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THE BIOSYNTHESIS OF MANUMYCIN TYPE METABOLITES

by

Yiding Hu

A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

University of Washington

2000

Program Authorized to Offer Degree: Organic Chemistry, Department of Chemistry

Doctoral Dissertation

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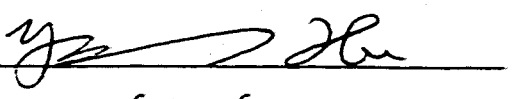
Signature 

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Heinz G. Floss

Reading Committee:

Heinz G. Floss

Tomikazu Sasaki

Craig C. Beeson

Date: 3/15/00

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Abstract

THE BIOSYNTHESIS OF MANUMYCIN TYPE METABOLITES

By Yiding Hu

Chairperson of the Supervisory Committee: Professor Heinz G. Floss
Department of Chemistry

The biosynthesis of manumycin type metabolites has been thoroughly studied, mainly with the asukamycin producer, *Streptomyces nodosus*. A complete biosynthetic pathway has been elucidated from two approaches: i) synthesizing and feeding labeled compounds to *S. nodosus* and the manumycin A producer, *S. parvulus*; ii) tracing and isolating pathway related metabolites by feeding an isotopically labeled precursor. 3-Amino-4-hydroxybenzoic acid (3,4-AHBA) was identified as the key precursor of the mC_7N unit of manumycins by synthesizing and feeding 3,4-[7-^{13}C]-AHBA. The discovery of a shunt metabolite, 7-(3-(N-acetylamino-4-hydroxyphenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienoic acid **(85)** and other type I manumycin (epoxyquinol mC_7N unit) co-metabolites produced by *S. nodosus* helped to elucidate the biosynthetic pathway which proceeds from 3,4-AHBA by the "lower" polyketide chain extension followed by attachment of the "upper" chain and subsequently of the C_5N unit. The quantitative studies on the composition of fatty acids and type I manumycins from *S. nodosus* revealed that the production of cyclohexanecarboxylic acid **(39)** is a rate limiting step of asukamycin biosynthesis. This limitation results in formation of shunt metabolite **85** and type I manumycin co-metabolites which differ from asukamycin at the starter unit of the "upper" chain by using branched chain starter units instead of **39**. The multi-step synthesis and feeding of N_1-(2-hydroxy-5-oxo-cyclopent-1-enyl)-7-[3-(7-cyclohexyl-hepta-(*2E,4E,6E*)-trienoyl)-amino-4-hydroxyphenyl]-[1,2-$^{13}C_2$]hepta-(*2E,4E,6E*)-trienamide **(71)** and its incorporation into asukamycin further demonstrated that oxidation / epoxidation is the last reaction on the biosynthetic pathway to the type I manumycins. The discovery of a series of new type II manumycins (hydroxyquinol mC_7N unit) and the study of the fermentation course indicated that the type II manumycins produced at a later stage are formed from the type I manumycins by reduction of the epoxide.

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LIST OF ABBREVIATIONS

ACP	acyl carrier protein
3,4-AHBA	3-amino-4-hydroxybenzoic acid
ALA	aminolevulinic acid
ATCC	American Type Culture Collection
BOC	*t*-butyl carbamate
BOP	(benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate
br	broad
CD	circular dichroism
CoA	coenzyme A
d, dd	doublet, double of doublets
δ	chemical shift
DCC	dicyclohexylcarbodiimide
DIBAL-H	American Type Culture Collection
DMAP	4-dimethylaminopyridine
DMF	N,N-dimethylformamide
DMSO	dimethyl sulfoxide
DTE	1,4-dithioerythritol
EDCI	1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride
EI	electron impact
ES	electrospray

FAB	fast atom bombardment
GC	gas chromatography
HPLC	high performance liquid chromatography
hr	hour
HR-MS	high resolution mass spectrometry
Hz	hertz
J	coupling constant, Hz
LC	liquid chromatography
M, mM	molar, micromolar
MS	mass spectrometry
m	multiplet
min	minute
MW	molecular weight
m/z	mass/charge
NAC	N-acetylcysteamine
NMR	nuclear magnetic resonance
PEP	phosphoenolpyruvate
PKS	polyketide synthase
PLP	pyridoxal phosphate
PMSF	phenylmethanesulfonyl fluoride
ppm	parts per million
PyBrOP	bromotripyrrolidino phosphonium hexafluorophosphate
q	quintet
R_f	relative mobility

s	singlet
sec	second
SIM	selective ion monitoring
t	triplet
TFA	trifluoroacetic acid
TFAA	trifluoroacetic anhydride
THF	tetrahydrofuran
TLC	thin layer chromatography
***p*-TsOH**	*p*-toluenesulfonic acid
UV	ultraviolet
TBDMS	*tert*-butyldimethylsilyl

ACKNOWLEDGMENTS

I sincerely thank my advisor, Professor Heinz G. Floss, for providing me the opportunity to work in this project and for his guidance and support throughout the research. I am grateful to my supervisory committee, Professor Frank Turecek, Professor Tomikazu Sasaki, Professor Craig C. Beeson and Professor Paul Yager for their time and help. A special thank goes to our collaborator, Dr. Steven J. Gould for his suggestions and discussions. Also I acknowledge former and current Floss group members for sharing their knowledge and helpful discussion with me. Last but not the least, thanks to my parents, Boxiong Hu and Chuyu Fan, for their support and encouragement, thanks to my wife, Jingqiu Wang, for her support and love along this journey.

This thesis is dedicated to my parents, Boxiong Hu and Chuyu Fan, and my wife, Jingqiu Wang

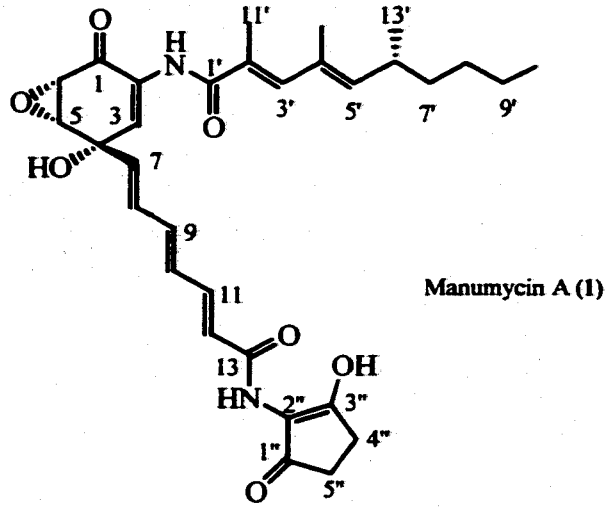
CHAPTER 1. INTRODUCTION

1.1 The Manumycin Family of Microbial Metabolites

1.1.1 General

The first member of the manumycin family of antibiotics, manumycin A **(1)** (Figure 1), produced by the bacterium *Streptomyces parvulus* Tü 64, was discovered in 1963 by Zähner and co-workers (Buzzetti *et al.*, 1963). Its constitutional structure was determined in 1973 (Schroder and Zeeck 1973) and its complete stereochemistry was reported in 1987 (Thiericke *et al.*, 1987) and revised in 1998 (Alcarz *et al.*, 1998). Manumycin A contains a "central" six-membered ring 2-amino-4-hydroxy-5,6-epoxycyclohex-2-enone structure (epoxyquinol moiety or mC_7N unit) and two *meta*-arranged polyketide chains ("upper" and "lower" chain). The "upper" chain consists of a methyl branched unsaturated fatty acid linked to the nitrogen of the mC_7N unit by an amide bond. The "lower" chain extends from the mC_7N unit as an all *trans* triene and terminates with a carboxyl group amide-linked to the five-membered ring 2-amino-3-hydroxycyclopent-2-enone moiety (C_5N unit).

In 1976, asukamycin **(2)** (Figure 1), produced by *Streptomyces nodosus* subsp. *asukaensis*, was discovered in Japan by Omura (Omura *et al.*, 1976). Asukamycin was the second known manumycin group metabolite. It shares with **(1)** the same structural elements of the mC_7N unit, the "lower" chain and the C_5N moiety, and only differs in the structure of



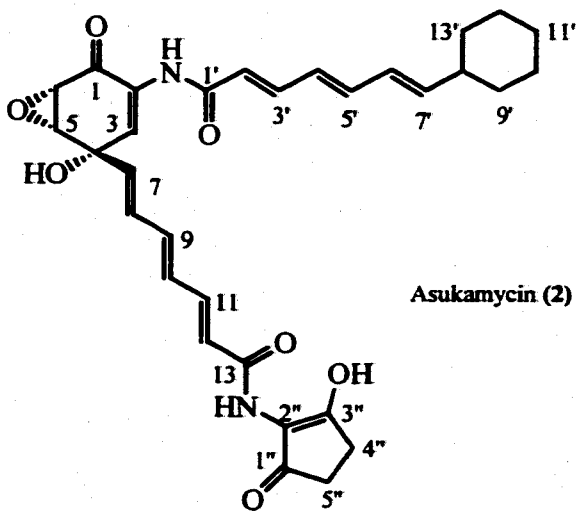


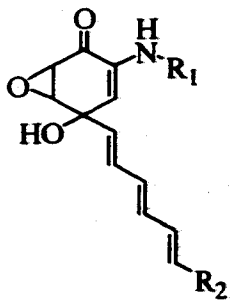
Figure 1. Manumycin A and Asukamycin

the "upper" chain. Instead of a methyl branched unsaturated fatty acid chain, asukamycin carries a linear triene "upper" chain terminating in a cyclohexane ring. Since then, more and more structurally closely related natural products have been discovered, structurally characterized and classified as members of the manumycin family of antibiotics. To date, this group of metabolites has grown to 30 members (Figure 2 and Table 1-3, enlarged and modified from Zeeck's review, (Sattler *et al.*, 1998)).

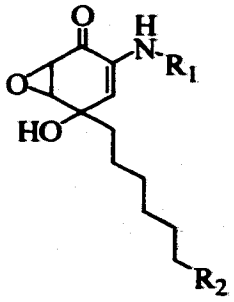
1.1.2 Structure and Classification

The manumycin group of microbial metabolites share the "mC_7N" unit as their most distinctive feature and are classified into two sub-classes. The compounds with an oxirane at C-5/C-6 are named type I manumycins. Those with a hydroxyethylene group at C-5/C-6 are referred to as type II manumycins. The mC_7N unit and the all *trans* triene "lower" chain are the most conserved structural elements in all manumycin type metabolites, except for colabomycin A (**5**) and D (**20**) with an all *trans* tetraene "lower" chain and U-62,162 (**19**) with a saturated "lower" chain. The C_5N unit is the second most characteristic moiety; it exists in most manumycin type compounds except nisamycin (**13**) and U-62,162 which terminate in a carboxyl group.

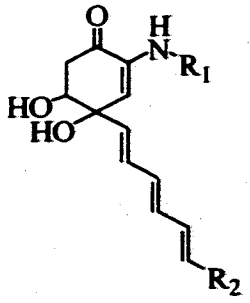
The "upper" chain of the manumycin family compounds displays the most structural variation. In general, it consists of an unsaturated fatty acid carrying from one to four double bonds, with or without methyl branches, and terminates in a saturated



Type IA and IB

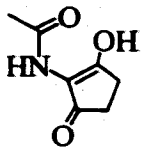


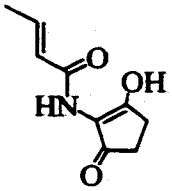
Type IC



Type IIA and IIB

R_2 =

a 

b 

c COOH

Figure 2. Classification of Manumycins

Table 1. Type IA Manumycin Type Metabolites

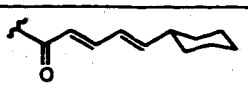
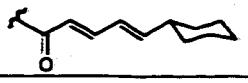
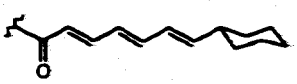
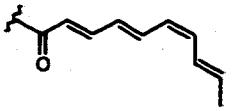
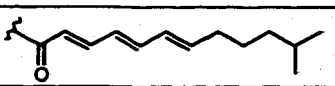
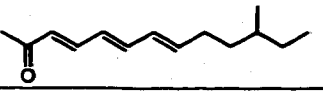
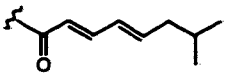
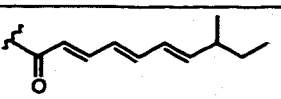
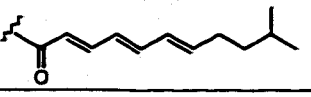
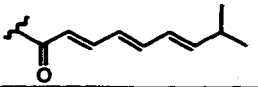
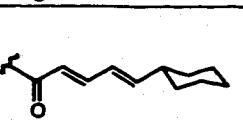
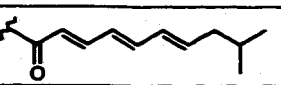
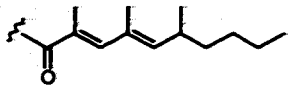
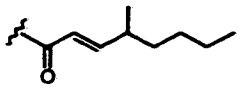
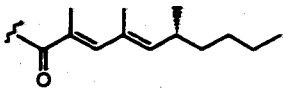
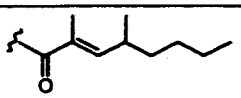
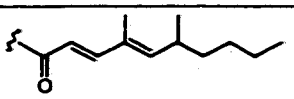
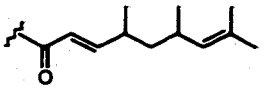
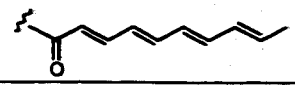
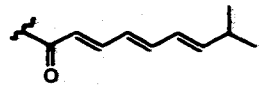
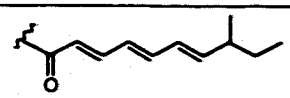
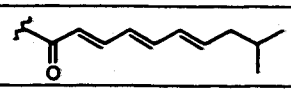
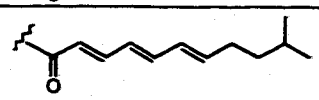
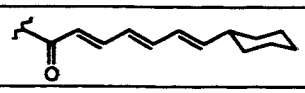
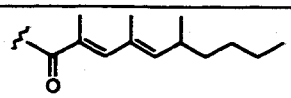
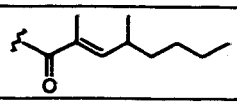
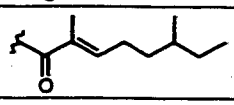
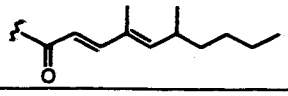
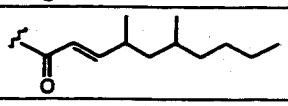
Compound	Stereochemistry	R^1	R^2	Reference
Alisamycin **3**	*4R, 5S, 6R*		a	(Franco *et al.*, 1991),(Hayashi *et al.*, 1994a),
Ent-alisamycin **4**	*4S, 5R, 6S*			(Tanaka *et al.*, 1996a)
Asukamycin **2**	*4S, 5R, 6S*			(Omura *et al.*, 1976), (Kakinuma *et al.*, 1979),(Cho *et al.*, 1993)
Colabomycin A **5**	*4S, 5R, 6S*		b	(Grote *et al.*, 1988b), (Grote *et al.*, 1988a)
Compound **6a**	Possibly *4S, 5R, 6S*		a	This thesis
Compound **6b**	Possibly *4S, 5R, 6S*			This thesis
Compound **7**	Possibly *4S, 5R, 6S*			This thesis
EI-1511-3 **8**	*4S, 5R, 6S*			(Tanaka *et al.*, 1996a; Tanaka *et al.*, 1996b),(Tanaka and Tsukuda 1995)
EI-1511-5 **9**	*4S, 5R, 6S*			(Tanaka *et al.*, 1996a),(Tanaka *et al.*, 1996b),(Tanaka and Tsukuda 1995)
Manumycin E **10**	*4S*, 5 and 6 not determined			(Shu *et al.*, 1994), (Patel and Shu 1995)
Manumycin F **11**	*4S*, 5 and 6 not determined			(Shu *et al.*, 1994), (Patel and Shu 1995)
Manumycin G **12**	*4S*, 5 and 6 not determined			(Shu *et al.*, 1994), (Patel and Shu 1995)
Nisamycin **13**	4*R*, 5*S*, 6*R*		c	(Hayashi *et al.*, 1993), (Hayashi *et al.*, 1994c),(Hayashi *et al.*, 1994b)
U-56, 407 **14**	*4S, 5R, 6S*		a	(Brodasky *et al.*, 1983)

Table 2. Type IB and IC Manumycin Metabolites

Compound	Stereochemistry	R^1	R^2	Reference
Type IB				
Compound **15**	not determined		c	(Izumi *et al.*, 1994)
EI-1625-2 **16**	*4S, 5R, 6S**		a	(Tanaka *et al.*, 1996a), (Tanaka *et al.*, 1996b), (Tanaka and Tsukuda 1995)
Manumycin A **1**	4S, 5R, 6S*			(Schroder and Zeeck 1973),(Zeeck *et al.*, 1987),(Kohno *et al.*, 1996),(Thiericke *et al.*, 1987),
Manumycin B **17**	*4S, 5R, 6S**			(Sattler *et al.*, 1993)
Manumycin C **18**	*4S, 5R, 6S*			(Sattler *et al.*, 1993), (Kohno *et al.*, 1996)
Type IC				
U-62, 162 **19**	not determined		c	(Slechta *et al.*, 1982)

* These compounds were originally assigned as 4*R*, 5*R*, 6*S*; the revised stereochemistry is based on the work of Taylor and co-workers on *ent*-manumycin A (Alcarz *et al.*, 1998).

Table 3. Type IIA and IIB Manumycin Metabolites

Compound	Stereochemistry	R^1	R^2	Reference
Type IIA				
Colabomycin D **20**	4*S*, 5*R*		b	(Sattler *et al.*, 1998)
Compound **21**	Possibly 4*S*, 5*R*		a	This thesis
Compound **22**	Possibly 4*S*, 5*R*			This thesis
Compound **23**	Possibly 4*S*, 5*R*			This thesis
Compound **24**	Possibly 4*S*, 5*R*			This thesis
Compound **25**	Possibly 4*S*, 5*R*			This thesis
Type IIB				
Manumycin D **26**	4*S*, 5*R*		a	(Kohno *et al.*, 1996),(Sattler *et al.*, 1993)
TMC-1A **27**	*4S, 5R*			(Kohno *et al.*, 1996)
TMC1B **28**	*4S, 5R*			(Kohno *et al.*, 1996)
TMC-1C **29**	*4S, 5R*			(Kohno *et al.*, 1996)
TMC-1D **30**	*4S, 5R*			(Kohno *et al.*, 1996)

hydrocarbon group ranging from methyl to branched hexane or cyclohexane. Based on the nature of the "upper" chain, manumycins are further divided into group A and group B. Type IA manumycins, which include asukamycin **(2)**, alisamycin **(3)**, *ent*-alisamycin **(4)**, colabomycin **(5)**, compound **6**, **7**, EI-1511-3 **(8)**, EI-1511-5 **(9)**, manumycin E **(10)**, F **(11)**, G **(12)**, nisamycin **(13)** and U56-407 **(14)**, and type IIA, which include colabomycin D **(20)**, compound **21**, **22**, **23**, **24** and **25**, contain linear polyene "upper" chains. Type IB, which include compound **14**, EI-1625-3 **(16)**, manumycin A **(1)**, B **(17)** and C **(18)**, and type IIB, which include manumycin D **(26)**, TMC-1A **(27)**, 1B **(28)**, 1C **(29)** and 1D **(30)** contain methyl branched polyene "upper" chains. Type IC is the rare case of a saturated "lower" chain which is found in U-62,162 **(19)** only.

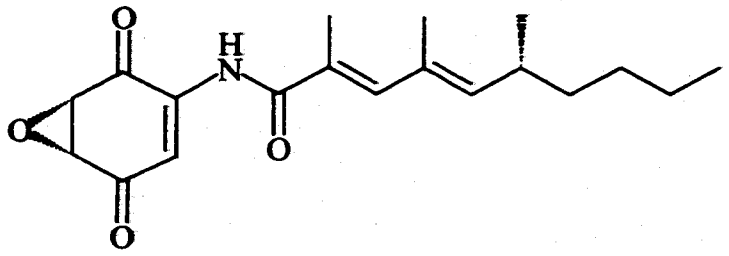
1.1.3 Stereochemistry at the mC_7N Unit

The stereochemistry at C-4, C-5 and C-6 of the mC_7N moiety was studied by circular dichroism (CD), ^{1}H-NMR aromatic solvent induced shift (ASIS), nuclear Overhauser enhancement (NOE) and chiral derivative methods.

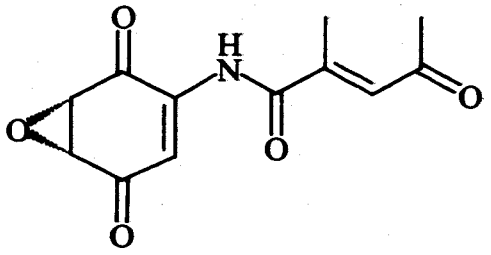
The absolute configuration at C-4 was first deduced in asukamycin by the exciton chirality method, and this method was later applied to deducing the C-4 stereochemistry in other manumycins as well. A positive Cotton effect at the longer wavelength indicated an *S*-configuration at C-4 for asukamycin and a negative one for manumycin A suggested an *R*-configuration for the latter.

The absolute configurations at C-5 and C-6 of the mC_7N unit were first deduced in manumycin A via its chromate oxidation products. Manumycin A was oxidized to the epoxyquinone derivatives **31** and **32** (Figure 3) with the removal of the "lower" chain and C_5N unit. The configurations at C-5 and C-6 were determined by comparing the CD spectrum of the oxidation products with that of antibiotic G7063-3 **(33)**. This method was also applied to colabomycin A **(5)**, alisamycin **(3)**, nisamycin **(13)**, EI-1511-3 **(7)**, EI-1511-5 **(9)**, EI-1625-3 **(16)**, manumycin G **(12)**, U-56, 407 **(14)** and *ent*-alisamycin **(4)**. Most manumycin type metabolites possess 4*S*, 5*R*, 6*S* configuration, except alisamycin and nisamycin which have the opposite 4*R*, 5*S*, 6*R* configuration. Overall most manumycins were deduced to have a *syn* 4-hydroxy/5,6-epoxide relative stereochemistry, except manumycin A, B and EI-1625-3 which were originally assigned to have an *anti* relationship between the 4-hydroxy group and the epoxide function.

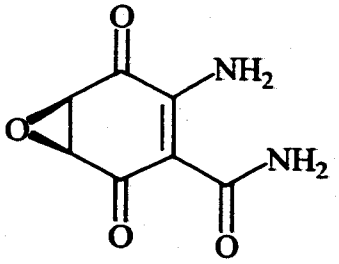
Recently, Taylor and co-workers synthesized *ent*-(+)-manumycin A with 4*R*, 5*S* 6*R* configuration and hence a *syn* 4-hydroxy/5,6-epoxide relationship (Alcarz *et al.*, 1998). This compound showed opposite stereochemical data to manumycin A (mirror image optical rotation and CD spectrum), indicating that the absolute configuration of manumycin A should be revised from 4*R* to 4*S*. Very likely, this change also applies to manumycin B and EI-1625-3. The error in the stereochemical assignment at C-4 from the CD spectra could be attributed to the structural variation in the "upper" chain, i.e., the linear or branched polyene. In type IB manumycin metabolites, the methyl branched



Degradation product from manumycin A **(31)**



Degradation product from manumycin A **(32)**



G7063-2 **(33)**

Figure 3. Structure of Manumycin A Derivatives and Antibiotic G7063-2

polyene may cause conformational changes which result in a realignment of the coupled "upper" and "lower" polyene chromophores. This work revealed that the *syn* 4-hydroxy/5,6-epoxide relationship is another unique feature for all manumycin type compounds.

1.2 Biological Activity

Most manumycins were discovered through biological screening. The manumycin family of compounds exhibits a broad range of diverse biological activities, including the inhibition of RAS farnesyltransferase (FTase), the inhibition of interleukin-1β converting enzyme (ICE) and the inhibition of Gram-positive bacteria, fungi and insects.

1.2.1 Antitumor Activity

In 1993 manumycin A was found to be an inhibitor of RAS protein farnesyltransferase (Hara *et al.*, 1993). The discovery of this important biological activity made manumycin A a potential anticancer drug. Therefore, it is the most intensively studied compound in this family, and it is now commercially available.

RAS genes, first discovered in 1978 at the National Cancer Institute, recently have been recognized to play one of the most crucial roles in human cell biology. RAS proteins generated from RAS genes are membrane-bound GTP-binding proteins that serve as

molecular switches in mitogenic signal transduction controlling the growth and differentiation of cells. This process is initiated by the addition of the farnesyl group to the C-terminal CAAX motif of RAS proteins, followed by the removal of the AAX residues and methylation at the carboxyl group of the remaining cysteine terminus (where C is cysteine, A is any aliphatic amino acid and X is methionine or serine). In the "switched on" GTP bound state, RAS proteins trigger a series of chemical processes which make cells divide. When they are in the "switched off" GDP bound state, this process is stopped. Mutated RAS proteins can not hydrolyze GTP and are constantly "switched on", causing unlimited cell growth and division thus leading to life threatening cancer (Der and Cox 1991, Gibbs *et al.*, 1994, Tamanoi 1993). Mutated RAS genes are believed to be involved in as many as one-third of all cancers, particularly in 90% of pancreatic cancers and in 50% of colon cancers. It is also reported that lung cancer patients bearing mutated RAS genes have a doubled recurrence and death rate.

RAS proteins thus undergo a series of post-translational modifications and then are anchored to the cell membrane before they can transmit their signal to start cell division. For that reason, RAS protein farnesyltransferase which catalyzes one of the modifications of RAS proteins, is an ideal target for cancer therapy. If RAS protein farnesyltransferase can be blocked, mutant RAS protein cannot be anchored to the cell membrane, as a result the tumor growth will cease. It is also important that this potential drug is able to selectively inhibit FTase but does not affect geranylgeranyltransferase (GGTase), because

geranylgeranylation of normal cellular proteins is five to ten times more prevalent than farnesylation.

Manumycin A **(1)**, as well as B **(17)** and C **(18)**, showed competitive inhibition of Ras farnesyltransferase (Hara *et al.*, 1993). The most potent one, manumycin A, has an *in vitro* level of activity of IC_{50} = 5 μM and K_i = 1.2 μM against yeast FTase. The structure-activity study revealed that the epoxyquinol (mC_7N) moiety is crucial to the inhibition. The "upper" chain contributes significantly to the antitumor activity due to its structural similarity to the methyl branched chain of farnesylpyrophosphate. The "lower" chain and C_5N moiety are not essential for the inhibition. Furthermore, manumycin A inhibits GGTase at a 30 times higher concentration than FTase. This could lead to the development of manumycin A into a potential drug against RAS-dependent tumors.

Since then, much effort has been put into studies of the inhibitory effect of manumycin A **1** on various tumor cells (Nagase *et al.*, 1996, Nagase *et al.*, 1997, Ito *et al.*, 1996, Kainuma *et al.*, 1997, Hara and Han 1995, Zou *et al.*, 1996). Kawata and co-workers reported that manumycin A selectively inhibits the proliferation of a human hepatoma cell line in a dose-dependent manner, and demonstrated that a non-farnesylated GTPase-defect RAS mutant could block the actions of oncogenic RAS (Nagase *et al.*, 1996). Kawata's group also found that manumycin A inhibited RAS farnesylation, colon cancer and hepatoma cell growth without disturbing the farnesylation and localization of the nuclear lamins. The colon cancer cell number was reduced to half at 5 μM and to only

10% at 15 μM of **1** in 72 hours. (Nagase *et al.*, 1997). Besides, manumycin A was found to be able to suppress the growth of human pancreatic cancer cells inoculated into nude mice (Ito *et al.*, 1996). It functioned in a dose-dependent manner and showed no apparent hepatotoxicity *in vivo*. At a 5 mg/kg daily injection level for 5 days, tumors shrunk to less than 90% of their original size, whereas the untreated tumors grew to more than 130% of their original size in mice.

Kainuma reported that manumycin A inhibited the growth and invasive activity of human pancreatic cancer cells, and suppressed the oncogenic RAS signal transduction more markedly than that of wild-type RAS (Kainuma *et al.*, 1997). The numbers of mutated cells were about 50% lower than that of wild-type cells upon treatment with 10 μM of **1**.

Most recently, it was found that manumycin A induced apoptosis of medulloblastoma cells, a malignant cerebellar tumor usually manifesting itself in childhood (Wang and Macaulay 1999).

Besides manumycin A **1**, the type II manumycins TMC-1A **(27)**, B **(28)**, C **(29)**, D **(30)** and manumycin D **(26)** also exhibit antitumor activity against various cancers, including colon and ovarian cancer, osteogenic sarcoma, promyelocytic leukemia, epitheloid carcinoma and lymphoid neoplasm (Kohno *et al.*, 1996). In general, type IIB manumycins possess from comparable to one order of magnitude less antitumor activity than their type IB counterparts with the same "upper" chain structure. For instance, the

IC_{50} is 11 μM on a colon adenocarcinoma cell line and 3.4 μM on lymphoid neoplasm for manumycin D, compared to 10.0 and 0.4 μM for manumycin A, respectively.

1.2.2 Antiinflammatory Activity

Interleukin-1 (IL-1), secreted by activated monocytes or macrophages, is involved in acute and chronic inflammation. IL-1 consists of two isoforms, IL-1α and IL-1β. The originally produced IL-β is inactive and has to be processed by interleukin-1β converting enzyme (ICE) to its active form. The active IL-1β, which is the major form of IL-1 in diseases, stimulates inflammation. ICE inhibitors might lead to the development of anti-inflammatory drugs.

EI-1511-3 **(8)**, EI-1511-5 **(9)**, EI-1625-2 **(16)** U-56, 407 **(14)**, manumycin G **(12)**, *ent*-alisamycin **(4)**, manumycin A **(1)** and manumycin B **(17)** were found to be biologically active in an *in vitro* screening of ICE inhibitors (Tanaka *et al.*, 1996b). The IC_{50} values of these type I manumycins vary from 0.09 to 11 μM, with EI-1511-3 **7** being the best inhibitor. The study also showed that removal of the "lower" chain and C_5N moiety by chromate oxidation did not change the ICE inhibitory potencies. The type IA linear polyene "upper" chain manumycin type metabolites exhibited better activity than the type IB methyl branched polyene "upper" chain compounds. All the tested manumycin compounds show inhibition of IL-1β secretion with IC_{50} values from 3.5 to 11 μM, and

the removal of the "lower" chain results in a great decrease of inhibitory potencies towards IL-β secretion to above 83 μM.

1.2.3 Antibacterial, Antifungal and Insecticidal Activity

Most type I manumycin family metabolites, including manumycin A **(1)**, asukamycin **(2)** (Omura *et al.*, 1976), U-56, 407 **(14)** (Brodasky *et al.*, 1983), U-62162 **(19)** (Slechta *et al.*, 1982), manumycin E **(10)**, F **(11)**, G **(12)** (Shu *et al.*, 1994), collabomycin A **(5)** (Grote *et al.*, 1988b), alisamycin **(3)** (Franco *et al.*, 1991), nisamycin **(13)** (Hayashi *et al.*, 1994b; Hayashi *et al.*, 1994c), EI-1511-3 **(8)**, EI-1511-5 **(9)** and EI-1625-2 **(16)** (Tanaka *et al.*, 1996b), exhibit antimicrobial activity against Gram-positive bacteria such as *Staphylococcus, Enterococcus faecalis* and *Bacillus subtilis.* No activity against Gram-negative bacteria, such as *Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris* and *Pseudomonas aeruginosa,* was observed with most of these compouds. Only manumycin E **(10)**, F **(11)** and G **(12)** possess minor activity against *Escherichia coli* (Shu *et al.*, 1994).

The type II manumycin family metabolites TMC-1A **(27)**, B **(28)**, C **(29)**, D **(30)** and manumycin D **(26)** have no activity against either Gram-positive or Gram-negative bacteria (Kohno *et al.*, 1996). This fact indicates that the epoxyquinol moiety is crucial to antibacterial activity. In a structure-activity study, it was shown that nisamycin **(13)** has 6-fold higher activity than alisamycin **(3)**, suggesting that the C_5N unit is not essential

for the antibacterial activity. Nisamycin derivatives with an R group such as an alkane, cycloalkane or arene, instead of the C_5N moiety showed that an increase in the hydrophobicity of the substituents caused decreases in antimicrobial activity (Figure 4) (Hayashi *et al.*, 1994b). For example, when R is cyclohexyl, the compound **37** was 10 times less active than **35** with an R group of cyclopropyl and 25 times less active than nisamycin.

Some of the manumycin type metabolites possess antifungal activity, such as manumycin A **(1)** (Zeeck *et al.*, 1987) and alisamycin **(3)** (Franco *et al.*, 1991) against *Candida albicans*, asukamycin **(2)** against *Trichophyton mentagrophyces*. Asukamycin also exhibits anticoccidial acitivity in chickens infected with *Eimeria tenella* (Omura *et al.*, 1976).

In experiments on insects, manumycin A was shown to restrict insect development in *Lepidoptera* and *Coleoptera* and to be a repellent to their larvae. Good insecticidal effects were achieved by spraying the eggs and larvae of *Pieris brassica* and *Epilachna varivestis* with low concentration manumycin A solution (0.05%) (Zeeck *et al.*, 1987).

1.3 Biosynthesis of Manumycin Type Metabolites

Studies on the biosynthesis of the manumycin family have mainly focused on manumycin A and asukamycin and have involved feeding radioactive and stable isotope-labeled

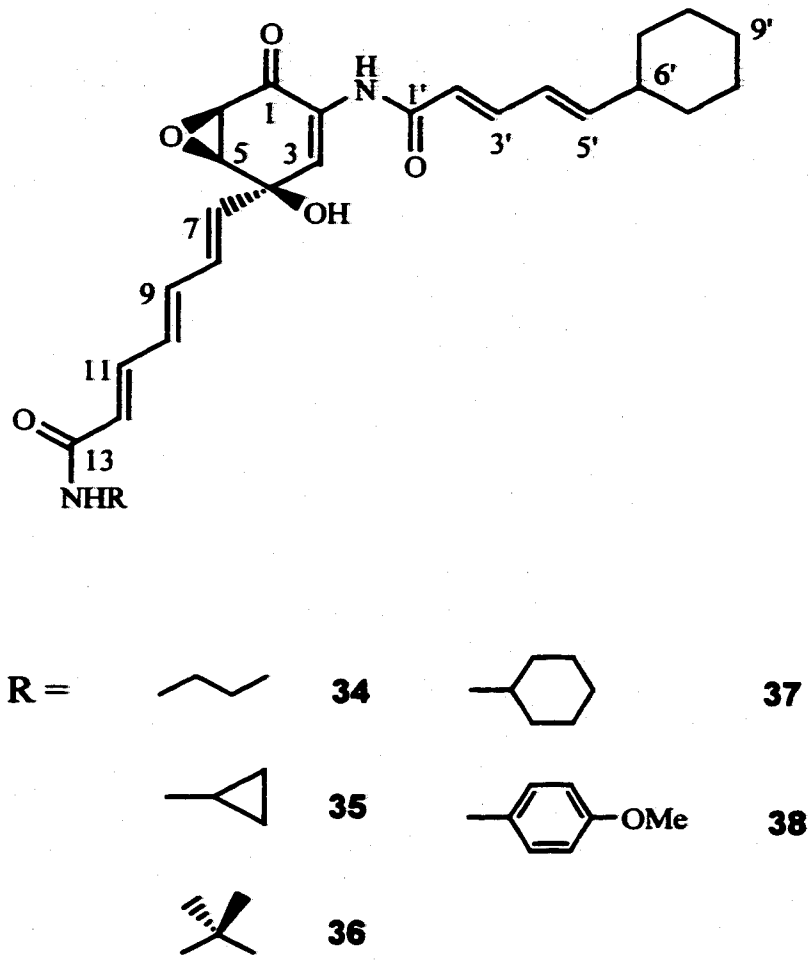


Figure 4. Nisamycin Derivatives

compounds. The most characteristic structural variation of manumycin type compounds is in the "upper" chain. The biosyntheses of manumycin A (type IB) and asukamycin (type IA) could thus be representative of the biosynthesis of the whole class of compounds. The results of the biosynthetic studies up to the work reported in this thesis are summarized below.

1.3.1 Biosynthesis of the "Upper" Chain

Singly and doubly ^{13}C-labeled acetate, malonate and propionate were fed to the manumycin producer, *Streptomyces parvulus* Tü 64, and the asukamycin producer, *Streptomyces nodosus*, to explore the biosynthesis of the carbon skeleton of these manumycin type metabolites (Figure 5) (Floss *et al.*, 1986),(Thiericke and Zeeck 1988a, Thiericke *et al.*, 1990). Two acetate and three propionate units were incorporated into the manumycin A "upper" chain and three acetate units were incorporated into the asukamycin "upper" polyene chain. No incorporation of acetate was observed into the cyclohexane ring. This labeling pattern indicates that the "upper" chain is formed via the polyketide pathway. The manumycin A "upper" chain is initiated by an acetyl-CoA and extended by one malonyl-CoA and three methylmalonyl-CoA building blocks. The asukamycin "upper" chain is initiated by a starter unit containing a cyclohexane ring which is elongated by three malonyl-CoA. Feeding experiments further revealed that the cyclohexane ring and the adjacent carbon are derived from cyclohexanecarboxylic acid **(39)**, which is activated as the CoA ester to initiate

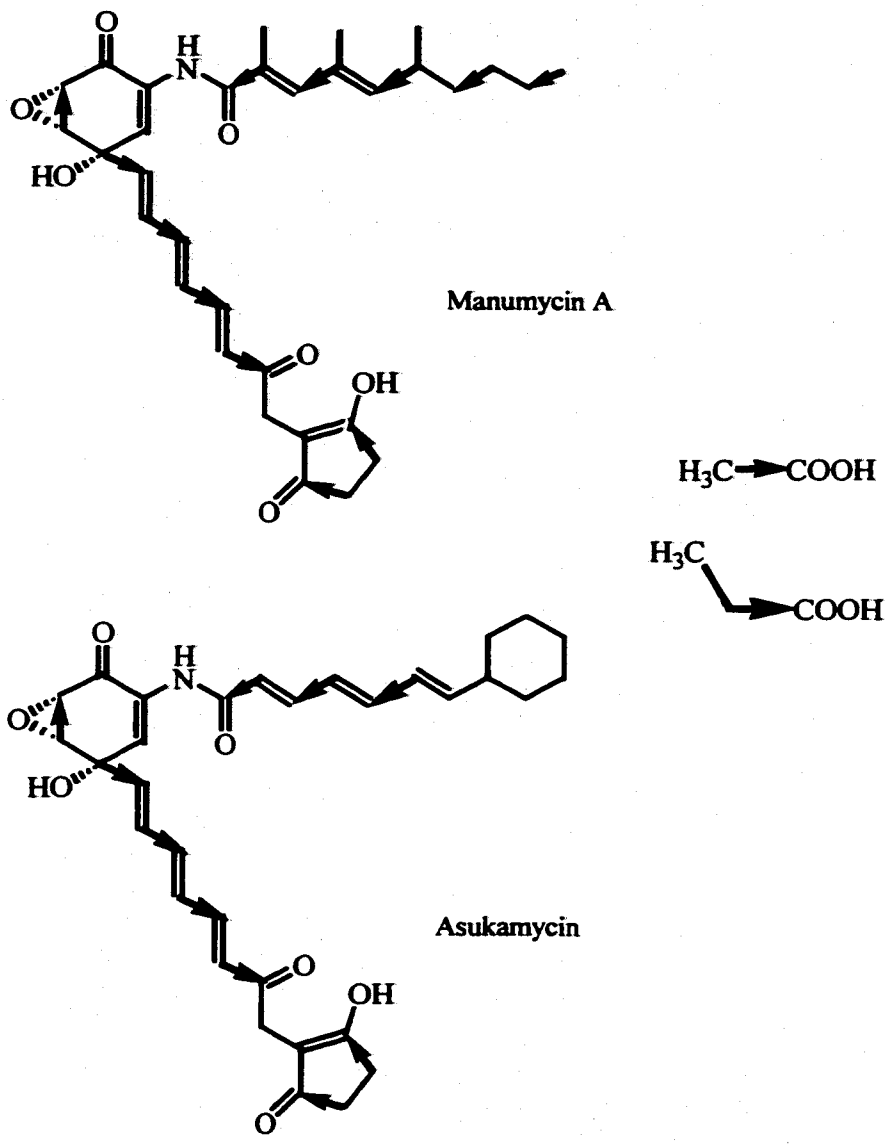


Figure 5. Labeling Pattern in Manumycin A and Asukamycin from [^{13}C]-Acetate and [^{13}C]-Propionate

subsequent "upper" chain elongation. Feeding experiments with [U-$^{13}C_3$]-glycerol further demonstrated that the cyclohexane ring and the adjacent carbon come from the shikimate pathway (Figure 6) (Thiericke *et al.*, 1990). The pathway of biosynthesis of cyclohexanecarboxylic acid from shikimic acid has been well established by investigations of ansatrienin and ω-cyclohexyl fatty acid biosynthesis (Figure 7) (Moore *et al.*, 1993a, Moore *et al.*, 1993b).

The biosynthesis of the "upper" chain distinguishes group A and group B manumycins. Group B manumycin compounds, including manumycin A **(1)**, B **(17)**, C **(18)** and EI-1625-3 **(16)**, start with acetyl-CoA and then use methylmalonyl-CoA and malonyl-CoA as polyketide building blocks. Group A manumycin compounds utilize various starter units, such as cyclohexanecarbonyl-CoA for asukamycin **(2)**, manumycin F **(11)**, nisamycin **(13)** and alisamycin **(3)**, deaminated and decarboxylated branched amino acids for manumycin E **(10)**, G **(12)**, EI-1511-3 **(8)**, EI-1511-5 **(9)** and U-56, 407 **(14)**. Only malonyl CoA is used in type A polyene chain extension.

1.3.2 Biosynthesis of the C_5N Unit

The ^{13}C-labeled acetate feeding experiments gave a "tail-to-tail" labeling pattern in the C_5N unit of manumycin A and asukamycin (Figure 5). It was suggested that carbons C-1", 3", 4" and 5" of the C_5N unit are derived from succinic acid via the TCA cycle, and this has been confirmed by the feeding of [1,4-$^{13}C_2$]- and [1,2-$^{13}C_2$]succinate (Figure 8)

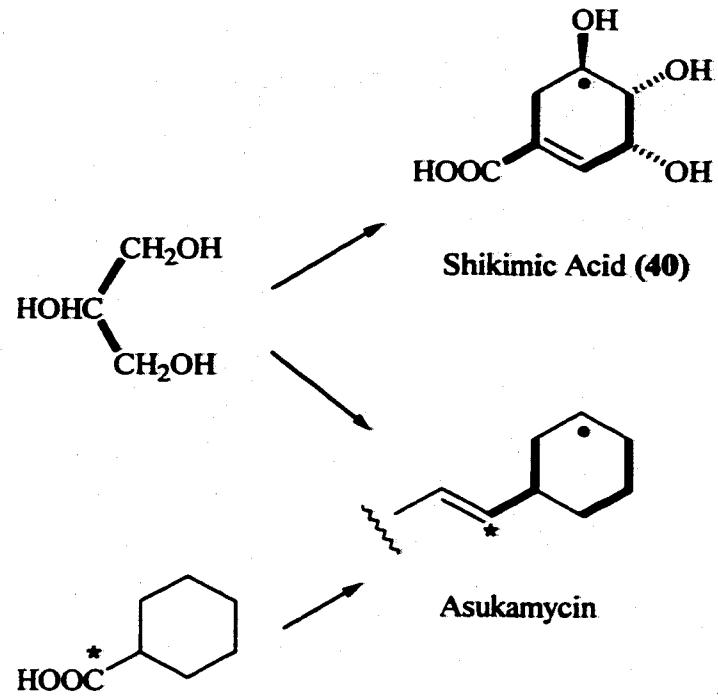


Figure 6. Biosynthetic Origin of the Cyclohexane moiety in Asukamycin

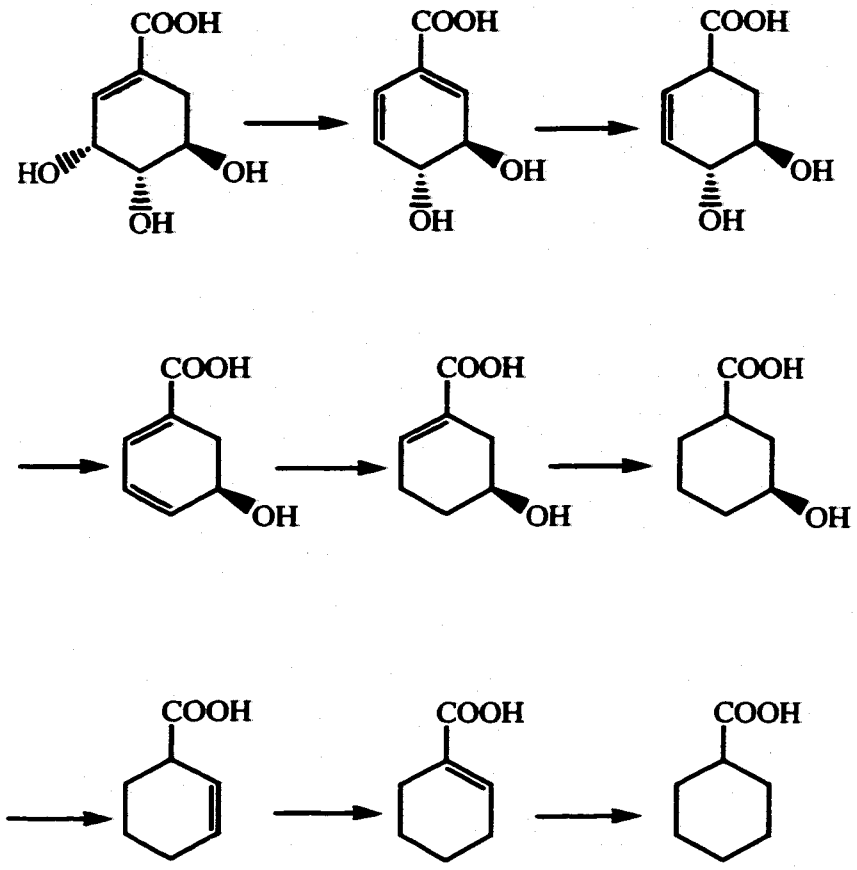


Figure 7. Biosynthesis of Cyclohexanecarboxylic Acid
(from Moore *et al.*, 1993)

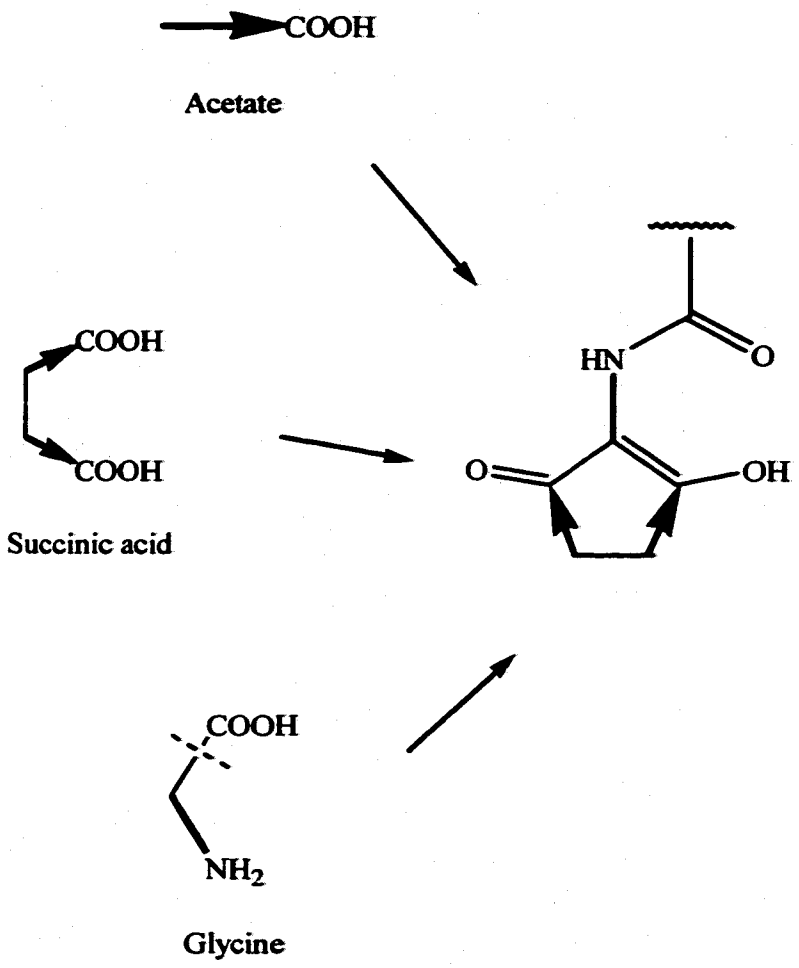


Figure 8. Biosynthesis of the C_5N Unit in Manumycin A and Asukamycin

(Nakagawa *et al.*, 1985, Thiericke *et al.*, 1990). Incorporation of [2-^{13}C, ^{15}N]glycine into C-2" and the adjacent nitrogen with retention of the ^{13}C-^{15}N coupling further suggested that the C_5N unit is formed from 5-aminolevulinic acid (5-ALA), which in turn is biosynthesized from succinyl-CoA and glycine by 5-aminoleulinic acid synthase. Finally, radiolabeled 5-[5-^{14}C]-aminolevulinic acid was also incorporated into asukamycin. At this point, the biosynthesis of the C_5N unit has been solved and a mechanism of intramolcular cyclization of 5-ALA has been proposed, in which a pyridoxal phosphate (PLP)-dependent enzyme is involved (Figure 9).

1.3.3 Biosynthesis of the mC_7N Unit and "Lower" Chain

The ^{13}C-labeled acetate feeding experiments with manumycin A and asukamycin clearly demonstrated that the "lower" chain is generated by a polyketide biosynthesis that is initiated by the mC_7N starter unit and involves chain extension by three malonyl-CoA (Figure 5) (Thiericke *et al.*, 1990). The feeding of ^{13}C-^{18}O labeled acetate further led to the incorporation of the intact ^{13}C-^{18}O assembly into the two amide carbonyl groups, which confirmed the polyketide character of the "upper" and "lower" polyene chains (Figure 10) (Thiericke *et al.*, 1989b).

The distinct mC_7N moiety in the manumycins is similar in structure to the mC_7N unit in other natural products, such as the ansamycins ansatrienin **(41)** or rifamycin **(42)** (Figure 11). Originally, manumycin A was regarded as an ansamycin metabolite with a broken

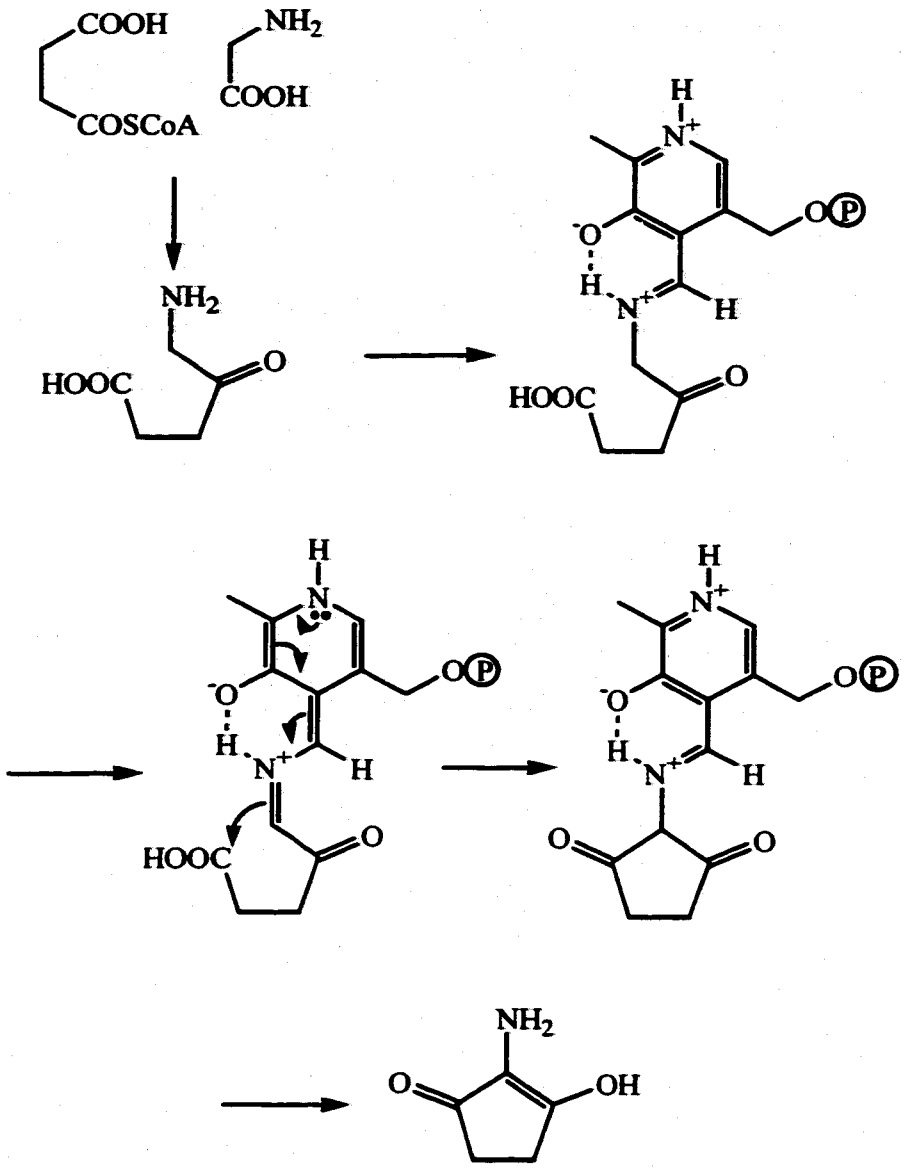


Figure 9. Proposed Mechanism of 5-ALA Cyclization in the Biosynthesis of the C_5N Unit of Manumycins

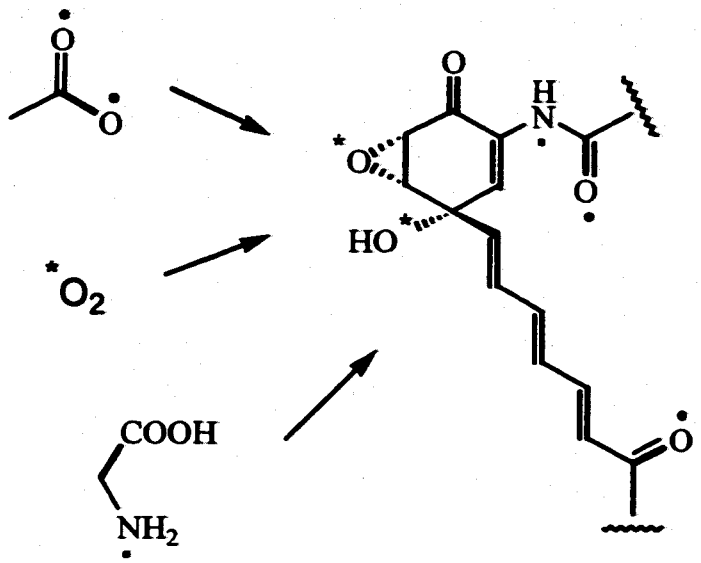


Figure 10. Biosynthetic Origin of the Heteratoms in Manumycin A and Asukamycin

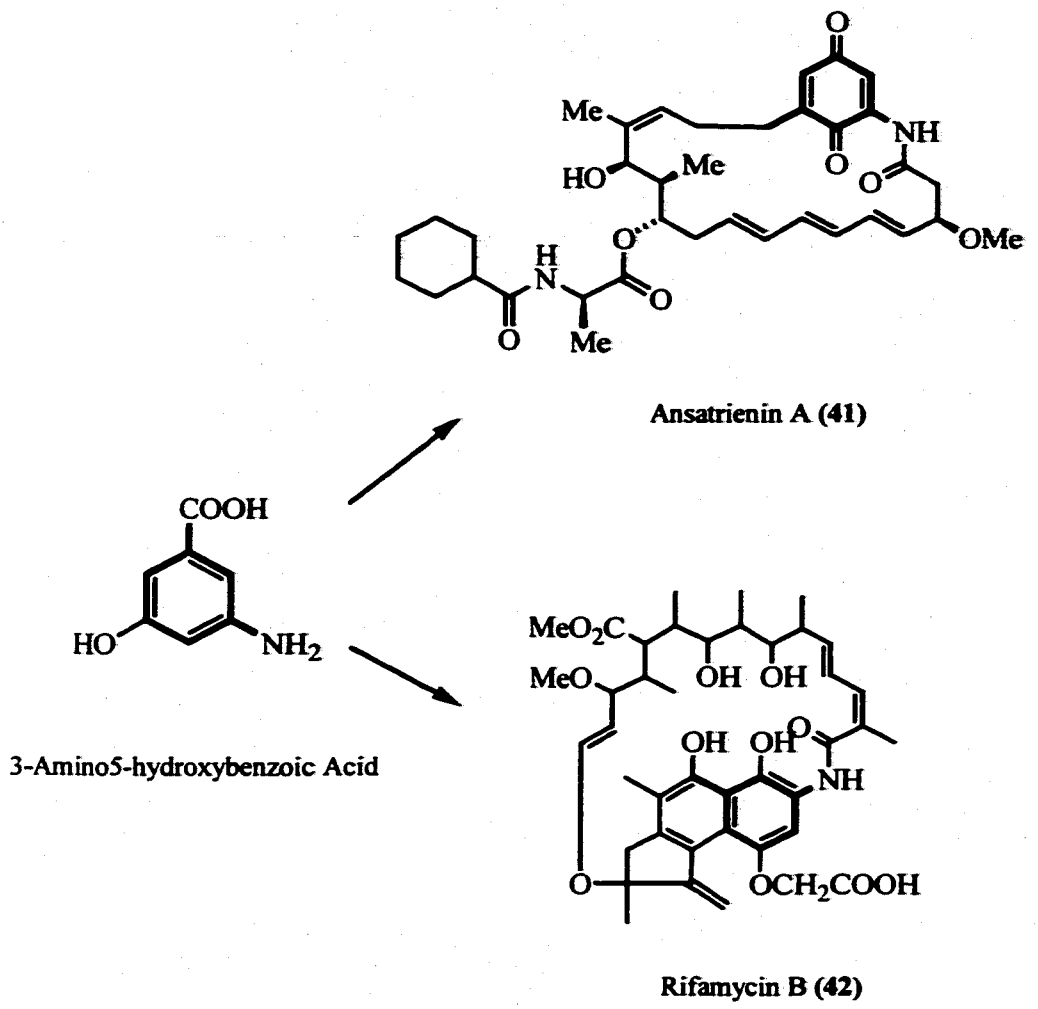


Figure 11. Ansatrienin A, Rifamycin B and the Biosynthetic Precursor of Their mC_7N Units

ansa chain. When it was found that the stereochemistry at C-4 of asukamycin was identical to that at C-1 of 3-dehydroquinic acid **(44)** (DHQ), it was suggested that asukamycin is a shunt product from DHQ in the shikimate pathway (Figure 12) (Kakinuma *et al.*, 1979). It is now known that the mC_7N unit in rifamycin and ansatrienin arises from a branch of the shikimate pathway via 3-amino-5-hydroxybenzoic acid (3,5-AHBA) **(46)** (Figure 11) (Kim *et al.*, 1996). ^{13}C-Labeled 3,5-AHBA has been fed to the manumycin A and asukamycin producers, but no incorporation into **1** or **2** was observed (Thiericke *et al.*, 1990). The "tail-to-tail" incorporation of two acetates into the mC_7N unit suggests formation from succinate via the TCA cycle, and this has been confirmed by the incorporation of labeled succinate (Figure 13) (Floss *et al.*, 1986, Thiericke and Zeeck 1988a, Thiericke *et al.*, 1990).

The origin of the remaining three carbons in the mC_7N unit was discovered when [U-$^{13}C_3$]-glycerol was found to be incorporated intact into C-1, C-2 and C-3. This demonstrates that the mC_7N moiety is formed from the condensation, and subsequent cyclization, of a four-carbon unit formed via the TCA cycle and a triose derived from carbohydrate metabolism (Thiericke *et al.*, 1990). Furthermore, chirally labeled glycerol was fed to the asukamycin producer. The ^{13}C-labeled carbon in (2S)-[1-^{13}C]glycerol was incorporated into the C-1 carbonyl position, which indicates that there is a clear stereochemical preference in the utilization of glycerol. The *pro*-S hydroxymethyl group of glycerol gives rise to the carbonyl group in the mC_7N moiety. But no incorporation was observed with deuterium labeled glycerol (Cho *et al.*, 1993), suggesting that all

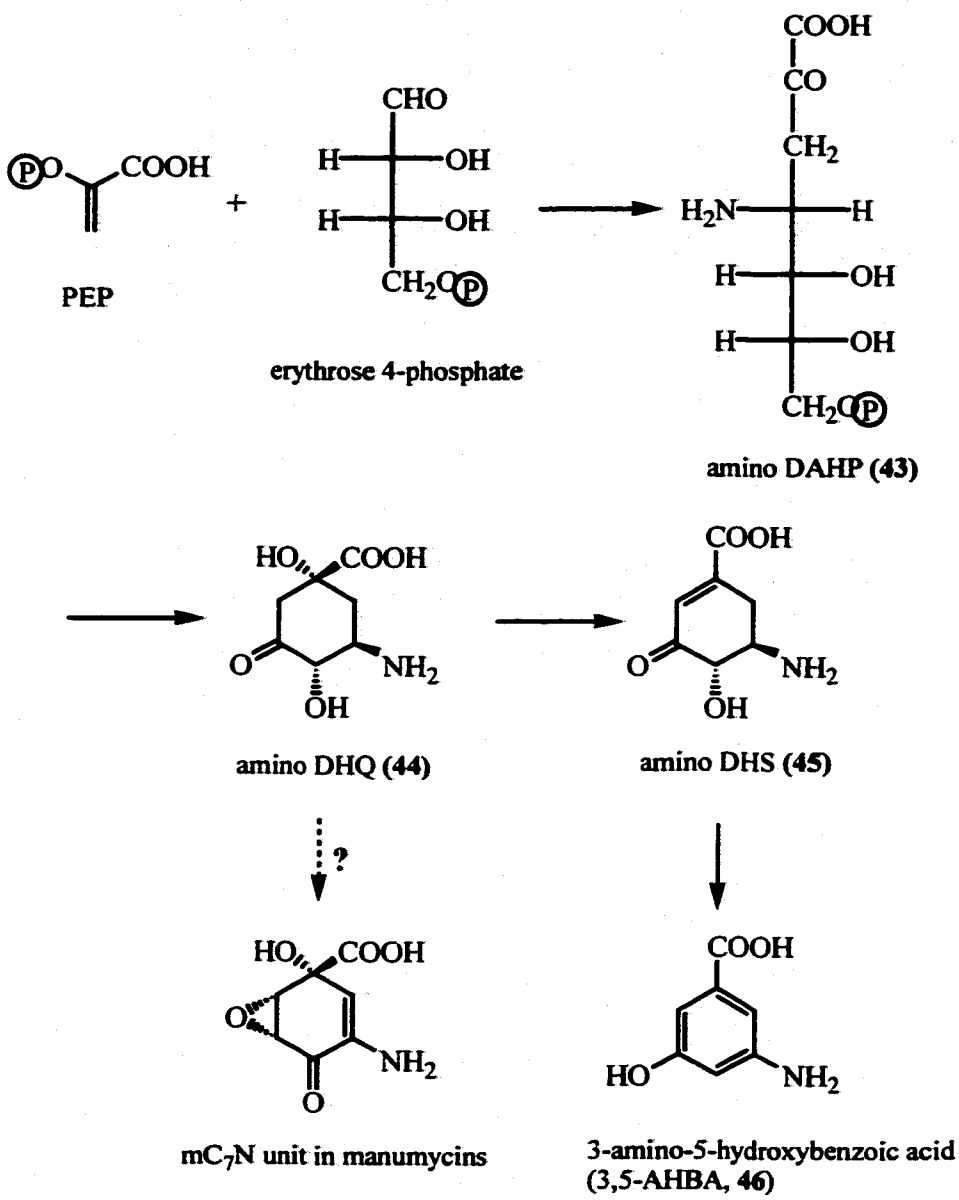


Figure 12. Biosynthesis of 3,5-AHBA via a Branch of the Shikimate Pathway

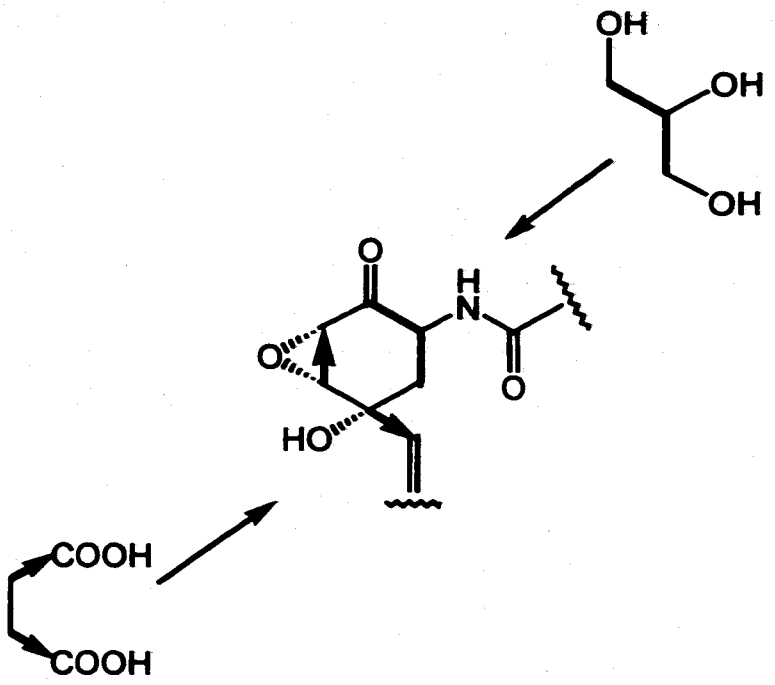


Figure 13. Biosynthetic Origin of the Carbon Skeleton of the mC_7N Unit of Manumycins

hydrogens of glycerol are lost during glycerol incorporation and metabolism and H-3 of the mC_7N unit is derived from other sources (Figure 14).

The origin of the heteroatoms on the mC_7N unit was investigated by feeding ^{15}N-labeled glycine to the manumycin A producing organism and by a fermentation in an $^{18}O_2$-containing atmosphere. ^{18}O incorporation was seen from molecular oxygen into both the epoxide and the hydroxy oxygen at C-4 (Figure 10). Incorporation of ^{15}N was observed into the nitrogen of the mC_7N unit. This might be explained by transamination between glycine and the general nitrogen pool and transfer of ^{15}N to a biosynthetic intermediate of the mC_7N unit derived from glycerol.

1.4 Research Goals

The biosynthetic origin of the "upper" chain, the C_5N unit and the "lower" chain has been well established. However, the biosynthesis of the multifunctional mC_7N epoxyquinol moiety that plays an important biological role remains a major mystery in the total biosynthesis of manumycin type metabolites. Based on the information obtained in the previous studies, a new pathway is expected for the biosynthesis of the mC_7N unit. On the other hand, it is now clear that the three structural components, the mC_7N unit with the "lower" chain, the C_5N moiety and the "upper" chain, are synthesized independently, and then assembled later. But the timing of this assembly is still not known. Our goals in this research therefore were:

1. Explore the intermediates or precursors in the biosynthesis of the mC_7N unit to help elucidate this new biosynthetic pathway.
2. Discover and identify new intermediates or pathway related metabolites to acquire information important for the elucidation of the biosynthetic pathway to the manumycin type metabolites.
3. Determine the timing of assembly of the three structural elements, the mC_7N unit with "lower" chain, the C_5N unit and the "upper" chain, to elucidate the total biosynthetic pathway to the manumycin family of compounds.

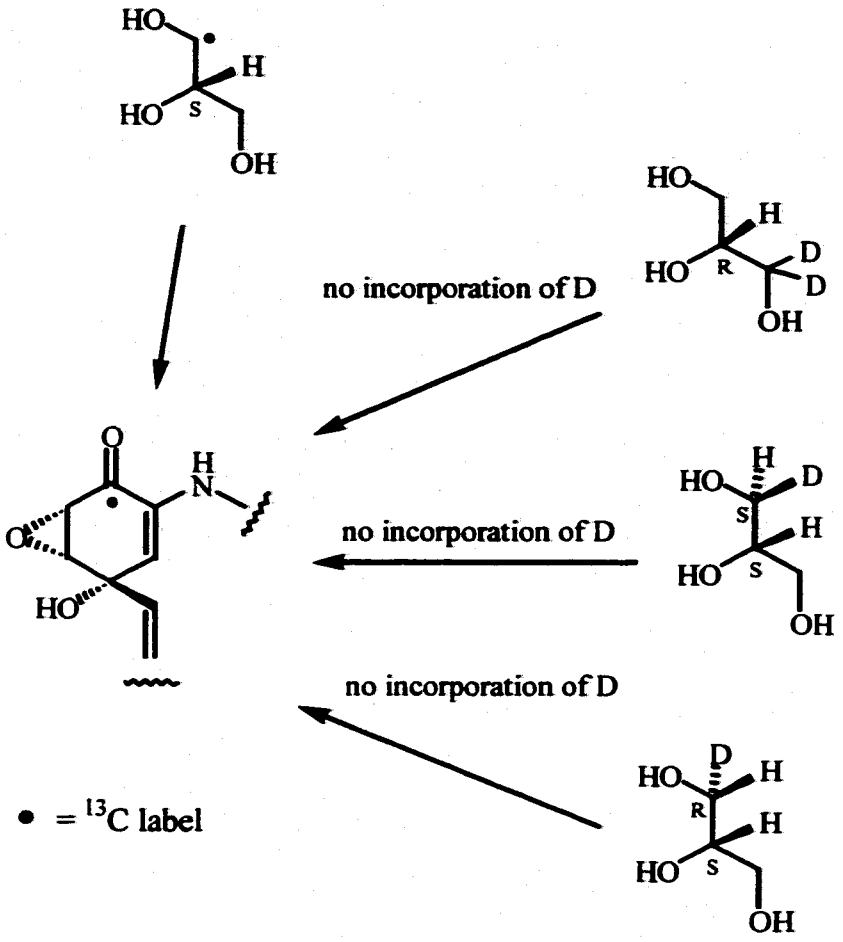


Figure 14. Incorporation of Labeled Glycerol into the mC_7N Unit of Asukamycin

CHAPTER 2. THE BIOSYNTHESIS OF THE mC_7N UNIT

2.1 Introduction

2.1.1 mC_7N Units in Other Antibiotics and Their Biosyntheses

Structurally similar mC_7N units are found in antibiotics other than the manumycins, such as ansatrienin A **(41)**, rifamycin B **(42)** and acarbose **(47)**. It was once speculated that some of these natural products shared the same biosynthetic pathway of the mC_7N unit with the manumycins. Since then, the biosynthesis of these compounds has been brought to light. The mC_7N unit in ansatrienin A and rifamycin B is derived from the shikimate pathway via 3-amino-5-hydroxybenzoic acid **(46)** as mentioned in Chapter 1 (Figure 11). The mC_7N unit in acarbose is formed via the pentose phosphate pathway by cyclization of sedoheptulose 7-phosphate (Figure 15). Feeding experiments showed that [U-$^{13}C_3$] glycerol was incorporated into the mC_7N unit of acarbose and the labeling and coupling pattern of the incorporated ^{13}C indicated that the six-membered ring is formed from the cyclization of a seven-carbon sugar (Degwert *et al.*, 1987). Sedoheptulose 7-phosphate was subsequently identified as the substrate of the enzyme catalyzing this cyclization (Mahmud *et al.*, 1999; Stratmann *et al.*, 1999).

