An Abstract of the Thesis of

Ben Shen for the degree of Doctor of Philosophy in Chemistry presented on November 27, 1990

Title: LL-C10037α and MM 14201: Structure, Biosynthesis and Enzymology of Two Epoxysemiquinone Antibiotics

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Abstract approved: _____

Dr. Steven J. Gould

Antibiotic LL-C10037α has now been shown to have the epoxysemiquinone structure **2**, with 4*S*, 5*S*, 6*S* stereochemistry. Two additional metabolites of *Streptomyces* LL-C10037 were isolated and characterized as **66** and **68**, respectively, which were obviously biogenetically related to **2**.

Antibiotic MM 14201 **4** has been established as the desacetyl enantiomer of **2**, having 4*R*, 5*R*, 6*R* stereochemistry. The enantiomeric relationship between the two epoxysemiquinones (**2** and **3**) and the two epoxyquinones (**70** and **77**) was established spectroscopically and confirmed by biosynthetic studies.

The pathway for the biosynthesis of **2** has been established by combined applications of *in vivo* and *in vitro* studies. Thus, it was shown that **65** is first hydroxylated to form **62**, which then decarboxylates to form **88**, followed by a second hydroxylation to form **85**. N-acetylation of **85** affords **78**, which is subsequently epoxidized to form **70**. Reduction of **70** finally yields **2**. The epoxidation of **78** is catalyzed by 2,5-dihydroxyacetanilide (DHA) epoxidase and the reduction of **70** is catalyzed by 2-acetamido-5,6-epoxy-1,4-benzoquinone (AEBQ) dehydrogenase.

Speculation on the biosynthesis of **4** was based on its structural similarity to **2**, and it was postulated that **4** could be derived simply via the same pathway as **2** (exclusive of absolute stereochemistry) with the addition of a deacetylation as the last step. This hypothesis was given strong support by the isolation of the DHA epoxidase which catalyzed the epoxidation of **78** to form **77**, the enantiomer of **70**. The two DHA epoxidases catalyze reactions of

same stoichiometry but display opposite facial specificity towards the planar substrate **78**.

Acetamido-1,4-benzoquinone (ABQ) dehydrogenase from *S.* LL-C10037 catalyzes the reduction of **80** to form **78** in the presence of NADH. The reduction is unidirectional under the conditions studied. The enzyme has an optimal pH of 6.5 and displays a remarkable thermostability.

DHA epoxidase from *S.* LL-C10037 has been purified to apparent homogeneity, with an overall purification factor of 640. The enzyme catalyzes the epoxidation of **78** to form **70**, requiring molecular oxygen but no added cofactors. The enzyme is a pentamer or hexamer with a molecular weight of 117K ± 10K. The molecular weight of the subunit is 22.3K. The enzyme has an optimal pH of 6.5. It follows classical Michaelis-Menten kinetics and the apparent V_{max} and K_m are 3.7 ± 0.2 mmol min^{-1} mg^{-1} and 8.4 ± 0.5 μM, respectively, with **78** showing inhibition at 150 μM. It is inhibited by CN^-, PCMBA and 1,10-phenanthroline and is a metalloenzyme. The enzyme is activated by Co^{2+}, Mn^{2+} and Ni^{2+}. Enzyme activity is totally reconstructed from its apoenzyme by addition of Co^{2+}, Mn^{2+} or Ni^{2+}; no other metals tested are effective.

DHA epoxidase from *Streptomyces.* MPP 3051 has been purified to near homogeneity, with an overall purification factor of 1489. The enzyme catalyzes the epoxidation of **78** to form **77**, requiring molecular oxygen but no added cofactors. The enzyme is a dimer with a molecular weight of 33K ± 2K. The molecular weight of the subunit is 16K. The enzyme has an optimal pH of 5.5. It follows classical Michaelis-Menten kinetics and the apparent K_m for **78** is 7.2 ± 0.4 μM, with **78** showing inhibition at 100 μM. It is inhibited by CN^-, CO, PCMBA and 1,10-phenanthroline. The enzyme is a metalloenzyme and its inhibition by 1,10-phenanthroline can be reversed by addition of Fe^{2+}, Cu^{2+} or Cu^{1+}. However, neither activation of the enzyme nor reconstruction of the epoxidase from the apoenzyme is observed by addition of all the metals tested.

AEBQ dehydrogenase from *S.* LL-C10037 catalyzes the reduction of **70** to form **2** in the presence of NADPH. The reaction is reversible. The enzyme has an optimal pH of 7.0 for the reduction of **70**. The molecular weight of the enzyme is estimated to be 100K ± 30K. The enzyme follows classical Michaelis-Menten kinetics, and the apparent K_m for **70** and NADPH are 8.2 ± 0.5 μM and 45 ± 3 μM, respectively.

LL-C10037α and MM 14201: Structure, Biosynthesis and Enzymology of Two Epoxysemiquinone Antibiotics

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What we call the beginning is often the end
And to make an end is to make a beginning
The end is where we start from

T. S. Eliot

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List of Abbreviations

ABA:	3-aminobenzoic acid
ABQ:	acetamido-1,4-benzoquinone
AEBQ:	2-acetamido-5,6-epoxy-1,4-benzoquinone
AHBA:	3-amino-5-hydroxybenzoic acid
CD:	circular dichroism
CFE:	cell-free extract
DHA:	2,5-dihydroxyacetanilide
DMAP:	dimethylamino pyridine
DTT:	dithiothreitol
FAD:	flavin adenine dinucleotide
FMN:	flavin mononucleotide
FPLC:	fast protein liquid chromatography
HPLC:	high performance liquid chromatigraphy
MW:	molecular weight
NAD^+:	nicotinamide adenine dinucleotide
NADH:	nicotinamide adenine dinucleotide, reduced form
$NADP^+$:	nicotinamide adenine dinucleotide phosphate
NADPH:	nicotinamide adenine dinucleotide phosphate, reduced form
NNM:	nanaomycin
PCC:	pyridinium chlorochromate
PCMBA:	*p*-chloromercuribenzoic acid
PMSF:	phenylmethylsulfonyl fluoride
PVPP:	polyvinylpolypyrrolidone
TLC:	thin layer chromatography

LL-C10037α and MM 14201: Structure, Biosynthesis and Enzymology of Two Epoxysemiquinone Antibiotics

Chapter 1

Introduction

Isolation and Structure Elucidation of LL-C10037α

Antitumor antibiotic LL-C10037α was isolated by Lee and coworkers in 1984 from cultures of *Streptomyces LL-C10037*.[1] As shown in Figure 1, its structure was first assigned based on spectral data as the γ-aminosemiquinone, **1**, which was believed to be the first example of this class of natural product.[1] As part of their initial effort to study the biosynthesis of LL-C10037α, Gould and coworkers revised its structure to **2** by re-evaluating the ^{1}H NMR data in DMSO-d_6 (400 MHz). The ^{1}H NMR data for **2** were identical to those reported in the literature.[1] Whereas Whittle and Gould assigned the doublet at 5.8 ppm (J = 6.4 Hz) to the OH proton and the broad singlet at 9.0 ppm to the NH proton, Lee had made the reverse assignments. The new structure was confirmed by an X-ray diffraction study.[2] While the absolute configuration of **2** remained unknown, the *cis* relative stereochemistry was clearly established.[2] Remarkably, therefore, (+)-MT 35214, **3**, obtained by acetylation of antibiotic MM 14201, **4**, produced by *Streptomyces sp.* NCIB 11813,[3] had the same gross structure and relative stereochemistry as **2**. The difference in the sign of their specific rotations indicated that they may form an enantiomeric pair.

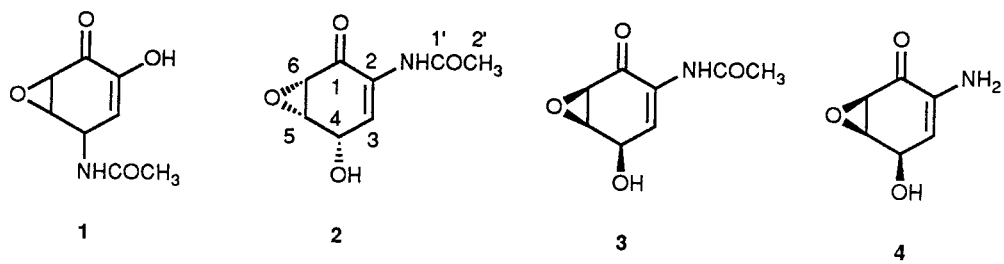


Figure 1. The Structure of LL-C10037α and Related Antibiotics

Chemical and Physical Properties of LL-C10037α

LL-C10037α is obtained as a non-crystalline, fluffy, white powder from water but crystallizes as colorless prisms from methanol. It is soluble in water and lower alcohols, fairly stable in acid and unstable in base. Listed in Table 1 are selected physical data of **2**.

Table 1. Physical Data of **2**.[1,4]

m.p. 153 °C (decomposed)
$[\alpha]^{26}_D$ = -155° (c 0.1, H_2O)
UV λ_{max} nm (ε) (in H_2O): 212 (10970), 275 (3510)
IR (KBr) cm^{-1}: 3260, 3070, 1690, 1655, 1545

Position	^{13}C NMR (DMSO-d_6) δ_C	Mult.	^1H NMR (DMSO-d_6) δ_H	Mult.	J (Hz)	^1H NMR (CH_3OH-d_4) δ_H	Mult.	J (Hz)
1	189.6	s						
2	128.3	s						
NH			9.04	bs				
3	128.3	d	7.04	dd	2.6, 2.7	7.19	bt	2.6
4	63.3	d	4.79	ddd	2.7, 3.1, 6.1	4.82	bt	2.8
OH			5.79	bd	6.4			
5	53.7	d	3.77	ddd	2.7, 3.1, 4.1	3.79	dd	2.8, 4.3
6	52.2	d	3.55	d	4.2	3.50	d	4.4
1'	169.5	s						
2'	23.7	q	2.01	s		2.08	s	

Biological Activity of LL-C10037α and MM 14201

LL-C10037α has poor activity against a limited number of Gram-positive and Gram-negative bacteria. However, it is active against murine leukemia P388, with a 29% increase in life-span of the treated mice relative to saline-treated controls at its optimal dose level.[1]

MM 14201 shows broad spectrum antibacterial activity and weak activity against *Candida albicans*. It has greatest activity against strains of *P. aeruginosa* and *Serratia marcescens*.[3]

Naturally Occurring Epoxyquinones and Epoxysemiquinones

A large number of epoxyquinone-class antibiotics as well as non-antibacterial metabolites have been reported.[5-51] This group of compounds is typically characterized by a core structure of epoxyquinone or the partially reduced form, the epoxysemiquinone, with different substitutions on the ring. In most cases the epoxyquinone and the epoxysemiquinone, as well as the

corresponding quinone, were found as co-metabolites. Listed in Table 2 are the benzoquinone-type compounds of this class.

Table 2. Naturally Occurring Epoxyquinones and Epoxysemiquinones

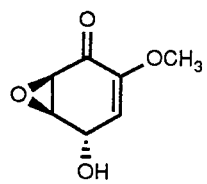
Name	Organism	Biosynth.	Ref.
Chaloxone (**5**)	*Chalara microspor*	unknown	6
2,3-Epoxy-6-hydroxy -5-methoxy-2-methyl -1,4-benzoquinone (**6**)	*Aspergillus fumigatus*	polyketide	7,8
(4S, 5R, 6R)-2-bromo -5,6-epoxy-4 -hydroxycyclohex -2-enone (**7**)	Acorn worms	unknown	9
2,5-Diphenyl-2,3-epoxy -6-hydroxy-1,4 -benzoquinone (**8**)	*Streptomyces sp.* AAA566	unknown	10
Epoxydon (Phyllosinol) (**9**)	*Phyllosticta* & *Panus sp. (basidiomycetes)*	polyketide	11,12
Isoepoxydon (**10**)	*Penicillium urticae*	polyketide	13,14
Terremutin (**11**)	*Aspergillus terreus*	polyketide	15,16
Terreic acid (**12**)	*Aspergillus terreus*	polyketide	17,18
Phyllostine (**13**)	*Phyllosticta*	polyketide	19
Epoformin (**14**)	*P. claviforme*	unknown	5,20
Isopanepoxydon (**15**)	*Panus sp.* (basidiomycetes)	unknown	21
Panepoxydon (**16**)	*Panus sp.* (basidiomycetes)	unknown	21
Panepoxydion (**17**)	*Panus sp.* (basidiomycetes)	unknown	21
Harveynone (**18**)	*Curvularia herveyi*	unknown	22
LL-C10037α (**2**)	*Streptomyces* LL-C10037	shikimate	1, 2 23
MM 14201 (**4**)	*Streptomyces* NCIB 11813	unknown	3,23
Enaminomycin A (**19**)*	*Streptomyces baarnensis* No 13120	unknown	24

Table 2 continued:

Enaminomycin B (**20**)	*Streptomyces baarnensis* No 13120	unknown	24
Enaminomycin C (**21**)	*Streptomyces baarnensis* No 13120	unknown	24
G 7063-2 (**22**)**	*Streptomyces* NCIB 11306	unknown	25
I-851 (**23**)*	*Streptomyces* No. 851	unknown	26
2061-A (**24**)**	*Streptomyces sp.*	unknown	27
2061-B (**25**)	*Streptomyces sp.*	unknown	27
Manumycin (**26**)	*Streptomyces parvulus*	C_4 Krebs & C_3 triose phosphate pool	28 -30
Asukamycin (**27**)	*Streptomyces nodosus subsp. asukaensis*		32 -34
U-56,407 (**28**)	*Streptomyces hagronensis strain* 360	unknown	35
U-62,162 (**29**)	*Streptomyces dietz sp. a.* (UC-8157)	unknown	36
Colabomycin A (**30**)	*Streptomyces griseoflavus*	unknown	37 38

*. The stereochemistry of **19** as shown is the absolute stereochemistry. The absolute stereochemistry of **23** was not determined. Unfortunately, no specific rotation data were collected for **23**, while **19** had a $[\alpha]^{20}_D$ -20.9° (c 0.83, MeOH).

. The stereochemistry of **22 as shown is the absolute stereochemistry, while that of **24** is the relative stereochemistry. No specific rotation data were available for either compounds.



