peaks from (^{13}C-^{13}C) incorporation, "IC" is the intensity of the singlet from (^{13}C) incorporation, and "f" is the ratio of singly to doubly labelled acetate fed to the microorganism ($C_{13,12}/C_{13,13}$= 1/99). It is noteworthy that the amount of [1,2-^{13}C] sodium acetate fed in this experiment was lower than previous experiments in order to decrease the probability of adjacent doubly labelled acetate incorporation (^{13}C-^{13}C-^{13}C-^{13}C). An alternative instrumental solution to the multiple labeling based spin echo Fourier transform techniques (SEFT) has been described.[27,28]

Acetate experiments illustrated the composition of one part of the molecule. In order to determine the other constituent [1,4-^{13}C] sodium succinate was fed.

[2.3.4] [1,4-$^{13}C_2$] Sodium succinate

Based on the proposed biosynthetic pathway (scheme 5) it was expected that feeding [1,4-$^{13}C_2$] succinate to the liquid cultures of *O. radicata* would result in signal enhancements in C-1, C-4 and C-9 (scheme 9).



Scheme 9: Proposed sites of succinate and diethyl succinate incorporation.

[1,4-$^{13}C_2$] sodium succinate dissolved in distilled water was fed to the liquid culture of *O. radicata*, in two equal portions. The ^{13}C NMR of oudenone demonstrated an incorporation in C-1 and C-4 (Table 4). Efforts made to incorporate [1,4-$^{13}C_2$] succinate were unsuccessful since production of oudenone would cease. Column 1 Table 4, illustrates the only successful feeding experiment using [1,4-$^{13}C_2$] succinate. Cane *et al.*[4] demonstrated that the use of diethyl succinate instead of the disodium salt could give better enrichments. This was most likely due to more efficient transport across the cell wall.

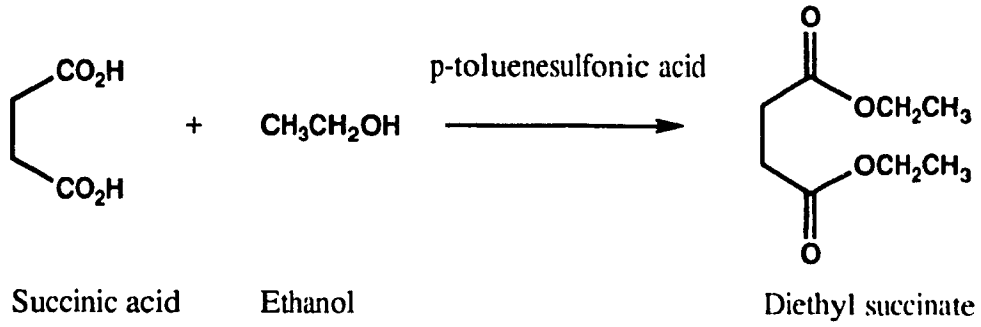
Table 4: Feeding experiments using [1,4-$^{13}C_2$] sodium succinate and [1,4-$^{13}C_2$] diethyl succinate.

	[1,4-^{13}C] sodium succinate	[1,4-^{13}C] diethyl succinate	[1,4-^{13}C] diethyl succinate	[1,4-^{13}C] diethyl succinate
δ(ppm)	I.R.*	I.R.*	I.R*	I.R *
13.87 (C12)	1.00	1.00	1.00	1.00
18.72 (C11)	1.13	1.31	0.84	1.07
26.91 (C8)	0.98	1.00	0.96	1.00
33.79 (C7)	1.03	0.88	1.14	1.01
34.67 (C2)	0.97	1.45	1.79	1.62
35.00 (C3)	1.48	1.33	1.51	1.41
36.81 (C10)	0.75	0.95	1.03	0.99
90.53 (C9)	1.16	1.00	1.19	1.09
109.34 (C5)	0.57	1.60	1.06	1.33
184.68 (C6)	1.29	0.90	1.10	1.00
200.90 (C1)	2.24	1.73	1.60	2.66
204.28 (C4)	2.35	1.92	2.57	2.24

* incorporation ratio = $\frac{\text{peak height ratio (Cn/C12) in labelled oudenone}}{\text{peak height ratio (Cn/C12) in unlabeled oudenone}}$

[2.3.5] [1,4-$^{13}C_2$] Diethyl succinate

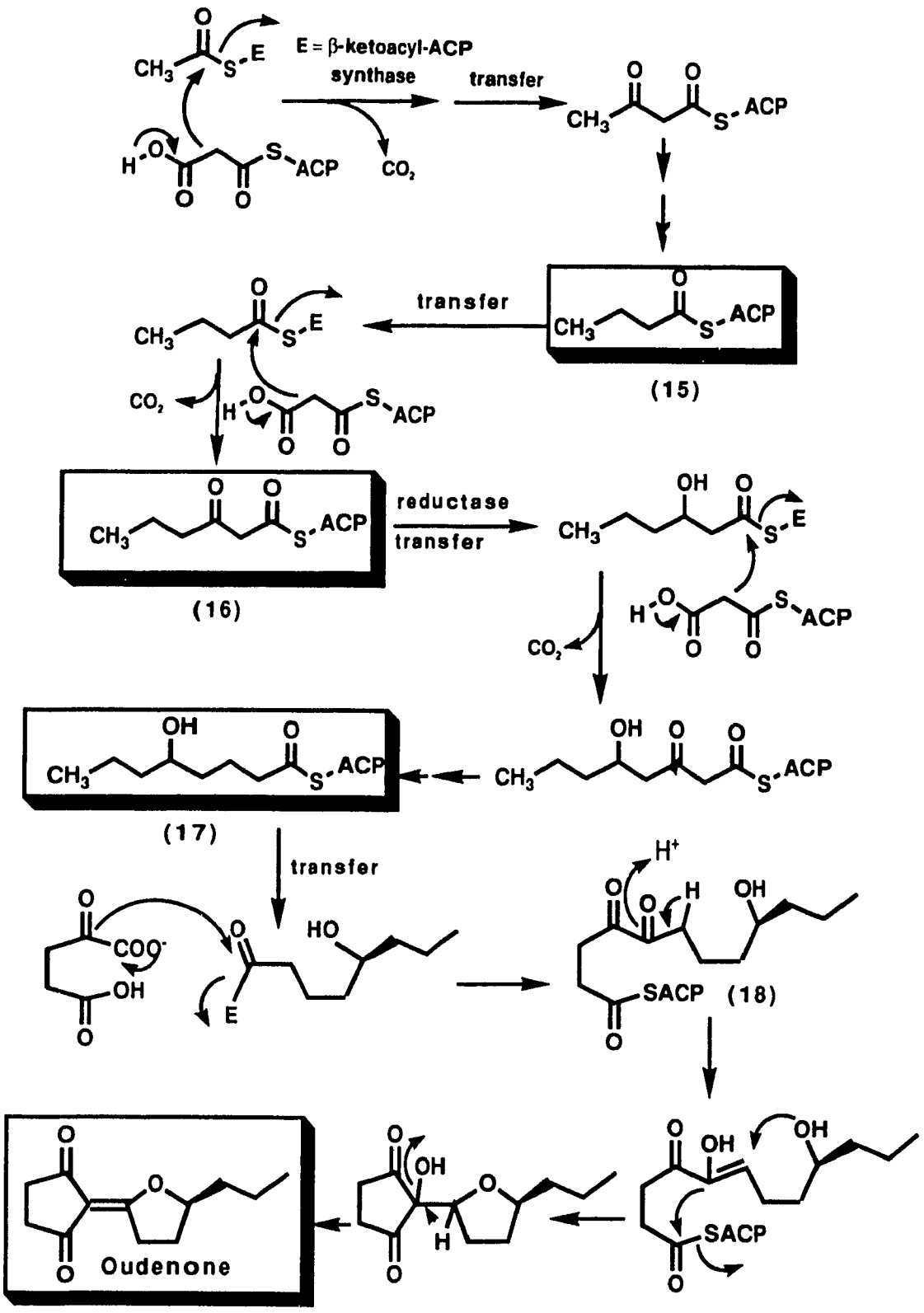
The diethyl ester was synthesized by reacting [1,4-$^{13}C_2$] succinate with ethanol in the presence of catalytic amount of p-toluenesulfonic acid (scheme 10).



Scheme 10: Synthesis of [1,4-$^{13}C_2$] diethyl succinate.

[1,4-$^{13}C_2$] Diethyl succinate dissolved in ethanol, was fed to the liquid culture of *O. radicata* in two equal portions. The NMR of purified oudenone demonstrated an incorporation of C-1 and C-4 (Table 4, column 2, 3, 4). Contrary to the [1,4-$^{13}C_2$] sodium succinate, the [1,4-$^{13}C_2$] diethyl succinate feeding did not stop the production of oudenone. Presumably feeding the ester will not alter the pH of the media as much as the acid.

Based on our experimental results it appears that oudenone is synthesized *via* the condensation of four acetate units and one succinate unit (scheme 9). Condensation of a malonate unit onto the enzyme-bound acetate produces a C_4 unit which undergoes reduction to



Scheme 11: Revised proposal for the biosynthesis of oudenone.

give the butyryl intermediate. Two malonyl units are then condensed with the intermediate to yield a tetraketide. This is followed by the condensation of a succinate onto the tetraketide to produce the open chain polyketide (**18**) which is believed to be cyclized by an enzyme to give oudenone.

[2.4] Incorporation studies of advance precursors

Simple precursor incorporation studies have established that the carbon skeleton of oudenone is composed of four C_2 units condensed onto an α-ketoglutarate. The proposed α-condensation in this pathway is quite uncommon among fungal metabolites. If our new proposal is correct, structures (**15**), (**16**), and (**17**) will be the intermediates in the biosynthesis of oudenone. The synthesis of the ^{13}C-labelled butyryl thioester of N-acetylcysteamine has been accomplished and the preliminary experiments on its incorporation will be discussed next.[29,30,31] Efforts were also made to synthesize the ^{13}C-labelled tetraketide (**17**). A proposed synthetic scheme will be briefly discussed.