Nevertheless, the feeding of ^{13}C-labeled 3-amino-5-hydroxybenzoic acid (3,5-AHBA) and [U-$^{13}C_3$] glycerol to manumycin A and asukamycin producers resulted in no incorporation or a coupling pattern in the mC_7N unit different from those in rifamycin B

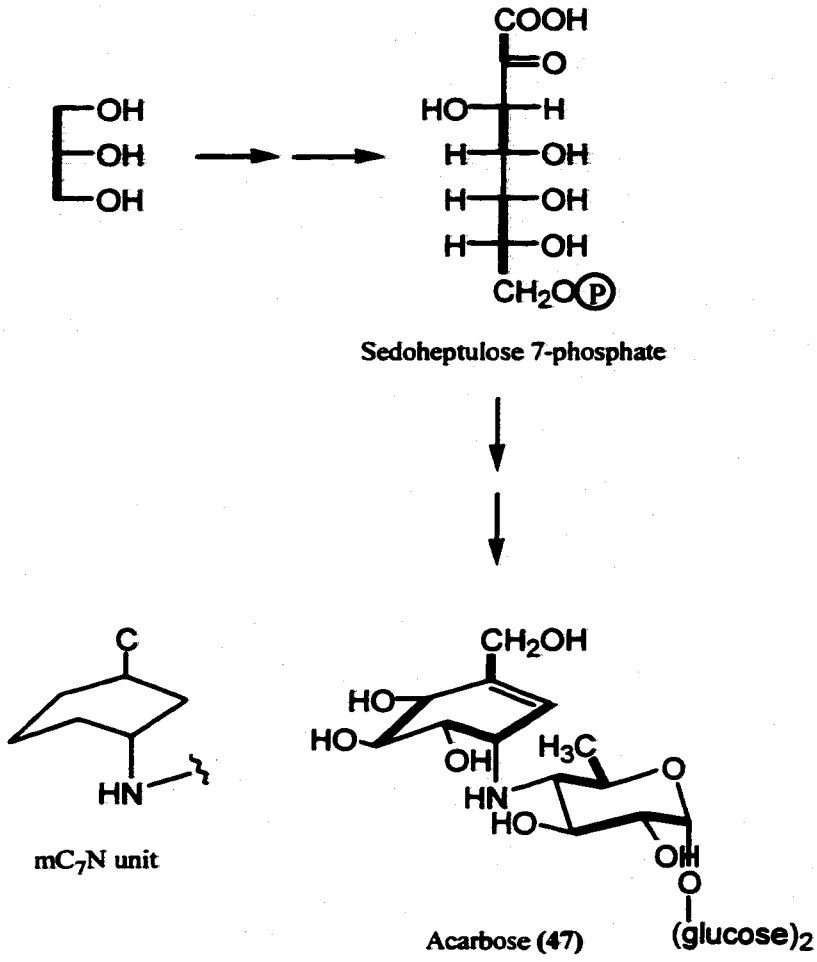
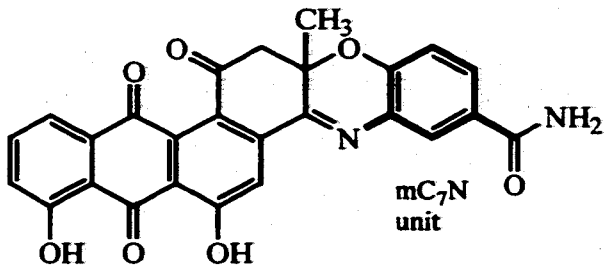


Figure 15. Proposed Heptulose Phosphate Cyclization

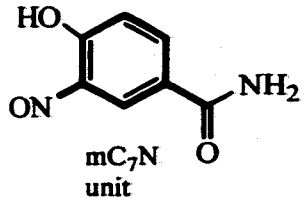
and acarbose, respectively (Figure 13). Based on these experiments, the shikimate pathway and heptulose phosphate cyclization have been ruled out for the biosynthesis of the mC_7N unit in manumycin type of metabolites (Thiericke *et al.*, 1990).

2.1.2 3-Amino-4-hydroxybenzoic Acid is Derived from the TCA Cycle

Streptomyces murayamaensis produces a variety of aromatic polyketides. Murayaanthraquinone **(48)** (Figure 16) is one of these aromatic polyketides which contains a mC_7N unit. In the biosynthetic study of these compounds, *S. murayamaensis* mutants MC2, MC3 and MC11, were generated in Gould's laboratory at Oregon State University by using UV light. It was found that these mutant strains produce and accumulate a compound containing a mC_7N moiety, 4-hydroxy-3-nitrosobenzamide **(49a)** (Figure 5) and its ferrous chelate **49b** (Cone *et al.*, 1995) (Figure 17). Furthermore, ammonium 3-amino-4-hydroxy-[2-^{2}H]benzoate **(50)** was fed to the *S. murayamaensis* mutant MC11 and the incorporation of deuterium into the ion-chelating 4-hydroxy-3-nitrosobenzamide was observed. This observation suggests that 3,4-AHBA **(50)** is the precursor of the mC_7N unit in 4-hydroxy-3-nitrosobenzamide and murayaanthraquinone. In the biosynthetic study of 4-hydroxy-3-nitrosobenzamide and its chelate, it was reported that ^{13}C-labeled acetate and succinate were incorporated into compound **49b**. The coupling patterns were identical to those in the mC_7N unit of manumycin A and asukamycin (Gould *et al.*, 1996) (Figure 17). These findings suggested that the mC_7N unit in manumycin type metabolites may share the same biosynthetic origin as the one in



Murayaanthraquinone (**48**)



4-hydroxy-3nitrosobenzamide (**49a**)

Figure 16. Structure of Murayaanthraquinone and 4-Hydroxy-3-nitrosobenzamide

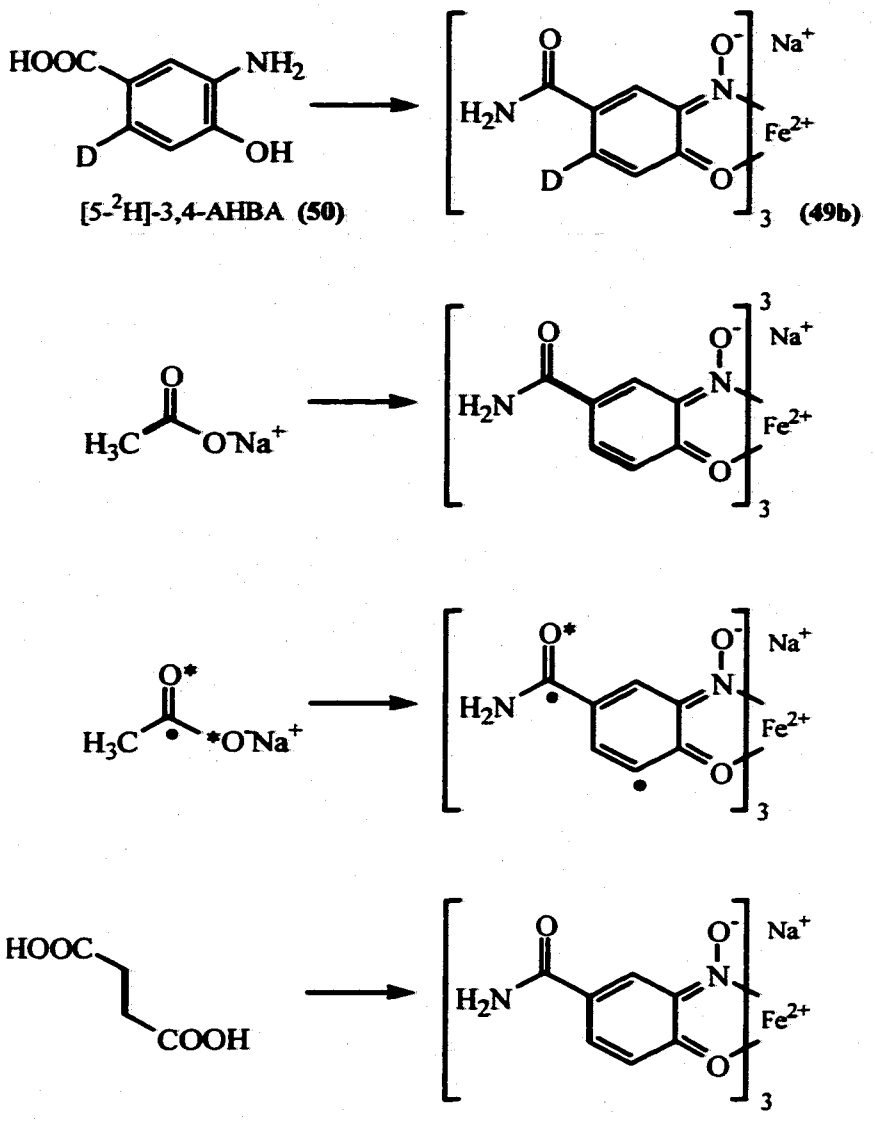


Figure 17. Labeling Pattern of 4-Hydroxy-3-nitrosobenzamide Ferrous Chelate **(49b)**

4-hydroxy-3-nitrosobenzamide and murayaanthraquinone, i.e., 3,4-AHBA may be the precursor of the mC_7N unit in manumycins. Labeled 3,4-AHBA should be fed to the manumycin producers *S. parvulus* and *S. nodosus* to confirm this hypothesis. We have collaborated with Dr. Gould in this part of the work.

2.2 Synthesis of 3,4-[7-^{13}C]-AHBA (50)

3-Amino-4-hydroxy-[7-^{13}C]benzoic acid **(50)** was synthesized from 4-hydroxy-[7-^{13}C]benzoic acid **(51)** by nitration and subsequent reduction (Figure 18). The nitration reaction of compound **51** was attempted under various conditions, including H_2SO_4/HNO_3, HNO_3/HOAc and $H_2SO_4/NaNO_2$ at a range of temperatures. The di-nitrated product is formed in large excess under the first two conditions. When 4-hydroxy-[7-^{13}C]benzoic acid (99% pure) was treated with $NaNO_2$ and sulfuric acid at 0 °C followed by heating to 90°C, the desired nitration product 4-hydroxy-3-nitro-[7-^{13}C]benzoic acid **(52)** was isolated in a yield of 88% (Kondo *et al.*, 1994). [7-^{13}C]-**52** was reduced with Raney nickel in the presence of hydrazine hydrate to afford 3,4-[7-^{13}C]-AHBA **(50)** in a yield of 85% (Balcom and Furst 1953). The final product [7-^{13}C]-**50** was characterized by ^{1}H- and ^{13}C-NMR spectrometry and high resolution mass spectroscopy.

2.3 Feeding of 3,4-AHBA to *Streptomyces nodosus*

Streptomyces nodosus ssp. *asukaensis* (from the American Type Culture Collection, ATCC 29757) was grown in seed medium for 2 days, and was subsequently transferred to production medium. Ammonium 3,4-[2-^{2}H]-AHBA dissolved in deionized water or 3,4-[7-^{13}C]-AHBA dissolved in 5% K_2CO_3 were fed after 24 hours in production medium and the cultures were harvested 48 hours after feeding. Asukamycin was obtained by extraction with ethyl acetate and purified by preparative TLC or by flash column chromatography with methanol and chloroform as the eluent. Further purification by HPLC eluting with a gradient of the solvents methanol / water or acetonitrile / water was performed. The ammonium salt of 3,4-[2-^{2}H]-AHBA (80% atom ^{2}H) was provided by Gould's lab and had been synthesized via deuterium exchange (Cone *et al.*, 1995).

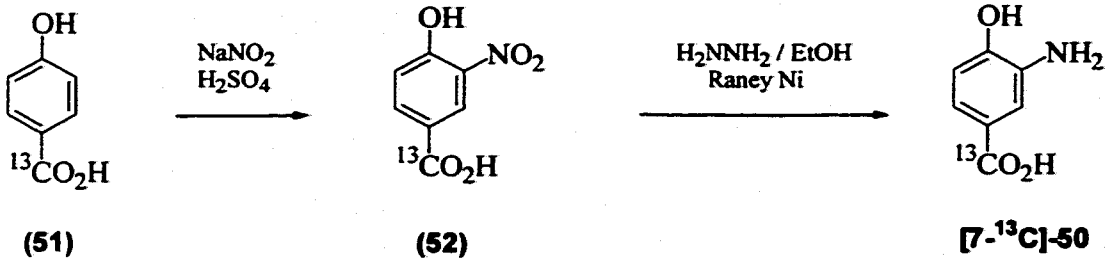
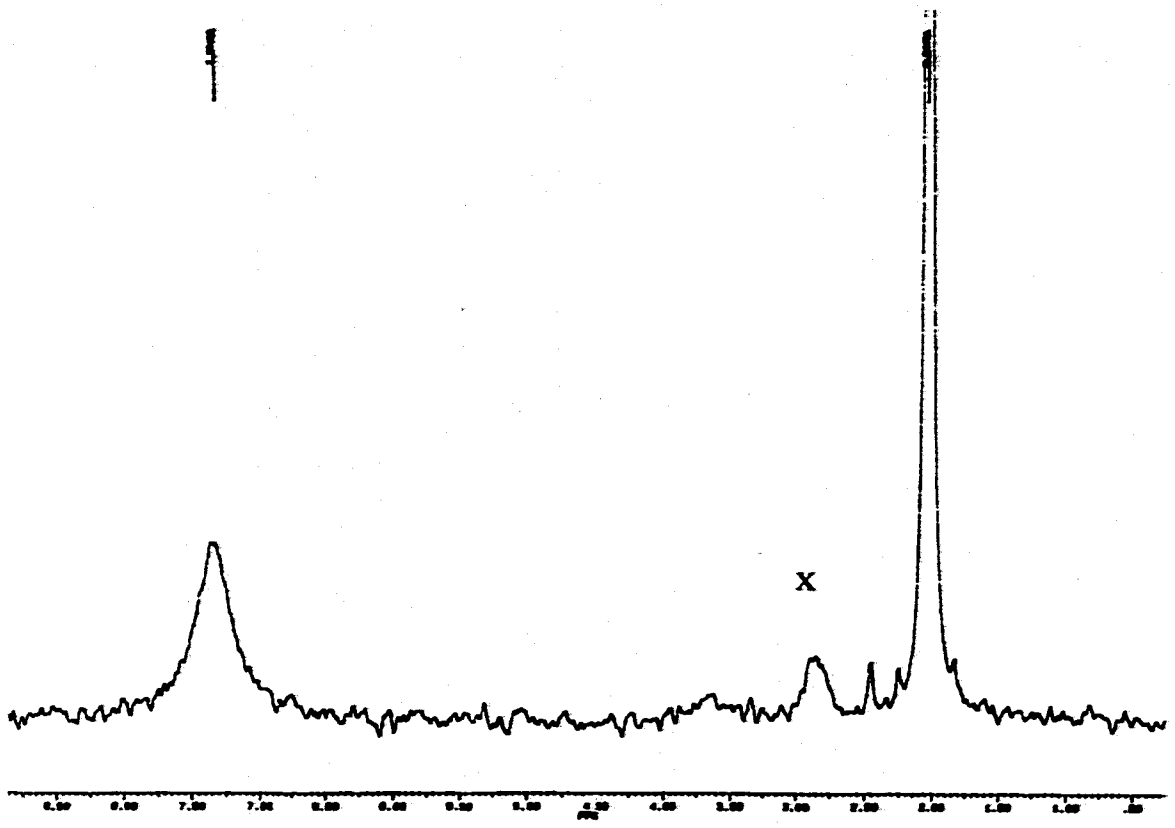


Figure 18. Synthesis of 3-Amino-4-hydroxy-[7-^{13}C]benzoic Acid

^{2}H-NMR was used to analyze the preliminarily purified manumycin type metabolites (on preparative TLC) from the fermentation with added ammonium 3,4-[2-^{2}H]-AHBA (Figure 19). A deuterium signal at 7.4 ppm, corresponding to H-2 of asukamycin, was observed. ^{13}C-NMR was used to analyze the asukamycin from the fermentation with



x Unknown

Figure 19. ^{2}H-NMR Spectrum of Manumycin Type Metabolites Derived from 3,4-[2-^{2}H]-AHBA

3,4-[7-^{13}C]-AHBA (Figure 20), revealing an enhanced ^{13}C signal at 136 ppm (in chloroform), corresponding to C-7 of asukamycin. The shift of the enriched ^{13}C signal from 165 ppm in the 3,4-[7-^{13}C]-AHBA to 136 ppm in the asukamycin derived from it demonstrated that the carboxyl group of 3,4-AHBA had been converted to a C-C double bonded carbon in asukamycin. SIM-ES/MS analysis indicated that both the deuterium and the ^{13}C incorporation was 16.5%.

2.4 Feeding of 3,4-[7-^{13}C]-AHBA to *Streptomyces parvulus*

S. parvulus Tü 64 obtained from Professor Zeeck at the University of Göttingen was grown in seed medium for 2 days, and subsequently transferred to production medium. 3,4-[7-^{13}C]-AHBA dissolved in 5% K_2CO_3 was fed after one day in production medium and the culture was harvested 2 days after feeding. The manumycins were obtained by extraction of the mycelium with acetone and purified by preparative TLC with methanol /chloroform as eluant. Further purification by HPLC eluting with a gradient of methanol / water was carried out. Pure manumycin A as the major component and manumycin B as the minor component were collected.

Enriched ^{13}C signals were observed by ^{13}C-NMR at 136 ppm in both manumycin A and manumycin B. SIM-ES/MS was used to determine the percentage ^{13}C incorporation into manumycin A as 5.4 % and into manumycin B as 5.7 % (Figure 21).

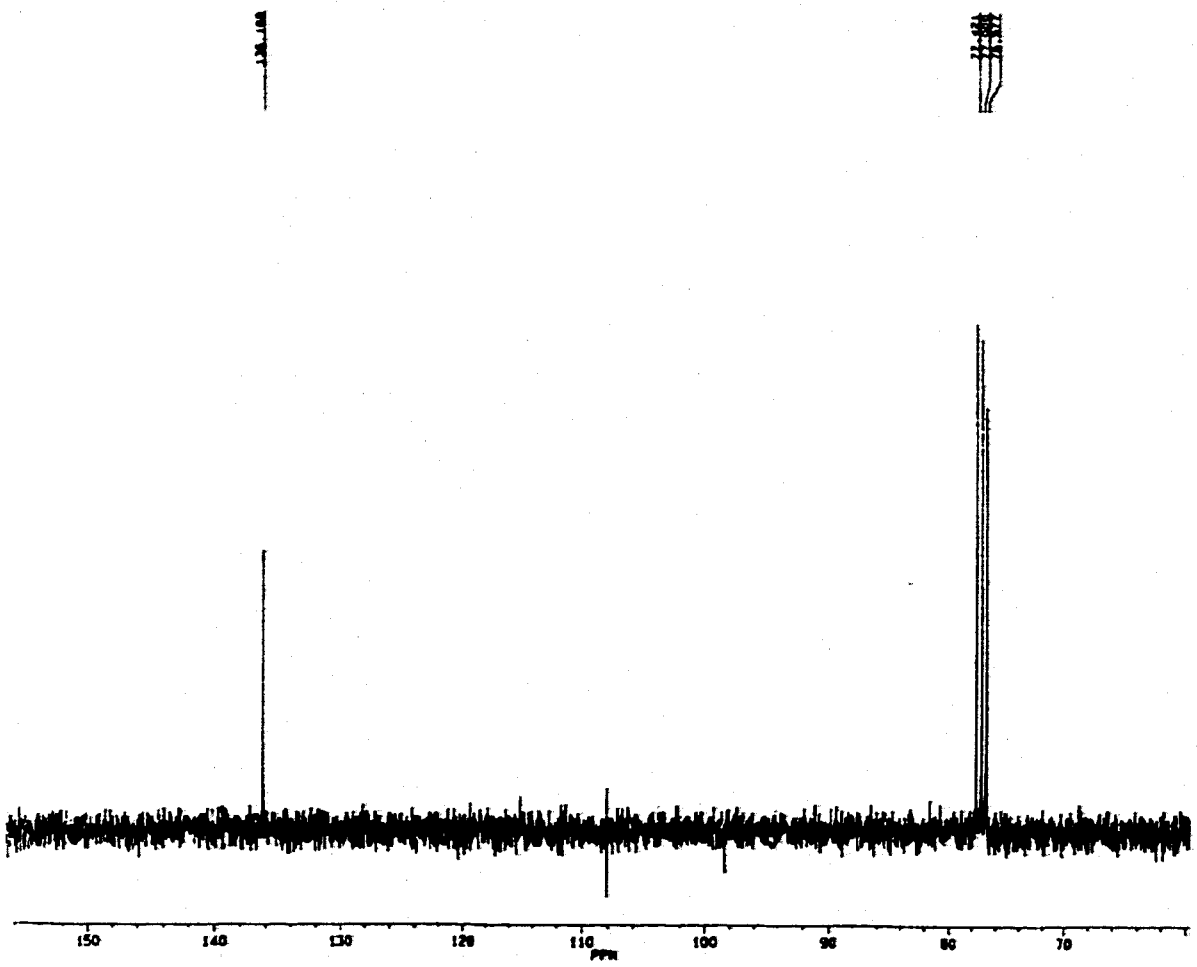


Figure 20. Enhanced Signal in ^{13}C-NMR Spectrum of Asukamycin Derived from 3,4-[7-^{13}C]-AHBA

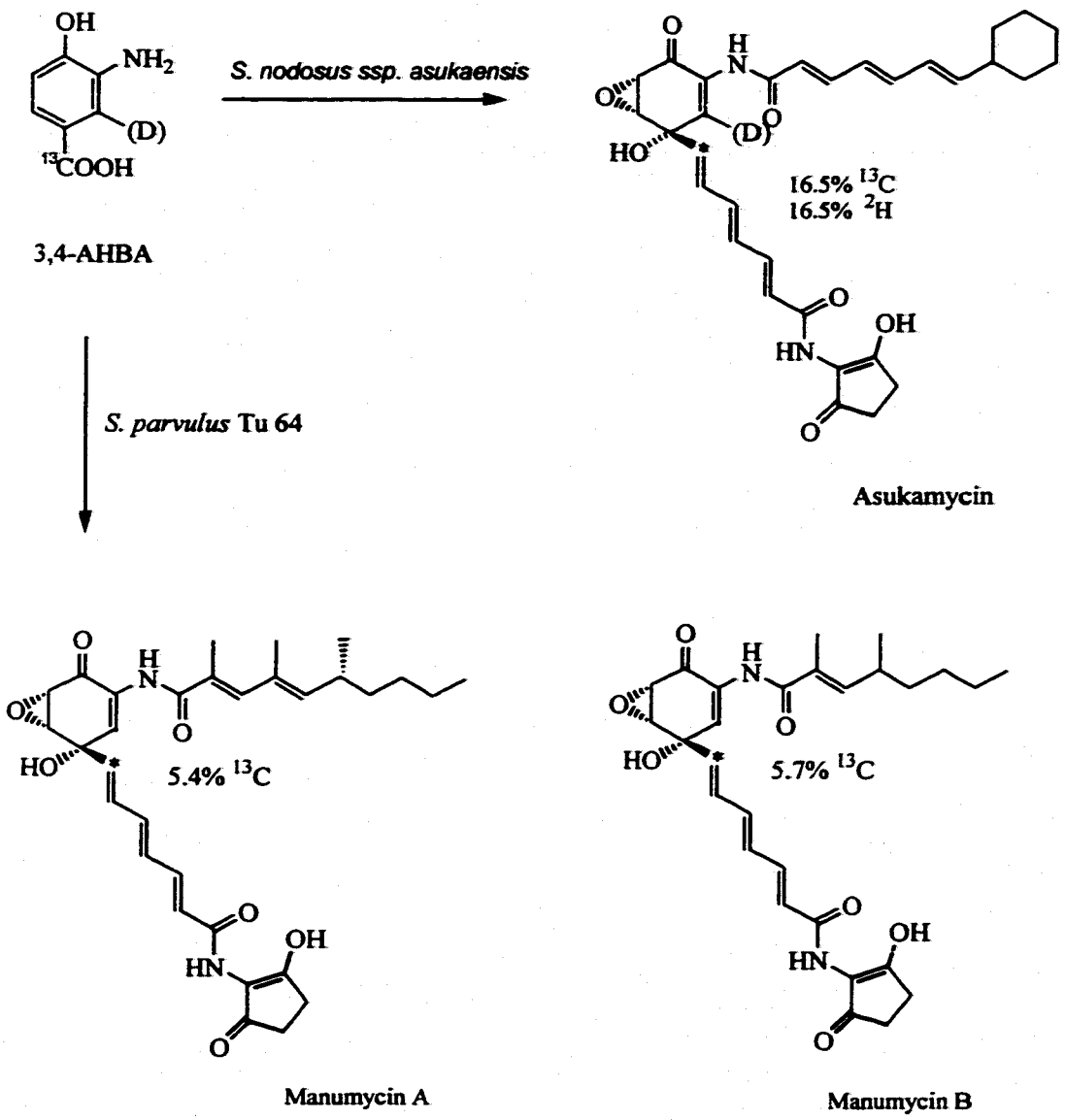


Figure 21. Labeled 3,4-AHBA Feeding to *S. nodosus* and *S. parvulus*

2.5 Conclusion

Through the feeding experiments with deuterium and ^{13}C-labeled 3,4-AHBA, it has been clearly demonstrated that 3,4-AHBA is the precursor of the mC_7N unit of manumycin A, manumycin B and asukamycin, providing the starter unit for the "lower" polyketide chain biosynthesis. 3,4-AHBA arises from the condensation and subsequent cyclization of a C_4 unit formed *via* the TCA cycle and a C_3 unit derived from a triose. The deuterium from 3,4-[2-^2H]-AHBA was retained at C-2 of asukamycin, which contrasts with the loss of all deuterium from ^2H-labeled glycerol (Figure 14), suggesting that the deuterium losses from glycerol occur during the condensation and cyclization reactions to form 3,4-AHBA.

Since 3,4-AHBA is the precursor of the mC_7N unit of manumycin A, B and asukamycin, it is expected that 3,4-AHBA is also the precursor of mC_7N units of other manumycin type metabolites. This newly discovered biosynthetic pathway may be a common secondary metabolism pathway in nature since it is found in different *Streptomycetes,* such as *S. parvulus*, *S. nodosus* and *S. murayamaensis,* as well as in different types of metabolites, including the manumycin family of antibiotics, 4-hydroxy-3-nitrosobenzamide and murayaanthraquinone.

2.6 Gould's Hypothesis of the Biosynthesis of 3,4-AHBA

Further feeding experiments have been conducted in Gould's laboratory to explore the biosynthesis of 4-hydroxy-3-nitrosobenzamide. ^{13}C-Labeled glutamic acid, aspartic acid and pyruvate were fed to *S. murayamaensis* mutant MC2 to investigate the biosynthetic origin of the mC_7N unit and the orientation of the condensation and cyclization of the C_4 unit with the C_3 unit (Figure 22). It was believed that [1,2-$^{13}C_2$]glutamic acid **(53)** is converted *in vivo* to α-ketoglutarate **(54)** and then decarboxylated to succinic acid **(55)**. The latter was incorporated into **49b** symmetrically (Figure 22(a)). [4-^{13}C]Aspartic acid **(56)** and [2-^{13}C]oxalacetic acid **(57)** were incorporated into **49b** unsymmetrically. It was deduced that aspartic acid is converted to oxalacetic acid before cyclization. Thus, the C_4 unit orientation in the condensation and cyclization is clear (Figure 22(b), (c)). The orientation of the C_3 unit in the condensation and cyclization was confirmed by feeding experiments with [2,3-$^{12}C_2$]-, and [1-^{13}C]pyruvates (Figure 22(d), (e)). The carboxyl group of pyruvate is converted into the phenolic carbon of 4-hydroxy-3-nitrosobenzamide. Based on these feeding experiments, a hypothesis regarding the formation of 3,4-AHBA has been proposed (Figure 23) (Gould *et al.*, 1996; Li and Gould 1997). It was rationalized that oxalacetate **(57)** formed from aspartic acid catalyzed by aspartate aminotransferase or from succinate via the TCA cycle condenses with PEP to afford **58**, followed by reduction and transamination to yield intermediate **60**. Alternatively, **57** could be reduced to **59** followed by condensation to **60**. Intermediate **60** undergoes cyclization, dehydration, tautomerization and hydrolysis to give 3,4-AHBA.

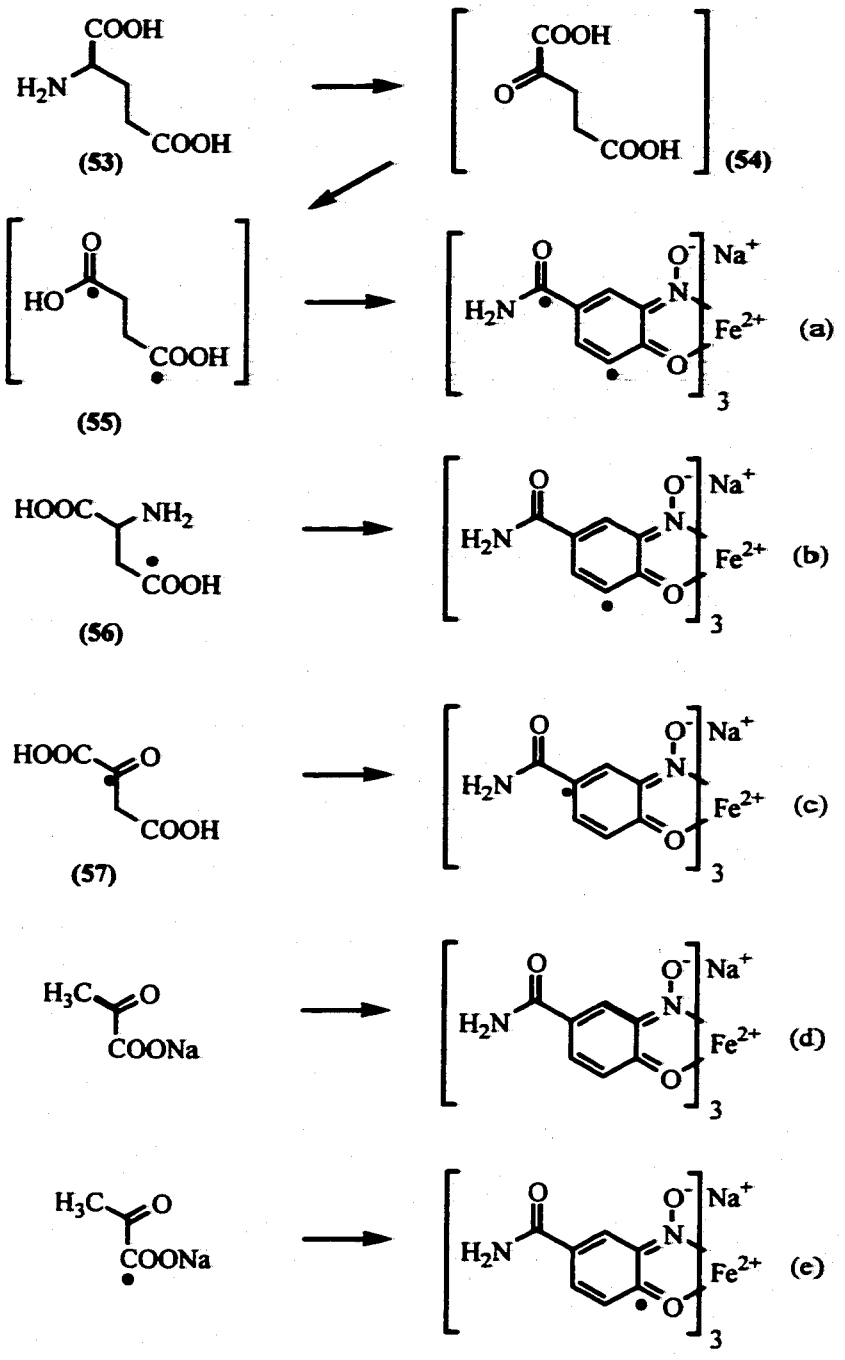


Figure 22. Labeling Pattern of 4-Hydroxy-3-nitrosobenzamide Ferrous Chelate After Feeding Various Labeled Precursors

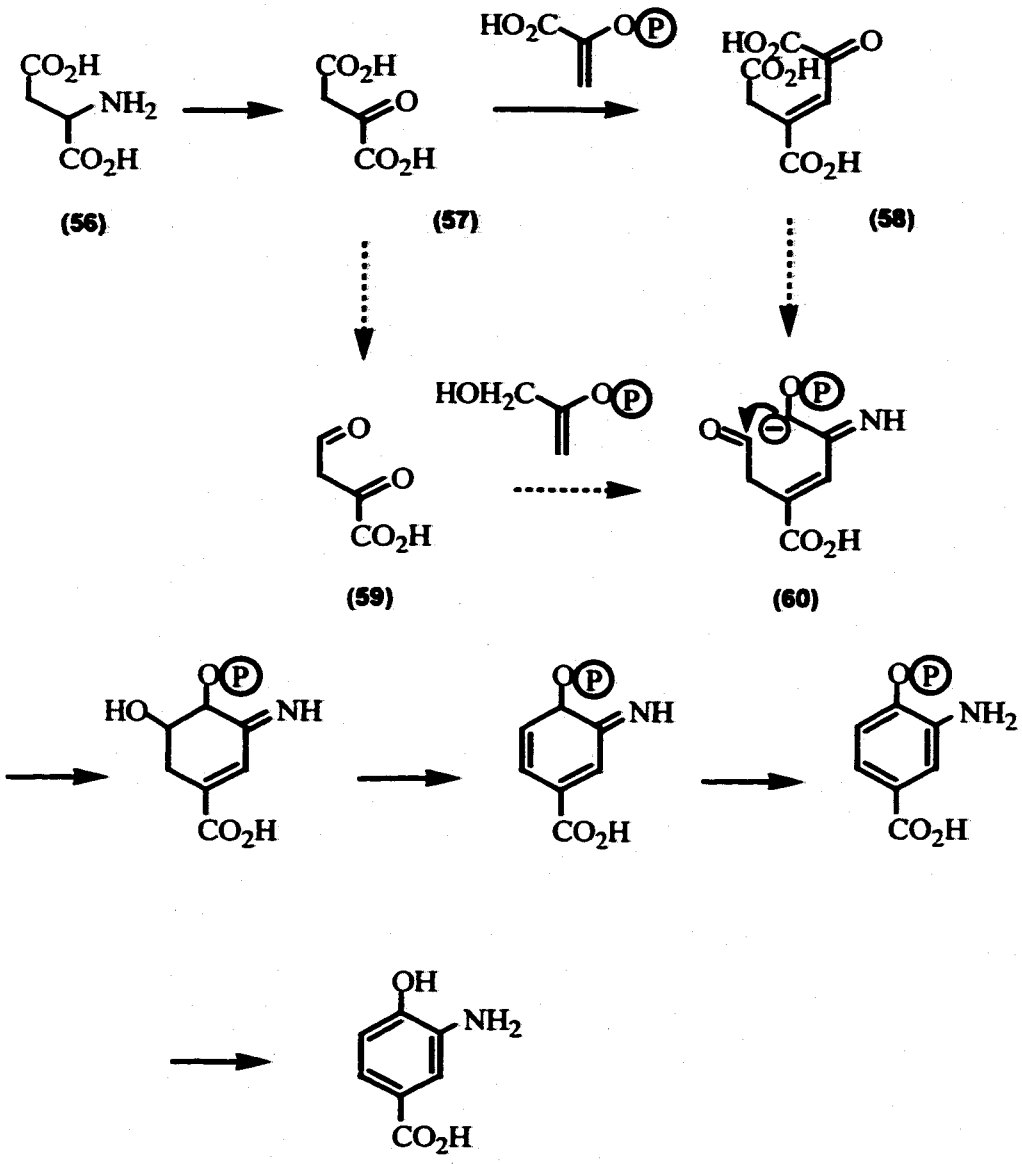


Figure 23. Mechanism for the Biosynthesis of 3,4-AHBA Proposed by Gould

CHAPTER 3. THE BIOSYNTHESIS OF THE "LOWER" POLYKETIDE CHAIN

3.1 Introduction

3.1.1 The Hypotheses of "Lower" Chain Biosynthesis

Prior to the work described in this thesis, it had been deduced by feeding experiments that the biosynthesis of the "lower" polyketide chain of manumycins is initiated by the mC_7N starter unit. The detailed structure of the mC_7N starter unit was unknown due to the lack of further pathway information. It was only recognized that the mC_7N starter unit must be an acid which is converted to its CoA thioester to initiate the polyketide biosynthesis. In Chapter 2, 3,4-AHBA was shown to be the specific precursor of the mC_7N unit, indicating that 3,4-AHBA, or a modified form of it, is the polyketide starter unit. Three possible routes for the biosynthesis of the "lower" polyketide chain are listed in Figure 24, which differ from each other by the structures of their various starter units. In route (a), 3,4-AHBA is converted to its CoA thioester prior to the "lower" polyketide chain synthesis. Oxidation of the aromatic ring occurs at a later stage. In contrast, route (b) involves epoxidation / oxidation catalyzed by a dioxygenase or two coordinated mono-oxygenases which take place on 3,4-AHBA, prior to polyketide synthesis. It is also possible that the "upper" chain is synthesized and attached to the amino group of 3,4-AHBA first, followed by "lower" chain synthesis initiated from the carboxyl group (route (c)).

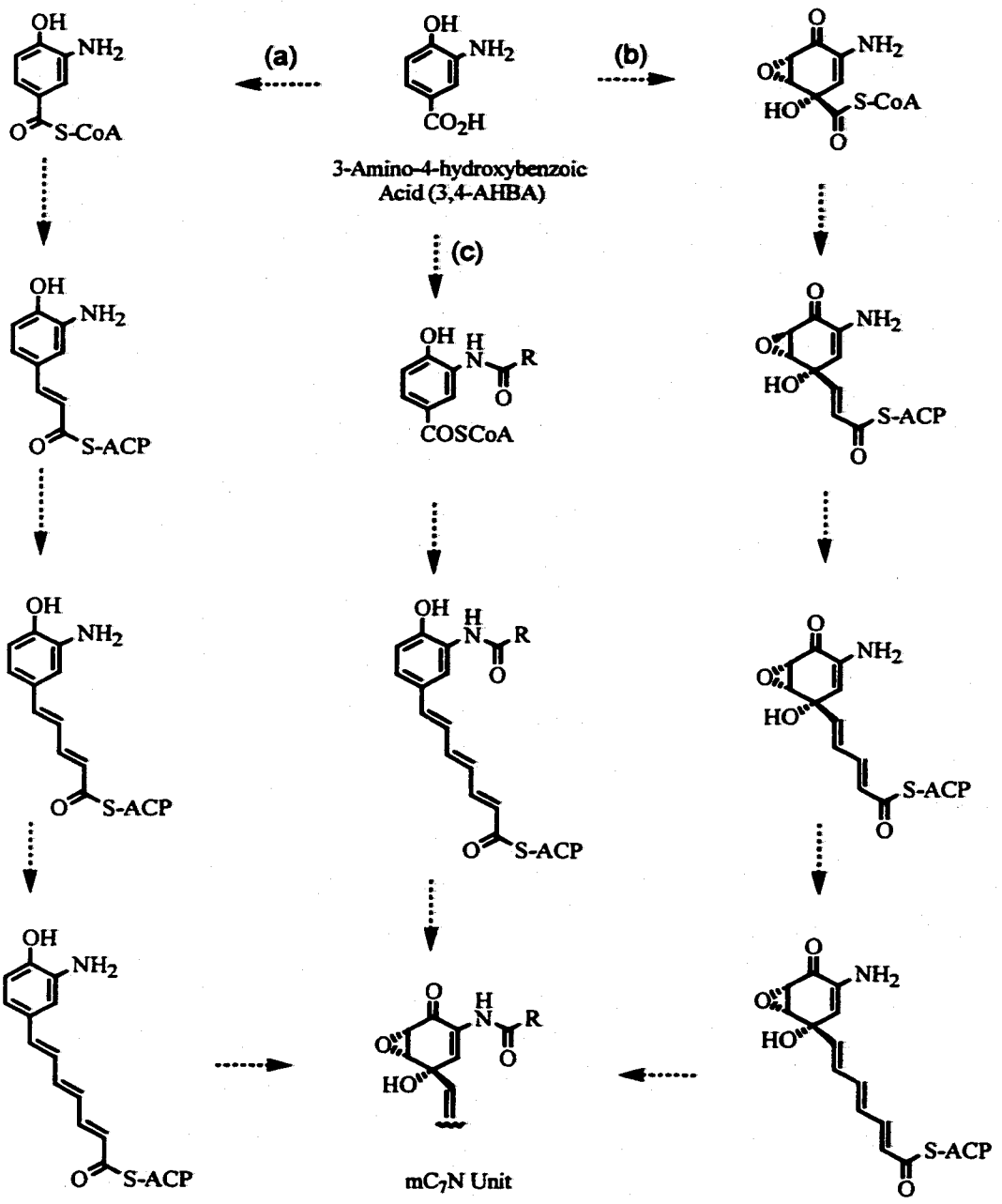


Figure 24. Possible Pathway of Polyketide Chain Extension and Oxidation of the mC_7N Unit of Manumycins

3.1.2 Evidence from Precursor-Directed Biosynthesis

Precursor-directed biosynthesis has been carried out on the manumycin A, asukamycin and colabomycin producers in an attempt to synthesize new artificial metabolites which display biological activities not seen with their parent natural products. A variety of aromatic acids were fed to the manumycin A producer *S. parvulus* at a high concentration and from these fermentations manumycin analogs with intact aromatic rings were isolated and identified. These resulting analogs were divided into three classes according to their structures (Figure 25) (Thiericke *et al.*, 1989a; Thiericke and Zeeck 1988b). Class 1 consists of six compounds which were found to carry the "lower" polyketide chain extended from the fed precursor and terminating with a C_5N moiety, while the "upper" chain is absent. Two Class 2 compounds were isolated with the "upper" chain identical to that of manumycin, but the "lower" polyketide chain and C_5N moiety are not present. Four Class 3 compounds were discovered which have both an "upper" and "lower" polyketide chain as well as a C_5N unit. 3-Aminobenzoic acid was also fed to the asukamycin producer *S. nodosus* and the colabomycin producer *S. griseoflavus*, resulting in one Class 1 product, asuka-mABA **65**, in *S. nodosus* (Cho *et al.*, 1993) and one Class 3 product, 2880-mABA **66**, in *S. griseoflavus* (Figure 26) (Sattler *et al.*, 1998). The production of precursor-directed manumycin analogs varies with the type of aromatic acid fed and the species of *Streptomyces* used. *S. parvulus* is most prone to accommodate false precursors, while *S. nodosus* failed to yield any manumycin analogs other than asuka-mABA. When the aromatic acids were fed in high concentrations (55 mM), the

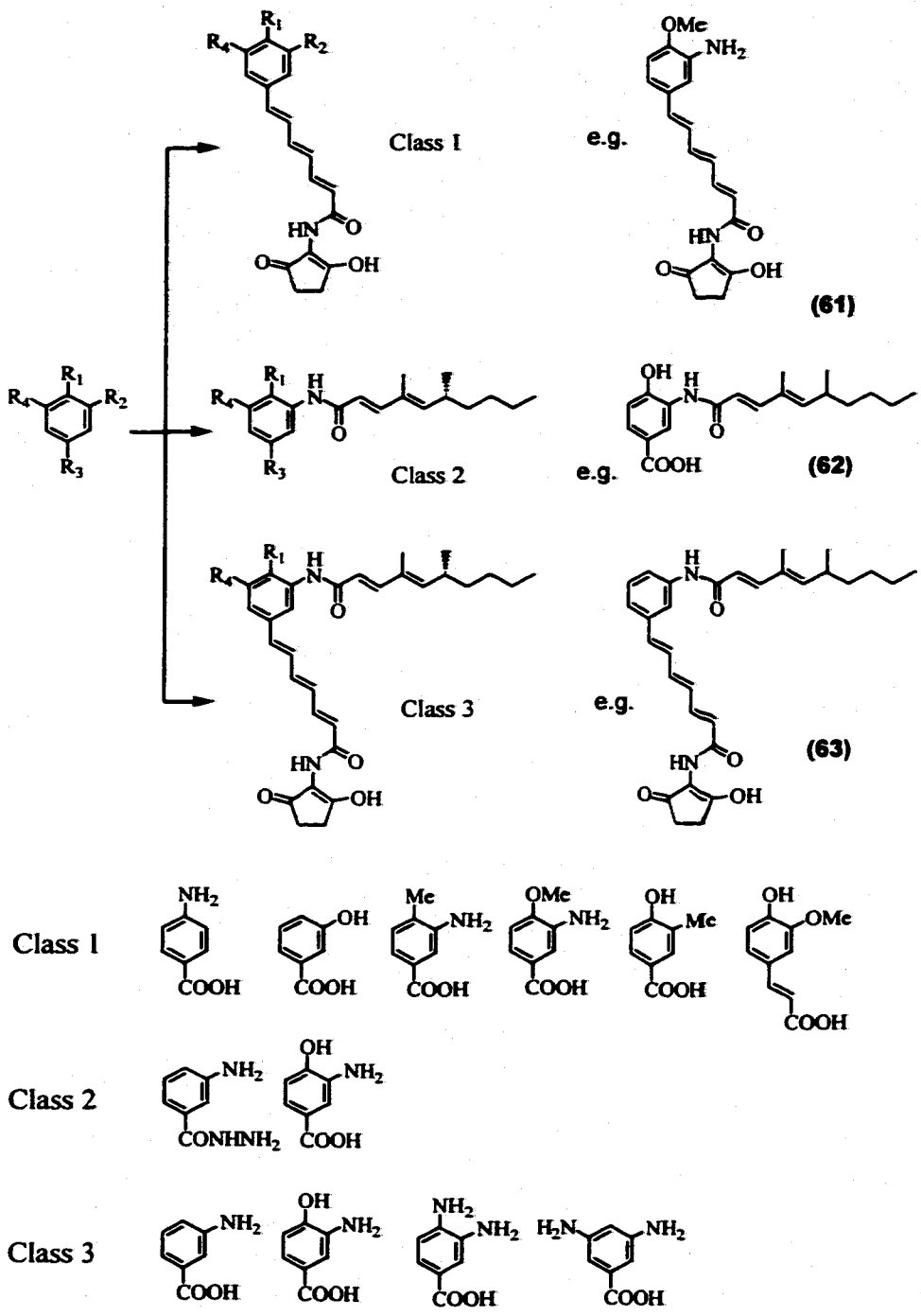


Figure 25. Manumycin Analogs Produced by *S. parvulus* via Precursor-directed Biosynthesis

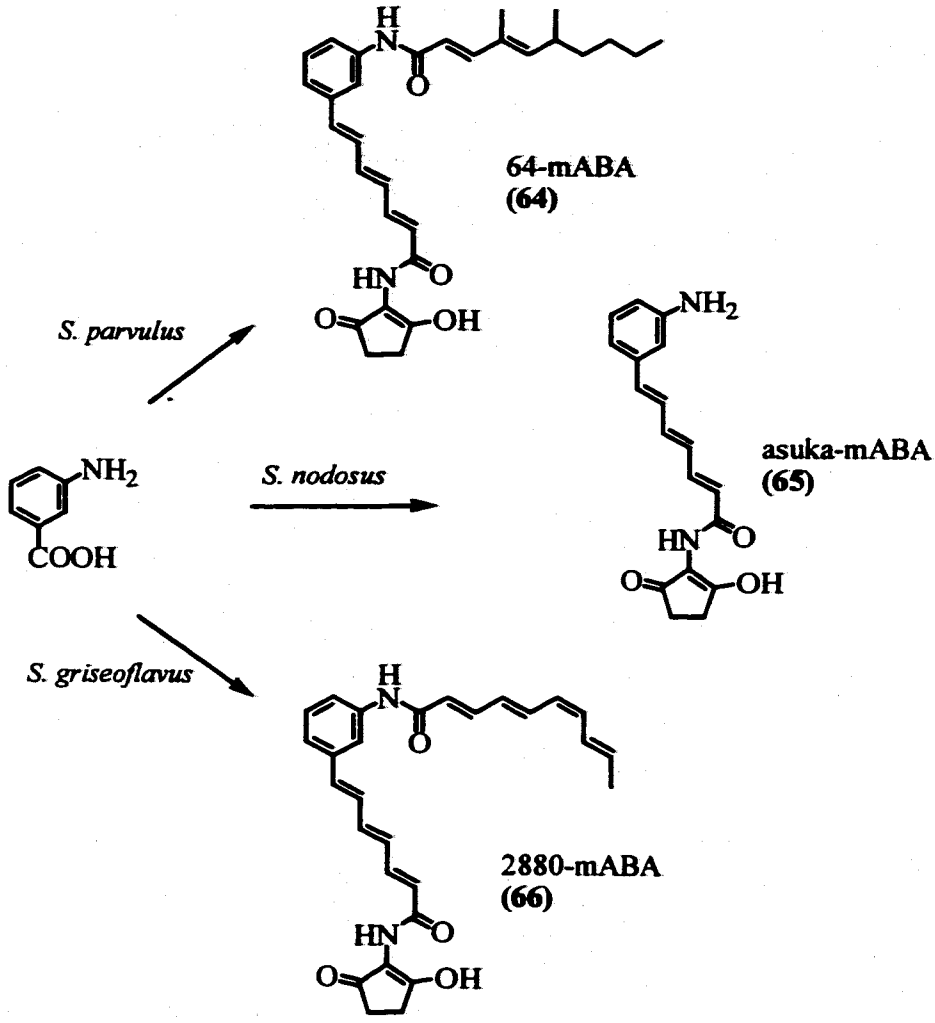


Figure 26. 3-Aminobenzoic Acid-Directed Biosynthesis of Manumycin Analogs in Different *Streptomycets*

production of normal manumycin antibiotics was shut down in most cases.

3.2 Proposed Normal Biosynthesis of Manumycins

Useful biosynthetic information has been extracted from the precursor directed biosynthesis experiments. The fact that the Class 1 and 3 analogs are predominate suggests that the "lower" polyketide chain is probably synthesized immediately after 3,4-AHBA is formed, followed by the attachment of the C_5N unit and then the "upper" chain. Oxidation of the aromatic portion of the mC_7N unit may be the last reaction. We are in favor of the route (a) in Scheme 17, because the products from the precursor directed biosynthesis largely point to this pathway for the natural biosynthesis of manumycin type metabolites. One problem may exist because when the unlabeled true precursor 3,4-AHBA was fed to *S. parvulus* in high concentration, one Class 2 metabolite, **62**, was produced, which has an "upper" chain but without a "lower" chain and C_5N moiety. This result indicates the possibility of route (c) in Scheme 17 as the pathway, contradicting the conclusions drawn from most of the other manumycin analogs produced the same way by precursor directed feeding.

Based on the precursor directed feeding results and previous biosynthetic studies, a biosynthetic pathway to manumycin type metabolites was proposed as our working hypothesis (Figure 27). 3,4-AHBA is derived from succinate or aspartic acid and glycerol via oxalacetate and pyruvate. Next, 3,4-AHBA is converted into its CoA

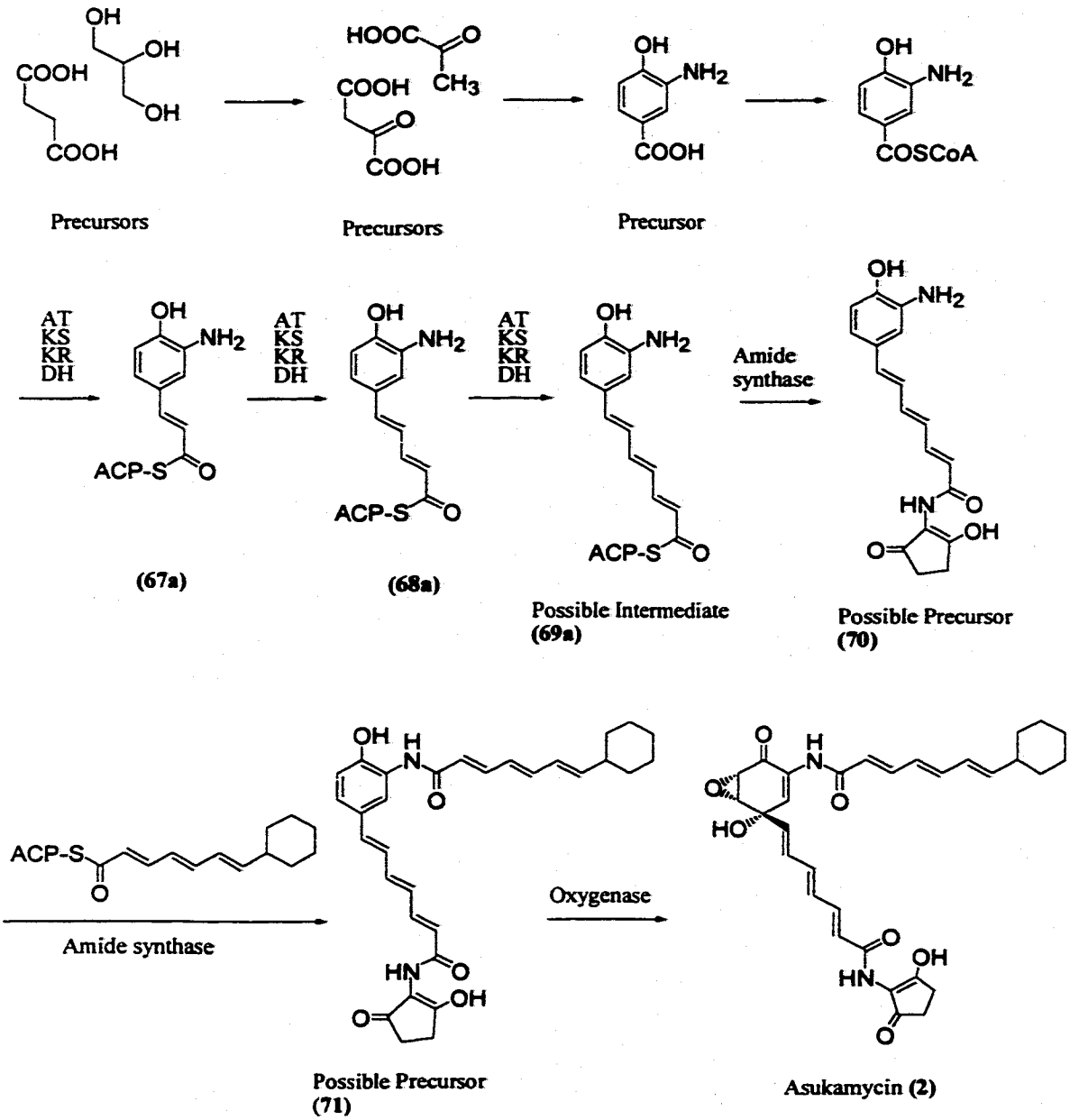
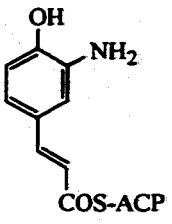


Figure 27. Possible Biosynthetic Pathway to Manumycins, for Instance, Asukamycin

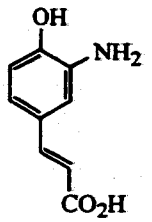
thioester and transferred to ketosynthase (KS) by acyltransferase (AT). The "lower" polyketide synthesis is initiated by the addition of malonyl-ACP (acyl carrier protein) thioester catalyzed by a ketosynthase (KS), followed by carbonyl reduction with a ketoreductase (KR) and formation of the double bond by a dehydratase (DH) (Hopwood 1997). This cycle is repeated three times to generate 7-(3-amino-4-hydroxyphenyl)-hepta-2,4,6-trienoic acid ACP thioester which is coupled with the C_5N moiety by an amide synthase. The "upper" chain is then attached to the aromatic amine group by a second amide synthase. In the final step, an oxygenase accomplishes the modification of the aromatic ring to the epoxyquinol structure. According to this hypothesis, 3-(3-amino-4-hydroxy-phenyl)-acrylic acid, 5-(3-amino-4-hydroxyphenyl)-penta-2,4-dienoic acid and 7-(3-amino-4-hydroxyphenyl)-hepta-2,4,6-trienoic acid ACP thioesters (**67a – 69a**, Figure 28) are intermediates on the biosynthetic pathway. To study the biosynthesis of the "lower" chain and to test the above hypothesis, these isotopically-labeled intermediates need to be synthesized and fed. Due to the difficulty to make the ACP thioester of these acids, the three free acids were synthesized and fed to bacteria in the hope that cellular enzymes may be able to convert them to their ACP thioesters. In the past, we have already succeeded in the feeding of 3,4-AHBA which must be converted to its CoA thioester by enzymes in the cell.

3.3 Synthesis and Feeding of Possible Precursors of "Lower" Polyketide Chain

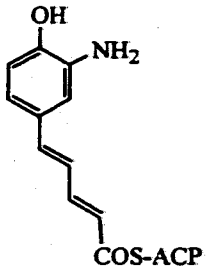
3.3.1 Synthesis of 3-(3-Amino-4-hydroxyphenyl)-*E*-[1,2-$^{13}C_2$]prop-2-enoic Acid ([1,2-$^{13}C_2$]-**67b**)



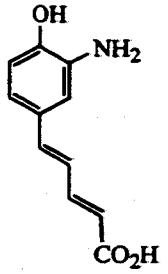
(67a)



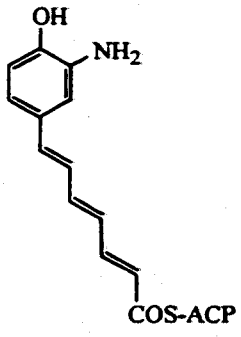
3-(3-Amino-4-hydroxyphenyl)-*E*-prop-2-enoic acid **(67b)**



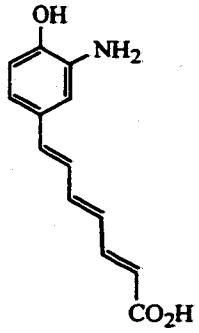
(68a)



5-(3-Amino-4-hydroxyphenyl)-(*2E,4E*)-penta-2,4-dienoic acid **(68b)**



(69a)



7-(3-Amino-4-hydroxyphenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienoic acid **(69b)**

Figure 28. Possible Precursors and Intermediates of the "Lower" Polyketide Chain

The synthesis of [1,2-$^{13}C_2$]-**67b** was designed and carried out as illustrated in Figure 29. The ^{13}C labels at C-1 and C-2 were introduced via a Knoevenagel condensation (Rajagopalan and Raman, 1955; Ressler *et al.*, 1979). It is worth noting that the nitrogen atom is introduced by nitration with NH_4NO_3 (Crivello 1981), a cheap and convenient method which provides an opportunity for introducing ^{15}N into the mC_7N unit to make a ^{13}C-^{15}N doubly labeled amide bond in a future synthesis of compound **71**.

Methoxybenzaldehyde **(72)** was treated with NH_4NO_3 in $CHCl_3$ in the presence of trifluoroacetic anhydride at room temperature. After work-up and purification by flash column chromatography, 4-methoxy-3-nitrobenzaldehyde **(73)** was obtained in a yield of 85%. Compound **73** was condensed with [U-$^{13}C_3$]-malonic acid followed by decarboxylation under mild basic conditions at 100 °C to afford 3-(4-methoxy-3-nitro-phenyl)-*E*-[1,2-$^{13}C_2$]prop-2-enoic acid [1,2-$^{13}C_2$]-**74** (93%), which was demethylated by reaction with lithium iodide in anhydrous 2,4,6-collidine under reflux (Harrison 1969) to produce 3-(4-hydroxy-3-nitrophenyl)-*E*-[1,2-$^{13}C_2$]prop-2-enoic acid ([1,2-$^{13}C_2$]-**75**) in a yield of 75%. The reduction of the nitro group to an amino group was accomplished with the mild reducing reagent tin(II) chloride in ethanol at 70 °C (Bellamy and Ou 1984) to give 3-(3-amino-4-hydroxyphenyl)-*E*-[1,2-$^{13}C_2$]prop-2-enoic acid ([1,2-$^{13}C_2$]-**67b**) (73%). [1,2-$^{13}C_2$]-**67b** was characterized by NMR and HR-MS.

3.3.2 Synthesis of 5-(3-Amino-4-hydroxyphenyl)-(*2E,4E*)-[1,2-$^{13}C_2$]penta-2,4-dienoic Acid ([1,2-$^{13}C_2$]-**68b**)

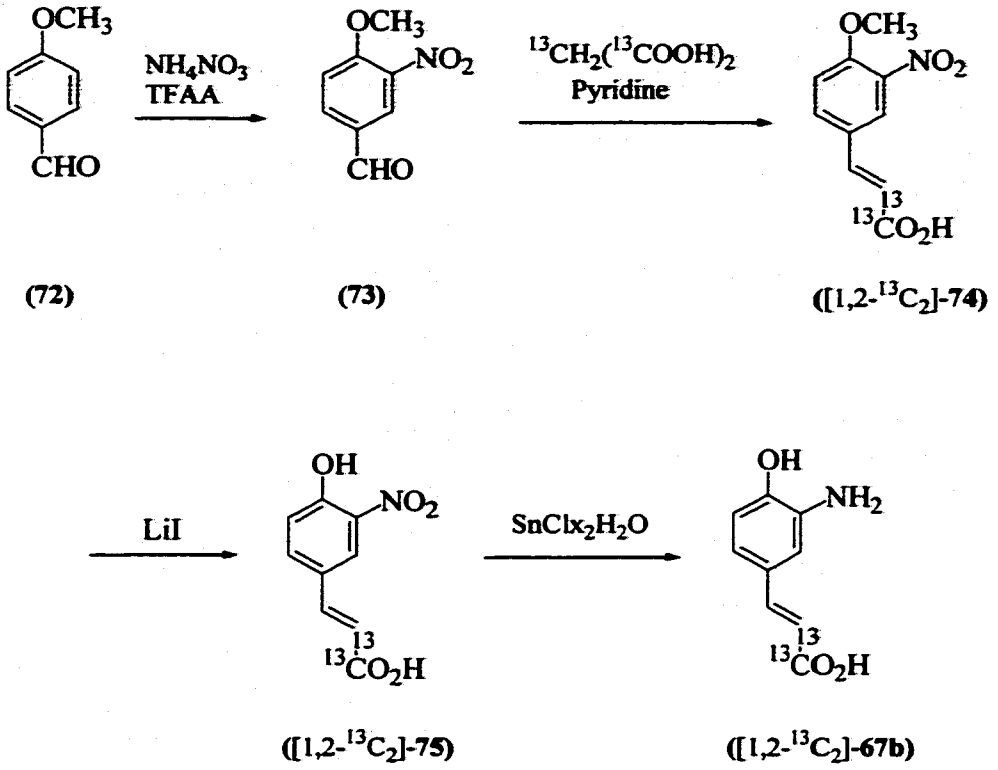


Figure 29. Synthesis of 3-(3-Amino-4-hydroxyphenyl) -*E*-[1,2-$^{13}C_2$]prop-2-enoic acid

The synthesis of [1,2-$^{13}C_2$]-**68b** is shown in Figure 30, using similar methodology as in the synthesis of [1,2-$^{13}C_2$]-**67b**, except that the commercially available 4-methoxycinnamaldehyde **76** served as starting material and the Wadsworth-Emmons reaction was used to extend the conjugated system and at the same time to introduce the ^{13}C label (Seguineau and Villieras 1988; Williams and White 1987).

4-Methoxycinnamaldehyde **(76)** was treated with NH_4NO_3 and trifluoroacetic anhydride. The crude product was purified by flash column chromatography to afford 4-methoxy-3-nitrocinnamaldehyde **(77)** in a yield of 60%. This yield is lower compared with the nitration of methoxybenzaldehyde **(72)**, probably due to side reactions with the double bond of compound **76**. The heterogeneous reaction of **77** with 1 equivalent triethyl phosphono-[1,2-$^{13}C_2$]acetate and 6M K_2CO_3 provided ethyl 5-(4-methoxy-3-nitro-phenyl)-(*2E, 4E*)-[1,2-$^{13}C_2$]penta-2,4-dienoate ([1,2-$^{13}C_2$]-**78**), which was purified by recrystallization and column chromatography (69%). The cleavage of the methyl ether and hydrolysis of the ethyl ester were completed in one step. Compound **78** was hydrolyzed by LiI to give 5-(4-hydroxy-3-nitrophenyl)-(*2E,4E*)-[1,2-$^{13}C_2$]penta-2,4-dienoic acid ([1,2-$^{13}C_2$]-**79**) (92%), followed by reduction of the nitro group with tin(II) chloride to yield 5-(3-amino-4-hydroxyphenyl)-(*2E,4E*)-[1,2-$^{13}C_2$]penta-2,4-dienoic acid ([1,2-$^{13}C_2$]-**68b**) (81%). [1,2-$^{13}C_2$]-**68b** was characterized by NMR and HR-MS.

3.3.3 Synthesis of 7-(3-Amino-4-hydroxyphenyl)-(*2E,4E,6E*)-[l, 2-$^{13}C_2$]-hepta-2,4,6-trienoic Acid ([1,2-$^{13}C_2$]-**69b**)

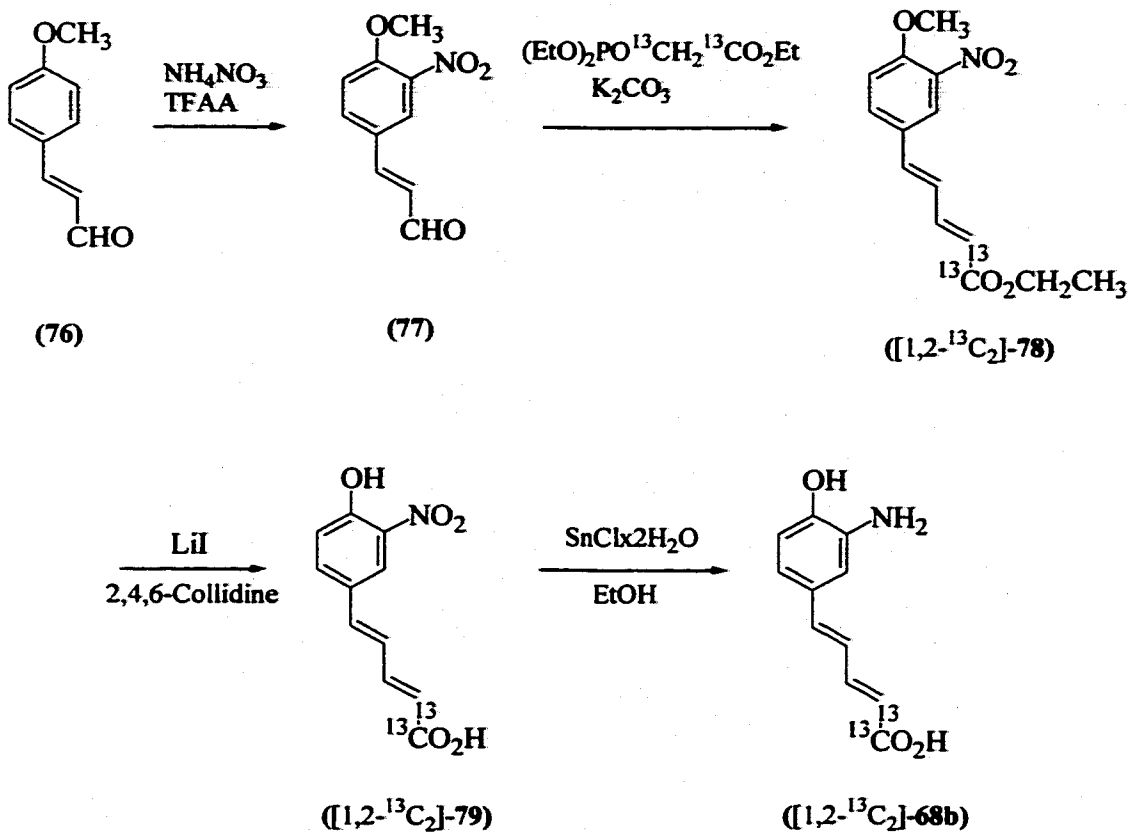


Figure 30. Synthesis of 5-(3-Amino-4-hydroxyphenyl)-(*2E,4E*)-[1,2-$^{13}C_2$]penta-2,4-dienoic Acid

[1,2-$^{13}C_2$]-**69b** was synthesized in a similar way as [1,2-$^{13}C_2$]-**68b** (Figure 31), but an additional double bond is required in the *trans* triene chain. Therefore, the Wadsworth-Emmons reaction has to be applied twice to extend the polyene chain in the synthesis. To synthesize 5-(4-methoxy-3-nitrophenyl)-(*2E,4E*)-penta-2,4-dienal **(81)**, two routes (a) and (b) were attempted. In attempt (a), the Wittig reaction was employed to introduce the additional double bond from **77** to **81** (Meyers *et al.*, 1986; Roush *et al.*, 1991; Schlessinger *et al.*, 1985). Aldehyde **77** and (triphenylphosphoranylidene)-acetateldehyde were heated under reflux in benzene to afford a 1:1 ratio of starting material **77** to product **81**, along with a minor amount of the over-reaction product 7-(4-methoxy-3-nitrophenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienal. Unfortunately, **77** and **81** could not be separated due to the similarity of their structures and properties. In attempt (b), a controlled reaction was pursued to reduce the ester to the aldehyde with a stoichiometric amount of reducing reagent, as reported previously (Garner and Park 1987; Kelly and Kaul 1983; Pearson *et al.*, 1990). An equimolar amount of diisobutylaluminum hydride was added slowly with vigorously stirring at -78 ^{0}C to a solution of **78**. Even though, a mixture of starting material **78** and over-reduced alcohol 5-(4-methoxy-3-nitrophenyl)-(*2E,4E*)-penta-2,4-dienol **(80)** was recovered. Therefore the attempt (c) was adopted to reduce ester **78** completely to alcohol **80** which is then oxidize to dienal **81**.