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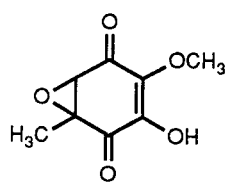

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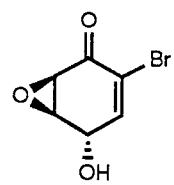

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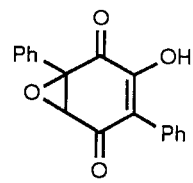

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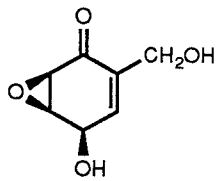

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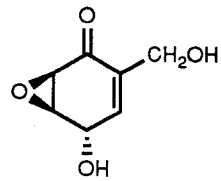

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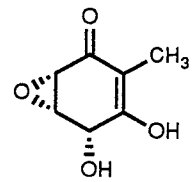

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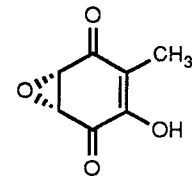

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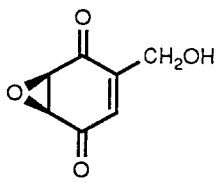

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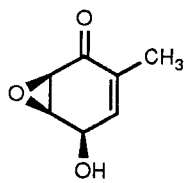

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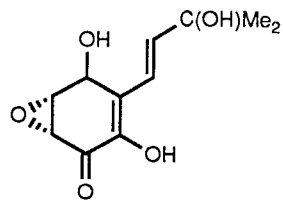

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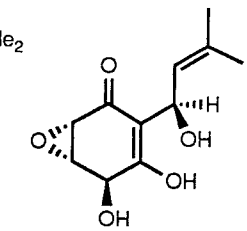

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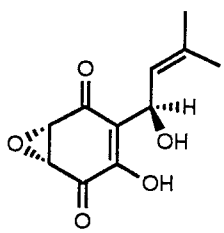

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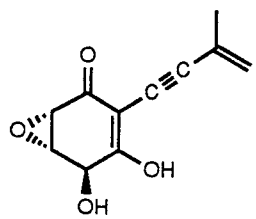

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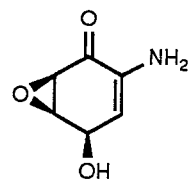

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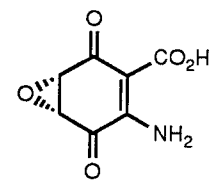

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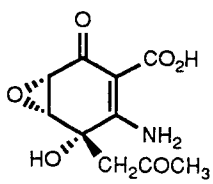

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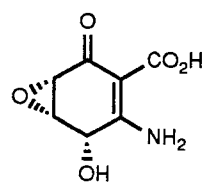

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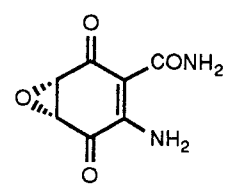

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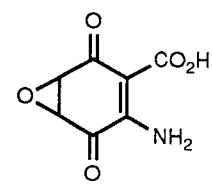

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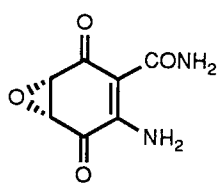

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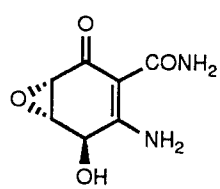

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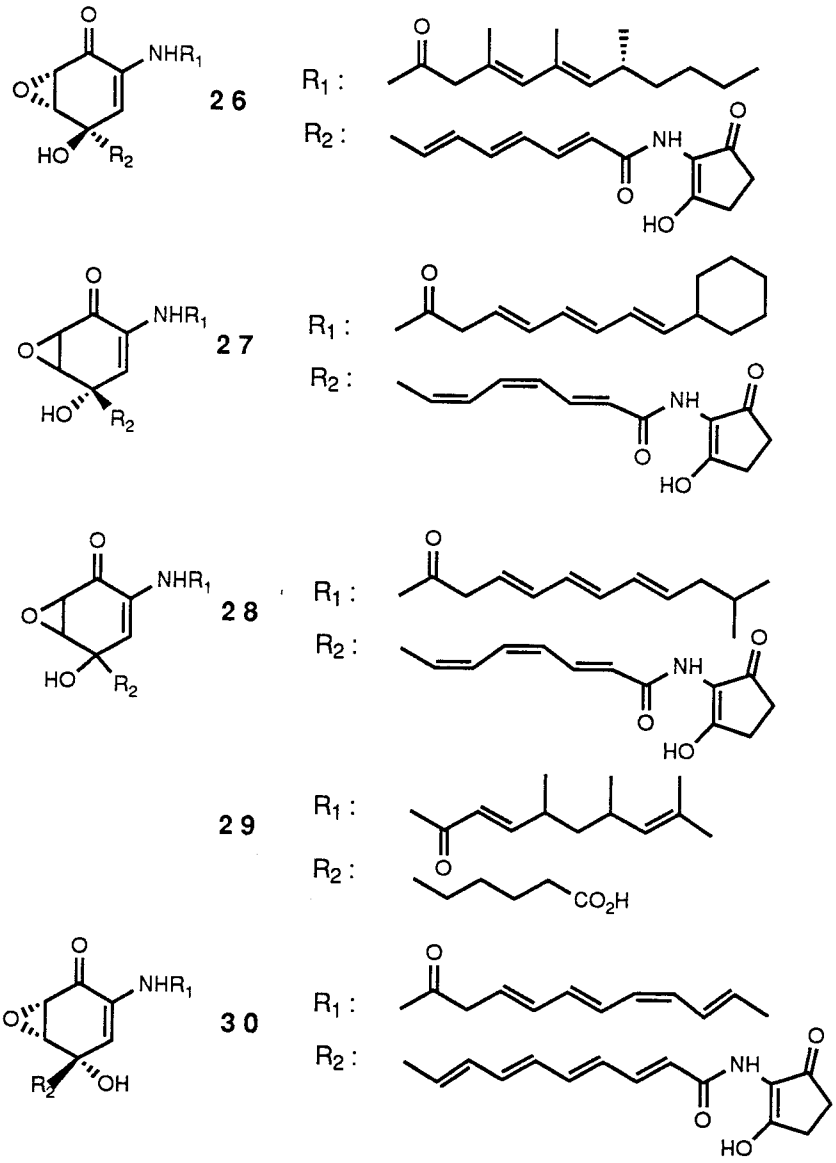
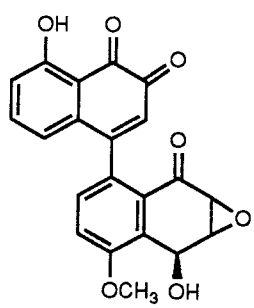


This epoxyquinone or epoxysemiquinone functionality is, of course, not limited to the family of benzoquinones. Epoxynaphthoquinones and epoxyanthraquinones have been reported, as well. Listed in Table 3 are some of those epoxyquinones and/or epoxysemiquinones.

Table 3. Naturally Occurring Epoxydated Naphthoquinones and Anthraquinones

Name	Organism	Ref.
Mycochrysone (**31**)	unnamed *inoperculate discomycete*	39,40
Diosquinone (**32**)	*Diospyros tricolor*	41
Naphthoxirene derivatives (**33**) & (**34**)	*Sesamum angolense* Welw. *(Pedaliaceae)*	42
Frenolicin (**35**)	*Streptomyces fradiae*	43
Nanamycin E (**36**) αE (**37**) βE (**38**)	*Streptomyces rosa* var. *notoensis*	44,45
Crisamicin C (**39**)	*Micromonospore purpureochromogenes* subs. *halotolerans*	46
Lactoquinomycin B (**40**)		47
Cerricarcin (**41**)	*Streptomyces ogaensis*	48,49
Julichromes Q_1-Q_3 (**42**) Q_1-Q_4 (**43**)	*Streptomyces shiodaensis*	50,51



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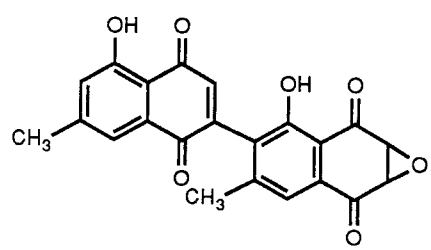

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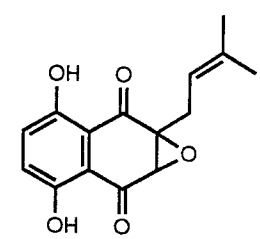

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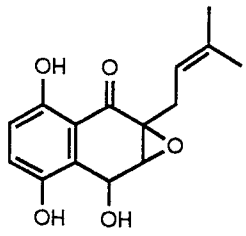

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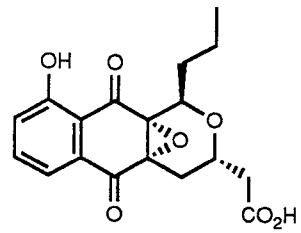

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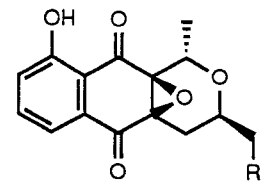
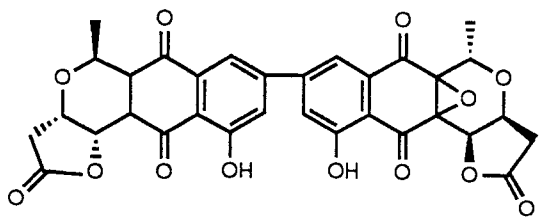


36 R: COOH
37 CO_2CH_3
38 CH_2OH



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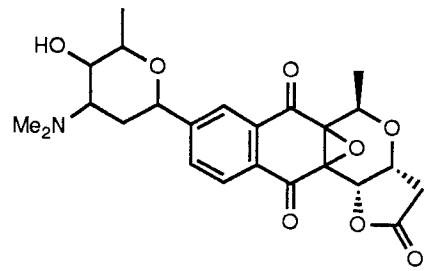

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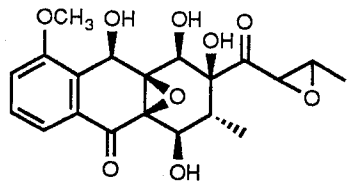

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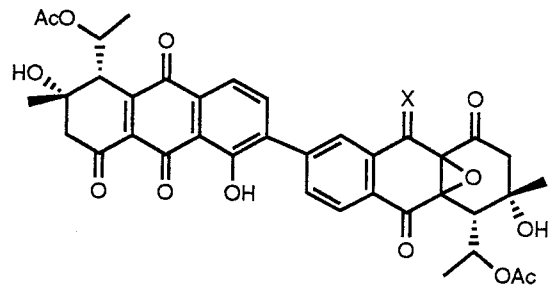


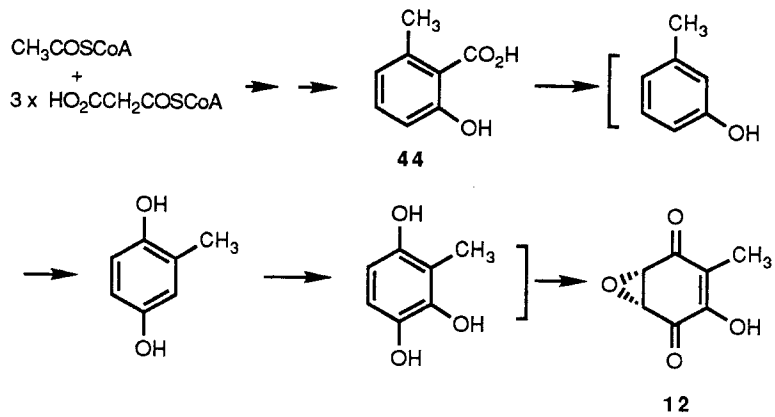
42 X: H, OH
43 X: O

Previous Biosynthetic Studies on Epoxyquinones and Epoxysemiquinones

Polyketide Origin

Terreic acid, **12**, was isolated from several cultures of *Aspergillus terrus*.[17,18] Numerous benzoquinone pigments of related structure have been isolated from species of *Aspergillus* [17,52] and there was substantial evidence that these were biosynthesized via a polyketide pathway.[53,54] By tracer studies using ^{14}C-labeled acetate, 6-methylsalicylic acid, **44**, and oresellinic acid (2,4-dihydroxy-6-methylbenzoic acid), Vining and coworkers demonstrated that

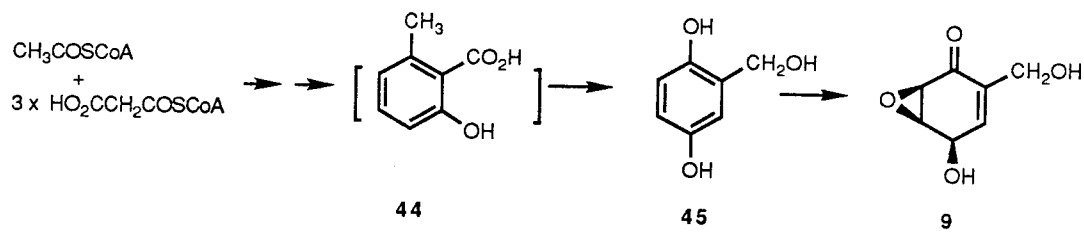
the carbon skeleton of **12** was derived from acetate and **44** was an intermediate (Scheme 1).[55]



Scheme 1. Proposed Pathway for the Biosynthesis of **12**

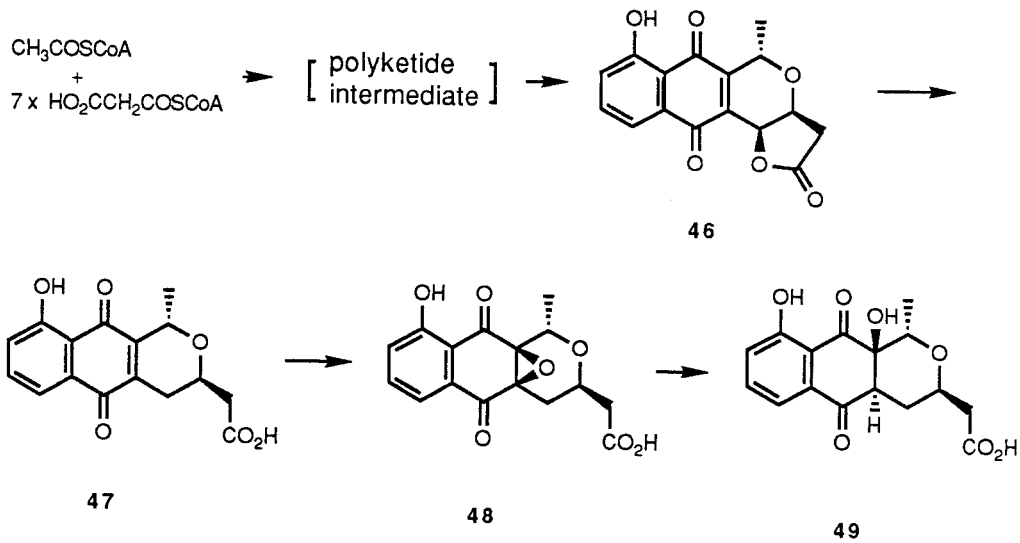
Although oresellinic acid was evidently not an intermediate in the biosynthesis of **12**, it was suggested as a precursor in the biosynthesis of fumigatin (3-hydroxy-4-methoxy-2,5-toluquinone) and related benzoquinone pigments produced by *Aspergillus fumigatus*,[8,56-58] indicating that a clear distinction existed between the biosynthesis of the structurally related compounds in the two species.

Sakamura and coworkers reported the biosynthesis of epoxydon, **9**, a major phototoxic and antitumor metabolite produced by *Phyllosticta sp.*[11,12] The labeling pattern of **9** obtained by feeding [1-^{13}C]- and [2-^{13}C]acetate, and the incorporation of [^{14}C]gentisyl alcohol, **45**, into **9**, led them to establish the polyketide origin of **9** (Scheme 2).[59,60]



Scheme 2. Proposed Pathway for the Biosynthesis of **9**

The nanaomycins (NNMs) are antifungal antimycoplasmal antibiotics produced by *Streptomyces rosa* var. *notoensis* OS-3966.[44,45,61-65] Omura and coworkers have studied the biosynthesis of the NNMs extensively. The carbon skeleton of the antibiotics was found to be derived from eight acetate units.[63] The biosynthetic relationship of the nanaomycin components was studied by a bioconversion method using the antibiotic cerulenin, a specific inhibitor of fatty acid and polyketide biosynthesis.[66] As a result, NNM-D, **46**, was considered to be the first component produced from the hypothetical "polyketide" intermediate, and the biosynthetic sequence for the NNMs was proposed to be: **46** to NNM-A, **47**, to NNM-E, **48**, to NNM-B, **49**.[67] Subsequently, cell-free studies and the isolation of NNM-D reductase, [68, 69] NNM-A monooxygenase [70] and NNM-B synthetase [70] led them to establish the biosynthetic pathway for the NNMs (Scheme 3).