[2.4.1] ^{13}C-labelled N-acetylcysteamine thioester of butyric acid

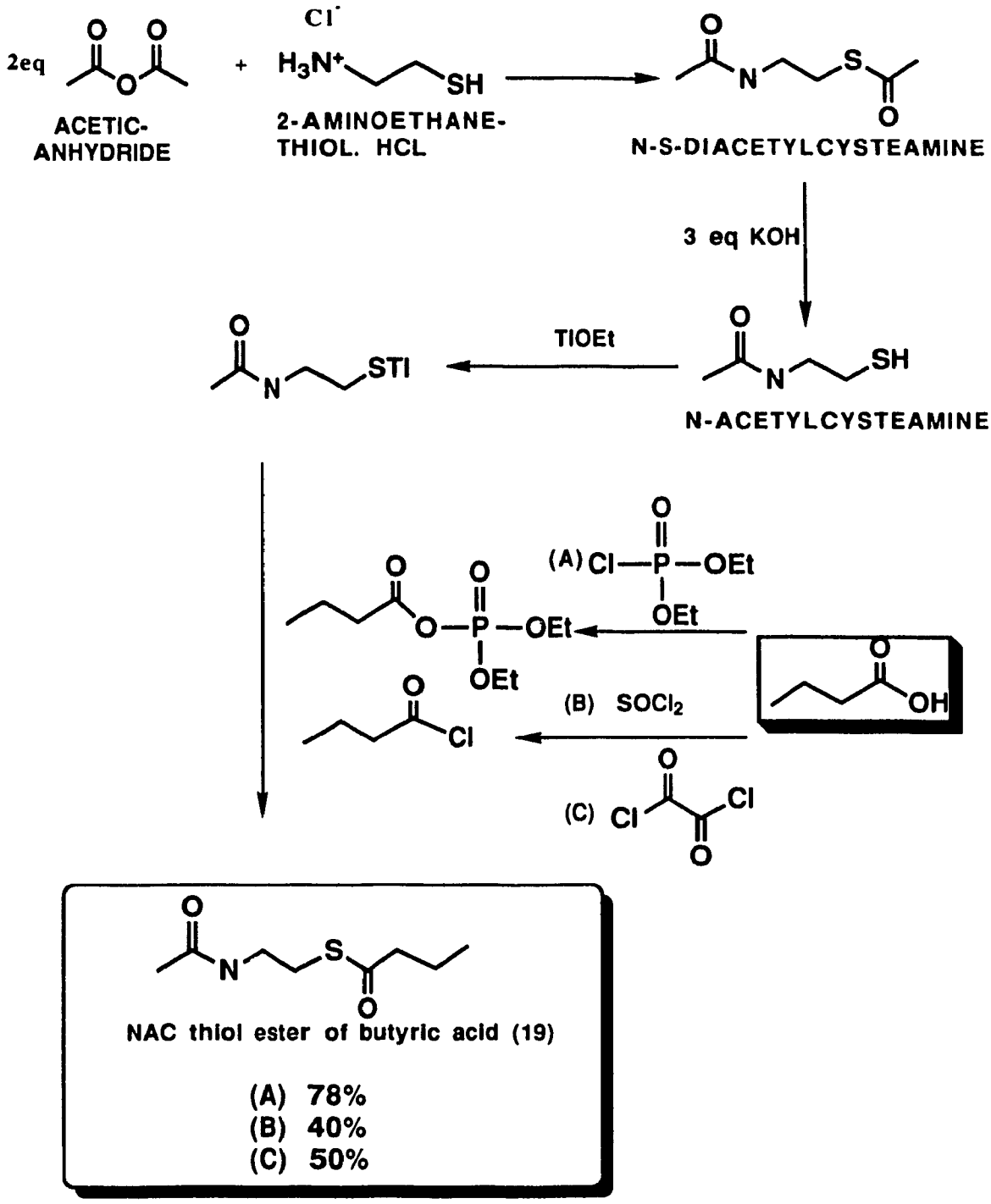
A scheme for the synthesis of the [1-^{13}C] N-acetylcysteamine thioester of butyric acid (**19**) was first devised using the unlabelled compound. Initially, we proposed that the reaction of sodium butyrate with excess thionyl chloride (as solvent) would yield butyryl chloride which could undergo nucleophilic attack by the thiol group of N-

acetylcysteamine to produce the desired product. This reaction gave a low yield (30%) when performed at r.t. and improved slightly (40%) when refluxed. The low yield convinced us to replace the thionyl chloride with oxalyl chloride.[32] Oxalyl chloride reacted with sodium butyrate at r.t. to give the desired product in a moderate yield (50%).

Reaction of sodium butyrate with 1 eq diethylchlorophosphate yielded the (diethoxy)phosphonoxybutanoate which was further reacted with the thallium salt of N-acetylcysteamine without further purification (scheme 12). This approach gave the highest overall yield of product (78 %) therefore [1-^{13}C] N-acetylcysteamine thioester of butyric acid was synthesized by the same procedure.

The ^{1}H NMR of ^{13}C labelled thioester of butyric acid demonstrated the coupling of labelled carbon with protons in close vicinity (^{13}C-^{1}H). For example multiplets appeared at δ 2.38 and 3.02 in place of the triplets in the unlabelled ^{1}H NMR (Figure 17). The ^{13}C NMR of ^{13}C-labelled thioester of butyric acid illustrated an intense peak at δ 199.5 due to the labelled thioester carbon.

The ^{13}C NMR also illustrated the ^{13}C-^{13}C coupling between adjacent carbons (Figure 18). In order to assign all ^{1}H-^{13}C couplings, a HETCOR NMR was performed (Figure 19). As observed, the triplet at δ 0.93 was coupled to two carbon peaks at δ 13.62 and 13.67. The multiplet at δ 1.67 was coupled to the carbon peaks at δ 19.45 and 19.48, while the multiplet at δ 2.50 was coupled to the carbon peaks at δ 45.86 and 46.55.



Scheme 12: Proposed scheme for the synthesis of N-acetylcysteamine thioester of butyric acid.

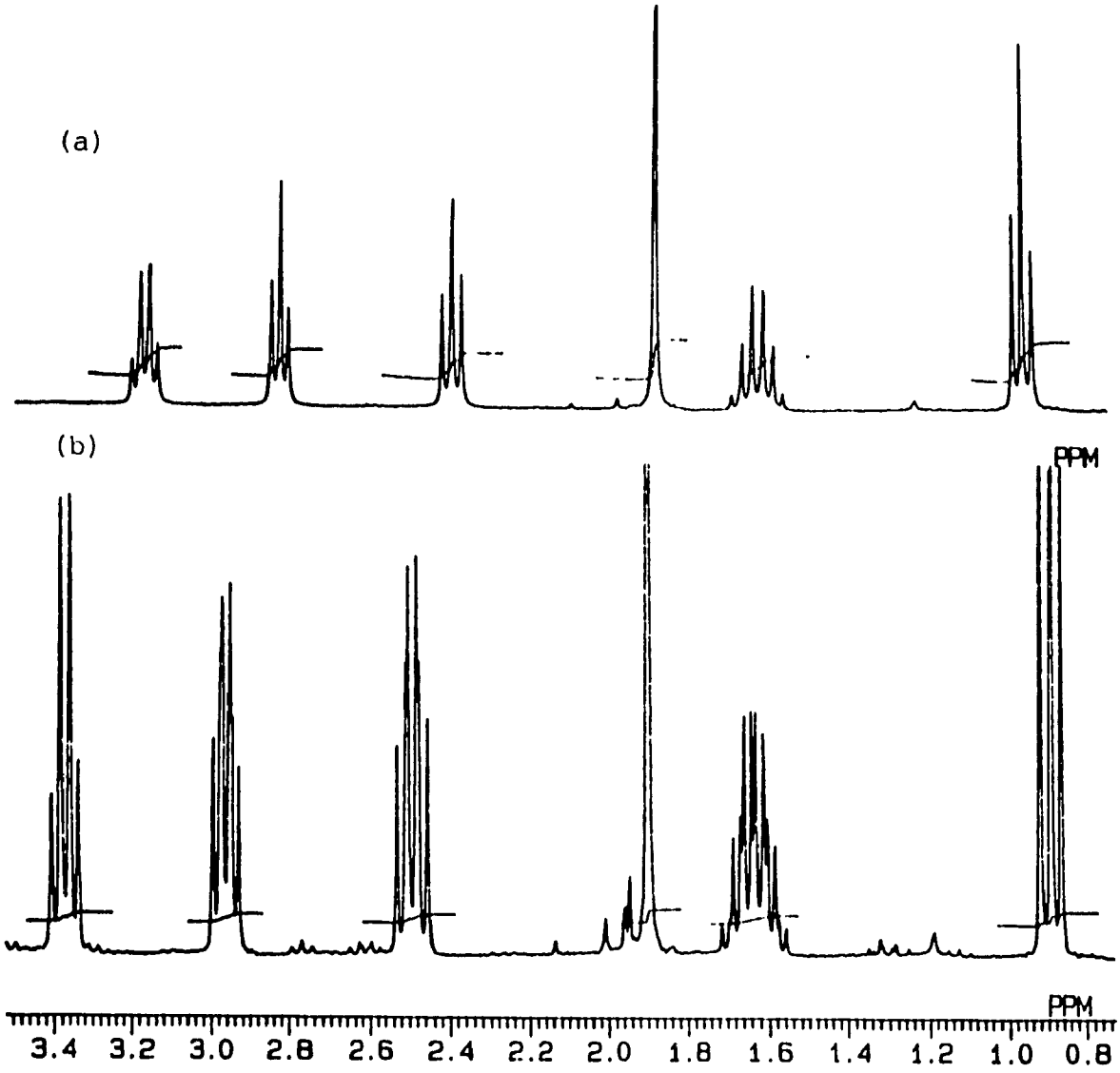


Figure 17: (a) ^{1}H NMR of normal and (b) ^{13}C-labelled thioester of butyric acid.