Compound **77** was reacted with an excess of triethyl phosphonoacetate to afford **78** in a yield of 87%. **78** was reduced with 2 equivalents of DIBAL-H to give dienol **80** (88%), which was subsequently oxidized to 5-(4-methoxy-3-nitrophenyl)-(*2E,4E*)-penta-2,4-

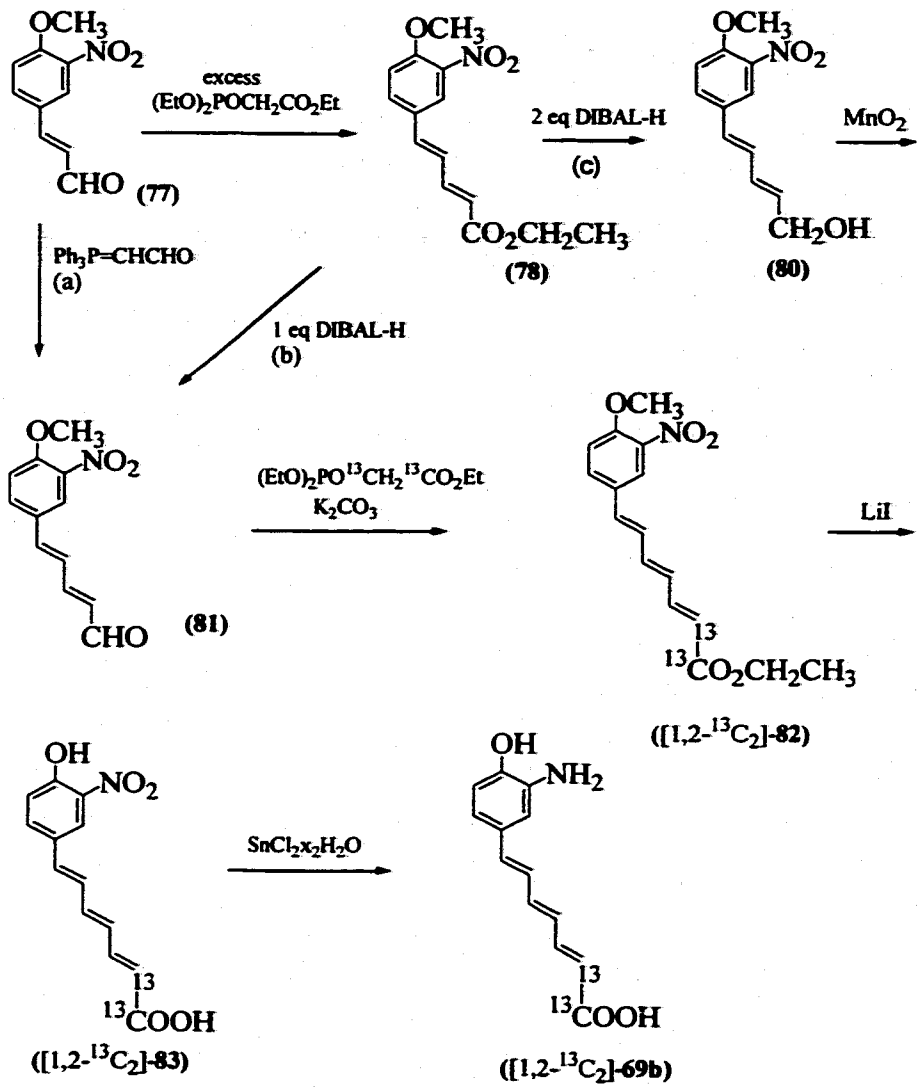


Figure 31. Synthesis of 7-(3-Amino-4-hydroxyphenyl)-(*2E,4E,6E*)-[1,2-$^{13}C_2$]-hepta-2,,4,6-trienoic Acid

dienal **(81)** by activated manganese dioxide at room temperature (96%) (Hoeger *et al.*, 1987; Rao *et al.*, 1986). The ^{13}C label was introduced in the second Wadsworth-Emmons reaction by mixing **81**, 1 equivalent of triethyl phosphono-[1,2-$^{13}C_2$]acetate and 6M K_2CO_3 to generate ethyl 7-(4-methoxy-3-nitrophenyl)-(*2E,4E,6E*)-[1,2-$^{13}C_2$]hepta-2,4,6-trienoate [1,2-C_2]-**(82)** in 64% yield. Deprotection of the methyl ether and ethyl ester was accomplished in the same way as in the dienoate **78** by LiI to provide 7-(4-hydroxy-3-nitrophenyl)-(*2E,4E,6E*)-[1,2-$^{13}C_2$]-penta-2,4,6-trienoic acid **(83)** (84%), followed by reduction with tin(II) chloride to 7-(3-amino-4-hydroxyphenyl)-(*2E,4E,6E*)-[1,2-$^{13}C_2$]-hepta-2,4,6-trienoic acid ([1,2-$^{13}C_2$]-**69b**) (84%).

The ^{13}C doubly labeled free acids [1,2-$^{13}C_2$]-**67b**, [1,2-$^{13}C_2$]-**68b** and [1,2-$^{13}C_2$]-**69b** were fed to *S. nodosus* and the asukamycin produced was isolated and purified. Unfortunately, no incorporation of ^{13}C from the three acids into asukamycin was observed by ^{13}C-NMR.

3.3.4 Synthesis and Feeding of 3-(3-Amino-4-hydroxyphenyl)-*E*-[1,2-$^{13}C_2$]prop-2-enoic Acid N-Acetylcysteamine Thioester ([1,2-$^{13}C_2$]-**84**)

Since the feeding of the three free acids [1,2-$^{13}C_2$]-**67b**, [1,2-$^{13}C_2$]-**68b** and [1,2-$^{13}C_2$]-**69b** resulted in no incorporation into asukamycin, it was rationalized that these acids can not be converted to their ACP thioesters by cellular enzymes. Therefore, it was considered to synthesize an analog of the ACP thioester as an activated form of the acid. The N-acetylcysteamine thioester is a good candidate that has been widely used in the study of

biosynthesis of natural products, especially polyketides. For example, a series of presumed intermediates of polyketide chain elongation have been synthesized as their N-acetylcysteamine thioesters and were successfully incorporated into methymycin, neomethymycin (Cane *et al.*, 1993a), nargenicin (Cane *et al.*, 1993b), oudenone (Tsantrizos *et al.*, 1995) and lovastatin (Witter and Vederas 1996). Moreover, a N-acetylcysteamine thioester was used to study the allylic rearrangement in the biosynthesis of unsaturated fatty acids by incubation with dehydratase (Schwab and Klassen 1984). Therefore it was attempted to synthesize and feed the N-acetylcysteamine thioester of **67b** to explore the biosynthesis of the "lower" chain of manumycins.

The synthesis of [1,2-$^{13}C_2$]-**84**, the N-acetylcysteamine thioester of **67b**, proved problematic. The phenolic hydroxyl group is much more reactive than an aliphatic hydroxyl group, thus causing serious problems such as side reactions at the phenolic hydroxyl group and the addition of the thiol to the acrylic double bond which were encountered also during the synthesis of 4-coumaroyl CoA. Most of the esterification methods failed in the synthesis of 4-coumaroyl CoA. Protection of the phenol was also unsuccessful, because either the protecting group was not stable during esterification or the resulting thioester was destroyed during deprotection. Consequently, an indirect two-step reaction was attempted in which 4-coumaric acid was converted to a phenyl thiol ester or a N-hydroxysuccinimide ester followed by ester exchange to the CoA ester (Johns 1974; Stockigt and Zenk 1975). Unfortunately, the overall yield for this process was lower than 10%.

67b carries an additional amino group compared to 4-coumaric acid. For this reason, it can be predicted that the thioester of [1,2-$^{13}C_2$]-**67b** is even more difficult to synthesize than that of 4-coumaric acid. A more favorable approach would be to protect the aromatic hydroxyl and amino groups by a single protecting reagent to form a five-membered ring, hence the free acid would be more easily esterified. It was reported that acetone condenses with *o*-aminothiophenol to form a five-membered heterocyclic ring, but it failed to form the same five-membered ring with *o*-aminophenol (Figure 32) (Erker 1989). This selectivity can be rationalized by considering the C-S and C-O bond length. Five-membered ring formation is favored with the larger sulfur atom and longer C-S bond compared to the shorter C-O bond. Other commonly used protecting groups such as TBDMS and BOC were placed on **67b**, but the following esterification failed to make thioester **84** or even the protection reaction did not give good yields due to the multiple functionalities of **67b**. Due to the failure of the attempts to protect the phenolic and amine groups, a direct synthesis of **84** was attempted using N,N'carbonyldiimidazole (Kawaguchi *et al.*, 1981; Moore *et al.*, 1995; Ohta *et al.*, 1982), Tl-NAC (Masamune *et al.*, 1975; Schwab and Klassen 1984), DCC/DMAP or DMAP-HCl, EDCI (Boden and Keck 1985; Dhaon *et al.*, 1982; Neises and Steglich 1978; Witter and Vederas 1996) as the coupling reagents (Figure 33).

Thus [1,2-$^{13}C_2$]-**67b** was reacted with N-acetylcysteamine, DCC (or EDCI) and DMAP at

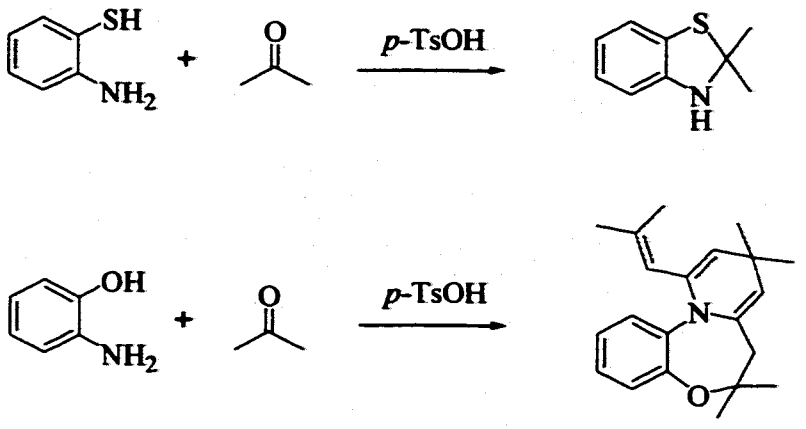
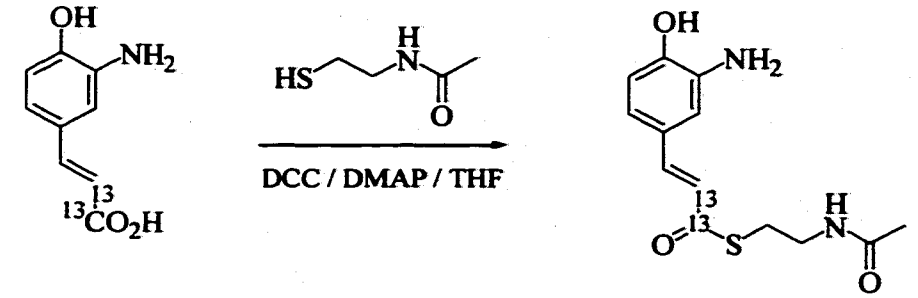


Figure 32. Condensation of Acetone with *o*-Aminophenol and *o*-Aminothiophenol



([1,2-$^{13}C_2$]-67b)

([1,2-$^{13}C_2$]-84)

Figure 33. Synthesis of 3-(3-Amino-4-hydroxyphenyl)-*E*-[1,2-$^{13}C_2$]prop-2-enoic Acid N-Acetylcysteamine Thioester

room temperature. The crude product was purified by normal phase and RP18 reverse phase column chromatography to give 3-(3-amino-4-hydroxyphenyl)-*E*-[1,2-$^{13}C_2$]prop-2-enoic acid N-acetylcysteamine thioester ([1,2-$^{13}C_2$]-**84**) in a 15% yield.

[1,2-$^{13}C_2$]-**84** dissolved in a minimal amount of DMSO was fed to *S. nodosus*. Unfortunately, no incorporation of [1,2-$^{13}C_2$]-**84** into asukamycin was observed by ^{13}C-NMR analysis.

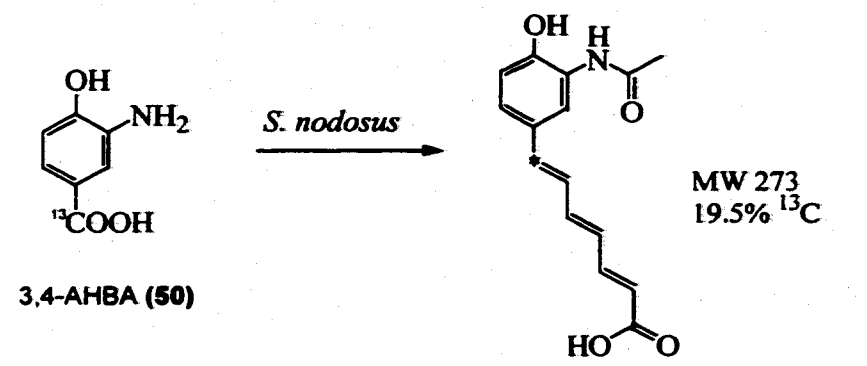
3.4 Isolation and Identification of Pathway-Related New Shunt Metabolite

^{13}C-NMR analysis of the crude extract from an asukamycin fermentation fed with 3,4-[7-^{13}C]-AHBA revealed an enhanced ^{13}C signal at 136 ppm in chloroform-*d*. Due to the increased solubility of the crude extract in acetone, two significantly enhanced ^{13}C signals were seen at 137 ppm and 139 ppm in acetone-*d*. The peak at 139 ppm was later identified as C-7 of asukamycin. This implies that the peak at 137 ppm should belong to a new compound which is derived from 3,4-AHBA. As 3,4-AHBA is a specific precursor of the mC_7N unit in manumycin type metabolites, this new mC_7N unit-containing compound is likely an intermediate or pathway related shunt metabolite of the manumycins. In particular the fact that the enhanced ^{13}C signal is shifted from the 165 ppm of a carboxyl carbon in 3,4-AHBA to the 137 ppm characteristic of a C-C double bonded carbon in the new compound implies polyketide synthesis. It was thought that the isolation and identification of this new compound might shed light on the

biosynthesis of the "lower" polyketide chain which was not solved by feeding the experiments with the ^{13}C labeled acids [1,2-$^{13}C_2$]-**67b**, [1,2-$^{13}C_2$]-**68b**, [1,2-$^{13}C_2$]-**69b** and thioester [1,2-$^{13}C_2$]-**84**.

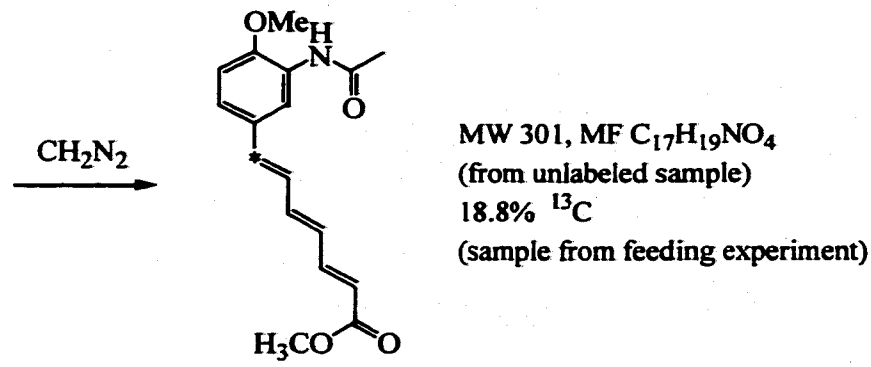
3.4.1 Isolation and Purification of the New Metabolite

Crude extracts containing the unknown compound were obtained from the supernatant of the fermentation broth by ethyl acetate extraction. Purification was performed by RP18 silica-gel column chromatography, eluting with acetone/water 20/80, followed by HPLC with water/2-propanol 72.5/27.5. ^{13}C-NMR spectra were taken to trace the new compound by the ^{13}C enhancement at 137 ppm in acetone-*d*. ^{1}H-NMR analysis of the new compound showed a conjugated polyene structure and the absence of saturated hydrogens except for a singlet methyl peak from an acetyl group. The molecular weight of this unknown was determined as 273 by FAB-MS. This compound was methylated by diazomethane and the molecular formular of the methylated derivative was determined as $C_{17}H_{19}NO_4$ (MW 301) by HR-MS. Two methyl groups were added to this unknown during methylation. Therefore, the molecular formala of the unknown was deduced as $C_{15}H_{15}NO_4$. Compared with that of acid **69b** ($C_{13}H_{13}NO_3$), the new compound seems to have an additional acetyl group. It is known that this new compound is derived from 3,4-AHBA and that it is polyketide in nature; on the other hand acetylation is a common way in organisms to detoxify, hence the unknown compound was assumed to be N-acetylated **69b** (**85** in Figure 34).



3,4-AHBA (**50**)

7-(3-N-Acetylamino-4-hydroxyphenyl)-
(*2E,4E,6E*)-hepta-2,4,6-trienoic Acid (**85**)



Methyl 7-(3-N-Acetylamino-4-methoxyphenyl)-
(*2E,4E,6E*)-hepta-2,4,6-trienoate (**86**)

Figure 34. New Shunt Metabolite Discovered in *S. nodosus*

3.4.2 Synthesis of Methyl 7-(3-N-Acetylamino-4-methoxyphenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienoate (**86**) and Identification of the Unknown Metabolite **85**

Compound **86** was synthesized from **82** by reduction, acetylation, hydrolysis and methylation (Figure 35). Ethyl 7-(3-nitro-4-methoxyphenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienoate (**82**) was reduced with tin(II) chloride in ethyl acetate to afford ethyl 7-(3-amino-4-methoxyphenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienoate (**87**) in 73% yield. Subsequent acetylation with acetic anhydride and triethylamine as catalyst gave ethyl 7-(3-N-acetylamino-4-methoxyphenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienoate (**88**) in 79% yield. The saponification of **88** by lithium hydroxide in methanol/water gave 7-(3-N-acetylamino-4-methoxyphenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienoic acid (**89**) in 57% yield (Corey *et al.*, 1977). Acid **89** was converted to its methyl ester by quantitative methylation with diazomethane to give methyl 7-(3-N-acetylamino-4-methoxyphenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienoate (**86**) (Black 1983). The resulting **86** was recrystallized from ethyl acetate/hexane solvent.

The ^{1}H-NMR spectrum of **86** was identical to that of the methylated new compound from the fermentation. Both gave two methyl peaks from methoxy groups at chemical shifts of 3.87 and 3.68 ppm, as well as one methyl peak from the acetyl group at 2.15 ppm. The comparison of the aromatic/conjugated polyene range is enlarged and illustrated in Figure 36. Thus the unknown metabolite was identified as 7-(3-N-acetylamino-4-hydroxyphenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienoic acid **85**.

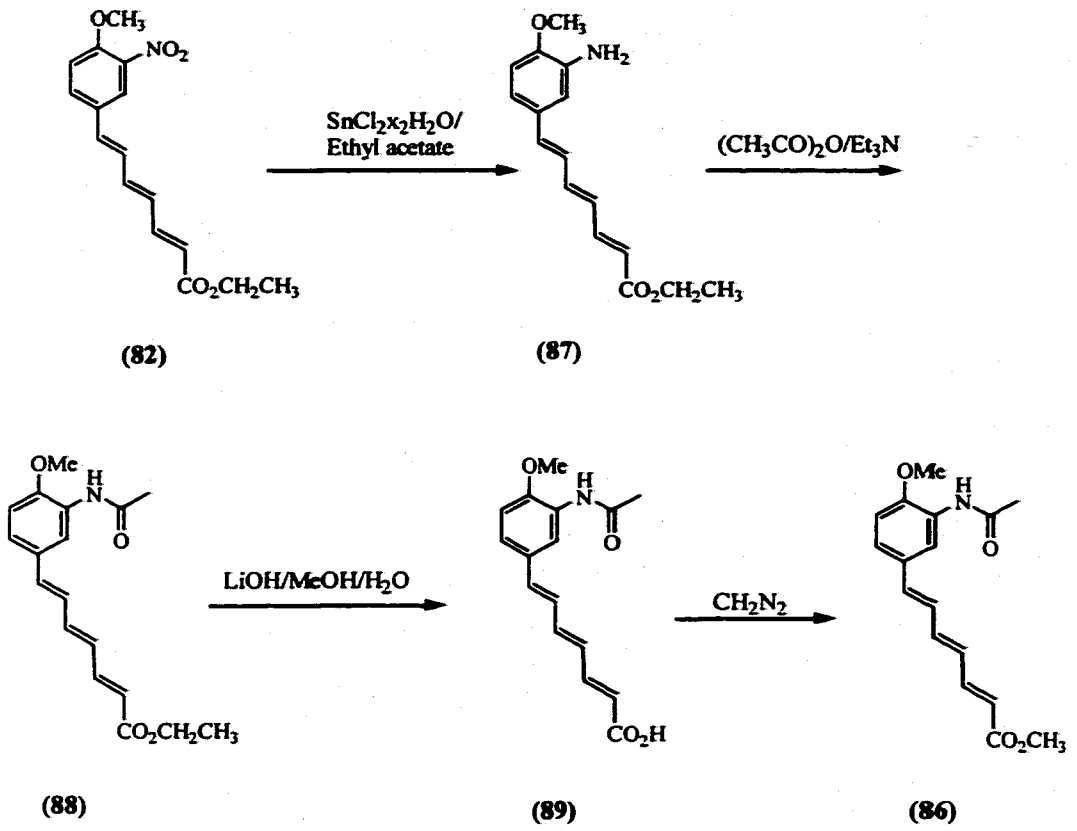


Figure 35. Synthesis of Methyl 7-(3-N-Acetylamino-4-methoxyphenyl)-hepta-2,4,6-trienoate

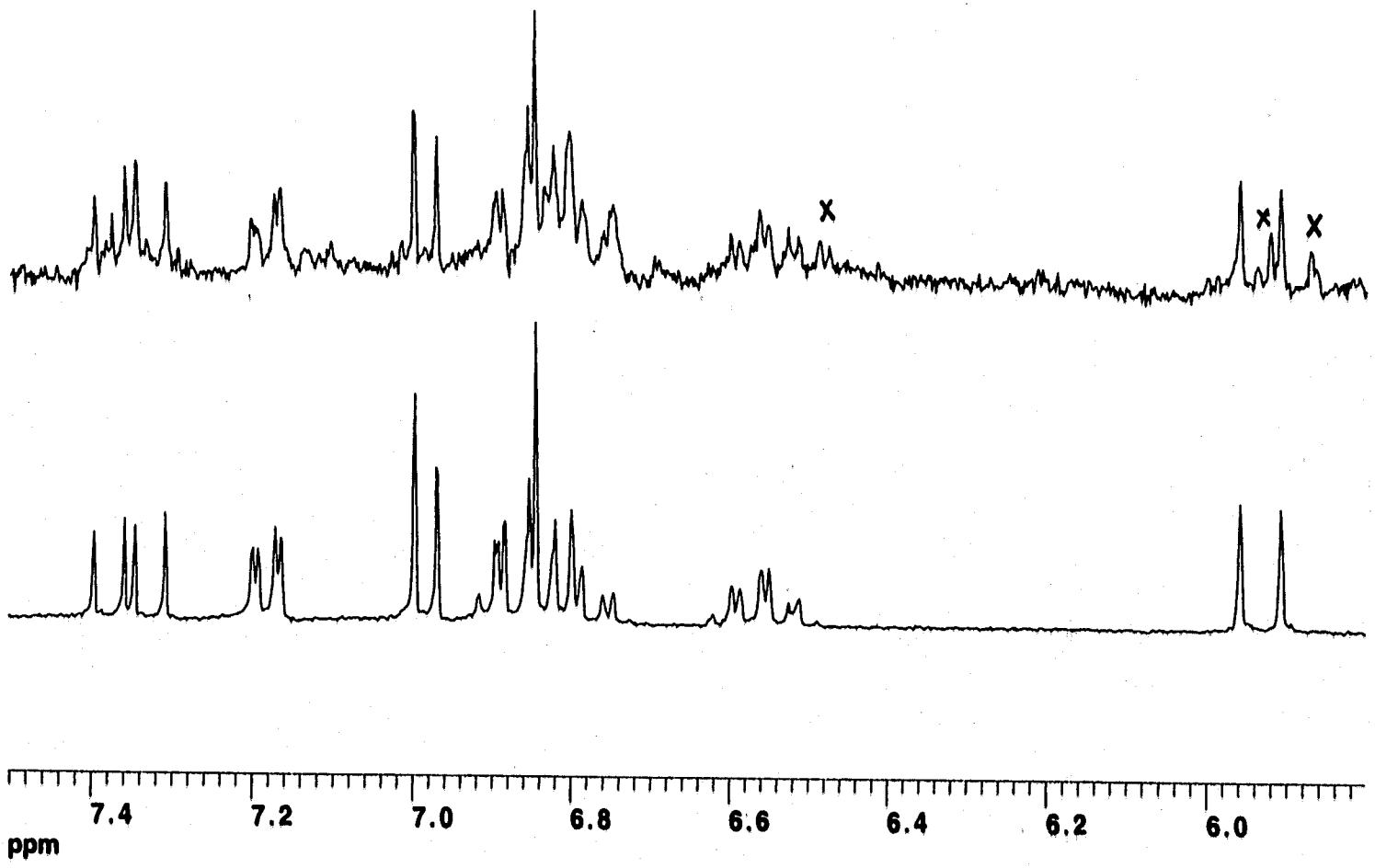


Figure 36. ^{1}H-NMR Comparison of Methyl 7-(3-N-Acetylamino-4-methoxyphenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienoate **(86)**, (above) From Fermentation and Isolation; (below) From Synthesis and Recrystallization.

SIM-MS gave an incorporation of 3,4-[7-^{13}C]-AHBA into **85** of 19.5% and into **86** of 18.8%. The difference is within reasonable error.

3.5 Synthesis and Feeding of [Acetyl-$^{2}H_3$, carboxyl-^{13}C]-3-N-acetylamino-4-hydroxybenzoic Acid and 7-(3-N-Acetylamino-4-hydroxyphenyl)-(*2E,4E,6E*)-[1,2-$^{13}C_2$]hepta-2,4,6-trienoic Acid

Since compound **85** contains a triene moiety, it can be presumed to be a metabolite related to the asukamycin biosynthetic pathway in *S. nodosus*, although it is not clear whether this new compound is a precursor of asukamycin or a shunt metabolite. If it is a shunt metabolite, at what point does it exit the pathway? Is the 3,4-AHBA acetylated before the "lower" polyketide chain is synthesized or does the "lower" chain extension arise first to yield **69a** or **69b**, followed by acetylation? The answers to these questions may reveal the pathway, or at least yield useful information about the pathway. To answer these questions, labeled 3-N-acetylamino-4-hydroxybenzoic acid **(90)** and **85** were synthesized by acetylation of the labeled 3,4-AHBA and **69b** and subsequently selective cleavage of the O-acetyl group (Figure 37).

3.5.1 Synthesis of [Acetyl-$^{2}H_3$, carboxyl-^{13}C]-3-N-acetylamino-4-hydroxybenzoic Acid ([Acetyl-$^{2}H_3$, carboxyl-^{13}C]-**90**)

3,4-[7-^{13}C]-AHBA was acetylated with excess deuterated acetyl chloride (CD_3COCl) and

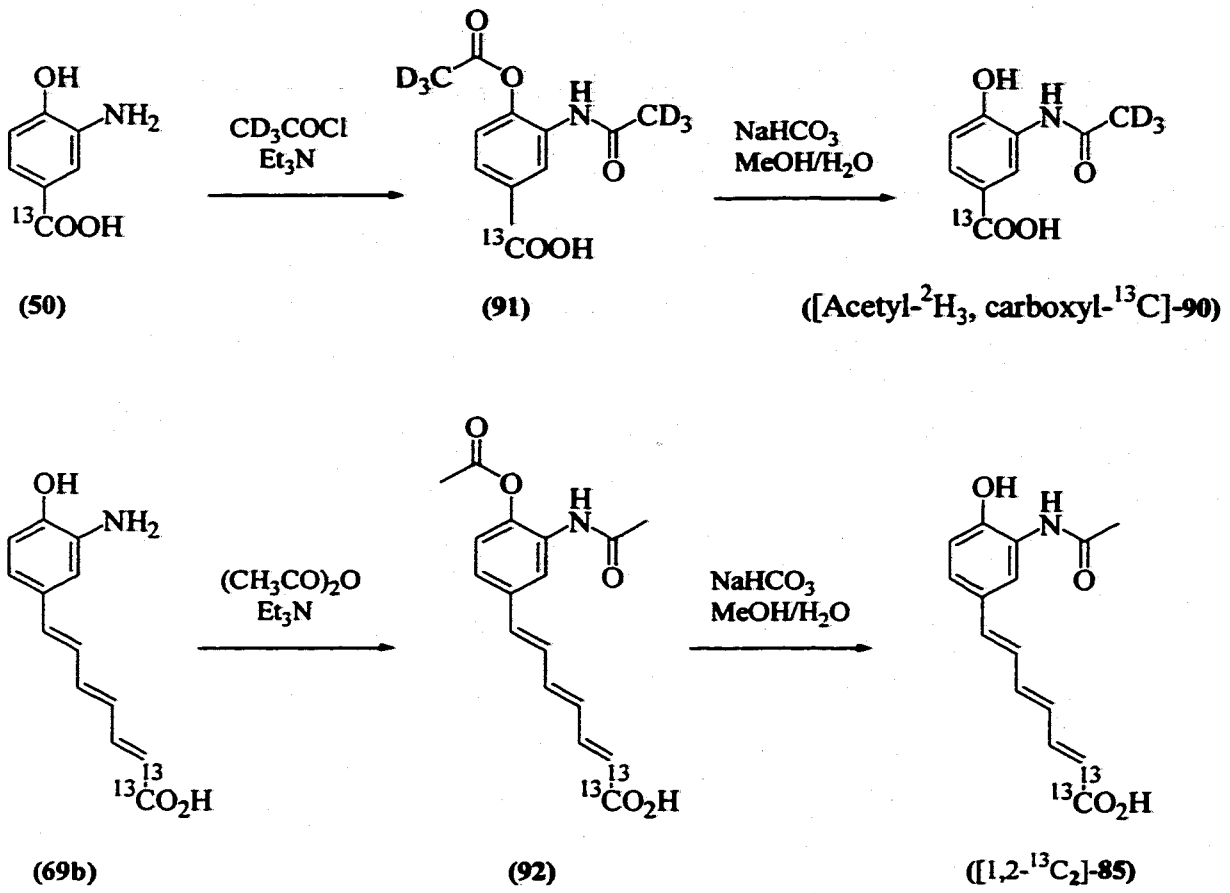


Figure 37. Synthesis of [Acetyl-$^{2}H_3$, carboxyl-^{13}C]-3-N-acetylamino-4-hydroxybenzoic Acid and 7-(3-N-Acetylamino-4-hydroxyphenyl)-(*2E,4E,6E*)-[1,2-$^{13}C_2$]-hepta-2,4,6-trienoic Acid

triethylamine. The resulting crude product of [acetyl-$^{2}H_3$,carboxyl-^{13}C]-3-N-acetylamino-4-acetoxybenzoic acid [7-^{13}C]-(**91**) was used directly in the next reaction. The O-acetyl group of [7-^{13}C]-**91** was removed by hydrolysis in saturated aqueous $NaHCO_3$/ methanol solution and the final product **90** was purified on a silica-gel column with CH_2Cl_2 / MeOH 100:5, and characterized by NMR and HR-MS.

3.5.2 Synthesis of 7-(3-N-Acetylamino-4-hydroxyphenyl)-(*2E,4E,6E*)-[1,2-^{13}C]-hepta-2,4,6-trienoic Acid ([1,2-^{13}C]-**85**)

[1,2-$^{13}C_2$]-**69b** was acetylated with excess acetic anhydride and triethylamine. The resulting 7-(3-N-acetylamino-4-acetoxyphenyl)-(*2E,4E,6E*)-[1,2-$^{13}C_2$]hepta-2,4,6-trienoic acid ([1,2-$^{13}C_2$]-**92**) was hydrolyzed in saturated aqueous $NaHCO_3$/ methanol solution. The product, 7-(3-N-acetylamino-4-hydroxyphenyl)-(*2E,4E,6E*)-[1,2-$^{13}C_2$]hepta-2,4,6-trienoic acid ([1,2-$^{13}C_2$]-**85**), obtained in this two-step reaction sequence in 46% yield, was characterized by NMR and high resolution mass spectrometry.

[Acetyl-$^{2}H_3$, carboxyl-^{13}C]-**90** and [1,2-$^{13}C_2$]-**85**, respectively, were fed to *S. nodosus*, and **85** and asukamycin were isolated from each fermentation and analyzed by ^{13}C-NMR. No incorporation of ^{2}H or ^{13}C from [acetyl-$^{2}H_3$,carboxyl-^{13}C]-**90** into **85** was observed, and no incorporation of [1,2-$^{13}C_2$]-**85** into asukamycin either. These results imply that **90** is not the precursor of **85**, and **85** is not a precursor of asukamycin. Free acid **69b** has never been discovered in the fermentation, although **85** is naturally produced in a

considerable amount. Therefore, **85** is evidently formed from the ACP thioester of 7-(3-amino-4-hydroxyphenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienoic acid **69a** by acetylation and then released to the medium.

3.6 Conclusion

In this chapter, a hypothesis for the complete biosynthesis of the manumycin type metabolites has been proposed. This hypothesis, especially the "lower" chain biosynthesis, has been examined by two approaches. Initially, three labeled acids [1,2-$^{13}C_2$]-**67b**, [1,2-$^{13}C_2$]-**68b** and [1,2-$^{13}C_2$]-**69b** were synthesized and fed to *S. nodosus*, but none was incorporated into asukamycin. Presumably these acids can not be converted into their ACP thioesters in the cell system, although 3,4-AHBA is efficiently converted into its CoA thioester under the same conditions. The next experiment therefore involved the synthesis and feeding of a labeled analog of one of the ACP thioester, the N-acetylcysteamine thioester of [1,2-$^{13}C_2$]-**84**. Unfortunately, [1,2-$^{13}C_2$]-**84** was again not incorporated into asukamycin by the bacterium. Possible reasons for this negative result include (a) that the polyketide is synthesized in a closed system and the analog of the ACP thioester has no opportunity to bind to the active site of the enzyme; or (b) that the N-acetylcysteamine thioester is not recognized by the enzyme as a true analog of the ACP thioester.

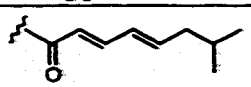
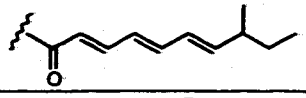
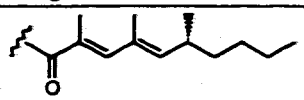
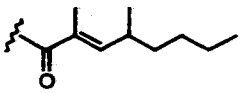
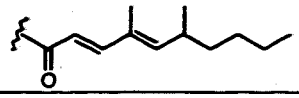
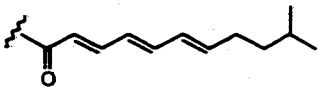
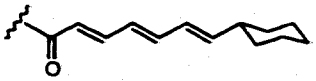
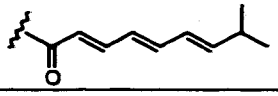
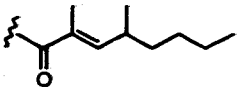
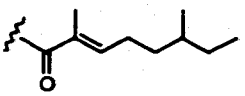
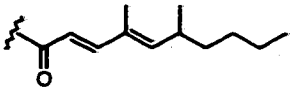
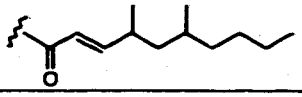
The second approach is to discover a pathway related new metabolite. In contrast to the negative results from the feeding experiments with labeled polyene acids and thioester, an important shunt metabolite **85** was discovered by tracing the enhanced ^{13}C signal of the fermentation products derived from 3,4-[7-^{13}C]-AHBA. Compound **85** is a naturally produced metabolite rather than a precursor directed product because it was isolated from non-supplemented fermentations as well, and the incorporation of 3,4-[7-^{13}C]-AHBA into **85** was determined as about 19%. The polyketide chain extension from 3,4-AHBA to **69a** occurs first, followed by acetylation of the amine group. Therefore, it has been demonstrated that the "lower" polyketide chain is synthesized before the attachment of the "upper" chain and the aromatic ring epoxidation/oxidation, as indicated in the working hypothesis proposed (Figure 27).

CHAPTER 4. THE BIOSYNTHESIS OF THE "UPPER" POLYKETIDE CHAIN

4.1 Introduction

The "upper" chain contains the most distinctive structural variations among the manumycin metabolites. It has been found that manumycin compounds are produced as a group of co-metabolites, i.e., a single strain produces more than one manumycin type metabolite (Table 4). These co-metabolites differ from each other only in the "upper" chain. The recently discovered manumycin type metabolites are all produced in such groups, e.g. manumycin E, F and G produced by *Streptomyces* sp. WB-8376 (Patel and Shu 1995; Shu *et al.*, 1994), EI-1511-3 and EI-1511-5 by *Streptomyces* sp. EI-1511 (Tanaka and Tsukuda 1995; Tanaka *et al.*, 1996a; Tanaka *et al.*, 1996b) and TMC-1A, 1B, 1C and 1D by *Streptomyces* sp. A-230 (Kohno *et al.*, 1996). The originally discovered manumycins were reported as single metabolites. Later, following extensive studies, manumycin A was found to be accompanied by minor co-metabolites, manumycin B and C (Sattler *et al.*, 1993). U-56,407 was also found to be formed along with other manumycins (Tanaka and Tsukuda 1995). Other early discovered manumycins, such as U-61,162 and compound **15**, have not been reported to be part of a group. Likewise, asukamycin, although extensively studied biosynthetically, has not been reported to have other co-metabolites.

Table 4. Manumycin Co-metabolites Produced by a Single Strain

Compound	Strain	"Upper" chain
EI-1511-3 **7**	*Streptomyces* sp. EI-1511	
EI-1511-5 **8**	*Streptomyces* sp. EI-1511	
Manumycin A **1**	*Streptomyces parvulus*	
Manumycin B **17**	*Streptomyces parvulus*	
Manumycin C **18**	*Streptomyces parvulus*	
Manumycin E **10**	*Streptomyces* sp. WB-8376	
Manumycin F **11**	*Streptomyces* sp. WB-8376	
Manumycin G **12**	*Streptomyces* sp. WB-8376	
TMC-1A **27**	*Streptomyces* sp. A-230	
TMC1B **28**	*Streptomyces* sp. A-230	
TMC-1C **29**	*Streptomyces* sp. A-230	
TMC-1D **30**	*Streptomyces* sp. A-230	

As the discovery of a series of manumycins produced by *S. parvulus* has been discussed (Sattler *et al.*, 1993), this chapter focuses on the discovery of manumycins produced by *S. nodosus*, and explores aspects of the diversity in "upper" chain biosynthesis.

4.2 Other type I Manumycins Produced by *Streptomyces nodosus*

4.2.1 Manumycin G Was Isolated from *Streptomyces nodosus*

Crude asukamycin obtained from the fermentation broth was purified by silica gel column chromatography with methanol / chloroform and further by reverse-phase HPLC with methanol / water or acetonitrile / water. HPLC purification yielded three fractions, corresponding to peaks 1, 2 and 4 in Figure 38. The fractions 1, 2 and 4 showed identical UV spectra, with an absorption maximum at 310 nm. The ^{13}C-NMR spectra of the three fractions resulting upon feeding 3,4-[7-^{13}C]-AHBA all showed the enhanced ^{13}C signal at δ_C 139 ppm. The CD spectra of the three fractions also displayed the identical chromophore with a positive Cotton effects at (+) 345 nm and a negative Cotton effects at (–) 305 nm. FAB-MS analysis demonstrated that the molecular masses of fractions 1, 2 and 4 were 506, 520 and 546, respectively. The molecular formula of each was determined by high resolution mass spectrometry. As a result, fraction 4 was identified as asukamycin. Fractions 2, 3 and 4 have major fragment ions at m/z of 149, 163 and 189, respectively (Figure 39), which correspond to the loss of mass 357 [M-357]. Fragment ion 357 represents the sum of the mC_7N unit, "lower" chain and C_5N unit. The fragment of 189 from asukamycin (fraction 4) was identified as the "upper" chain

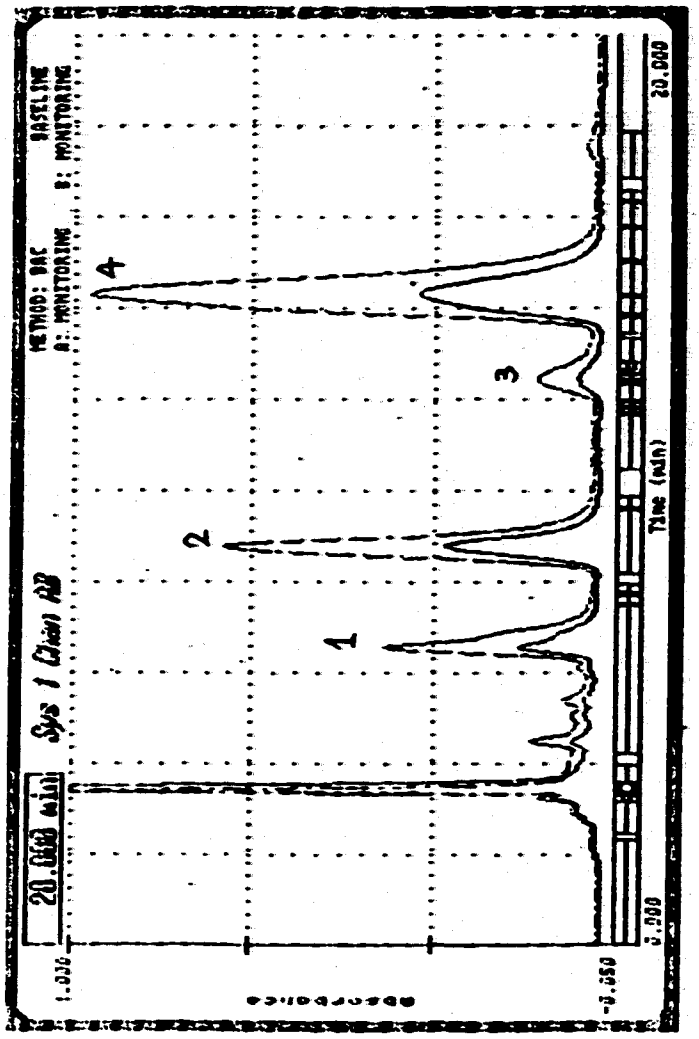


Figure 38. HPLC Chromatogram from Purification of Type I Manumycins

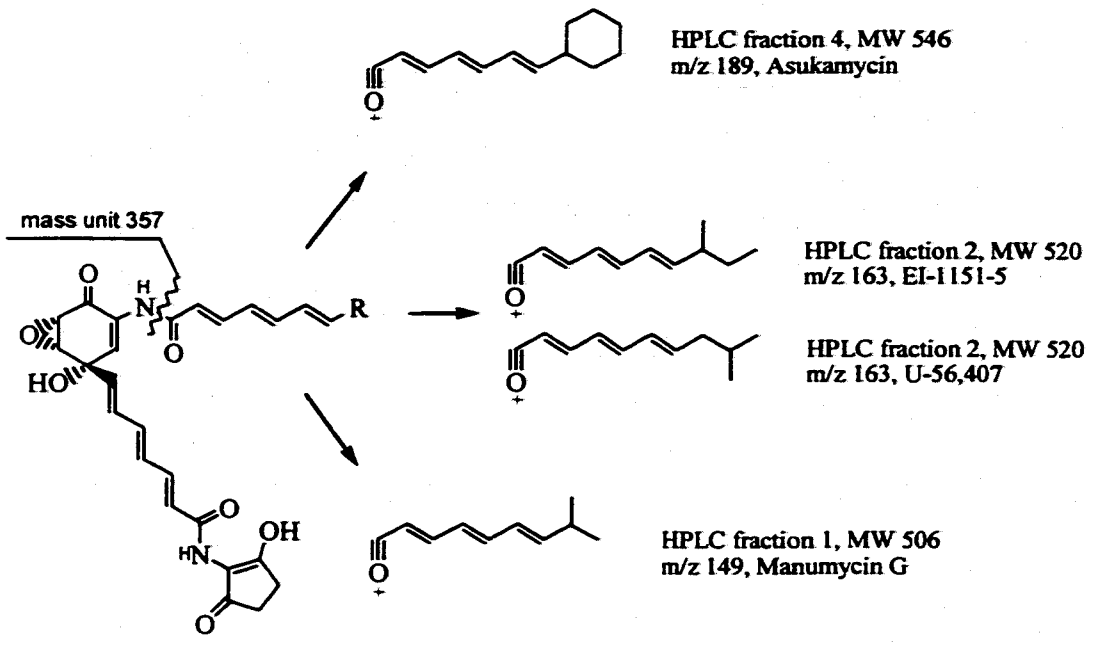


Figure 39. "Upper" Chain Fragmentation from Asukamycin and Its Co-metabolites

fragment (Shu *et al.*, 1994). Usually the "upper" chain fragment exhibits a high intensity in positive ionization mass spectrometry. Therefore, fractions 2 and 3 should have the same mC_7N and C_5N units as asukamycin, with the only difference in the "upper" chain. The ^{13}C-NMR spectrum of fraction 1 (in acetone-d_6) is identical to the published data for manumycin G (for comparison see Table 5) (Shu *et al.*, 1994). Thus, fraction 1 was identified as manumycin G.

4.2.2 U-56, 407 and EI-1511-5 Were Isolated and Identified from *S. nodosus*

Manumycin G differs from asukamycin only by utilizing a different starter unit in the "upper" chain. Therefore it was expected that fraction 2 also has the same *trans* triene in the "upper" chain, but a different starter unit. Thus its starter unit is limited to three butyl groups; n-butyl, isobutyl or anteisobutyl. U-56,407, a manumycin family compound possessing an isobutyl starter unit in its "upper" chain, was discovered in 1983. However the ^{13}C-NMR data that are reported are incomplete (Brodasky *et al.*, 1983). While this research was in progress, a new manumycin type compound, EI-1511-5, was reported having an anteisobutyl "upper" chain starter unit (Tanaka *et al.*, 1996b). The ^{13}C-NMR signals of fraction 2 were compared with those of U-56,407 and EI-1511-5, as listed in Table 6. The ^{13}C signals of fraction 2 closely matched those of EI-1511-5, despite the solvent effect caused by using different NMR solvents. However, an extra carbon signal at 23 ppm from fraction 2 could not be attributed to EI-1511-5. Upon reviewing the aliphatic range of the ^{1}H-NMR spectrum of fraction 2, it was found that there was more

Table 5. ^{13}C NMR Data Comparison of Manumycin G and Fraction 1 from *Streptomyces nodosus* (acetone-d_6)

Carbon Position	Manumycin G (ppm)	Fraction 1 (ppm)
1	189.9	189.9
2	129.2	129.2
3	128.1	128.2
4	71.9	71.9
5	57.8	57.8
6	53.4	53.4
7	139.5	139.4
8	131.4	131.4
9	140.7	140.5
10	132.2	132.3
11	143.8	143.5
12	122.9	123.1
13	167.1	166.9
1'	165.6	165.6
2'	124.1	124.1
3'	142.8	142.8
4'	129.2	129.2
5'	141.6	141.5
6'	128.2	128.2
7'	147.1	147.0
8'	32.1	32.1
9'	22.3	22.3
10'	22.3	22.3
1''	196.8[a]	-[b]
2''	115.8	115.5
3''	172.9[a]	-[b]
4''	26.0	-[b]
5''	32.9	-[b]

a). Detected in the mixed solvent of chloroform / acetone
b). Not Detectable because of coalescence phenomena

Table 6. ^{13}C NMR Data Comparison of EI-1511-5(2), U-56, 407 and Fraction 2 from *Streptomyces nodosus*

Carbon position	EI-1511-5 $CDCl_3$	Fraction 2 DMSO	U-56, 407 DMSO	Fraction 2 DMSO
1	188.6	189.5	189.6	-
2	128.2	128.5	128.2	-
3	126.3	128.1	130.1	-
4	71.3	70.7	70.8	-
5	57.5	57.3	56.6	-
6	53.0	52.4	52.6	-
7	136.3	139.2		-
8	131.7	130.0		-
9	139.6	139.5		-
10	131.8	131.3		-
11	143.6	142.0		-
12	121.6	122.5		-
13	165.4	165.9	166.1	-
1'	165.1	164.9	165.0	-
2'	121.6	123.6		-
3'	143.7	141.2		-
4'	127.5	129.0		-
5'	142.1	140.4		-
6'	128.2	123.3		-
7'	146.7	144.9	138.3	-
8'	38.8	38.9	41.8	-
9'	29.5	28.9	28.0	-
10'	11.7	11.7	22.3	23.0
11'	19.7	19.9	22.3	23.0
1"	197.3	-[a]	198.0	-
2"	114.9	114.7	115.0	-
3"	173.8	-[a]	175.8	-
4"	25.7	-[a]	25.5	-
5"	32.2	-[a]	32.2	-

a). Not Detectable because of coalescence phenomena

than one compound in fraction 2. Proton decoupling experiments established that two sets of alkyl protons could be attributed to an isobutyl and an anteisobutyl group (Figure 40). Hence it is clear that fraction 2 contains two manumycin type compounds, U-56,407 and EI-1511-5. The ratio between the two compounds was determined by ^{1}H-NMR as 1 / 3.7 in favor of EI-1511-5. This also provided a rationale for the extra ^{13}C signal at 23.0 ppm of fraction 2, which belongs to the two *geminal* methyl groups of U-56,407. Now it can be concluded that the type I manumycins produced by *Streptomyces nodosus* and purified by HPLC are manumycin G (MW 506, fraction 1), U-56,407 (MW 520, fraction 2), EI-1511-5 (MW 520, fraction 2) and asukamycin (MW 546, fraction 4).

4.3 The Fatty Acid Composition in the Membrane of *S. nodosus*

4.3.1 The Fatty Acid Composition

The discovery of manumycin G, U-56,407 and EI-1511-5 as co-metabolites of asukamycin indicated that the "upper" chain variations occur solely at the terminus. The "upper" polyketide chain is initiated by the CoA thioester of cyclohexanecarboxylic acid or of branched chain acids such as isobutyric acid, 3-methylbutyric acid and 2-methylbutyric acid. These branched chain acids are known starter units for branched chain fatty acid synthesis. It is probable that these branched chain acids serve as starter units for the synthesis of both fatty acids and the "upper" polyketide chain. Analysis of the fatty acid composition in the membrane of *S. nodosus* may explain the diversity of the "upper" chain of manumycins.

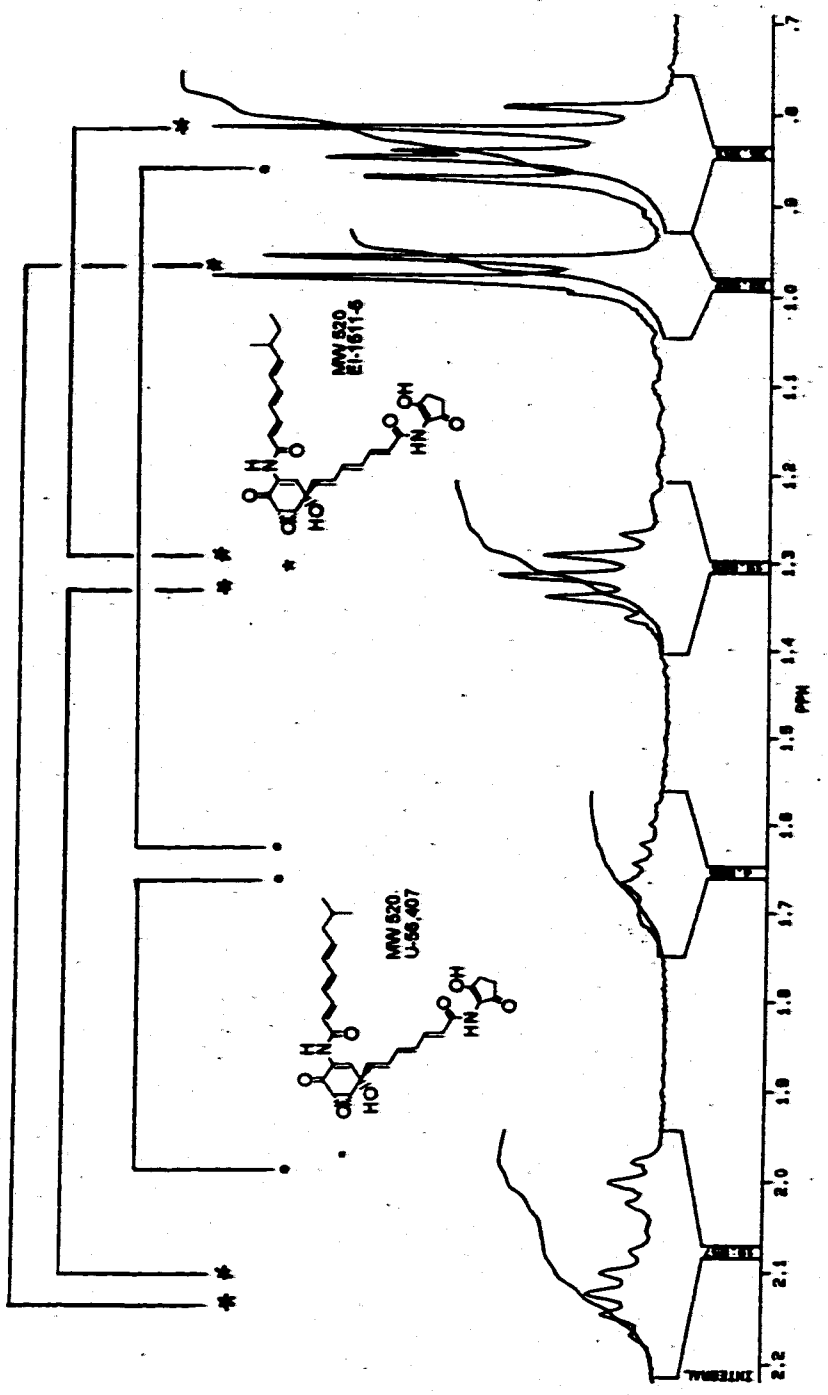


Figure 40. ^{1}H-NMR Spectrum of HPLC Fraction 2 (U-56,407 and EI-1511-5)
Produced by *S. nodosus*

Fatty acids were obtained from the mycelium of *S. nodosus* by saponification and extraction, followed by methylation using diazomethane and analyzed by GC-MS (Table 7). C_{14} through C_{18} fatty acids were found in the membrane of *S. nodosus*, mainly consisting of the C_{15} to C_{17} acids. The analysis demonstrated that the branched chain fatty acids account for over 80% of the total amount. Besides the straight chain fatty acids, all types of the branched chain fatty acids, including *iso*-C_{2n}, *iso*-C_{2n+1} and *anteiso*-C_{2n+1}, were found in the membranes. Surprisingly, about 3.4% of ω-cyclohexylundecanoic acid (C_{17}) was also found.

4.3.2 The Origin of the Fatty Acid Starter Units

It is well known that the synthesis of common branched chain fatty acids, *iso*-C_{2n}, *iso*-C_{2n+1}, and *anteiso*-C_{2n+1}, starts from isobutyryl-CoA, 2-methylbutyryl-CoA and 3-methylbutyryl-CoA, respectively, followed by repeated cycles of addition of two carbon units from malonyl-CoA (Kaneda 1977; Massey *et al.*, 1976; Okazaki *et al.*, 1973; Rezanka *et al.*, 1991; Suutari and Laakso 1992; Wallace *et al.*, 1995). Subsequent reduction, dehydration and hydrogenation provide long chain saturated fatty acids. The starter units of the branched chain fatty acids are derived from branched chain amino acids such as valine, leucine and isoleucine *via* transamination and oxidative decarboxylation (Figure 41).

Table 7. Fatty Acid Composition of *Streptomyces nodosus*

Fatty acid	Trial 1 (%)	Trial 2 (%)	Average (%)
iso-C_{14}	4.9	2.6	3.8
normal-C_{14}	0.8	1.1	1.0
iso-C_{15}	6.6	6.8	6.7
anteiso-C_{15}	13.2	14.2	13.7
normal-C_{15}	1.6	1.4	1.5
iso-C_{16}	29.1	30.9	30.0
normal-C_{16}	12.9	10.2	11.5
iso-C_{17}	5.3	5.9	5.6
anteiso-C_{17}	18.7	19.7	19.2
normal-C_{17}	1.7	1.4	1.6
cyclohexyl-C_{17}	2.6	4.3	3.4
iso-C_{18}	1.8	0.8	1.3
normal-C_{18}	0.7	0.7	0.7

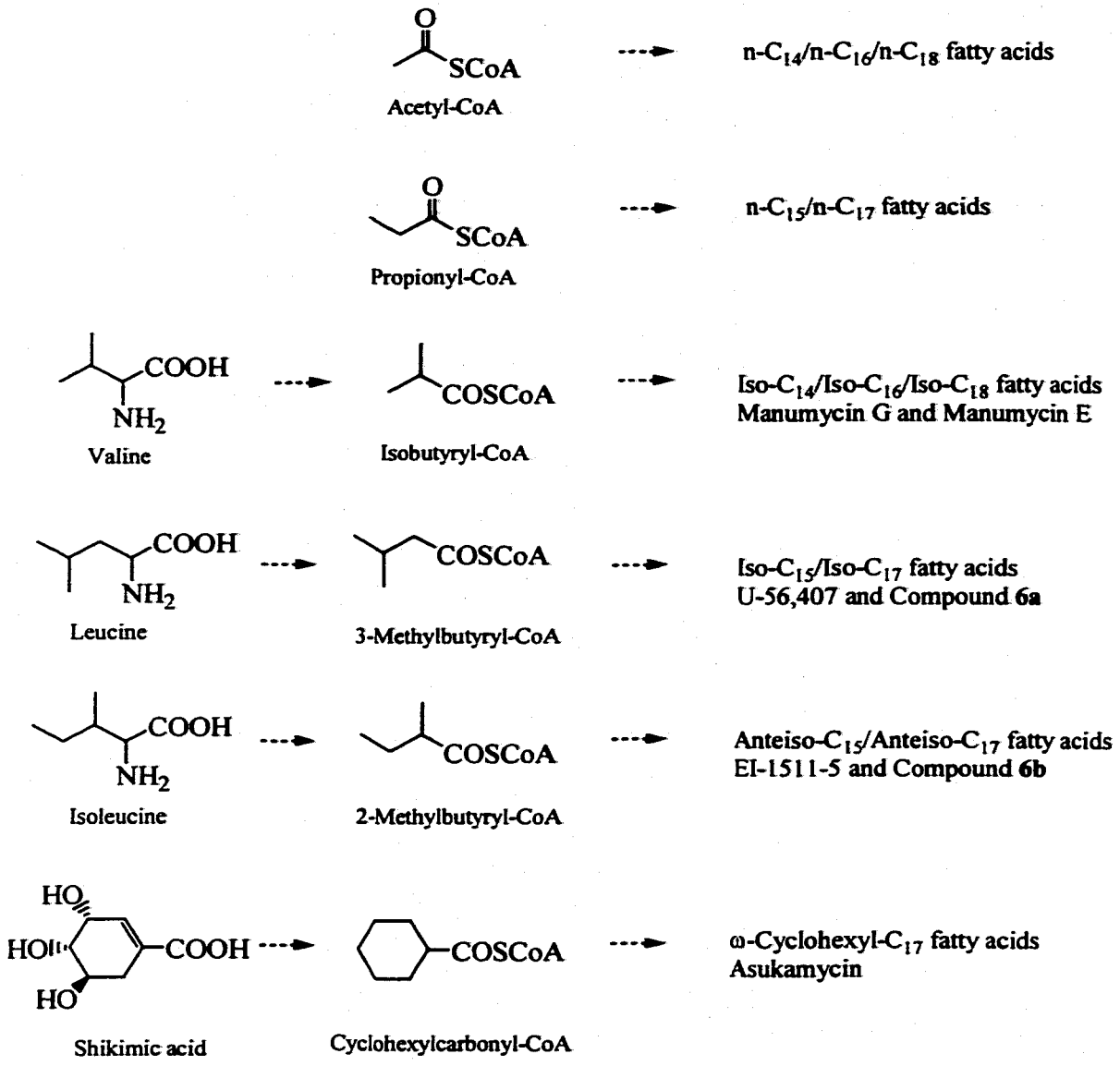


Figure 41. Starter Units of Fatty Acids and the Manumycin "Upper Chain" in *Streptomyces nodosus*

It is known that the even numbered straight chain fatty acids (C_{14}, C_{16} and C_{18}) utilize acetyl-CoA as a starter unit. The odd numbered straight chain fatty acids (C_{15} and C_{17}) originate from propionyl-CoA. The even numbered *iso*-branched fatty acids (C_{14}, C_{16} and C_{18}) are derived from valine *via* isobutyryl-CoA, whereas the odd numbered *iso*-branched fatty acids (C_{15} and C_{17}) are formed from leucine *via* 3-methylbutyryl-CoA. The *anteiso*- odd number fatty acids (C_{15} and C_{17}) come from isoleucine *via* 2-methylbutyryl-CoA. The C_{17} ω-cyclohexylundecanoic acid is derived from shikimic acid *via* cyclohexylcarbonyl-CoA.

4.4 Manumycin E and New Manumycin Type Metabolites **6a** and **6b** from *S. nodosus*

In the purification of asukamycin, manumycin G, U-56,407 and EI-1511-5, minor components in HPLC peak 3 (Figure 38) were observed, having the same UV absorption characteristics as known manumycins. Manumycin G differs from U-56, 407 and EI-1511-5 by one carbon atom (methylene group), whereas U-56, 407 and EI-1511-5 differ from asukamycin by two carbon units. The minor manumycin type compounds observed by UV may differ from known members of the group by virtue of extra methylenes in the "upper" polyketide chain. As it was difficult to acquire sufficient material of the minor components for NMR analysis, LC-MS was used as a tool to identify new manumycin type metabolites. As a result, more manumycins were detected in addition to the known type I manumycin compounds from *S. nodosus* with molecular masses of 534 and 548. MS/MS spectra of the two compounds revealed that their structural variation was in the

"upper" chain, with fragment masses of 177 and 191, respectively. The former was assigned to the known type I manumycin class compound, manumycin E, because its "upper" chain has an even number (C_6) branched chain starter unit, which is derived from valine *via* isobutyryl-CoA followed by the addition of an acetate unit.

No manumycins known to date have a molecular mass of 548. Comparing the MS/MS spectrum of this compound with that of asukamycin (Figure 42 and 43), the "upper" chain of the new compound has two more mass units than asukamycin. This suggests that the "upper" chain of the new compound has a saturated and non-cyclic starter unit, most likely a methyl branched odd numbered (C_7) starter unit. Both odd numbered starter units, *iso-* and *anteiso-*, have been demonstrated in U-56,407 and EI-1511-5. Therefore, it is speculated that m/z 548 consists of two structurally isomeric manumycin type compounds **6a** and **6b** of identical molecular mass. Their "upper" chain starter units are derived from leucine and isoleucine *via* 3-methylbutyryl- and 2-methylbutyryl-CoA followed by the addition of an acetate unit. A complete list of type I manumycins discovered in *S. nodosus* is shown in Figure 44.

4.5 Fatty Acids and "Upper" Chains Share the Same Starter Units in *S. nodosus*

4.5.1 Starter Units of "Upper" Chains in Asukamycin Producer

S. nodosus seems to produce a series of manumycin type metabolites, including

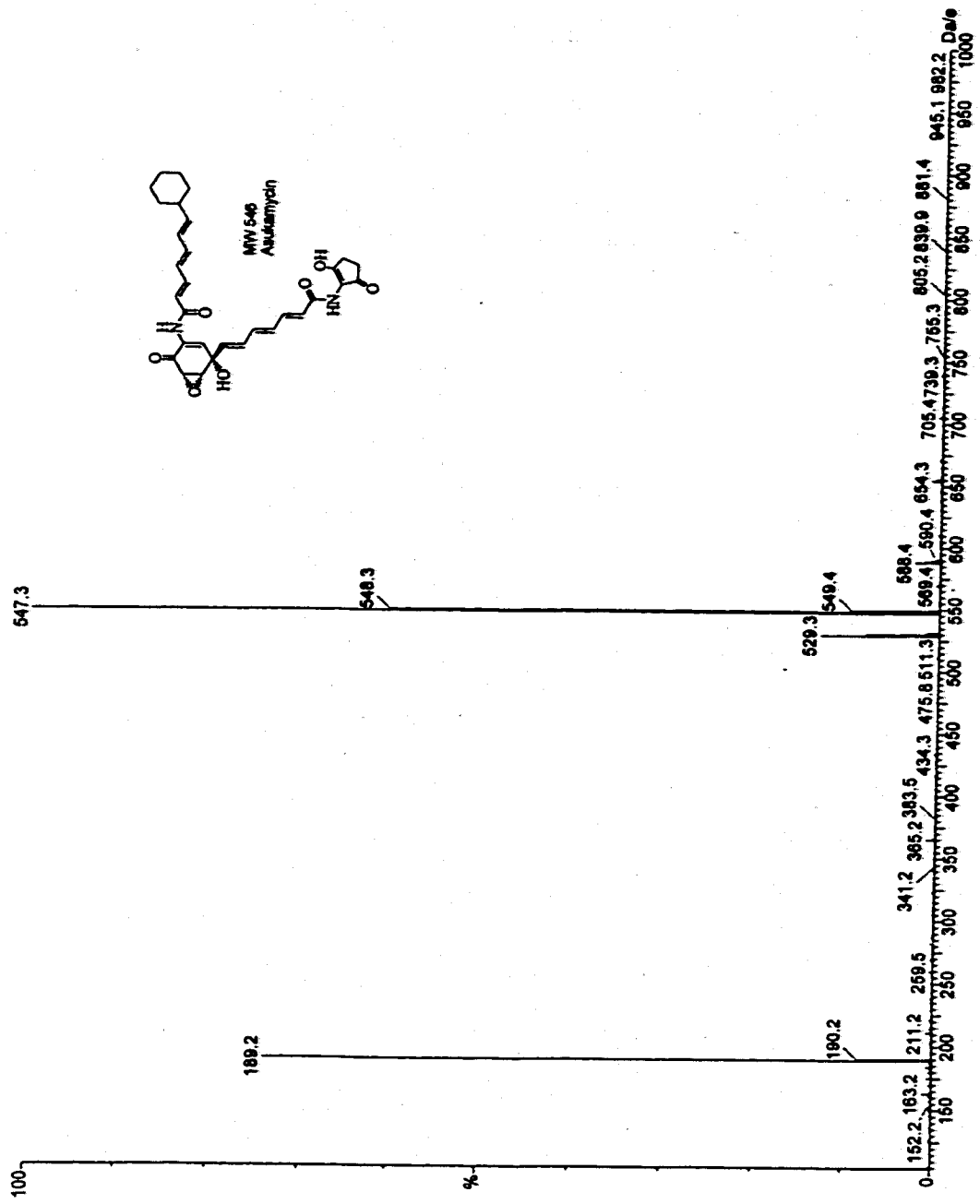


Figure 42. ES-MS/MS Spectrum of Asukamycin

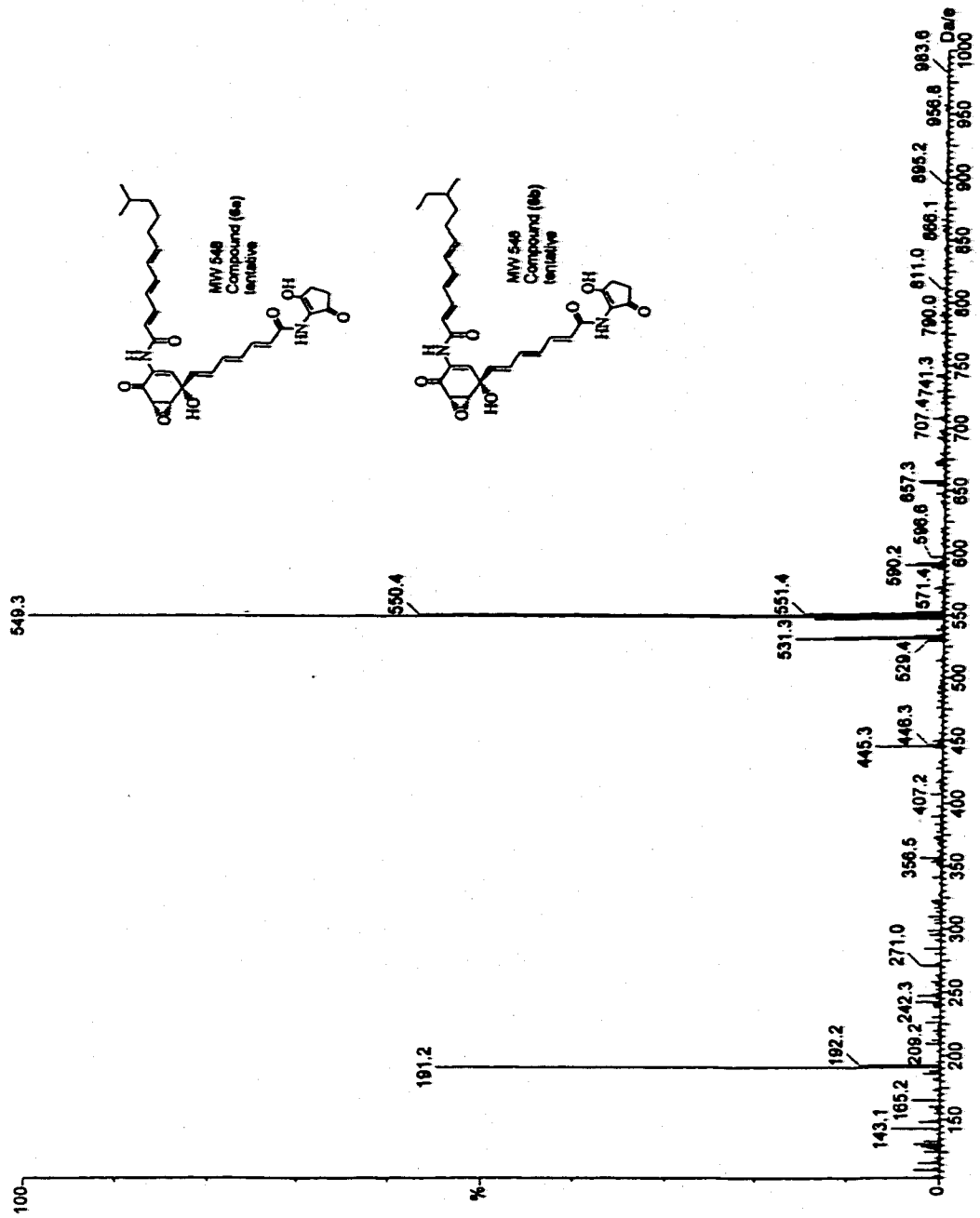
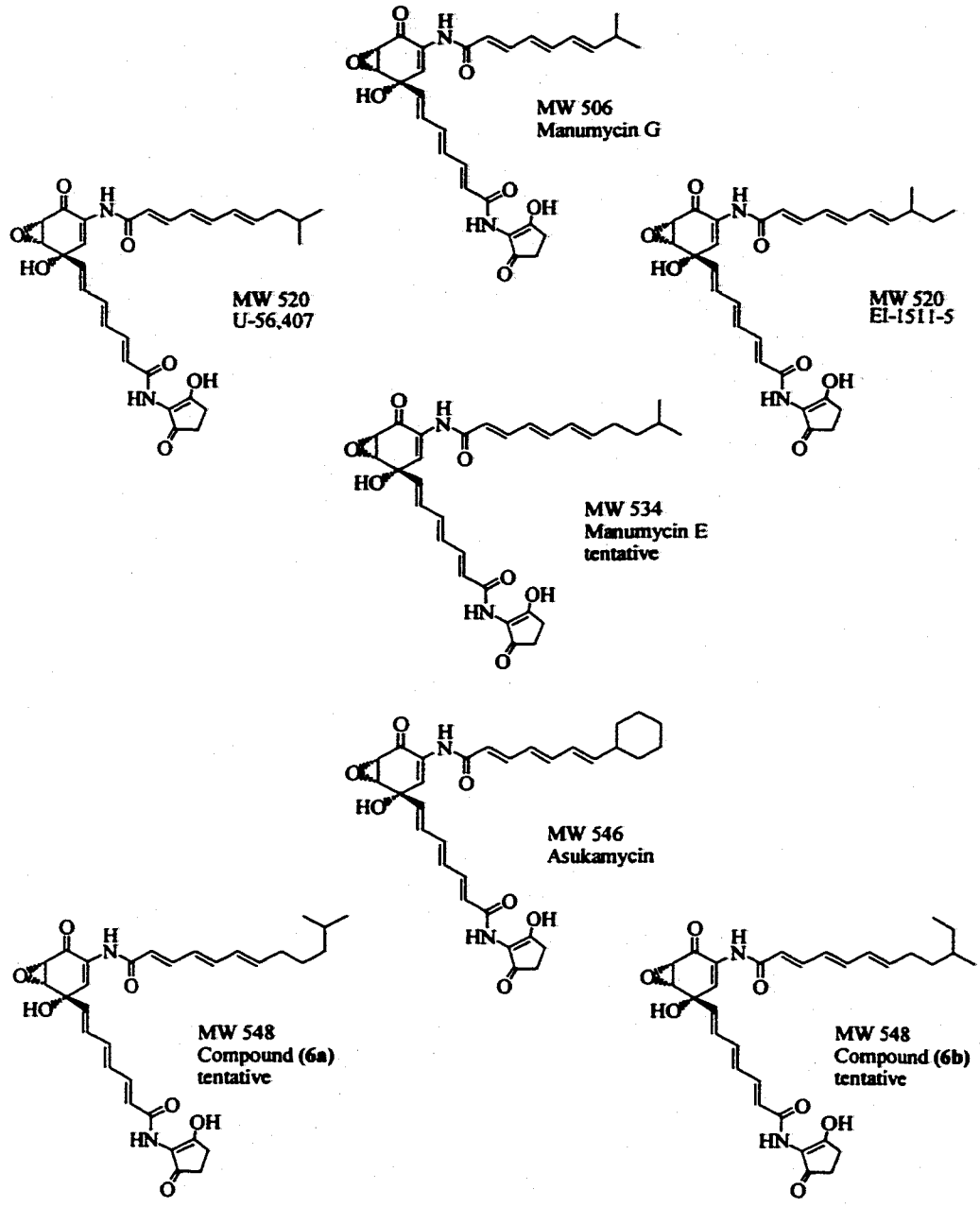


Figure 43. ES-MS/MS Spectrum of New Type I Manumycins **6a** and **6b**



Scheme 31. Type I Manumycin Class Antibiotics Identified from *Streptomyces nodosus*

asukamycin, manumycin G, US-56,407, EI-1511-5, manumycin E and compound **6a** and **6b**. The relative amounts of these compounds were determined by ion-trap mass spectrometry, as listed in Table 8. Asukamycin accounts for less than half of the total amount of manumycins produced, indicating that in the biosynthesis of the "upper" polyketide chain the acyltransferase efficiently accepts various branched chain acids as starter units besides cyclohexanecarboxylic acid.