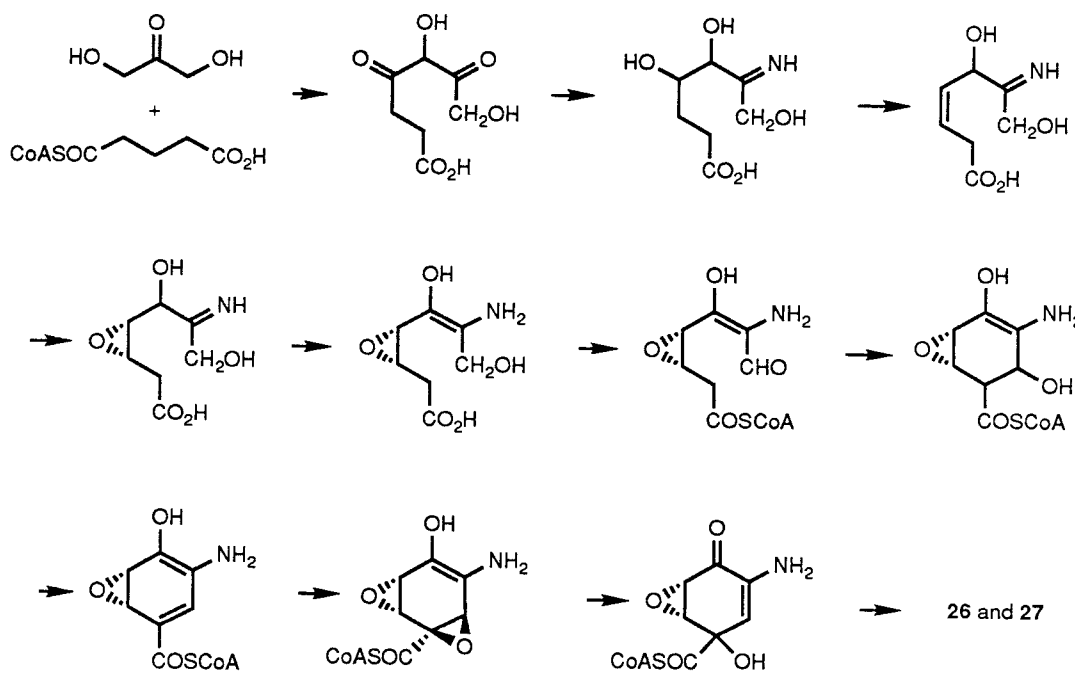


Scheme 3. Proposed Pathway for the Biosynthesis of NNMs

Krebs Cycle and Triose Phosphate Origin

The manumycin group of antibiotics, represented by **26**,[28-31] **27**,[33,34] **28**,[35] **29**,[36] and **30**[37,38] contains as a central element a multifunctional *m*-C_7N unit. The biosyntheses of other *m*-C_7N units has been shown to proceed either via a branch of the shikimic acid pathway,[71] with 3-amino-5-hydroxybenzoic

acid (AHBA), or 3-aminobenzoic acid (ABA), as proximate precursor, or via a sedoheptulose from the pentose phosphate pathway,[72,73] or via polyketide metabolism from acetate.[74] The research groups of Floss, Omura and Zeeck have taken **26** and **27** as representives of this group of antibiotics to explore their biosynthesis. By a series of elegant feeding experiments of ^{2}H, ^{13}C, ^{15}N, ^{18}O, single- or multiple-labeled putative precursors, they have demonstrated[32,75-77] that the central *m*-C_7N unit, which served as the starter unit for a short polyketide chain, was biosynthesized from a C_4 intermediate of the Krebs cycle and a C_3 intermediate related to triose phosphate by a pathway distinct from the shikimate, polyketide, or pentose phosphate routes leading to other *m*-C_7N units in Nature. A pathway for the assembly of the *m*-C_7N unit in the manumycin group of antibiotics has been proposed, although little experimental evidence was available to support the detailed individual steps proposed (Scheme 4).[32]



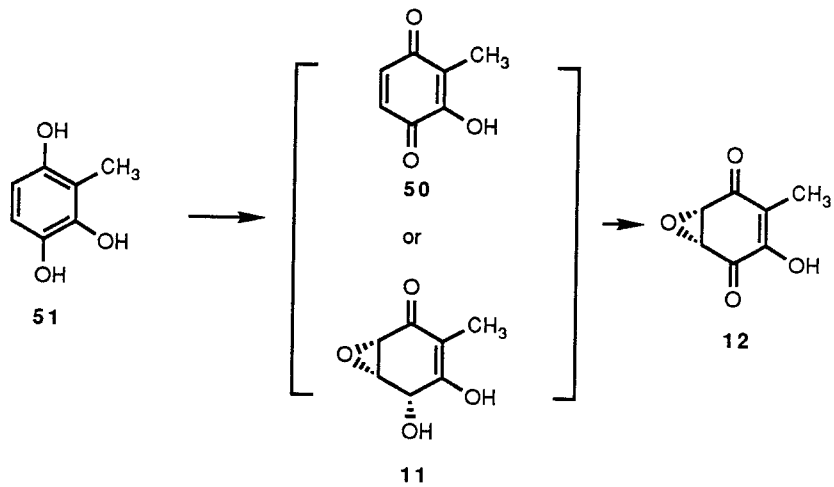
Scheme 4. Proposed Pathway for the Biosynthesis of **26** and **27**

Mechanism for the Formation of the Oxirane Functionality

The origin of the oxygen of the oxirane functionality of epoxyquinones and epoxysemiquinones has been a key issue in the investigation of the biosynthesis of this class of metabolites. Although the oxygen could be derived from a primary organic precursor or from water, in all cases studied, the oxygen comes from molecular oxygen,[32,55,59,60,70,75,77-79] presumably via an enzymatic process involving a monooxygenase.

With the origin of the oxygen established, the question of the identity of the epoxidation substrate --- a quinone or a hydroquinone --- was raised. Although one would prefer that nature had developed a single process for the biosynthesis of the oxirane of all the epoxyquinones and epoxysemiquinones, both the quinone and the hydroquinone have been postulated as the substrate for the epoxidation.

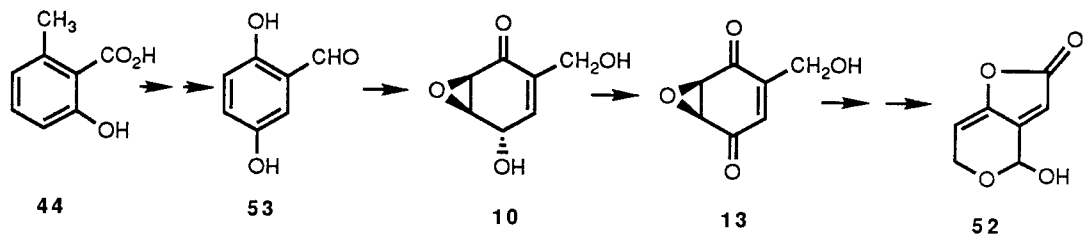
In the biosynthesis of **12** in *Aspergillus terrus*, Vining and coworkers proposed the pathway in which the immediate intermediate was the quinone, **50**.[55] On the other hand, the isolation of **11**, a metabolite from a mutant strain of *Aspergillus terrus*,[15,16] led them to postulate an alternative in which the hydroquinone, **51**, was epoxidized (Scheme 5).[55]



Scheme 5. Proposed Mechanism for the Oxirane Formation of **12**

Sakamura and coworkers proposed the biosynthetic pathway for **9** in *Phyllosticta sp.*[59,60] shown in Scheme 2, implying the direct epoxidation of **45**

without specifying any mechanistic detail. In the biosynthesis of patulin, **52**, a toxin produced by a number of fungal species, notably those of *Penicillium* and *Aspergillus*,[80] Gaucher and coworkers have identified **10** and **13** as intermediates, although the nature of the epoxidation remained a matter of speculation. There have been conflicting reports on the relationship of gentisaldehyde, **53**, and gentisyl alcohol **45** to **52**.[13,14,56,81-85] Combining results from their feeding studies, mutant studies and cell-free studies, Gaucher and coworkers suggested **53** and **45** were obligatory biosynthetic intermediates of **52**, indicating the direct epoxidation of **53**. Unfortunately, they were not able to find the enzyme activity responsible for the epoxidation of **53**, although several enzyme activities on the proposed biosynthetic pathway for **52** have been isolated (Scheme 6).[85]

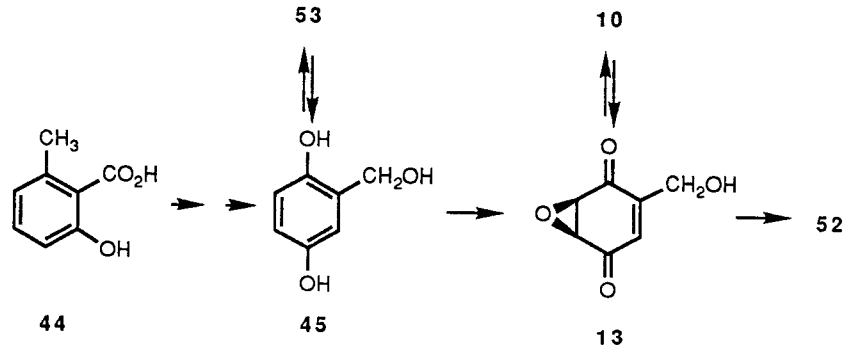


Scheme 6 Part of the Proposed Pathway for the Biosynthesis of **52**

Most recently, however, Light and Priest have isolated a crude extract from cultures of *Penicillium patulum* that catalyzed the epoxidation of **45** to **13**, as well as epoxidation of toluquinone, **54**, but not **53**, suggesting that both **53** and **10** were shunt metabolites of the biosynthesis of **52**.[78,79] Therefore, they proposed a linear biosynthetic pathway for **52** from **44** (Scheme 7).

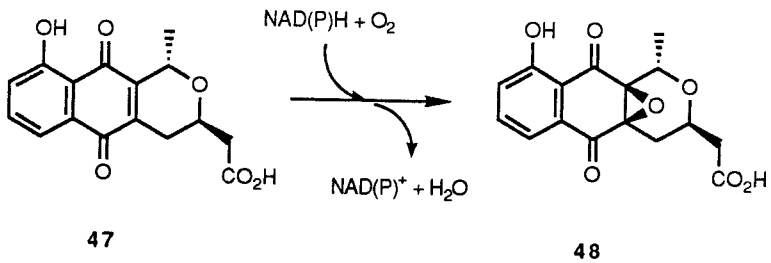
The enzymatic epoxidation of **45** required molecular oxygen but did not involve any loosely bound cofactors. It was inhibited by 1,10-phenanthroline, EDTA and 4-chloromercuribenzoic acid, indicating a requirement of a metal ion, and possibly a sulfhydryl group. However, it seems that a question remains whether the enzyme activity isolated is related to the biosynthesis of **52**, especially due to the fact that the natural substrate **45** had a larger K_m (K_m = 0.24 mM) than **54** (K_m = 0.17 mM), which was epoxidized to give desoxyphollostine, **55**, a quinone epoxide not previously reported in the fungal

cultures. Further study of this enzyme has so far been limited by the particulate nature of the cell-free preparation.



Scheme 7 Part of the Proposed Pathway for the Biosynthesis of **52**

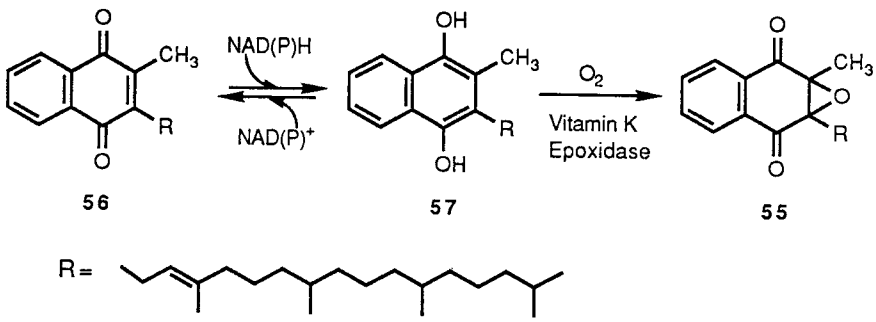
Quite some time before Light's work was published, Omura and coworkers reported data on the mechanism of the oxirane formation of **48** (Scheme 8).[70] An enzyme activity was isolated that catalyzed the epoxidation of **47** to **48**. This process required NAD(P)H and molecular oxygen, and the enzyme was described as a monooxygenase. Unfortunately, no attempt to purify the enzyme activity was apparently made, which prevented further investigation of the nature of the epoxidation. However, the apparent difference of the specifity of the substrate and the requirement of the nicotinamide cofactor in the latter case suggest that the epoxidase activities isolated from *Penicillium patulum* and from *Streptomyces rosa* var. *notoensis*, respectively, may be mechanistically different.



Scheme 8. Enzymatic Formation of **48** form **47**

It would be incomplete in this context not to mention the chemistry of vitamin K epoxides. Matschiner and coworkers first described the existence of

the 2,3-epoxide of vitamin K_1, **55**, in rat liver,[86] and the *in vitro* formation of **55** was demonstrated by Willingham and Matschiner in microsomal preparation.[87] Several other vitamins K were subsequently found to be epoxidized also, as reported by Friedman and Smith, who found that the rates of epoxidation of menaquinone-2 and menaquinone-3 were more rapid than that of vitamin K_1, **56**.[88] Various efforts have been devoted to study the mechanism of the vitamin K epoxidation and its relationship to the vitamin K-dependent carboxylation of glutamic acid residues and to the biosynthesis of prothrombin.[89-92] The origin of the oxygen of the oxirane of **55** from molecular oxygen was established by incubation under $^{18}O_2$ and the enrichment of ^{18}O in the oxirane was easily observed in its mass spectrum.[89]



Scheme 9. Mechanism for the Epoxidation of **56** in Rat Liver Microsomes

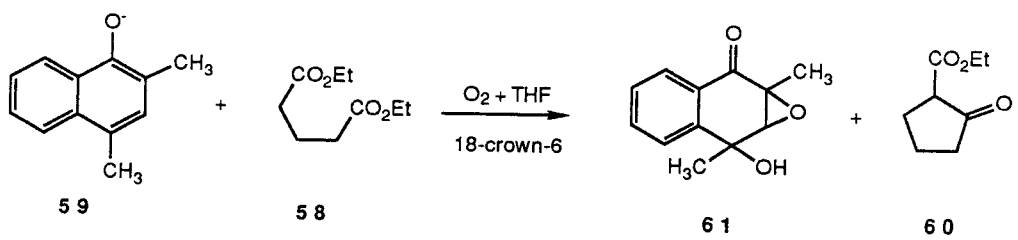
Suttie and coworkers examined very carefully the requirement of various subcellular fractions for the epoxidation of **56** in postmitochondrial supernatants from vitamin K deficient rat livers.[89] When **56** was incubated with the microsome preparation, no epoxidation of **56** occurred unless either NADH or NADPH was included. However, the requirement of reduced pyridine nucleotide for the epoxidation of **56** was eliminated if the vitamin was supplied to the incubation mixture as the chemically reduced hydroquinone, **57**. The choice of **57** as the substrate for the epoxidation was further confirmed by competitive epoxidation of a mixture of **57** and **56**, labeled with ^3H and ^{14}C respectively. In the absence of NADH, only ^3H could be detected in the isolated **55**, while when NADH was added to the same incubation, **55** contained both ^3H and ^{14}C. Therefore, it was concluded that NAD(P)H was needed to reduce **56** to **57**, which can be epoxidized to yield **55** in the absence of either NADH or

NADPH (Scheme 9).[89,90] Since no inhibition of vitamin K epoxidation was demonstrated by the addition of any of the cytochrome P_{450} inhibitors, it was established that the epoxidation of **56** was not mediated by cytochrome P_{450}.[89]

Although it was not investigated if NAD(P)H can reduce **56** to **57** chemically, it has been reported that this reduction can be carried out either by NAD(P)H dehydrogenase in the presence of NAD(P)H,[93] or possibly by vitamin K reductase in the presence of dithiothreitol.[94]

Purification of the vitamin K epoxidase by traditional methods had not been successful because of problems with its solubilization and stability.[95-102] However, very recently, Furie and coworkers purified the vitamin K-dependent carboxylase to homogeneity by using affinity chromatography based on a synthetic propeptide containing the γ-carboxylation recognition site.[103] Although it had been suspected on the basis of the analysis of crude and partially purified vitamin K-dependent carboxylase and vitamin K epoxidase activities, that the carboxylation of glutamic acid residues and the epoxidation of **56** are reactions that are coupled,[102,104] with the pure enzyme Furie and coworkers[103] demonstrated that the vitamin K-dependent carboxylation and vitamin K epoxide formation were catalyzed by the same enzyme.