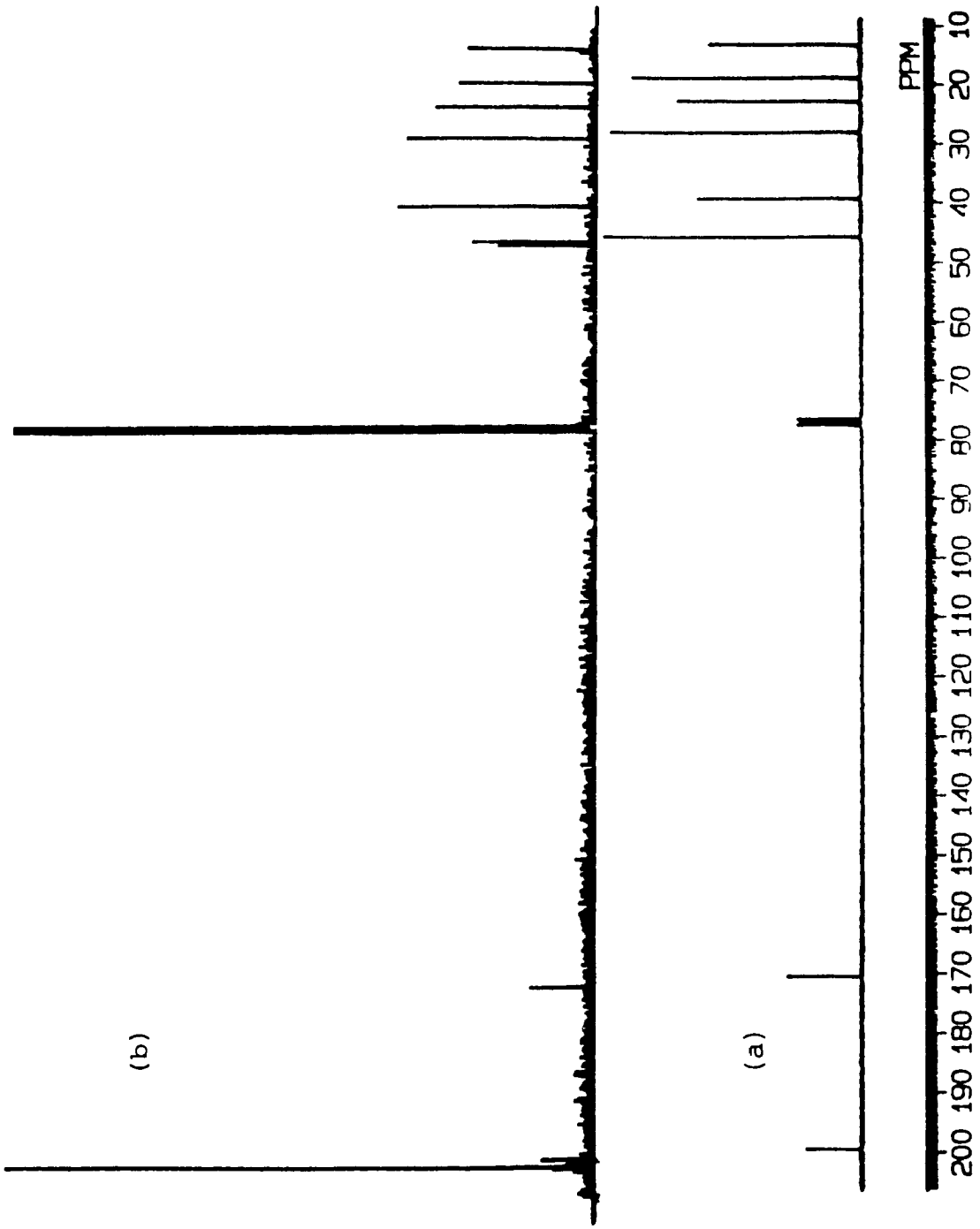


Figure 18: (a) ^{13}C NMR of normal and (b) ^{13}C-labelled thioester of butyric acid.

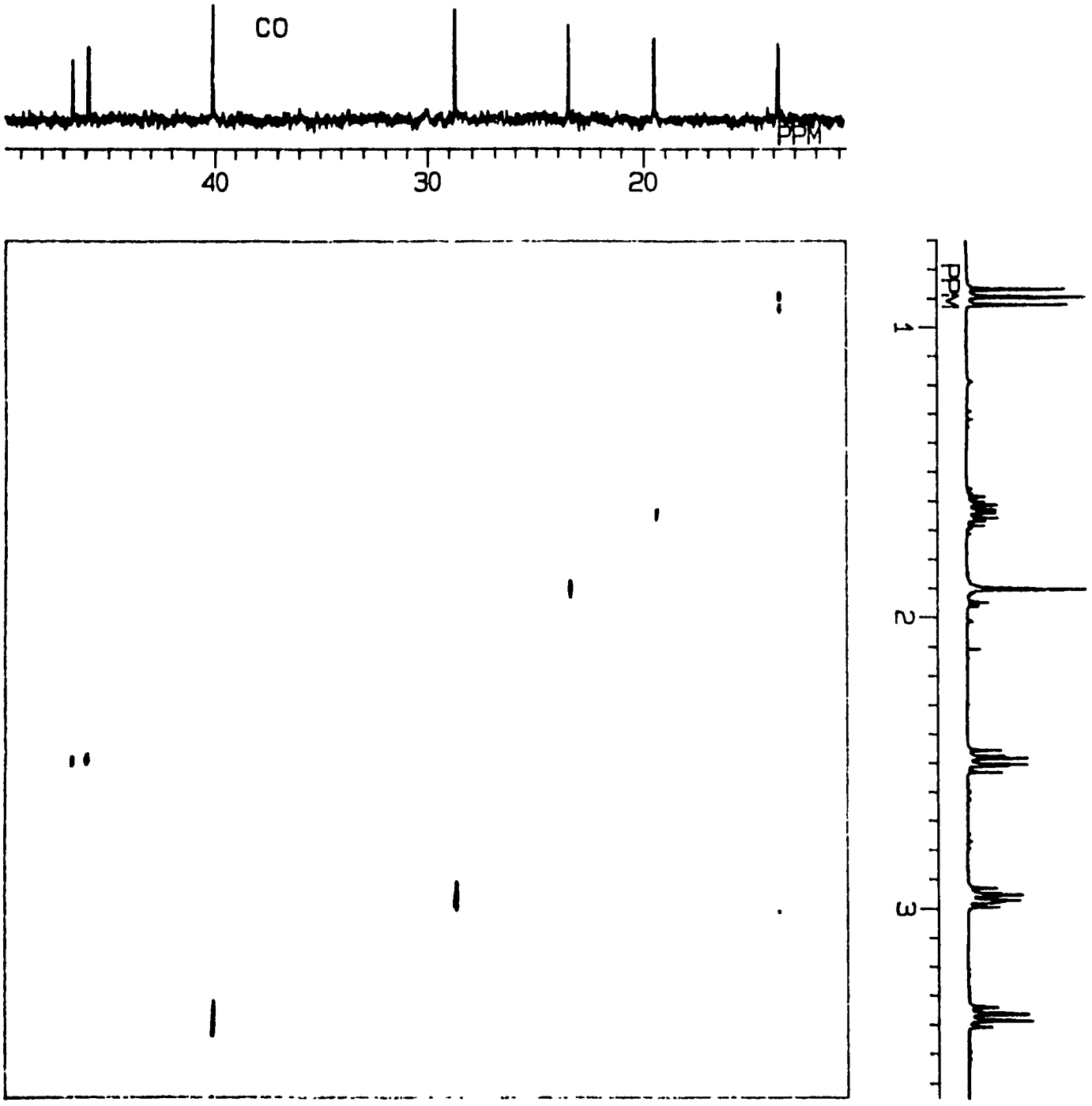


Figure 19: HETCOR NMR spectra of ^{13}C-labelled thioester of butyric acid.

To a 500 mL liquid culture of *O. radicata*, ^{13}C labelled thioester of N-acetylcysteamine (88 mg) along with the ethyl ester of 3-(tetradecylthio) propionic acid dissolved in 99% ethanol, was fed in two portions. The ^{13}C NMR of oudenone demonstrated random incorporation in C-11, C-9, C-7, C-5, C-4, C-1 signifying total breakdown of the substrate by β-oxidase enzyme. β-oxidation of polyketide precursor is commonly known. However the use of β-oxidase inhibitor along with replacement culture would increase the chance of the incorporation of the precursors.[8,12]

Four different experiments were performed in order to determine the highest possible incorporation of the advance precursor.

(1) To a 100 mL replacement culture of *O. radicata*, ^{13}C-labelled thioester of N-acetylcysteamine (40 mg), along with ethyl ester of 3-(tetradecylthio) propionic acid dissolved in 99% ethanol was added. Column 1 of the Table 5 illustrates the NMR results of this experiment.

(2) To a 100 mL replacement culture of *O. radicata*, [1-^{13}C] sodium butyrate (40 mg), dissolved in a solution consisting of water and 99% ethanol, along with ethyl ester of 3-(tetradecylthio) propionic acid dissolved in of 99% ethanol was fed. Column 2 in table 5 illustrates the NMR results of this experiment.

(3) To a 100 mL replacement culture of *O. radicata*, [1-^{13}C] sodium butyrate (40 mg), dissolved in a solution consisting of water and 99% ethanol, along with ethyl ester of 3-(tetradecylthio) propionic acid dissolved in 99% ethanol was fed in three equal portions five days after transferring the mycelia from cultures into the replacement media. Column 3 in table 5 illustrates the NMR results of this experiment.

(4) To a 100 mL liquid culture of *O. radicata* [1-^{13}C] sodium butyrate (40 mg) dissolved in a solution consisting of water and 99% ethanol, along with ethyl ester of 3-(tetradecylthio) propionic acid dissolved in 99% ethanol, was fed in two portions. Column 4 in table 5 illustrates the NMR results of purified oudenone.

Table 5: The NMR results of the advance precursor feeding experiments.