4.5.2 Fatty Acid and "Upper Chain" Share the Same Starter Units

Cyclohexyl ring and methyl branched starter units were found both in the "upper" chains of manumycins and in fatty acids produced by *S. nodosus*. This indicates that fatty acids and "upper" chains share the same starter units. The starter unit of cyclohexyl fatty acids, cyclohexanecarboxylic acid **(39)**, originates in the secondary metabolic asukamycin biosynthetic pathway. The starter units of manumycin G, E, U-56,407, EI-1511-5, compound **6a** and **6b** are generated in primary metabolism. The cross utilization of branched chain and cyclic acids in manumycin and fatty acid synthesis establishes a connection between the biosynthesis of primary and secondary metabolites.

The consumption of branched and cyclic acids in the synthesis of primary and secondary metabolites is competitive. This relationship was studied quantitatively (Table 9). The amounts of branched chain fatty acids and manumycins produced by *S. nodosus* were correlated. For the manumycins and fatty acids derived from the same starter unit, a high

Table 8. Relative Percentages of Manumycins Produced by *S. nodosus*

Manumycins	%[a]
Manumycin G	10.1
U56, 407	6.9[b]
EI-1511-5	25.4[b]
Manumycin E	11.8
Asukamycin	45.8

a: Percentage was determined by ion-trap MS
b: U56,407 and EI-1511-5 total 32.3%. The ratio of the two was determined by ^{1}H-NMR

Table 9. Biosynthetic Origins for Manumycin "Upper" Chain and Fatty Acids Produced by *Streptomyces nodosus*

Source	Fatty acid	Fatty acid (%)	Antibiotic	Manumycins (%)
Acetate	$n_{(2n)}$	13.2	no	-
Propionate	$n_{(2n+1)}$	3.1	no	-
Valine	$iso_{(2n)}$	35.1	Manumycin G Manumycin E	21.9
Leucine	$iso_{(2n+1)}$	12.3	U-56, 407 Compound **6a**	6.9
Isoleucine	$antiiso_{(2n+1)}$	32.9	EI-1511-5 Compound **6b**	25.4
Shikimic acid	$cyclohexyl_{(2n+1)}$	3.4	Asukamycin	45.8

percentage of that fatty acid in the membrane correlated to a high percentage of the relevant manumycin produced in the fermentation. For example, valine and isoleucine derived fatty acids were present in comparable amounts of 33-35%, as opposed to the production of manumycin G and E *vs.* EI-1511-5 and Compound **6b** (22-25%). Leucine derived fatty acids were present at 12%, whereas the corresponding U-56,407 and Compound **6a** accounted for 7% of the total amount of type I manumycins from *S. nodosus*. Although fatty acids derived from the straight chain starter units acetate and propionate comprised 16% of the total amount of fatty acids in the membrane, no corresponding straight chain starter units were found in the manumycin "upper" chain. It is known that *Streptomycetes* produce predominantly branched chain and a small proportion of straight chain fatty acids (Saddler *et al.*, 1987; Suutari and Laakso 1992; Vancura *et al.*, 1987; Wallace *et al.*, 1995). Genetic studies showed that type II fatty acid synthase (FAS) operates in the fatty acid biosynthesis in *Streptomycetes* (Revill and Leadlay 1991; Summers *et al.*, 1995). It was also found that the FabH gene, encoding β-ketoacyl-acyl carrier protein (β-ketoacyl-ACP) synthase (KAS III), is responsible for initiating both straight and branched chain fatty acid biosynthesis in *Streptomyces glaucescens* (Han *et al.*, 1998). Furthermore, KAS III prefers to utilize branched chain acids rather than straight chain acids as starter units. The addition of thiolactomycin, an inhibitor of FabH, reduced the percentage of branched chain fatty acids but increased that of straight chain fatty acids, indicating a FabH-independent pathways for initiation of straight chain fatty acid biosynthesis (Han *et al.*, 1998; Wallace *et al.*, 1997). One

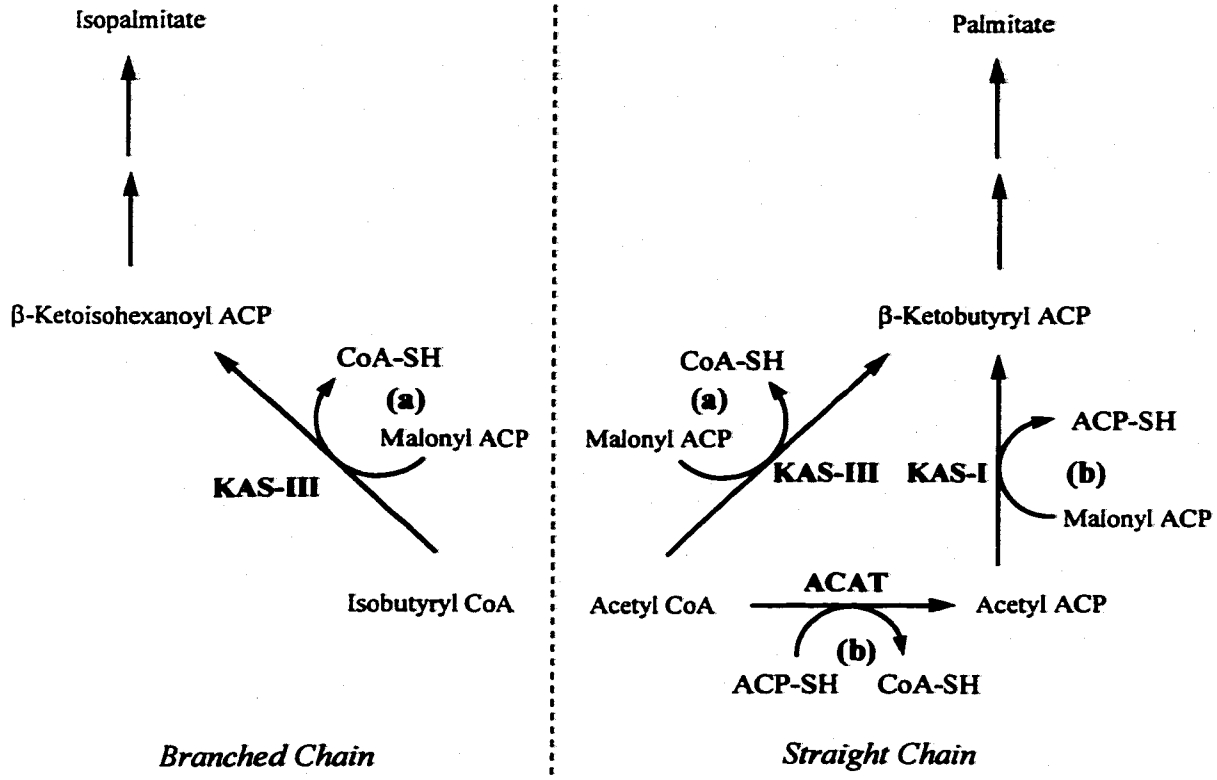


Figure 45. Potential Pathways for Initiation of Fatty Acid Biosynthesis in *Streptomycetes* (a) FabH-dependent Pathway; (b) FabH-independent Pathway

possible explanation is that there are two different initiation pathways for straight chain fatty acids synthesis in *Streptomycetes* (Figure 45). In route (a), 3-ketoacyl synthase III (KAS III) catalyzes the condensation of acetyl-CoA with malonyl-ACP to form β-ketobutyryl-ACP. In route (b), acetyl-ACP is transacylated to acetyl-CoA catalyzed by acyl-CoA/ACP transacylase (ACAT), and then condensed with malonyl-ACP catalyzed by 3-ketoacyl synthase I (KAS I). Accordingly, branched chain and straight chain fatty acids are synthesized by different FAS's in *Streptomyces nodosus*, but the FAS which mainly produces branched chain fatty acids is predominant. In contrast, the "upper" chain polyketide synthase (PKS) has a strong preference for cyclohexanecarboxylic acid **(39)** as its normal starter unit; it can replace **39** with branched chain acids, but not with straight chain acids. Approximately 3% of ω-cyclohexylundecanoic acid derived from acid **39** was detected among the fatty acids, compared to asukamycin representing almost half of the manumycins produced. This suggests that cyclohexanecarboxylic acid is a specific substrate for the asukamycin "upper" chain biosynthesis. Therefore, the diversity of the manumycin "upper" chain is the result of the diversity of the starter units in fatty acid biosynthesis and of an insufficient supply of cyclohexanecarboxylic acid. This suggests that the biosynthesis of cyclohexanecarboxylic acid is the limiting step for the overall biosynthesis of asukamycin. The formation of shunt metabolite **85** also supports this notion.

4.6 The Biosynthesis of an Artificial Manumycin and Fatty Acid, a Proof that Fatty Acids and Manumycin "Upper" Chains Share the Same Starter Units

The hypothesis that the "upper" chain and fatty acids share the same starter units can be tested by feeding an artificial branched or cyclic acid to observe if that acid is incorporated into both the fatty acids and manumycins. Cyclopentanecarboxylic acid **(93)** was chosen for this purpose, as it is not produced by *S. nodosus*.

4.6.1 The Fatty Acid Composition in the Membrane of *S. nodosus* upon Feeding of [1-^{13}C]-**93**

[1-^{13}C]Cyclopentanecarboxylic acid **(93)** was synthesized from bromocyclopentane *via* a Grignard reaction with ^{13}C-labeled carbon dioxide. [1-^{13}C]-**93** was neutralized with aqueous sodium bicarbonate and administed to the culture. The resulting fatty acid composition of *S. nodosus* is shown in Table 10. Fatty acids range from C_{14} to C_{18}. Two ω-cyclopentyl fatty acids were detected and identified as ω-cyclopentylundecanoic acid (C_{16}) and ω-cyclopentyltridecanoic acid (C_{18}). These two artificial fatty acids accounted for about 35% of the total amount. The naturally produced ω-cyclohexylundecanoic acid was decreased to 0.7%, from 3.4% without feeding. This demonstrates that the fatty acid synthase is able to accept branched chain or medium sized cyclic acids as starter unit, and that the fatty acid composition can be greatly affected by the feeding of external acids.

4.6.2 Directed Biosynthesis of a New Manumycin by Feeding of [6-^{13}C]cyclopentanecarboxylic Acid

Table 10. Fatty Acid Composition of *Streptomyces nodosus* After [6-^{13}C]Cyclopentanecarboxylic Acid Feeding

Fatty acid	(%)
iso-C_{14}	2.7
normal-C_{14}	0.4
iso-C_{15}	5.3
anteiso-C_{15}	11.4
normal-C_{15}	0.8
iso-C_{16}	20.9
normal-C_{16}	9.1
cyclopentyl-C_{16}	18.2
iso-C_{17}	3.2
anteiso-C_{17}	11.0
normal-C_{17}	trace
cyclohexyl-C_{17}	0.7
iso-C_{18}	trace
normal-C_{18}	-
cyclopentyl-C_{18}	16.3

The incorporation of [6-^{13}C]-**93** into a manumycin type metabolite was observed by ^{13}C-NMR and mass spectrometry. An artificial manumycin, compound **7**, was produced. The relative amounts of the manumycins produced by *S. nodosus* in this experiment were determined from an ion-trap mass spectrum (Table 11). This showed that the new metabolite accounted for 72% of the total amount of manumycins produced. The relative amount of asukamycin was reduced to 23%, about half of that in a control experiment without feeding. The total amount of other manumycins was reduced to 6% from 54%.

4.6.3 Proof that Fatty Acid and "Upper" Chain Share the Same Starter Unit

The correlated production of manumycins and fatty acids upon feeding [1-^{13}C]-**93** was studied (Table 12, Figure 46). High incorporation of the fed acid into both the fatty acids and compound **7** was observed. The manumycins derived from branched chain acids were greatly suppressed, although the corresponding fatty acids were present in considerable amounts. This indicates that the "upper" chain polyketide synthase is more likely to accept medium ring size cyclic acids than branched chain acids, compared to the fatty acid synthase. Although only 0.7% of ω-cyclohexylundecanoic acid was found in the membrane, as much as 23% of the corresponding asukamycin was produced. This indicates that cyclohexanecarboxylic acid is produced and channeled preferentially into asukamycin biosynthesis.

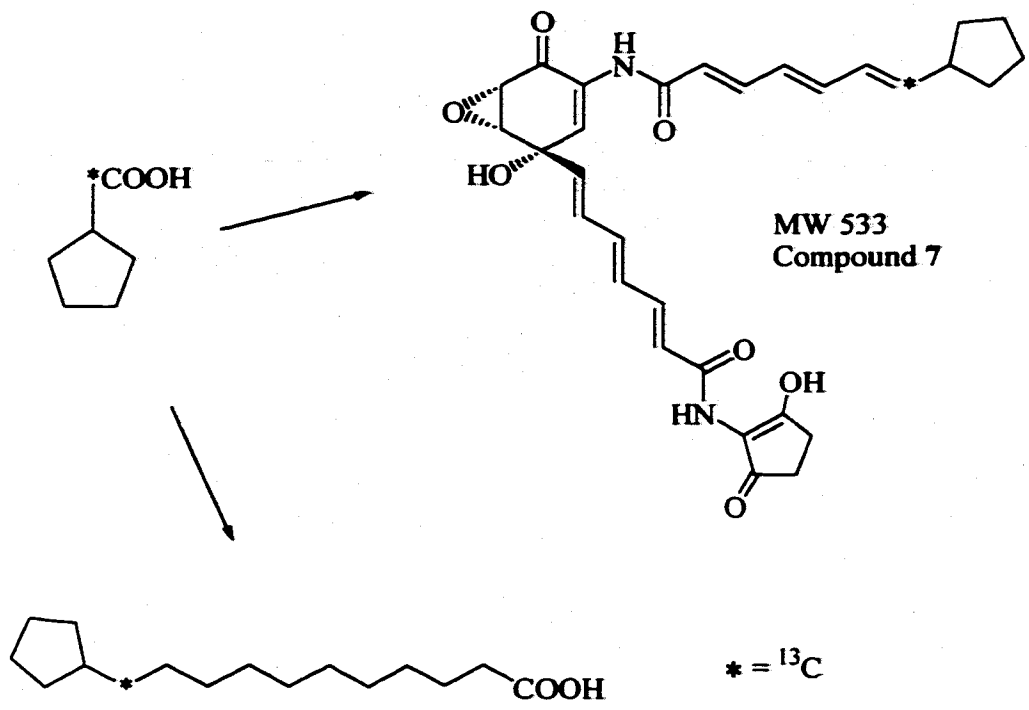
Table 11. Manumycins Produced by *Streptomyces nodosus* After [6-^{13}C]Cyclopentanecarboxylic Acid Feeding

Manumycins	%[a]
Manumycin G	1
U56, 407	1[b]
EI-1511-5	3[b]
Compound **7**	72[c]
Manumycin E	1[c]
Asukamycin	23

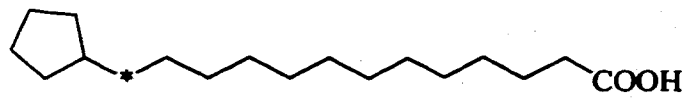
a: Percentage was determined by ion-trap MS
b: U56,407 and EI-1511-5 total 3.7%. The ratio of the two was determined by ^{1}H-NMR
c: Compound **7** and manumycin E total 73%, the amount of manumycin E was determined by its percentage in a non-feeding samples (see Table 9)

Table 12. Biosynthetic Origins of Manumycin "Upper" Chain and Fatty Acids Produced by *Streptomyces nodosus* After [6-^{13}C]Cyclopentanecarboxylic Acid Feeding

Source	Fatty acid	Fatty acid (%)	Manumycins	Manumycins (%)
Acetate	$n_{(2n)}$	9.5	None	-
Propionate	$n_{(2n+1)}$	0.8	None	-
Valine	$iso_{(2n)}$	23.6	Manumycin G Manumycin E	~2
Leucine	$iso_{(2n+1)}$	8.5	U56, 407 Compound **6a**	~1
Isoleucine	$antiiso_{(2n+1)}$	22.4	EI-1511-5 Compound **6b**	~3
Shikimic acid	$cyclohexyl_{(2n+1)}$	0.7	Asukamycin	23
[6-^{13}C]Cyclo-pentane-carboxylic acid	$Cyclopentyl_{(2n)}$	34.5	Compound **7**	72



ω-Cyclopentylundecanoic acid



ω-Cyclopentyltridecanoic acid

Figure 46. Incorporation of [6-^{13}C]Cyclopentanecarboxylic Acid into Manumycin Type Metabolite and Fatty Acids

This feeding experiment showed that the addition of an exogenous acid could generate new artificial antibiotics by directed biosynthesis. The addition of a natural external acid may enhance the production of one of the natural products and eliminate others. For example, if cyclohexanecarboxylic acid were fed to *S. nodosus*, the production of manumycins other than asukamycin should be very much reduced, thus resulting in asukamycin as the major or only product.

4.7 Differences in the "Upper" Chain between Type IA and Type IB Manumycins

The major differences between type IA and type IB manumycins are variations in the starter unit and "upper" chain polyene structure.

In type IA manumycins the starter unit varies. The ACP acyltransferase involved in their synthesis accepts various cyclic acids and branched acids. The ketosynthase may only use malonyl-CoA as building block to generate a *trans* conjugated polyene. Type IB manumycins demonstrate differences in the "upper" polyene chain. The acyltransferase exclusively utilizes butyryl-CoA (or one acetyl-CoA followed by chain extension of one acetate unit) as starter unit, but the subsequent polyketide synthesis is irregular. The ketosynthase may accept methylmalonyl-CoA, as well as malonyl-CoA as polyene building block.

The same applies to type IIA and IIB manumycins. In addition, the type IIB manumycins TMC-1B and TMC-1D contain a saturated propionate unit in the polyketide chain, implying the existence of a functioning enoyl reductase. Overall, the type IB / IIB polyketide synthase (PKS) shows more flexibility in the polyketide extension process, but not in the starter units. It is likely that the type IA / IIA and type IB / IIB manumycins are produced by different types of polyketide synthases.

CHAPTER 5. THE BIOSYNTHESIS OF THE TYPE II MANUMYCINS

5.1 Introduction

Compounds of the manumycin family are defined as type I or type II by virtue of the structure of their mC_7N unit. 17 Type I manumycins have been discovered from 10 *Streptomycetes* and 6 type II manumycins from 3 *Streptomycetes* in which both type I and type II manumycins are produced. No type II manumycin has been isolated from an organism that does not also produce the corresponding type I manumycin. Type I and type II manumycins share some pharmacological properties, such as anticancer activity (Hara *et al.*, 1993; Hara and Han 1995; Ito *et al.*, 1996; Kainuma *et al.*, 1997; Kohno *et al.*, 1996; Nagase *et al.*, 1996; Nagase *et al.*, 1997; Zou *et al.*, 1996). But they may have different biological activities as well. For example, the type I manumycins have antibacterial activity but the type II manumycins do not (Kohno *et al.*, 1996). These findings revealed the biological importance of the mC_7N unit and highlighted the necessity to investigate the biosynthesis of type II manumycins. No type II manumycins have been previously reported in the *Streptomyces nodosus* fermentation.

All type II manumycins are found to co-exist with the type I manumycins, indicating a close biosynthetic relationship between type I and type II compounds. In some cases, the corresponding type I and type II manumycins that have identical carbon skeletons are produced by a single strain, e.g. manumycin A (type I) and D (type II) are produced by

Streptomyces parvulus and *Streptomyces* sp. A-230 (Kohno *et al.*, 1996; Sattler *et al.*, 1993; Schroder and Zeeck 1973), manumycin C and TMC-1C by *Streptomyces* sp. A-230 (Scheme 32) (Kohno *et al.*, 1996). Colabomycin A and D are produced by *Streptomyces griseoflavus*, even though the configurational assignment at the terminal double bond of the "upper" chain is different (Grote *et al.*, 1988a; Grote *et al.*, 1988b; Sattler *et al.*, 1998). Manumycin B and TMC-1A are type I and type II counterparts discovered in different *Streptomyctes* (Figure 47).

A series of type II manumycin compounds produced by *S. nodosus* have been discovered in this thesis work. These type II manumycins are counterparts of the type I manumycins discussed in Chapter 4. It is believed that type II manumycins are formed from type I manumycins *via* reduction.

5.2 New Type II Manumycins Discovered in *S. nodosus*

In the fermentation of *S. nodosus* supplemented with 3,4-[7-^{13}C]-AHBA **50**, ^{13}C-NMR was used to monitor the course of antibiotic production. It was observed that an enhanced ^{13}C signal appeared at 141 ppm (in acetone-d_6) besides the enhanced ^{13}C signals at 137 ppm and 139 ppm belonging to the shunt metabolite **85** and the type I manumycins (including asukamycin), respectively (Figure 48). The compounds having this new signal were traced in an attempt to discover new metabolites related to the manumycin biosynthetic pathway.

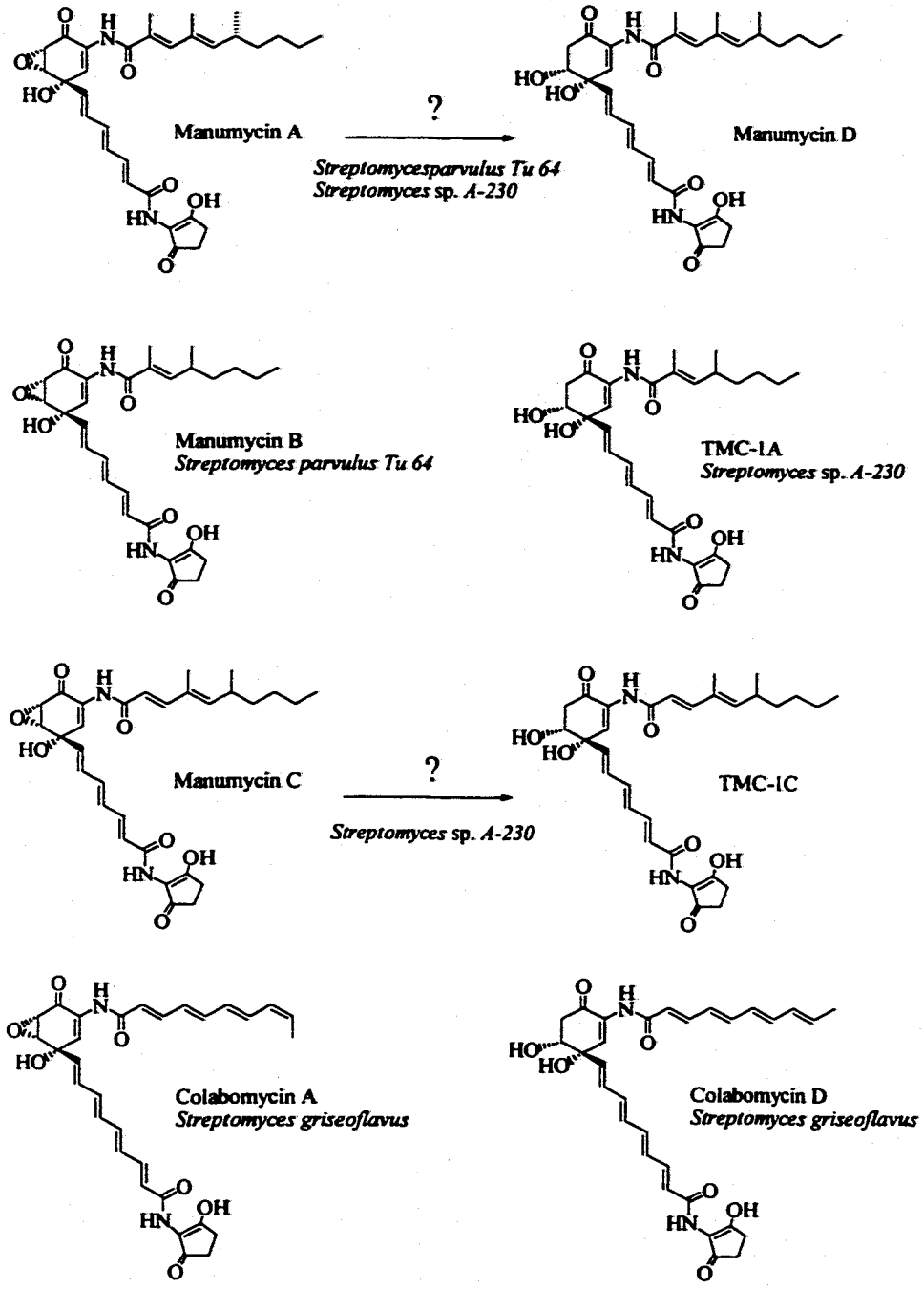


Figure 47. Type I and Type II Manumycin Co-metabolites

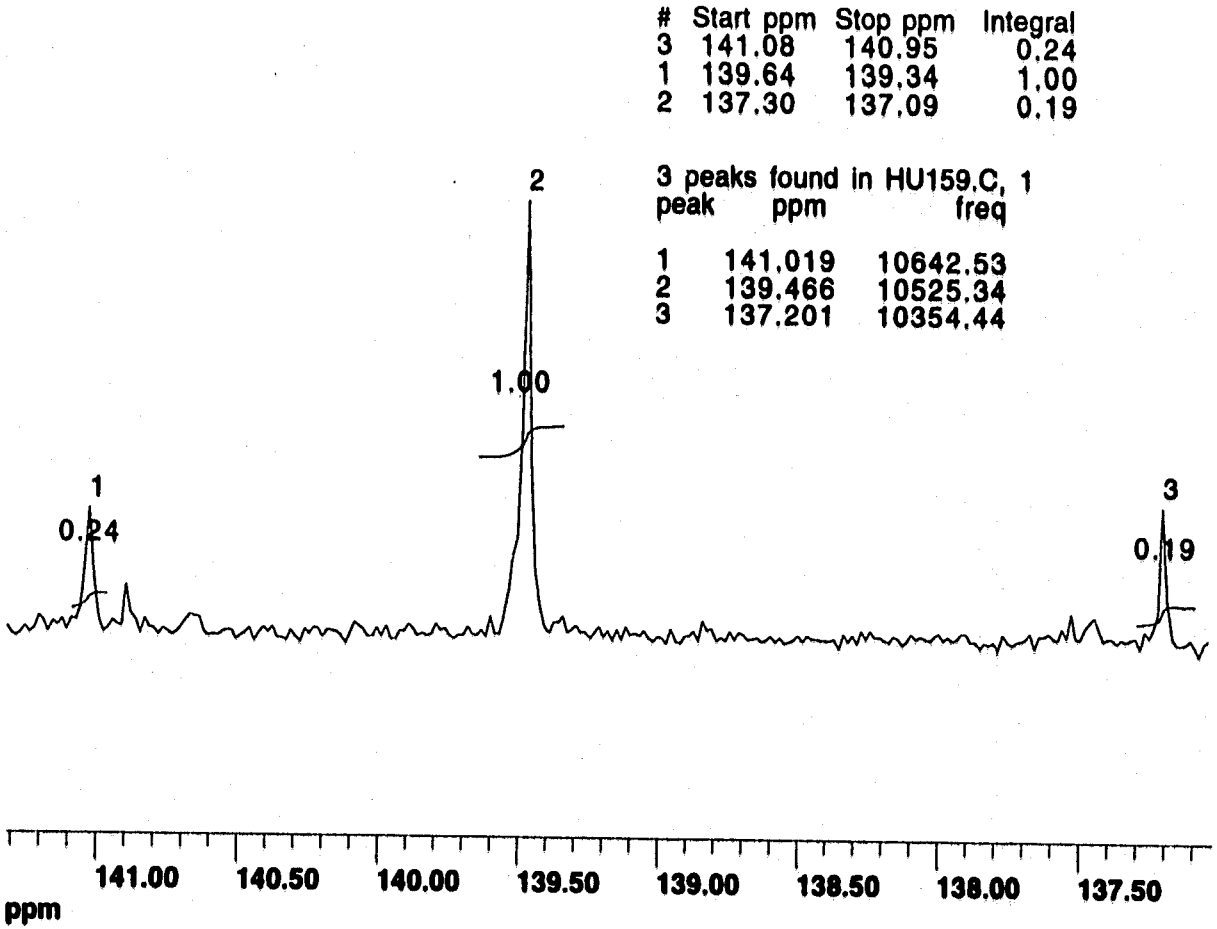


Figure 48. Enhanced ^{13}C Signals of Shunt Metabolite, Type I and Type II Manumycins After Feeding [7-^{13}C]-**50**

The crude fermentation extract was purified by column chromatography (methanol / methylene chloride), and further purified on reverse-phase HPLC using gradient elution with acetonitrile / water. Four small fractions were collected and ^{13}C-NMR spectroscopy was used to trace the new compounds. ^{1}H-NMR, LC-MS, LC-MS/MS and high resolution mass spectra were used to elucidate the structures of these new compounds.

^{13}C-NMR spectroscopy of the HPLC fractions 1 – 4 (Figure 49) showed the enhanced ^{13}C signal at 141 ppm, which indicated that they were structurally-related compounds. ^{1}H-NMR spectroscopy showed that each fraction displayed signals with almost identical chemical shift values in polyene region (5.8 – 8.1 ppm), which integrated to thirteen protons. These signals correspond to the "upper" and "lower" polyketide chains plus H-3 from the mC_7N unit (Figure 50). The major differences between these compounds and type I manumycins in ^{1}H-NMR spectra are the absence of the two adjacent oxrine protons (H-5 dd and H-6 d from the mC_7N unit of type I manumycins) at 3.8 and 3.7 ppm and the appearance of one proton (H-6 from the mC_7N unit of type II manumycins) at 4.1 ppm. These features match with those of type II manumycins (Kohno *et al.*, 1996; Sattler *et al.*, 1993). The only differences among these compounds are located in the saturated hydrocarbon region from 2.4 to 0.8 ppm, which is attributed to the starter unit of the "upper" polyketide chain. The molecular mass of each new compound was determined by LC-MS as 508 (fraction 1), 522 (fraction 2), 536 (fraction 3) and 548 (fraction 4) (Figure 51), all two mass units higher than the type I manumycin series produced by *S. nodosus*. MS/MS spectra gave significant fragments of the "upper" chain

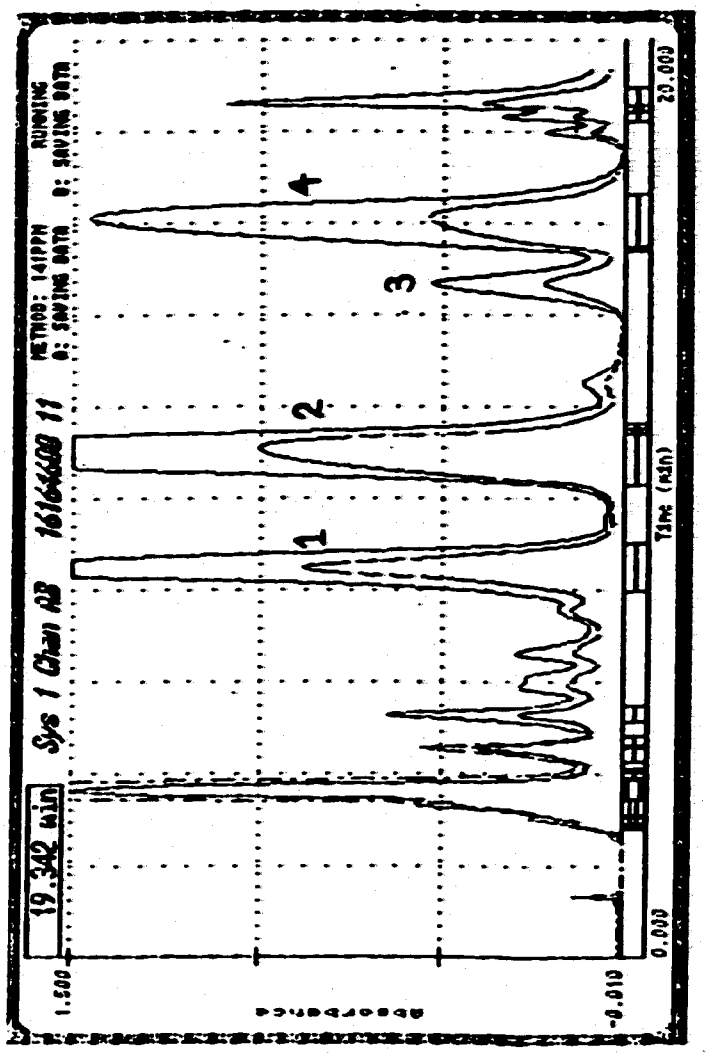


Figure 49. HPLC Chromatogram from Purification of Type II Manumycins

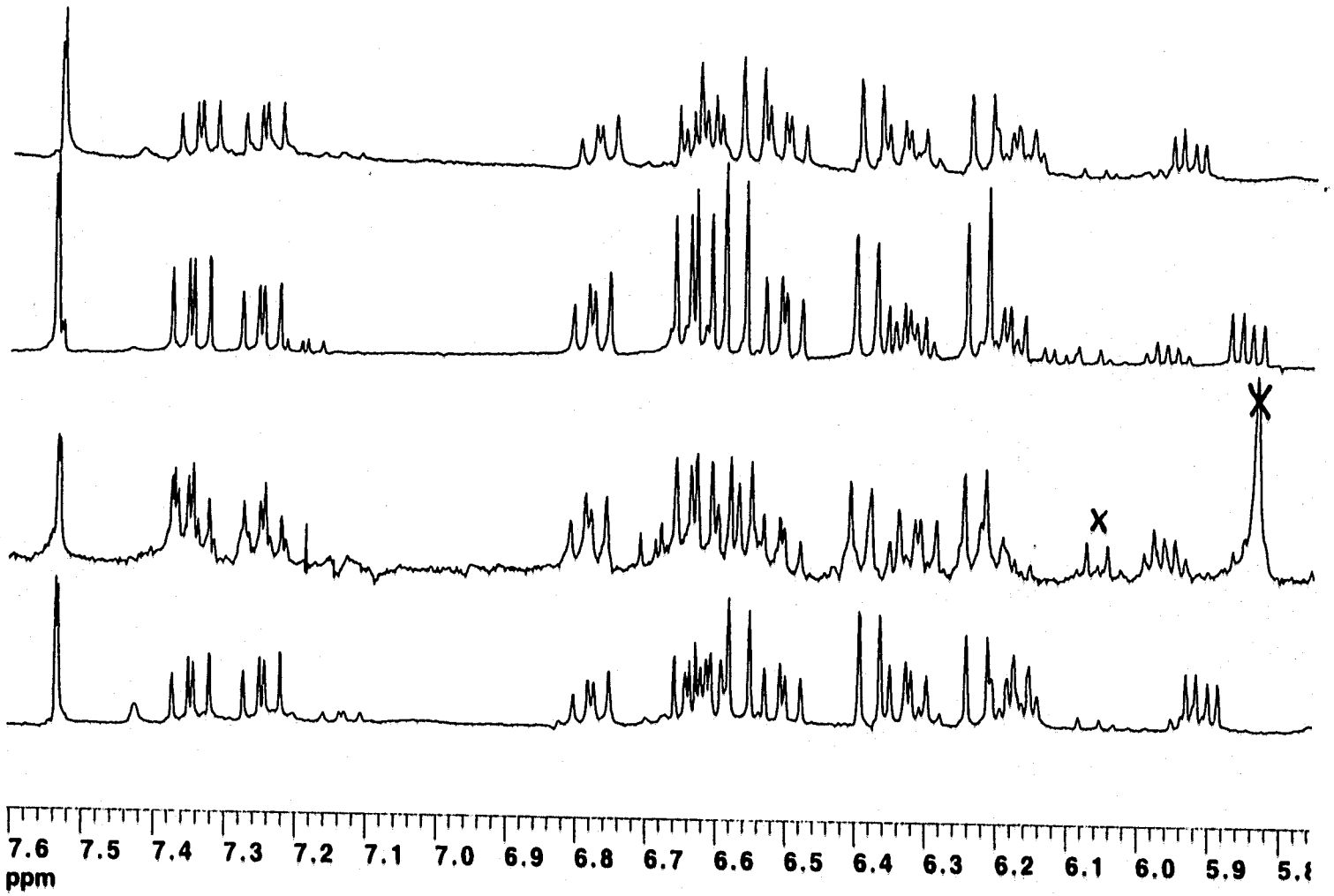


Figure 50. ^{1}H-NMR Comparison (Polyene Region) of Type II manumycins Produced by *Streptomyces nodosus*, from top to bottom: HPLC fraction 1, 2, 3 and 4.

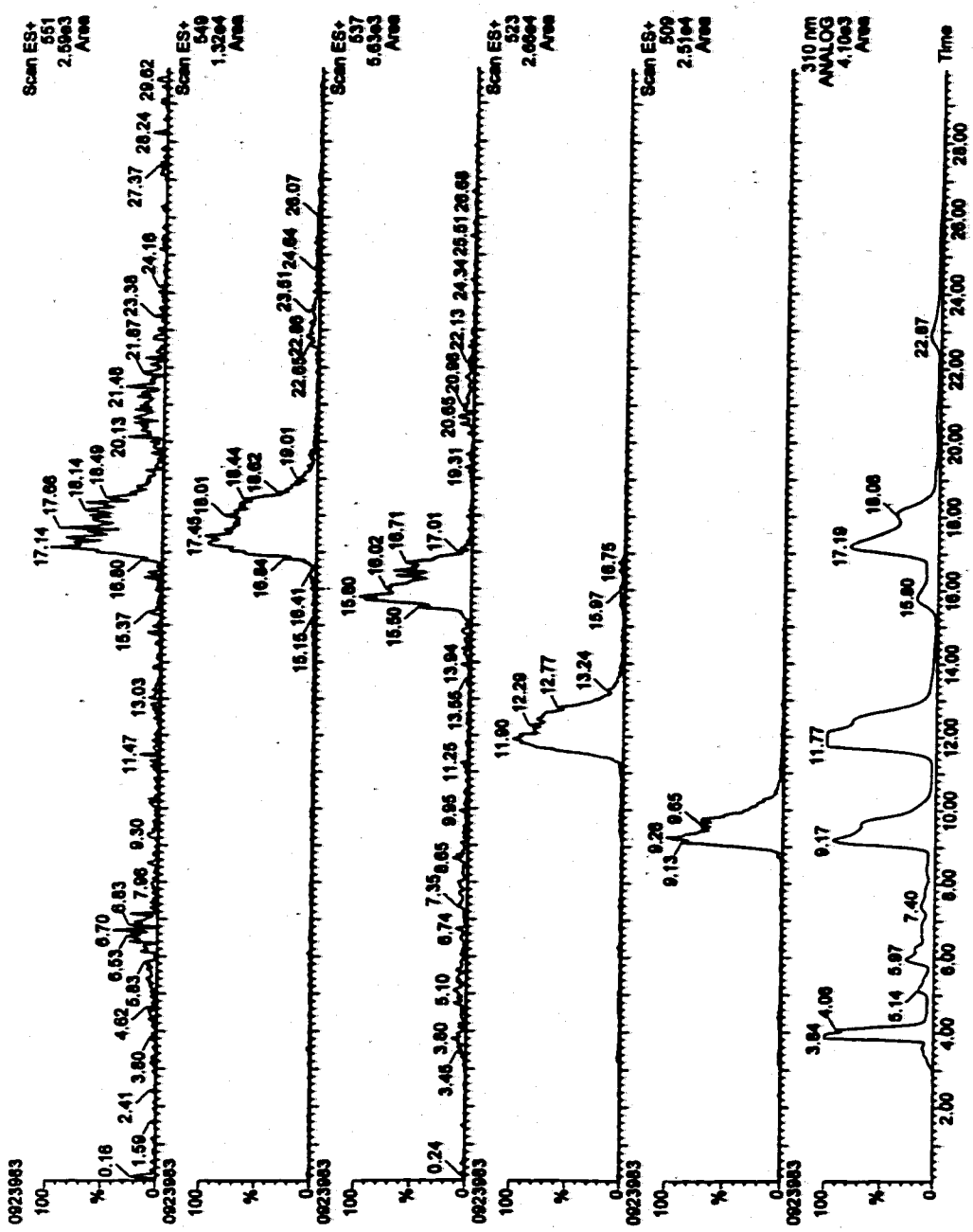


Figure 51. LC-MS Chromatogram of Detection of Type II Manumycins Produced by *S. nodosus*

at m/z 149, 163, 177 and 189, corresponding to fraction 1 to 4. These fragments are identical to the "upper" chains of manumycin G, U-56,407, EI-1511-5, manumycin E and asukamycin. The two extra mass units therefore reside in a part of the molecule other than the "upper" chain. The MS/MS spectrum of fraction 4 was compared with those of the type I manumycins asukamycin and compound **6a/6b** (Figure 42, 43 and 52). The new compound has a relatively more intense fragment resulting from loss of water (M-18). This suggests that the new metabolite contains a hydroxyethylene mC_7N unit, which is easier to dehydrate than an oxirane mC_7N unit. Furthermore, analysis of the ^{1}H-NMR spectra of the "upper" chain starter unit of these compounds indicates that fraction 1 contains an isopropyl group, fraction 2 contains a mixture of an isobutyl and an anteisobutyl groups which resemble to the ^{1}H-NMR pattern of U-56,407 and EI-1511-5 (Figure 40), fraction 3 contains two *geminal* methyl groups, and fraction 4 contains a cyclohexane ring moiety. Based on the NMR and mass spectroscopy analyses, the new metabolites from the four HPLC fractions were established as type II manumycins corresponding to their type I manumycin counterparts manumycin G, U-56,407, EI-1511-5, manumycin E and asukamycin (Figure 53). It is instructive to compare the MS/MS spectrum of compound **25** (fraction 4) with those of asukamycin and compound **6a/6b** (Figure 42, 43 and 52). Also, the molecular mass of compound **25** is two mass units higher than that of asukamycin, but both compounds have the same "upper" chain fragment, suggesting the two hydrogens are added to the mC_7N unit to afford a hydroxyethylene structure. The molecular mass of compound **25** is identical to that of

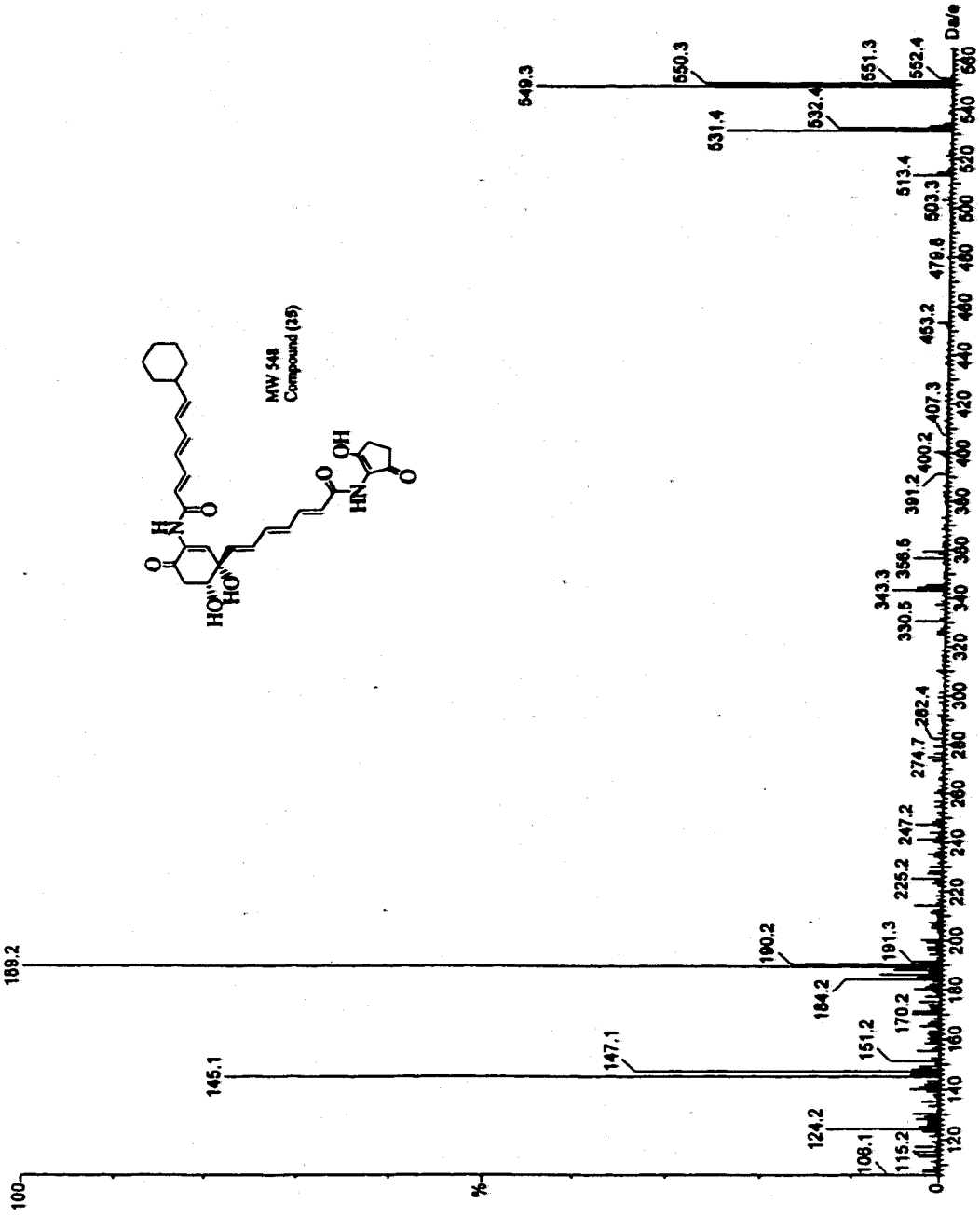


Figure 52. ES-MS/MS Spectrum of Type II Manumycin **25**

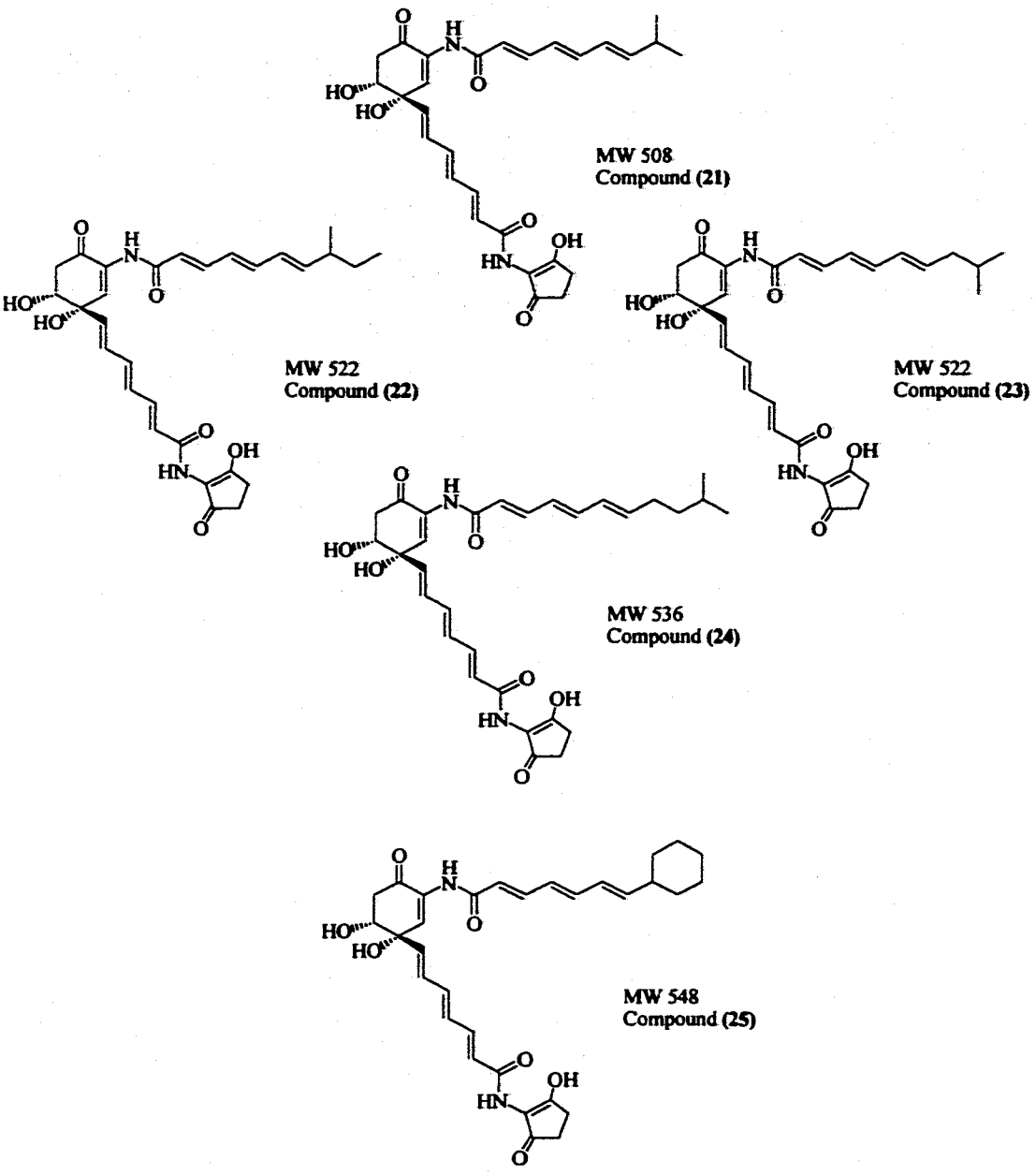


Figure 53. New Type II Manumycin Antibiotics from *S. nodosus*

compound **6a/6b**, but their "upper" chain fragments are different, indicating that compound **25** contains a cyclohexane ring at its "upper" chain terminus (Figure 54).

5.3 The Relative Production of Shunt Metabolite **85**, Type I Manumycins and Type II Manumycins

The incorporation of 3,4-[7-^{13}C]-AHBA gave enhanced ^{13}C-NMR signals at 137 ppm, 139 ppm and 141 ppm (in acetone-d_6), corresponding to shunt metabolite **85**, type I and type II manumycins. A time course study of the fermentation revealed that at the early and middle stage (one to two days in production medium), only shunt metabolite **85** and type I manumycins were produced (Table 13 and Figure 55). The type II manumycins were detected in the middle stage (after two days in production medium). The production of the type I manumycins declined and the type II manumycins increased with the processof fermentation. The early appearance of the shunt metabolite indicated that 3,4-AHBA was consumed and that the "lower" chain polyketide biosynthesis operated in the early fermentation stage. The late appearance of the type II manumycins suggests that the type II manumycins might be formed from the type I manumycins *via* reduction of the epoxide group. The discovery of the shunt metabolite and the type II manumycins provides useful information regarding the manumycin biosynthetic pathway and supports the assumption that the "lower" polyketide chain is synthesized before the attachment of the "upper" chain, and that therefore the epoxidation / oxidation is followed by reduction of the aromatic ring.

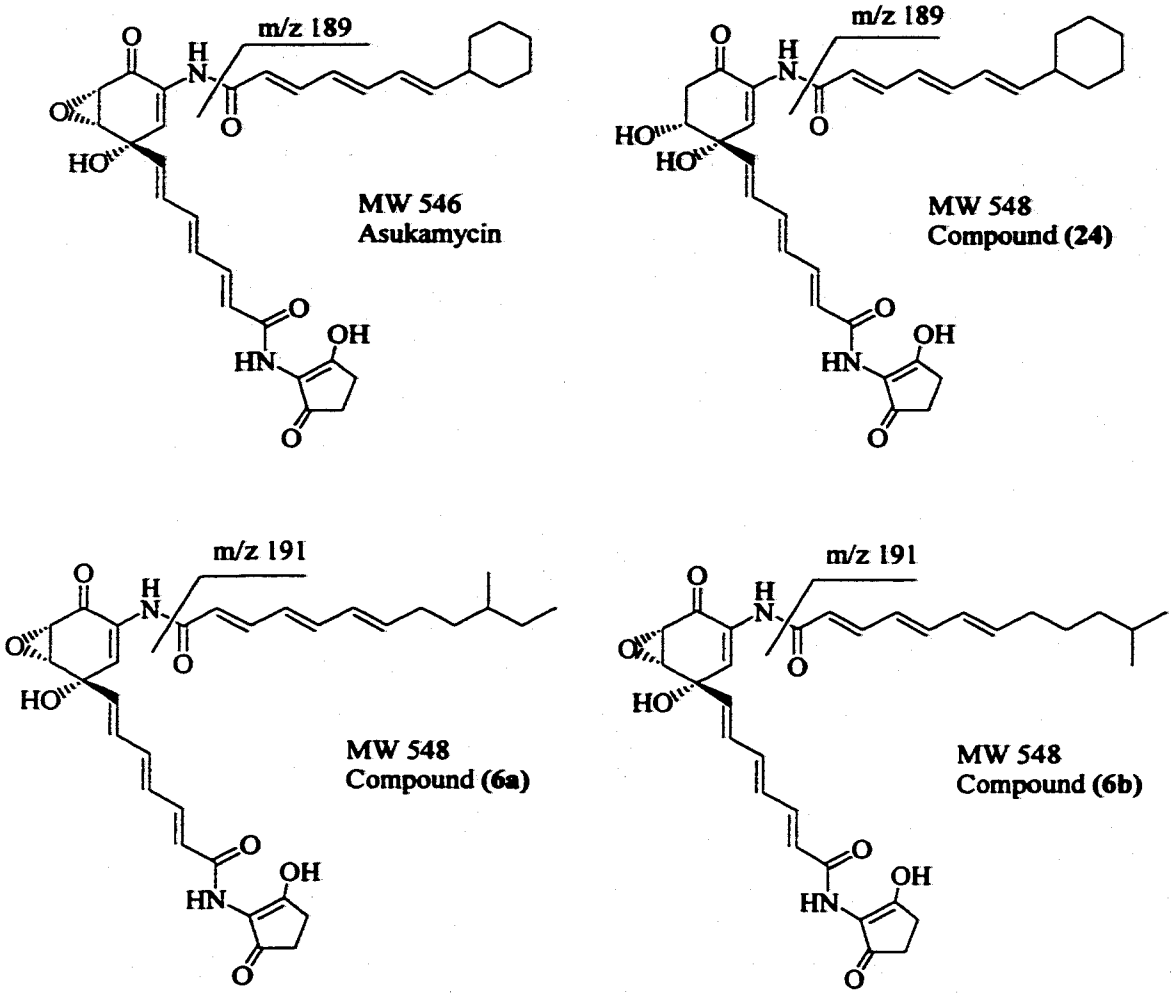


Figure 54. The "Upper" Chain Fragments from MS/MS

Table 13. Relative Production of Shunt Metabolite **85**, Type I and II Asukamycins[a]

Time (hr) [b]	Shunt Metabolite	Type I Manumycins	Type II Manumycins
24	0.15	1	0
24	0.22	1	0
30	0.25	1	0
48	0.19	1	0.11
54	0.19	1	0.19
66	0.28	1	0.19
68	0.19	1	0.24
72	0.37	1	0.29
90	0.24	1	0.68

a) Relative amount was determined by the integration of enhanced ^{13}C signals
b) Hours of fermentation in production medium

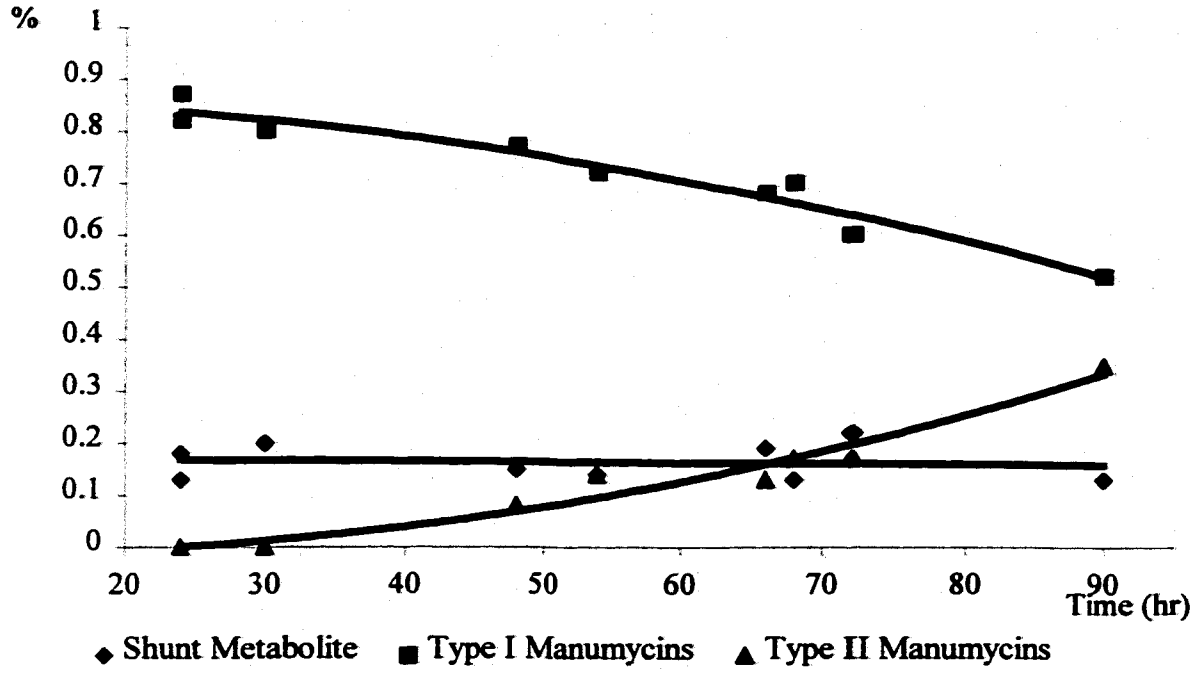


Figure 55. Relative Production of Shunt Metabolite, Type I and Type II Manumycins in *Streptomyces nodosus*

5.4 Mechanistic Hypotheses for the Formation of Type II Manumycins

The mechanism of formation of type II manumycins is not yet known. Hypotheses have been proposed by Zeeck and by Gould based on the oxidation of the mC_7N unit or similar aromatic systems. Zeeck proposed two possible oxidation / epoxidation hypotheses (Figure 56) (Sattler *et al.*, 1998). In the first, one oxygen molecule is attached to the aromatic ring catalyzed by a dioxygenase to form a four-membered ring intermediate which then may yield both type I and type II manumycins. The second is a two-step monooxygenase catalyzed mechanism. Two monooxygenases attack the aromatic ring in a coordinated manner to form type I manumycins, which are converted to type II manumycins by subsequent reduction.

From the time course of type I and type II manumycin production, it follows that type II manumycins may be derived from their type I counterparts, because they are produced in the later stages of the fermentation. Accordingly, type II manumycins are formed in a two-step process. As the stereochemistry of manumycin A was reassigned by Taylor (Alcarz *et al.*, 1998), all the manumycins seem to have a *syn* relationship between the epoxide and hydroxy group, indicating a dioxygenase catalyzed formation of type I manumycins (the mechanism I route (a), proposed by Gould (Cone *et al.*, 1995)).

5.5 Attempt to Discover the Oxirane Reductase

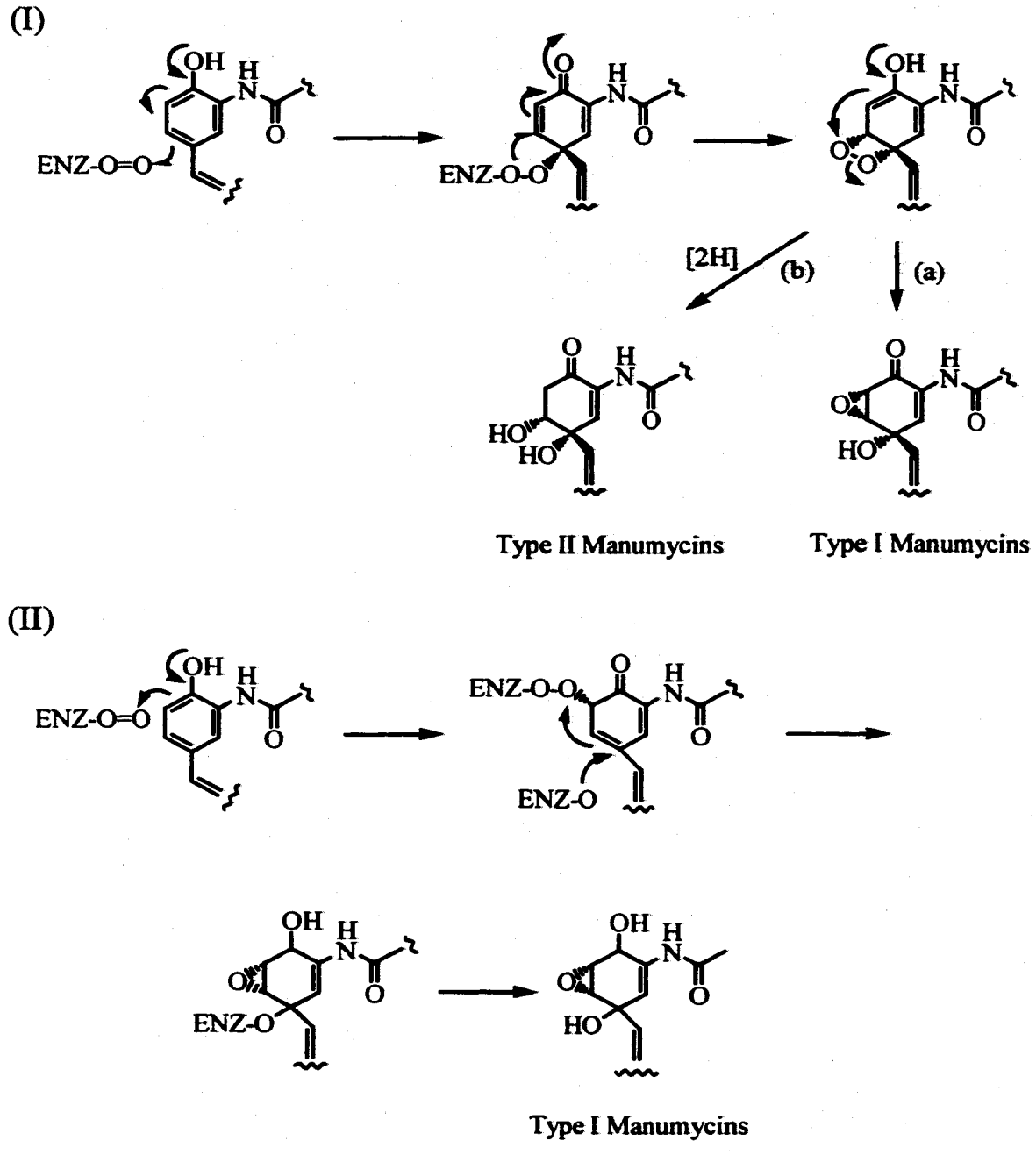


Figure 56. Oxygenation Mechanisms Proposed by Zeeck *et al.* (I) Dioxygenase Mechanism; (II) Mixed Mechanism with a Di- and a Mono-Oxygenase

Since the type I and type II manumycins have different biological activities, it might be important to study the oxirane reduction mechanism and find a way to control this process. This reduction mechanism may apply to other *Streptomycetes* strains which produce both type I and II manumycin class of antibiotics. Whether the conversion of type I manumycins to type II manumycins is an enzymatic reaction or not remains unknown. Incubations with cell-free extracts were conducted to investigate this reduction. Cell-free extracts were prepared using a French Press from *Streptomyces nodosus* cells after growing for two days in production medium. No conversion of the type I asukamycin to its type II compound **24** was observed in the assay in the presence or absence of the co-factor NADH or NADPH. Surprisingly, the epoxide group of asukamycin was easily reduced to the hydroxyethylene group by the addition of DTE (1, 4-dithioerythritol) even without the cell-free extracts. It has been reported that the epoxide could be reduced chemically by $Na[PhSeB(OEt)_3]$ in the total synthesis of colabomycin D (Wei *et al.*, 1999). It is not known if the *in vivo* reduction of type I manumycins to type II manumycins is a spontaneous chemical process or an enzyme-catalyzed reaction. It is likely to be an enzymatic reaction, because the fermentation broth is acidic during the middle and late fermentation period. This is not a favorable condition for spontaneous reduction. On the other hand, an enzyme with cysteine groups at its active site may catalyze this reduction effectively.

CHAPTER 6. TIMING OF THE ASSEMBLY OF THE C_7N UNIT WITH THE "LOWER" CHAIN, THE C_5N UNIT AND THE "UPPER" CHAIN

6.1 Introduction

The biosynthesis of the manumycin type antibiotics is of considerable of interest due to the fact that they consist of several component and thus more than one pathway is involved. Moreover, these pathways are closely associated and must function coordinately. For instance, asukamycin is synthesized by the operation of three independent main pathways: i) the mC_7N unit originates from the TCA cycle and a triose *via* cyclization followed by "lower" chain polyketide synthesis; ii) the C_5N unit is formed from glycine and succinic acid *via* 5-aminolevulinic acid followed by cyclization; and iii) the "upper" chain is derived from the shikimic acid pathway followed by polyketide synthesis. These three pathways must interact with each other to assemble the final product in a well-defined order.

In Chapter 3, a biosynthetic pathway for manumycin type compounds has been proposed based on previous precursor directed feeding experiments (Figure 27). In this proposed pathway, the "lower" polyketide chain is derived from chain extension of 3,4-AHBA, followed by the attachment of the C_5N unit, and then the "upper" chain. It has been shown in Chapter 3 that the biosynthesis of the "lower" polyketide is initiated by 3,4-AHBA. It has also been implied in Chapter 5 that the oxidation / epoxidation followed by reduction of the aromatic ring are the last two reactions on the biosynthetic pathway.

Therefore, the remaining question is the timing of the assembly of three components: the mC_7N unit with the "lower" chain, the C_5N unit and the "upper" chain. The assembly process presumably takes place in a well-defined order. Accordingly, compounds **70** and **71** are possible precursors on the pathway (Figure 57), and are the synthetic targets to be labeled and fed to bacteria to verify the proposed pathway. Precursor directed biosynthesis by feeding a high concentration of 3,4-AHAB to *S. parvulus* which had resulted in compound **62** (Figure 25) (Thiericke *et al.*, 1989a), a compound composed of 3,4-AHBA coupled with the "upper" chain, provided an alternative order of assembly in which the attachment of the "upper" chain occurs prior to that of the C_5N unit. Therefore labeled compound **94a** (Figure 57) is also considered a synthetic target. The synthesis and feeding of labeled compounds **70**, **71** and **94a** will be discussed in this chapter. This resulted in the elucidation of the complete biosynthetic pathway to the manumycin type metabolites.

Since compound **69b** had been synthesized earlier (Chapter 3), it was expected that compounds **70**, **71** and **94a** could be synthesized *via* direct coupling of **69b**, 7-cyclohexyl-hepta-(*2E,4E,6E*)-trienoic acid **(95)** and 2-amino-3-hydroxy-2-cyclopenten-1-one hydrochloride **(96)** (Figure 57).