Ham and Dowd recently reported that when a THF solution of diethyl adipate, **58**, was treated at room temperature with potassium 2,4-dimethyl-1-naphthoxide, **59**, and molecular oxygen in the presence of 18-crown-6, condensation (internal carboxylation) occurred to yield the 1-oxo-cyclopentane-2-carboxylate **60** and the keto epoxide **61** (Scheme 10).[105] This reaction mimics the essential features of the vitamin K-dependent carboxylation of glutamic acid residues and could be used as a nonenzymatic model to study the reaction mechanism.

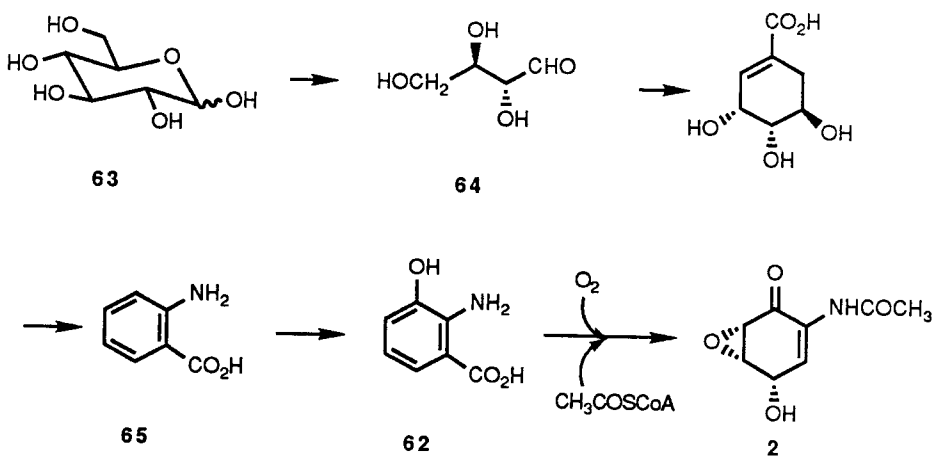


59 58 61 60

Scheme 10. Nonenzymatic Model for the Vitamin K-Dependent Carboxylation

Previous Biosynthetic Studies on LL-C10037α

In their preliminary study of the biosynthesis of **2**, Gould and Whittle reported that **2** was derived via the shikimic acid pathway with 3-hydroxyanthranilic acid, **62**, as the key intermediate.[2] [2-^{13}C]acetate was shown to label **2** only in the acetamide methyl group, revealing that the carbocycle was not polyketide in origin. On the other hand, a feeding of [1-^{13}C]-D-glucose, **63a**, gave specific labeling at C-2 and C-4, clearly indicative of a shikimate-type pathway. The correct orientation of the apparent shikimic acid precursor relative to **2** was determined by feeding [1-^{13}C]-D-erythrose, **64a**, which resulted in specific labeling at C-1, rather than at C-5. Thus, the nitrogen substitutent of **2** had been introduced at C-6 of the shikimate rather than at C-2. Fermentation under an atmosphere containing $^{18}O_2$ labeled **2** at the secondary alcohol and the oxirane ring, implying that these oxygens were introduced by oxidative processes while the oxygen at C-1 had been retained from the organic precursor. Although [^{14}C]anthranilic acid, **65a**, gave poor incorporation,[106] [5-^{2}H]-3-hydroxyanthranilic acid, **62a**, very effectively labeled **2** specifically at C-5, which confirmed that the biosynthesis of **2** represented a novel aromatic amino acid metabolism (Scheme 11).



Scheme 11. Summary of the Feeding Results for the Biosynthesis of **2**

Purpose of the Present Studies

The preliminary biosynthetic study of **2** had revealed its origin from the shikimic acid pathway via a distinctive route leading to the epoxysemiquinone antibiotic.[2] The revised structure of **2** had indicated that **4** may be the desacetyl enantiomer of **2**. To extend these, the initial purpose of the subsequent research had three goals. The first goal was the isolation and structure elucidation of other co-metabolites in cultures of *Streptomyces* LL-C10037, which, as in many other instances, might have provided information relative to the biosynthesis of the major metabolite. The second goal was to confirm the enantiomeric relationship between **2** and **3** and to then determine the absolute stereochemistry of **2** and related metabolites. The third goal was to establish the biosynthetic pathway of **2** from **69**, mainly by the whole-cell feeding of putative precursors, in which either radioactive or stable isotope label(s) had been introduced strategically at specific position(s).

As the project developed, the enantiomeric relationship between **2** and **3** was confirmed, the absolute stereochemistry of **2** was determined, and all the intermediates for the biosynthesis of **2** were identified. It then became attractive to attempt to isolate important enzyme activities involved in the biosynthesis of **2**. Therefore, cell-free studies were initiated and subsequently proved to be necessary for the unambiguous establishment of the pathway for the biosynthesis of **2**.

Once the pathway for the biosynthesis of **2** was established with isolations of related enzyme activities, it was then possible to isolate, purify, and characterize the critical enzymes in order to study the individual steps in greater detail. The most intriguing enzyme was the one which generated the epoxide functionality. This was particularly attractive because of the enantiomeric relationship between **2** and **4**. It was possible that **4** could be derived either by a pathway totally different from the biosynthesis of **2** or --- more simply --- by the same pathway (exclusive of the stereochemistry) with addition of a deacetylation as the last step. The latter implied the opportunity to isolate complementary epoxidases from two organisms showing opposite enantiomeric specificity. The final goal of the research, therefore, became the isolation, purification and characterization of the two epoxidases from *S.* LL-

C10037 and *S.* MPP 3051, and the study of their mechanisms of enzymatic epoxidation.

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Chapter 2

Structures and Stereochemistry of Metabolites from *Streptomyces* LL-C10037

Part of this work has been published.

Shen, Ben.; Whittle, Y. G.; Gould, S. J.; Keszler, D. A. *J. Org. Chem.* **1990**, *55*, 4422.

Isolation

The procedure for the isolation of **2** and related metabolites is depicted in Figure 2. Thus, the fermentation broth (typically 400 mL in a 2-L Erlenmeyer flask) was cooled to ice-water temperature and separated from mycelia by filtration though cheesecloth and celite. The filtrate was adjusted to pH 4.0-4.5 with the addition of solid KH_2PO_4 and then saturated with solid $(NH_4)_2SO_4$. The subsequent steps involved EtOAc extraction (typically 400 mL broth was extracted with 10 x 200 mL EtOAc), SiO_2 chromatography (20 g flash grade SiO_2 was used for 200 mg crude mixture), preparative TLC, and finally recrystallization to give pure **2**, **66**, **67** and **68**.

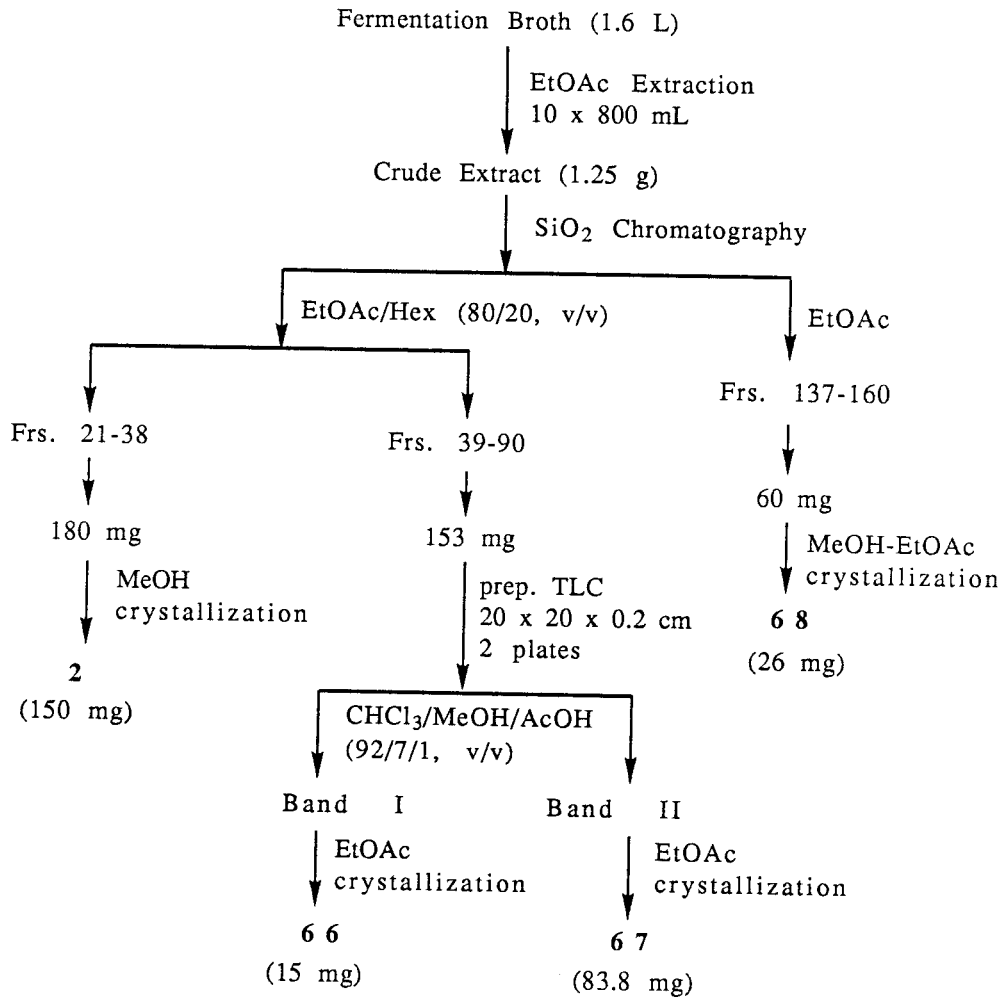
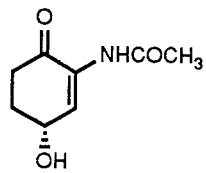


Figure 2. Process for the Isolation of **2**, **66**, **67** and **68**

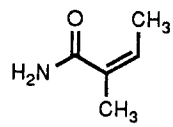
Structural Elucidation

The physico-chemical data acquired for **2** were identical to those reported.[1,2] Two new metabolites, very closely related to **2**, were also isolated from *S.* LL-C10037. One, more polar than **2**, has been named as LL-C10037β, **68**, and the other, slightly less polar than **2**, has been named as LL-C10037γ, **66**.

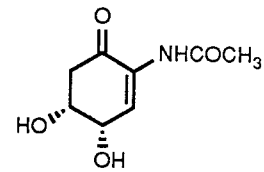
From the ^{1}H NMR and ^{13}C NMR spectral data of **66** and **68**, a resemblance to **2** was very clear. The UV and IR spectra indicated that each was also a 2-acetamidocyclohexenone. From the presence of one methylene adjacent to the ketone carbonyl (δ 2.67 and δ 2.78), the diol structure **68** was assigned to the more polar metabolite. The *cis* stereochemistry was derived from the H_4/H_5 coupling constant (3.4 Hz). The ^{1}H NMR spectrum of the less polar compound contained resonances from two adjacent methylenes, one next to the ketone (δ 2.46 and δ 2.64), and structure **66** was therefore assigned. The full assignments of both compounds came from the analysis of $^{1}H/^{1}H$ COSY and $^{1}H/^{13}C$ HETCOR spectra.



66



67



68

The structure of **67** was a little more elusive. Mass spectrum data and elemental analysis assigned the molecular formula of **67** to be C_5H_9NO, requiring two degrees of unsaturation. The ^{13}C NMR spectrum showed a carbonyl peak at 175.8 ppm, characteristic of an α,β-unsaturated amide. This was further supported by corresponding absorptions at 1675, 1654 and 1624 cm^{-1} in its IR spectrum and the absorption at 204 nm in its UV spectrum.

Two methyl groups and one olefinic proton in the ^{1}H NMR spectrum, combined with the information above, suggested that **67** may be 2- or 3-methyl-2-butenamide. This was further supported by single frequency decoupling experiments of its ^{1}H NMR spectrum. Based on its coupling constant, it was more reasonable to assign **67** to be 2-methyl-2-butenamide, which was a known compound. (*E*)-2-methyl-2-butenamide has a melting point of 73 - 74 ^{o}C[3] and (*Z*)-2-methyl-2-butenamide has melting point of 127 -

128 °C.[4a] Therefore, **67**, having a melting point of 129.5 - 130.5 °C, was assigned as (*Z*)-2-methyl-2-butenamide or angelamide, which was obviously not so closely related to the biosynthesis of **2**. Angelamide had been isolated from *L. hispidum*.[4b]

Absolute Stereochemistry

Inverse Quadrant Rule

Numerous epoxyquinones and epoxysemiquinones have been isolated from natural sources.[5-39] Generally, the absolute stereochemistry of the epoxide ring has been determined from circular dichroism (CD) data. An empirical correlation of the sign of the R band (from the n --> π^* transition of the C=O) at approximately 340 nm of an epoxycyclohexenone with compounds of known absolute stereochemistry (steroid derivatives) resulted in formation of an "inverse quadrant" rule, with the sign of R band dictated by the quadrant in which the oxirane oxygen atom lay.[40,41] The "inverse quadrant" rule states that cyclohexenones with oxirane rings in the upper left or lower right quadrants cause a negative Cotton effect, and those in the upper right and lower left quadrant cause a positive Cotton effect for the n --> π^* transition (Figure 3).

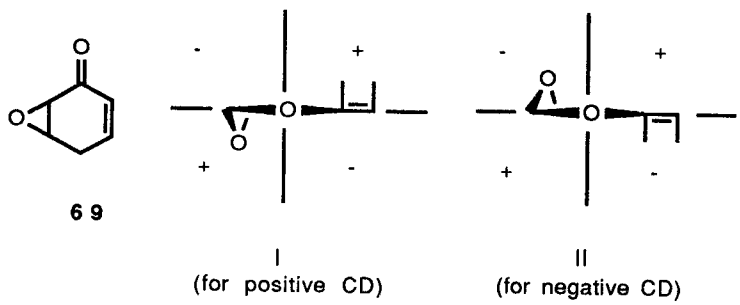


Figure 3. Rules for R-band CD of Epoxycyclohexenone **69**

In Table 4 the CD data for **2** and some of these other metabolites are given. Since the R band of **2** is negative (ΔE_{326} = -5.59), similar to **11** (ΔE_{341} = -1.64), **2** should have the oxirane oxygen lying in the upper left quadrant, leading to the structure as shown. Having previously established that **2** was a

cis stereoisomer, its absolute stereochemistry would be 4*S*, 5*S*, 6*S*. However, although numerous epoxysemiquinones have been assigned in this manner, the method is an empirical correlation and the stereochemistry of the standard most often chosen, **11**, was itself deduced by application of this helicity rule.[21,42] Thus, at this point in our work, the absolute stereochemistry of the simple epoxysemiquinones had not been established by a non-empirical approach.