δ (ppm)		Exp (1) I.R.*	Exp (2) I.R.*	Exp (3) I.R.*	Exp (4) I.R.*
13.87	(C-12)	1.00	1.00	1.00	1.00
18.72	(C-11)	2.22	5.40	2.54	1.40
36.81	(C-10)	1.14	1.41	0.97	1.13
90.53	(C-9)	1.78	3.43	2.13	1.00
26.91	(C-8)	1.16	1.02	0.76	1.01
33.79	(C-7)	1.90	3.30	2.02	0.96
184.68	(C-6)	0.97	1.43	0.77	0.80
109.34	(C-5)	1.80	3.11	0.60	0.81
204.28	(C-4)	3.2	5.81	2.50	1.28
35.00	(C-3)	2.13	5.26	1.77	1.89
34.67	(C2)	2.50	3.22	0.51	0.77
200.90	(C-1)	2.80	5.10	2.50	1.33

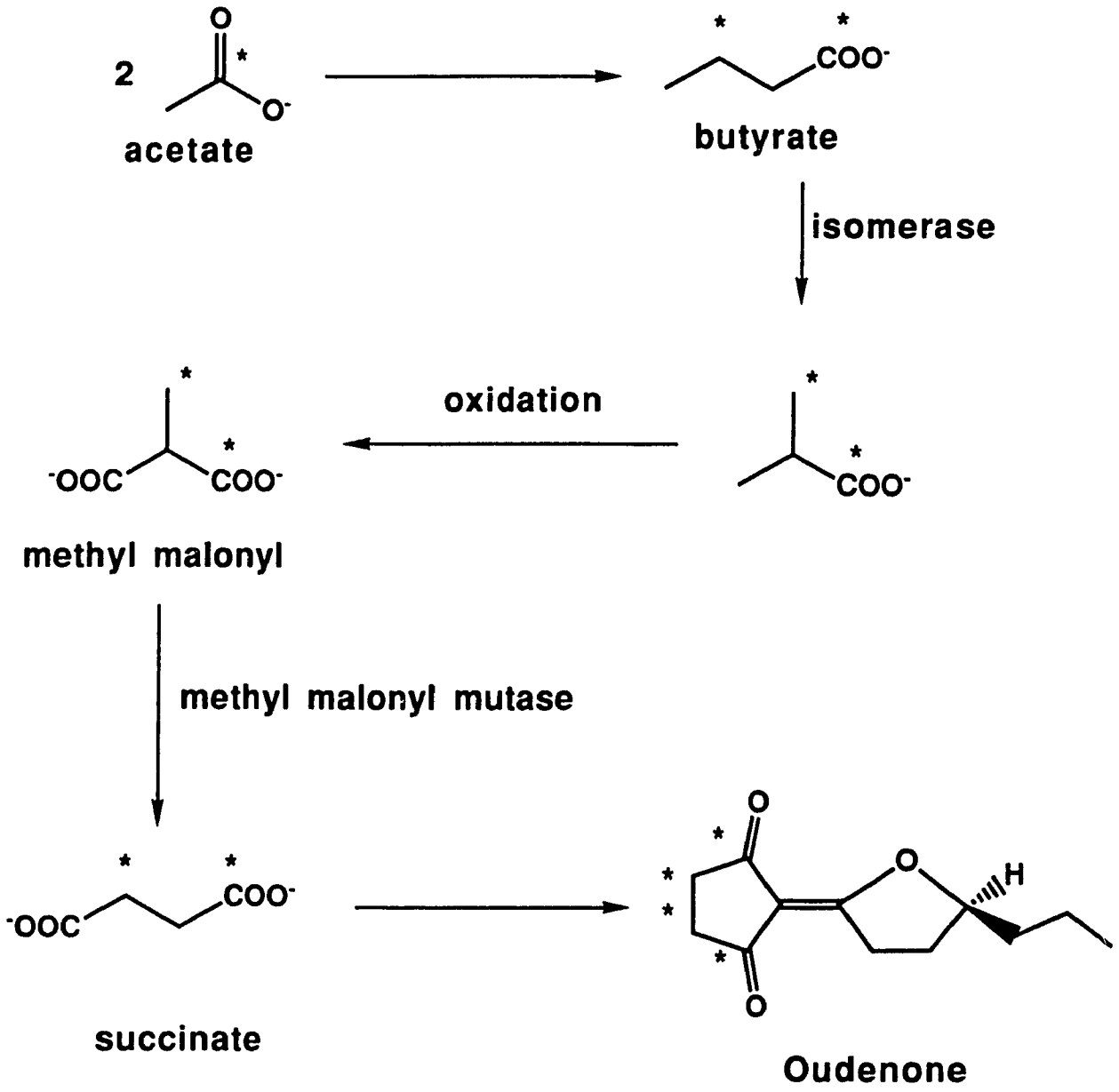
* incorporation ratio = $\frac{\text{peak height ratio (Cn/C12) in labelled oudenone}}{\text{peak height ratio (Cn/C12) in unlabeled oudenone}}$

These experiments were plagued by rapid degradation of the labelled precursor to acetate by efficient β-oxidation. This indicated

that the ethyl ester of 3-(tetradecylthio)propionic acid is not an efficient inhibitor of the β-oxidation reaction in *O. radicata*. The observation by Vederas *et al.*[12] of an exceptional recovery of unchanged precursors using 3-(tetradecylthio)propionic acid, prompted us to repeat the feeding experiments while replacing the ester with the acid. The use of 3-(tetradecylthio)propionic acid as the inhibitor did not stop the β-oxidation either.[33]

As observed in Table 5, there was no specific incorporation observed at C-9. Only random incorporation at C-11, C-9, C-7, C-4, C-3, C-2, C-1 was observed. The maximum incorporation was observed in C-11 since it was at the beginning of the growing chain. [The probability of incorporation at the beginning of the growing chain is usually the highest.] Almost the same incorporation was observed in C-9 and C-7, indicating the breakdown of butyrate into acetate. The incorporations in C-1 and C-4 were the result of acetate incorporation into the Krebs cycle. The incorporation in C-2 and C-3 can be explained by the fact that butyrate was degraded into acetate units labelled at C-1. Two of these acetates were combined to produce the butyrate labelled at C-1 and C-3 (scheme 13). In some microorganisms,[34,35,36] possibly including *O. radicata*, butyryl-CoA can be converted into 2-methyl propionate. The 2-methyl propionate would then be oxidized to methyl malonyl-CoA and converted to succinyl-CoA labelled at C-1 and C-3. The incorporation of this succinate into oudenone could have resulted in the enrichments observed in C-2 and C-3.

Despite the fact that using replacement media and a β-oxidase inhibitor did not produce the desired results, some useful information was obtained. We have shown that the use of replacement medium leads to a two-fold increase in the percent incorporation of ^{13}C label in to oudenone.



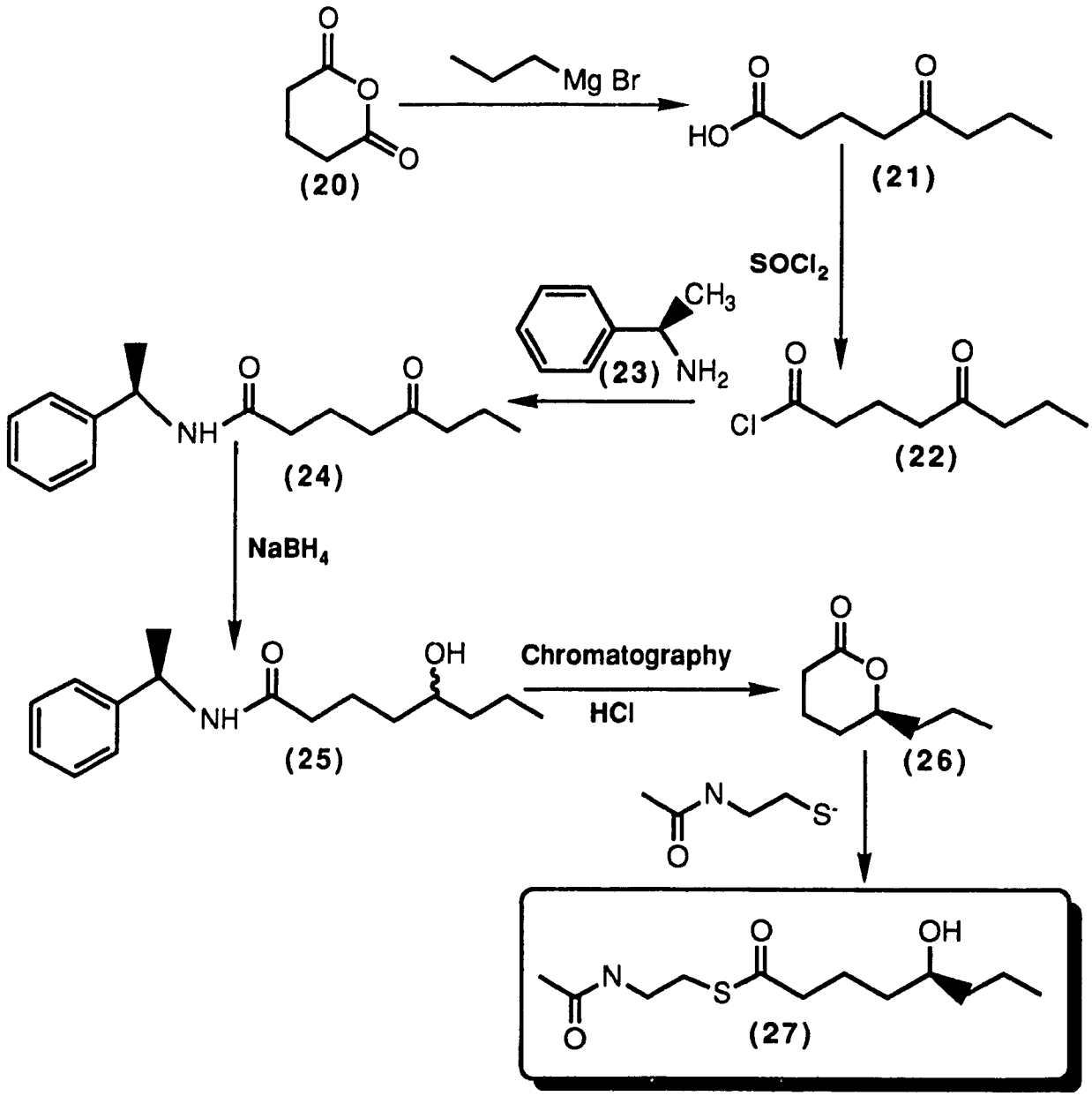
Scheme 13, The proposed incorporation pathway in C-1, C-2, C-3, C-4 of oudenone. Symbol (*) indicates ^{13}C enriched carbon.