6.2 Synthesis of 7-Cyclohexyl-hepta-(*2E,4E,6E*)-trienoic Acid **(95)**

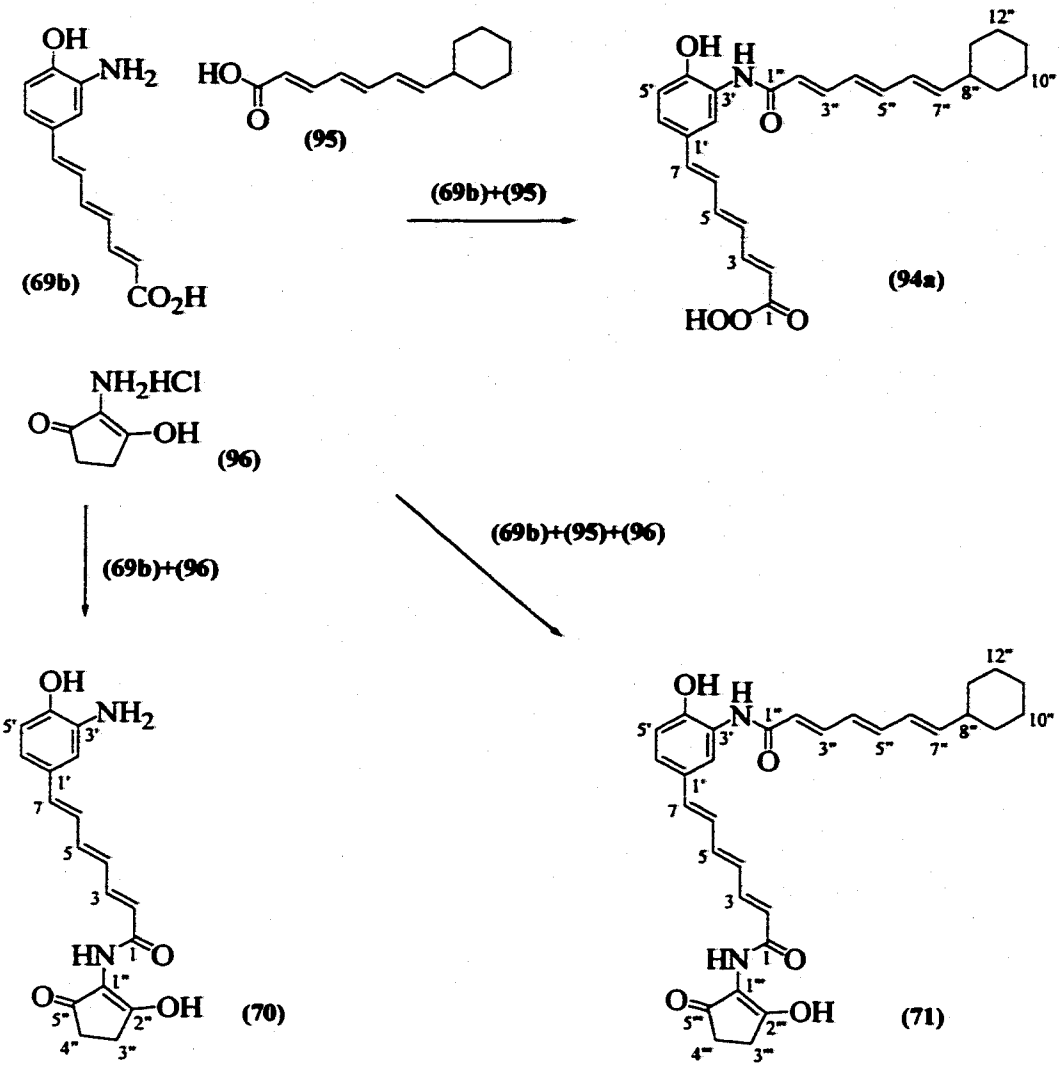


Figure 57. Three Possible Pathway Intermediates

The "upper" chain of asukamycin, compound **95**, was synthesized *via* repeated cycles of the Wadsworth-Emmons reaction which had proved to be highly effective in the synthesis of the "lower" chain *trans* triene portion of **69b** (Figure 58).

6.2.1 7-Cyclohexyl-(*2E,4E,6E*)-hepta-2,4,6-trienoic Acid (**95**)

Cyclohexanecarboxaldehyde **(97)** was treated with excess triethyl phosphonoacetate and 6 M K_2CO_3 to yield ethyl 3-cyclohexyl-*E*-acrylate **(98)** quantitatively (greater than 95% *E*). Ester **98** was reduced with 2 equivalents of diisobutylaluminum hydride (DIBAL-H) to afford 3-cyclohexyl-*E*-prop-2-en-1-ol **(99)**. Alcohol **99** was oxidized without purification to 3-cyclohexyl-*E*-propenal **(100)** with activated manganese dioxide, and the resulting product was purified by silica gel column chromatography to give **100** in a yield of 47% for the two steps. Aldehyde **100** reacted with triethyl phosphonoacetate and K_2CO_3 to give a quantitative yield of ethyl 5-cyclohexyl-(*2E,4E*)-penta-2,4-dienoate **(101)**, which was subsequently reduced with DIBAL-H to afford 5-cyclohexyl-penta-2,4-dien-1-ol **(102)** (77%). Oxidization of alcohol **102** with activated manganese dioxide gave 5-cyclohexyl-(*2E,4E*)-penta-2,4-dienal **(103)** in 93% yield. The third Wadsworth-Emmons reaction was performed on aldehyde **103** with excess triethyl phosphonoacetate to produce ethyl 7-cyclohexyl-(*2E,4E,6E*)-hepta-2,,4,6-trienoate **(104)** and 6 M K_2CO_3 in a yield of 70%. ^{13}C could be introduced in this step if a doubly labeled ^{13}C-^{15}N amide bond is desired. Finally, ester **104** was saponified with LiOH in methanol/water (50/50) to give 7-cyclohexyl-(*2E,4E,6E*)-hepta-2,4,6-trienoic acid **(95)** (56%).

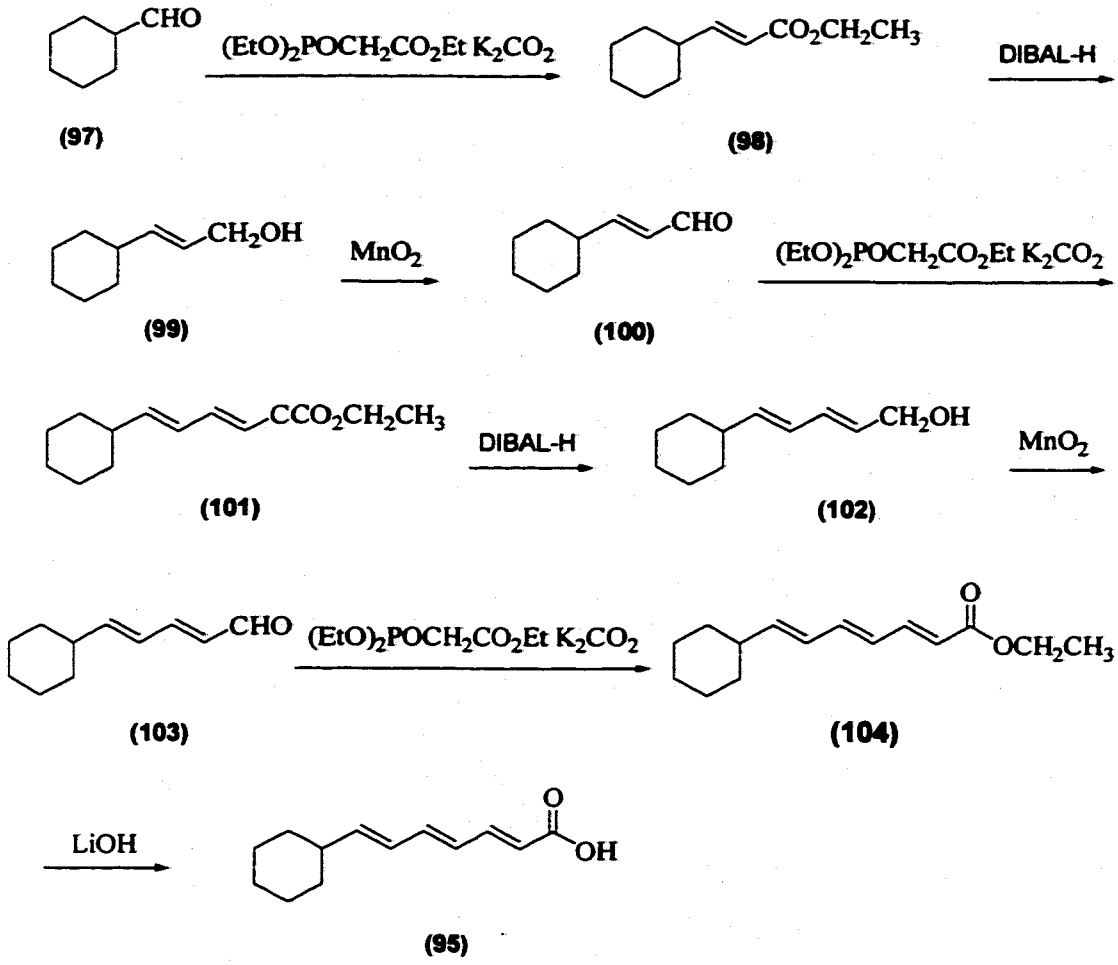


Figure 58. Synthesis of 7-Cyclohexyl-hepta-(*2E,4E,6E*) -trienoic Acid (**95**)

6.2.2 Alternative Synthesis of 5-Cyclohexyl-(*2E,4E*)-penta-2,4-dienal (**103**)

Similar *trans* polyene compounds have been synthesized *via* organozirconocenes or glutaconaldehyde (Lewis *et al.*, 1991; Wipf and Coish 1997). A shorter synthesis of 5-cyclohexyl-penta-2,4-dienal **(103)** was adopted to minimize the synthetic steps towards compound **95** (Figure 59).

Pyridinium-1-sulfonate **(105)** was reacted with KOH in water. The resulting potassium glutaconaldehyde **(106)** was obtained in a yield of 72% (Becher ; Becher 1980b). Silylation of **106** with tert-butyldimethylsilyl chloride (TBDMS-Cl) in the presence of triethylamine and 4-dimethylaminopyridine led to intermediate **107**, which was treated with the Grignard reagent cyclohexyl magnesium bromide **(109)** followed by acid hydrolysis to afford **103** (Lewis *et al.*, 1991). Compound **103** was purified by column chromatography using hexane / ethyl acetate as eluent to give a 45% yield of product.

6.3 First Attempt to Synthesize **70** via Direct Coupling

6.3.1 Synthesis of 2-Amino-3-hydroxy-2-cyclopenten-1-one Hydrochloride (**96**)

The synthesis of 2-amino-3-hydroxy-2-cyclopenten-1-one hydrochloride **(96)** was described by Ebenezer in 1991 (Ebenezer 1991) (Figure 60). In this synthesis, ^{15}N could be introduced using $^{15}NO_2$ to form the ^{13}C-^{15}N doubly labeled amide bond of compound **70**, if desired.

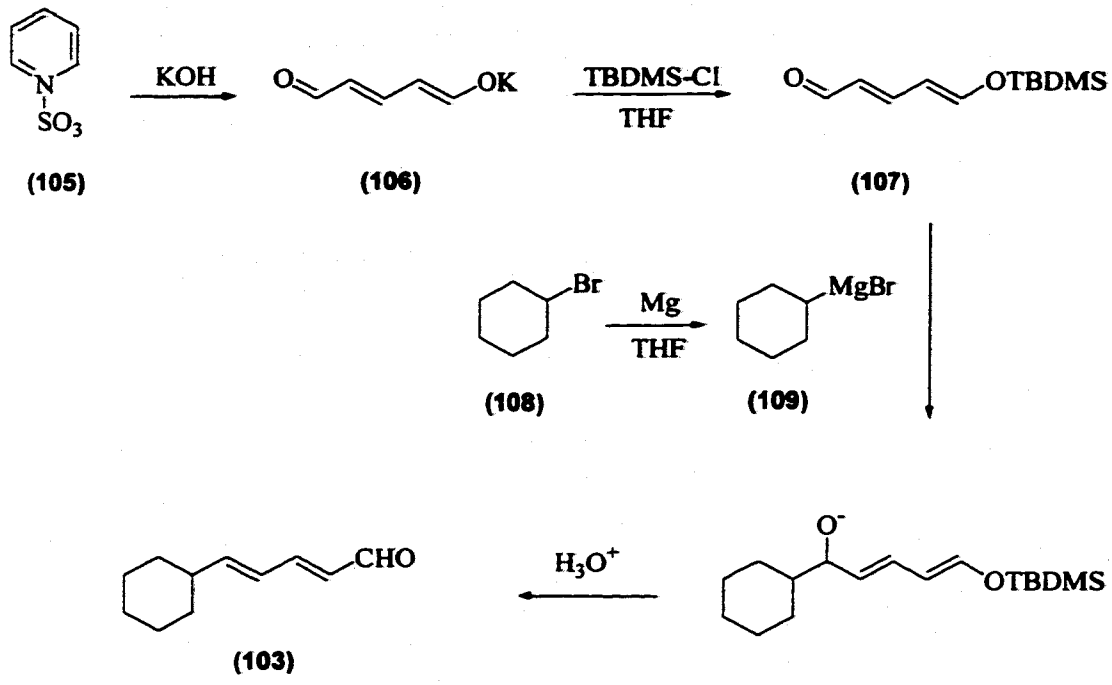
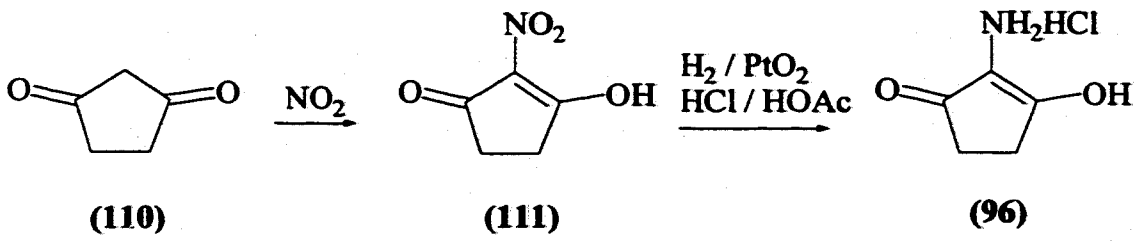


Figure 59. Alternative Synthesis of 5-Cyclohexyl-penta-2,4-dienal



(110) **(111)** **(96)**

Figure 60. Synthesis of C_5N unit

3-Hydroxy-2-nitrocyclopent-2-en-1-one **(111)** was obtained in 90% yield by passing nitrogen dioxide through a suspension of 1,3-cyclopentanedione **(110)** in anhydrous diethyl ether. Reduction of the nitro group of **111** was achieved *via* hydrogenation with Adam's catalyst in aqueous HCl and glacial acetic acid. The resulting 2-amino-3-hydroxycyclopent-2-en-1-one hydrochloride **(96)** was quite unstable and was used immediately in the coupling reaction.

6.3.2 Attempts of Direct Coupling to Synthesize Compound **70**

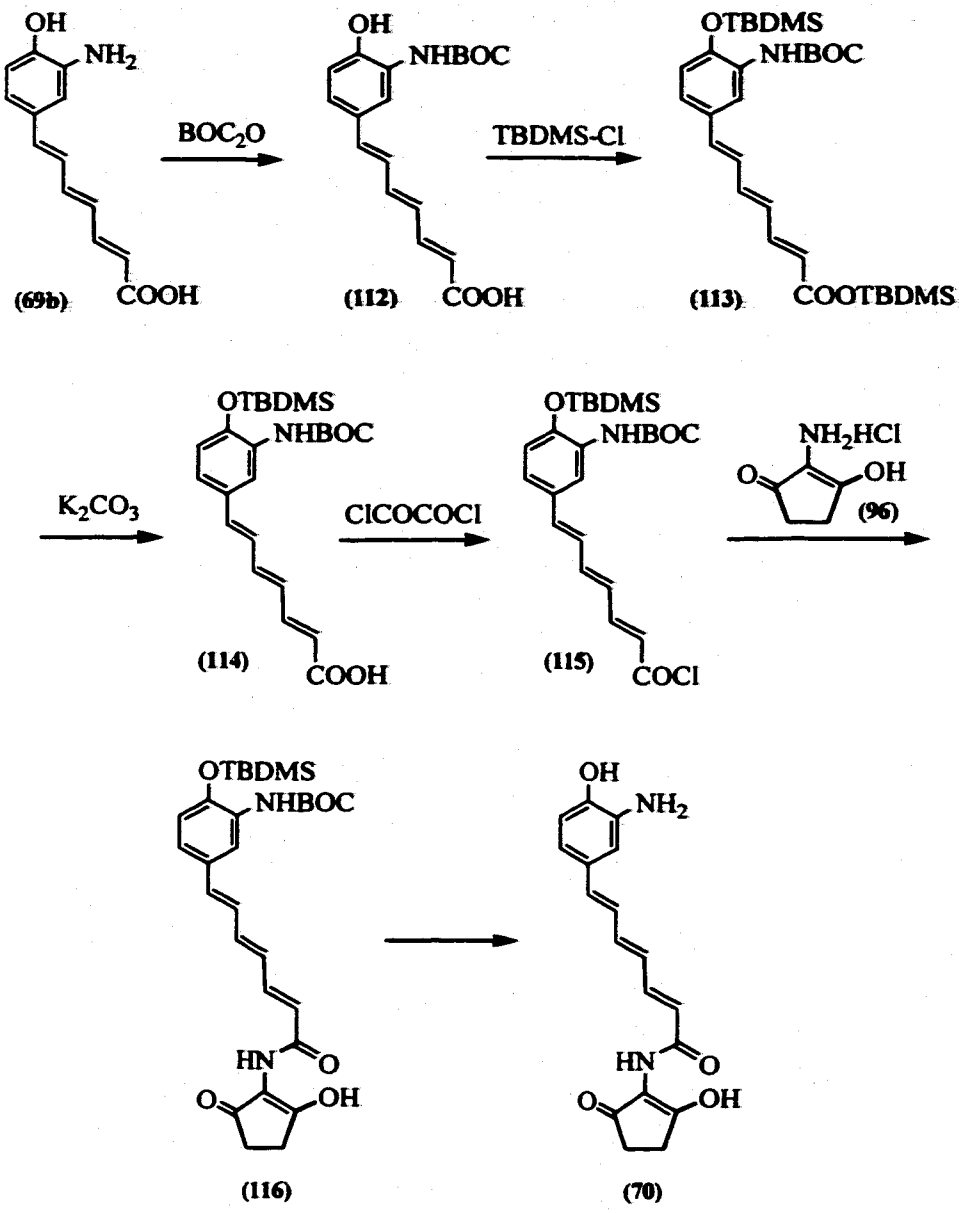
The direct coupling of **69b** with **96** to synthesize **70** was attempted. Coupling reagents such as DCC, EDCI/ DMAP failed due to the poor reactivity of **96**. The acid chloride had been employed in such a coupling in the synthesis of alisamycin (Alcarz *et al.*, 1996) and asuka-mABA (Macdonald *et al.*, 1998b; Macdonald *et al.*, 1996). But in this case, the phenolic hydroxy and amino groups of **69b** would interfere with the formation of the acid chloride by forming by-products. As another approach it was considered to protect

the hydroxy and amino groups with TBDMS and BOC protecting groups in successive reactions, followed by mild hydrolysis to release free acid **114** (Figure 61). A similar strategy had been applied successfully in the synthesis of protected tyrosine (Grieco and Perez-Medrano 1988; Morton and Thompson 1978). This approach was attempted on the model compound 3,4-AHBA. Unfortunately, the reactions placing protective groups on 3,4-AHBA proved difficult. Mono-, di- and tri- TBDMS or BOC substituted 3,4-AHBA were generated with low yield of the derived compound. Even worse, the protecting groups were cleaved during acid chloride formation. Therefore, the synthesis of **70** by direct coupling of **69b** or its derivatives with **96** was considered not viable.

6.4 Second Attempt to Synthesize **70** *via* a Stille Approach

Recently, asuka-mABA **(65)** was synthesized by Taylor's group *via* a Stille coupling (Figure 62) (Macdonald *et al.*, 1998b; Macdonald *et al.*, 1996). Amine **96** was coupled with 5-bromopentadienoic acid **(118)** to give vinyl bromide **119**. **119** underwent Stille coupling with vinylstannane **123** followed by deprotection to afford asuka-mABA **(65)**. Accordingly, the synthesis of compound **70** was proposed as shown in Figure 63. It is expected that amine **96** reacts with 7-bromoheptatrienoic acid **126** to yield vinyl bromide **127**, which is coupled with arylstannane **129** or **132** to proceed to **70**. Some progress has been made, but more problems were encountered in this approach.

On the way to synthesize vinyl bromide **127**, the bromination of potassium



Figurer 61. Attempted Synthesis of **70** via Protected **69b**

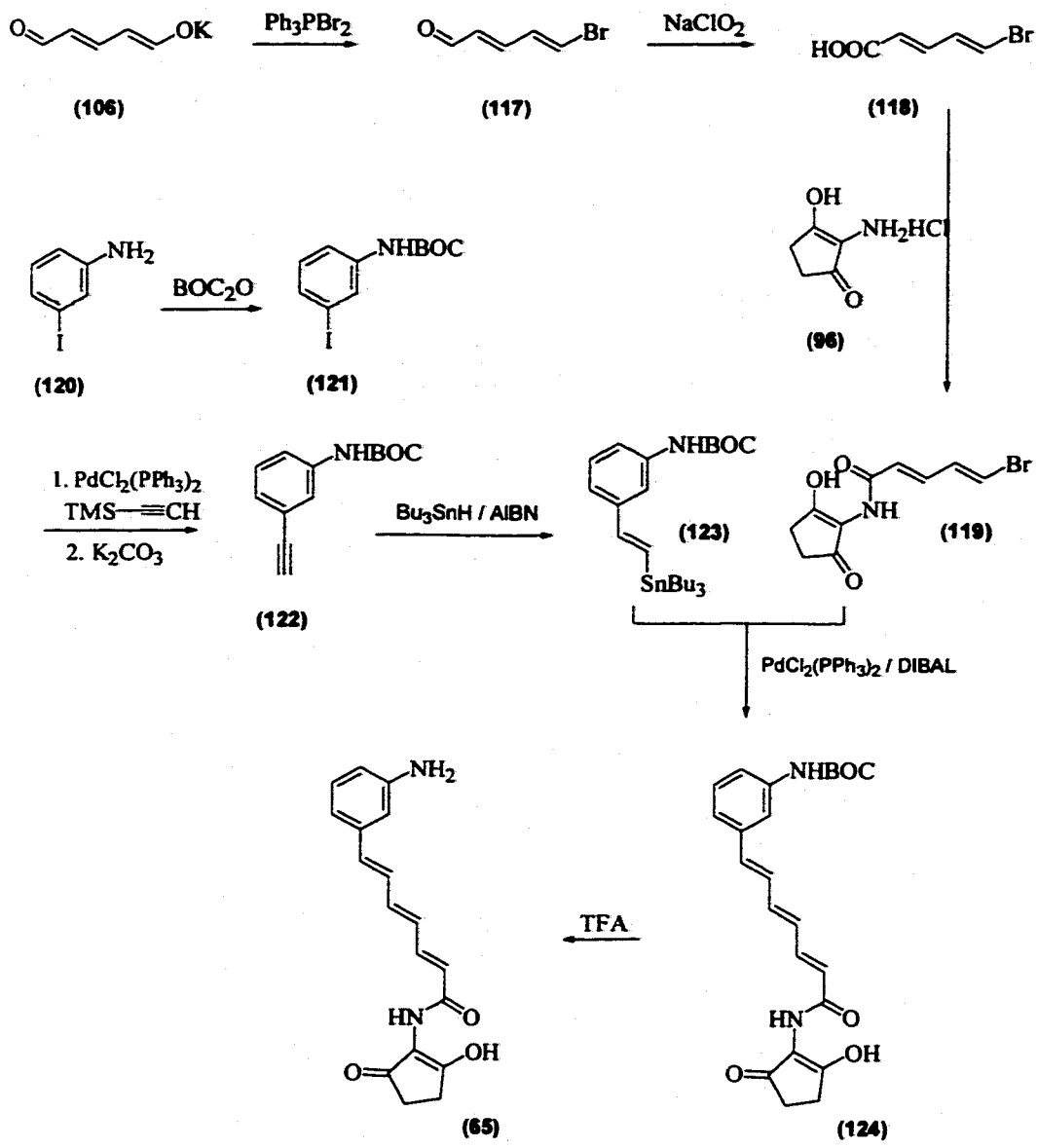


Figure 62. Synthesis of Asuka-mABA (65) by Taylor's Group

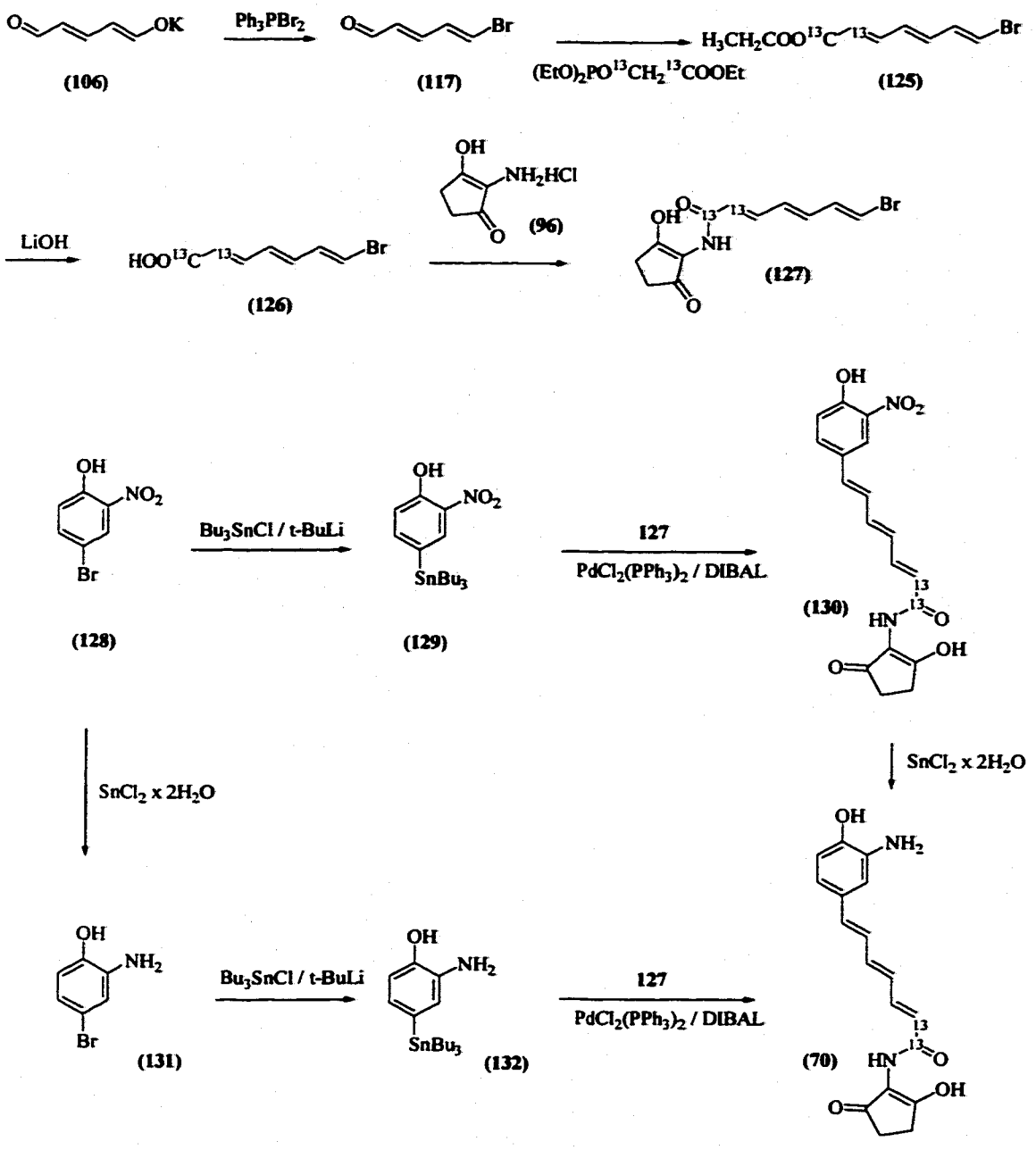


Figure 63. Attempted Stille Approach to the Synthesis of (70)

glutaconaldehyde **(106)** was performed with dibromotriphenylphosphorane to give bromopentadienal **(117)** (Soullez *et al.*, 1995).

In parallel, studies were performed on the synthesis of the arylstannanes. 4-Bromo-2-nitrophenol **(128)** was reduced to 2-amino-4-bromophenol **(131)** with tin(II) chloride dihydrate in 91% yield. The direct synthesis of arylstannanes **129** and **132** from **128** and **131** with tributyltin chloride was unsuccessful. It was then attempted to protect the hydroxy and amino groups of **128** and **131** using two different protecting groups, thus allowing the protecting groups to be removed under different conditions later in the synthesis (Figure 64).

4-Bromo-2-nitrophenol **(128)** was silylated with TBDMS-Cl catalyzed by DMAP. The resulting 4-bromo-2-nitrophenol TBDMS ether **(132)** was purified by flash column chromatography with ethyl acetate / hexane as eluent to afford 42% yield of product. Compound **132** proved to be unstable and the TBDMS protecting group was cleaved during storage. Ultrasound-promoted reaction of **132** with magnesium was initiated by 1,2-dibromoethane and the resulting Grignard reagent was coupled with $(Bu_3Sn)_2O$ in a one-pot reaction (Lee and Dai 1996). Column chromatography and preparative TLC purification with ether / hexane yielded a trace amount of 2-nitro-4-(tributylstannyl)-phenol TBDMS ether **(133)**. This reaction yielded a mixture of tin-containing by-products, and product **133** was easily decomposed. The prospects for improvement of this reaction are not encouraging.

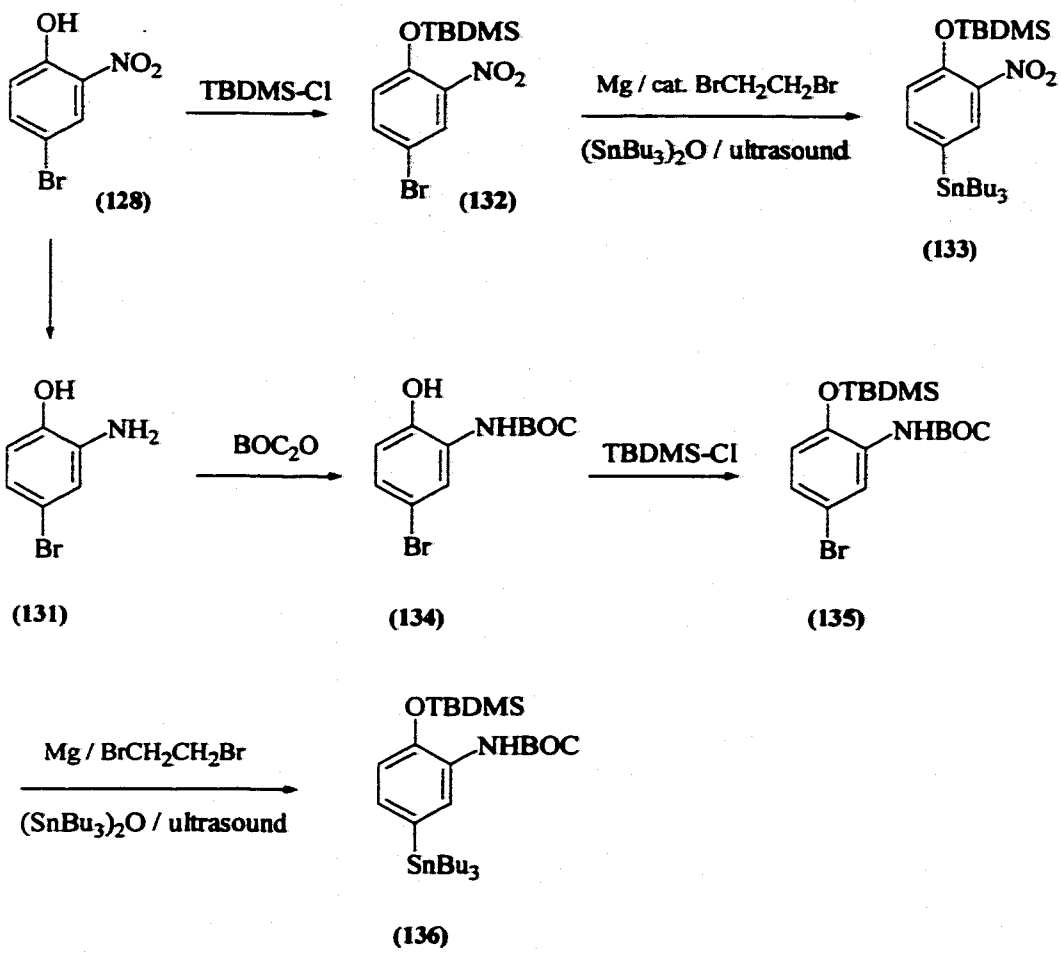


Figure 64. Synthesis of Arylstannanes

The *t*-butoxycarbonylation of 2-amino-4-bromophenol **131** with di-*t*-butyl dicarbonate and triethylamine or imidazole resulted in a mixture of O- and N- substitution products (Ponnusamy *et al.*, 1986). It was found that without base a slow reaction would generate uniquely the N-substituted compound **134**. Thus, 2-amino-4-bromophenol **(131)** was reacted with Boc_2O in anhydrous THF at room temperature for 3 days, followed by recrystallization from hexane to give 2-N-Boc-amino-4-bromophenol **(134)** (86%). Compound **134** was silylated with TBDMS-Cl in the presence of imidazole to afford 2-N-Boc-amino-4-bromophenol TBDMS ether **(135)** in a yield of 91% after purification by column chromatography with ethyl acetate / hexane (Kendall *et al.*, 1979; Ronald *et al.*, 1982). However, the effort to convert **135** to arylstannane failed. Overall, the Stille approach to compound **70** was not successful.

6.5 Synthesis of Labeled Compounds **70**, **71** and **94a**

6.5.1 Synthesis of N_1-(2-Hydroxy-5-oxo-cyclopent-1-enyl)-7-(3-amino-4-hydroxy-phenyl)-[1,2-$^{13}C_2$]hepta-(*2E,4E,6E*)-trienamide ([1,2-$^{13}C_2$]-**70**)

It has been reported that manumycin slowly decomposes on silica gel (Zeeck *et al.*, 1987), and that amine **96** is highly unstable (Ebenezer 1991). It is believed that the instability of the manumycins is attributable to their C_5N moiety. Therefore, in the previously attempted synthesis of compound **70**, harsh reaction conditions were avoided for the coupling of **69b** and **96**. Judging from the experience in these unsuccessful attempts, it seems necessary to have more stable hydroxy and amino protecting groups on compound

69b to survive the coupling of **69b** and **96**. The most convenient method would be to make the amide bond between **82** and **96**, followed by demethylation and reduction of the nitro group to yield **70**, as shown in Figure 65. It would be challenging for the C_5N moiety and its amide bond to survive demethylation and reduction. A mild reagent is needed to cleave the methyl aryl ether but not the amide bond. It was reported that BBr_3 can cleave ether and ester bonds, and in some cases it will cleave the ether only, depending on the structure of substrates and reaction conditions (Fraser *et al.*, 1976; Kopcho and Schaeffer 1986; McOmie *et al.*, 1968; Vickery *et al.*, 1979). BBr_3 was tested on compound **82**. It cleaved the methyl ether while the ethyl ester remained intact. Therefore, this approach (in Figure 65) looked promising and was pursued despite low yield in some reactions.

Ethyl 7-(4-methoxy-3-nitro-phenyl)-[1,2-$^{13}C_2$]hepta-(*2E,4E,6E*)-trienoate ([1,2-$^{13}C_2$]-**82**) was hydrolyzed with LiOH in a mixed solvent of THF, methanol and water. Purification was performed by column chromatography with methanol / CH_2Cl_2 to give 7-(4-methoxy-3-nitrophenyl)-[1,2-$^{13}C_2$]hepta-(*2E,4E,6E*)-trienoic acid ([1,2-$^{13}C_2$]-**137**) in 92% yield. Acid [1,2-$^{13}C_2$]-**137** was converted to 7-(4-methoxy-3-nitrophenyl)-[1,2-$^{13}C_2$]hepta-(*2E,4E,6E*)-trienoyl chloride ([1,2-$^{13}C_2$]-**138**) with oxalyl chloride followed by coupling with 2-amino-3-hydroxycyclopent-2-enone hydrochloride **96** in the presence of DMAP in pyridine. N_1-(2-hydroxy-5-oxo-cyclopent-1-enyl)-7-(4-methoxy-3-nitrophenyl)-[1,2-$^{13}C_2$]hepta-(*2E,4E,6E*)-trienamide ([1,2-$^{13}C_2$]-**139**) was obtained in a yield of 78% after purification by column chromatography eluting with methanol /

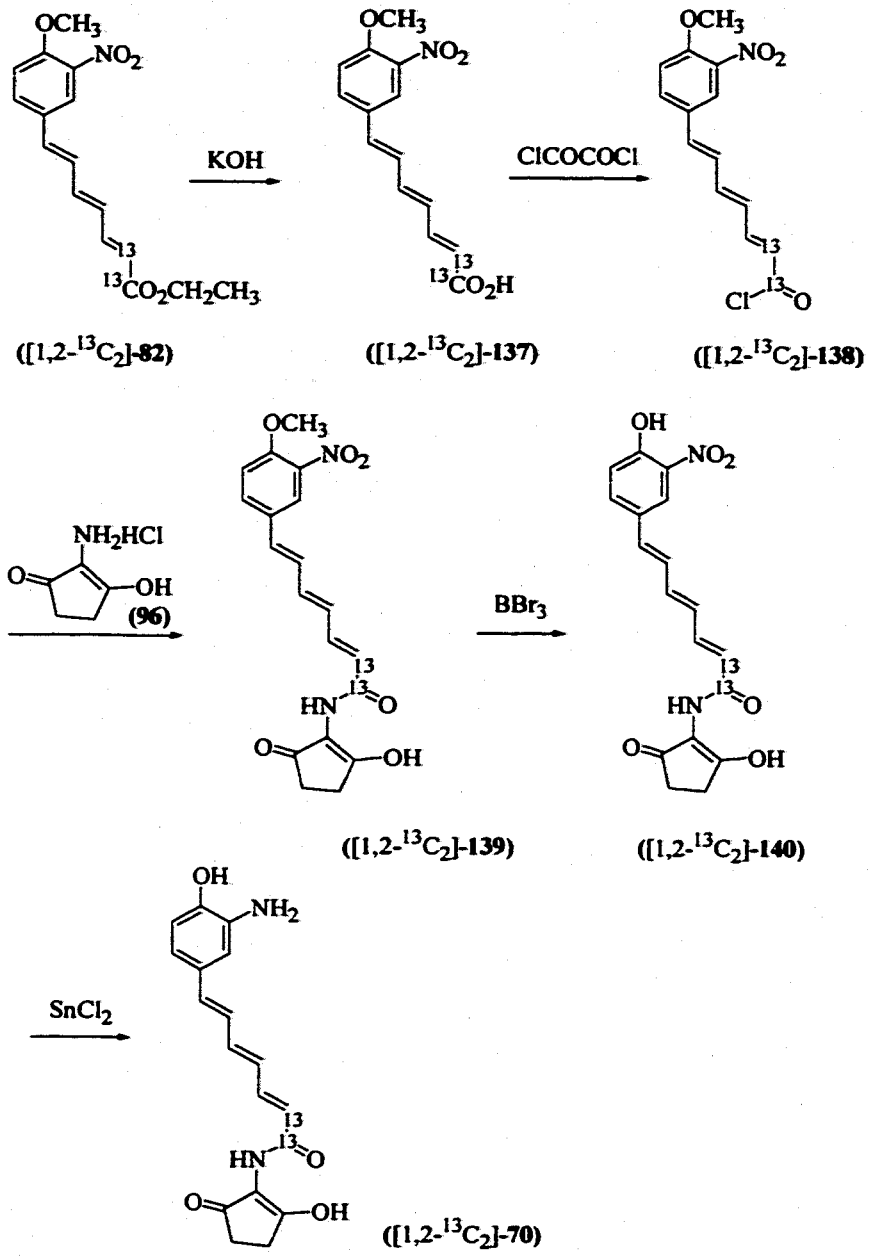


Figure 65. Synthesis of N_1-(2-Hydroxy-5-oxo-cyclopent-1-enyl)-7-(3-amino-4-hydroxy-phenyl)-[1,2-$^{13}C_2$]-hepta-(*2E,4E,6E*)-trienamide

methylene chloride. It was found that this coupling reaction had to be terminated within two hours, as degradation occurred due to the instability of the C_5N moiety under the reaction conditions.

[1,2-$^{13}C_2$]-**139** was demethylated with BBr_3 at –78 °C to afford N_1-(2-hydroxy-5-oxo-cyclopent-1-enyl)-7-(4-hdyroxy-3-nitrophenyl)-[1,2-$^{13}C_2$]hepta-(*2E,4E,6E*)-trienamide ([1,2-$^{13}C_2$]-**140**) after purification by column chromatography with methanol / methylene chloride (21%). The nitro group of [1,2-$^{13}C_2$]-**140** was reduced with tin(II) chloride dihydrate . Purification on a flash column with methanol / methylene chloride gave N_1-(2-hydroxy-5-oxo-cyclopent-1-enyl)-7-(3-amino-4-hydroxyphenyl)-[1,2-$^{13}C_2$]hepta-(*2E,4E,6E*)-trienamide ([1,2-$^{13}C_2$]-**70**) (28%). Compound **70** was characterized by NMR, ion trap MS/MS and high resolution mass spectroscopy.

6.5.2 Synthesis of 7-[3-(7-Cyclohexyl-hepta-(*2E,4E,6E*)-trienoyl)-amino-4-hydroxyphenyl]-[1,2-$^{13}C_2$]hepta-(*2E,4E,6E*)-trienoic Acid (**94a**)

The synthesis of compound [1,2-$^{13}C_2$]-**94a** was carried out as shown in Figure 66. The key steps in this synthesis are the coupling of acid chloride **141** with the amino group of **87** and the selective hydrolysis of the ethyl ester.

Ethyl 7-(4-methoxy-3-nitrophenyl)-(*2E,4E,6E*)-[1, 2-C_2]-hepta-2,4,6-trienoate ([1,2-$^{13}C_2$]-**82**) was reduced with tin(II) chloride dihydrate. The crude product was purified by

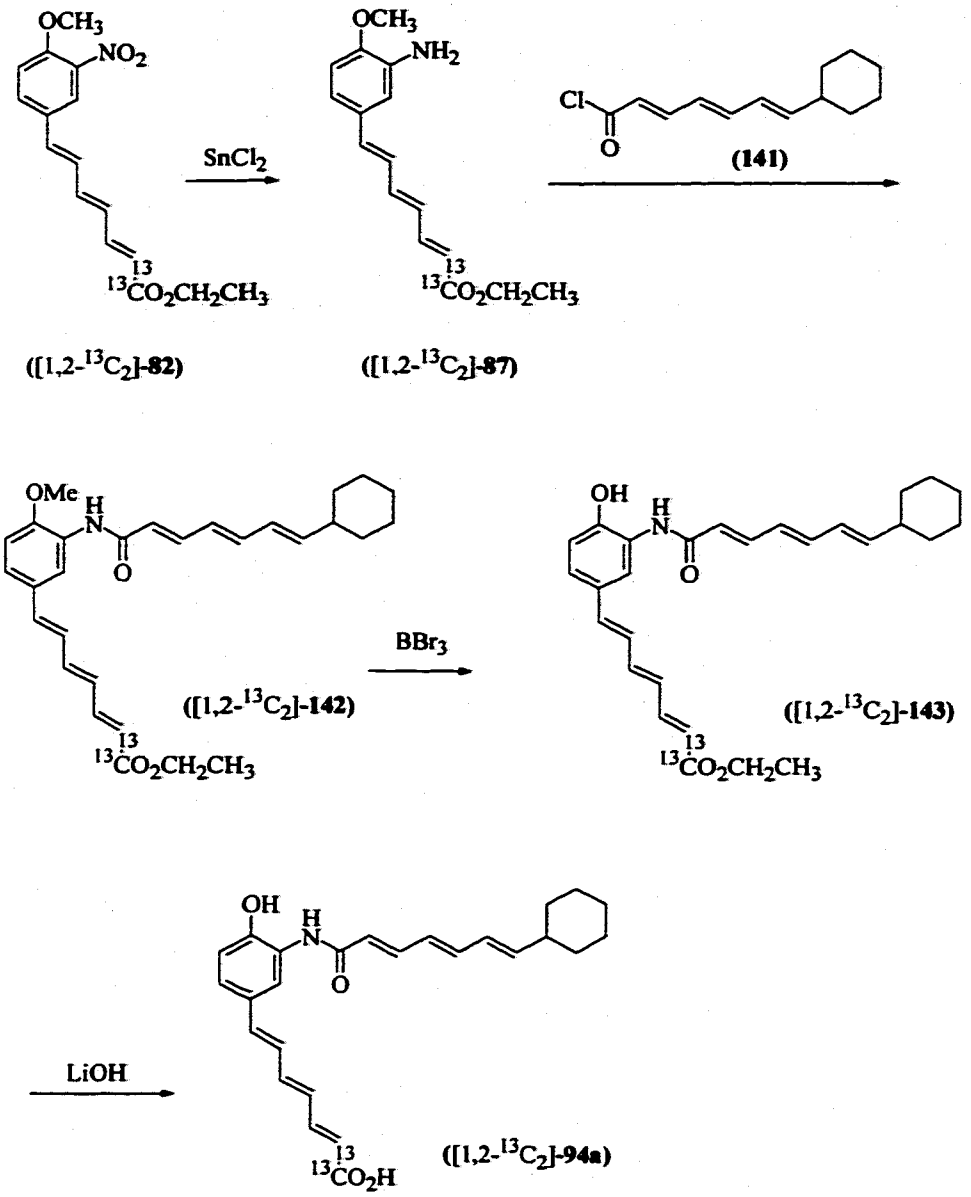


Figure 66. Synthesis of 7-[3-(7-Cyclohexyl-hepta-(*2E,4E,6E*)-trienoyl)-amino-4-hydroxyphenyl]-[1,2-$^{13}C_2$]-hepta-(*2E,4E,6E*)-trienoic Acid

column chromatography with ethyl acetate / hexane to afford ethyl 7-(3-amino-4-methoxyphenyl)-(*2E,4E,6E*)-[1, 2-C_2]-hepta-2,4,6-trienoate ([1,2-$^{13}C_2$]-**87**) in a yield of 76%.

Since the amino group of **87** is very unreactive towards acylation, normal peptide synthesis coupling reagents will not be effective. This type of coupling should be accomplished by using the acid chloride in the presence of DMAP or *tert*-BuOLi (Alcarz and Taylor 1998; Kapfer *et al.*, 1996; Macdonald *et al.*, 1998a; Taylor *et al.*, 1998; Wipf and Goish 1999).

7-Cyclohexyl-hepta-(*2E,4E,6E*)-trienoic acid (**95**) was converted to 7-cyclohexyl-hepta-(*2E,4E,6E*)-trienoyl chloride (**141**) with oxalyl chloride. **141** was coupled with [1,2-$^{13}C_2$]-**87** in pyridine with DMAP as catalyst. The product, ethyl 7-[3-(7-cyclohexyl-hepta-(*2E,4E,6E*)-trienoyl)-amino-4-methoxyphenyl]-[1,2-$^{13}C_2$]hepta-(*2E,4E,6E*)-trienoate ([1,2-$^{13}C_2$]-**142**), was purified by column chromatography with methanol / methylene chloride (61%). [1,2-$^{13}C_2$]-**142** was demethylated with BBr_3 at –78 °C, and the resulting product was purified by column chromatography with ethyl acetate / hexane to afford ethyl 7-[3-(7-cyclohexyl-hepta-(*2E,4E,6E*)-trienoyl)-amino-4-hydroxyphenyl]-[1,2-$^{13}C_2$]hepta-(*2E,4E,6E*)-trienoate ([1,2-$^{13}C_2$]-**143**) in a yield of 34%. Compound [1,2-$^{13}C_2$]-**143** was hydrolyzed with LiOH in THF / water to afford 7-[3-(7-cyclohexyl-hepta-(*2E,4E,6E*)-trienoyl)-amino-4-hydroxyphenyl]-[1,2-$^{13}C_2$]hepta-(*2E,4E,6E*)-trienoic acid ([1,2-$^{13}C_2$]-**94a**). Purification was conducted on a flash column eluted with methanol /

methylene chloride to give a 49% yield of the product. Significant deacylation (cleavage of the "upper" chain) was observed in the hydrolysis step. The reaction proceeded very slowly under mild basic conditions (pH 8), but deacylation increased with the increase of pH. Compound [1,2-$^{13}C_2$]-**94a** was characterized by NMR, ion trap MS/MS and high resolution mass spectroscopy.

6.5.3 Synthesis of N_1-(2-Hydroxy-5-oxo-cyclopent-1-enyl)-7-[3-(7-cyclohexyl-hepta-(*2E,4E,6E*)-trienoyl)-amino-4-hydroxyphenyl]-[1,2-$^{13}C_2$]hepta-(*2E,4E,6E*)-trienamide ([1,2-$^{13}C_2$]-**71**)

The synthesis of [1,2-$^{13}C_2$]-**71** is illustrated in Figure 67. In the first approach compound **142** was hydrolyzed with LiOH in THF/water. Purification by column chromatography with methanol / methylene chloride afforded 7-[3-(7-cyclohexyl-hepta-(*2E,4E,6E*)-trienoyl)-amino-4-methoxyphenyl]-hepta-(*2E,4E,6E*)-trienoic acid (**144**). **144** reacted with oxalyl chloride followed with **96**. This coupling did not produce a significant amount of compound **146**, probably due to the deacylation (loss of the "upper" chain). This route was not pursued any further because of the deacylation which resulted in low yields.

In the second approach, N_1-(2-hydroxy-5-oxo-cyclopent-1-enyl)-7-(4-methoxy-3-nitrophenyl)-[1,2-$^{13}C_2$]hepta-(*2E,4E,6E*)-trienamide ([1,2-$^{13}C_2$]-**139**) was reduced to N_1-(2-hydroxy-5-oxo-cyclopent-1-enyl)-7-(3-amino-4-methoxyphenyl)-[1,2-$^{13}C_2$]hepta-

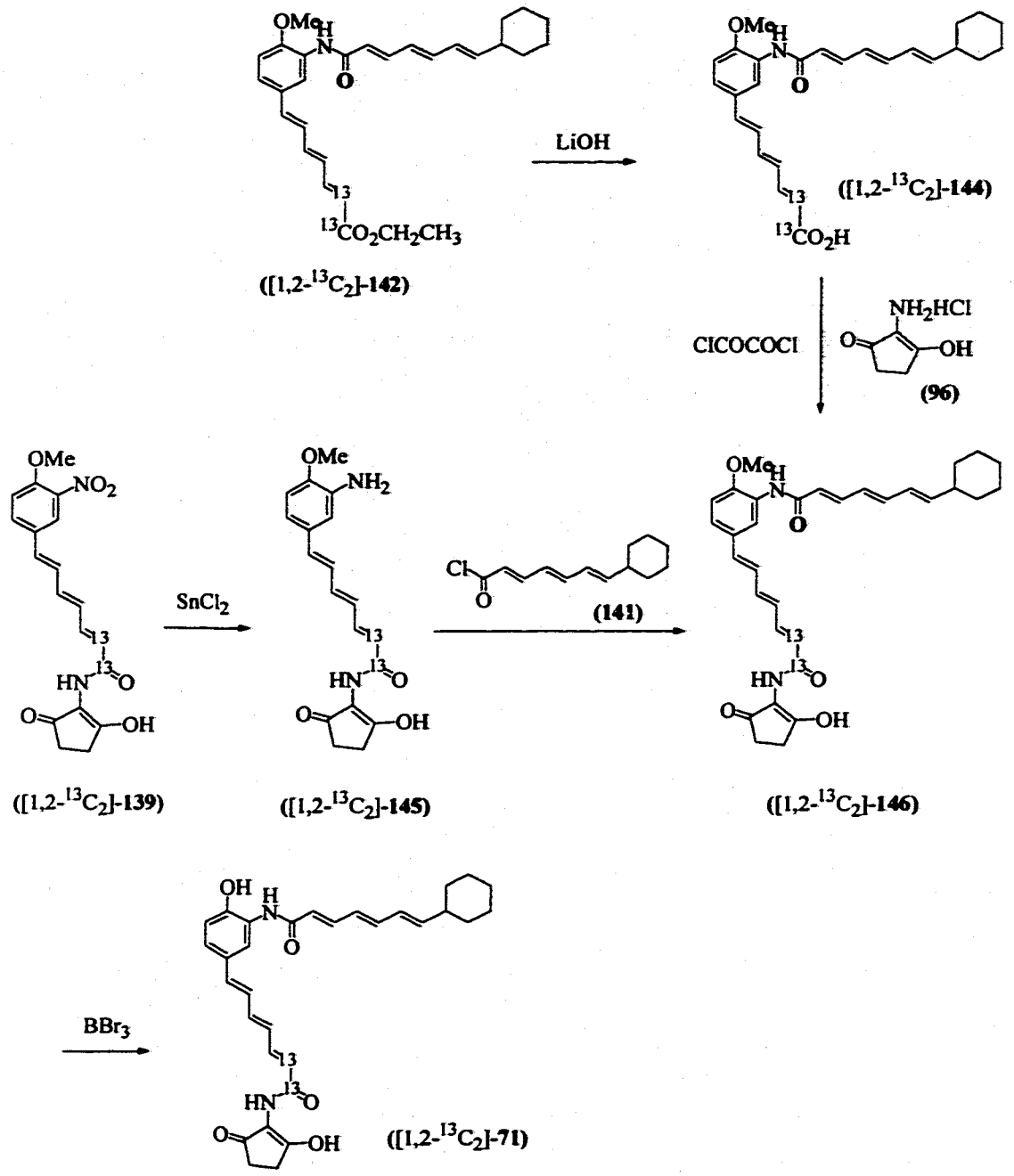


Figure 67. Synthesis of N_1-(2-Hydroxy-5-oxo-cyclopent-1-enyl)-7-[3-(7-cyclohexyl-hepta-(*2E, 4E, 6E*)-trienoyl)-amino-4-hydroxyphenyl]-[1,2-$^{13}C_2$]hepta-(*2E, 4E, 6E*)-trienamide

(*2E,4E,6E*)-trienamide ([1,2-$^{13}C_2$]-**145**) with tin(II) chloride dihydrate in a yield of 30% after column chromatographic separation.

Since the C_5N moiety decomposes under the acid chloride coupling conditions, the coupling of the "upper" chain was first tested on acid **95** and **145** with peptide coupling reagents DCC (Neises and Steglich 1978), EDCI (Dhaon *et al.*, 1982), BOP (Castro *et al.*, 1975), and PyBrOP. The latter reagent works for inert N-methylated amino acids (Coste *et al.*, 1991), but unfortunately none of these coupling reagents worked for the coupling of **145** and **95**. Thus, there were no choice but to use the acid chloride method. [1,2-$^{13}C_2$]-**145** was coupled with **142** in pyridine with DMAP as catalyst. The product N_1-(2-hydroxy-5-oxo-cyclopent-1-enyl)-7-[3-(7-cyclohexyl-hepta-(*2E,4E,6E*)-trienoyl)-amino-4-methoxyphenyl]-[1,2-$^{13}C_2$]hepta-(*2E,4E,6E*)-trienamide ([1,2-$^{13}C_2$]-**146**) was purified by column chromatography with methanol / methylene chloride (79%). Compound [1,2-$^{13}C_2$]-**146** was then demethylated with BBr_3 to afford N_1-(2-hydroxy-5-oxo-cyclopent-1-enyl)-7-[3-(7-cyclohexyl-hepta-(*2'E,4'E,6'E*)-trienoyl)-amino-4-hydroxyphenyl]-[1,2-$^{13}C_2$]hepta-(*2E,4E,6E*)-trienamide ([1,2-$^{13}C_2$]-**71**), which was purified by column chromatography with methanol / methylene chloride (51%). Compound **71** was characterized by NMR, ion trap MS/MS and high resolution mass spectroscopy.

6.6 Feeding of [1,2-$^{13}C_2$]-**70**, [1,2-$^{13}C_2$]-**71** and [1,2-$^{13}C_2$]-**94a** to *Streptomyces nodosus*

The ^{13}C doubly labeled compounds, [1,2-$^{13}C_2$]-**70**, [1,2-$^{13}C_2$]-**71** and [1,2-$^{13}C_2$]-**94a**, were fed to *Streptomyces nodosus*. Asukamycin was isolated from the fermentation broth and purified by column chromatography with methanol / methylene chloride and HPLC with methanol / water, and analyzed by ^{13}C-NMR and mass spectrometry. No incorporation of [1,2-$^{13}C_2$]-**70** and [1,2-$^{13}C_2$]-**94a** into asukamycin was observed. Also no aromatic ring oxidization / epoxidation of [1,2-$^{13}C_2$]-**70** and [1,2-$^{13}C_2$]-**94a** was seen. The amount of asukamycin produced upon feeding of [1,2-$^{13}C_2$]-**94a** was greatly reduced. The incorporation of compound **71** into asukamycin was clearly seen in the mass spectrum with a significantly enhanced ion at mass M + 2 and the appearance of ^{13}C coupled signals for C-1 and C-2 of the "lower" chain (δ 167.1 and 129 ppm, d, J = 65.9 Hz, Figure 68). The specific incorporation was determined by selective ion monitoring mass spectroscopy as 30% (Figure 69).

6.7 Conclusion

The fact that compound **71** was incorporated into asukamycin at a high efficiency demonstrates that oxidation / epoxidation is the last step on the biosynthetic pathway to type I manumycins. To our surprise, neither compound [1,2-$^{13}C_2$]-**70** nor [1,2-$^{13}C_2$]-**94a** was incorporated into asukamycin. If oxidation / epoxidation is the last reaction on the pathway, the second to last reaction has to be the attachment of either the "upper" chain or the C_5N moiety. Since [1,2-$^{13}C_2$]-**71** is incorporated into asukamycin, the permeability of the similar compounds **70** and **94a** through the cell membrane should not be a

4 peaks found in HU286.C, 9/27/99

peak	ppm	freq	amp
1	167.494	12642.35	252541.89
2	166.621	12576.43	368378.72
3	123.343	9309.83	264734.62
4	122.469	9243.91	191738.66

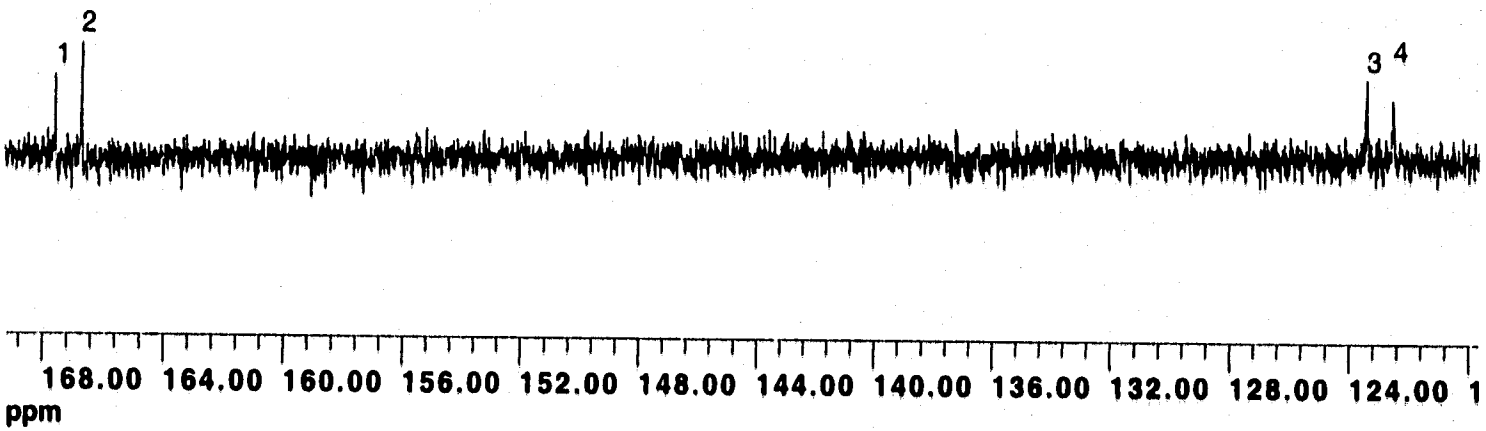


Figure 68. Enhanced Signals in ^{13}C-NMR Spectrum of Asukamycin After Feeding [1,2-$^{13}C_2$]-71

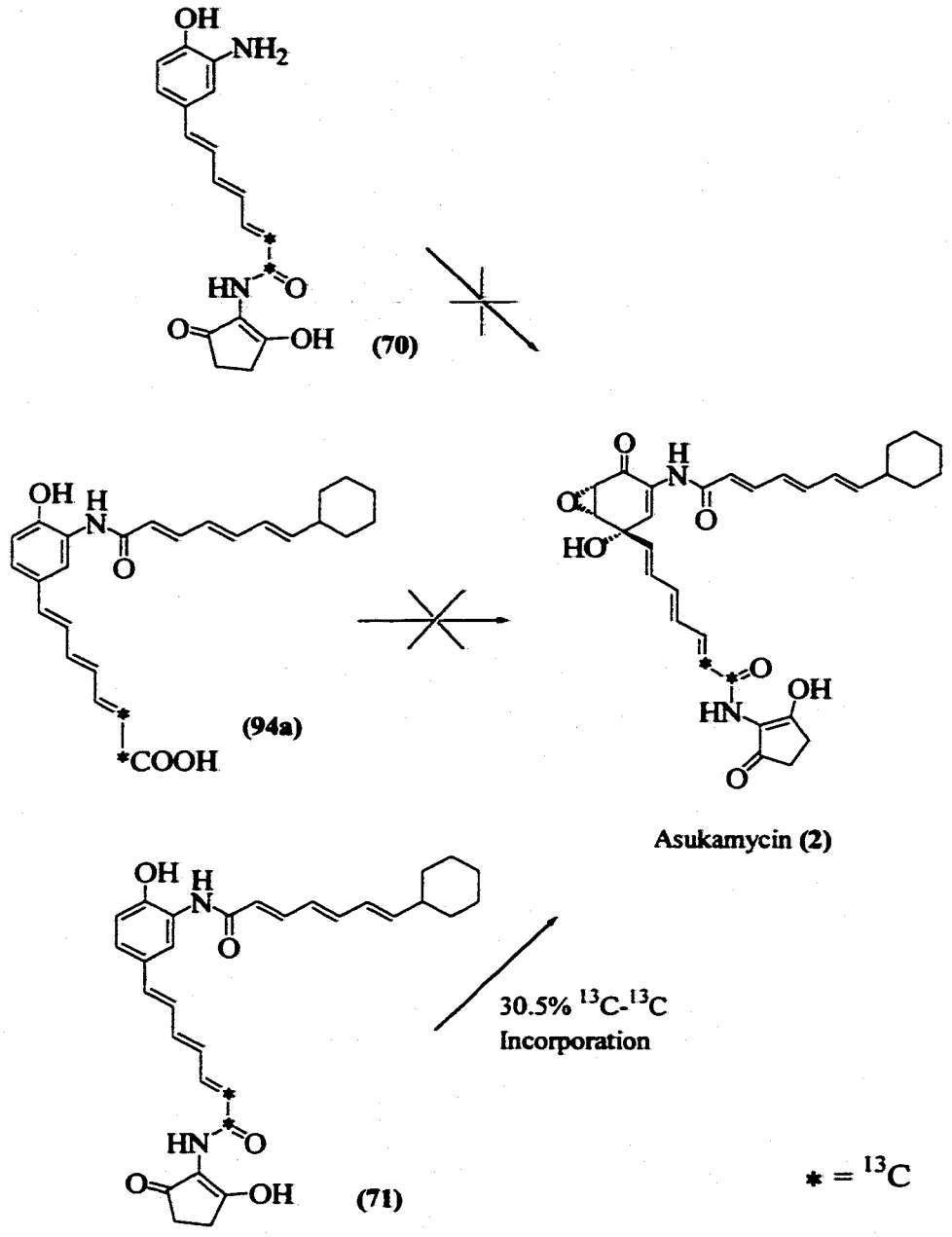


Figure 69. Feeding of ^{13}C Doubly-labeled **70**, **94a** and **70** to *S. nodosus*

problem. One possible explanation is that the "lower" chain is synthesized on the polyketide synthase and remains anchored there on its ACP through a thioester bond. The "upper" chain is then attached to the aromatic amino group by an amide synthase or acyltransferase, followed by formation of the amide bond to the C_5N unit by a second amide synthase with release of the product from the polyketide synthase enzyme. The intermediate is an enzyme-bound thioester of **94b**, rather than free acid **94a**. Therefore, **70** and **94a** are not free intermediates and could not be incorporated into asukamycin. A revised pathway hypothesis is illustrated in Figure 70. It is proposed that all the steps of the synthesis of the two polyketide chains and the assembly of all the components into the immediate asukamycin precursor, **71**, take place on one or more enzymes without any free intermediates between 3,4-AHBA (**50**), cyclohexanecarboxylic acid (**39**) and **96**, and the product **71**.

The following evidence supports the hypothesis that the "upper" chain is attached prior to the C5N unit. First, compound **94a** behaves as an inhibitor towards the production of asukamycin. It inhibits some enzyme(s) on the pathway, possibly the second amide synthase. In contrast, compound **70** has no effect on the production of asukamycin. Second, the discoveries of shunt metabolite **85** from *S. nodosus*, and compound **62**, 3,4-AHBA coupled with the "upper" chain from precursor directed biosynthesis experiments in *S. parvulus*, indicate the propensity for coupling between the aromatic amino group and an acylating reagent. Third, the fact that a relatively low percentage (3%) of ω-cyclohexyl fatty acids is present in the membrane and a relatively high percentage (54%)

of manumycins other than asukamycin is produced by *S. nodosus* suggests that the supply of cyclohexanecarboxylic acid for asukamycin production is insufficient. Therefore, the biosynthesis of cyclohexanecarboxylic acid, and maybe the subsequent synthesis of the "upper" chain as well, is the limiting step in the overall biosynthesis of asukamycin. Intermediate **69a** is therefore over-produced. The excess **69a** is acetylated and then released as shunt metabolite **85** exiting from the biosynthetic pathway. Fourth, the type I manumycin compound alisamycin (with a C_5N moiety) and nisamycin (without a C_5N moiety) are produced by a single strain, *Streptomyces* sp. K106 (Figure 71). This suggests that the attachment of the "upper" chain occurs prior to that of the C_5N moiety. It is noted that the oxygenase in alisamycin biosynthesis is different from the one in asukamycin biosynthesis, because the former is able to oxidize acid **147**, along with **148**, to alisamycin and nisamycin with opposite stereochemistry of the mC_7N unit.

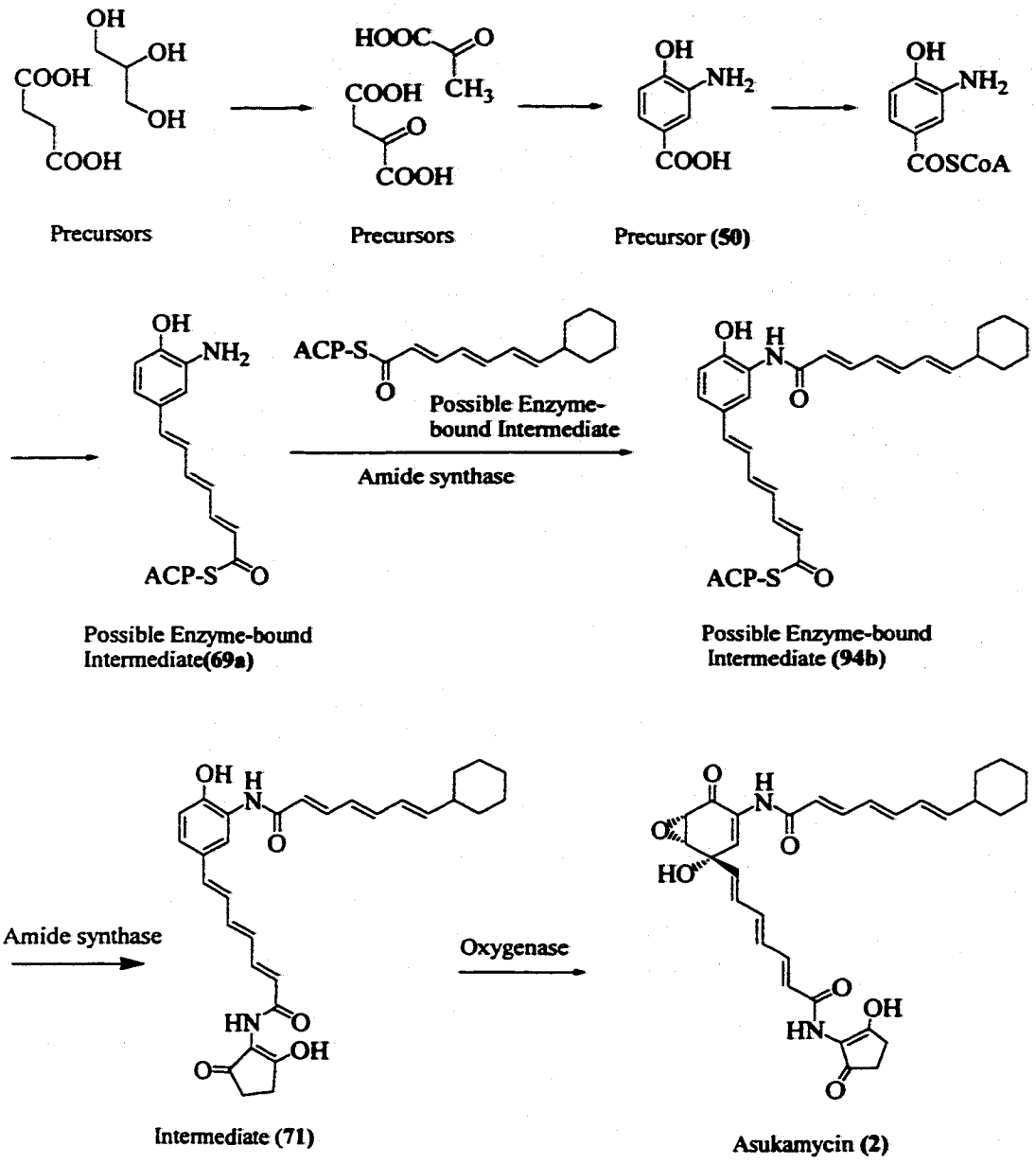


Figure 70. Revised Biosynthetic Pathway of Asukamycin

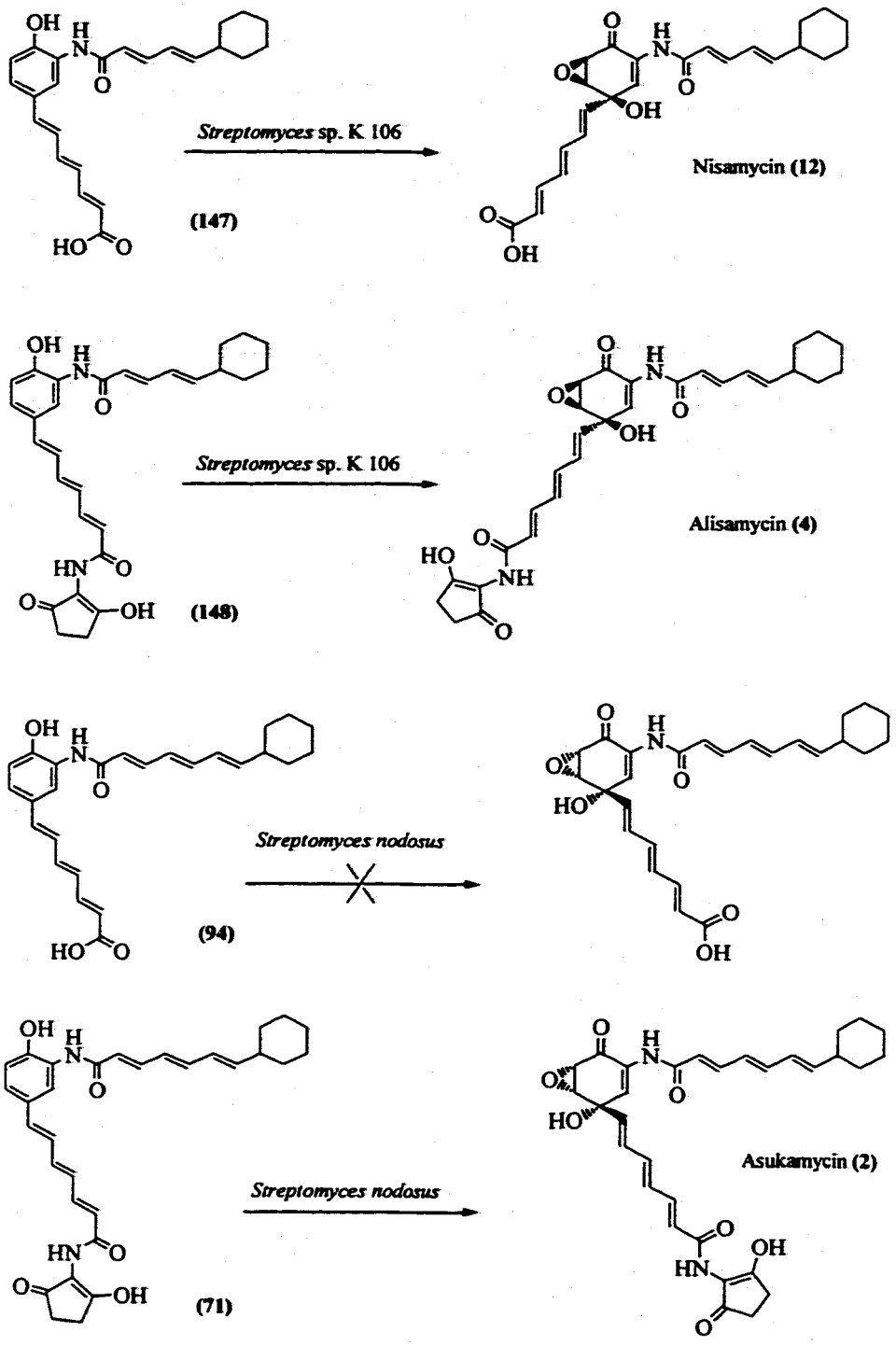


Figure 71. Oxidation / Epoxidation of mC_7N Unit in *Streptomyces* sp. K 106 and *Streptomyces nodosus*

CHAPTER 7. SUMMARY AND FUTURE PROSPECT

7.1 Summary

7.1.1 Overall Biosynthetic Pathway to the Manumycins

Prior to the work in this thesis, extensive biosynthetic studies on manumycin A and asukamycin had been accomplished. Although the origin of the carbon skeleton, the C_5N unit and the "upper" chain had been elucidated and the sources of the heteroatoms identified, a number of questions still remained. The biosynthesis of the mC_7N unit, the oxidation / epoxidation mechanism and stereochemistry, and the order of the "lower" polyketide chain extension, the "upper" polyketide chain formation and attachment, the C_5N unit attachment, and epoxyquinol formation were all unknown.