Table 4. CD Values of **2** and Other Epoxysemiquinones

Compound	Solvent	λ_{max} (ΔE)
11[15]	dioxane	318 (-1.64), 265 (-0.42)
26[32]	CH_3CN	320 (-13.76), 286 (+10.23), 259 (+3.48)
	MeOH	314 (-11.87), 271 (+13.33)
16[21]	dioxane	341 (-1.81), 274 (+0.49), 246 (-4.85)
2	MeOH	326 (-5.59), 246 (+3.10)
9[12]	dioxane	341 (+3.60), 245 (-3.16)
5[6]	ethanol	330 (+2.88), 270 (+7.5)

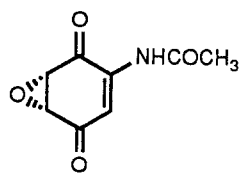
The epoxyquinone **70**, which was prepared by pyridinium chlorochromate (PCC) oxidation of **2**, was also analyzed by CD and the data were compared with those of other epoxyquinones in the literature. A standard has been **12**, whose absolute stereochemistry was established by chemical correlation with **11**.[15] Such compounds exhibit two Cotton effects for the n --> π^* transition between 300 to 400 nm. These transitions have been associated with the two individual carbonyls, and the difference in the band positions has been ascribed to the transitions from the energetically higher n orbital of the two carbonyl groups into the π^* orbital. Additionally, there may be some intramolecular hydrogen bonding at one of the two carbonyl groups.[15,32]

In Table 5 are CD data of **70** and some of the other epoxyquinones. The positive Cotton effect of **70** (ΔE_{364} = + 6.78), is the same as that of **71** (ΔE_{356} = + 4.59), a degradation product of **26**,[32] and opposite to that of **12** (ΔE_{351} = - 1.34), indicating that the former has 5*R*, 6*S* stereochemistry, consistent with that assigned to **2**. However, both assignments are based on the same underlying empirical rule since **12** was assigned by comparison with **11**.[15] We, therefore, chose to establish the absolute stereochemistry unequivocally with a

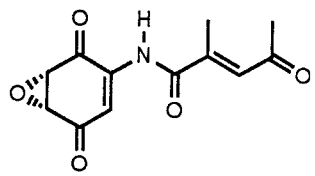
nonempirical method:[43] either the exciton chirality method, pioneered by Harada and Nakanishi,[44-46] or single-crystal X-ray crystallographic analysis.

Table 5. CD Values of **70** and Other Epoxyquinones

Compound	Solvent	λ_{max} (ΔE)
12[18]	MeOH	351 (-1.34), 313 (+1.87)
	CH_3CN	362 (-2.00), 314 (+2.29), 241 (-3.33), 220 (+4.87)
21[26b]	CH_3CN	376 (-6.58), 327 (+10.53), 233 (+4.60), 198(-8.20)
70	MeOH	364 (+6.78), 308 (-10.00), 242 (+6.29), 224(-6.61)
71[32]	CH3CN	363 (+5.16), 307 (-9.68), 240 (+6.51), 221 (-7.78)
	MeOH	356 (+4.59), 300 (-8.61), 237 (+5.52), 218 (-5.30)



70



71

Exciton Chirality Method

The chiral interaction between two or more isolated but spatially close chromophores, which gives rise to Davydov-split Cotton effects[47,48] (exciton chirality method),[44-46] is one of the most reliable, nonempirical optical methods for studies of absolute configuration or conformation. Prerequisities for this method are: 1. the separation of two chromophores by at least one or two sigma bonds; 2. strong individual transition moments; 3. not too large a difference in transition energies; 4. a chiral arrangement of these two transition moments. If the direction of the transition moment in the chromophore is established, the signs of the two Cotton effects enable one to establish the absolute stereochemistry of the two chromophores in space. In other words, the absolute configuration of a compound can be determined by the sign of the Cotton effects on the basis of the chiral exciton coupling mechanism (Figure 4).

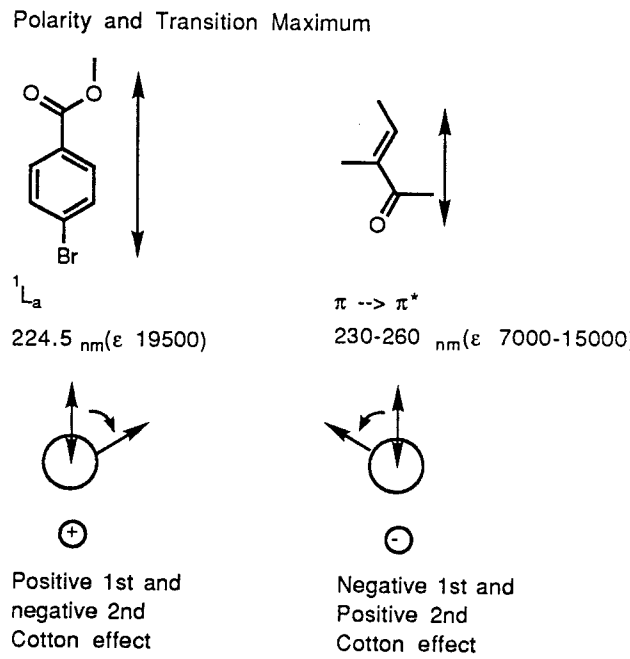


Figure 4. Definition of Exciton Chirality for a Binary System[45,46]

In order to apply the exciton chirality method, a second chromophore had to be introduced into **2**. Thus, the *p*-bromobenzoate of **2**, **72**, was prepared in 75% yield by treating **2** with *p*-bromobenzoyl chloride, triethylamine, and a catalytic amount of (dimethylamino)pyridine (DMAP) in tetrahydrofuran (THF). The *p*-bromobenzoates **73** and **74** were prepared in the same manner from **66** and **68** in 20% and 26% yield, respectively. The CD spectra of **72**, **73** and **74** in methanol were recorded as shown in Figure 5. In Table 6 are CD data for **72**, **73**, **74**, as well as the benzoate of (*S*)-2,6,6-trimethyl-4-hydroxylcyclohexen-2-one, **75**.[49] While **75** displayed a negative CD-couplet (ΔE_{max} = - 10.0 at 238, + 3.2 at 219), a positive CD-couplet was found (ΔE_{max} = + 1.90 at 286, - 9.20 at 244) for **72**, indicative of the opposite stereochemistry at C-4. The positive CD-couplet for **72** is consistent only with the configuration as shown, thus proving the absolute configuration at C-4 of **2** to be 4*S*. The same positive CD-couplet was displayed by the CD spectra of **73** (ΔE_{max} = + 0.55 at 280, - 3.79 at 252) and **74** (ΔE_{max} = + 1.46 at 274, - 1.70 at 240), which confirmed the biogenetic expectation that these compounds should have the same absolute stereochemistry as **2**.

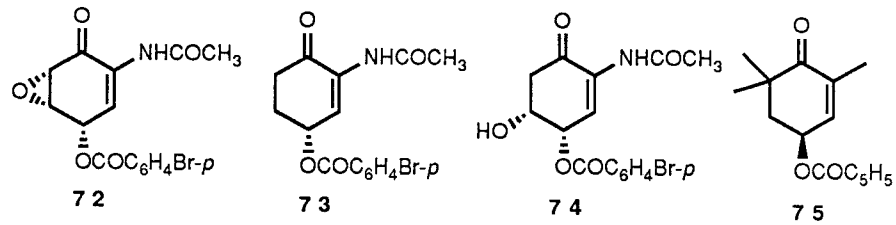


Table 6. CD-couplet Values of **72**, **73**, **74** and **75**

Compound	Solvent	λ_{max} (ΔE)	Chirality
72	MeOH	280 (+1.90), 244 (-9.20)	positive
73	MeOH	280 (+0.55), 252 (-3.79)	positive
74	MeOH	274 (+1.46), 240 (-1.70)	positive
75	MeOH	238 (-10.0), 219 (+3.2)	negative

Single Crystal X-ray Crystallography

While the application of the empirical "inverse quadrant" rule and the non-empirical exciton chirality method had resulted in the stereochemistry of **2** at the epoxide carbons and the carbinol center, respectively, we simultaneously prepared a number of urethanes of **2** with optically active isocyanates with the intention to establish the stereochemistry of **2** by X-ray crystallographic analysis. One of these, **76**, obtained by treating **2** with (*S*)-(α)-methylbenzylisocyanate in THF at reflux, was carefully recrystallized from toluene. This yielded a crystal suitable for X-ray analysis. The ORTEP drawing in Figure 6 clearly shows the 4*S*, 5*S*, 6*S*, 10*S* stereochemistry.

Numerous other naturally occurring epoxyquinones and epoxysemiquinones have been reported in the past thirty years, but in no case, until recently,[43] while our work was in progress, had the absolute stereochemistry of any of these been established by an unambiguous, non-empirical analysis. Absolute stereochemistry for the epoxide carbons had only been inferred from empirical correlations of the signs of Cotton effect in the CD spectra ("inverse quadrant" rule). With our work, and that of Scheuer's group, this rule may perhaps now be more reliably invoked.

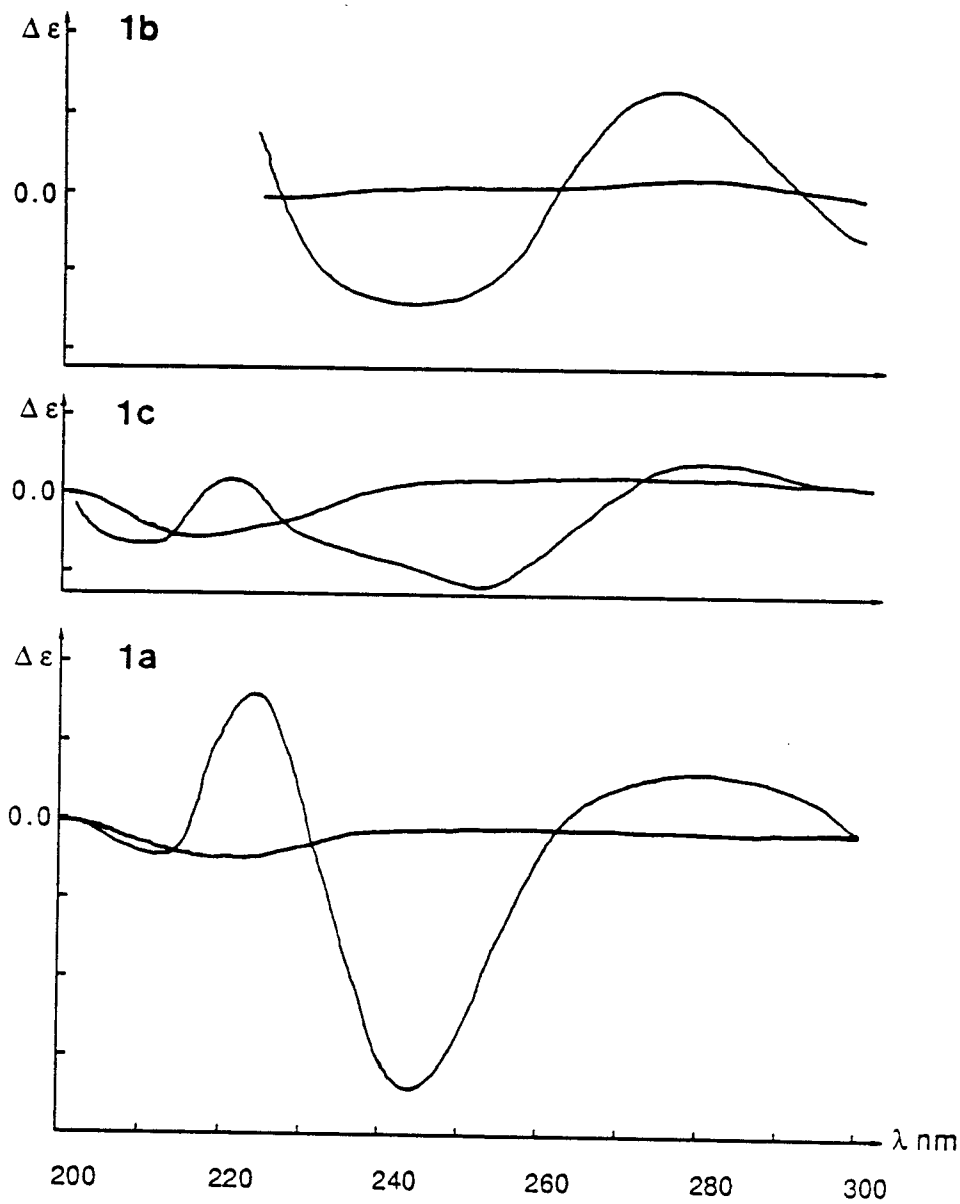
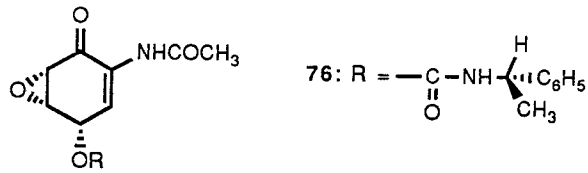


Figure 5. CD Spectra of **72** (a), **73** (b) and **74** (c) in Methanol



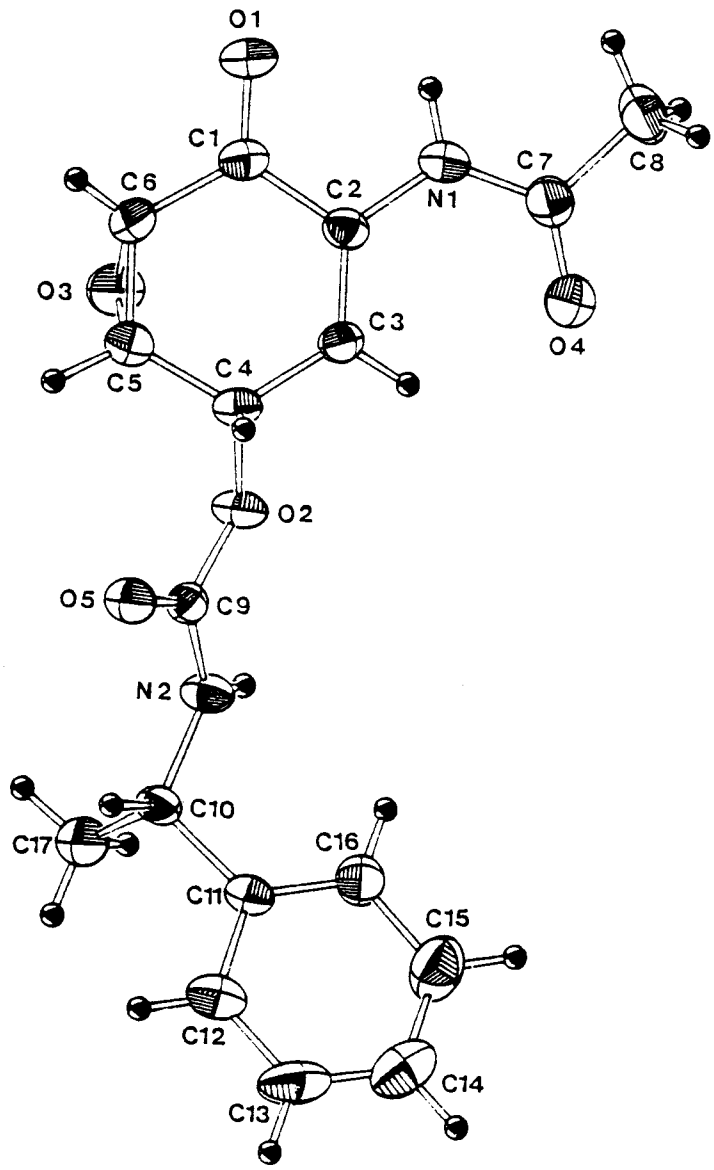


Figure 6. X-ray Crystal Structure of **76**

Absolute Stereochemistry of MM 14201, MT 35214 and MT 36531

During screening of soil cultures, **4** was discovered by Box and coworkers from *Streptomyces sp.* NCIB 11813.[24] It was found that **4** was very unstable and readily decomposed upon concentration. However, its N-acetyl derivative, **3**, was relatively stable and was isolated.[24] Remarkably, **3**, $[\alpha]^{20}_D$ +104° (c 1.0, MeOH),[24] has the same gross structure and relative stereochemistry as **2**, $[\alpha]^{20}_D$ - 202° (c 0.334, MeOH),[1] The difference in the sign of their specific rotations indicated that they may form an enantiomeric pair.[50] To provide further evidence in support of the enantiomeric relationship, **2** was oxidized to **70**, and its specific rotation was compared to that which was reported for the corresponding epoxyquinone MT 36531 **77**. The same trend of opposite sign of the specific rotations was observed. In this case the absolute magnitude of the two specific rotations was much closer, and $[\alpha]^{20}_D$ + 115.6° (c 0.5, MeOH) of **70** and $[\alpha]^{20}_D$ - 99° (c 0.5, MeOH) of **77**[24] confirmed them as enantiomers. Because the absolute stereochemistry of **2** has been established as shown,[23] **3** was then assigned to have the 4*R*, 5*R*, 6*R* absolute stereochemistry.