Excess random incorporation has masked the validity of the incorporation studies using singly labelled [1-^{13}C] sodium butyrate and ^{13}C- labelled thioester of butyric acid. It is necessary to synthesize the [2,3-^{13}C] labelled thioester of butyric acid. If there is an intact incorporation, (^{13}C-^{13}C) coupling between adjacent carbons can be observed. Vederas *et al.*[10] have synthesized N-acetylcysteamine thioester of (S)-[2,3-$^{13}C_2$]-3-hydroxybutyrate. Their experiments illustrate enrichments on every carbon as well as carbon-coupled signals at two specific sites indicating the incorporation of a four carbon chain without cleavage of C-2 to C-3 bond. Similar experiments should support our incorporation studies.

[2.4.2] Attempts in the synthesis of tetraketide 17

Oudenone has been shown to be in the (S) configuration at the chiral carbon (C-9), regardless of skeletal changes between structures **14 A** and **14 B**. The reason is that the ring opening mechanism of oudenone is a Michael addition of water followed by opening of the ether linkage to allow the retention of the asymmetric center at C-9.[21]

Considering that only a few commercially available ^{13}C compounds exist, we proposed to synthesize the (S)-tetraketide (**17**) in the following manner: a Grignard[37] reagent of bromopropane and magnesium can react with glutaric anhydride (**20**) to produce 5-keto-octanoic acid (**21**). The acid may be transformed into an acid chloride (**22**) using thionyl chloride.[38] The reaction of (S)-α-methylbenzylamine (**23**) with the acid-chloride will produce



Scheme 14: Proposed scheme for the synthesis of the tetraketide portion of oudenone.

(S)-(-)-α-methylbenzyl-(5-keto-octanoic) amide. The reduction of the intermediate (**24**) using sodium borohydride yields two diastereomers (**25**) that may be separated chromatographically.[39] Hydrolysis of the desired isomer produces the (S)-lactone (**26**). The reaction of the thallium salt of N-acetylcysteamine with this lactone should yield the N-acetylcysteamine-bound tetraketide (**27**, scheme 14).

A Grignard reagent was prepared from bromopropane and magnesium in dry tetrahydrofuran (THF). The reaction mixture was added into a solution of glutaric anhydride in THF. TLC analysis of the reaction in the first hour indicated the production of three compounds which were isolated by flash column chromatography. One of these compounds, 5-keto-octanoic acid, was produced in less than 20% yield. The second compound, 5-propyl-5-hydroxy octanoic acid (M^++1=203), was obtained in low yield (20-30%). The third compound, an alcohol (M^++1=176) whose structure was not completely assigned, was obtained in less than 10%. The production of 5-propyl-5-hydroxy octanoic acid is proposed to be from the attack of the Grignard reagent onto C-5 ketone of 5-keto-octanoic acid. The attempts to optimize the yield by slow addition and dilution of the reaction mixture were not successful.

The above procedure was modified by opening the anhydride with (S)-(-)-α-methylbenzylamine and triethylamine as the base. A pure product was obtained without further purification. The attempt to transform the acid into an acid chloride followed by a Grignard reaction was unsuccessful since no major product was obtained. A possible explanation is that the acid chloride is very unstable and undergoes rearrangements to give multiple products. The high costs of ^{13}C compounds oblige us to be very concerned about the yields of reactions hence we abandoned this pathway for one with a more

favorable yield. The synthesis of the tetraketide using the new pathway is underway in Dr. Tsantrizos' laboratory.[33]

[2.5] Conclusions

The ^{1}H and ^{13}C NMR chemical shifts of oudenone were assigned. The production curve of oudenone was determined to illustrate the optimum feeding period. Originally we proposed that oudenone is biosynthesized by the condensation of two succinate and three acetate units (Scheme 5). Using ^{13}C labelled precursors, oudenone was shown to be biosynthesized *via* the condensation of four acetate and one succinate unit therefore our proposed biosynthetic pathway is modified (Scheme 11). The preliminary feeding experiment using N-acetylcysteamine thioester of butyric acid and sodium butyrate was masked by the excessive breakdown of precursors into acetate thus a better b-oxidase inhibitor for this system needs to be found. The synthesis of doubly labelled precursors should further illustrate the validity of these experiments.

CHAPTER 3: Experimental

[3.1] General Methods & Experimental

[3.1.1] Reagents and Chemicals

All chemicals necessary for the preparation of media were purchased from Difco.

[3.1.2] Chromatography

Silica gel chromatography was carried out on Merck Kieselgel 60 using flash chromatography.[40] Reversed phase flash column chromatography was performed on silica gel (Merck Kieselgel 60) reacted with *n*-octadecyltrichlorosilane.[37] Thin layer chromatography (TLC) was carried out on Merck Kieselgel 60 F_{254} plates (0.2 mm thickness).

[3.1.3] Spectra

Ultraviolet spectra were recorded on a Hewlett Packard 8452A DIODE ARRAY spectrophotometer. Nuclear Magnetic Resonance spectra were obtained at 23-24 °C using JEOL 270, Gemini 200, Varian XL-200 and Varian XL-300 MHz instruments. ^{1}H and ^{13}C NMR shifts are quoted in ppm and are referenced to the internal deuterated solvent. C.I. (NH_3) mass spectra were conducted at the Biomedical Mass Spectrometry Unit, McGill University.

[3.2.1] Cultivation of the *Oudemansiella radicata* [16]

O. radicata was grown on a plate containing 2.0 g glucose, 0.5 g dry yeast extract, and 1.5 g agar in 100 mL of nanopure water for 8-15 days. A small plug of *O. radicata* mycelium was used to inoculate a seed culture containing 5 g cellulose, 2 g glucose, and 0.5 g yeast in 100 mL of water. The seed culture was shaken for 17 days at 26.3°C and 140 rpm. An aliquot of seed culture (10 ml) was transferred into a 2800 ml flask containing 500 mL media composed of 10 g dextrose, 2.5 g bactopeptone, 1.5 g dry yeast extract, 1.5 g KH_2PO_4, 0.5 g $MgSO_4.7H_20$, in 500 mL nanopure water. Oudenone production was usually observed on days 5-7 and reached its maximum on days 7-11.

In feeding experiments using butyrate, an aliquot of seed culture (3 mL) was used to inoculate 100 mL of culture medium. The cultures were grown for 5-7 days, at which time the production of oudenone was observed. The liquid cultures were then transferred into a 250 mL sterilized centrifuge bucket and centrifuged at 10,000 rpm for 10 minutes. The mycelia were resuspended in 100 mL replacement media containing 10 g glucose, 0.3 g KH_2PO_4, and 0.1 g $MgSO_4.7H_20$.

[3.2.2] Isolation of oudenone

Liquid cultures (500 mL) were filtered through cheesecloth and extracted with butanol (3 x 300 mL). The butanol was removed by evaporation under reduced pressure. The syrup was dissolved in ethyl acetate (50 mL) and extracted with pH 7.0 water (3 x 100 mL). The pH of the aqueous layer was adjusted to 3.0 with 1N HCl and extracted with

ethyl acetate (3 x 200 mL). The ethyl acetate was dried over magnesium sulfate, and evaporated under reduced pressure to give 50 to 200 mg of crude oudenone.

Oudenone was purified by reversed phase flash chromatography using a linear gradient from 100% H_2O and 0.1% CH_3COOH to 50% methanol (MeOH) in H_2O and 0.1% CH_3COOH. Fractions containing the compound were identified by UV spectroscopy. Pure oudenone (14 A) 30-100 mg was obtained from 500 mL of liquid cultures.

[3.2.3] UV assay for monitoring oudenone production

A 3 mL sample was withdrawn from an actively fermenting culture, and filtered through cheesecloth. An aliquot (1 mL) was diluted to 10 mL with water in a volumetric flask. Two aliquots (1 mL each) were transferred into 4 mL of phosphate buffer (pH 7.0) and into 4 mL of 0.1N HCl. The production of oudenone was estimated by the difference in the UV absorbance at 246 nm of the crude extract in buffer and in acid.

[3.3] Feeding experiments using simple precursors

[3.3.1] [1-^{13}C] Sodium Acetate

To a 500 mL liquid culture, [1-^{13}C] sodium acetate (100 mg), dissolved in 2 mL distilled water, was added in two equal portions on days 6 and 7 through a millex disposable sterile filter unit. The production of oudenone was followed by UV spectroscopy. The increase in UV (difference between buffer (pH 7) and acid at 246 nm) was 0.65

and 0.02 absorbance units at 24 hours and 48 hours after the first feeding, respectively.

[3.3.2] [1-^{13}C] thioester of N,S-diacetylcysteamine

N,S-Diacetylcysteamine (160 mg) dissolved in 2 mL of 99% EtOH was fed in two equal portions on days 6 and 7 to a 500 mL liquid culture. An increase of 0.27 absorption units at 24 hours and 0.08 absorption units at 48 hours in the UV absorbance was observed after the first feeding of N,S-diacetylcysteamine. Isolation and purification of oudenone yielded 40 mg of pure compound.

[3.3.3] [1,2-$^{13}C_2$] Sodium acetate

To a 500 mL liquid culture, [1,2-$^{13}C_2$] sodium acetate (70 mg), dissolved in 2 mL distilled water, was fed in two equal portions on days 5 and 6 through a millex disposable sterile filter. An increase of 0.01 absorption units at 24 hours and 0.4 absorption units at 48 hours in the UV absorbance was observed after the first feeding.

[3.3.4] [1,4-$^{13}C_2$] Sodium succinate

To a 500 mL liquid culture, [1,4-$^{13}C_2$] sodium succinate (70 mg), dissolved in 2 mL distilled water, was fed in two equal portions on days 5 and 6 through a millex disposable sterile filter. The increase in UV absorbance was 0.15 and 0.2 absorbance units at 24 hours and 48 hours after the first feeding, respectively.