In this thesis, the investigation of the biosynthesis of manumycin type metabolites has been carried out mainly with the asukamycin producer, *Streptomyces nodosus*. The biosynthetic pathway was elucidated in two approaches: i) by synthesizing and feeding labeled compounds to *S. nodosus* and *S. parvulus*; ii) by tracing and isolating pathway related metabolites after feeding isotope labeled compounds to reveal the biosynthetic pathway. Based on the work in this thesis, a complete biosynthetic pathway to the manumycin type metabolites has been pinpointed (Figure 72)

7.1.2 Summary of This Work

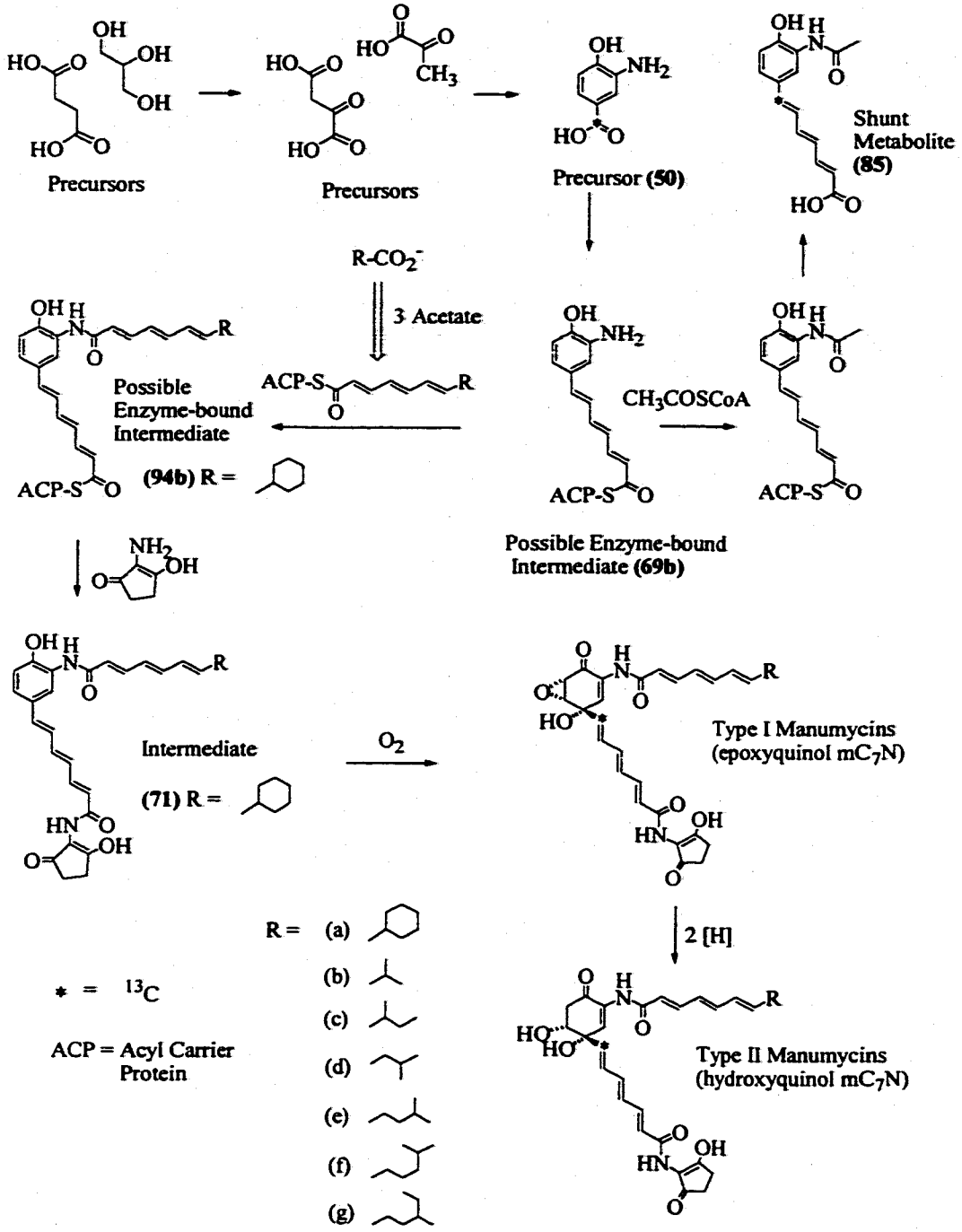


Figure 72. Complete Biosynthetic Pathway of Manumycins Produced by *Streptomyces nodosus*

Work in this thesis is summarized as follows, along with the illustration of the biosynthetic pathway in Figure 72:

1. 3-Amino-4-hydroxybenzoic acid (3,4-AHBA) has been revealed as the key precursor to the mC_7N unit. 3,4-[7-^{13}C]-AHBA was synthesized and fed to *Streptomyces nodosus* and *Streptomyces parvulus*. 3,4-[7-^{13}C]-AHBA and 3,4-[2-^{2}H]-AHBA were incorporated into asukamycin and manumycin A and B.
2. The biosynthetic pathway proceeds with "lower" polyketide chain extension which is initiated by an activated 3,4-AHBA unit. This was indicated by the discovery of a shunt metabolite, 7-(3-N-acetylamino-4-hydroxyphenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienoic acid **85**. [Acetyl-$^{2}H_3$, carboxyl-^{13}C]-3-N-acetylamino-4-hydroxybenzoic acid **(90)** and [1,2-$^{13}C_2$]-**85** were synthesized and fed to *S. nodosus*, resulting in no incorporation of [Acetyl-$^{2}H_3$, carboxyl-^{13}C]-**90** into **85** and no incorporation of [1,2-$^{13}C_2$]-**85** into asukamycin. This comfirms that **85** is a shunt metabolite and suggests that the acetylation of the aromatic amino group occurs after the "lower" polyketide chain extension.
3. The synthesis and feeding of ^{13}C-doubly labeled acids, 3-(3-amino-4-hydroxy-phenyl)-*E*-prop-2-enoic acid **(67b)**, 5-(3-amino-4-hydroxyphenyl)-(*2E,4E*)-penta-2,4-dienoic acid **(68b)** and 7-(3-amino-4-hydroxyphenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienoic acid **(69b)** and 3-(3-amino-4-hydroxyphenyl)-*E*-prop-2-enoic acid N-acetylcysteamine thioester **(84)** produced no incorporation into asukamycin. This

suggests that the "lower" polyketide chain is synthesized in a closed system with ACP thioesters as the enzyme-bound substrates.

4. Six type I manumycins were discovered in *S. nodosus* along with asukamycin, two of them are new natural products. These co-metabolites differ from asukamycin in the starter unit of the "upper" chain. The diversity of the "upper" chain of manumycins is attributed to the branched and cyclic acids used as the starter units for the biosynthesis of both primary metabolite, fatty acid, and secondary metabolite, manumycin. This argument was supported by feeding labeled cyclopentanecarboxylic acid **93**, which resulted in the formation of two new fatty acids and one new manumycin type compound, **5**.

5. The quantitative studies on the composition of fatty acids and manumycins produced by *S. nodosus* indicated that the biosynthesis of cyclohexanecarboxylic acid **39** is the limiting step of asukamycin production, further implying that the biosynthesis of the "upper" chain may be a limiting step as well, with the consequence that the excess intermediate **69a** is acetylated and released from the enzyme as the accumulated shunt metabolite **85**.

6. Multi-step syntheses of ^{13}C doubly labeled compounds **70**, **71** and **94a** were designed and accomplished. The feeding of the three compounds and the incorporation of **71** revealed that oxidation / epoxidation is the last reaction on the pathway to the type I manumycins. The fact that both **70** and **94a** were not incorporated indicates that the "upper" chain attachment occurs prior to that of the C_5N unit, and the resulting intermediate is the enzyme-bound ACP thioester **94b**

rather than the free acid **94a**. The relative production of manumycin type compounds versus shunt metabolite **85** is determined by the N-acylation of intermediate **69a**.

7. Five new type II manumycins were discovered. The study of the fermentation time course showed that shunt metabolite **85** is produced early in the fermentation, and the type II manumycins are produced at a later stage. The type II manumycins are formed from the type I manumycins by reduction of the epoxide.

Overall, this research studied various aspects of the biosynthesis of manumycins produced by *Streptomyces nodosus* and revealed the complete biosynthetic pathway. This pathway presumably applies also to manumycins produced by other *Streptomycetes*. The mechanism of 3,4-AHBA formation from PEP and oxalacetic acid is still unknown, and since the postulated intermediates in Gould's proposed mechanism (Scheme 16) are impossible to synthesize due to their likely instability, this mechanism can only be elucidated through a biochemical or genetic approach.

7.2 Future Prospects

Unlike the production of rifamycin, in which the biosynthesis is accomplished by a main pathway followed by modifications (e.g. methylation, acetylation), the biosynthesis of the manumycins involves three closely entangled pathways which make the biosynthesis more complicated and its investigation more interesting.

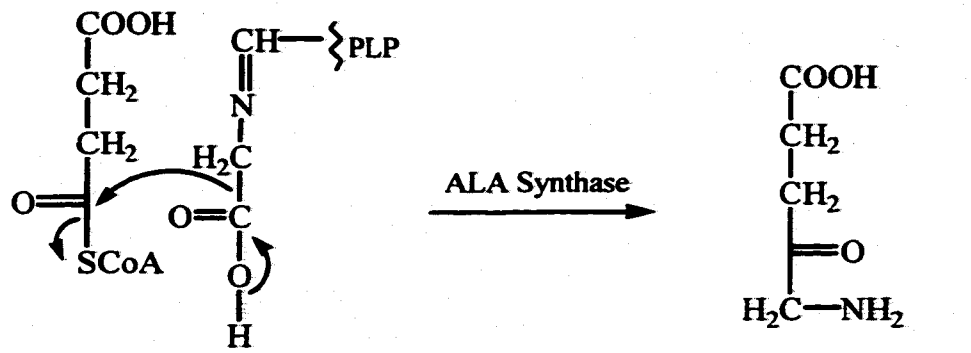
Future biosynthetic studies on this pathway should focus on its molecular biology and enzymology. The extensive pathway studies and the synthesis of labeled precursors and isolation of new pathway related metabolites have laid a solid foundation and provided useful tools for future genetic and enzymology work.

7.2.1 Future Research in Molecular Biology

The future genetic work would involve the location, analysis and expression of the asukamycin biosynthetic gene cluster and should lead to the elucidation of the mechanism of 3,4-AHBA formation, which is not amenable to chemical approaches. Available probes for two known pathway genes encoding 5-aminolevulinate synthase and 1-cyclohexenylcarbonyl CoA reductase could be used to locate and identify the genes involved in the biosynthesis of asukamycin. The insertional mutation technique can be employed to verify the identity of the gene cluster and later the function of the individual gene. Isolation and isotope tracing techniques developed in this thesis could also be used. For example, the intermediates downstream from 3,4-AHBA accumulated in mutants can be identified by feeding ^{13}C-labeled 3,4-AHBA and isolated by the techniques used in the isolation of shunt metabolite **85** and type II manumycins. Individual gene (s) of particular interests are the ones involved in the assembly of 3,4-AHBA from its basic building blocks, the two polyketide synthases, and the epoxyquinol-forming dioxygenase.

7.2.1.1 Locate the asukamycin biosynthetic gene cluster in *S. nodosus*

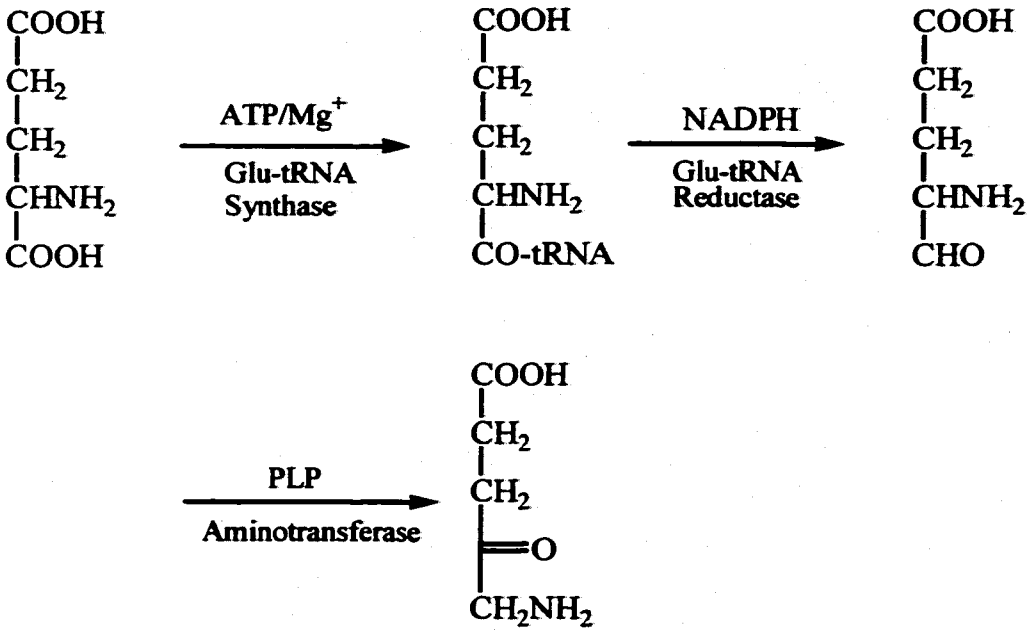
Since biosynthetic genes in *Streptomycetes* are usually clustered, the identification of a specific gene in the cluster should lead to the discovery of the whole gene cluster. Two specific gene probes could be used to locate the asukamycin biosynthetic gene cluster from a cosmid library of *S. nodosus* ssp. *asukaensis* genomic DNA. The first one is a probe for the 5-aminolevulinic acid (ALA) synthase gene from this organism, which was obtained from Dr. Petricek of the Institute of Microbiology, Academy of Science of the Czech Republic. As the earlier feeding experiments have shown, the C_5N unit is formed by intramolecular cyclization of 5-aminolevulinic acid (ALA) which is derived from the Shemin (C_4) pathway via the condensation of succinyl-SCoA and a PLP activated glycine, with simultaneous decarboxylation (Bohinski 1987; Shemin and Rittenberg 1946; Shemin and Rittenberg 1951; Torssell 1997). Another ALA biosynthetic pathway is found in nature, the glutamate (C_5) pathway, in which glutamic acid is activated by ligation to t-RNA in an ATP requiring reaction catalyzed by glutamyl-tRNA synthase, and then reduced by a nucleotide-linked Glu-tRNA reductase, followed by transamination catalyzed by a PLP-dependent aminotransferase to yield ALA (Beals 1990) (Figure 73). Dr. Petricek has examined various *Streptomycetes* for the presence of the two pathways by hybridization with specific gene probes, the Glu-t-RNA reductase gene for the C_5 pathway and the ALA synthase gene for the Shemin pathway (C_4). It was found that all *Streptomycetes* examined had the C_5 pathway but the asukamycin producer additionally had the Shemin pathway. This C_4 pathway gene probably functions



Succinyl-SCoA Glycine-PLP

5-aminolevulinic acid

Shemin (C_4) Pathway



Glutamate (C_5) Pathway

Figure 73. Two Biosynthetic Pathways of 5-Aminolevulinic Acid

specifically in asukamycin biosynthesis and is part of the biosynthetic gene cluster.

Formation of the "upper" chain of asukamycin is initiated by cyclohexanecarboxylic acid CoA ester derived from shikimic acid. The last enzyme on the pathway from shikimate, 1-cyclohexenylcarbonyl-CoA reductase, has been isolated and cloned by Professor Reynolds at the University of Maryland from the ansatrienin A producer, *S. collinus* Tü 1892. The second probe thus is the *chcA* gene which encodes the 1-cyclohexenylcarbonyl-CoA reductase and was obtained from Professor Reynolds. Hybridization of *chcA* to genomic DNA of *S nodosus* has been demonstrated (Yu 1996).

Other probes, such as polyketide synthase gene probes (type I and II PKS), could be used to locate the PKS genes of asukamycin production. It was reported that preliminary hybridization studies with the polyketide synthase genes *actI* and *actIII* on *Streptomyces nodosus* yielded positive results (Sherman *et al.*, 1988).

One of these genes could be used to isolate by colony hybridization cosmid clones or BAC clones carrying this gene. These clones should be mapped and probed with the other probes to confirm their presence in the same region of DNA. If it is found that the ALA synthase gene, two sets of PKS genes and *chcA* are all located in one cluster, it probably means that this is the asukamycin biosynthesis gene cluster.

7.2.1.2 Prove the biosynthetic gene cluster

The targeted pathway genes can be confirmed by insertional inactivation of genes within the cluster and demonstration of the loss of asukamycin production. The isolation and identification of the products from these mutants would identify the function of the mutated genes, e.g., i) the accumulation of 3,4-AHBA implies that the insertional inactivation occurs at the "lower" chain polyketide synthase; ii) the accumulation of compounds similar to **69b**, or even shunt metabolite **85** would suggest that the insertional inactivation is at the "upper" chain polyketide synthase or genes involved in the C_5N unit biosynthesis; iii) if the manumycins except asukamycin are detected, probably genes involved in the biosynthesis of cyclohexanecarboxylic acid **39** are inactivated, and the production of asukamycin should be restored upon feeding **39**; iv) if asukamycin is produced only upon feeding of 3,4-AHBA, the insertional inactivation must have occured in a gene involved in 3,4-AHBA synthesis; v) the accumulation of **71** would indicate that an oxygenase is mutated. All these accumulated intermediates could be easily isolated and identified by the separation techniques and isotope tracing methods described in this thesis.

7.2.1.3 Express interesting genes and elucidate the asukamycin biosynthetic pathway by the genetic approach

Once the biosynthetic gene cluster has been identified, the PKS region(s) should be sequenced to identify the functions of the individual components of the PKSs by

homologous alignment with available PKS genes in the data banks. This is the easiest approach to decode the asukamycin biosynthetic gene cluster due to the extensive genetic studies on polyketide biosynthesis. The "upper" and "lower" chain PKSs synthesize exclusively *trans* triene acids. The "lower" chain PKS may play a role in the assembly of the "upper" chain and C_5N unit. The "upper" chain PKS, which is able to accommodate a variety of starter units, has potential application in combinatorial biosynthesis.

By mutational analysis, specific locations within the gene cluster can be inactivated to create asukamycin non-producing mutants. Complementary feeding with ^{13}C-labeled 3,4-AHBA or cyclohexanecarboxylic acid to these mutants and the isolation of accumulated intermediates will identify the functions of the inactivated gene, therefore pinpointing aspects of the biosynthetic pathway which are not accessible from a chemical approach. For instance, the insertional inactivation of genes involved in the C_5N unit biosynthesis, especially the ALA cyclase, may result in intermediate **94**, confirming the hypothesis of the attachment of the "upper" chain prior to that of the C_5N unit.

Another unknown area in the biosynthetic pathway is the formation of 3,4-AHBA, which involves condensation of a C_3 and a C_4 component followed by cyclization. By mutation and complementary feeding of 3,4-AHBA, all the genes required for 3,4-AHBA biosynthesis can be identified and eventually expressed. It is suggested to construct a recombinant enzyme system for 3,4-AHBA synthesis using PEP and oxalacetic acid as substrates, representing the C_3 and C_4 components, respectively, plus a nitrogen donor.

Once the formation of 3,4-AHBA has been established, the reconstituted system can be dissected to establish the role of the individual components and to study their reaction mechanisms.

7.2.2 Future research in Enzymology

It is known that the epoxyquinol structure in the mC_7N unit is important to the biological activity of manumycin type metabolites. For this reason the oxygenase which catalyzes the oxidation / epoxidation reaction, is of particular interest. It was proposed by Gould and supported by Taylor's work that it is a concerted di-oxygen insertion reaction catalyzed by a dioxygenase to provide a *syn* epoxyquinol structure (Alcarz *et al.*, 1998; Cone *et al.*, 1995). It should be pointed out that the dioxygenase in *Streptomyces* sp. K 106, the alisamycin and nisamycin producer, yields the completely opposite stereochemistry in the mC_7N unit, compared to the rest of the manumycin type metabolites. Another notable characteristic is that the dioxygenase in *Streptomyces* sp. K 106 may catalyze the oxidation / epoxidation on compound **148** to afford nisamycin, whereas the dioxyenase in *S. nodosus* can not oxidize compound **94** (Figure 71).

Since compound **71** has been incorporated into asukamycin, the investigation on oxidation / epoxidation could proceed with an available enzyme assay. Compound **71** is the substrate for the enzyme and could be used in the assay and subsequent isolation and purification of the dioxygenase from a cell-free extract. The oxidation / epoxidation

mechanism and stereochemistry can be studied using the purified dioxygenase. It is particularly interesting to conduct this study in parallel with the dioxygenase from *Streptomyces* sp. K 106. Both dioxygenases are useful in biosynthesizing biological active compounds in future applications.

CHAPTER 8. EXPERIMENTAL

8.1 Materials

Streptomyces nodosus ssp. asukaensis (ATCC 29757) was purchased from American Type Culture Collection, *Streptomyces parvulus* Tü 64 was obtained from Professor Axel Zeeck, University of Göttingen, Germany. Fermentation ingredients were purchased from Difco and Sigma. Chemicals were purchased from Aldrich and Lancaster, nitrogen dioxide was purchased from Sigma. 4-Hydroxy-[7-^{13}C]benzoic acid was purchased from Cambridge Isotope Laboratories, triethyl phosphono-[1,2-$^{13}C_2$]acetate was purchased from Sigma and ^{13}C-labeled carbon dioxide from Isotec. 3-Amino4-hydroxy-[2-^{2}H]benzoic acid was obtained from Professor Steven J. Gould, Oregon State University. [U-^{13}C]Malonic acid had been synthesized previously. All chemicals and solvents were of reagent or HPLC grade and were used without further purification unless otherwise noted.

8.2 Instrumentation

^{1}H, ^{2}H and ^{13}C-NMR spectra were obtained on Bruker AF 300 and AM 500 spectrometers in deuterated solvents $CDCl_3$, acetone-d_6, DMSO-d_6 and pyridine-d_6. Chemical shifts are given in parts per million (ppm) and are adjusted to the TMS scale by reference to the solvent signal. Data are reported as follows: chemical shift, multiplicity,

integration, coupling constant (Hertz), assignment. EI-MS was carried out on a Kratos Profile HV-3 mass spectrometer and GC-MS on a Hewlett-Packard 5790A series gas chromatograph with a Kratos Profile HV-3 mass spectrometer. Electrospray mass spectra (positive ion mode) were recorded on a Kratos Profile HV-4 double focusing magnetic sector mass spectrometer. Electrospray mass spectra (negative ion mode) and tandem mass spectra were recorded on a Bruker Esquire ion trap mass spectrometer. LC-MS was carried out with Shimadzu LC-10AD pumps (2), a SPD-10AV UV-Vis variable detector and a Micromass Quattro II Tandem Quadrupole mass spectrometer. High resolution mass spectra were obtained on a micromass 70SEQ tandem hydrid mass spectrometer. The mass spectra and tandem mass spectra of the series of type I and type II manumycins were obtained by LC-MS. The isotopic distributions in asukamycin, manumycin A and B, 7-(3-N-acetylamino-4-hydroxyphenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienoic acid and new type II manumycins were determined by selected ion monitoring (SIM) on a micromass Quattro II tandem quadrupole mass spectrometer. Fermentations were carried out in a New Bruswick G25 controlled environment incubator shaker or in an Adolf Kühner ISF-4-V rotary shaker cabinet. Sterilization of solutions, fermentation medium and glassware was carried out in an Amsco 2332 isothermal steam autoclave operating at 121 °C and 18 psi. pH was measured on a pH electrode. Centrifugations were done in a Sorvall RC5C refrigerated centrifuge operating at 4 °C using a GSA rotor. Small scale centrifugations were performed with a IEC model CL centrifuge. Cell-free extracts were prepared using an American Instrument French Press. UV spectra were recorded on a Hewlett-Packard 8452 diode array spectrophotometer.

8.3 Chromatography

Analytical TLC was performed on precoated silica gel plates (aluminum backing, 0.25 mm layer, UV-254 fluorescence) from EM Science. Compounds on the plates were visualized under UV light at 254 and/or 310 nm, or spotted in an iodide chamber or immersed in a ninhydrin solution and heated with a heat-gun. Mobilities are quoted relative to the solvent front (R_f). Preparative TLC was carried out on precoated silica gel plates (glass backing, 2.0 mm layer, UV-254 fluorescence) from EM Separations Technology. Flash column chromatography was performed on 230-400 mesh silica gel from Aldrich, and reverse-phase C_{18} silica gel from Whatman. HPLC was conducted with a Beckman model 116 isocratic pump and Beckman model 166 absorbance detector using C_{18} reverse-phase analytical or semi-preparative columns.

8.4 Fermentation and Feeding Experiments

8.4.1 Fermentation and Feeding Experiments with *Streptomyces nodosus*

Streptomyces nodosus ssp. *asukaensis* was grown on yeast-malt extract agar plates and incubated at 28 °C for 4 days, then was stored at 0 °C. A loop of *S. nodosus* was transferred into 100 mL of culture medium in a 500 mL baffled Erlenmeyer flask and grown on a rotary shaker at 300 rpm for 2 days at 28 °C. Ten mL seed culture was used to inoculate 100 mL culture medium. Culture medium contained glucose 20 g, Bacto

Peptone 5 g, K_2HPO_4 0.25 g, $MgSO_4$ 0.25 g, trace elements $(NH_4)_6Mo_7O_{24}x4H_2O$ 5 mg, $FeSO_4x7H_2O$ 50 mg, $CuSO_4x5H_2O$ 5 mg, $ZnSO_4x7H_2O$ 5 mg, $MnCl_2x4H_2O$ 10 mg, deionized water 1000 mL, pH 7.0. Both media were sterilized for 20 min at 121 °C in an autoclave.

In general, single doses of labeled compounds were administered to the production culture after 24 hour. The labeled compounds were added in the amounts indicated per culture volume: ammonium salt of 3,4-[2-^{2}H]-AHBA dissolved in DI H_2O, 15 mg/100 mL (0.877 mM); 3,4-[7-^{13}C]-AHBA dissolved in 5% K_2CO_3, 10 mg/100 mL (0.649 mM); 3-(3-amino-4-hydroxyphenyl)-*E*-[1,2-$^{13}C_2$]prop-2-enoic acid ([1,2-$^{13}C_2$]-**67b**) dissolved in 5% K_2CO_3, 15 mg/100 mL (0.829 mM); 5-(3-amino-4-hydroxyphenyl)-(*2E,4E*)-[1,2-$^{13}C_2$]penta-2,4-dienoic acid ([1,2-$^{13}C_2$]-**68b**) dissolved in 5% K_2CO_3, 15 mg/100 mL (0.725 mM); 7-(3-amino-4-hydroxyphenyl)-(*2E,4E,6E*)-[l, 2-$^{13}C_2$]hepta-2,4,6-trienoic acid ([1,2-$^{13}C_2$]-**69b**) dissolved in 5% K_2CO_3, 20 mg/100 mL (0.858 mM); 3-(3-amino-4-hydroxyphenyl)-*E*-[1,2-$^{13}C_2$]prop-2-enoic acid N-acetylcysteamine thioester ([1,2-$^{13}C_2$]-**84**) dissolved in DMSO, 20 mg/100 mL (0.707 mM); N_1-(2-hydroxy-5-oxo-cyclopent-1-enyl)-7-(3-amino-4-hydroxyphenyl)-[1,2-$^{13}C_2$]hepta-(*2E,4E,6E*)-trienamide ([1,2-$^{13}C_2$]-**70**) dissolved in 5% K_2CO_3, 7 mg/60 mL (0.356 mM); 7-[3-(7-cyclohexyl-hepta-(*2E,4E,6E*)-trienoyl)-amino-4-hydroxyphenyl]-[1,2-$^{13}C_2$]hepta-(*2E,4E,6E*)-trienoic acid (**94a**) dissolved in 5% K_2CO_3, 9 mg/60 mL (0.356 mM); N_1-(2-hydroxy-5-oxo-cyclopent-1-enyl)-7-[3-(7-cyclohexyl-hepta-(*2E,4E,6E*)-trienoyl)-amino-4-hydroxyphenyl]-[1,2-$^{13}C_2$]hepta-(*2E,4E,6E*)-trienamide ([1,2-$^{13}C_2$]-**71**) dissolved in 5% K_2CO_3, 11 mg/60 mL

(0.356 mM). In the fermentation time course study, 3,4-[7-^{13}C]-AHBA was administered to *Strepomyces nodosus* at the time when the production culture was inoculated.

8.4.2 Fermentation and Feeding Experiments with *Streptomyces parvulus*

Streptomyces parvulus Tü 64 was grown on yeast-malt extract agar plates, incubated at 28 °C for 4 days, and subsequently stored at 0 °C. A loop of *S. parvulus* was transferred into 100 mL of culture medium in a 500 mL baffled Erlenmeyer flask and grown on a rotary shaker at 300 rpm for 2 days at 28 °C. Ten mL of seed culture was used to inoculate 100 mL of culture medium. The Culture medium contained soybean meal 20 g, mannitol 20 g, deionized water 1000 mL, pH 7.0. Both media were sterilized for 15 min at 121 °C in an autoclave.

Ten mg of 3,4-[7-^{13}C]-AHBA dissolved in 5% K_2CO_3 was administered to the production culture at 24 hour, based on per 100 mL of production medium.

8.5 Isolation, Purification and Identification of Manumycins and Shunt Metabolite

8.5.1 Isolation, Purification and Identification of Type I Manumycins

Streptomyces nodosus or *Streptomyces parvulus* production cultures were harvested 72 hours after inoculation. The fermentation broth was centrifuged at 9000 rpm for 25 min. The supernatant was saturated with NaCl and extracted three times with ethyl acetate.

The mycelium was extracted with acetone, and the acetone extracts were concentrated. The residue was extracted with ethyl acetate. The ethyl acetate extracts from supernatant and mycelium were combined, dried and the ethyl acetate was evaporated *in vacuo* to afford the crude products.

The crude products were purified on 2.0 mm preparative TLC plates which were developed three times with chloroform/methanol (9:1), or on a silica gel column eluting with methylene chloride/methanol (100:3) {type I manumycins R_f 0.42 [silica gel, MeOH/$CHCl_3$ (10:1)]}. The products were further purified using semi-preparative C_{18} reverse-phase HPLC eluting with methanol / water or acetonitrile / water. The HPLC purification chromatogram of the type I manumycins from *Streptomyces nodosus* is shown in Figure 38 (Chapter 4). Three fractions (1, 2 and 4) were collected, and identified as following compounds.

Manumycin G **(12)** (HPLC fraction 1): Tr 6.4 min (acetonitrile / water 70:30, flow rate 4 mL/min). ^{13}C-NMR see Chapter 4 Table 5. ES-MS/MS see Appendix A Figure 74, $[M+H]^+$ 507, daughter ion 489, 149.

U-56,407 **(14)** and EI-1511-5 **(9)** (HPLC fraction 2): Tr 8.2 min (acetonitrile / water 70:30, flow rate 4 mL/min). ^{13}C-NMR see Chapter 4 Table 6. ^{1}H-NMR spectrum (the "upper" chain terminus) see Figure 40. ES-MS/MS see Appendix A Figure 75, $[M+H]^+$ 521, daughter ion 503, 163.

Asukamycin **(2)** (HPLC fraction 4): Tr 12.1 min (acetonitrile / water 70:30, flow rate 4 mL/min). ^{13}C-NMR (300 MHz, acetone-d_6) δ_C 190.0, 166.7, 165.6, 145.9, 143.2, 142.8, 141.7, 140.3, 139.3, 132.4, 131.4, 129.2, 128.6, 128.4, 124.1, 123.6, 115.3, 71.9, 57.9, 53.4, 41.8, 33.3, 26.7, 26.5. ES-MS/MS see Chapter 4 Figure 42, $[M+H]^+$ 547, daughter ion 529, 189.

Other minor components detected by LC-MS/MS are tentatively assigned as:

Manumycin E **(10):** ES-MS/MS see Appendix A Figure 76, $[M+H]^+$ 535, daughter ion 517, 177.

New type I manumycin metabolites **6a** and **6b**: ES-MS/MS see Chapter 4 Figure 43, $[M+H]^+$ 549, daughter ion 531, 191.

The feeding of [6-^{13}C]cyclopentanecarboxylic acid [6-^{13}C]-**93** (4 mM, standard feeding condition as described in 8.4.1) resulted in the new artificial type I manumycin compound [7"-^{13}C]-**7**, which is inseparable from asukamycin by HPLC. Compound [7"-^{13}C]-**7** was characterized by ion trap ES-MS.

Compound [7"-^{13}C]-**7**: ion-trap ES-MS/MS (Appendix A Figure 77) m/z 532 $[M-H]^-$, daughter ions 514, 401, 357, 339, 322, 216, 178. HR-MS m/z for $C_{29}{}^{13}CH_{32}N_2O_7Na$,

$[M+Na]^+$ calculated 556.2141, found 556.2131. The percentage of compound [7"-^{13}C]-7 in the mixture of type I manumycins see Chapter 4 Table 11.

8.5.2 Isolation, Purification and Identification of Type II Manumycins

The *Streptomyces nodosus* production culture was harvested after 3 ½ days. After extraction of the supernatant and mycelium, crude extracts were purified on a silica-gel column by elution with methylene chloride/methanol 50:1. The fractions containing unknown compounds {TLC R_f 0.30 [silica gel, MeOH/$CHCl_3$ (10:1)]} with an enhanced ^{13}C-NMR signal at 141 ppm were further purified by HPLC eluting a with gradient of acetonitrile / water 57.5:42.5 to 62.5:37.5 over 15 min at flow rate 4 mL/min. Four fractions were collected (see Chapter 5 Figure 49).

^{13}C-NMR spectra were taken to trace these unknown compounds during purification and ^{1}H-NMR, LC-MS, ES-MS/MS and HR-MS were used to reveal their structures. This series of new natural products were assigned as the following type II manumycins.

Compound 21 (HPLC fraction 1, Tr 8.5 min): ^{1}H-NMR (500 MHz, acetone-d_6) δ_H 7.53 (d, 1H, J = 1.9 Hz, H-3), 7.34 (dd, 1H, J = 14.8, 11.4 Hz, H-11), 7.25 (dd, 1H, J = 14.8, 11.1 Hz, H-3'), 6.79 (dd, 1H, J = 14.8, 11.1 Hz, H-9), 6.64 (dd, 1H, J = 15.1, 11.1 Hz, H-8), 6.62 (dd, 1H, J = 14.8, 10.8 Hz, H-5'), 6.58 (d, 1H, J = 14.8 Hz, H-12), 6.50 (dd, 1H, J = 14.8, 11.4 Hz, H-10), 6.38 (d, 1H, J = 14.8 Hz, H-2'), 6.30 (dd, 1H, J = 14.8, 11.2 Hz,

H-4'), 6.22 (d, 1H, J = 14.8 Hz, H-7), 6.18 (dd, 1H, J = 15.5, 10.8 Hz, H-6'), 5.93 (dd, 1H, J = 15.5, 7.4 Hz, H-7'), 4.04 (m, 1H, H-5), 2.46 (br s, 4H, H-4", H-5"), 2.40 (m, 1H, H-8'), 1.01 (d, 6H, J = 6.8 Hz, H-9'). ES-MS/MS (from LC-MS, Appendix B, Figure 78) $[M+H]^+$ 509, daughter ions 491, 149.

Compound 22 (major) and **23** (minor) (HPLC fraction 2, Tr 11.1 min), data from the major component (**22**): ^{1}H-NMR (500 MHz, acetone-d_6) δ_H 7.54 (d, 1H, J = 1.9 Hz, H-3), 7.36 (dd, 1H, J = 14.8, 11.7 Hz, H-11), 7.26 (dd, 1H, J = 14.8, 11.4 Hz, H-3'), 6.79 (dd, 1H, J = 14.8, 11.4 Hz, H-9), 6.65 (dd, 1H, J = 14.8, 11.1 Hz, H-8), 6.65 (dd, 1H, J = 14.8, 11.1 Hz, H-5'), 6.58 (d, 1H, J = 14.8 Hz, H-12), 6.51 (dd, 1H, J = 14.8, 11.4 Hz, H-10), 6.39 (d, 1H, J = 14.8 Hz, H-2'), 6.33 (dd, 1H, J = 14.8, 11.4 Hz, H-4'), 6.23 (d, 1H, J = 14.8 Hz, H-7), 6.19 (dd, 1H, J = 15.5, 11.1 Hz, H-6'), 5.85 (dd, 1H, J = 15.5, 7.4 Hz, H-7'), 4.04 (m, 1H, H-5), 2.74 (m, 2H, H-6_{eq}, H-6_{ax}, obscured by H_2O peak), 2.47 (br s, 4H, H-4", H-5"), 2.10 (m, 1H, H-8', beneath acetone peak), 1.35 (m, 2H, H-9'), 1.00 (d, 3H, J = 6.8 Hz, H-11'), 0.85 (t, 3H, J = 7.4 Hz, H-10'), for minor component (**23**), 2.04 (m, 1H, H-8', beneath acetone peak), 1.71 (m, 1H, H-9'), 0.89 (d, 3H, J = 6.8 Hz, H-10'), HR-MS [M+Na] for $C_{29}H_{34}N_2O_7Na$, calculated 545.2264, found 545.2286. 24.0% ^{13}C incorporation (not calibrated against unlabeled sample). ES-MS/MS (from LC-MS, Appendix B, Figure 79) $[M+H]^+$ 523, daughter ions 505, 163.

Compound 24 (HPLC fraction 3, Tr 14.6 min): ^{1}H-NMR (500 MHz, acetone-d_6) δ_H 7.54 (d, 1H, J = 1.9 Hz, H-3), 7.35 (dd, 1H, J = 14.8, 11.1 Hz, H-11), 7.25 (dd, 1H, J = 14.8,

11.7 Hz, H-3'), 6.78 (dd, 1H, J = 14.8, 10.8 Hz, H-9), 6.63 (dd, 1H, J = 14.8, 10.8 Hz, H-8), 6.61 (dd, 1H, J = 15.1, 10.5 Hz, H-5'), 6.57 (d, 1H, J = 14.8 Hz, H-12), 6.51 (dd, 1H, J = 14.8, 11.1 Hz, H-10), 6.39 (d, 1H, J = 14.8 Hz, H-2'), 6.31 (dd, 1H, J = 15.1, 11.7 Hz, H-4'), 6.23 (d, 1H, J = 14.8 Hz, H-7), 6.19 (m, 1H, H-6'), 5.97 (dd, 1H, J = 15.5, 8.0 Hz, H-7'), 4.04 (m, 1H, H-5), 2.77 (m, 2H, H-6_{eq}, H-6_{ax}, obscured by H_2O peak), 2.47 (br s, 4H, H-4", H-5"), 2.16 (m, 2H, H-8', beneath acetone peak), 1.57 (m, 1H, H-10'), 1.30 (m, 2H, H-9'), 0.88 (d, 6H, J = 6.8 Hz, H-11'). ES-MS/MS (from LC-MS, Appendix B, Figure 80) $[M+H]^+$ 537, daughter ion 521, 177.

Compound 25 (HPLC fraction 4, Tr 16.0 min): R_f 0.30 [silica gel, MeOH/$CHCl_3$ (10:1)]. ^{1}H-NMR (500 MHz, acetone-d_6) δ_H 7.54 (d, 1H, J = 1.9 Hz, H-3), 7.35 (dd, 1H, J = 14.8, 11.7 Hz, H-11), 7.25 (dd, 1H, J = 14.8, 11.1 Hz, H-3'), 6.78 (dd, 1H, J = 14.8, 10.5 Hz, H-9), 6.63 (dd, 1H, J = 14.8, 10.5 Hz, H-8), 6.61 (dd, 1H, J = 14.8, 10.5 Hz, H-5'), 6.57 (d, 1H, J = 14.8 Hz, H-12), 6.50 (dd, 1H, J = 14.8, 11.1 Hz, H-10), 6.38 (d, 1H, J = 14.8 Hz, H-2'), 6.32 (dd, 1H, J = 14.8, 11.2 Hz, H-4'), 6.23 (d, 1H, J = 14.8 Hz, H-7), 6.18 (dd, 1H, J = 15.5, 10.5 Hz, H-6'), 5.91 (dd, 1H, J = 15.5, 7.4 Hz, H-7'), 4.04 (m, 1H, H-5), 2.77 (m, 2H, H-6_{eq}, H-6_{ax}, obscured by H_2O peak), 2.47 (br s, 4H, H-4", H-5"), 2.08 (m, 1H, H-8', beneath acetone peak), 1.71 (m, 4H, H-10'), 1.28 (m, 2H, H-11'), 1.20-1.10 (m, 4H, H-9'). ES-MS/MS (from LC-MS, Chapter 5 Figure 52) $[M+H]^+$ 549, daughter ions 531, 189. HR-MS [M+Na] for $C_{31}H_{36}N_2O_7Na$, calculated 571.2420, found 571.2424. 21.2% ^{13}C incorporation (not calibrated against unlabeled sample).

8.5.3 Isolation, Purification and Identification of Shunt Metabolite **85**

The *Streptomyces nodosus* culture was harvested after 2 ½ days in production medium. The supernatant extracts were purified on an RP18 silica-gel column eluted with acetone / water 20:80. ^{13}C-NMR spectra were taken to trace the unknown compound with an enhanced ^{13}C signal at 137 ppm. Further purification was performed on HPLC with water / 2-propanol 72.5 / 27.5 as eluent. ^{1}H-, ^{13}C-NMR and FAB-MS spectra were taken to elucidate the structure of this unknown compound. The compound was methylated with diazomethane and the molecular formula of the resulting derivative was determined by HR-MS. This unknown was identified as a shunt metabolite, 7-(3-N-acetylamino-4-hydroxyphenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienoic acid **(85)**.

7-(3-N-Acetylamino-4-hydroxyphenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienoic acid (85): R_f 0.21 [silica gel, CH_2Cl_2/MeOH (10:1)]. UV absorption maximum 368 nm (MeOH). ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H 7.90 (d, 1H, J = 2.0 Hz, H-2'), 7.23 (dd, 1H, J = 15.1, 11.2 Hz, H-3), 7.12 (dd, 1H, J = 8.3, 2.0 Hz, H-6'), 6.84 (d, 1H, J = 8.3 Hz, H-5'), 6.80-6.6.68 (m, 3H, H-5, H-6, H-7), 6.49 (dd, 1H, J = 14.2, 11.2 Hz, H-4), 5.86 (d, 1H, J = 15.1 Hz, H-2), 2.09 (s, 3H, CH_3), ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C 169.0, 167.7, 148.6, 144.3. 141.2, 137.1 (enriched, C-7), 136.6 (enriched, C-7), 129.0, 127.6, 126.7, 126.6, 125.6, 123.6, 120.8, 115.9, 23.7. FAB-MS m/z 274 $[M+H]^+$. HR-MS for methylated-**85** $[M]^+$ $C_{17}H_{19}NO_4$, calculated 301.1322, found 301.1314.

8.5.4 Type I Manumycins Incubated with Cell-free Extracts

Streptomyces nodosus fermentation cultures (200 mL) were harvested after 2 days in production medium. The culture broth was centrifuged at 8000 rpm for 30 min, the cell mass was washed with 20 ml sodium phosphate buffer (pH 7.0) and centrifuged again for 20 min. The washed mycelium was re-suspended in phosphate buffer (20 mL) and cells were broken by French Press (18,000 psi) and centrifuged. The supernatant was used as cell-free extract for enzyme incubations.

The phosphate buffer consists of 50 mM sodium phosphate (NaH_2PO_4/Na_2HPO_4) at pH 7.0, 2 mM PMSF (phenylmethanesulfonyl fluoride), 1 mM DTE (1,4-dithioerythritol) and 15 % glycerol.

The protein concentration in the cell-free extract was determined according to the protocol of Sigma Procedure No. TPRO-562 using the bicinchoninic acid protein assay kit purchased from Sigma.

The incubations were conducted with cell-free extract (1 ml), the mixture of type I manumycins (1 mg, from *Streptomyces nodosus* and purified on silica gel column), and NADH or NADPH (1 mg) at 30 °C for 2 to 16 hr. The control experiment used cell-free extract which had been boiled for 5 min. The resulting mixture was extracted with ethyl acetate and analyzed by TLC and HPLC. The results are listed in Table 14.

8.6 Fatty Acid Analysis

A loop of S. *nodosus* was transferred into 100 mL of culture medium in a 500 mL Erlenmeyer flask and grown in a rotary shaker at 300 rpm for 2 days at 28 °C. Ten mL of this seed culture was used to inoculate 100 mL of production culture and the cells were harvested at 72 hr.

Table 14. Cell-free Incubation with Type I Manumycins

Protein boiled	NADH	NADPH	DTE	Formation of type II manumycins
-	-	-	-	-
-	+	-	-	-
-	-	+	-	-
-	-	-	+	+
+	-	-	+	+
without cell-free extract	-	-	+	+

The cell cultures were centrifuged at 7,500 rpm for 25 min. The wet cells were suspended in 2.5% KOH in 20 mL vials and heated in an oven at 80 °C overnight. The solution was filtered, the filtrate extracted with 10 mL of n-hexane and centrifuged. The aqueous layer was acidified with 6 M HCl, re-extracted with 2 x 10 mL of n-hexane and centrifuged.

The combined acidic hexane extracts were dried over Na_2SO_4 and the hexane was removed by evaporation. The fatty acids were methylated by diazomethane. The fatty acid composition of *Streptomyces nodosus* was analyzed by GC-MS of the methyl esters. The initial column temperature was 100 °C, the injector and detector temperature were both 250 °C. After 2 min, the column temperature was raised 10 °C/min to a final temperature of 290 °C. Helium was used as the carrier gas.

The signals of the methyl esters of straight-chain, *iso*- and *anteiso*-branched chain fatty acids on GC chromatogram were identified with genuine samples purchased from Sigma (Appendix C Figure 81). Two ω-cyclopentyl fatty acid methyl ester were detected from *Streptomyces nodosus* fed with [1-^{13}C]Cyclopentanecarboxylic acid [1-^{13}C]-**(93)**.

Methyl ω-cyclopentyl-[1-^{13}C]undecanoate (149): Tr 17 min 17 sec. GC-MS m/z (relative intensity) 269 (M^+, 24), 226 (14), 200(13), 143 (36), 87 (88), 74 (100) (Appendix C Figure 82).

Methyl ω-cyclopentyl-[1-^{13}C]tridecanoate (150): Tr 21 min 7 sec. GC-MS m/z (relative intensity) 297 (M^+, 29), 254 (11), 143 (21), 87 (84), 74 (100) (Appendix C Figure 83).

8.7 Synthesis of Intermediates and Labeled Precursors

8.7.1 3-Amino-4-hydroxy-[7-^{13}C]benzoic Acid (**50**)

(1). **4-Hydroxy-3-nitro-[7-^{13}C]benzoic acid (52)** --- To a mixture of 4-hydroxy-[7-^{13}C]benzoic acid **(51)** (1 g, 7.2 mmol), $NaNO_2$ (2 g, 30 mmol) and H_2O (2 mL) was added H_2SO_4 (5 mL) at 0 °C. The resulting mixture was heated to 70 °C for 1 hr, and poured over crushed ice. The precipitate was filtered and washed with cold water to give **52** as a yellow solid (1.16 g, 6.3 mmol, 88%): ^{1}H-NMR (300 MHz, DMSO) δ_H 8.35 (dd, 1H, J = 4.2, 2.1 Hz, H-2), 8.03 (ddd, 1H, J = 8.8, 3.6, 2.1 Hz, H-6), 7.21 (dd, 1H, J = 8.8, 3.6 Hz, H-5). ^{13}C-NMR (125 MHz, DMSO) δ_C 165.4 (enriched, C-7), 155.4 (C-4), 136.5 (d, J = 4.8 Hz), 135.3 (d, J = 3.2 Hz), 126.7 (d, J = 3.2 Hz), 121.6 (d, J = 74.6 Hz, C-1), 119.1 (d, J = 4.8 Hz).

(2) **3-Amino-4-hydroxy-[7-^{13}C]benzoic acid (50)** --- To 4-hydroxy-3-nitrobenzoic acid (0.5 g, 2.7 mmol) dissolved in ethanol (10 mL) was added hydrazine hydrate (0.82 g, 16 mmol). The solution was heated on a steam-bath and when warm a small amount of Raney Ni was added. As the reaction proceeded, the color changed from orange to colorless. More Raney Ni was added to decompose the excess hydrazine and the solution was heated to boiling to drive off the dissolved gases. The hot solution was filtered to remove the Ni and [7-^{13}C]-**50** was obtained by evaporating the remaining solvent (0.36 g, 2.3 mmol, 85%): ^{1}H-NMR (300 MHz, DMSO) δ_H 7.16 (dd, 1H, J = 4.4, 2.2 Hz, H-2), 7.02 (ddd, 1H, J = 8.1, 3.9, 2.2 Hz, H-6), 6.61(dd, 1H, J = 8.1, 1.0 Hz, H-5). ^{13}C-NMR (125 MHz, DMSO) δ_C 168.2 (enriched, C-7), 147.9 (C-4), 136.0 (d, J = 4.8 Hz), 123.1 (d,

J = 71.4 Hz, C-1), 118.9 (d, J = 3.2 Hz), 115.2, 113.4 (d, J = 4.8 Hz). HR-MS m/z for $C_6{}^{13}CH_8NO_3$, $[M+H]^+$ calculated 155.0536, found 155.0538.

8.7.2 3-(3-Amino-4-hydroxyphenyl)-*E*-[1,2-$^{13}C_2$]prop-2-enoic Acid [1,2-$^{13}C_2$]-**67b**

(3) **4-Methoxy-3-nitrobenzaldehyde (73)** --- To the suspension of methoxybenzaldehyde **(72)** (15.00 g, 0.11 mol) and NH_4NO_3 (8.8 g, 0.11 mol) in $CHCl_3$ (110 mL) was added trifluoroacetic anhydride (55 mL). The mixture was stirred at room temperature until the NH_4NO_3 had dissolved. The solution was poured into 100 mL of water and extracted with $CHCl_3$ (3x150 mL). The combined $CHCl_3$ extracts were dried over Na_2SO_4, filtered and the $CHCl_3$ was evaporated *in vacuo*. The oily residue was purified by silica gel flash column chromatography with a gradient of hexane/ethyl acetate as eluent to afford **73** (16.89 g, 0.933 mol, 85%): R_f 0.32 [silica gel, ethyl acetate/hexane (1:1)]. ^{1}NMR (500 MHz, acetone-d_6) δ_H 10.00 (s, 1H, C*H*O), 8.34 (d, 1H, J = 1.9 Hz, H-2), 8.18 (dd, 1H, J = 8.7, 1.9 Hz, H-6), 7.54 (d, 1H, J = 8.7 Hz, H-5), 4.11 (s, 3H, OCH_3). ^{13}C-NMR (125 MHz, DMSO) δ_C 189.9, 157.2, 140.7, 135.4, 130.0, 126.8, 115.2, 57.6. EI-MS m/z (relative intensity) 181 (M^+, 100), 151 (36), 134 (69), 119 (78), 105 (56), 77 (87).

(4) **3-(4-Methoxy-3-nitrophenyl)-*E*-prop-2-enoic acid (74)** --- 4-Methoxy-3-nitrobenzaldehyde (1 g, 5.5 mmol) and malonic acid (0.57 g, 5.5 mmol) in pyridine (1.5 mL) were heated on a steam bath for 2 hr. The resulting mixture was cooled and 6M HCl

was added to acidify the solution to pH < 2. A white solid formed and the mixture was cooled in an ice-bath, and the product was filtered and washed with ice-water, and air dried to give **74** (1.21 g, 5.4 mmol, 98%): R_f 0.40 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H 8.24 (d, 1H, J = 2.1 Hz, H-2'), 8.02 (dd, 1H, J = 8.8, 2.1 Hz, H-6'), 7.49 (d, 1H, J = 16.1, H-3), 7.40 (d, 1H, J = 8.8 Hz, H-5'), 6.57 (d, 1H, J = 16.1 Hz, H-2). ^{13}C-NMR (75 MHz, DMSO-d_6) δ_C 167.4, 152.7, 141.5, 139.7, 133.8, 127.0, 124.4, 119.6, 114.6, 57.1. EI-MS [M+] 223, fragments m/z 206, 162, 147, 89, 77, 63, 51.

(5) **3-(4-Methoxy-3-nitrophenyl)-*E*-[1,2-$^{13}C_2$]prop-2-enoic acid ([1,2-$^{13}C_2$]-74)** --- By the same procedure as in (4), 4-methoxy-3-nitrobenzaldehyde (1.79 g, 9.9 mmol) and [U-$^{13}C_3$]malonic acid (1 g, 9.4 mmol) in pyridine (2 mL) was heated by steam bath for 2 hr. The resulting mixture was cooled and 6M HCl gave [1,2-$^{13}C_2$]-**74** (2.07g, 9.3 mmol, 93%): R_f 0.40 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H 8.21 (d, 1H, J = 2.1 Hz, H-2'), 7.98 (dd, 1H, J = 8.8, 2.1 Hz, H-6'), 7.57 (ddd, 1H, J = 16.1, 6.74, 2.59 Hz, H-3), 7.37 (d, 1H, J = 8.8 Hz, H-5'), 6.54 (ddd, 1H, J = 161.9, 16.1, 2.08 Hz, H-2). ^{13}C-NMR (75 MHz, DMSO) δ_C 167.43 (enriched, d, J = 72.0 Hz, C-1), 152.8, 141.5 (d, J = 60.8 Hz, C-3), 139.7, 133.8 (d, J = 4.9 Hz), 127.0 (d, J = 7.3 Hz), 119.6 (enriched, d, J = 72.0 Hz, C-2), 114.6, 57.0.

(6) **3-(4-Hydroxy-3-nitrophenyl)-*E*-prop-2-enoic acid (75)** --- 3-(4-Methoxy-3-nitrophenyl)-*E*-prop-2-enoic acid **(74)** (300 mg, 1.35 mmol), LiI (360 mg, 2.69 mmol) and

anhydrous 2,4,6-collidine (5 mL) was heated to reflux under argon for 3.5 hr. The mixture was cooled and acidified with 6M HCl, diluted with water and extracted with ethyl acetate (4x15 mL). The extracts were combined, dried over Na_2SO_4 and ethyl acetate was evaporated *in vacuo* to give **75** (254 mg, 1.22 mmol, 90%): R_f 0.37 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H 8.18 (d, 1H, J = 2.1 Hz, H-2'), 7.88 (dd, 1H, J = 8.8, 2.1 Hz, H-6'), 7.55 (d, 1H, J = 15.8 Hz, H-3), 7.14 (d, 1H, J = 8.3 Hz, H-5'), 6.47 (d, 1H, J = 15.8 Hz, H-2), ^{13}C-NMR (75 MHz, DMSO) δ_C 167.6, 153.2, 141.9, 137.4, 133.9, 125.8, 119.5, 118.7.

(7) **3-(4-Hydroxy-3-nitrophenyl)-*E*-[1,2-$^{13}C_2$]prop-2-enoic acid ([1,2-$^{13}C_2$]-75)** --- By the same procedure as in (6), 3-(4-methoxy-3-nitrophenyl)-*E*-[1,2-$^{13}C_2$]prop-2-enoic acid ([1,2-$^{13}C_2$]-**74**) (0.5 g, 2.22 mmol), LiI (0.7 g, 5.23 mmol) and anhydrous 2,4,6-collidine (5 mL) gave [1,2-$^{13}C_2$]-**75** (0.35g, 1.66 mmol, 75%): R_f 0.37 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H 8.17 (d, 1H, J = 2.1 Hz, H-2'), 7.88 (dd, 1H, J = 8.7, 2.1 Hz, H-6'), 7.55 (ddd, 1H, J = 16.0, 6.8, 2.9 Hz, H-3), 7.13 (d, J = 8.7 Hz, H-5'), 6.46 (ddd, 1H, J = 161.5, 16.0, 2.5 Hz, H-2). ^{13}C-NMR (75 MHz, DMSO) δ_C 167.5 (enriched, d, J = 72.3 Hz, C-1), 153.2, 141.9 (d, J = 70.4 Hz, C-3), 137.4, 133.9 (d, J = 4.1 Hz), 125.6 (d, J = 3.2 Hz), 119.5, 118.8 (enriched, d, J = 72.3 Hz, C-2).

(8) **3-(3-Amino-4-hydroxy-phenyl)-*E*-prop-2-enoic acid (67b)** --- A mixture of **75** (0.20 g, 0.96 mmol), $SnCl_2x2H_2O$ (1.08 g, 4.79 mmol) and ethanol (4 mL) was heated at 70 °C under argon for 0.5 hr. The mixture was then cooled and poured onto ice. The pH

was adjusted to 4 with 2M NaOH and extracted with ethyl acetate (4x10 mL). The combined extracts were washed with brine, dried over Na_2SO_4 and filtered. Evaporation of the solvent gave **67b** (0.13 g, 0.726 mmol, 77%): R_f 0.23 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H 7.36 (d, 1H, J = 15.8 Hz, H-3), 6.87 (d, 1H, 2.2 Hz, H-2'), 6.71 (dd, 1H, J = 8.0, 2.2 Hz, H-6'), 6.65 (d, 1H, J = 8.0 Hz, H-5'), 6.10 (d, 1H, J = 15.8 Hz, H-2). ^{13}C-NMR (75 MHz, DMSO) δ_C 167.9, 146.8, 145.1, 136.9, 125.5, 118.7, 114.3, 114.2, 112.5.

(9) **3-(3-Amino-4-hydroxyphenyl)-*E*-[1,2-$^{13}C_2$]prop-2-enoic acid ([1,2-$^{13}C_2$]-67b)** --- A mixture of [1,2-$^{13}C_2$]-**75** (0.30 g, 1.42 mmol), $SnCl_2x2H_2O$ (1.60 g, 7.10 mmol) and ethanol (8 mL) was heated at 70 °C under argon for 0.5 hr. The mixture was then cooled and poured onto ice. The pH was adjusted to 4 by 2M NaOH and extracted with ethyl acetate (5x30 mL). The combined extracts were washed with brine, dried over Na_2SO_4 and filtered. Evaporation of the solvent gave [1,2-$^{13}C_2$]-**67b** (0.19 g, 1.05 mmol, 73%): R_f 0.23 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H 7.36 (m, 1H, H-3), 6.87 (d, 1H, 2.0 Hz, H-2'), 6.71 (dd, 1H, J = 8.3, 2.0 Hz, H-6'), 6.65 (d, 1H, J = 8.3 Hz, H-5'), 6.10 (ddd, 1H, J = 159.8, 15.6, 3.1 Hz, H-2). ^{13}C-NMR (75 MHz, DMSO) δ_C 168.2 (enriched, d, J = 73.2 Hz, C-1), 147.0, 145.4 (d, J = 69.6 Hz, C-3), 137.2, 125.8 (d, J = 7.3 Hz), 118.9 (d, J = 4.9 Hz), 114.5 (enriched, d, J = 73.2 Hz, C-2), 114.4 (d, J = 7.3 Hz), 112.8. EI-MS (relative intensity) m/z 181 (M^+, 14), 136 (55), 45 (100). HR-MS m/z for $C_7{}^{13}C_2H_{10}NO_3$, $[M+H]^+$ calculated 182.0728, found 182.0713.

8.7.3 5-(3-Amino-4-hydroxyphenyl)-(*2E,4E*)-[1,2-$^{13}C_2$]penta-2,4-dienoic Acid ([1,2-$^{13}C_2$]-**68b)**

(10) **4-Methoxy-3-nitrocinnamaldehyde (77)** --- To a solution of 4-Methoxycinnamaldehyde **(76)** (20 g, 0.123 mol) and NH_4NO_3, (10 g, 0.125 mol) in $CHCl_3$ (120 mL) was added trifluoroacetic anhydride (65 mL). The reaction mixture was stirred at room temperature until the NH_4NO_3 had dissolved. The solution was poured into 50 mL water and extracted with $CHCl_3$ (3x150 mL). The organic phase was dried over Na_2SO_4. $CHCl_3$ was evaporated *in vacuo*. The oily residue was purified by silica gel flash column chromatography with a gradient of hexane / ethyl acetate to give 4-methoxy-3-nitrocinnamaldehyde **77** (15.31 g, 0.074mol, 60%): R_f 0.26 [silica gel, ethyl acetate/hexane (1:1)]. ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 9.69 (d, 1H, J = 7.3 Hz, C*H*O), 8.18 (d, 1H, J = 2.3 Hz, H-2'), 8.01 (dd, 1H, J = 8.8, 2.3 Hz, H-6'), 7.67 (d, 1H, J = 16.1 Hz, H-3), 6.78 (dd, 1H, J = 16.1, 7.3 Hz, H2), ^{13}C-NMR (75 MHz, acetone-d_6) δ_C 193.8, 154.8, 150.6, 141.2, 134.6, 129.6, 127.9, 125.7, 115.3, 57.4. EI-MS m/z (relative intensity) 207 (M^+, 100), 190 (90), 131 (87), 118 (47), 102 (74), 77 (91).

(11) **Ethyl 5-(4-methoxy-3-nitrophenyl)-(*2E,4E*)-penta-2,4-dienoate (78)** --- A suspension of **77** (5.00 g, 24.2 mmol) and triethyl phosphonoacetate (10.82 g, 0.483 mol) in aqueous 6M K_2CO_3 (20 mL) was stirred vigorously at room temperature for 3 days. The mixture was diluted with water and extracted with ethyl acetate (3x150 mL). The combined extracts were dried over $MgSO_4$, ethyl acetate was evaporated *in vacuo*. The

crude product was washed with a small amount of cold methanol to afford **78** (5.8 g, 20.9 mmol, 87%): R_f 0.23 [silica gel, hexane/ethyl acetate (2:1)]. ^{1}H-NMR (500 MHz, acetone-d_6) δ_H 8.00 (d, 1H, J = 2.4 Hz, H-2'), 7.79 (dd, 1H, J = 8.7, 2.4 Hz, H-6'), 7.39 (m, 1H, H-3), 7.32 (d, 1H, J = 8.7 Hz, H-5'), 7.06 (m, 2H, H-4, H-5), 6.03 (ddd, 1H, J = 163.4, 15.1, 2.6 Hz, H-2), 4.16 (q, 2H, J = 7.4 Hz, OCH_2CH$_3$), 3.99 (s, 3H, OCH_3), 1.25 (t, 3H, J = 7.4 Hz, OCH$_2$CH_3). ^{13}C-NMR (125 MHz, acetone-d_6) δ_C 166.6, 153.1, 144.6, 140.9, 138.1, 133.1, 129.7, 127.5, 123.8, 122.4, 114.9, 60.6, 57.1, 14.6.

(12) **Ethyl 5-(4-methoxy-3-nitrophenyl)-(*2E,4E*)-[1,2-$^{13}C_2$]penta-2,4-dienoate ([1,2-$^{13}C_2$]-78)** — A suspension of **77** (0.92 g, 4.44 mmol) and [l,2-$^{13}C_2$]-triethyl phosphonoacetate (1.0 g, 4.42 mmol) in aqueous 6M K_2CO_3 (30 mL) was stirred vigorously at room temperature for 30 hr. The mixture was diluted with water, extracted with ethyl acetate (3x100 mL). The combined extracts were dried over Na_2SO_4, and ethyl acetate was evaporated *in vacuo* to afford [1,2-$^{13}C_2$]-**78**. [1,2-$^{13}C_2$]-**78** was further purified on a silica gel column with hexane/ethyl acetate as eluent (0.86 g, 3.08 mmol, 69%): R_f 0.23 [silica gel, hexane/ethyl acetate (2:1)]. ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 8.00 (d, 1H, J = 2.1 Hz, H-2'), 7.80 (dd, 1H, J = 8.8, 2.1 Hz, H-6'), 7.39 (dd, 1H, J = 15.5, 9.9 Hz, H-3), 7.32 (d, 1H, J = 8.8 Hz, H-5'), 7.05 (m, 2H, H-4, H-5), 6.04 (d, 1H, J = 15.5 Hz, H-2), 4.16 (qd, 2H, J = 7.3, 3.1 Hz, OCH_2CH$_3$), 3.99 (s, 3H, OCH_3), 1.25 (t, 3H, J = 7.3 Hz, OCH$_2$CH_3), ^{13}C-NMR (75 MHz, acetone-d_6) δ_C 166.8 (enriched, d, J = 75.7 Hz, C-1), 153.3, 144.8 (d, J = 69.6 Hz, C-3), 141.0, 138.2 (d, J = 8.5 Hz), 133.2, 129.9,

127.7 (d, J = 8.5 Hz), 124.0 (d, J = 2.4 Hz), 122.6 (enriched, d, J = 75.7 Hz, C-2), 115.1, 60.6, 57.1, 14.6.

(13) **5-(4-Hydroxy-3-nitrophenyl)-(*2E,4E*)-penta-2,4-dienoic acid (79)** --- A suspension of **78** (0.120 mg, 0.433 mmol), LiI (0.57 g, 4.26 mmol) and anhydrous 2,4,6-collidine (4 mL) was heated to reflux under argon for 6 hr. The mixture was cooled and acidified by 6M HCl, diluted with water and extracted with ethyl acetate (3x20 mL). The extracts were combined, dried over Na_2SO_4 and filtered. The solvent was evaporated *in vacuo* to afford **79** (91 mg, 0.387 mmol, 89%): R_f 0.36 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H 8.27 (d, 1H, J = 2.2 Hz, H-2'), 7.97 (dd, 1H, J = 8.8, 2.2 Hz, H-6'), 7.45 (dd, 1H, J = 15.3, 9.3 Hz, H-3), 7.24 (d, 1H, J = 8.8 Hz, H-5'), 7.14 (m, 2H, H-4, H-5), 6.09 (d, 1H, J = 15.3 Hz, H-2), ^{13}C-NMR (75 MHz, DMSO-d_6) δ_C 167.5, 152.3, 144.1, 137.5, 137.1, 133.0. 127.6, 126.2, 123.8, 122.1, 119.5.

(14) **5-(4-Hydroxy-3-nitrophenyl)-(*2E,4E*)-[1,2-$^{13}C_2$]penta-2,4-dienoic acid ([1,2-$^{13}C_2$]-79)** --- A suspension of [1,2-$^{13}C_2$]-**78** (0.6 g, 2.15 mmol) and LiI (0.86 g, 6.43 mmol) in anhydrous 2,4,6-collidine (4 mL) was heated to reflux under argon for 6 hr. The mixture was cooled and acidified by 6M HCl, diluted with water and extracted with ethyl acetate (3x20 mL). The extracts were combined, dried over Na_2SO_4 and filtered. Ethyl acetate was evaporated *in vacuo* to afford [1,2-$^{13}C_2$]-**79** (0.47 g, 1.98 mmol, 92%): R_f 0.36 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H 8.04 (d, 1H, J = 2.1 Hz, H-2'), 7.74 (dd, 1H, J = 8.6, 2.1 Hz, H-6'), 7.29 (m, 1H, H-3), 7.13 (d, 1H, J

= 8.6 Hz, H-5'), 5.97 (ddd, 1H, J = 161.9, 15.1, 3.1 Hz, H-2), ^{13}C-NMR (75 MHz, DMSO-d_6) δ_C 167.6 (enriched, d, J = 73.3 Hz, C-1), 152.4, 144.0 (d, J = 68.4 Hz, C-3), 137.6 (d, J = 9.8 Hz), 137.2, 133.1, 127.7, 126.3 (d, J = 8.5 Hz), 123.8, 122.1 (enriched, d, J = 73.3 Hz, C-2), 119.6.

(15) **5-(3-Amino-4-hydroxyphenyl)-(*2E,4E*)-penta-2,4-dienoic acid (68b)** --- A suspension of **79** (120 mg, 0.510 mmol) and $SnCl_2x2H_2O$ (570 mg, 2.53 mmol) in ethanol (4 mL) was heated to 70 °C under argon for 1 hr. The mixture was cooled and poured onto ice. The pH was adjusted to 4 with 2M NaOH and the resulting solution was extracted with ethyl acetate (3x20 mL). The combined extracts were washed with brine, dried over Na_2SO_4 and filtered. Evaporation of the solvent gave **68b** (92 mg, 0.449 mmol, 88%): R_f 0.21 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H 7.30 (dd, 1H, J = 15.1, 10.7 Hz, H-3), 6.87-6.65 (m, 5H, H-3, H-4, H-2', H-5' H-6'), 5.88 (d, 1H, J = 15.1 Hz, H-2), ^{13}C-NMR (75 MHz, DMSO-d_6) δ_C 167.9, 146.0, 145.3, 141.5, 136.6, 127.6, 122.6, 119.6, 117.5, 114.4, 112.4.