Experimental

General Procedures

^{1}H NMR spectra (400 MHz) and ^{13}C NMR spectra (100.6 MHz) were taken on a Bruker AM 400 spectrometer. All ^{13}C NMR spectra were broadband decoupled. Five-mm NMR tubes were used for all NMR measurements. ^{1}H and ^{13}C NMR samples were referenced with TMS or *t*-BuOH. IR spectra were recorded on a Nicolet 5DXB FTIR spectrometer. Low resolution mass spectra were taken on a Varian MAT CH-7 spectrometer. High resolution mass spectra were taken on a Kratos MS 50 TC spectrometer. Optical rotation was measured on a Perkin-Elmer model 243 polarimeter. UV spectra were recorded on an IBM 9420 UV-Visible spectrophotometer and CD spectra were recorded on a Durrum JASCO Model J-10 Circular Dichroism spectrometer. X-Ray crystal diffraction analysis was carried out on a Rigaku AFC6R diffractometer.

Melting points were taken on a Büchi melting point apparatus and are uncorrected. Flash chromatography was carried out on silica gel (EM Reagents, Keiselgel 60, 230-400 mesh). Silicar CC-4 was purchased from Mallinckrodt. Analytical thin layer chromatography (TLC) was carried out on precoated Keiselgel 60 F_{254} (either 0.2-mm aluminum sheets or 0.25-mm glass plates) and visualized by long and/or short wave UV. Anhydrous solvents were prepared by distillation over sodium or lithium aluminum hydride. S-(-)-methylbenzyl isocyanate was purchased from Aldrich.

Standard Culture Conditions

S. LL-C10037 was maintained at 4 °C as spores on sterile soil. A loopful of this material was used to inoculate 50 mL of seed medium containing 1.0% glucose, 2.0% soluble potato starch, 0.5% yeast, 0.5% N-Z Amine A 59027, and 0.1% $CaCO_3$ in glass distilled water, all adjusted to pH 7.2 with 2% KOH. The seed inoculum contained in a 250-mL Erlenmeyer flask, was incubated for 3 days at 28 °C, 200 rpm. Production broths (200 mL in 1-L Erlenmeyer flasks), consisting of 1.0% glucose, 0.5% bactopeptone, 2.0% molasses (Grandma's famous light unsulfured), and 0.1% $CaCO_3$ in glass distilled water and adjusted to pH 7.2 with 10% HCl prior to sterilization, were subsequently inoculated 5% (v/v) with vegetative inoculum from seed broths. The production broths were incubated for 120 h.

Isolation

As depicted in Figure 2, the fermentation mixture was filtered through cheesecloth and celite. The filtrate was adjusted to pH 4.7 with solid KH_2PO_4 saturated with $(NH_4)_2SO_4$, and extracted repeatedly with EtOAc (typically 10 times). After concentration *in vacuo*, the residue was dissolved in a minimum volume of methanol and adsorbed onto a small quantity of silica gel. This was applied to the top of a column of flash grade silica gel (25 g/200 mL fermentation) prepared in 40% hexane/EtOAc. After low polarity colored impurities had been eluted the solvent was changed to 20% hexane/EtOAc and elution yielded fractions containing **2**, and **66** and **67**, respectively. Once **2**, **66** and **67** were eluted, the solvent was changed to EtOAc and **68** was now eluted.

The fractions containing **2** were pooled and concentrated to yield the crude compound, which was recrystallized from methanol to yield crystalline **2**. The fractions containing **66** and **67** were pooled and concentrated to dryness. The residue was further separated by preparative silica gel TLC plates (2 mm, 20x20 cm) developed with $CHCl_3/CH_3OH/AcOH$ (92:7:1). The bands containing **66** and **67** were collected and eluted with EtOAc, respectively. The eluted EtOAc solutions were concentrated *in vacuo* to give **66** and **67**, each of which were recrystallized from EtOAc. The fractions containing **68** were pooled and concentrated to yield the crude compound, which was recrystallized from EtOAc-methanol.

LL-C10037γ (66)

Table 7. Physico-chemical Properties and Spectral Data of **66**

Appearance:	colorless flat crystals (from EtOAC)
m.p.:	122.5 - 123.5 °C
$[\alpha]^{22}_D$	+ 20.3 (c 0.249, MeOH)
Anal:	for $C_8H_{11}NO_3$
	Calcd: C 56.83 H 6.56 N 8.28
	Found: C 57.05 H 6.33 N 8.38
MS:	low-resolution (EI):
	169, 127, 126 (100), 110, 98, 82, 71, 70, 53
UV:	λ_{max} (ε): 265 (51500), 212 nm (71800)
IR (KBr):	3350, 3329, 1687, 1676, 1649, 1629, 1560, 1540, 1535, 1373, 1343 cm^{-1}

Table 8. ^1H NMR and ^{13}C NMR Assignments of **66**

Position	^{13}C NMR (100.6 MHz, methanol-*d*4) δ_c	mult.	^1H NMR (400 MHz, methanol-*d*4) δ_H	mult.	*J* (Hz)
1	194.9	s			
2	133.0	s			
3	134.9	d	7.65	dd	1.2, 2.9
4	66.5	d	4.60	ddd	8.8, 4.9, 2.9
5	32.7	t	2.27(e)	ddddd	17.0, 4.9, 4.8, 4.7, 1.2
			1.89(a)	dddd	17.0, 12.3, 8.8, 4.6
6	35.1	t	2.64(e)	ddd	17.0, 4.8, 4.6
			2.46(a)	ddd	17.0, 12.3, 4.7
1'	172.2	s			
2'	29.3	q	2.09	s	

Angelamide (67)

Table 9. Physico-chemical Properties and Spectral Data of **67**

Appearance:	colorless needle crystals (from EtOAc)		
m.p.:	129.5 - 130.5 °C (sealed capillary, sublimes at about 100 °C)		
$[\alpha]^{22}_D$	0		
Anal:	for C_5H_9NO		
	Calcd:	C 60.62	H 9.16 N 14.14
	Found:	C 60.37	H 9.24 N 14.45
MS:	low-resolution (EI): 99, 83, 82, 69, 56, 55(100), 54, 53, 51		
UV:	λ_{max} (ε): 204 nm (42900)		
IR (KBr):	3339, 3188, 3183, 3179, 1675, 1654, 1624, 1457		

Table 10. ^1H NMR and ^{13}C NMR Assignments of **67**

	^{13}C NMR (100.6 MHz, methanol-*d*4)		^1H NMR (400 MHz, methanol-*d*4)		
Position	δ_C	mult.	δ_H	mult.	*J* (Hz)
1	175.8	s			
2	133.9	s			
3	128.3	d	5.61	q q	7.0, 1.2
4	15.3	t	1.77	d q	6.7, 1.4
5	20.9	t	1.87	p	1.4

LL-C10037β (68)

Table 11. Physico-chemical Properties and Spectral Data of **68**

Appearance:	colorless flat crystals (from EtOAc-MeOH)		
m.p.:	160.5 - 161.5 °C		
$[\alpha]^{22}_D$	+ 26.3 (c 0.268, MeOH)		
Anal:	for $C_8H_{11}NO_4$		
	Calcd:	C 51.92	H 5.99 N 7.57
	Found:	C 51.93	H 5.95 N 7.51
MS:	low-resolution (EI): 185, 156, 144, 143, 125, 114, 96, 71, 70 (100)		
UV:	λ_{max} (ε): 265 (52700), 209 nm (81800)		
IR (KBr):	3431, 3427, 3355, 3311, 1670, 1664, 1656, 1650, 1533, 1529, 1375 cm^{-1}		

Table 12. ^{1}H NMR and ^{13}C NMR Assignments of **68**

Position	^{13}C NMR (100.6 MHz, methanol-*d*4) δ_C	mult.	^{1}H NMR (400 MHz, methanol-*d*4) δ_H	mult.	*J* (Hz)
1	193.7	s			
2	133.7	s			
3	129.8	d	7.51	dd	1.3, 3.8
4	68.6	d	4.59	dd	3.8, 3.4
5	70.1	d	4.18	dddd	6.5, 3.5, 3.4, 1.3
6	42.9	t	2.78(e)	dd	16.6, 6.5
			2.67(a)	dd	16.6, 3.5
1'	172.2	s			
2'	29.3	q	2.10	s	

***p*-Bromobenzoate of 2 (72)**

To a cold (0 °C) stirred solution of **2** (40.0 mg, 0.219 mmol) and triethylamine (24.3 mg, 0.24 mmol) in THF (2.0 mL), *p*-bromobenzoyl chloride (60.0 mg, 0.273 mmol) in THF (1.0 mL) was slowly added. DMAP (1.4 mg, 0.012 mmol) in THF (0.7 mL) was then added and the reaction solution was allowed to warm to room temperature. After 17h, the reaction was quenched with H_2O and extracted with $CHCl_3$ (5 x 3.0 mL). The combined organic solution was washed with H_2O (2 x 1.0 mL), dried over Na_2SO_4 and evaporated to give a white solid (85.0 mg). Recrystallization from hexane/EtOAc afforded **72** as colorless needles (70 mg, 75%): m.p. 155-157 °C; IR (KBr) 3290, 1738, 1682, 1528, 1312, 1265, 1085, 1013, 877, 752 cm^{-1}; UV max (ε) 201 nm (19600), 246 nm (14700); ^{1}H NMR (400 MHz, acetone-*d6*) δ 2.21 (s, 3H), 3.66 (d, 1H, *J* = 4.3 Hz), 4.13 (ddd, 1H, *J* = 2.6, 2.9, 4.3 Hz), 6.25 (dd, 1H, *J* = 2.9, 3.0 Hz), 7.40 (dd, 1H, *J* = 2.9, 3.0 Hz), 8.02, 8.13 (AA'BB', 4H, *J* = 8.7 Hz), 8.4 (bs, 1H); ^{13}C NMR (100.6 MHz, acetone-*d6*) δ 189.3, 170.3, 165.6, 132.9, 132.4, 131.2, 129.7, 128.9, 120.5, 66.8, 52.8, 52.1, 24.2; CD (CH_3OH) Δε 330 nm = - 2.49, Δε 280 nm = + 1.9, Δε 244 nm = - 9.2, Δε 223 nm = + 6.4; Low resolution mass spectrum (EI) 186.0, 185.0, 184.0, 183.0 (100%), 157.0, 155.0, 140.0, 124.0, 110.0, 43.0; Anal. calc. C 49.20, H 3.30, N 3.83, found C 49.23, H 3.06, N 3.75.

p-Bromobenzoate of 66 (73)

To a cold (0° C) stirred solution of **66** (28.0 mg, 0.167 mmol) in CH_2Cl_2 (5.0 mL), triethylamine (18.9 mg, 0.20 mmol), *p*-bromobenzoyl chloride (43.98 mg, 0.20 mmol) in THF (1.0 mL) and DMAP (1.0 mg) were added. The reaction solution was allowed to warm to room temperature. After 30h, the reaction mixture was cooled and quenched by adding crushed ice. The organic layer was separated and the remaining aqueous solution was extracted with CH_2Cl_2 (3 x 2.0 mL). The combined organic solution was washed with H_2O (3 x 1.0 mL) and dried over Na_2SO_4. The dried extract was concentrated *in vacuo* to afford a solid (64.0 mg) which was further purified on a silica gel 60 column (10 x 1.5 cm) eluting with 50% hexane/EtOAc to give partially pure **73** (240 mg). This material was chromatographed again with 25% hexane/EtOAc as eluting solvent to afford **73** as a colorless solid (12.0 mg, 20%): m.p. 96-99 °C; IR (KBr) 3349, 1718, 1684, 1667, 1591, 1508, 1323, 1282, 1289, 1245, 1118, 1105, 897, 847, 757 cm^{-1}; UV max (ε) 240 nm (30600), 246.8 nm(24800); ^1H NMR (400 MHz, acetone-*d6*) δ 2.11 (s, 3H), 2.2 (m, 1H), 2.5 (m, 1H), 2.64 (ddd, *J* = 4.7, 9.9, 17.3 Hz), 2.80 (ddd, 1H, *J* = 4.8, 7.0, 17.2 Hz), 5.92 (ddd, *J* = 3.8, 4.2, 7.6 Hz), 7.78 (d, 1H, *J* = 3.7 Hz), 7.72, 7.98 (AA'BB', 4H, *J* = 8.3 Hz), 8.39 (bs, 1H); ^{13}C NMR (100.6 MHz, acetone-*d6*) d 193.4, 169.9, 165.5, 134.8, 132.7, 132.2, 130.3, 128.5, 124.9, 69.7, 34.3, 34.0, 28.7; CD (CH_3OH) Δε 280 nm = + 0.55, Δε 252 nm = - 3.79, Δε 220 nm = + 2.01; High resolution mass spectrum (EI) calc. 351.01062, 353.00862, found 351.01040, 353.00860; Low resolution mass spectrum (EI) 353.0, 351.0, 311.0, 309.0, 202.0, 200.0, 185.0 (100%), 183.0, 167.8, 157.0, 155.0, 126.0, 110.0, 109.0, 43.0.

p-Bromobenzoate of 68 (74)

To a cold (0° C) stirred solution of **68** (43.0 mg, 0.232 mmol) and triethylamine (23.5 mg, 0.232 mmol) in THF (2.0 mL), *p*-bromobenzoyl chloride (51.0 mg, 0.232 mmol) in THF (1.0 mL) was slowly added. DMAP (1.0 mg) in THF (0.05 mL) was then added and the reaction solution was allowed to warm to room temperature. After 28h, the reaction mixture was cooled, diluted with CH_2Cl_2 (2.0 mL) and quenched by adding crushed ice. The organic layer was separated; the remaining aqueous solution was extracted with EtOAc (3 x 2.0 mL). The combined organic solution was dried over Na_2SO_4 and concentrated

in vacuo to afford a solid (94.0 mg), which was further purified on a Silicar CC-4 column eluting with 50% hexane/EtOAc to afford 74 as a glass (22.0 mg, 26%): IR (KBr) 3442, 3349, 1720, 1675, 1590, 1516, 1372, 1328, 1267, 1104, 1008, 7566 cm^{-1}; UV max (ε) 245 nm(17000); ^1H NMR (400 MHz, acetone-*d6*) δ 2.13 (s, 3H), 2.90 (dd, 1H, *J* = 7.8, 16.8 Hz), 2.96 (dd, 1H, *J* = 4.0, 16.8 Hz), 4.58 (1H, m), 4.71 (1H, d, *J* = 4.7 Hz), 5.99 (1H, dd, *J* = 3.5, 3.8 Hz), 7.60 (1H, bd, *J* = 3.8 Hz), 7.71, 8.04 (AA'BB', 4H, *J* = 8.4 Hz), 8.39 (bs, 1H); ^{13}C NMR (100.6 MHz, acetone-*d6*) δ 192.6, 169.9, 165.7, 135.1, 132.6, 132.4, 130.3, 128.5, 121.5, 72.4, 67.8, 42.9, 24.3; CD (CH_3OH) Δε 315 nm = - 0.97, Δε 274 nm = + 1.46, Δε 240 nm = - 1.70; Low resolution mass spectrum (EI) 202.0, 200.0 185.0, 183.0 (100%), 167.0, 157.0, 155.0, 125.0, 43.0.