[3.3.5] [1,4-$^{13}C_2$] Diethyl succinate

To a 500 mL liquid culture, [1,4-$^{13}C_2$] diethyl succinate (70 mg), dissolved in 1 mL of 99% ethanol, was fed in two equal portions on days 6 and 7. The increase in UV absorbance was 0.5 and 0.1 absorbance units at 24 hours and 48 hours after the first feeding, respectively.

[3.4] Feeding experiments using the advanced precursor

(1) To a 100 mL liquid replacement culture, ^{13}C-labelled thioester of N-acetylcysteamine (40 mg) dissolved in 1 mL 99% ethanol, along with ethyl ester of 3-(tetradecylthio) propionic acid (16 mg) dissolved in 1 mL of 99% ethanol, was fed in four equal portions at 12-hour intervals. The increase in UV was 0.25 absorbance units 24 hours after the last feeding.

(2) To a 100 mL liquid replacement culture [1-^{13}C] sodium butyrate (40 mg) dissolved in a solution consisting of 0.1 mL water and 1-mL of 99% ethanol, along with ethyl ester of 3-(tetradecylthio) propionic acid (16 mg) dissolved in 1 mL of 99% ethanol, was fed in four equal portions at 12 hour intervals. The increase in the UV absorbance was 0.25 absorbance units 24 hours after the last feeding.

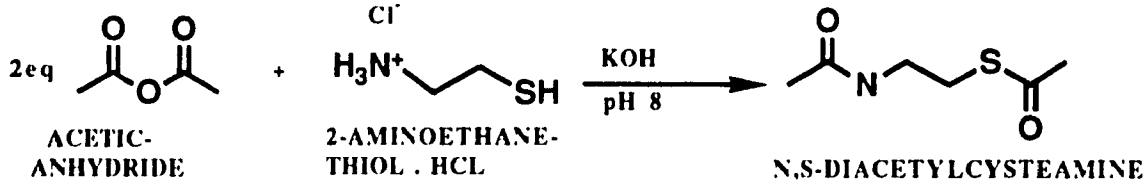
(3) To a 100 mL liquid replacement culture [1-^{13}C] sodium butyrate (40 mg) dissolved in a solution consisting of 0.1 mL water and 1 ml of 99% ethanol, along with ethyl ester of 3-(tetradecylthio) propionic acid (16 mg) dissolved in 1 mL of 99% ethanol, was fed in three equal portions at 12 hours intervals, five days after transferring the mycelia from vegetative cultures into the replacement media. The increase in the UV absorbance was 0.25 absorbance units 24 hours after the last feeding.

(4) To a 100 mL vegetative culture [1-^{13}C] sodium butyrate (40 mg) dissolved in a solution consisting of 0.1 mL water and 1-ml of 99%

ethanol, along with ethyl ester of 3-(tetradecylthio) propionic acid (16 mg) dissolved in 1 mL of 99% ethanol, was fed in two equal portions on days 6 and 7. The increase in UV was 0.05 and 0.08 absorbance units 24 hours and 48 hours after the first feeding, respectively.

[3.5] Synthesis Section

[3.5.1] N,S-diacetylcysteamine [41]

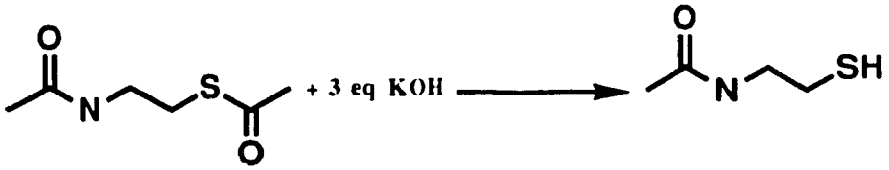


2-Aminoethanethiol.HCl (2.7 g, 35 mmol) was dissolved in 30 mL of water in a 250 mL three-neck flask. The reaction was placed in an icebath and the pH was adjusted to 8.0 with 1N KOH. Acetic anhydride (10.8 g, 105 mmol, 3 eq) was added dropwise along with sufficient KOH to maintain the pH at 8. After all the acetic anhydride was added the pH was adjusted to 7.0 with 1N HCl and the reaction was stirred for 1 hour. The reaction mixture was saturated with NaCl and extracted with CH_2Cl_2 (3x50 mL). The combined mixture was dried with anhydrous $MgSO_4$, and concentrated under vacuum to yield 80% pure product.

^{1}H NMR (200 MHz, $CDCl_3$) : δ 1.90 (s, 3H, $C\underline{H}_3CON$), 2.25 (s, 3H, $C\underline{H}_3COS$), 2.90 (t, J=6 Hz, 2H, $C\underline{H}_2S$), 3.25 (dt, J=6.6 Hz, 2H, $C\underline{H}_2NH$), 6.88 (s, 1H, N$\underline{H}$).

^{13}C NMR (270 MHz, $CDCl_3$) : δ 22.3 (CH_3CON), 28.1 (CH_2S), 30.0 (CH_3COS), 38.6 (CH_2NH), 170.3 (CON), 195.0(COS) .

[3.5.2] N-acetylcysteamine [41]



N,S-DIACETYLCYSTEAMINE N-ACETYLCYSTEAMINE

N,S-Diacetylcysteamine (1 g, 6.2 mmol, 1 eq) and KOH (1.15 g, 20.5 mmol, 3.3 eq) were dissolved in 30.0 mL water and stirred at 0°C in a 250 mL flask for 30 min. The pH was then adjusted to 7.0 with 1N HCl. The reaction mixture was saturated with NaCl, extracted with ethyl acetate, concentrated to clear oil, and dried under vacuum overnight to give a yield of 70%. Since the product was pure by NMR it was used in subsequent steps without further purification.

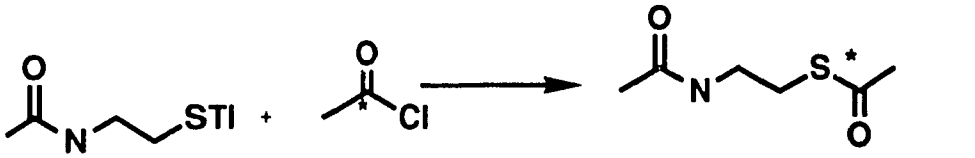
^{1}H NMR (270 MHz, $CDCl_3$) : δ 1.46 (t, J=8.6,1H, SH), 2.05 (s, 3H, $C\underline{H}_3$-CON), 2.70 (d of t, J=8.6 and 5.9 Hz, 2H, $C\underline{H}_2$-SH), 3.46 (d of t, J=5.9 Hz 2H, $C\underline{H}_2$-NH), 6.60 (s, 1H, NH).

[3.5.3] Thallium salt of N-acetylcysteamine [41]

N-ACETYLCYSTEAMINE

Thallium ethoxide (1.75 g, 0.50 mL, 1 eq) was added to THF (75 mL) in a 100 mL flask with stirring at r.t. N-acetylcysteamine (0.84 g, 7 mmol, 1 eq) was added dropwise and the solution turned bright yellow after 20 min. This solution was used in subsequent steps.

[3.5.4] ^{13}C labelled N,S-diacetylcysteamine



N,S-DIACETYLCYSTEAMINE

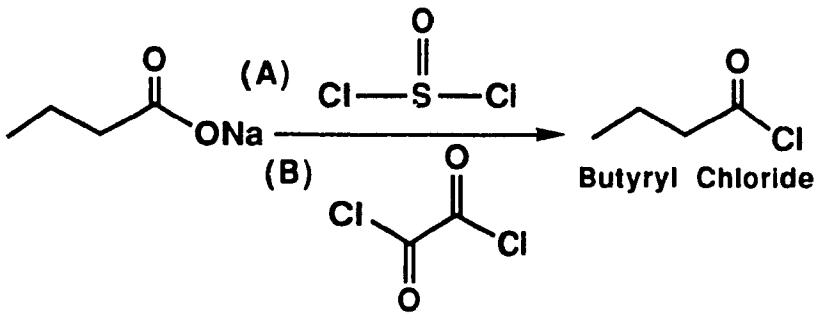
^{13}C labelled acetyl chloride (0.5 g, 7.0 mmol, 1 eq) was added dropwise *via* syringe into thallium salt solution of NAC (7 mmol, 1 eq) at 0°C. The reaction was then left to stir for one hour after which the bright yellow color changed to pale yellow or milky white. THF was evaporated and the compound was dissolved in water and the pH was adjusted to 7.0. The solution was passed through celite and then extracted with dichloromethane (3x50 mL). The organic layers were collected, dried with anhydrous $MgSO_4$, and evaporated to give the crude product. Flash

column chromatography was performed using 3:1 EtOAc and CH_2Cl_2 as the solvent system resulting in an 85% yield of ^{13}C labelled compound.

^1H NMR (270 MHz, $CDCl_3$) : δ 1.99 (s, 3H, $C\underline{H}_3CON$), 2.35 (d, 3H, $C\underline{H}_3COS$, $^2J_{C1\text{-}H2}$=6 Hz), 3.03 (m, 2H, J=6.6 $C\underline{H}_2S$), 3.43 (d of t, J=5.9 and 6.6 Hz, 2H, $C\underline{H}_2$-NCO), 6.74 (s, 1H, NH).

^{13}C NMR (270 MHz, $CDCl_3$) : δ 22.9 (CH_3CON), 28.5 (CH_2S), 30.0 (d, $^2J_{C1\text{-}C2}$=47.3 Hz CH_3COS), 39. 2 (CH_2NH), 170.6 (CON), 195.9 (^{13}COS) .

[3.5.5] Butyryl Chloride



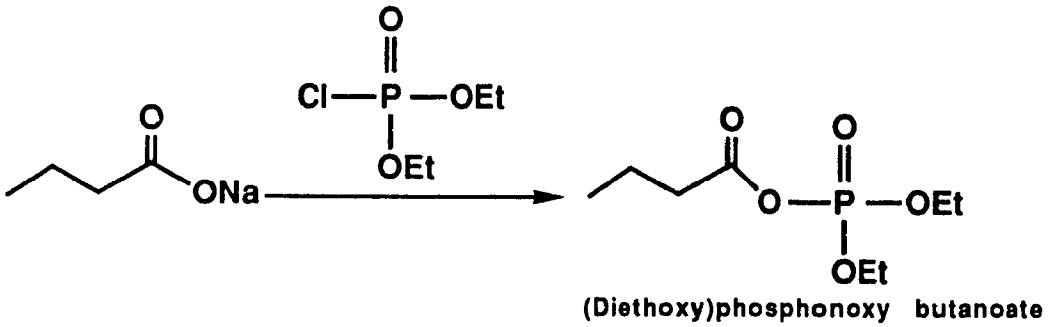
Route 1:

Sodium butyrate (964 mg, 1 mL, 10 mmol, 1 eq) was refluxed neat with freshly distilled thionyl chloride (8.155 g, 5 mL, 68 mmol, 6.8 eq) for 5 hours. The excess thionyl chloride was evaporated, the reaction mixture was dissolved in 5 mL THF and transferred *via* syringe to the solution mixture of the thallium salt of N-acetylcysteamine.