(16) **5-(3-Amino-4-hydroxyphenyl)-(*2E,4E*)-[1,2-$^{13}C_2$]penta-2,4-dienoic acid ([1,2-$^{13}C_2$]-68b)** --- A suspension of [1,2-$^{13}C_2$]-**79** (300 mg, 1.27 mmol) and $SnCl_2x2H_2O$ (1.43 g, 6.36 mmol) in ethanol (8 mL) was heated to 70 °C under argon for 1 hr. The mixture was cooled and poured onto ice. The pH was adjusted to 4 with 2M NaOH and the resulting solution was extracted with ethyl acetate (4x30 mL). The combined extracts were washed with brine, dried over Na_2SO_4 and filtered. Evaporation of the solvent gave

[1,2-$^{13}C_2$]-**68b** (213 mg, 1.03 mmol, 81%): R_f 0.21 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (500 MHz, DMSO-d_6) δ_H 7.30 (m, 1H, H-3), 6.85-6.65 (m, 4H, H-4, H-2', H-5' H-6'), 6.83 (d, 1H, J = 14.8 Hz, H-5), 5.87 (ddd, 1H, J = 161.3, 15.5, 3.1 Hz, H-2), ^{13}C-NMR (75 MHz, DMSO-d_6) δ_C 167.7 (enriched, d, J = 73.0 Hz, C-1), 146.3, 145.1 (d, J = 68.2 Hz, C-3), 141.2 (d, J = 9.5 Hz), 135.4, 127.4, 122.6, 119.6 (enriched, d, J = 73.0 Hz, C-2), 118.1, 114.5, 113.0. HR-MS m/z for $C_9{}^{13}C_2H_{12}NO_3$, $[M+H]^+$ calculated 208.0884, found 208.0871.

8.7.4 7-(3-Amino-4-hydroxyphenyl)-(*2E,4E,6E*)-[1, 2-$^{13}C_2$]-hepta-2,4,6-trienoic Acid ([1,2-$^{13}C_2$]-**69b**)

(17) **5-(4-Methoxy-3-nitrophenyl)-(*2E,4E*)-penta-2,4-dienol (80)** --- Diisobutylaluminum hydride (27.8 mL, 1.5 M in toluene) was added slowly to the solution of ethyl 5-(4-methoxy-3-nitrophenyl)-(*2E,4E*)-penta-2,4-dienoate **(78)** (5.50 g, 19.9 mmol) in toluene (200 mL) under argon at -78 ^{0}C. The reaction mixture was stirred at -78 ^{0}C for 1 hr and warmed to room temperature. After stirring for 1 hr, water was added and the mixture extracted with ethyl acetate (4x400 mL). The combined extracts were dried over $MgSO_4$, filtered and the solvent was removed *in vacuo* to afford **80** (4.10 g, 17.4 mmol, 88%): R_f 0.17 [silica gel, hexane/ethyl acetate (1:1)]. ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 7.88 (d, 1H, J = 2.3 Hz, H-2'), 7.70 (dd, 1H, J = 8.8, 2.3 Hz, H-6'), 7.28 (d, 1H, J = 8.8 Hz, H-5'), 6.92 (dd, 1H, J = 16.1, 10.9 Hz, H-4), 6.55 (d, 1H, J = 16.1 Hz, H-5), 6.42 (m, 1H, H-3), 6.00 (dt, 1H, J = 15.1, 5.2 Hz, H-2), 4.17 (m, 1H, CH_2OH), 3.86 (t,

1H, J = 5.7 Hz, O*H*), ^{13}C-NMR (75 MHz, acetone-d_6) δ_C 152.2, 141.2, 136.4, 132.2, 131.4, 130.4, 130.1, 129.4, 122.8, 115.0,62.8, 57.1. EI-MS m/z (relative intensity) 235 (M^+, 60), 218 (13), 179 (73), 128 (54), 115 (100), 91 (49), 77 (36).

(18) **5-(4-Methoxy-3-nitrophenyl)-(*2E,4E*)-penta-2,4-dienal (81)** --- To **80** (3.60 g, 15.3 mmol) dissolved in $CHCl_3$ (200 mL) was added activated manganese dioxide (13.33 g, 85% purity). The mixture was stirred at room temperature for 4 hr. Upon the completion of the reaction, the mixture was filtered through Celite and the filter washed with $CHCl_3$. The combined filtrate and washings were concentrated *in vacuo*. The resulting residue was purified by flash silica gel chromatography with hexane/ethyl acetate as eluents to afford **81** (3.43 g, 14.7 mmol, 96%): R_f 0.23 [silica gel, hexane/ethyl acetate (1:1)]. ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 9.62 (d, 1H, J = 8.0 Hz, C*H*O), 8.07 (d, 1H, J = 2.3 Hz, H-2'), 7.88 (dd, 1H, J = 8.8, 2.3 Hz, H-6'), 7.44 (dd, 1H J = 15.1, 9.9 Hz, H-3), 7.38 (d, 1H, J = 8.8 Hz, H-5'), 7.25 (m, 2H, H-4, H-5), 6.26 (dd, 1H, J = 15.1, 7.8 Hz, H-2), 4.00 (s, 3H, OCH_3), ^{13}C-NMR (75 MHz, acetone-d_6) δ_C 193.8, 153.7, 152.3, 139.9, 133.6, 132.9, 129.7, 128.0, 124.4, 115.3, 57.3. EI-MS m/z (relative intensity) 233 (M^+, 86), 158 (27), 144 (32), 128 (100), 115 (93).

(19) **Ethyl 7-(4-methoxy-3-nitrophenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienoate (82)** --- A suspension of **81** (2.25 g, 9.66 mmol) and triethyl phosphonoacetate (6.70 g, 29.9 mmol) in aqueous 6 M K_2CO_3 (50 mL) was stirred vigorously at room temperature for 3 days. The resulting mixture was diluted with water and extracted with ethyl acetate (3x120 mL).

The organic phase was dried over $MgSO_4$, filtered and ethyl acetate was evaporated *in vacuo*. Pure **82** was obtained by washing with a small amount of cold methanol and filtering the resulting crystals (2.60 g, 8.58 mmol, 89%): R_f 0.21 [silica gel, hexane/ethyl acetate (2:1)]. ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 7.97 (d, 1H, J = 2.1 Hz, H-2'), 7.78 (dd, 1H, J = 8.8, 2.1 Hz, H-6'), 7.33 (m, 1H, H-3), 7.32 (d, 1H, J = 8.8 Hz, H-5'), 7.06 (dd, 1H, J = 16.1, 10.9 Hz, H-6), 6.84 (m, 2H, H-5, H-7), 6.57 (dd, 1H, J = 15.1, 10.9 Hz, H-4), 5.95 (d, 1H, J = 15.1 Hz, H-2), 4.15 (q, 2H, J = 7.3 Hz, OCH_2CH_3), 3.98 (s, 3H, OCH_3), 1.24 (t, 3H, J = 7.3 Hz, OCH_2CH_3). ^{13}C-NMR (75 MHz, acetone-d_6) δ_C 167.0, 152.9, 144.9, 141.3, 141.2, 134.5, 132.8, 131.8, 130.7, 129.7, 123.5, 122.0, 115.1, 60.6, 57.1, 14.6.

(20) **Ethyl 7-(4-methoxy-3-nitrophenyl)-(*2E,4E,6E*)-[1,2-$^{13}C_2$]hepta-2,4,6-trienoate [1,2-C_2]-(82)** --- A suspension of **81** (0.8 g, 3.43 mmol) and [1,2-$^{13}C_2$]triethyl phosphonoacetate (0.74 g, 3.27 mmol) in aqueous 6M K_2CO_3 (25 mL) was stirred vigorously at room temperature for 30 hr. The resulting mixture was diluted with water and extracted with ethyl acetate (4x30 mL). The organic phase was dried over Na_2SO_4, filtered and ethyl acetate was evaporated *in vacuo*. [1,2-$^{13}C_2$]-**82** was obtained after purification by flash column chromatography with hexane/ethyl acetate as eluents (0.67 g, 2.20 mmol, 64%): R_f 0.21 [silica gel, hexane/ethyl acetate (2:1)]. ^{1}H-NMR (500 MHz, acetone-d_6) δ_H 7.98 (d, 1H, J = 2.5 Hz, H-2'), 7.79 (dd, 1H, J = 8.7, 2.5 Hz, H-6'), 7.34 (m, 1H, H-3), 7.32 (d, 1H, J = 8.7 Hz, H-5'), 7.07 (dd, 1H, J = 15.5, 10.5 Hz, H-6), 6.85 (m, 2H, H-5, H-7), 6.58 (td, 1H, J = 15.5, 11.7 Hz, H-4), 5.95 (ddd, 1H, J = 163,5, 4.7,

2.4 Hz, H-2), 4.15 (qd, 2H, J = 7.4, 3.1 Hz, OCH_2CH_3), 3.98 (s, 3H, OCH_3), 1.24 (t, 3H, J = 7.4 Hz, OCH_2CH_3). ^{13}C-NMR (125 MHz, acetone-d_6) δ_C 166.7 (enriched, d, J = 76.2 Hz, C-1), 152.9, 144.8 (d, J = 69.8 Hz, C-3), 141.2, 141.1, 134.3, 132.6, 131.6 (d, J = 7.9 Hz), 130.5, 129.5, 123.4, 121.9 (enriched, d, J = 76.2 Hz, C-2), 115.0, 60.5, 57.1, 14.6.

(21) **7-(4-Hydroxy-3-nitrophenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienoic acid (83)** --- A suspension of ethyl **82** (100 mg, 0.330 mmol) and LiI (200 mg, 1.49 mmol) in 2,4,6-collidine (3 mL) was heated to reflux under argon for 6 hr. The mixture was cooled and acidified by 6M HCl, diluted with water and extracted with ethyl acetate (4x25 mL). The extracts were combined, dried over Na_2SO_4 and filtered. Ethyl acetate was evaporated *in vacuo* to afford **83** (64 mg, 0.245 mmol, 74%): R_f 0.35 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (500 MHz, DMSO-d_6) δ_H 8.00 (d, 1H, J = 2.2 Hz, H-2'), 7.73 (dd, 1H, J = 8.7, 2.3 Hz, H-6'), 7.26 (dd, 1H, J = 15.5, 11.7 Hz, H-3), 7.10 (d, 1H, J = 8.7 Hz, H-5'), 6.99 (dd, 1H, J = 16.1, 11.1 Hz, H-6), 6.83 (m, 1H, H-5), 6.77 (d, 1H, J = 16.1 Hz, H-7), 6.53 (dd, 1H, J = 16.1, 11.7 Hz, H-4), 5.91 (d, 1H, J = 15.5 Hz, H-2). ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C 167.5, 151.8, 144.0, 140.4, 137.0, 133.8, 132.6, 130.4, 128.1, 128.0, 123.2, 121.6, 119.4.

(22) **7-(4-Hydroxy-3-nitrophenyl)-(*2E,4E,6E*)-[1,2-$^{13}C_2$]hepta-2,4,6-trienoic acid [1,2-$^{13}C_2$]-(83)** --- A suspension of ethyl [1,2-$^{13}C_2$]-**82** (400 mg, 1.31 mmol) and LiI (0.8 g, 5.98 mmol) in 2,4,6-collidine (4 mL) was heated to reflux under argon for 6 hr. The mixture was cooled and acidified by 6M HCl, diluted with water and extracted with ethyl

acetate (4x15 mL). The extracts were combined, dried over Na_2SO_4 and filtered. Ethyl acetate was removed *in vacuo* to afford [1,2-$^{13}C_2$]-**83** (289 mg, 1.10 mmol, 84%): R_f 0.35 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (500 MHz, DMSO-d_6) δ_H 7.99 (d, 1H, J = 2.2 Hz, H-2'), 7.73 (dd, 1H, J = 8.7, 2.3 Hz, H-6'), 7.26 (m, 1H, H-3), 7.10 (d, 1H, J = 8.7 Hz, H-5'), 6.99 (dd, 1H, J = 15.5, 11.1 Hz, H-6), 6.82 (m, 1H, H-5), 6.77 (d, 1H, J = 15.5 Hz, H-7), 6.53 (m, 1H, H-4), 5.91 (dd, 1H, J = 161.3, 15.5 Hz, H-2). ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C 167.4 (enriched, d, J = 73.0 Hz, C-1), 151.8 (d, J = 14.3 Hz), 144.0 (d, J = 69.8 Hz, C-3), 140.4 (d, J = 9.5 Hz), 137.0, 133.8, 132.6, 130.4 (d, J = 7.9 Hz), 128.1, 128.0, 123.2, 121.6 (enriched, d, J = 73.0 Hz, C-2), 119.4.

(23) **7-(3-Amino-4-hydroxyphenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienoic acid (69b)** --- A suspension of **83** (50 mg, 0.190 mmol) and $SnCl_2x2H_2O$ (205 mg, 0.911) in ethanol (3 mL) was heated at 70 °C under argon for 0.5 hr. The mixture was cooled and poured onto ice, adjusted to pH 4 with 2M NaOH and extracted with ethyl acetate (4x15 mL). The combined extracts were washed with brine, dried over Na_2SO_4 and filtered. Evaporation of the solvent gave **69b** (33 mg, 0.143 mmol, 75%): R_f 0.21 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (500 MHz, DMSO-d_6) δ_H 7.24 (dd, 1H, J = 14.8, 11.7 Hz, H-3), 6.80 (m, 2H, H-6, H-2'), 6.70-6.60 (m, 4H, H-5, H-5', H-6'), 6.60 (d, 1H, J = 15.5 Hz, H-7), 6.44 (dd, 1H, J = 14.2, 11.7 Hz, H-4), 5.84 (d, 1H, J = 14.8 Hz, H-2). ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C 167.6, 145.4, 144.5, 141.6, 137.3, 136.2, 128.1, 127.9, 124.4, 120.1, 117.2, 114.4, 112.1.

(24) **7-(3-Amino-4-hydroxyphenyl)-(*2E,4E,6E*)-[l, 2-$^{13}C_2$]-hepta-2,4,6-trienoic acid ([1,2-$^{13}C_2$]-69b)** --- By the same procedure as in (23), [1,2-$^{13}C_2$]-**83** (211 mg, 0.803 mmol) and $SnCl_2x2H_2O$ (960 mg, 4.27 mmol) in ethanol (6 mL) gave [1,2-$^{13}C_2$]-**69b** (157 mg, 0.674 mmol, 84%): R_f 0.21 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (500 MHz, DMSO-d_6) δ_H 7.24 (m, 1H, H-3), 6.79 (m, 2H, H-6, H-2'), 6.69-6.59 (m, 4H, H-5, H-7, H-5', H-6'), 6.59 (d, 1H, J = 15.5 Hz, H-7), 6.43 (ddd, 1H, J = 14.8, 11.7, 3.7 Hz, H-4), 5.84 (ddd, 1H, J = 161.3, 14.8, 2.5 Hz, H-2). ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C 167.7 (enriched, d, J = 73.0 Hz, C-1), 145.4, 144.5 (d, J = 68.2 Hz, C-3), 141.6 (d, J = 9.5 Hz), 137.7, 136.4, 128.2 (d, 7.9 Hz), 128.0, 124.4, 120.2 (enriched, d, J = 73.0 Hz, C-2), 117.1, 114.4, 112.1. HR-MS m/z for $C_{11}{}^{13}C_2H_{14}NO_3$, $[M+H]^+$ calculated 234.1041, found 234.1049.

8.7.5 3-(3-Amino-4-hydroxyphenyl)-*E*-[1,2-$^{13}C_2$]prop-2-enoic Acid N-Acetylcysteamine Thioester ([1,2-$^{13}C_2$]-**84**)

(25) **3-(3-Amino-4-hydroxyphenyl)-*E*-prop-2-enoic acid N-acetylcysteamine thioester (84)** --- To a stirred solution of 3-(3-amino-4-hydroxy-phenyl)-*E*-prop-2-enoic acid **(67b)** (90 mg, 0.390 mmol) in anhydrous THF (3 mL) was added a catalytic amount of DMAP and N-acetylcysteamine (100 mg, 0.840 mmol). DCC (125 mg, 0.607 mmol) was added to the reaction mixture at 0°C, which was then stirred under argon at room temperature overnight. The precipitated dicyclohexyl urea was removed by filtration and the filtrate was evaporated *in vacuo*. The residue was purified by silica gel flash column

chromatography eluting with a gradient of methylene chloride/methanol followed by reverse phase silica gel column chromatography with acetone/water as eluent to afford **84** (11 mg, 0.039 mmol, 8%): R_f 0.23 [silica gel, MeOH/CH_2Cl_2 (10:1)]. ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 7.39 (d, 1H, J = 15.6 Hz, H-3), 6.95 (d, 1H, J = 2.0 Hz, H-2'), 6.83 (dd, 1H, J = 8.1, 2.0 Hz, H-6'), 6.68 (d, 1H, J = 8.1 Hz, H-5'), 6.57 (d, 1H, J = 15.6 Hz, H-2), 3.21 (m, 2H, SCH_2CH_2N), 3.00 (t, 2H, J = 6.8 Hz, SCH_2CH_2N), 1.80 (s, 3H, CH_3). ^{13}C-NMR (75 MHz, acetone-d_6) δ_C 188.2, 169.3, 147.7, 142.0, 137.2, 125.0, 120.4, 119.8, 114.3, 112.9, 38.4, 28.0, 22.5. EI-MS m/z (relative intensity) 280 (M^+, 8), 254 (7), 202 (13), 162 (79), 136 (72), 118 (100).

(26) **3-(3-Amino-4-hydroxyphenyl)-*E*-[1,2-$^{13}C_2$]prop-2-enoic acid N-acetylcysteamine thioester ([1,2-$^{13}C_2$]-84)** --- To a stirred solution of 3-(3-amino-4-hydroxyphenyl)-*E*-[1,2-$^{13}C_2$]prop-2-enoic acid ([1,2-$^{13}C_2$]-**67b**) (150 mg, 0.644 mmol) in anhydrous THF (5 mL) was added a catalytic amount of DMAP and N-acetylcysteamine (170 mg, 1.43 mmol). DCC (210 mg, 1.02 mmol) was added to the reaction mixture at 0°C, which was then stirred under argon at room temperature overnight. The precipitated urea was removed by filtration and the filtrate was evaporated *in vacuo*. Purification as in (25) afforded [1,2-$^{13}C_2$]-**84** (30 mg, 0.106 mmol, 15%). R_f 0.23 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 7.38 (m, 1H, H-3), 6.94 (d, 1H, J = 2.0 Hz, H-2'), 6.81 (d, 1H, J = 8.1 Hz, H-6'), 6.68 (d, 1H, J = 8.1 Hz, H-5'), 6.58 (ddd, 1H, J = 160.2, 16.1, 5.4 Hz, H-2), 3.20 (m, 2H, SCH_2CH_2N), 3.00 (m, SCH_2CH_2N), 1.79 (s, 3H, CH_3). ^{13}C-NMR (75 MHz, acetone-d_6) δ_C 188.2 (enriched, d, J = 64.7 Hz,

C-1), 169.3, 147.7, 141.9 (d, J = 68.4 Hz, C-3), 137.1, 125.0 (d, J = 7.3 Hz), 120.5 (enriched, d, J = 64.7 Hz, C-2), 119.9, 114.3, 38.4, 28.0, 22.5. HR-MS m/z for $C_{11}{}^{13}C_2H_{17}N_2O_3S$, $[M+H]^+$ calculated 283.1027, found 283.1034.

8.7.6 Methyl 7-(3-N-Acetylamino-4-methoxyphenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienoate **(86)**

(27) **Ethyl 7-(3-amino-4-methoxyphenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienoate (87)** --- A suspension of ethyl 7-(4-methoxy-3-nitrophenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienoate **(82)** (1.60 g, 5.28 mmol) and $SnCl_2x2H_2O$ (6.0 g, 26.7 mmol) in ethyl acetate (20 mL) was stirred at 70 °C under argon for 2 hr until educt disappeared on TLC. The mixture was cooled to room temperature and then poured on ice. The pH was adjusted to neutral and the mixture was extracted three times with ethyl acetate. The combined extracts were dried over $MgSO_4$, filtered and the ethyl acetate evaporated *in vacuo*. Further purification on a silica gel column eluting with hexane/ethyl acetate afforded **87** (1.06 g, 3.88 mmol, 73%): R_f 0.22 [silica gel, hexane/ethyl acetate (2:1)]. ^{1}H-NMR (500 MHz, acetone-d_6) δ_H 7.33 (dd, 1H, J = 15.1, 11.7 Hz, H-3), 6.91 (d, 1H, J = 1.9 Hz, H-2'), 6.84-6.74 (m, 4H, H-5, H-6, H-5', H6'), 6.75 (d, 1H, J = 14.8 Hz, H-7), 6.46 (m, 1H, H-4), 5.89 (d, 1H, J = 15.1 Hz, H-2), 4.14 (q, 2H, J = 7.1 Hz, $COCH_2CH_3$), 3.82 (s, 3H, OCH_3), 1.24 (t, 3H, J = 7.1 Hz, OCH_2CH_3). ^{13}C-NMR (125 MHz, acetone-d_6) δ_C 166.9, 148.5, 145.3, 142.4, 138.4, 138.3, 130.5, 129.3, 126.1, 120.4, 117.9, 112.2, 110.9, 60.4, 55.7,

14.6. EI-MS m/z (relative intensity) 273 (M^+, 100), 244 (66), 200 (92), 184 (40), 169 (38), 156 (31), 156 (28).

(28) **Ethyl 7-(3-N-acetylamino-4-methoxyphenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienoate (88)** --- Compound **87** (85 mg, 0.311 mmol) was acetylated by adding acetic anhydride (156 mg, 1.56 mmol) and triethylamine (31 mg, 0.311 mmol). The mixture was stirred at room temperature overnight. Water was added and the mixture was extracted with ethyl acetate. The organic layer was dried over Na_2SO_4 and the solvent evaporated *in vacuo* to afford **88** (77 mg, 0.244 mmol, 79%): R_f 0.18 [silica gel, hexane/ethyl acetate (1:1)]. ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 8.54 (br, 1H, H-2'), 7.34 (dd, 1H, J = 15.1, 11.4 Hz, H-3), 7.18 (dd, 1H, J = 8.6, 2.1 Hz, H-6'), 6.97 (d, 1H, J = 8.6 Hz, H-5'), 6.90-6.75 (m, 3H, H-5, H-6, H-7), 6.54 (m, 1H, H-4), 5.92 (d, 1H, J = 15.1 Hz, H-2), 4.14 (q, 2H, J = 7.3 Hz, COOCH_2CH$_3$), 3.86 (s, 3H, OCH_3), 2.15 (s, 3H, COCH_3), 1.24 (s, 3H, COOCH$_2$CH_3). ^{13}C-NMR (75 MHz, acetone-d_6) δ_C 169.1, 167.2, 149.7, 145.4, 142.3, 137.8, 130.3, 130.2, 129.5, 127.2, 123.7, 121.0, 118.7, 111.4, 60.5, 56.2, 24.4, 14.6.

(29) **7-(3-N-Acetylamino-4-methoxyphenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienoic acid (89)** --- Compound **88** (25 mg, 0.079 mmol) was hydrolyzed with lithium hydroxide (8 mg, 0.49 mmol) in methanol/water (50/50) at room temperature overnight. The mixture was acidified and extracted with ethyl acetate three times. The combined extracts were dried over $MgSO_4$, filtered and the solvent evaporated *in vacuo* to give **89** (13 mg, 0.045 mmol, 57%).

(30) **Methyl 7-(3-N-acetylamino-4-methoxyphenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienoate (86)** --- The above compound **89** was quantitatively methylated to **86** by diazomethane (in ether). The resulting product was recrystallized from ethyl acetate/hexane as solvent: R_f 0.17 [silica gel, hexane/ethyl acetate (1:1)]. ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 8.53 (br, 1H, H-2'), 7.35 (dd, 1H, J = 15.3, 10.9 Hz, H-3), 7.18 (dd, 1H, J = 8.6, 2.1 Hz, H-6'), 6.98 (d, 1H, J = 8.6 Hz, H-5'), 6.90-6.6.75 (m, 3H, H-5, H-6, H-7), 6.55 (m, 1H, H-4), 5.93 (d, 1H, J = 15.3 Hz, H-2), 3.86 (s, 3H, OCH_3), 3.68 (s, 3H, COOCH_3), 2.15 (s, 3H, COCH_3). ^{13}C-NMR (75 MHz, acetone-d_6) δ_C 167.6, 167.4, 149.8, 145.6, 142.4, 137.8, 130.3, 130.1, 129.6, 127.2, 123.7, 120.5, 119.1, 111.4, 56.3, 51.5, 14.5.

8.7.7 [Acetyl-$^2H_3$, carboxyl-^{13}C]-3-N-acetylamino-4-hydroxybenzoic Acid [Acetyl-$^2H_3$, carboxyl-^{13}C]-**(90)**

(31) **3-N-acetylamino-4-acetoxybenzoic acid (91)** --- 3-Amino-4-hydroxybenzoic acid (500 mg, 3.27 mmol) was acetylated with an excess acetyl chloride CH_3COCl catalyzed by triethylamine. The mixture was stirred at room temperature overnight, and quenched with water. Excess acetic acid and triethylamine were removed by co-distillation with toluene. Crude **91** was used in the next reaction without purification.

(32) **[Acetyl-$^2H_3$, carboxyl-^{13}C]-3-N-acetylamino-4-acetoxybenzoic acid [Acetyl-$^2H_3$, carboxyl-^{13}C]-(91)** --- 3-Amino-4-hydroxy-[7-^{13}C]benzoic acid (154 mg, 1 mmol) was

acetylated with an excess acetyl chloride CD_3COCl catalyzed by triethylamine. The mixture was stirred at room temperature overnight, then quenched with water. Excess acetic acid and triethylamine were removed by co-distillation with toluene. Crude [acetyl-$^2H_3$, carboxyl-^{13}C]-**(91)** was used in next reaction without purification.

(33) **3-N-Acetylamino-4-hydroxybenzoic acid (90)** --- Compound **91** was hydrolyzed with saturated aqueous $NaHCO_3$/ methanol (1:1) solution at room temperature for 6 hr. The mixture was acidified and extracted with ethyl acetate. The resulting product was purified on a silica gel column with CH_2Cl_2/MeOH as eluent to afford **(90)** (322 mg, 1.65 mmol, 50% in two steps): R_f 0.19 [silica gel, MeOH/CH_2Cl_2 (10:1)]. ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H 8.41 (d, 1H, J = 1.9 Hz, H-2), 7.55 (dd, 1H, J = 8.3, 1.9 Hz, H-6), 6.90 (d, 1H, J = 8.3 Hz, H-5), 2.09 (s, 3H, CH_3). ^{13}C-NMR (75 MHz, DMSO-d_6) δ_C 168.9, 167.1, 151.9, 126.2, 126.1, 123.7, 121.3, 115.0, 23.7.

(34) **[Acetyl-$^2H_3$, carboxyl-^{13}C]-3-N-acetylamino-4-hydroxybenzoic acid [acetyl-$^2H_3$, carboxyl-^{13}C]-(90)** --- Under the same conditions as in (33), compound [acetyl-$^2H_3$, carboxyl-^{13}C]-**(91)** was hydrolyzed to afford [acetyl-$^2H_3$, carboxyl-^{13}C]-**90**: R_f 0.19 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H 8.40 (m, 1H, H-2), 7.54 (m, 1H, H-6), 6.90 (d, 1H, J = 8.3 Hz, H-5). ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C 168.9 ($COCD_3$), 167.1 (enriched, C-7), 151.8, 126.3 (d, J = 3.2 Hz), 126.1 (d, J = 4.8 Hz), 123.6 (d, J = 4.8 Hz), 121.2 (d, J = 73.0 Hz, C-1), 115.0 d, J = 4.8 Hz), 23.3 (br, $COCD_3$). HR-MS m/z for $C_8{}^{13}CH_6{}^2H_3NO_4Na$, $[M+Na]^+$ calculated 222.0651, found 222.0646.

8.7.8 7-(3-N-Acetylamino-4-hydroxyphenyl)-(*2E,4E,6E*)-[1,2-^{13}C]hepta-2,4,6-trienoic Acid [1,2-^{13}C]-**(85)**

(35) **7-(3-N-Acetylamino-4-acetoxyphenyl)-(*2E,4E,6E*)-[1,2-$^{13}C_2$]hepta-2,4,6-trienoic acid ([1,2-$^{13}C_2$]-92)** --- [1,2-$^{13}C_2$]-7-(3-Amino-4-hydroxyphenyl)-(*2E,4E,6E*)-hepta-2,4,6-trienoic acid ([1,2-$^{13}C_2$]-**69b**) (120 mg, 0.515 mmol) was acetylated with acetic anhydride (258 mg, 2.58 mmol) and triethylamine (52 mg, 0.515 mmol). The mixture was stirred at room temperature overnight. Excess acetic anhydride and triethylamine were removed by co-distillation with toluene. The residue was used directly in next reaction.

(36) **7-(3-N-Acetylamino-4-hydroxyphenyl)-(*2E,4E,6E*)-[1,2-^{13}C]hepta-2,4,6-trienoic acid [1,2-^{13}C]-(85)** --- [1,2-$^{13}C_2$]-**92** was hydrolyzed in saturated aqueous $NaHCO_3$/ methanol solution at room temperature for 6 hr. The mixture was acidified and extracted with ethyl acetate. The combined extracts were dried over $MgSO_4$, filtered and the solvent was evaporated *in vacuo*. [1,2-^{13}C]-**(85)** (65 mg, 0.236 mmol) was obtained (46% yield over the two steps): R_f 0.21 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (500 MHz, DMSO-d_6) δ_H 7.91 (br s, 1H, H-2'), 7.24 (m, 1H, H-3), 7.12 (dd, 1H, J = 8.0, 1.9 Hz, H-6'), 6.84 (d, 1H, J = 8.0 Hz, H-5'), 6.83-6.6.69 (m, 2H, H-5, H-6), 6.69 (d, 1H, J = 14.8 Hz, H-7), 6.49 (ddd, 1H, J = 14.8, 11.1, 3.1 Hz, H-4), 5.85 (ddd, 1H, J = 161.3, 15.5, 3.1 Hz, H-2), 2.09 (s, 3H, CH_3), ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C 168.8, 167.5

(enriched, d, J = 71.4 Hz, C-1), 148.5, 144.3 (d, J = 68.2 Hz, C-3). 141.2 (d, J = 9.5 Hz), 136.6, 128.9 (d, J = 7.9 Hz), 127.5, 126.6, 126.5, 125.4, 123.5, 120.6 (enriched, d, J = 71.4 Hz, C-2), 115.8, 23.7. HR-MS $[M+Na]^+$ for $C_{13}{}^{13}C_2H_{15}NO_4$, calculated 298.0966, found 298.0973.

8.7.9 [6-^{13}C]Cyclopentanecarboxylic Acid [6-^{13}C]-**(93)**

(37) **[6-^{13}C]Cyclopentanecarboxylic acid [6-^{13}C]-(93)** --- Cyclopentyl bromide (1.5 g, 9.93 mmol) was added to a mixture of magnesium shavings (0.36 g, 15 mmol) in anhydrous THF (10 mL) under argon at 50 °C. One drop of 1,2-dibromoethane was added to initiate the reaction. After 1 hr, the mixture was cooled to 0 °C and 240 mL of $^{13}CO_2$ (99 atom%) was bubbled from a syringe. The reaction mixture was stirred for 0.5 hr and poured into water, acidified and extracted with diethyl ether. The ether layer was washed with brine. Rvaporation of the diethyl ether provided [6-^{13}C]-**(93)** (0.62 g, 5.34 mmol, 54%): ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 2.72 (m, 1H, H-1), 1.90-1.50 (m, 8H, H-2 – H-5). ^{13}C-NMR (75 MHz, acetone-d_6) δ_C 177.6 (enriched, C-6), 44.0 (d, J = 56.2 Hz, C-1), 30.6, 26.3 (d, J = 3.7 Hz). EI-MS m/z (relative intensity) 115 (M^+, 42), 87 (71), 74 (94), 69 (82), 43 (100).

8.7.10 7-Cyclohexyl-(*2E,4E,6E*)-hepta-2,4,6-trienoic Acid **(95)**

(38) **Ethyl 3-cyclohexyl-*E*-acrylate (98)** --- A suspension of cyclohexanecarboxaldehyde **(97)** (10.1 g, 90.1 mmol) and triethyl phosphonoacetate (24 g, 107 mmol) in aqueous 6 M K_2CO_3 (45 mL) was stirred at room temperature for 24 hour. The reaction mixture was diluted with water and extracted with ethyl acetate (2x200 mL). The combined extracts were dried over $MgSO_4$, and ethyl acetate was removed *in vacuo*. Compound **98** was obtained in quantitative yield (16.32 g, 89.7 mmol): R_f 0.66 [silica gel, hexane/ethyl acetate (2:1)]. ^{1}H-NMR (300 MHz, $CDCl_3$) δ_H 6.91 (dd, 1H, J = 15.6, 6.8 Hz, H-3), 5.76 (dd, 1H, J = 15.6, 1.0 Hz, H-2), 4.18 (q, 2H, J = 7.2 Hz, OCH_2CH_3), 2.14 (m, 1H, H-1'), 1.72 (m, 4H, H-3'), 1.35-1.12 (m, 9H, H-4', H-2', OCH_2CH_3). ^{13}C-NMR (75 MHz, $CDCl_3$) δ_C 167.0, 154.1, 118.8, 59.9, 40.4, 31.6, 25.7, 25.6, 14.1.

(39) **3-Cyclohexyl-*E*-prop-2-en-1-ol (99)** --- Diisobutylaluminum hydride (DIBAL-H, 180 mL, 1 M in toluene) was added slowly to a solution of **98** (14.81 g, 81.4 mmol) in toluene (40 mL) under argon at –78 °C. After 1 hour at –78 °C and 1 hour at room temperature, methanol was added slowly to destroy excess DIBAL-H. The resulting mixture was poured into water and extracted with ethyl acetate (3x200 mL). The combined organic layer was washed with brine, dried over $MgSO_4$ and filtered. Ethyl acetate was removed *in vacuo* to give **99** which was used without further purification: ^{1}H-NMR (300 MHz, $CDCl_3$) δ_H 5.59 (m, 2H, H-2, H-3), 4.06 (d, 2H, J = 4.3 Hz, H-1), 1.95 (m, 1H, H-1'), 1.70 (m, 4H, H-3'), 1.35-1.05 (m, 6H, H-4', H-2'). ^{13}C-NMR (75 MHz, $CDCl_3$) δ_C 138.8, 126.3, 63.6, 40.2, 32.7, 26.0, 25.9.

(40) **3-Cyclohexyl-*E*-propenal (100)** --- To a solution of **99** (11.82 g, 84.4 mmol) in chloroform (250 mL) was added activated manganese dioxide (70 g). The mixture was stirred at room temperature for 5 hours, filtered through Celite and the solvent was removed *in vacuo*. Further purification was conducted on a silica gel flash column with hexane/ethyl acetate as eluent to give **100** (5.49 g, 39.8 mmol, 47% yield over the two steps): R_f 0.45 [silica gel, hexane/ethyl acetate (2:1)]. ^{1}H-NMR (300 MHz, $CDCl_3$) δ_H 9.50 (d, 1H, J = 8.0 Hz, H-1), 6.84 (dd, 1H, J = 15.6, 6.8, H-3), 6.07 (ddd, 1H, J = 15.6, 8.0, 1.5, H-2), 2.30 (m, 1H, H-1'), 1.80 (m, 4H, H-3'), 1.36-1.16 (m, 6H, H-4', H-2'). ^{13}C-NMR (75 MHz, $CDCl_3$) δ_C 194.3, 163.6, 130.3, 40.7, 31.3, 25.7, 25.4.

(41) **Ethyl 5-cyclohexyl-(*2E,4E*)-penta-2,4-dienoate (101)** --- A suspension of 3-cyclohexyl-propenal (2.44 g, 17.6 mmol) and triethyl phosphonoacetate (3.96 g, 17.6 mmol) in aqueous 6 M K_2CO_3 (9 mL) was stirred at room temperature for 24 hours. The mixture was diluted with water and extracted with ethyl acetate (2x50 mL). The extracts were dried over $MgSO_4$, filtered, and the ethyl acetate was removed *in vacuo* to afford **101** (quantitative, 3.67 g, 17.6 mmol): R_f 0.68 [silica gel, hexane/ethyl acetate (2:1)]. ^{1}H-NMR (300 MHz, $CDCl_3$) δ_H 7.25 (dd, 1H, J = 15.0, 9.9 Hz, H-3), 6.10 (m, 2H, H-4, H-5), 5.79 (d, 1H, J = 15.0, H-2), 4.18 (q, 2H, J = 6.8 Hz, OCH_2CH_3), 2.10 (m, 1H, H-1'), 1.75 (m, 4H, H-3'), 1.40-1.10 (m, 9H, H-4', H-2', OCH_2CH_3). ^{13}C-NMR (75 MHz, $CDCl_3$) δ_C 167.2, 150.0, 145.4, 125.7, 119.2, 60.0, 41.0, 32.2, 25.9, 25.7, 14.2.

(42) **5-Cyclohexyl-(*2E,4E*)-penta-2,4-dien-1-ol (102)** --- Diisobutylaluminum hydride (39 mL, 1 M in toluene) was added slowly to a solution of **101** (3.66 g, 17.6 mmol) in toluene (20 mL) under argon at –78 °C. After 1 hour at –78 °C and 1 hour at room temperature, methanol was added slowly to destroy excess DIBAL-H. The resulting mixture was poured into water and extracted with ethyl acetate 3x100 mL. The combined organic layers were washed with brine, dried over $MgSO_4$ and filtered. Ethyl acetate was removed *in vacuo* to give **102** (2.28 g, 13.7 mmol, 77%).

(43) **5-Cyclohexyl-(*2E,4E*)-penta-2,4-dienal (103)** --- To a solution of **102** (2.28 g, 13.7 mmol) in chloroform (100 mL) was added activated manganese dioxide (21 g). The reaction mixture was stirred at room temperature for 5 hour and was filtered through Celite. Chloroform was removed and the resulting crude product was purified on a silica gel column with hexane/ethyl acetate as eluent to afford **103** (2.09 g, 12.7 mmol, 93%): R_f 0.39 [silica gel, hexane/ethyl acetate (10:1)]. ^{1}H-NMR (300 MHz, $CDCl_3$) δ_H 9.53 (d, 1H, J = 7.8 Hz, H-1), 7.08 (dd, 1H, J = 15.1, 9.8, H-3), 6.25 (m, 2H, H-4, H-5), 6.09 (dd, 1H, J = 15.1, 7.8 Hz, H-2), 2.15 (m, 1H, H-1'), 1.75 (m, 4H, H-3'), 1.35-1.05 (m, 6H, H-4', H-2'). ^{13}C-NMR (75 MHz, $CDCl_3$) δ_C 193.9, 153.3, 152.6, 130.1, 126.1, 41.3, 32.1, 25.9, 25.7. EI-MS m/z (relative intensity) 164(M^+, 8), 135 (5), 95 (22), 81 (100), 67 (46).

(44) **Ethyl 7-cyclohexyl-(*2E,4E,6E*)-hepta-2,4,6-trienoate (104)** --- A suspension of **103** (2.09 g, 12.7 mmol) and triethyl phosphonoacetate (3.43 g, 15.2 mmol) in aqueous 6 M K_2CO_3 (10 mL) was stirred at room temperature for 3 days. The mixture was diluted

with water and extracted with ethyl acetate (2x100 mL). The extract was dried over $MgSO_4$, and the ethyl acetate removed *in vacuo* to afford **104** (2.07 g, 8.85 mmol, 70%): R_f 0.66 [silica gel, hexane/ethyl acetate (2:1)]. ^{1}H-NMR (300 MHz, $CDCl_3$) δ_H 7.32 (dd, 1H, J = 15.5, 11.4 Hz, H-3), 6.51 (dd, 1H, J = 15.1, 10.5 Hz, H-5), 6.22 (dd, 1H, J = 15.1, 11.4 Hz, H-4), 6.10 (dd, 1H, J = 15.1, 10.5 Hz, H-6), 5.87 (dd, 1H, J = 15.1, 6.7, H-7), 5.83 (d, 1H, J = 15.5 Hz, H-2), 4.19 (q, 2H, J = 7.2 Hz, OCH_2CH_3), 2.08 (m, 1H, H-1'), 1.72 (m, 4H, H-3'), 1.29 (t, 3H, J = 7.2 Hz, OCH_2CH_3), 1.12 (m, 6H, H-4', H-2'). ^{13}C-NMR (75 MHz, $CDCl_3$) δ_C 167.2, 146.1, 144.8, 141.5, 127.8, 119.8, 60.1, 41.0, 32.4, 26.0, 25.8, 14.2.

(45) **7-Cyclohexyl-(*2E,4E,6E*)-hepta-2,4,6-trienoic acid (95)** --- **104** (2.07 g, 8.85 mmol) was hydrolyzed with lithium hydroxide (1.06 g, 44.2 mmol) in 50 mL methanol/water (50/50) at room temperature overnight. The mixture was acidified and extracted with ethyl acetate (3x250 mL). The combined extracts were dried over $MgSO_4$, filtered and the ethyl acetate was evaporated *in vacuo*. The crude product was re-dissolved in 1 M NaOH and washed with ethyl acetate. The aqueous layer was acidified and extracted with ethyl acetate to give pure **95** (1.011 g, 4.91 mmol, 56%): R_f 0.41 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H 7.19 (dd, 1H, J = 15.3, 11.4 Hz, H-3), 6.64 (dd, 1H, J = 15.1, 10.4 Hz, H-5), 6.33 (dd, 1H, J = 15.1, 11.4 Hz, H-4), 6.14 (dd, 1H, J = 15.1, 10.4 Hz, H-6), 5.91 (dd, 1H, J = 15.1, 6.8 Hz, H-7), 5.83 (d, 1H, J = 15.3 Hz, H-2), 2.04 (m, 1H, H-1'), 1.67 (m, 4H, H-3'), 1.35-1.05 (m, 6H, H-4', H-2'). ^{13}C-NMR (75 MHz, DMSO-d_6) δ_C 167.6, 145.4, 144.4, 141.1, 128.2, 127.5, 120.9, 40.3,

32.0, 25.6, 25.4. EI-MS m/z (relative intensity) 206 (M^+, 80),161 (81), 124 (53), 79 (100).

8.7.11 5-Cyclohexyl-penta-2,4-dienal **(103)** - Alternative Synthesis

(46) **Potassium glutaconaldehyde (106)** --- KOH (28 g, 0.5 mol) and H_2O (72 mL) were placed in a 500 mL round-bottomed flask. With magnetic stirring at –20 °C, pyridinium-1-sulfonate **(105)** (20 g, 126 mmol) was added in one portion. The mixture was stirred for 1 hr during which it turned yellow. The mixture was gradually warmed to room temperature for 4 hr. The solid product was filtered and washed with acetone (3x80 mL) to give compound **106** (12.31 g, 90.5 mmol, 72%): ^{1}H-NMR (500 MHz, DMSO-d_6) δ_H 8.65 (d, 2H, J = 9.3 Hz, H-1, H-5), 7.03 (t, 1H, J = 13.0, H-3), 5.10 (dd, 2H, J = 13.0, 9.3 Hz, H-2, H-4). ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C 184.3 (C-1, C-5), 159.8 (C-3), 106.2 (C-2, C-4).

(47) **5-Cyclohexyl-penta-2,4-dienal (103)** --- Triethylamine (30 drops) and 4-dimethylaminopyridine (60 mg) were added to a slurry of anhydrous potassium glutaconaldehyde (6 g, 44.1 mmol) in anhydrous THF (75 mL) at room temperature under argon. The mixture was stirred and tert-butyldimethylsilyl chloride (46 mL, 1 M in THF) was added. After vigorous stirring for 3 hr the reaction mixture was cooled to 0 °C and a freshly prepared solution of cyclohexyl magnesium bromide in THF (9.14 g bromocyclohexane, 2.15 g Mg and 60 mL THF) was added. The reaction was warmed to

room temperature for 2 hr and 3 M HCl (60 mL) was added. The mixture was stirred for a further 2 hr and subsequently extracted with diethyl ether (3x200 mL). The combined ether extracts were washed with brine, dried over Na_2SO_4, and the ether was removed *in vacuo*. The crude product was purified by flash column chromatography with hexane/ethyl acetate (10:1) as eluent to afford **103** (3.24 g, 19.8 mmol, 45%).

8.7.12 2-Amino-3-hydroxy-2-cyclopenten-1-one Hydrochloride **(96)**

(48) **3-Hydroxy-2-nitrocyclopent-2-en-1-one (111)** --- Nitrogen dioxide gas was passed through a suspension of 1,3-cyclopentanedione **(110)** (0.4 g, 4.08 mmol) in anhydrous diethyl ether with stirring at room temperature for 1 hour. Ether was evaporated in a stream of air, and the solid product was filtered and washed with diethyl ether to afford **111** (0.52 g, 3.64 mmol, 90%): ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H 2.39 (br, s, 4H, H-4, H-5). ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C 191.1 (C-1, C-3), 125.9 (C-2), 29.7 (C-4, C-5). EI-MS m/z (relative intensity) 143 (M^+, 86), 126 (52), 110 (18), 70 (46), 55 (100).

(49) **2-Amino-3-hydroxy-2-cyclopenten-1-one hydrochloride (96)** --- A mixture of 3-hydroxy-2-nitro-2-cyclopenten-1-one (40 mg, 0.28 mmol), platinum dioxide (4 mg) and 2N HCl (1 mL) in glacial acetic acid (10 mL) was stirred under hydrogen gas at room temperature for 2 hr. The reaction mixture was filtered and the filtrate was evaporated by azeotropic distillation with toluene to leave **96** as a dark solid: ^{1}H-NMR (300 MHz, methanol-d_6) δ_H 2.62 (s, 4H, H-4, H-5). ES-MS m/z $[M+H]^+$ 114.

8.7.13 Arylstannanes - Stille Approach

(50) **Bromopentadienal (117)** --- Glutaconaldehyde potassium salt (1.5 g) was added to dibromotriphenylphosphorane (3.12 g) in anhydrous methylene chloride (15 mL) under argon at 0 °C. The reaction mixture was stirred at 0 °C for 2 hr and at room temperature for 8 hr and was subsequently filtered. The filtrate was concentrated to afford a dark brown solid. EI-MS and ^{1}H-NMR showed product (MW 160 and 162) at low yield together with tribromo- substituted by-product.

(51) **2-Amino-4-bromophenol (131)** --- A suspension of 4-bromo-2-nitrophenol **(128)** (6 g, 27.5 mmol) and $SnCl_2x2H_2O$ (31 g, 138 mmol) in ethanol (75 mL) was heated at 70 °C under argon for 1 hr. The reaction mixture turned from yellow to white at 0.5 hr. The reaction mixture was cooled, diluted with water and adjusted to pH 7 with 40% NaOH, and extracted with ethyl acetate (3x300 mL). The combined extracts were dried over Na_2SO_4 and ethyl acetate was evaporated *in vacuo* to give **131** (4.72 g, 25.1 mmol, 91%): R_f 0.23 [silica gel, hexane/ethyl acetate (2:1)]. ^{1}H-NMR (500 MHz, acetone-d_6) δ_H 6.83 (d, 1H, J = 2.5 Hz, H-3), 6.64 (d, 1H, J = 8.7 Hz, H-6), 6.56 (dd, 1H, J = 8.7, 2.5 Hz, H-5).

(52) **2-N-BOC-amino-4-bromophenol (134)** --- A solution of **131** (3.5 g, 18.6 mmol), BOC_2O (4.87 g, 22.3 mmol) in anhydrous THF (20 mL) was stirred at room temperature

under argon for 3 days. THF was removed by rotary evaporation to give a sticky dark brown liquid. Hexane was added to precipitate the product, followed by filtration and evaporation of solvent to give **134** (4.6 g, 16.0 mmol, 86%): R_f 0.41 [silica gel, hexane/ethyl acetate (2:1)]. ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 8.07 (d, 1H, J = 2.3 Hz, H-3), 7.00 (dd, 1H, J = 8.6, 2.3 Hz, H-5), 6.82 (d, 1H, J = 8.6 Hz, H-6), 1.49 (s, 9H, CH_3). ^{13}C-NMR (75 MHz, acetone-d_6) δ_C 153.7, 145.9, 129.6, 126.1, 122.3, 117.4, 112.1, 81.0, 28.3.

(53) **2-N-BOC-amino-4-bromophenol TBDMS ether (135)** --- A solution of **134** (3.9 g, 13.5 mmol), imidazole (1.84 g) and *tert*-butyldimethylsilyl chloride (TBDMS-Cl, 13.5 mL, 1 M in THF) in anhydrous THF (20 mL) was stirred at room temperature under argon for 3 days. The mixture was filtered and the filtrate concentrated. Purification was performed on a silica gel column eluting with a gradient of hexane/ethyl acetate to afford **135** (4.84 g, 12.3 mmol, 91%): R_f 0.71 [silica gel, hexane/ethyl acetate (2:1)]. ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 8.21 (d, 1H, J = 2.3 Hz, H-3), 7.03 (dd, 1H, J = 8.6, 2.3 Hz, H-5), 6.81 (d, 1H, J = 8.6 Hz, H-6), 1.50 (s, 9H, OC(CH_3)$_3$), 1.03 (s, 9H, SiC(CH_3)$_3$), 0.27 (s, 6H, Si(CH_3)$_2$). ^{13}C-NMR (75 MHz, acetone-d_6) δ_C 152.6, 143.8, 132.5, 125.6, 122.1, 120.3, 114.2, 80.7, 28.3, 26.0, 18.7, -4.4. EI-MS m/z (relative intensity) 401 (M^+, 48), 403 (M^+, 48), 348 (18), 346 (18), 288 (81), 290 (81), 244 (77), 246 (75), 228 (68), 230 (68), 165 (62), 73 (73 (76), 57 (100).

(54) **4-Bromo-2-nitrophenol TBDMS ether (132)** — A solution of 4-bromo-2-nitrophenol **(128)** (1.00 g, 4.59 mmol), TBDMS-Cl (0.83 g, 5.91 mmol) and DMAP (1.12 g) in anhydrous THF (20 mL) was stirred under argon at room temperature for 1 day. The resulting product was purified on a silica gel column eluting with a gradient of hexane/ethyl acetate to afford **132** as light yellow oil (0.64 g, 1.99 mmol, 42%): R_f 0.69 [silica gel, hexane/ethyl acetate (2:1)]. ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 7.96 (d, 1H, J = 2.6 Hz, H-3), 7.68 (dd, 1H, J = 8.8, 2.6 Hz, H-5), 7.12 (d, 1H, J = 8.8 Hz), 0.98 (s, 9H, SiC(CH_3)$_3$), 0.97 (s, 6H, Si(CH_3)$_2$). ^{13}C-NMR (75 MHz, acetone-d_6) δ_C 148.7, 140.6, 137.3, 128.3, 124.6, 112.9, 25.8, 18.6, -4.4. EI-MS m/z (relative intensity) 334 (M^+, 11), 332 (M^+, 11), 318 (37), 316 (37), 376 (96), 374 (96), 260 (71), 258 (67), 246 (50), 244 (52), 231 (68), 229 (68), 216 (56), 214 (55), 104 (72), 57 (100).

(55) **2-Nitro-4-(tributylstannyl)-phenol TBDMS ether (133)** — A suspension of **132** (83 mg, 0.258 mmol), magnesium shavings (14 mg, 0.583 mmol), $(Bu_3Sn)_2O$ (149 mg, 25.5 mmol) and 1,2-dibromoethane (52 mg, 0.275 mmol) in anhydrous THF (1 mL) was heated at 45 °C under argon in an ultrasonicator (Branson B-22-4, 50/60 Hz) for 1.5 hr, after which no starting material was visible on TLC. The reaction mixture was added to water and extracted with ethyl acetate (3x10 mL), the extract dried over Na_2SO_4 and filtered. Ethyl acetate was removed *in vacuo*. Column chromatography on silica gel with hexane/diethyl ether (50:1) afforded a mixture of arylstannane products (27 mg). Further separation on preparative TLC was performed eluting with hexane/ethyl acetate (50:1) three times. The unstable compound **133** was obtained in very low yield (1 mg, 0.002

mmol): EI-MS m/z 541, 543, 545 (M^+ tin isotope pattern), 427, 429, 431 (tin isotope pattern, loss of TBDMS).

8.7.14 N_1-(2-Hydroxy-5-oxo-cyclopent-1-enyl)-7-(3-amino-4-hydroxyphenyl)-hepta-(*2E,4E,6E*)-trienamide ([1,2-$^{13}C_2$]-**70**)

(56) **7-(4-Methoxy-3-nitrophenyl)-hepta-(*2E,4E,6E*)-trienoic acid (137)** --- A suspension of ethyl 7-(4-methoxy-3-nitro-phenyl)-hepta-(*2E,4E,6E*)-trienoate **(82)** (1.0 g, 3.30 mmol), LiOH (0.69 g, 28.8 mmol) in THF (40 mL), methanol (20 mL) and water (20 mL) was stirred at room temperature for 48 hr. The reaction mixture was acidified and extracted with ethyl acetate (3x500 mL). The organic layer was dried over $MgSO_4$, and ethyl acetate was removed *in vacuo*. Further purification by silica gel flash column chromatography with CH_2Cl_2/methanol (10:1) afforded **137** (0.60 g, 2.18 mmol, 66%): R_f 0.36 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H 8.04 (d, 1H, J = 2.3 Hz, H-2'), 7.80 (dd, 1H, J = 8.8, 2.3 Hz, H-6'), 7.34 (d, 1H, J = 8.8 Hz, H-5'), 7.26 (dd, 1H, J = 15.1, 11.4 Hz, H-3), 7.06 (dd, 1H, J = 15.6, 10.4 Hz, H-6), 6.81 (m, 2H, H-5, H-7), 6.54 (dd, 1H, J = 15.1, 11.4, H-4), 5.93 (d, 1H, J = 15.1 Hz, H-2), 3.92 (s, 3H, OCH_3). ^{13}C-NMR (75 MHz, DMSO-d_6) δ_C 167.7, 151.5, 144.0, 140.3, 139.7, 133.5, 132.3, 131.0, 129.5, 128.9, 122.7, 122.1, 114.7, 56.9. EI-MS m/z (relative intensity) 275 (M^+, 78), 258 (28), 245 (11), 184 (44), 153 (100), 115 (83), 77 (54).

(57) **7-(4-Methoxy-3-nitrophenyl)-[1,2-$^{13}C_2$]hepta-(*2E,4E,6E*)-trienoic acid ([1,2-$^{13}C_2$]-137)** --- A suspension of ethyl 7-(4-methoxy-3-nitro-phenyl)-[1,2-$^{13}C_2$]hepta-(*2E,4E,6E*)-trienoate ([1,2-$^{13}C_2$]-**82**) (0.7 g, 2.30 mmol), LiOH (0.5 g, 20.8 mmol) in the mixed solvent of THF (30 mL) and water (15 mL) was stirred at room temperature for 5 days. The reaction mixture was acidified and extracted with ethyl acetate (3x500 mL). The organic layer was dried over $MgSO_4$, and ethyl acetate was removed *in vacuo*. The resulting solid was washed with cold ethyl acetate to afforded [1,2-$^{13}C_2$]-**137** (0.59 g, 2.13 mmol, 92%): R_f 0.36 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H 8.04 (d, 1H, J = 2.1 Hz, H-2'), 7.81 (dd, 8.8, 2.1 Hz, H-6'), 7.35 (d, 1H, J = 8.8 Hz, H-5'), 7.26 (m, 1H, H-3), 7.07 (dd, 1H, J = 15.6, 10.4 Hz, H-6), 7.79 (m, 2H, H-5, H-7), 6.55 (m, 1H, H-4), 5.95 (ddd, 1H, J = 161.9, 15.6, 3.1 Hz, H-2), 3.93 (s, 3H, OCH_3). ^{13}C-NMR (75 MHz, DMSO-d_6) δ_C 167.6 (enriched, d, J = 72.0 Hz, C-1), 151.5, 144.0 (d, J = 69.6 Hz, C-3), 140.4 (d, J = 9.8 Hz), 139.7, 133.5, 132.3, 130.9, 129.4, 128.9, 122.7, 122.0 enriched, (d, J = 72.0 Hz, C-2), 114.7, 56.9. EI-MS m/z (relative intensity) 277 (M^+, 86), 260 (28), 247 (78), 185 (100), 154 (87), 116 (82), 77 (63).

(58) **7-(4-Methoxy-3-nitrophenyl)-hepta-(*2E,4E,6E*)-trienoyl chloride (138)** --- To a mixture of **137** (190 mg, 0.691 mmol), methylene chloride (8 mL) and DMF (0.5 mL) was added oxalyl chloride (97 mg, 0.764 mmol) in methylene chloride (12 mL) at 0 °C under argon over 15 min. The suspension was stirred at room temperature for 2 hr and turned into a clear light yellow solution. This solution of compound **138** was used directly in the coupling reaction.

(59) **7-(4-Methoxy-3-nitrophenyl)-[1,2-$^{13}C_2$]hepta-(*2E,4E,6E*)-trienoyl chloride ([1,2-$^{13}C_2$]-138)** --- To a mixture of [1,2-$^{13}C_2$]-**137** (550 mg, 1.99 mmol), methylene chloride (20 mL) and DMF (1mL) was added oxalyl chloride (380 mg, 2.99 mmol) in methylene chloride (20 mL) at 0 °C under argon in 20 min. The suspension was stirred at room temperature for 2 hr and turned into a clear light yellow solution. This solution of compound [1,2-$^{13}C_2$]-**138** was used directly in coupling reaction.

(60) **N_1-(2-Hydroxy-5-oxo-cyclopent-1-enyl)-7-(4-methoxy-3-nitrophenyl)-hepta-(*2E,4E,6E*)-trienamide (139)** --- The solution of **138** was added to the mixture of 2-amino-3-hydroxy-2-cyclopenten-1-one hydrochloride **(96)** (freshly made from 120 mg 3-hydroxy-2-nitrocyclopent-2-en-1-one), DMAP (45 mg) and anhydrous pyridine (15 mL) at 0 °C under argon over 15 min. The reaction mixture was stirred at room temperature for 2 hr, after which the solvent was removed by co-distillation with toluene. Flash column chromatography with methylene chloride/methanol (40:1) as eluent afforded **139** (225 mg, 0.608 mmol, 88%): R_f 0.53 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H 8.04 (d, 1H, J = 2.1 Hz, H-2'), 7.81 (dd, 1H, J = 8.8, 2.1 Hz, H-6'), 7.36 (d, 1H, J = 8.8 Hz, H-5'), 7.29 (dd, 1H, J = 15.1, 11.4 Hz, H-3), 7.13 (dd, 1H, J = 15.7, 10.6 Hz, H-6), 6.87 (dd, 1H, J = 14.5, 10.6 Hz, H-5), 6.81 (d, 1H, J = 15.7 Hz, H-7), 6.58 (d, 1H, J = 15.1 Hz, H-2), 6.54 (dd, 1H, J = 14.5, 11.4 Hz, H4), 3.93 (s, 3H, OCH_3), 2.45 (s, 4H, H-4", H-5"). EI-MS m/z (relative intensity) 370 (M^+, 29), 353 (17), 258 (62), 192 (73), 184 (100), 169 (44), 153 (65), 115 (51), 77 (43).

(61) **N_1-(2-Hydroxy-5-oxo-cyclopent-1-enyl)-7-(4-methoxy-3-nitrophenyl)-[1,2-$^{13}C_2$]-hepta-(*2E,4E,6E*)-trienamide ([1,2-$^{13}C_2$]-139)** --- The solution of [1,2-$^{13}C_2$]-**138** was added to the mixture of 2-amino-3-hydroxy-2-cyclopenten-1-one hydrochloride **(96)** (freshly made from 340 mg 3-hydroxy-2-nitrocyclopent-2-en-1-one), DMAP (90 mg) and anhydrous pyridine (30 mL) at 0 °C under argon over 20 min. The reaction mixture was stirred at room temperature for 2 hr, after which the solvent was removed by co-distillation with toluene. Flash column chromatography with a gradient of methylene chloride/methanol as eluent afforded [1,2-$^{13}C_2$]-**139** (577 mg, 1.55 mmol, 78%): R_f 0.53 [silica gel, MeOH/CH_2Cl_2 (10:1)].

(62) **N_1-(2-hydroxy-5-oxo-cyclopent-1-enyl)-7-(4-hydroxy-3-nitrophenyl)-hepta-(*2E,4E,6E*)-trienamide (140)** --- To a suspension of **139** (50 mg, 0.135 mmol) in anhydrous chloroform (3 mL) was added BBr_3 (4 mL, 1 M in methylene chloride) at –78 °C under argon. The solution immediately turned dark brown. After 1 hr at –78 °C, the reaction was maintained at room temperature for 9 hr. Water was added to destroy excess BBr_3 and the mixture was extracted with ethyl acetate, the extract dried over Na_2SO_4, and the ethyl acetate evaporated *in vacuo*. Purification by silica gel flash column chromatography with methylene chloride/methanol (40:1) afforded **140** (11 mg, 0.031 mmol 23%): R_f 0.51 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (500 MHz, DMSO-d_6) δ_H 8.01 (d, 1H, J = 1.9 Hz, H-2'), 7.73 (dd, 1H, J = 8.7, 1.9 Hz, H-6'), 7.29 (dd, 1H, J = 14.8, 11.7 Hz, H-3), 7.11 (d, 1H, J = 8.7 Hz, H-5'), 7.07 (dd, 1H, J = 15.5,

11.1 Hz, H-6), 6.86 (dd, 1H, J = 14.8, 11.1 Hz, H-5), 6.79 (d, 1H, J = 15.5 Hz, H-7), 6.57 (d, 1H, J = 14.8 Hz, H-2), 6.53 (dd, 1H, J = 14.8, 11.7 Hz, H-4), 2.45 (br, 4H, H-4", H-5"). ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C 165.9, 151.7, 142.3, 140.8, 137.1, 133.8, 132.7, 130.6, 128.2, 123.1, 122.1, 121.6, 119.4, 114.8. EI-MS m/z (relative intensity) 356 (M^+, 79), 338 (18), 244 (94), 178 (54), 170 (100).

(63) N_1-**(2-Hydroxy-5-oxo-cyclopent-1-enyl)-7-(4-hydroxy-3-nitrophenyl)-[1,2-$^{13}C_2$]-hepta-(*2E,4E,6E*)-trienamide ([1,2-$^{13}C_2$]-140)** --- To a suspension of [1,2-$^{13}C_2$]-**139** (250 mg, 0.672 mmol) in anhydrous chloroform (15 mL) was added BBr_3 (20 mL, 1 M in methylene chloride) at –78 °C under argon. The solution turned dark brown right away. After 1 hr at –78 °C, the reaction was maintained at room temperature for 9 hr. Water was added to destroy excess BBr_3 and the mixture was extracted with ethyl acetate, the extract dried over Na_2SO_4, and the ethyl acetate evaporated *in vacuo*. Purification by silica gel flash column chromatography with a gradient of methylene chloride/methanol afforded [1,2-$^{13}C_2$]-**140** (51 mg, 0.142 mmol, 21%): R_f 0.51 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (500 MHz, DMSO-d_6) δ_H 7.99 (d, 1H, J = 2.3 Hz, H-2'), 7.70 (dd, 1H, J = 8.7, 2.3 Hz, H-6'), 7.28 (m, 1H, H-3), 7.08 (d, 1H, J = 8.7 Hz, H-5'), 7.04 (dd, 1H, J = 15.5, 10.5 Hz, H-6), 6.85 (dd, 1H, J = 14.8, 10.5 Hz, H-5), 6.78 (d, 1H, J = 15.5 Hz, H-7), 6.56 (ddd, 1H, J = 161.9, 14.8, 4.3 Hz, H-2), 6.52 (ddd, 1H, J = 14.8, 11.7, 4.3 Hz, H-4), 2.45 (br, 4H, H-4", H-5"). ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C 165.9 (enriched, d, J = 65.0 Hz, C-1), 152.5, 142.3 (d, J = 69.8 Hz, C-3), 140.9 (d, J = 9.5 Hz), 137.0, 134.0, 132.6, 130.3 (d, J = 7.9 Hz), 127.9, 123.3, 122.1, 121.4 (enriched, d, J = 65.0 Hz, C-2),

119.8, 114.8. EI-MS m/z (relative intensity) 358 (M^+, 22), 246 (54), 171 (100), 142 (28), 116 (38). ES-MS/MS (ion-trap) $[M-H]^-$ 357, daughter ions 339, 311, 244, 218, 215, 192, 180, 112.

(64) **N_1-(2-Hydroxy-5-oxo-cyclopent-1-enyl)-7-(3-amino-4-hydroxyphenyl)-hepta-(*2E,4E,6E*)-trienamide (70)** --- A suspension of **140** (10 mg, 0.028 mmol) and $SnCl_2x2H_2O$ (126 mg, 0.56 mmol) in ethyl acetate (1 mL) was heated to 70 °C under argon for 4.5 hr. The reaction was worked up by adding water, adjusting the pH to neutral and extracting with ethyl acetate (3x10 mL). The combined extracts were dried over Na_2SO_4, filtered and the solvent evaporated *in vacuo*. Purification by silica gel flash column chromatography with a gradient of methylene chloride/methanol gave **70** (2 mg, 0.006 mmol, 22%): R_f 0.31 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (500 MHz, DMSO-d_6) δ_H 7.26 (dd, 1H, J = 14.8, 11.7 Hz, H-3), 6.83 (dd, 1H, J = 14.2, 10.5 Hz, H-5), 6.76 (d, 1H, J = 1.9 Hz, H-2'), 6.72 (dd, 1H, J = 15.5, 10.5 Hz, H-6), 6.63-6.57 (m, 3H, H-7, H-5', H-6'), 6.50 (d, 1H, J = 14.8 Hz, H-2), 6.43 (dd, 1H, J = 14.2, 11.7 Hz, H-4), 2.45 (s, 4H, H-4", H-5"). ^{13}C-NMR (75 MHz, DMSO-d_6) δ_C 166.4, 145.5, 143.1, 142.3, 138.1, 136.9, 128.3, 124.6, 120.1, 116.9, 115.1, 114.4, 112.0. ES-MS/MS (ion-trap) $[M-H]^-$ 325, daughter ions 216, 213, 137, 112. HR-MS m/z for $C_{18}H_{19}N_2O_4$, $[M+H]^+$ calculated 327.1345, found 327.1351.

(65) **N_1-(2-Hydroxy-5-oxo-cyclopent-1-enyl)-7-(3-amino-4-hydroxyphenyl)-[1,2-$^{13}C_2$]-hepta-(*2E,4E,6E*)-trienamide ([1,2-$^{13}C_2$]-70)** --- Using the same procedure as in

(64), [1,2-$^{13}C_2$]-**140** (50 mg, 0.140 mmol) and $SnCl_2x2H_2O$ (330 mg, 1.47 mmol) in ethyl acetate (4 mL) gave [1,2-$^{13}C_2$]-**70** (13 mg, 0.040 mmol, 28%): R_f 0.31 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (500 MHz, DMSO-d_6) δ_H 7.26 (m, 1H, H-3), 6.84 (m, 1H, H-5), 6.76-6.68 (m, 2H, H-2', H-6), 6.64-6.54 (m, 3H, H-7, H-5', H-6'), 6.50 (ddd, 1H, J = 163.2, 15.0, 3.9 Hz, H-2), 6.42 (ddd, 1H, J = 14.5, 11.4, 3.9 Hz, H-4), 2.45 (s, 4H, H-4", H-5"). ^{13}C-NMR (75 MHz, DMSO-d_6) δ_C 166.3 (enriched, d, J = 65.9 Hz, C-1), 145.4, 143.1 (d, J = 69.6 Hz, C-3), 142.3 (d, J = 8.6 Hz), 138.0, 136.9, 128.1, 124.6, 120.0 (enriched, d, J = 65.9 Hz, C-2), 116.9, 115.0, 114.4, 112.0. ES-MS/MS (ion-trap) $[M-H]^-$ 327, daughter ions 218, 215, 139, 112. HR-MS m/z for $C_{16}{}^{13}C_2H_{19}N_2O_3$, $[M+H]^+$ calculated 329.1412, found 329.1409.

8.7.15 7-[3-(7-Cyclohexyl-hepta-(*2E,4E,6E*)-trienoyl)-amino-4-hydroxyphenyl]-hepta-(*2E,4E,6E*)-trienoic Acid ([1,2-$^{13}C_2$]-**94a**)

(66) **Ethyl 7-(3-amino-4-methoxyphenyl)-(*2E,4E,6E*)-[1,2-$^{13}C_2$]hepta-2,4,6-trienoate ([1,2-$^{13}C_2$]-87)** --- A suspension of ethyl 7-(4-methoxy-3-nitrophenyl)-(*2E,4E,6E*)-[1,2-$^{13}C_2$]hepta-2,4,6-trienoate ([1,2-$^{13}C_2$]-**82**) (0.4 g, 1.31 mmol) and $SnCl_2x2H_2O$ (2.2 g, 9.78 mmol) in 5 mL ethyl acetate was stirred at 70 °C under argon for 2 hr until the educt disappeared on TLC. The resulting mixture was cooled and then poured on ice. The pH was adjusted to neutral and the mixture was extracted three times with ethyl acetate. The combined extracts were dried over $MgSO_4$, filtered and the ethyl acetate evaporated *in vacuo*. Further purification on a silica gel column eluting with hexane/ethyl acetate gave

[1,2-$^{13}C_2$]-**87** (0.27 g, 0.98 mmol, 76%): R_f 0.22 [silica gel, hexane/ethyl acetate (2:1)]. ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 7.33 (m, 1H, H-3), 6.91 (d, 1H, J = 2.1 Hz, H-2'), 6.87-6.74 (m, 4H, H-5, H-6, H-5', H6'), 6.67 (d, 1H, J = 15.0 Hz, H-7), 6.46 (m, 1H, H-4), 5.89 (ddd, 1H, J = 162.4, 15.1, 2.6 Hz, H-2), 4.14 (qd, 2H, J = 7.3, 3.1 Hz, $COCH_2CH_3$), 3.83 (s, 3H, OCH_3), 1.24 (t, 3H, J = 7.3 Hz, OCH_2CH_3). ^{13}C-NMR (75 MHz, acetone-d_6) δ_C 167.2 (enriched, d, J = 75.7 Hz, C-1), 148.7, 145.4 (d, J = 70.8 Hz, C-4), 142.6 (d, J = 8.5 Hz), 138.6 (d, J = 9.8 Hz), 130.7, 129.5 (d, J = 8.5 Hz), 126.3, 120.6 (enriched, d, J = 75.7 Hz, C-3), 118.1, 112.3, 111.1, 60.4, 55.8, 14.6.

(67) **7-Cyclohexyl-(*2E,4E,6E*)-hepta-2,4,6-trienoyl chloride (141)** --- To a solution of 7-cyclohexyl-(*2E,4E,6E*)-hepta-2,4,6-trienoic acid **(95)** (1.2 equivalent to that of **87** or [1,2-$^{13}C_2$]-**87**) and DMF (0.5 to 1 mL) in methylene chloride was added oxalyl chloride (1.2 eq in methylene chloride) at 0 °C under argon in 15 min. The mixture was stirred at room temperature for 1.5 hr and was then used directly in the coupling reaction.