(*S*)-(-)-α-Methylbenzylurethane of 2 (76)

To a dry 10 mL two-necked round-bottomed flask, equipped with a condenser and a septum cap, a solution of **2** (44 mg, 0.24 mmol) in THF (4.0 mL) and (*S*)-(-)-α-methylbenzyl isocyanate (35.4 mg, 0.24 mmol) were introduced via the septum cap. The resulting solution was heated at reflux under a nitrogen atmosphere for 24 h. The wine-colored reaction mixture was diluted with CH_2Cl_2 (20.0 mL) and decolorized with active charcoal. Filtration and concentration *in vacuo* yielded a residue that was recrystallized from CH_2Cl_2 to give **76** as fine white needles (76 mg, 96%): m.p. 173-175 °C; IR(KBr) 3339, 3320, 1692, 1534, 1373, 1243, 1063, 875, 702 cm^{-1}; UV_{max}(ε): 274 nm (43000), 218 nm(72000); ^1H NMR (400.13 MHz, $CDCl_3$) δ 7.9 (1H, s, exch.), 7.2-7.4 (6H, m), 6.0 (1H, d, *J* = 7.4 Hz, exch.), 5.8 (1H, m), 4.9 (1H, d, *J* = 7.2 Hz), 4.0 (1H, m), 3.6 (1H, d, *J* = 3.8 Hz), 2.1(3H, s), 1.5 (3H, d, *J* = 6.8 Hz); ^{13}C NMR δ (100.6 MHz, $CDCl_3$) 188.3, 168.0, 154.3, 142.9, 129.2, 128.8, 127.6, 125.9, 121.3, 66.5, 51.6, 51.4, 51.1, 24.6, 22.4; Anal. calc. C 61.81, H 5.49, N 8.48; found C 61.56, H 5.44, N 8.18; $[\alpha]_D^{20}$ = - 125.6 (c, 0.05 in MeOH).

2-Acetamido-5,6-epoxy-1,4-benzoquinone (77)

To a CH_2Cl_2 solution (50 mL) containing **2** (200 mg, 1.1 mmol) was added NaOAc (90 mg, 1.1 mmol) and PCC (355 mg, 1.65 mmol). The resulting solution was stirred at room temperature for 1.5 h. The brown reaction mixture was then filtered through a celite pad and the residue was washed with CH_2Cl_2. The

combined CH_2Cl_2 filtrate was concentrated *in vacuo* to give a brown residue, which was further purified on a silica gel column (1.5x15 cm) eluted with CH_2Cl_2. The fractions containing **77** were combined and concentrated *in vacuo* to provide a yellow solid, which was recrystallized from EtOAc to yield **77** as bright yellow crystals (100 mg, 50%): m.p. 135-136 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 7.89 (1H, bs), 7.51 (1H, d, J = 2.2 Hz), 3.91 (1H, d, J = 3.7 Hz), 3.83 (1H, dd, J = 3.7, 2.2 Hz), 2.22 (3H, s); $[\alpha]^{22}_D$ = + 115.6 (c, 0.5 in MeOH).

X-Ray work on $C_{17}NO_5H_{17}$ (76)

A crystal of dimensions 0.20 x 0.10 x 0.05 mm was used for collection of data. Unit cell parameters were refined from a least-squares analysis of the angle settings of 13 reflections in the range 22° < 27 < 35°. Intensity data were collected with the ω-2θ scan technique and a scan speed of 32° min^{-1} in ω. The intensities of three standard reflections monitored throughout the data collection exhibited an average fluctuation of 2.1%. From 1802 reflections measured to $(\sin \theta/\lambda)_{max}$ = 0.5947 $Å^{-1}$ with the range of indices $0 \leq h \leq 8$, $0 \leq k \leq 43$, and $0 \leq l \leq 5$, 1235 unique data having $F_o^2 \geq 3\sigma (F_o^2)$ were obtained.

All calculations were performed on a μVAX II computer with programs from the TEXRAY crystallographic software package.[51] Atomic positions for all nonhydrogen atoms were derived from the direct methods program MITHRIL.[52] Hydrogen atoms attached to C atoms were placed in calculated positions (C-H = 0.95 Å). Following two cycles of least-squares refinement, the remaining hydrogen atoms, H(8) and H(11), were located from a difference electron density map; the positional parameters and an isotropic thermal parameter were subsequently refined for each of these atoms. Final refinement on F_o with 224 variable, 1235 observations, and $F_o^2 > 3\sigma (F_o^2)$ afforded the residuals R = 0.038 and R_w = 0.049 where the weights were derived from counting statistics and a value of p = 0.05. In the final cycle Δ/σ = 0.01 and the maximum excursion in the final difference electron density map = 0.32 $eÅ^{-3}$. The data were not corrected for absorption.

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Chapter 3

In Vivo Studies on the Biosynthesis of LL-C10037α

Part of this work has been published.

Gould, S. J.; Shen, B.; Whittle, Y. G. *J. Am. Chem. Soc.* **1989**, *111*, 7932.

Methodology in *In Vivo* Studies

In spite of a number of revolutionary changes in experimental techniques which have been introduced in recent years, the basic approach to biosynthetic investigation has remained largely unaltered.[1,2] Essentially all natural products result from multistep pathways by which some substrate(s) of primary metabolism, be it an amino acid, sugar, or aliphatic acid, is converted by a series of discrete enzyme-catalyzed steps to complex metabolites. The initial stage of any biosynthetic problem is the identification of the early ("primary") precursor(s). Conceptually these precursors are often recognized by "comparative anatomy" in which the essential building blocks themselves are discerned within the final structure. Precursor-product relationships are often inferred from structural analogies to metabolites whose biogenetic origins are already known. Biogenetic reasoning by analogy, while in fact a common exercise, can nevertheless be misleading in some cases.

Eventually, precursor identification almost always requires the use of isotopic labeling. Classically such experiments have involved the preparation of appropriate precursors, substituted at one or more sites with an isotopic form of carbon, hydrogen, nitrogen, or oxygen, or in some instances phosphorus, sulfur or even chlorine. Such labeled precursors are then administered to the producing organism and the resulting labeled material is subsequently isolated for analysis. Until recently the vast majority of such experiments have been carried out with live organisms, whether intact plants, excised organs, or suspensions of whole cells. The externally adminstered precursor, therefore, must traverse one cellular barrier or more in order to reach the site of active biosynthesis where it can join the mainstream biosynthetic pathway along with *de novo*-produced intermediates. At the end of a suitable incubation period, metabolites of interest are isolated by standard methods and subjected to thorough purification. While various chromatographic procedures have often been used, when radioisotopes are employed the most reliable, if not completely infallible technique, involves recrystallization of the metabolite itself or a suitable derivative to constant specific radioactivity. When radioactive tracers are being used, these purification procedures are frequently expedited by the addition of an

unlabeled sample ("carrier") of the metabolite under study in order to provide sufficient mass for the multiple recrystallizations.

It is traditional to describe the effectiveness of a given precursor in terms of two different, although related, measures of incorporation efficiency. The first, termed the percent incorporation, refers to the percentage of externally administered precursor which is actually utilized in the biosynthesis of any given metabolite. This value is based on the ratio of total radioactivity in the purified product to the total radioactivity of the labeled precursor. Low incorporation values may reflect the efficiency or rate at which the externally added precursor is taken up by the cell, competition between anabolic and catabolic pathways, and incomplete conversion to final product, as well as simple mechanical losses in administration of the labeled precursor and isolation of the resulting metabolite. Percent incorporation values, therefore, are not by themselves a reliable measure of relative precursor effectiveness.

The second method of describing precursor utilization is often called specific incorporation and is a measure of the ultimate proportion of the product which is derived from the labeled precursor. Specific incorporation is the ratio of specific activity of the product to the specific activity of the labeled precursor. Specific incorporation values are most relevant for stable isotope incorporation experiments since they indicate what proportion of the product is actually labeled, as distinguished from how much of total label has been recovered. The measured specific incorporation is a function not only of how much precursor is utilized but also the size of endogenous pools of precursors, intermediates, and products, and the relative proportions of prior, concurrent, and subsequent biosynthesis from unlabeled endogenous substrate. An alternative method of expressing specific incorporation is the dilution value, which is the simple inverse of the specific incorporation and indicates the extent to which the precursor has been diluted by endogenous substrates in the final product. It is also convenient to distinguish between specific incorporation and enrichment. The latter term refers to the percentage of (usually stable) isotope at each site and is derived either from direct measurement or from the specific incorporation by correction for the number of labeled sites in the product as well as the isotopic enrichment of the precursor.

Reliable evaluation of precursor effectiveness always requires some set of criteria more rigorous than simple measurement of total or specific incorporation. The most important one of these criteria is based on the unambiguous determination of the sites and relative distribution of isotopic label in the product. The manner in which such determinations are made depends directly on the isotope which has been employed. The commonly used radioisotopes (^{3}H, ^{14}C, ^{32}P) confer no useful physical property on any chemical sample in which they are present other than radioactivity detectable as emitted β or γ particles of energies characteristic of each isotope. Simple measurement of radioactivity, even in a presumably rigorously purified sample conveys no direct information as to the molecular location of such isotopic labels. In order to establish rigorously the position of isotopic labeling, each sample must be subjected to a sequence of chemical degradations and the relative activities of each derived fragment determined.

Most radioisotopic labels are used at only trace levels. By employing stable isotopes, however, it becomes possible to achieve substrate enrichments approaching 100%, thereby allowing the use of two additional analytical tools: mass spectrometry and nuclear magnetic resonance (NMR) spectrometry. The first of these methods is based on the fact that the commonly used stable isotopes (^{2}H, ^{13}C, ^{15}N, ^{17}O, ^{18}O) have atomic weights one or two mass units higher than the normal isotopic forms which they replace. The mass spectrum corresponding to a given labeled substrate will exhibit molecular ion peaks and fragments of increased m/e in proportion to the relative quantities of each isotope in the labeled sample. NMR has been by far the more powerful technique.[3,4] The NMR method is based on the fact that certain stable isotopes (^{2}H, ^{13}C, ^{15}N, as well as ^{3}H) have characteristic nuclear magnetic resonances which result from their net nuclear spin. The advantages of NMR methodology for biosynthetic studies are enormous. Besides obviating the need for lengthy degradation procedures, NMR can be used to monitor the time course of biosynthetic transformations. Such dynamic, non-invasive techniques not only allow the measurement of metabolic flux, but in some instances can can also lead to the recognition and preliminary characterization of previously undetected intermediates. The combination of stable isotope NMR and multinuclear labeling can be used to detect the making and breaking of carbon-carbon and carbon-heteroatom bonds and to distinguish between

inter- and intramolecular structural reorganizations, experiments[4] which would be difficult, if not impossible, using trace-level radioisotopes.

The Use of Deuterium in Biosynthesis

Deuterium has proven invaluable in its use in the study of chemical and biological processes.[5] Although its use in kinetic isotope effect studies has been well documented, its magnetic properties are generally of greater importance to the biosynthetic investigators.[6,7]