Route 2:

Sodium butyrate (250 mg, 2.25 mmol, 1 eq) was reacted neat with freshly distilled oxalyl chloride (4.35 g, 3 mL, 34.4 mmol, 34 eq) for 5 hours at room temperature. The excess oxalyl chloride was evaporated, the reaction mixture dissolved in 5 mL THF and transferred *via* syringe to the solution mixture of the thallium salt of N-acetylcysteamine.

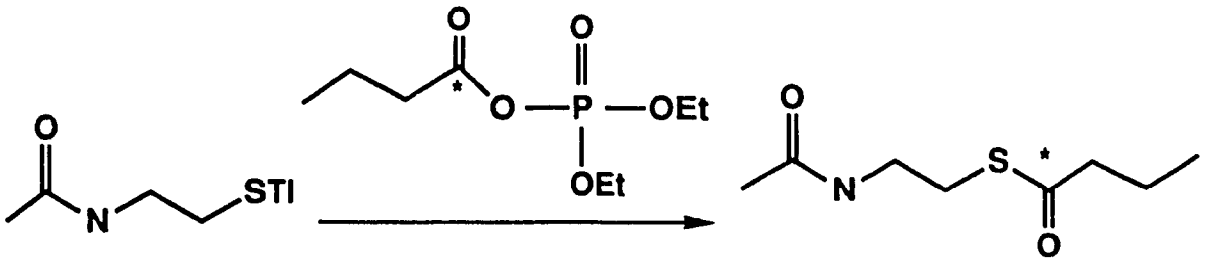
[3.5.6] (Diethoxy)phosphonoxy butanoate



(Diethoxy)phosphonoxy butanoate

Sodium butyrate (200 mg, 1.8 mmol, 1 eq) was added to 10 mL dry THF in a 25 mL flask under nitrogen. To this solution diethyl chlorophosphate (0.26 mL, 1.8 mmol, 1 eq) was added and the reaction was left to stir for five hours. This solution was used without further purifications in the following step.

[3.5.7] ^{13}C labelled N-acetylcysteamine thioester of butyric acid.[9]



NAC thioester of butyric acid

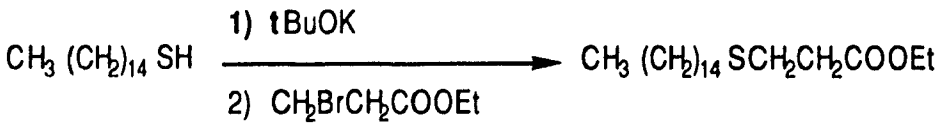
The above ^{13}C labelled (diethoxy)phosphonoxy butanoate was added dropwise *via* syringe into a solution of thallium salt of N-acetylcysteamine (1.8 mmol, 1 eq) in THF at r.t. The reaction was then left to stir for 12 hours after which the bright yellow color changed to milky white. THF was evaporated, the reaction mixture was dissolved in water and the pH was adjusted to 7.0. The solution was passed through celite and then extracted with CH_2Cl_2 (3x50 mL). The extracts were combined, dried with anhydrous $MgSO_4$, and evaporated to give ^{13}C-labelled N-acetylcysteamine thioester of butyric acid. The product was then purified by reversed phase flash column chromatography using a linear gradient from 100% water to 100% methanol and giving an isolated yield of 78%.

^1H NMR (270 MHz, $CDCl_3$) δ 0.93 (t, J=7.3 Hz, 3H, $C\underline{H}_3CH_2$) , 1.67 (m, J=7.3 and 4.6 Hz, 2H, $CH_3C\underline{H}_2$), 1.97 (s, 3H, $C\underline{H}_3CON$) , 2.50 (m, 2H, $C\underline{H}_2COS$) , 3.02 (m, 2H, $C\underline{H}_2S$), 3.6 (m, 2H, $C\underline{H}_2N$), 6.8 (s, 1H, NH).

^{13}C NMR (270 MHz, CDCl3) : δ 13.2 (d, $^3J_{C1\text{-}C4}$=3.3 Hz, $\underline{C}H_3CH_2$) , 18.9 (d, $^2J_{C1\text{-}C3}$=2.2 Hz, $CH_3\underline{C}H_2$) , 22.9 ($\underline{C}H_3CON$) , 28.1 ($\underline{C}H_2S$) , 39.4 ($C\underline{H}_2N$), 45.7 (d, $^3J_{C1\text{-}C4}$=45.2 Hz, $\underline{C}H_2COS$), 170.4 (CON), 199.5 (^{13}COS).

MS C.I, (NH_3) direct inlet: 190 ($M^+ +1$).

[3.5.8] The ethyl ester of (Tetradecylthio)propionic acid [42,43]

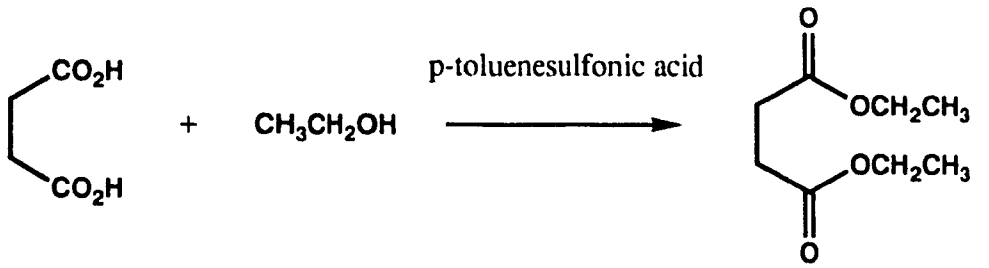


Tetradecanethiol (0.92 g, 1.08 mL, 4 mmol) was added to 40 mL dry THF in a 100 mL flask under nitrogen. Potassium *t*-butoxide (4.4 mL, 4.4 mmol, 1.1 eq) was added dropwise *via* syringe at room temperature and the reaction was left to stir for 1/2 hr (the reaction mixture was thick and cloudy). Ethyl 3-bromopropionate (0.72 g, 4 mmol, 1 eq), dissolved in 2 mL dry THF, was added dropwise and the reaction was left to stir at room temperature for 3 hours. THF was then evaporated and the reaction mixture was dissolved in 50 mL of water and extracted with diethyl ether (3x50 mL). The organic layers were combined, dried over $MgSO_4$, and evaporated under reduced pressure. The product was then purified by flash column chromatography using 5% ethyl acetate in hexane to give an isolated yield of 70%.

^{1}H NMR (270 MHz, $CDCl_3$) : δ 0.9 (t, J=6.6 Hz, 3H, $\underline{C}H_3$-CH_2) , 1.2 (s, 22H, $\underline{C}H_2$), 1.5 (m, 2H, $\underline{C}H_2$-CH_2S) , 2.5 (m, J=6.9 and 7.2 Hz 4H, $\underline{C}H_2$-S) , 2.7 (t, J=7.2 Hz, 2H, $\underline{C}H_2$ COO) ,4.1(q, 2H, O-$\underline{C}H_2$).

^{13}C NMR (270 MHz, $CDCl_3$) : δ 13.9, 13.8, 22.3, 26.6, 287.5, 28.9, 29.0, 29.2, 29.31, 29.34, 29.37, 31.6, 31.7, 34.6, 60.0, 171.2.

[3.5.9] Diethyl Succinate [44,45]

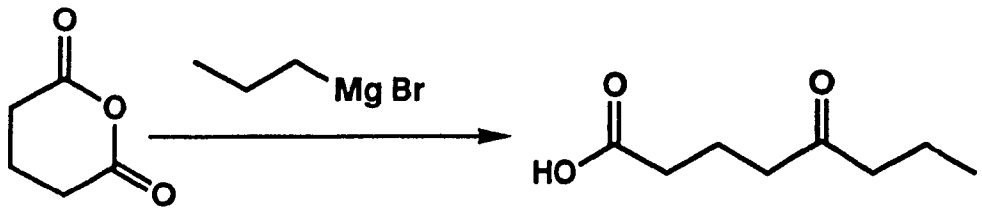


Succinic acid Ethanol Di-ethyl succinate

Succinic acid, 0.80 g (6.8 mmol), was dissolved in 50 mL of 99% ethanol with a catalytic amount of *p*-toluene sulfonic acid. The mixture was refluxed vigorously overnight. To the reaction mixture was added 100 mL of saturated NaCl water and 100 mL of chloroform. The chloroform layer was removed and the aqueous layer washed with 3x75 mL of fresh chloroform. The chloroform extracts were then combined and washed with 100 mL of saturated $NaHCO_3$. The chloroform was then dried and evaporated to give diethyl succinate in an isolated yield of 87.7%.

^{1}H NMR (270 MHz, $CDCl_3$) : δ 1.2 (m, 4H, $\underline{C}H_3$-CH_2), 2.5 (4H, $\underline{C}H_2COO$), 4.1 (m, 2H, CH_3-$\underline{C}H_2$).

[3.5.10] 5-Keto-octanoic acid



5-Keto-octanoic acid

A Grignard reagent was prepared from bromopropane (2.4 mL, 26.3 mmol, 1.0 eq) and magnesium turnings (640 mg, 26.3 mmol, 1.0 eq) in dry THF (20 mL). Complete formation of the Grignard reagent required 2 hours of refluxing. This solution was transferred *via* syringe over a period of 30 minutes into a stirred solution of glutaric anhydride (2.96 g, 26.0 mmol, 0.95 eq) in THF (20 mL). TLC analysis of the reaction indicated that three products were formed in the first hour. The reaction was quenched using water. The THF was evaporated, and the pH of the reaction mixture was adjusted to 3.0. The reaction mixture was then extracted using EtOAc and dried over $MgSO_4$ to yield the crude product (60 %). The crude was purified by flash column chromatography eluting with 5 % EtOAc/CH_2Cl_2 and 0.1 % CH_3COOH to give the product in a 10 % isolated yield.