(68) **Ethyl 7-[3-(7-cyclohexyl-hepta-(*2E,4E,6E*)-trienoyl)-amino-4-methoxyphenyl]-hepta-(*2E,4E,6E*)-trienoate (142)** --- To a solution of **87** (0.5 g, 1.83 mmol) and DMAP (45 mg) in anhydrous pyridine (5 mL) was added a solution of **141** (1.2 eq, in methylene chloride) dropwise over 20 min under argon at 0 °C. The mixture was stirred at room temperature overnight, and worked up by removing solvent *in vacuo*, adding water and extracting with ethyl acetate. The organic layer was dried over Na_2SO_4, filtered and the ethyl acetate was removed *in vacuo*. Purification was performed by silica gel flash

column chromatography with a gradient of hexane/ethyl acetate to afford **142** (0.50 g, 1.08 mmol, 59%): R_f 0.31 [silica gel, hexane/ethyl acetate (2:1)]. ^{1}H-NMR (500 MHz, acetone-d_6) δ_H 8.70 (d, 1H, J = 1.9 Hz, H-2'), 7.35 (dd, 1H, J = 15.1, 11.7 Hz, H-3), 7.32 (dd, 1H, J = 14.5, 11.2 Hz, H-3"), 7.19 (dd, 1H, J = 8.0, 1.9 Hz, H-6'), 7.00 (d, 1H, J = 8.0 Hz, H-5'), 6.88 (m, 2H, H-5, H-6), 6.79 (d, 1H, J = 14.2 Hz, H-7), 6.63 (dd, 1H, J = 14.8, 10.5 Hz, H-5"), 6.56 (m, 1H, H-4), 6.40 (d, 1H, J = 14.5 Hz, H-2"), 6.34 (dd, 1H, J = 14.8, 11.2 Hz, H-4"), 6.18 (dd, 1H, J = 15.1, 10.5 Hz, H-6"), 5.93 (d, 1H, J = 15.1 Hz, H-2), 5.91 (dd, 1H, J = 15.1, 7.4 Hz, H-7"), 4.13 (q, 2H, J = 7.4 Hz, COOCH_2CH$_3$), 3.89 (s, 3H, OCH_3), 2.06 (m, 1H, H-8"), 1.70 (m, 4H, H-10"), 1.30-1.23 (m, 2H, H-11"), 1.25 (t, 3H, J = 7.3 Hz, COOCH$_2$CH_3), 1.19-1.13 (m, 4H, H-9"). ^{13}C-NMR (125 MHz, acetone-d_6) δ_C 166.9, 164.6, 149.7, 145.4, 145.2, 142.1, 141.1, 137.6, 130.2, 130.1, 129.5, 129.1, 128.5, 127.1, 124.7, 123.6, 120.9, 120.8, 118.6, 111.3, 60.4, 56.2, 41.8, 33.3, 26.7, 26.5, 14.6. EI-MS/MS [M$^+$] 461, daughter ions 409, 383, 273, 256, 227, 200.

(69) **Ethyl 7-[3-(7-cyclohexyl-hepta-(*2E,4E,6E*)-trienoyl)-amino-4-methoxyphenyl]-[1,2-^{13}C$_2$]hepta-(*2E,4E,6E*)-trienoate ([1,2-^{13}C$_2$]-142)** --- Under the same conditions as in (68), [1,2-^{13}C$_2$]-**87** (0.25 g, 0.91 mmol) and DMAP (20 mg) in anhydrous pyridine (3 mL) was added a solution of **141** (1.2 eq, in methylene chloride) gave [1,2-^{13}C$_2$]-**142** (0.26 g, 0.56 mmol, 61%): R_f 0.31 [silica gel, hexane/ethyl acetate (2:1)]. ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 8.70 (d, 1H J = 2.1 Hz, H-2'), 7.35 (m, 1H, H-3), 7.32 (dd, 1H, J = 14.5, 11.4 Hz, H-3"), 7.19 (dd, 1H, J = 8.8, 2.1 Hz, H-6'), 7.00 (d, 1H, J = 8.8 Hz, H-5'), 6.85 (m, 2H, H-5, H-6), 6.78 (d, 1H, J = 15.0 Hz, H-7), 6.62 (dd, 1H, J = 15.1, 10.4 Hz,

H-5"), 6.56 (m, 1H, H-4), 6.40 (d, 1H, J = 14.5 Hz, H-2"), 6.34 (dd, 1H, J = 15.1, 11.4 Hz, H-4"), 6.18 (dd, 1H, J = 15.1, 10.4 Hz, H-6"), 5.93 (ddd, 1H, J = 162.4, 15.6, 3.1 Hz, H-2), 5.90 (dd, 1H, J = 15.1, 7.3 Hz, H-7"), 4.14 (qd, 2H, J = 7.3, 3.1 Hz, $COOCH_2CH_3$), 3.88 (s, 3H, OCH_3), 2.06 (m, 1H, H-8"), 1.70 (m, 4H, H-10"), 1.32-1.23 (m, 2H, H-11"), 1.25 (t, 3H, J = 7.3 Hz, $COOCH_2CH_3$), 1.19-1.10 (m, 4H, H-9"). ^{13}C-NMR (75 MHz, acetone-d_6) δ_C 167.1 (enriched, d, J = 75.7 Hz, C-1), 164.8, 149.8, 145.6, 145.3 (d, J = 69.6, C-3), 142.2, 141.2, 137.7, 130.4, 130.2 (d, J = 8.6 Hz), 129.6, 129.2, 128.6, 127.3, 124.9, 123.7, 121.0 (enriched, d, J = 75.7 Hz, C-2), 120.9, 118.7, 111.4, 60.4, 56.3, 41.8, 33.2, 26.7, 26.5, 14.6.

(70) **Ethyl 7-[3-(7-cyclohexyl-hepta-(*2E,4E,6E*)-trienoyl)-amino-4-hydroxyphenyl]-hepta-(*2E,4E,6E*)-trienoate (143)** --- To a suspension of **142** (50 mg, 0.108 mmol) in anhydrous methylene chloride (5 mL) was added BBr_3 (2.2 mL, 1 M in methylene chloride) under argon at –78 °C. After 3 hr, no more starting material was detected on TLC (methylene chloride/methanol 10:1). The reaction was worked up by adding water and extracting with ethyl acetate (2x50 mL). The extract was dried over $MgSO_4$, the solvent removed *in vacuo* and the crude product purified on a silica gel column eluting with a gradient of hexane/thyl acetate to afford **(143)** (16 mg, 0.036 mmol, 33%): R_f 0.30 [silica gel, hexane/ethyl acetate (2:1)]. ^{1}H-NMR (500 MHz, acetone-d_6) δ_H 7.65 (d, 1H, J = 2.5 Hz, H-2'), 7.38 (dd, 1H, J = 14.8, 11.1 Hz, H-3''), 7.34 (dd, 1H, J = 15.5, 11.1 Hz, H-3), 7.25 (dd, 1H, J = 8.0, 2.5 Hz, H-6'), 6.90 (d, 1H, J = 8.0 Hz, H-5'), 6.84 (m, 2H, H-5, H-7), 6.75 (m, 1H, H-6), 6.70 (dd, 1H, J = 14.8, 11.3 Hz, H-5"), 6.51 (m, 1H, H-4),

6.38 (d, 1H, J = 14.8 Hz, H-2"), 6.38 (dd, 1H, J = 14.8, 11.1 Hz, H-4"), 6.20 (dd, 1H, J = 15.5, 11.3 Hz, H-6"), 5.94 (dd, 1H, J = 15.5, 6.8 Hz, H-7"), 5.91 (d, 1H, J = 15.5 Hz, H-2), 4.14 (q, 2H, J = 7.4 Hz, $COOCH_2CH_3$), 2.10 (m, 1H, H-8"), 1.71 (m, 4H, H-10"), 1.31-1.22 (m, 2H, H-11"), 1.24 (t, 3H, J = 7.4 Hz, $COOCH_2CH_3$), 1.21-1.11 (m, 4H, H-9"). ^{13}C-NMR (125 MHz, acetone-d_6) δ_C 166.9, 166.3, 150.0, 146.2, 145.1, 143.6, 142.2, 142.0, 137.0, 129.9, 129.6, 128.9, 128.4, 127.8, 126.9, 125.4, 122.7, 121.2, 120.8, 119.2, 60.4, 41.8, 33.2, 26.7, 26.5, 14.6. ES-MS/MS (ion-trap) $[M-H]^-$ 446, daughter ion 258.

(71) **Ethyl 7-[3-(7-cyclohexyl-hepta-(*2E,4E,6E*)-trienoyl)-amino-4-hydroxyphenyl]-[1,2-$^{13}C_2$]hepta-(*2E,4E,6E*)-trienoate ([1,2-$^{13}C_2$]-143)** --- Under the same conditions as in (70), [1,2-$^{13}C_2$]-**142** (150 mg, 0.324 mmol) in anhydrous methylene chloride (15 mL) and BBr_3 (6.5 mL, 1 M in methylene chloride) produced [1,2-$^{13}C_2$]-**143** (50 mg, 0.111 mmol, 34%): R_f 0.30 [silica gel, hexane/ethyl acetate (2:1)]. ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 7.64 (d, 1H, J = 2.1 Hz, H-2'), 7.38 (dd, 1H, J = 14.5 Hz, H-3"), 7.33 (m, 1H, H-3), 7.25 (dd, 1H, J = 8.3, 2.1 Hz, H-6'), 6.90 (d, 1H, J = 8.3 Hz, H-5'), 6.82 (m, 2H, H-5, H-7), 6.75 (m, 1H, H-6), 6.70 (dd, 1H, J = 14.8, 10.9 Hz, H-5"), 6.50 (m, 1H, H-4), 6.38 (d, 1H, J = 14.5 Hz, H-2"), 6.37 (dd, 1H, J = 14.8, 11.4 Hz, H-4"), 6.19 (m, 1H, H-6"), 5.94 (dd, 1H, J = 15.0, 7.3 Hz, H-7"), 5.91 (ddd, 1H, J = 163.2, 15.6, 3.1 Hz, H-2), 4.13 (qd, 2H, J = 7.3, 3.1 Hz, $COCH_2CH_3$), 2.08 10 (m, 1H, H-8"), 1.70 (m, 4H, H-10"), 1.32-1.22 (m, 2H, H-11"), 1.24 (t, 3H, J = 7.4 Hz, $COOCH_2CH_3$), 1.20-1.07 (m, 4H, H-9"). ^{13}C-NMR (125 MHz, acetone-d_6) δ_C 166.9 (enriched, d, J = 76.2 Hz, C-1), 166.3, 150.0, 146.2, 145.1 (d, J = 68.4 Hz, C-3), 143.6, 142.2, 142.0, (d, J = 9.5 Hz) 137.0,

129.9 (d, J = 9.5 Hz), 129.6, 128.9, 128.4, 127.8, 126.9, 125.4, 122.7, 121.2, 120.8 (enriched, d, J = 76.2 Hz, C-2), 119.2, 60.4, 41.8, 33.2, 26.7, 26.5, 14.6.

(72) **7-[3-(7-Cyclohexyl-hepta-(*2E,4E,6E*)-trienoyl)-amino-4-hydroxyphenyl]-hepta-(*2E,4E,6E*)-trienoic acid (94a)** --- Compound **143** (10 mg, 0.022 mmol) was hydrolyzed with 0.1 M LiOH in THF/water (3:1, 15 mL) at room temperature for 3 days. The resulting solution was diluted, acidified and extracted with ethyl acetate (2x50 mL). The combined extracts were dried over $MgSO_4$ and ethyl acetate was removed *in vacuo*. Purification by silica gel flash column chromatography with a gradient of methylene chloride/methanol afforded **94a** (5 mg, 0.012 mmol, 53%): R_f 0.31 [silica gel, MeOH/CH_2Cl_2 (10:1)]. ^{1}H-NMR (500 MHz, pyridine-d_6) δ_H 8.83 (br s, 1H, H-2'), 7.83 (dd, 1H, J = 14.8, 11.7 Hz, H-3), 7.75 (dd, 1H, J = 14.8, 11.1 Hz, H-3"), 7.28 (dd, 1H, J = 8.7, 1.9 Hz, H-6'), 7.18 (d, 1H, J = 8.7 Hz, H-5'), 6.94 (ddd, 1H, J = 15.5, 11.1 Hz, H-5), 6.79-6.70 (m, 3H, H-6, H-7, H-4), 6.61 (dd, 1H, J = 14.8, 10.5 Hz, H-5"), 6.43 (dd, 1H, J = 14.8, 11.1 Hz, H-4"), 6.42(d, 1H, J = 14.8 Hz, H-2), 6.32 (d, 1H, J = 14.8 Hz, H-2"), 6.15 (dd, 1H, J = 15.5, 10.5 Hz, H-6"), 5.86 (dd, 1H, J = 15.5, 6.7 Hz, H-7"), 1.98 (m, 1H, H-8"), 1.67-1.52 (m, 4H, H-10"), 1.17 (m, 2H, H-11"), 1.05 (m, 4H, H-9"). ^{1}H-NMR (500 MHz, DMSO-d_6) δ_H 8.06 (br s, 1H, H-2'), 7.28 (dd, 1H, J = 14.8, 11.4 Hz, H-3), 7.23 (dd, 1H, J = 14.8, 11.4 Hz, H-3"), 7.18 (dd, 1H, J = 8.0, 1.9 Hz, H-6'), 6.88 (d, 1H, J = 8.0 Hz, H-5'), 6.88-6.78 (m, 2H, H-5, H-6), 6.74 (d, 1H, J = 15.5 Hz, H-7), 6.66 (dd, 1H, J = 14.8, 10.5 Hz, H-5"), 6.53 (dd, 1H, J = 14.2, 11.4 Hz, H-4), 6.48 (d, 1H, J = 14.8 Hz, H-2"), 6.38 (dd, 1H, J = 14.8, 11.4 Hz, H-4"), 6.21 (dd, 1H, J = 15.5, 10.5 Hz, H-6"),

5.94 (dd, 1H, J = 15.5, 8.7 Hz, H-7"), 5.90 (d, 1H, J = 14.8 Hz, H-2), 2.10 (m, 1H, H-8"), 1.73-1.62 (m, 4H, H-10"), 1.27 (m, 2H, H-11"), 1.12 (m, 4H, H-9"). ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C 167.6, 164.2, 148.5, 144.6, 144.3, 141.2, 141.0, 140.9, 140.3, 140.2, 136.5, 128.9, 128.4, 127.6, 126.7, 125.6, 123.8, 123.7, 120.6, 115.9, 40.4, 32.1, 25.6, 25.4. ES-MS/MS (ion-trap) $[M-H]^-$ 418, daughter ions 374, 230, 184. HR-MS m/z for $C_{26}H_{30}NO_4$, $[M+H]^+$ calculated 420.2175, found 420.2185

(73) **7-[3-(7-Cyclohexyl-hepta-(*2E,4E,6E*)-trienoyl)-amino-4-hydroxyphenyl]-[1,2-$^{13}C_2$]-hepta-(*2E,4E,6E*)-trienoic acid ([1,2-$^{13}C_2$]-94a)** --- Under the same conditions as in (72), compound [1,2-$^{13}C_2$]-**143** (50 mg, 0.111 mmol) was hydrolyzed by 0.2 M LiOH in THF/water (3:1, 40 mL) at room temperature for 5 days to give [1,2-$^{13}C_2$]-**94a** (23 mg, 0.055 mmol, 49%): R_f 0.31 [silica gel, MeOH/CH_2Cl_2 (10:1)]. ^{1}H-NMR (500 MHz, DMSO-d_6) δ_H 8.03 (br s, 1H, H-2'), 7.25 (m, 1H, H-3), 7.19 (dd, 1H, J = 15.5, 11.1 Hz, H-3"), 7.14 (dd, 1H, J = 8.3, 1.9 Hz, H-6'), 6.85 (d, 1H, J = 8.3 Hz, H-5'), 6.83-6.73 (m, 2H, H-5, H-6), 6.70 (d, 1H, J = 14.8 Hz, H-7), 6.63 (dd, 1H, J = 14.8, 10.5 Hz, H-5"), 6.51 (m, 1H, H-4), 6.44 (d, 1H, J = 15.5 Hz, H-2"), 6.35 (dd, 1H, J = 14.8, 11.1 Hz, H-4"), 6.18 (dd, 1H, J = 15.5, 10.5 Hz, H-6"), 5.90 (dd, 1H, J = 15.5, 6.8 Hz, H-7"), 5.86 (ddd, 1H, J = 161.3, 14.8, 2.5 Hz, H-2), 2.06 (m, 1H, H-8"), 1.70-1.60 (m, 4H, H-10"), 1.24 (m, 2H, H-11"), 1.09 (m, 4H, H-9"). ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C 167.6 (enriched, d, J = 73.0 Hz, C-1), 164.2, 148.5, 144.6, 144.2 (d, J = 69.8 Hz, C-3), 141.1, 141.0 (d, J = 7.9 Hz), 140.8, 140.3, 140.2, 136.5, 129.0 (d, J = 6.4 Hz), 128.4, 127.6, 126.7, 125.5, 123.8, 123.7, 120.8 (enriched, d, J = 73.0 Hz, C-2), 115.9, 40.3, 32.1, 25.6,

25.4. ES-MS/MS (ion-trap) $[M-H]^-$ 420, daughter ions 375, 232, 185. HR-MS m/z for $C_{24}{}^{13}C_2H_{30}NO_4$, $[M+H]^+$ calculated 422.2242, found 422.2249.

8.7.16 N_1-(2-Hydroxy-5-oxo-cyclopent-1-enyl)-7-[3-(7-cyclohexyl-hepta-(*2E,4E,6E*)-trienoyl)-amino-4-hydroxyphenyl]-[1,2-$^{13}C_2$]-hepta-(*2E,4E,6E*)-trienamide ([1,2-$^{13}C_2$]-**71**)

(74) **N_1-(2-Hydroxy-5-oxo-cyclopent-1-enyl)-7-(3-amino-4-methoxyphenyl)-hepta-(*2E,4E,6E*)-trienamide (145)** --- A suspension of N_1-(2-hydroxy-5-oxo-cyclopent-1-enyl)-7-(4-methoxy-3-nitrophenyl)-hepta-(*2E,4E,6E*)-trienamide **(139)** (200 mg, 0.541 mmol) and $SnCl_2x2H_2O$ (2.5 g, 11.1 mmol) in ethyl acetate (10 mL) was heated at 70 °C under argon for 3 hr. The reaction mixture was worked up by adding water and extracting with ethyl acetate (3x200 mL). The combined organic layers were washed with brine and dried over $MgSO_4$. Ethyl acetate was removed *in vacuo*. Silica gel flash column chromatography with a gradient of methylene chloride/methanol provided **145** (91 mg, 0.268 mmol, 49%): R_f 0.49 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (500 MHz, DMSO-d_6) δ_H 7.26 (dd, 1H, J = 14.8, 11.8 Hz, H-3), 6.84 (d, 1H, J = 14.2, 10.5 Hz, H-5), 6.79-6.74 (m, 3H, H-6, H2', H-5'), 6.70 (dd, 1H, J = 8.0, 1.9 Hz, H-6'), 6.64 (d, 1H, J = 14.8 Hz, H-7), 6.51 (d, 1H, J = 14.8 Hz, H-2), 6.46 (dd, 1H, J = 14.2, 11.8 Hz, H-4), 3.76 (s, 3H, OCH_3), 2.45 (br, 4H, H-4", H-5"). ^{13}C-NMR (75 MHz, DMSO-d_6) δ_C 166.3, 147.3, 142.9, 142.0, 137.8, 137.6, 129.3, 128.8, 125.5, 120.5, 116.5, 115.0, 111.1, 110.5, 55.3. ES-MS/MS (ion-trap) $[M-H]^-$ 341, daughter ions 374, 326, 214, 204, 182, 179, 112.

(75) **N_1-(2-Hydroxy-5-oxo-cyclopent-1-enyl)-7-(3-amino-4-methoxyphenyl)-[1,2-$^{13}C_2$]-hepta-(*2E,4E,6E*)-trienamide ([1,2-$^{13}C_2$]-145)** --- Under the same conditions as in (74), N_1-(2-hydroxy-5-oxo-cyclopent-1-enyl)-7-(4-methoxy-3-nitrophenyl)-[1,2-$^{13}C_2$]-hepta-(*2E,4E,6E*)-trienamide **([1,2-$^{13}C_2$]-139)** (260 mg, 0.699 mmol) and $SnCl_2x2H_2O$ (1.2 g, 5.33 mmol) in 8 mL of ethyl acetate provided [1,2-$^{13}C_2$]-**145** (71 mg, 0.208 mmol, 30%): R_f 0.49 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (500 MHz, DMSO-d_6) δ_H 7.26 (m, 1H, H-3), 6.85 (d, 1H, J = 14.8, 11.1Hz, H-5), 6.84-6.74 (m, 3H, H-6, H2', H-5'), 6.70 (dd, 1H, J = 8.7, 2.5 Hz, H-6'), 6.64 (d, 1H, J = 14.8 Hz, H-7), 6.51 (ddd, 1H, J = 159.0, 14.8, 3.7 Hz, H-2), 6.46 (ddd, 1H, J = 14.8, 11.7, 3.7 Hz, H-4), 3.77 (s, 3H, OCH_3), 2.45 (br, 4H, H-4", H-5"). ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C 166.1 (enriched, d, J = 66.7 Hz, C-1), 147.1, 142.8 (d, J = 69.8 Hz, C-3), 141.9 (d, J = 9.5 Hz), 137.7, 137.4, 129.2, 128.7 (d, J = 7.9 Hz), 125.4, 120.3 (enriched, d, J = 66.7 Hz, C-2), 116.4, 114.9, 111.0, 110.4, 55.2.

(76) **N_1-(2-Hydroxy-5-oxo-cyclopent-1-enyl)-7-[3-(7-cyclohexyl-hepta-(*2E,4E,6E*)-trienoyl)-amino-4-methoxyphenyl]-hepta-(*2E,4E,6E*)-trienamide (146)** --- To a solution of **145** (30 mg, 0.088 mmol, 1 eq) and DMAP (10 mg) in anhydrous pyridine (3 mL) was added a solution of **141** (1.6 eq, in methylene chloride) dropwise over 20 min under argon at 0 °C. The reaction mixture was then stirred at room temperature for 2 hr, and the solvents were removed by co-distillation with toluene. Purification was performed by silica gel flash column chromatography with a gradient of methylene chloride/methanol to afford **146** (30 mg, 0.057 mmol, 65%): R_f 0.65 [silica gel,

CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (500 MHz, DMSO-d_6) δ_H 8.25 (br s, 1H, H-2'), 7.27 (dd, 1H, J = 14.8, 11.7 Hz, H-3), 7.24 (m, 1H, H-6'), 7.18 (dd, 1H, J = 15.1, 11.7 Hz, H-3"), 7.04 (d, 1H, J = 8.7 Hz, H-5'), 6.88 (m, 2H, H-5, H-6), 6.75 (d, 1H, 14.2 Hz, H-7), 6.62 (dd, 1H, J = 14.8, 10.5 Hz, H-5"), 6.54 (d, 1H, J = 14.8 Hz, H-2), 6.51 (m, 1H, H-4), 6.47 (d, 1H, J = 15.1 Hz, H-2"),6.34 (dd, 1H, J = 14.8, 11.7 Hz, H-4"), 6.18 (dd, 1H, J = 15.5, 10.5 Hz, H-6"), 5.89 (dd, 1H, J = 15.5, 7.8 Hz, H-7"), 3.85 (s, 3H, OCH_3), 2.45 (br s, 4H, H-4"', H-5"'), 2.08 (m, 1H, H-8"), 1.68 (m, 4H, H-10"), 1.24 (m, 2H, H-11"), 1.09 (m, 4H, H-9"). ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C 166.0, 163.9, 149.9, 144.5, 142.6, 141.4, 140.6, 140.1, 139.2, 136.3, 129.5, 128.9, 128.8, 128.5, 127.5, 126.5, 124.2, 120.9, 119.8, 114.9, 111.2, 55.9, 40.4, 32.1, 25.6, 25.4.

(77) **N_1-(2-Hydroxy-5-oxo-cyclopent-1-enyl)-7-[3-(7-cyclohexyl-hepta-(*2E,4E,6E*)-trienoyl)-amino-4-methoxyphenyl]-[1,2-$^{13}C_2$]hepta-(*2E,4E,6E*)-trienamide** ([1,2-$^{13}C_2$]-**146**) --- Under the same conditions as in (76), [1,2-$^{13}C_2$]-**145** (60 mg, 0.175 mmol, 1 eq) and DMAP (30 mg) in 6 mL of anhydrous pyridine with 1.6 equivalent of **141** gave [1,2-$^{13}C_2$]-**146** (73 mg, 0.138 mmol, 79%): R_f 0.65 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (500 MHz, DMSO-d_6) δ_H 8.25 (br s, 1H, H-2'), 7.27 (m, 1H, H-3), 7.23 (dd, 1H, J = 8.4, 1.9 Hz, H-6'), 7.18 (dd, 1H, J = 14.8, 11.7 Hz, H-3"), 7.04 (d, 1H, J = 8.4 Hz, H-5'), 6.87 (m, 2H, H-5, H-6), 6.75 (d, 1H, 14.8 Hz, H-7), 6.62 (dd, 1H, J = 14.8, 10.5 Hz, H-5"), 6.54 (ddd, 1H, J = 161.9, 14.8, 3.7 Hz, H-2), 6.53 (m, 1H, H-4), 6.47 (d, 1H, J = 14.8 Hz, H-2"), 6.34 (dd, 1H, J = 14.8, 11.7 Hz, H-4"), 6.18 (dd, 1H, J = 15.5, 10.5 Hz, H-6"), 5.90 (dd, 1H, J = 15.5, 6.8 Hz, H-7"), 3.85 (s, 3H, OCH_3), 2.45 (br s, 4H, H-4"',

H-5'''), 2.07 (m, 1H, H-8"), 1.68 (m, 4H, H-10"), 1.24 (m, 2H, H-11"), 1.09 (m, 4H, H-9"). ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C 165.9 (enriched, d, J = 65.1 Hz, C-1), 163.9, 149.9, 144.5, 142.6 (d, J = 71.4 Hz, C-3), 141.4, 140.7, 140.0, 139.5, 136.3, 129.5 (d, J = 7.9 Hz), 129.0, 128.7, 128.4, 127.6 (d, J = 6.4 Hz), 126.5, 124.2, 120.8 (enriched, d, J = 65.1 Hz, C-2), 119.8, 114.9, 111.2, 55.9, 40.3, 32.1, 25.6, 25.4. ES-MS/MS (ion-trap) $[M-H]^-$ 529, daughter ion 341.

(78) **N_1-(2-hydroxy-5-oxo-cyclopent-1-enyl)-7-[3-(7-cyclohexyl-hepta-(*2'E,4'E,6'E*)-trienoyl)-amino-4-hydroxyphenyl]-hepta-(*2E,4E,6E*)-trienamide (71)** --- To a suspension of **146** (15 mg, 0.028 mmol) in anhydrous methylene chloride (2 mL) was added BBr_3 (0.6 mL, 1 M in methylene chloride) under argon at –78 °C. After 1 hr, TLC (methylene chloride/methanol 10:1) showed the disappearance of the starting material. The reaction was worked up by adding water and extracting with ethyl acetate (2x100 mL). The combined organic layers were dried over $MgSO_4$ and ethyl acetate was removed *in vacuo*. Crude product was purified on a silica gel column eluting with a gradient of methylene chloride/methanol and **71** was obtained (8 mg, 0.016 mmol, 55%): R_f 0.57 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (300 MHz, pyridine-d_6) δ_H 8.92 (br s, 1H, H-2'), 7.83 (dd, 1H, J = 14.5, 11.2 Hz, H-3"), 7.70 (dd, 1H, J = 14.8, 11.2 Hz, H-3), 7.26 (dd, 1H, J = 8.3, 2.1 Hz, H-6'), 7.15 (d, 1H, J = 8.3 Hz, H-5'), 6.96-6.76 (m, 3H, H-5, H-6, H-7), 6.69 (d, 1H, J = 14.5 Hz, H-2), 6.69 (d, 1H, J = 14.5 Hz, H-2"), 6.59 (dd, 1H, J = 14.5, 10.4 Hz, H-5"), 6.41 (dd, 1H, J = 14.5, 11.2 Hz, H-4), 6.38 (dd, 1H, J = 14.5, 11.2 Hz, H-4"), 6.13 (dd, 1H, J = 15.0, 10.4 Hz, H-6"), 5.82 (dd, 1H, J = 15.0, 7.3

Hz, H-7"), 2.40 (br s, 4H, H-4"', H-5"'), 1.95 (m, 1H, H-8"), 1.65-1.50 (m, 4H, H-10"), 1.25 (m, 2H, H-11"), 1.05 (m, 4H, H-9"). ^{1}H-NMR (500 MHz, DMSO-d_6) δ_H 8.03 (br s, 1H, H-2'), 7.27 (dd, 1H, J = 14.8, 11.7 Hz, H-3), 7.20 (dd, 1H, J = 14.8, 11.1 Hz, H-3"), 7.14 (dd, 1H, J = 8.0, 1.9 Hz, H-6'), 6.90-6.80 (m, 3H, H-5, H-6, H-5'), 6.71 (d, 1H, J = 14.8 Hz, H-7), 6.63 (dd, 1H, J = 14.8, 10.5 Hz, H-5"), 6.53 (d, 1H, J = 14.8 Hz, H-2), 6.46 (m, 2H, H-4, H-2"), 6.35 (dd, 1H, J = 14.8, 11.1 Hz, H-4"), 6.19 (dd, 1H, J = 15.5, 10.5 Hz, H-6"), 5.90 (dd, 1H, J = 15.5, 6.8 Hz, H-7"), 2.45 (br s, 4H, H-4"', H-5"'), 2.08 (m, 1H, H-8"), 1.70-1.60 (m, 4H, H-10"), 1.24 (m, 2H, H-11"), 1.10 (m, 4H, H-9"). ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C 166.1, 164.2, 148.5, 144.6, 142.7, 141.6, 141.0, 140.8, 140.2, 136.6, 129.0, 128.4, 127.6, 127.5, 126.7, 125.7, 123.8, 120.8, 120.6, 115.9, 114.9, 40.3, 32.1, 25.6, 25.4. ES-MS/MS (ion-trap) $[M-H]^-$ 513, daughter ions 400, 374, 325, 296, 216. HR-MS m/z for $C_{31}H_{34}N_2O_5Na$, $[M+Na]^+$ calculated 537.2365, found 537.2371.

(79) **N_1-(2-hydroxy-5-oxo-cyclopent-1-enyl)-7-[3-(7-cyclohexyl-hepta-(*2E,4E,6E*)-trienoyl)-amino-4-hydroxyphenyl]-[1,2-$^{13}C_2$]hepta-(*2E,4E,6E*)-trienamide ([1,2-$^{13}C_2$]-71)** --- Under the same conditions as in (78), [1,2-$^{13}C_2$]-146 (60 mg, 0.113 mmol) with BBr_3 (3.4 mL, 1 M in methylene chloride) gave [1,2-$^{13}C_2$]-**71** (30 mg, 0.058 mmol, 51%): R_f 0.57 [silica gel, CH_2Cl_2/MeOH (10:1)]. ^{1}H-NMR (500 MHz, DMSO-d_6) δ_H 8.03 (br s, 1H, H-2'), 7.27 (m, 1H, 11.7 Hz, H-3), 7.19 (dd, 1H, J = 14.8, 11.1 Hz, H-3"), 7.14 (dd, 1H, J = 8.7, 1.9 Hz, H-6'), 6.90-6.80 (m, 3H, H-5, H-6, H-5'), 6.71 (d, 1H, J = 14.2 Hz, H-7), 6.63 (dd, 1H, J = 14.8, 11.1 Hz, H-5"), 6.53 (dm, J ~ 150 Hz, H-2), 6.47

(m, 2H, H-4, H-2"), 6.35 (dd, 1H, J = 14.8, 11.1 Hz, H-4"), 6.18 (dd, 1H, J = 15.5, 11.1 Hz, H-6"), 5.90 (dd, 1H, J = 15.5, 6.8 Hz, H-7"), 2.45 (br s, 4H, H-4"', H-5"'), 2.08 (m, 1H, H-8"), 1.70-1.60 (m, 4H, H-10"), 1.24 (m, 2H, H-11"), 1.10 (m, 4H, H-9"). ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C 166.1 (enriched, d, J = 65.1 Hz, C-1), 164.2, 148.5, 144.6, 142.7 (d, J = 69.8 Hz, C-3), 141.7 (d, J = 9.5 Hz), 141.0, 140.9, 140.2, 136.7, 129.0 (d, J = 6.4 Hz), 128.4, 127.6, 127.5, 126.7, 125.7, 123.8, 120.9, 120.5 (enriched, d, J = 65.1 Hz, C-2), 115.9, 114.9, 40.4, 32.1, 25.6, 25.4. ES-MS/MS (ion-trap) $[M-H]^-$ 515, daughter ions 404, 375, 327, 296, 218. HR-MS m/z for $C_{29}{}^{13}C_2H_{34}N_2O_5Na$, $[M+Na]^+$ calculated 539.2433, found 539.2441.

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APPENDIX A: MS/MS SPECTRA OF TYPE I MANUMYCINS PRODUCED BY *STREPTOMYCES NODOSUS*

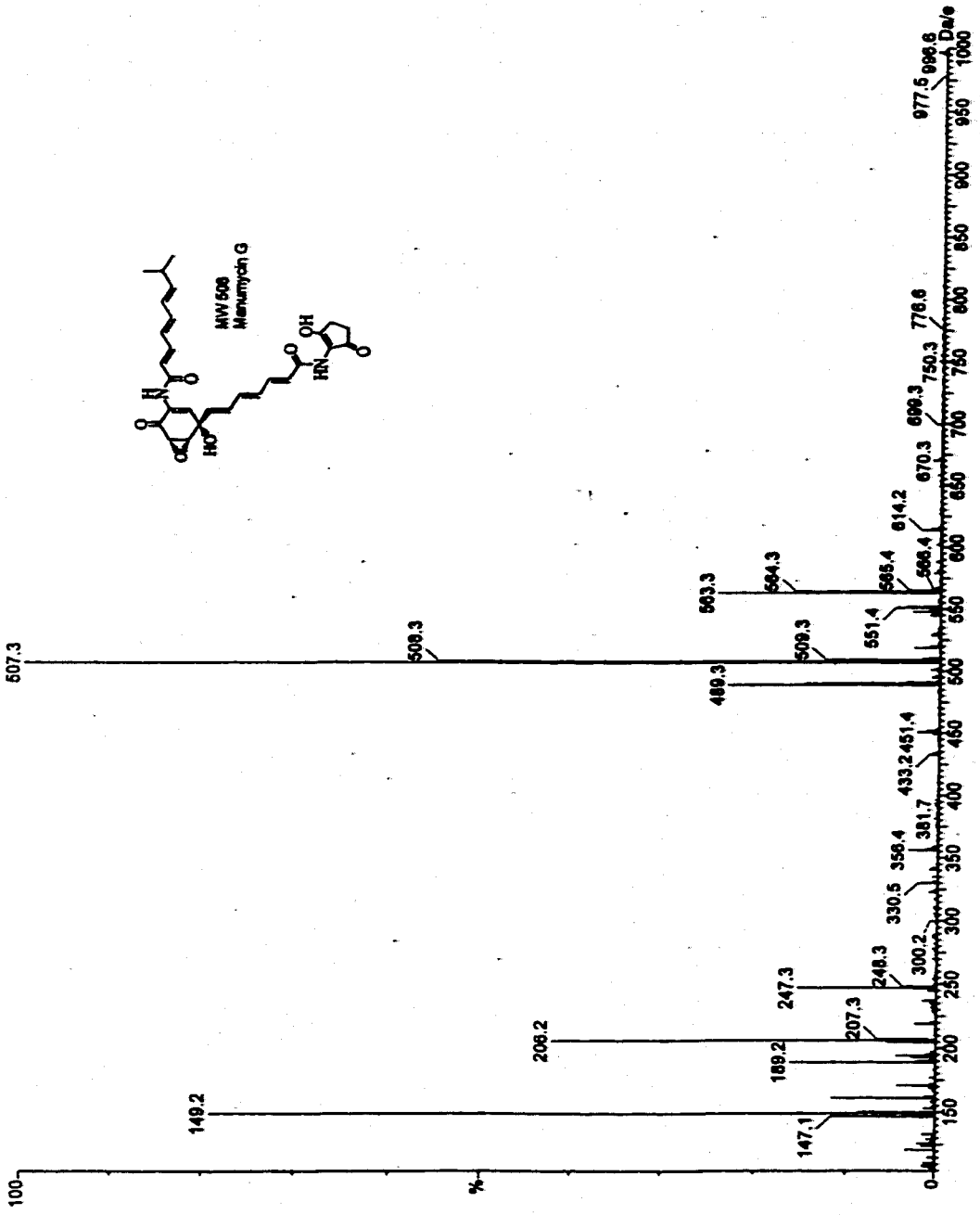


Figure 74. ES-MS/MS Spectrum of Manumycin G Produced by *S. nodosus*

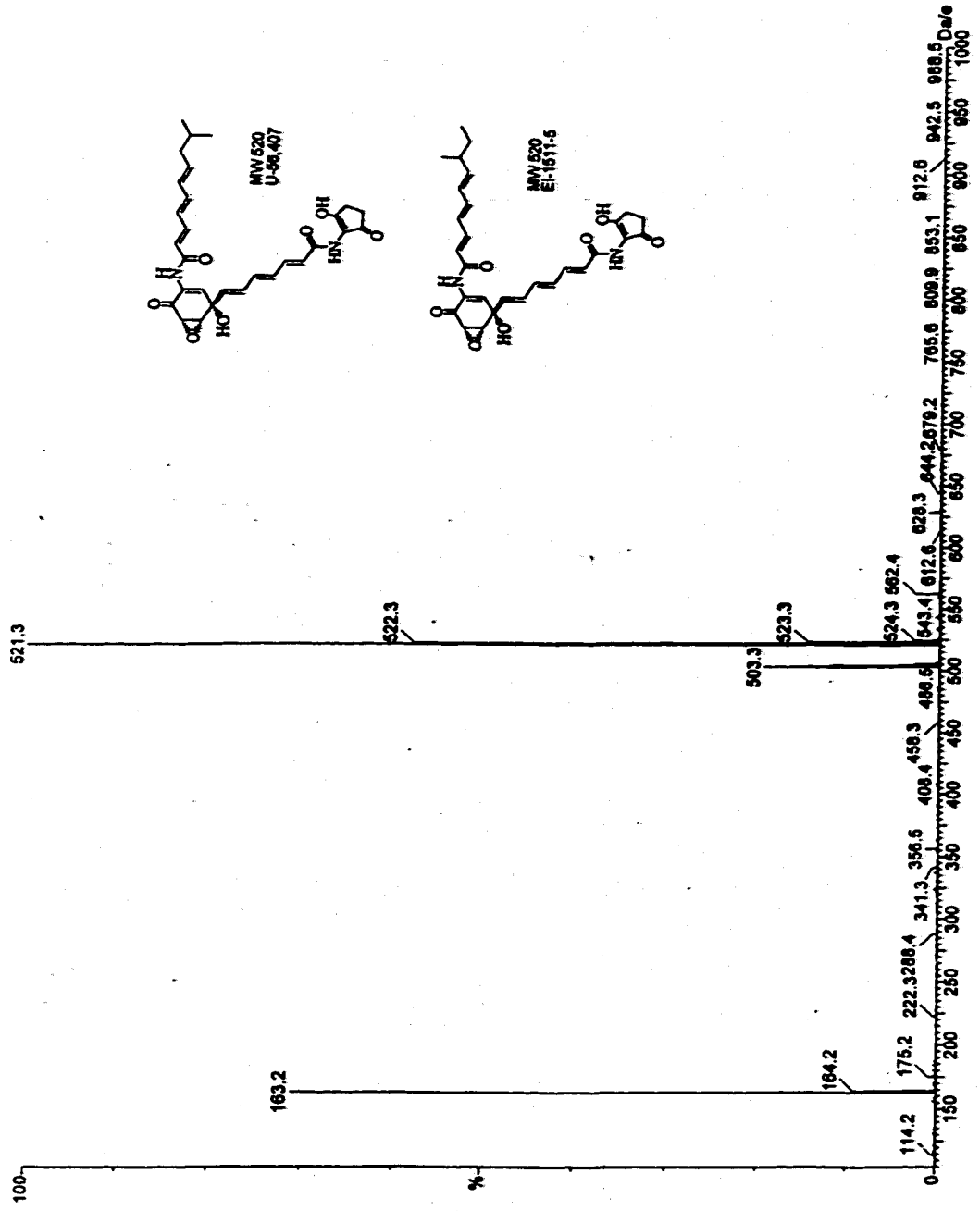


Figure 75. ES-MS/MS Spectrum of U-56,407 and EI-1511-5 Produced by *S. nodosus*

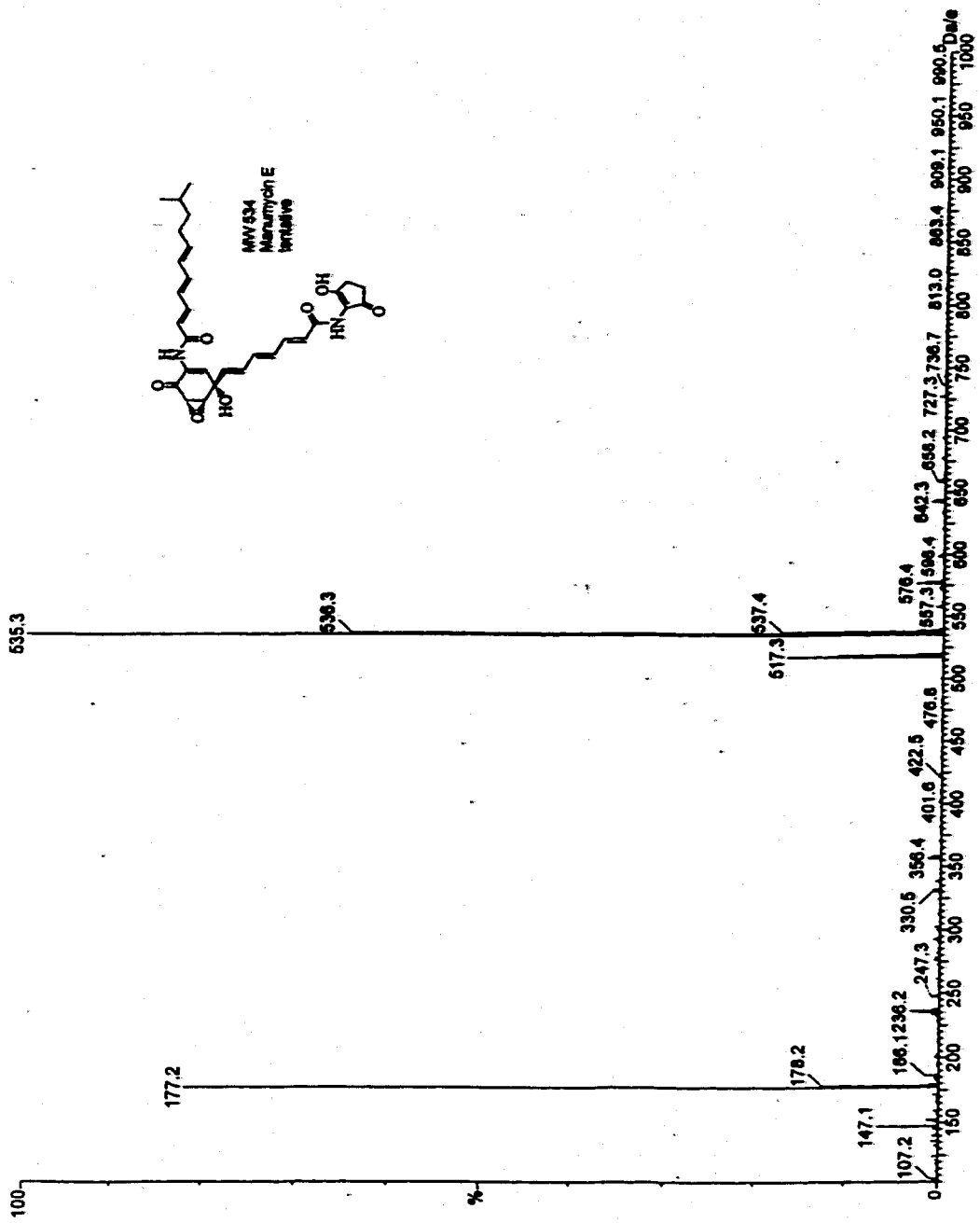


Figure 76. ES-MS/MS Spectrum of Manumycin E Produced by *S. nodosus*

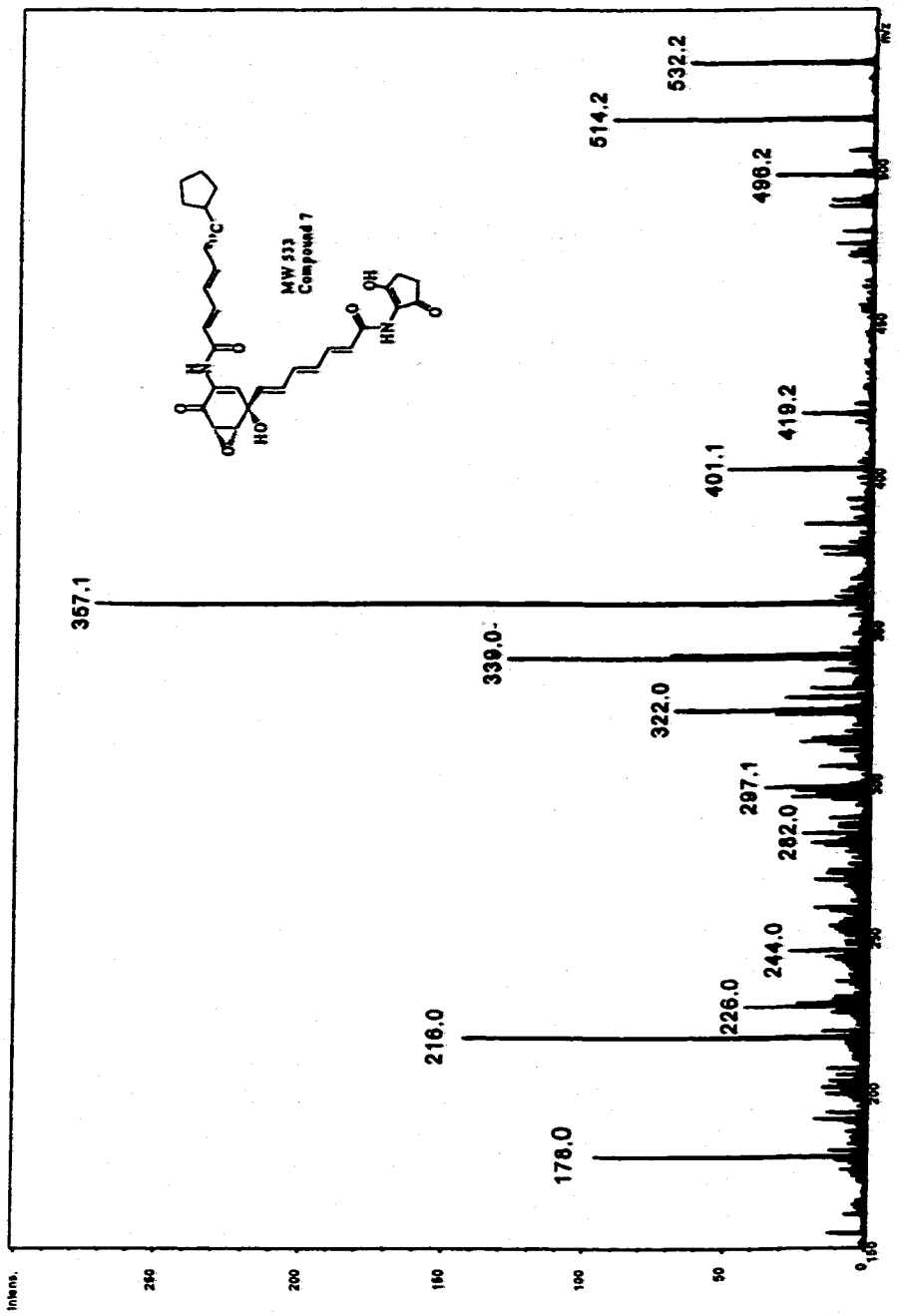


Figure 77. ES-MS/MS (Negative Ion Mode) Spectrum of Compound [7"-^{13}C]-7
Produced by *S. nodosus*

APPENDIX B: MS/MS SPECTRA OF TYPE II MANUMYCINS PRODUCED BY *STREPTOMYCES NODOSUS*

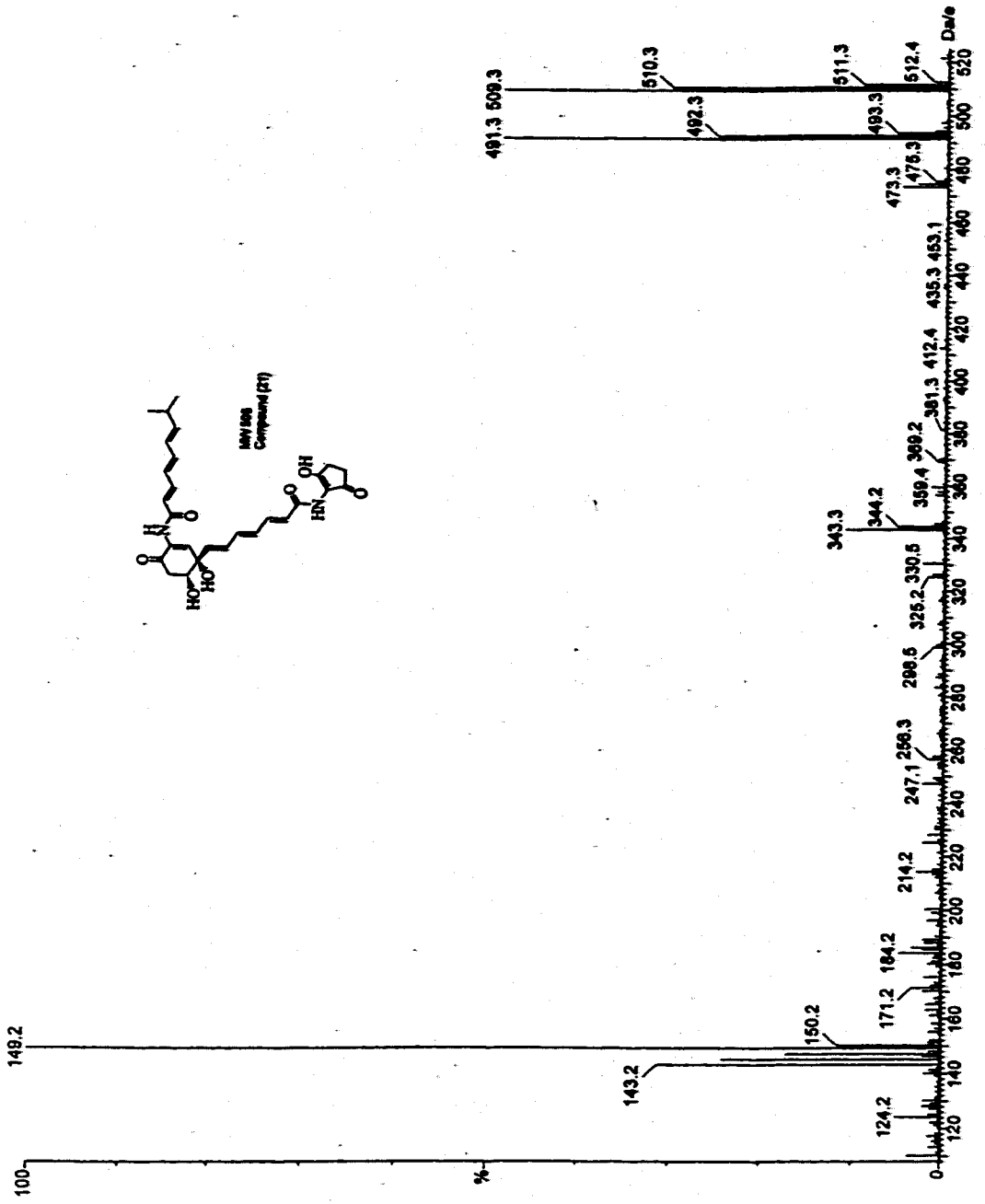


Figure 78. ES-MS/MS Spectrum of Type II Manumycin 21 Produced by *S. nodosus*

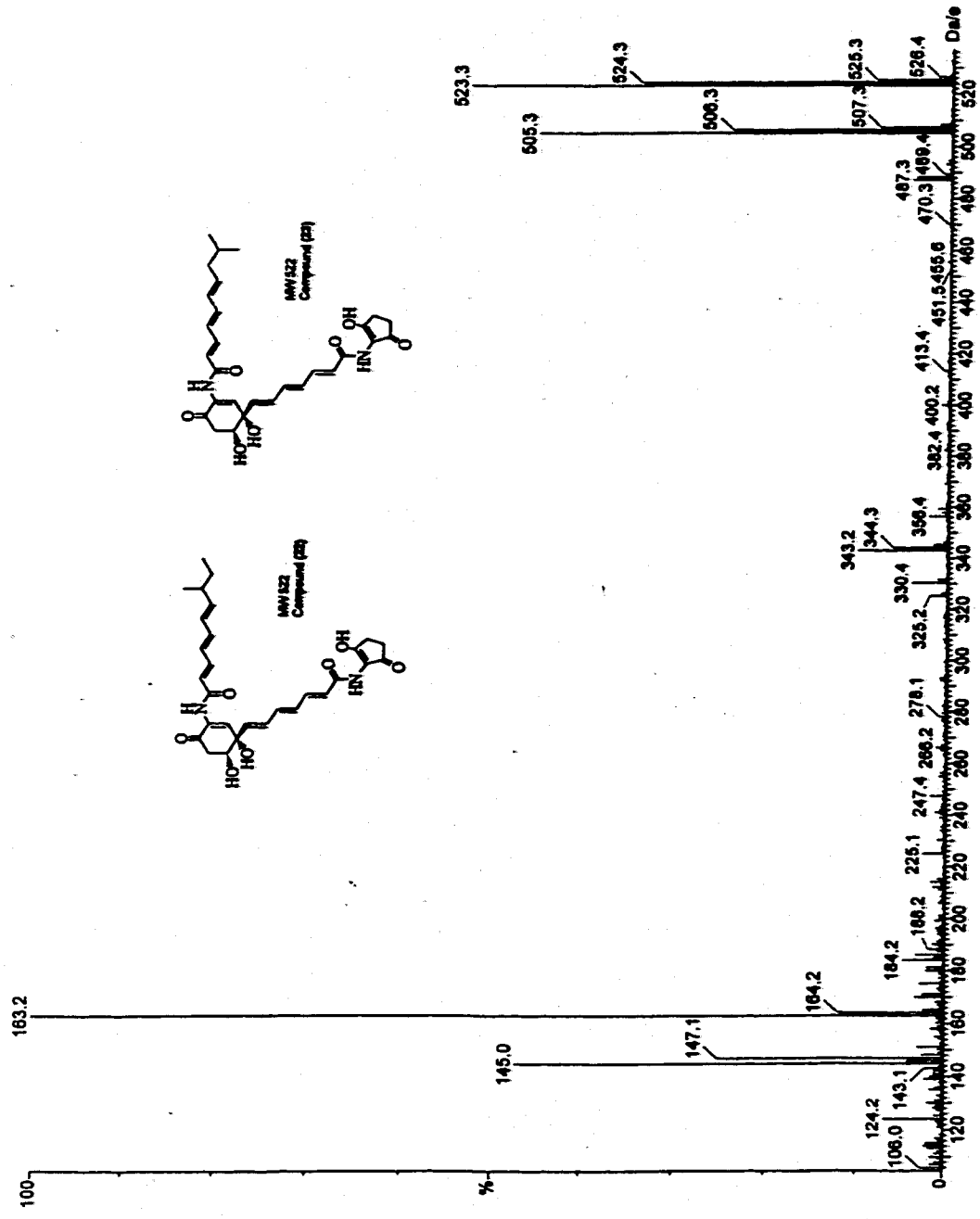


Figure 79. ES-MS/MS Spectrum of Type II Manumycins **22** and **23** Produced by *S. nodosus*

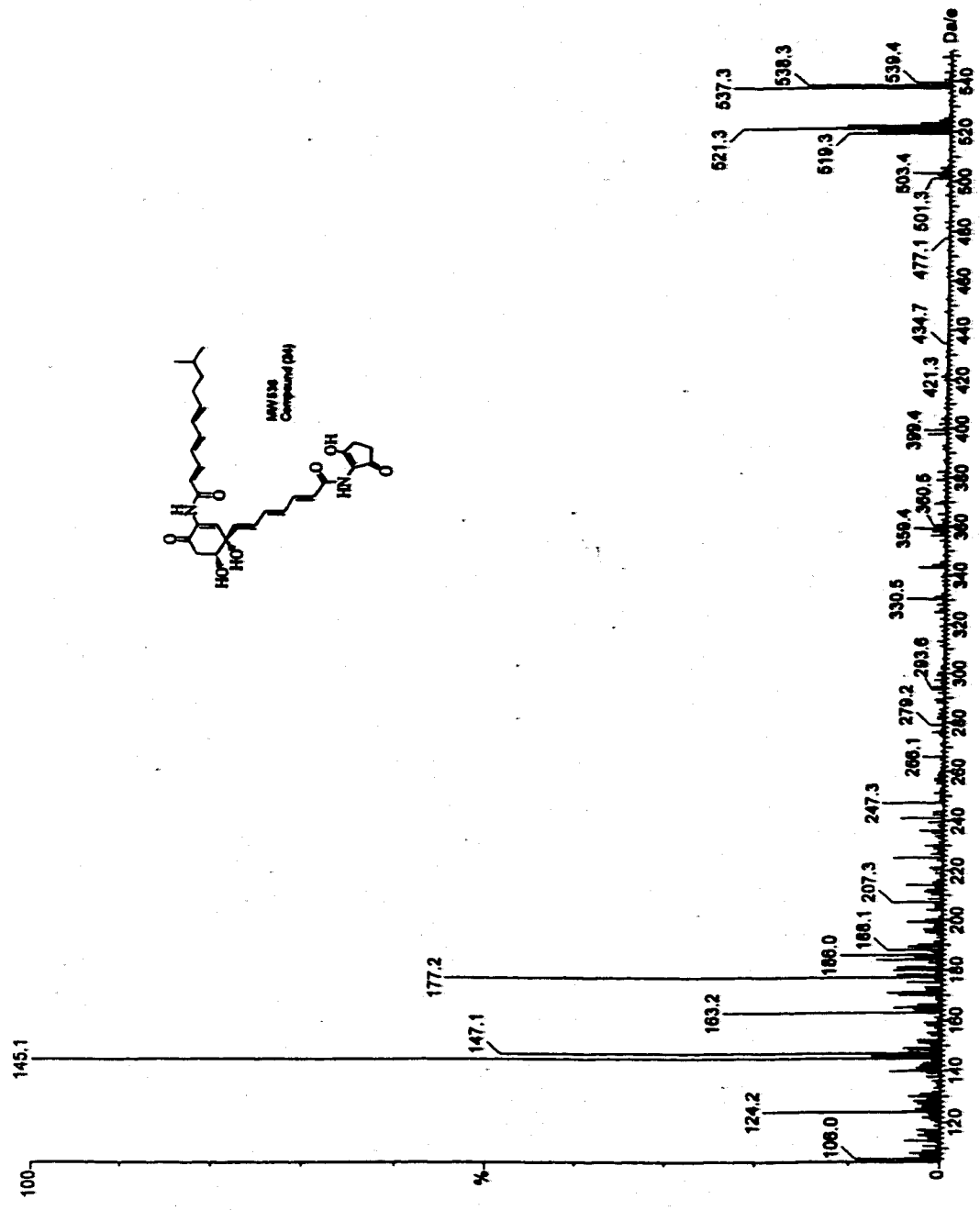
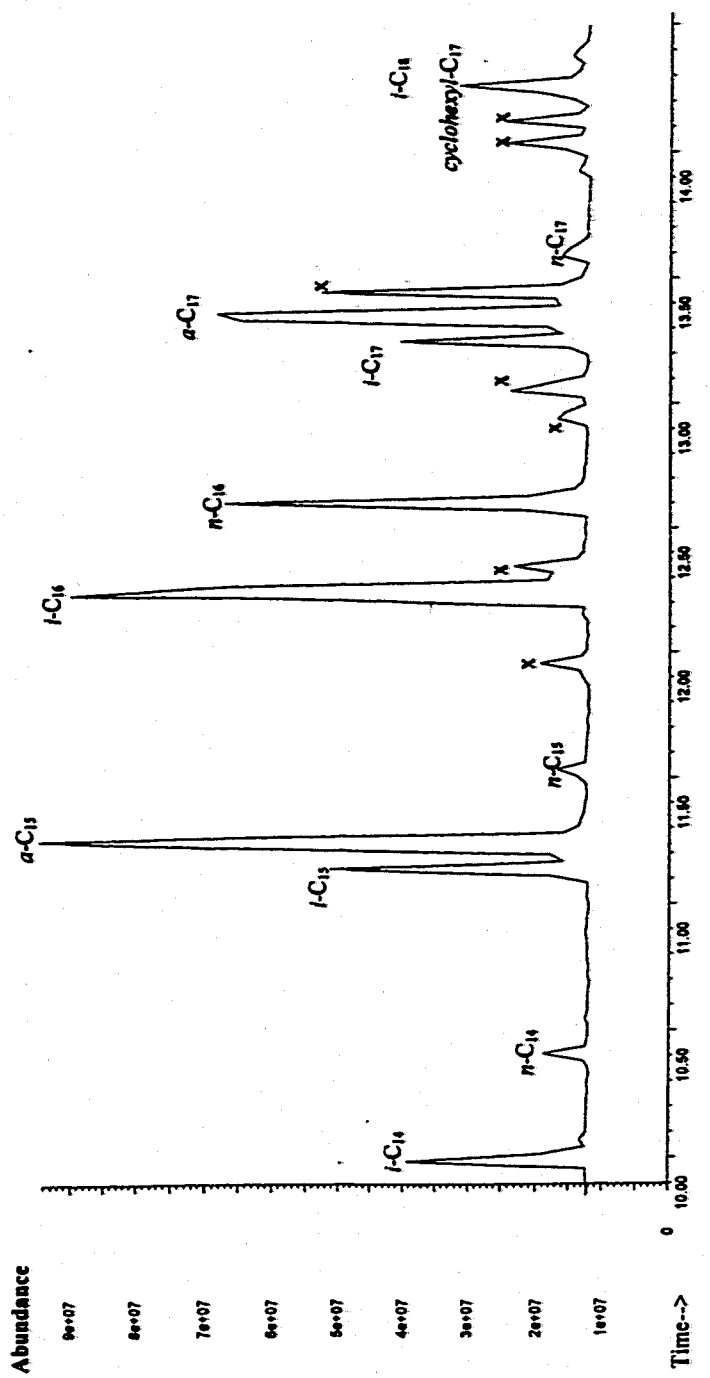


Figure 80. ES-MS/MS Spectrum of Type II Manumycin **24** Produced by *S. nodosus*

APPENDIX C: FATTY ACID ANALYSIS



X: unknown

Figure 81. GC Chromatogram of the Methyl Esters of Fatty Acid in *S. nodosus* Membrane

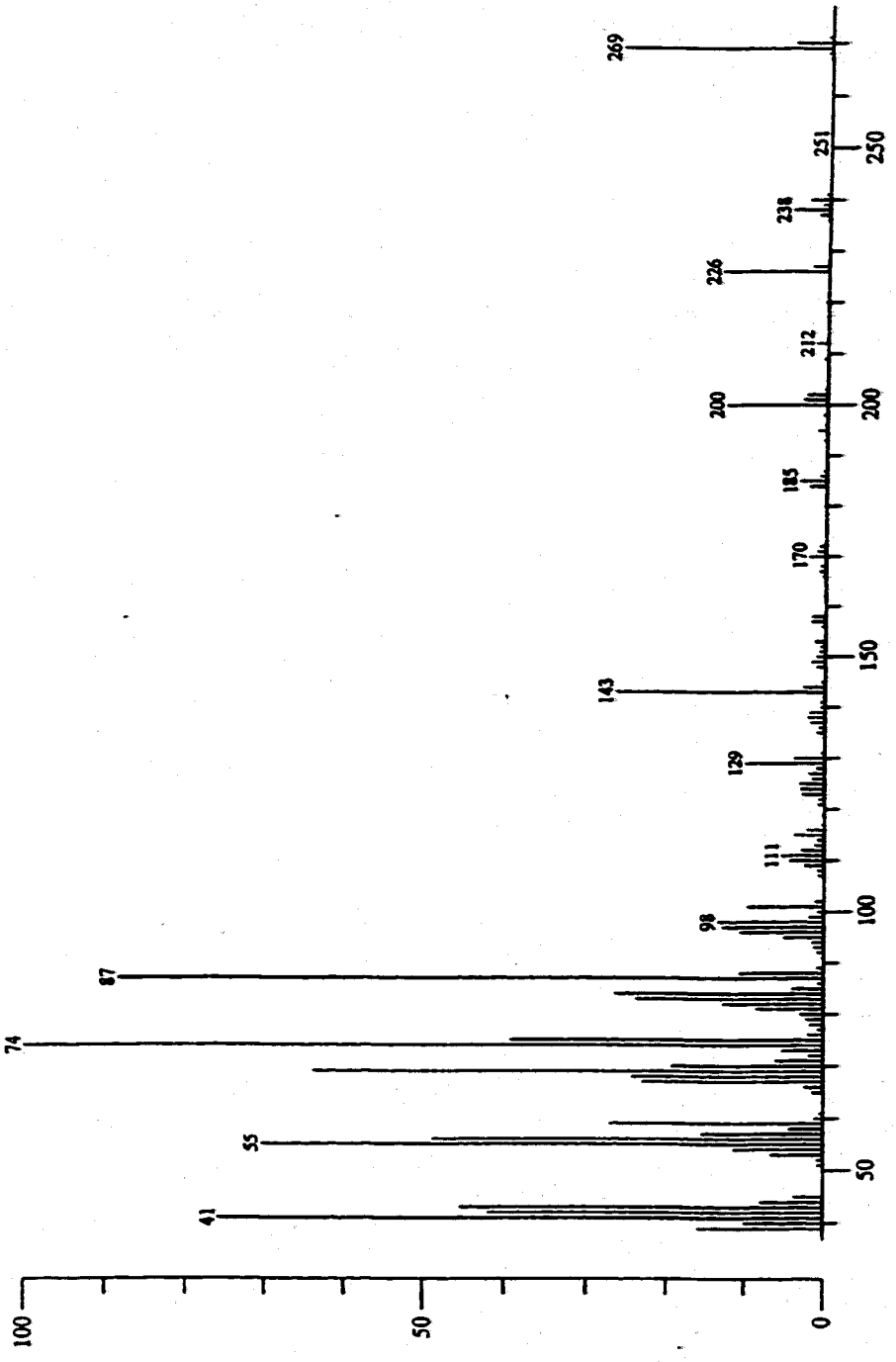


Figure 82. GC-MS Spectrum of Methyl ω-Cyclopentyl-[11-^{13}C]undecanoate (**149**)

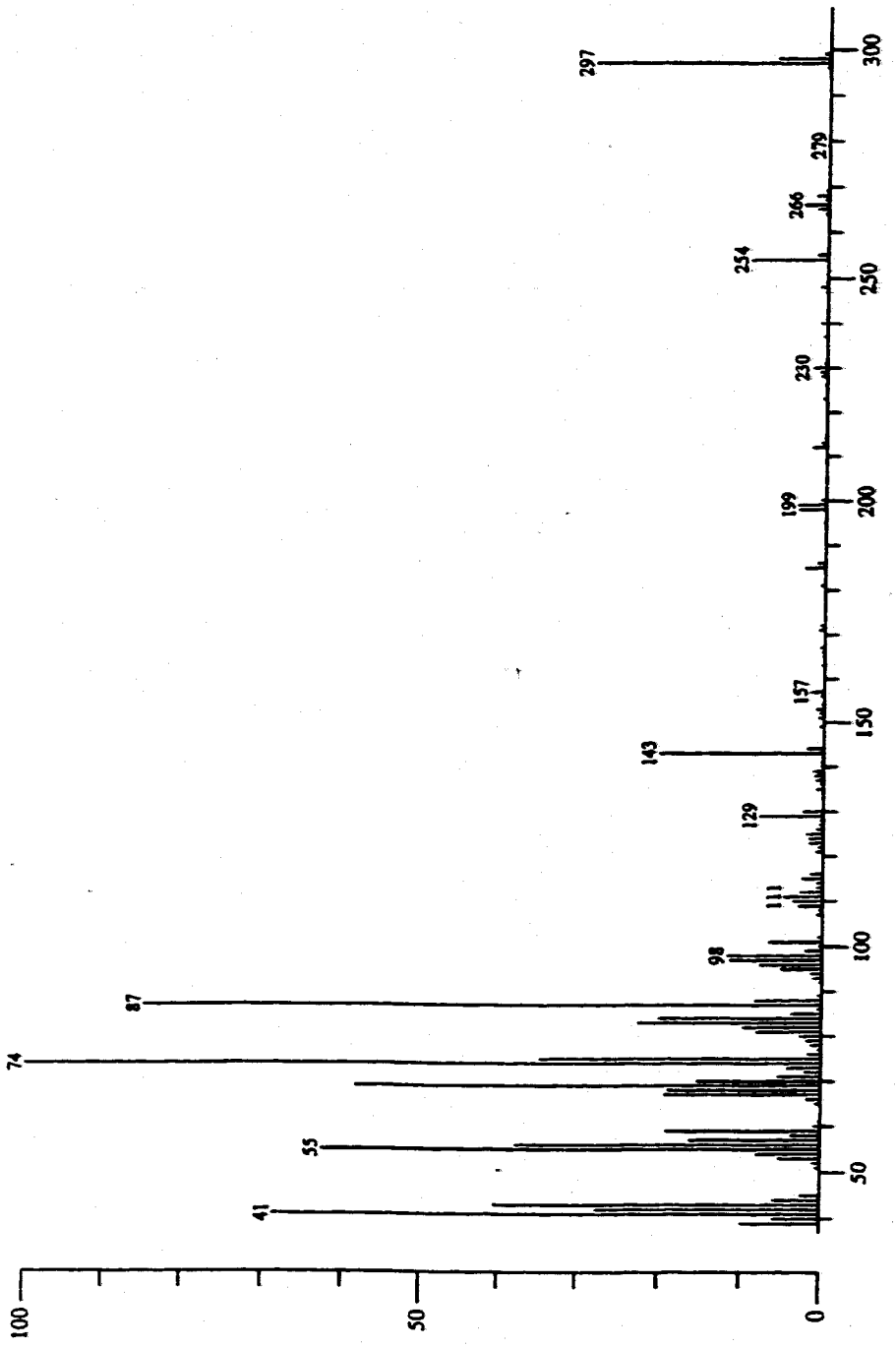


Figure 83. GC-MS Spectrum of Methyl ω-Cyclopentyl-[13-^{13}C]tridecanoate (**150**)

Vita

Yiding Hu was born October 20, 1962 in Beijing, China. He attended the Beijing University of Chemical Technology, earned his Bachelor of Science in 1984 and Master of Engineering in 1987, majored in Polymer Science. Then he had worked in the Institute of Chemistry, Chinese Academy of Science as a Research Scientist until 1991. He joined the University of Maine and received his Master of Science in Organic Chemistry in 1994. In March 2000, he completed the requirements for the Doctor of Philosophy degree in Chemistry at University of Washington.