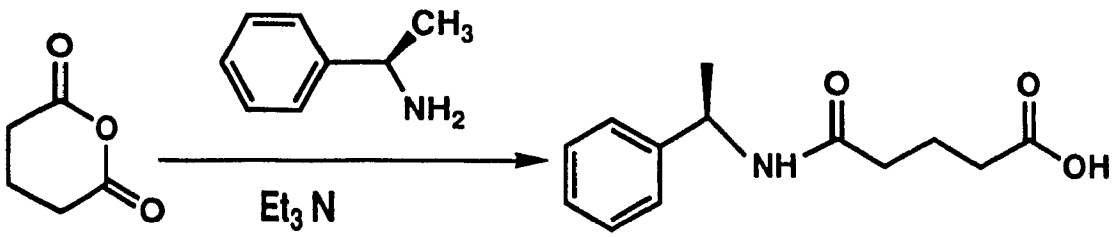
^{1}H NMR (270 MHz, $CDCl_3$) : δ 0.85 (t, J= Hz, 3H, $C\underline{H}_3CH_2$) , 1.57 (m, 2H, $CH_3C\underline{H}_2$), 1.87 (m, 2H, $C\underline{H}_2CH_2COOH$) , 2.40 (t, 4H, $C\underline{H}_2C0C\underline{H}_2$) , 2.48 (t, 2H, $C\underline{H}_2COOH$), 9.7 (s, 1H, OH).

^{13}C NMR (270 MHz, $CDCl_3$) : δ 13.6, 17.2, 18.5, 33.0, 41.3, 44.7, 179.2, 210.4.

MS(C.I, (NH_3) direct inlet: 159 (M^+ +1).

The side products of this reaction were an unknown alchol (M^++1=176) and 5-propyl-5-hydroxy octanoic acid (M^++1=203) which were isolated in 10% and 28% yield respectively.

[3.5.11] (S)-(-)-α-Methylbenzyl-(pentanoic acid) amide



(S)-(-)-α-Methylbenzylamine (630 mg, 5.2 mmol, 1 eq), with triethylamine (52.6 mg, 0.52 mmol, 0.1 eq), was added to a solution of glutaric anhydride (590 mg, 5.2 mmol, 1 eq) in THF (50 mL). The reaction was left to stir for 5 hours at r.t. The THF was evaporated and the pH of the reaction mixture adjusted to 3.0. The reaction mixture was then extracted using EtOAc and dried over $MgSO_4$ to give the pure product in an 82 % yield.

^{1}H NMR (270 MHz, $CDCl_3$) : δ 1.34 (d, J= 7.26 Hz, 3H, $C\underline{H}_3C(ph)HN$) , 1.82 (m, J=7.26 and 7.92 Hz) 2H, $C\underline{H}_2CH_2COOH$), 2.16 (t, J=7.92 Hz, 2H, $C\underline{H}_2CO$), 2.25 (t, J=7.26, 2H, $C\underline{H}_2COOH$) , 4.97 (q, J=7.26 Hz, 1H, C(Ph)$\underline{H}$N), 6.37 (d, 1H, NH), 7.17 (m, 5H, ph), 10.9 (s, 1H, OH).

^{13}C NMR (270 MHz, $CDCl_3$) : δ 20.7, 21.7, 33.0, 35.2, 48.8, 126.1, 127.2, 128.5, 143.0, 172.0, 177.4.

REFERENCES

[1] R.B. Hebert, The biosynthesis of secondary metabolites. 2nd ed. Chapman and Hall, New York. 1-5 , (1989).

[2] J. Baldwin, M. Bradley, *Chem. Rev*, 1079, (1990).

[3] D. E. Cane, H. Hasler, P. B. Taylor. T. Liang. *Tetrahedron.* **39**, No 21, 3449, (1983).

[4] J. M. Weber, J. O. Leung, S. J. Swanson. K. B. Idler, and J. B. McAlpine. *Science.* **252**, 114, (1991).

[5] J. Rohr, M. Schonewolf, G. Udvarnoki, K. Eckardt, G.Schumann, and C. Wagner. *J. Org. Chem.* **58**, 2547, (1993).

[6] G. Udvarnoki, T. Henkel, R. Machinek, and J. Rohr. *J. Org. Chem.* **57**, 1274, (1992).

[7] J. N. Collie, *J. Am. Chem. Soc.* **91**, 1806, (1907).

[8] D. E. Cane, R. H. Lambalot, P. C. Prabhakaran, and W. R. Ott. *J. Am. Chem. Soc.* **115**, 522, (1993).

[9] I. Wang, L.Vining, J.Walter, and G. Mcinnes, J. Antibiot. **39**, No 9, 1281, (1986).

[10] Y. Yoshizawa, Z. Li, P. B. Reese, And J. C. Vederas. *J. Am. Chem. Soc.* **112**, 3212, (1990).

11 S. Takenaka, Y. Sekiguchi, Y. Shimazaki, K. Ogazawara, *Tetrahedron Lett*, **30**, 4001, (1989).

12 Z. Li, F. M. Martin, and J. C. Vederas, *J. Am. Chem. Soc.* **114**, 1531, (1992).

13 H. Oikawa, A. Ichihara, S. Sakamura, *J. Chem. Soc. Chem. Commun.* 600, (1988).

14 L.Stryer. Biochemistry. 3rd ed. Freeman. New York. 355-363, (1988).

15 S. J. Wakil, J. K. Stoops, and V. C. Joshi, *Ann. Rev. Biochem.* **52**, 537, (1983).

16 H. Umezawa, T. Takeuchi, H. Linuma, K. Suzuki, M. Ito, and M. Matsuzaki. *J. Antibio.* **23**, No 10, 514, (1970).

17 T. Nagatsu, K. Mizutani, I. Nagatsu, H. Umezawa, M. Matsuzaki, and T. Takeuchi. *J. Molecular & Cellular Biochemistry.* **1**, No 1, 107, (1973).

18 J. W. Blunt, V. L. Cadler, G. Fenwick, R. J. Lake, J. D. McCombs, M. Munro and N. Perry, *J. Natural products*, **50**, No 2, 290, (1987).

19 Y. S. Tsantrizos, K. K. Ogilvie, A. K. Watson. *Can. J. Chem.* **70**, No 8, 2276, (1992).

20 B. Evans, D.Dale, C. J. Little, *Chromatographia.* **13**, No 1, 6, (1980).

[21] M.Ohno, M. Okamoto, N. Kawabe, H. U mezawa, T. Takeuchi, H. Linuma, S. Takahashi, *J. Am. Chem. Soc* . **93**, No 5, 1285, (1971).

[22] H. A. Bates, J. Farina. *J. Org. Chem.* **50**, 3843, (1985).

[23] T. Sakai, K. Iwata, M. U. Take, and A. Taketa. *Bull. Chem. Soc. Jpn.* **60**, 1161, (1987).

[24] H. A. Krebs. and W. A. Johnson. *Enzymologia.* **4**, 148, (1937).

[25] J. Staunton, A. C. Sutkowski, *J. Chem. Soc. Chem. Commun.* 1110, (1991) and accompanying papers.

[26] J. L. C. Wright, L. C. Vining, A. G. McInnes, D. G. Smith, and J. A. Walter. *J. Org. Chem.* **57**, 4452, (1992).

[27] J. G. Hill, T. T. Nakashima, and J. Vederas. *J. Am. Chem. Soc.* **104**, 1745, (1982).

[28] T. T. Nakashima, and J. Vederas. *J. Chem. Soc. Chem. Commun.* **206**, (1982).

[29] D. E. Cane, P. C. Prabhakaran, W. Tan, and R. W. Ott. *Tetrahedron lett.* **32**, 5457, (1991).

[30] D. E. Cane, C. Yang. *J. Am. Chem. Soc.* **109**, 1255, (1987).

[31] S. Yue, J. S. Duncan, Y. Yamamato, C. R. Hutchison. *J. Am. Chem. Soc.*
109, 1253, (1987).

[32] K. Parker, C. Coburn. *J. Org. Chem.* **56**, 4600, (1991).

[33] Fei Shou, M. Sc. thesis unpublished results.

[34] S. Omura, A. Nakagawa, H. Takeshima, J. Miyazawa, C. Kito, *Tetrahedron lett.* **50**, 4503, (1975).

[35] S. Omura, H. Takeshima, A. Nakagawa, N. Kamemato, and G. Lukacs,
Bioorganic Chemistry, **5**, 451, (1976).

[36] J. Omura, K. Tsuzuki, Y. Tamaka, H. Sakakibara, M. Aizawa, and G. Luckacs. *J. Antibio.* **5**, 614, (1983).

[37] V. Grignard. Noble prize winner in 1912 for the discovery of organomagnesium halides.

[38] Kice, Hanson. *J. Org. Chem.* **38**, 1410, (1973).

[39] S. Miki, Y. Sato, H. Tabuchi, H. Oikawa, A. Ichihara, and S. Sakamura. *J. Chem. Soc. Perkin Trans.* **1**, 1228, (1990).

[40] W. C. Still, M. Khan, and A. Mitra. *J. Org. Chem.* **43**, No 14, 2923, (1978).

[41] J. M. Schwab, J. B. Klassen, *J. Am. Chem. Soc.* **106**, 7217, (1984).

[42] O. Sydevold, *J. Biochim. Biophys. Acta* **1003**, 72, (1989).

[43] J. Skorve, B. Ruyter, A.C. Rustan, E. N. Christiensen, C. A. Dreven, and R. K. Berge. *J. Biochem. pharmacol.* **40**, 2005, (1990).

44 M. E. Volpin. *J. Organometallic React.* **5**, 313, (1975).

45 Paul Tiseo. Honors project. Chemistry, Concordia University. (1992).