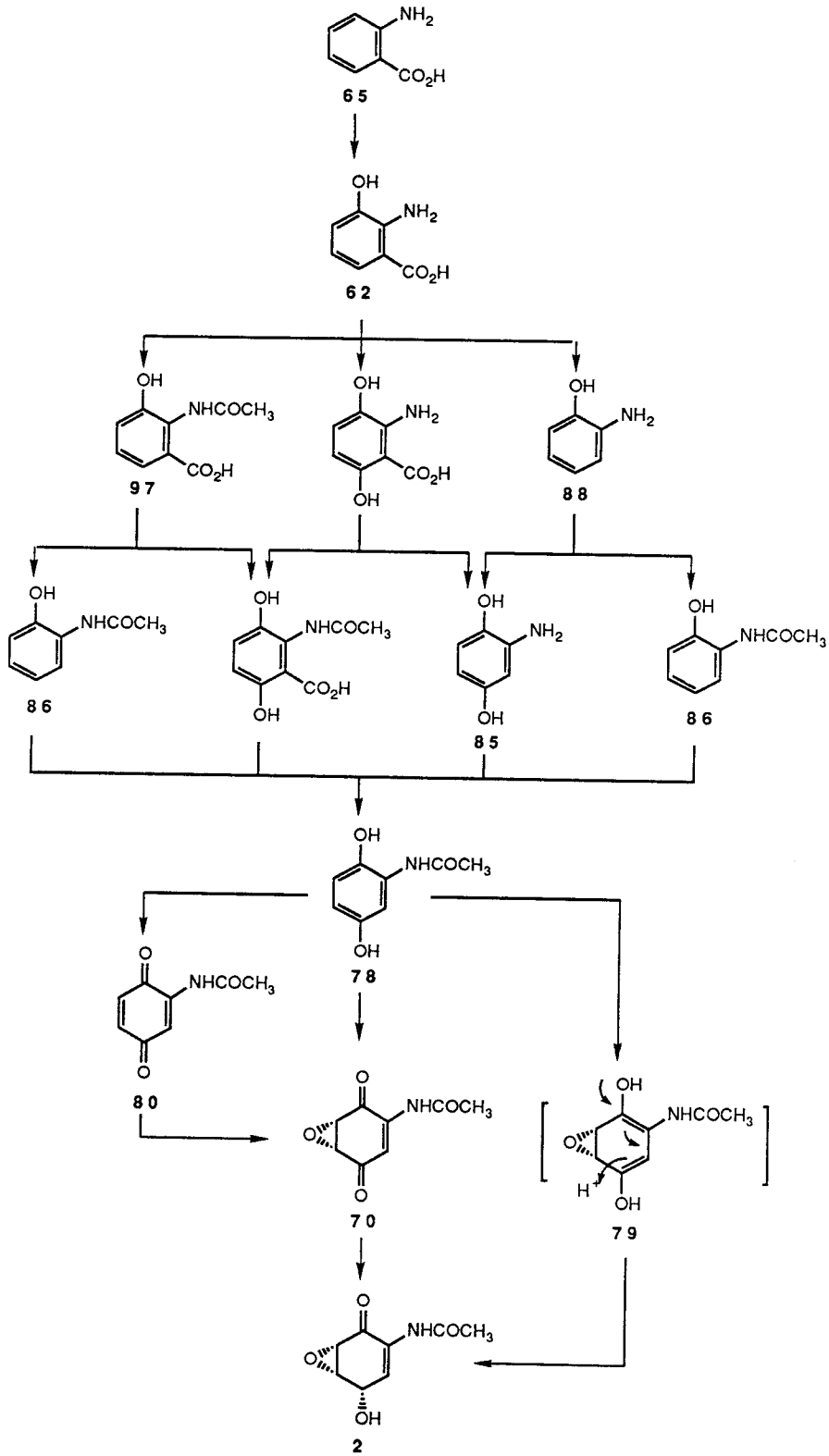
The magnetic properties of deuterium and the advantages and drawbacks of ^{2}H NMR spectroscopy in biosynthetic applications are extensively discussed in a review by Garson and Staunton.[3] Briefly, the major disadvantages are the inherently low sensitivity for detection of nuclear magnetic resonance (relative to ^{1}H or ^{13}C), the poor dispersion of chemical shifts (the range, in Hz, is about 15% that of ^{1}H NMR), and broad signals (due to the nuclear quadrupole of ^{2}H). However, deuterium is inexpensive and non-radioactive. Its low natural abundance (0.0156%) allows very low levels of labelling to be detected, while compounds commercially available that are enriched with >99 atom % ^{2}H make both the introduction and detection of ^{2}H labels much easier. The short relaxation times of deuterium and the lack of problems arising from NOE permit the ^{2}H content to be determined accurately by integration.[3] With the advent of high-field NMR spectrometers there has been considerable interest in the analysis of variations in the distribution of deuterium in samples that have been obtained from different sources and in which the deuterium is present at its natural abundance.[8-10] Since ^{2}H chemical shifts (in ppm) correspond very closely to ^{1}H chemical shifts, a complete assignment of the ^{1}H NMR spectrum allows deuterium resonances to be identified provided that any overlap of these broader signals does not interfere. Usually, such problems of overlap can be overcome by chemical transformation into a derivative that has better spectral properties, by using lanthanide shift reagents, or by using two-dimensional NMR methods. The detection of ^{2}H through isotope shift induced in ^{13}C NMR spectra,[3,4] and by mass spectrometry [11] are also widely utilized.

Biogenesis of LL-C10037α

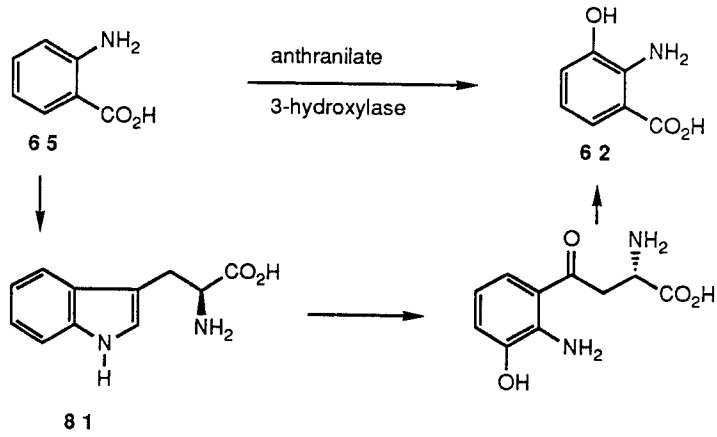
Gould and Whittle had suggested that **2** was derived via the shikimic acid pathway with **62** as the key intermediate.[12] The biosynthesis of **62** could be a process of direct hydroxylation of **65**,[13] or via trytophan degradation.[14] The subsequent metabolism of **62** implied by the structure of **2** required minimally oxidation at C-6, decarboxylation at C-1, acetylation at the nitrogen atom and epoxidation at C-4/C-5. Scheme 12 outlines these possibilities. The direct epoxidation of the hydroquinone **78**, followed by tautomerization of the arene oxide intermediate **79**, could yield **2**. The direct epoxidation of a hydroquinone to form an arene epoxide such as **79** would be consistent either with the mechanism for the hydroxylation of aromatic compounds,[15-18] or with the mechanism for the epoxidation of olefins.[19-23] Alternatively, both **78** and the corresponding acetamido-1,4-benzoquinone, **80**, could be epoxidized to form **70**, which could be further reduced to **2**. The inclusion of this additional redox chemistry would be consistent with the known --- but extremely inefficient --- chemical oxidation of **80**,[24] and the dehydrogenase-catalyzed reversible reaction between epoxyquinone and epoxysemiquinone.[25] Epoxidation of a hydroquinone or a quinone to yield an epoxyquinone moiety has been postulated in the biosynthesis of other epoxyquinones and epoxysemiquinones as well.[26-29]

The Origin of 3-Hydroxyanthranilic Acid

The biosynthesis of **62** from **65** has been well documented in the literature.[13,14] As shown in Scheme 13, **62** could be the product of the direct hydroxylation of **65**. Two enzymes have been reported to be associated with this hydroxylation,[30-35] anthranilate 3-hydroxylase and anthranilate 2,3-hydroxylase (deaminating), which formed **62** in addition to the main product 2,3-dihydroxybenzoic acid. However, this kind of enzyme is very rare in microorganisms and has not been found in *Streptomyces sp.* to date. Alternatively, **62** could be biosynthesized from **65** via tryptophan, **81**, degradation. This process is more common than the direct hydrolysis of **65** and can be tested by feeding **81**.



Scheme 12. Potential Biogenetic Pathways to **2**



Scheme 13. Biosynthesis of **62** from **65**

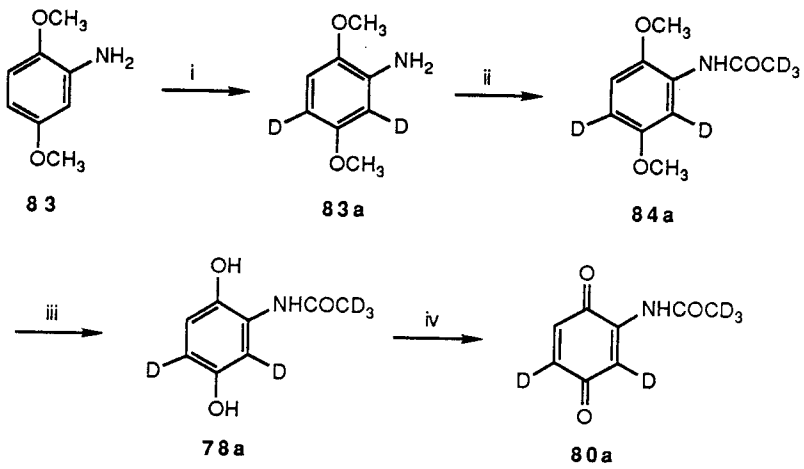
[5-^{2}H]Tryptophan, **81a**, was synthesized from 5-bromotryptophan, **82**, by Pd-C catalyzed hydrogenolysis under an atmosphere of D_2 in 100% yield. To a 200-mL fermentation broth of *S.* LL-C10037, four days after inoculation of the broth, 10 mg of **81a** were fed in pulse fashion (see Table 14). After a total of 120 hours, the fermentations were worked up and the product, **2a**, was analyzed by ^{2}H NMR. No significant incorporation of **81** into **2** was observed. In order to rule out the possibility that the level of **81a** fed was too low to label **2**, 50 mg of **81a** was fed again to a 200-mL fermentation broth under the same conditions. As expected, normal amount of **2a** was produced but ^{2}H NMR analysis of **2a** showed no incorporation of **81** into **2**, suggesting that **62** was not derived via degradation of **81**. Therefore, consistent with the low but specific incorporation of [^{15}N]**65** into **2**[12b] an anthranilate 3-hydroxylase may be involved in the hydroxylation of **65**.

Conversion from 3-Hydroxyanthranilic Acid to 2,5-Dihydroxyacetanilide

2,5-Dihydroxyacetanilide

Having obtained evidence for the direct hydroxylation of **65** to **62**, **78** was viewed as a likely key intermediate from **64** to **2** as shown in Scheme 12. [2',2',2',4,6-$^2H_5$]**78a** had been chosen as the precursor to test this hypothesis. This particular labeling pattern had been used so that the deuterium

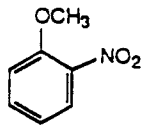
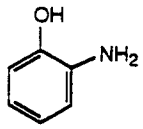
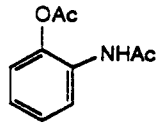
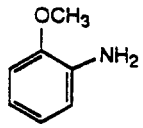
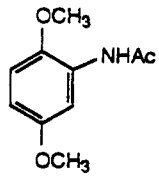
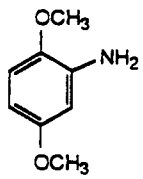
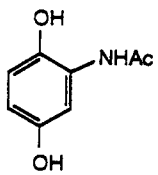
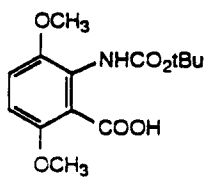
enrichments on the carbocyclic ring and on the acetamide moiety could reflect the intact incorporation of **78** into **2**. Thus, [2',2',2',4,6-$^2H_5$]**78a** was synthesized from 2,5-dimethoxyaniline, **8**, as shown in Scheme 14.[36] This approach was chosen after an investigation of the exchangeability of various nitrogen-substituted aromatic compounds under neutral and acidic conditions. Table 13 summarizes these results. In the event, exchange of **83** with deuterated trifluoroacetic acid at 80 °C led to 90% deuteration at H-4 and H-6, with quantitative mass recovery. This was then acetylated with [$^2H_3$]acetyl chloride in the presence of triethylamine to give the acetamide **84a** in 92% yield. A variety of reagents were examined for the demethylation of **84a**. Neither trimethylsilyl iodide, purchased or generated *in situ*,[37-39] lithium thiomethoxide,[40] nor cerric ammonium nitrate[41] gave a clean reaction. However, boron tribromide[42,43] gave **78a** in 83% yield; some loss of deuterium occurred in this step. Thus the final enrichments at H-4 and H-6 were 60 and 89%, respectively. This compound was prone to oxidation, but could be purified by careful recrystallization.



i: D_2O/TFA-d_1/MeOD/80 °C; ii: $CD_3COCl/Et_3N/CH_2Cl_2$;
iii: BBr_3/CH_2Cl_2, -78 °C to r.t.; iv: PbO_2/CH_2Cl_2-Et_2O.

Scheme 14. Synthesis of **78a** from **83**

Table 13. Hydrogen Exchange of Some Nitrogen/Oxygen-Substituted Benzenes under Neutral and Acidic Conditions

Substrate	CH_3OD/D_2O r.t./24 h	$CH_3OD/D_2O/TFA\text{-}d_1$ a,b,c,d	4M DCl 110 °C	3M DCl 80 °C
	NR	NR (a,b)	-	-
	NR	NR (a,b)	-	-
	NR	NR (a,b)	-	-
	NR	NR (a) H-4, H-6 (d)	H-4, H-6 0.5 h	
	NR	NR (a,b)		
	NR	H-4 (a) H-4, H-6 (c)		
	-	-	-	H-4, H-6 0.5h
	-	NR (d)		

a. r.t./24 h; b. 60 °C/24 h; c. 80 °C/12 h; d. 80 °C/48 h.

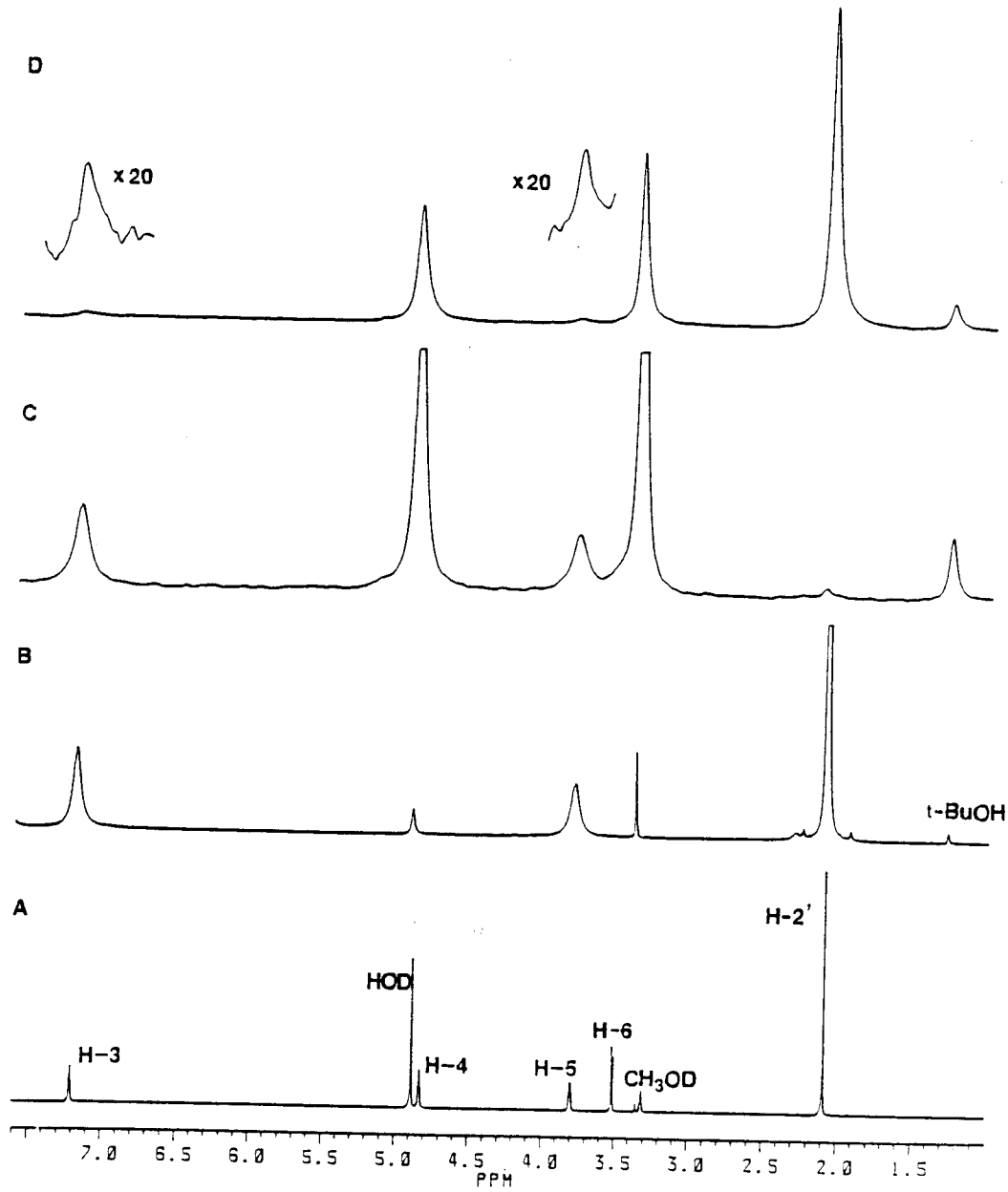
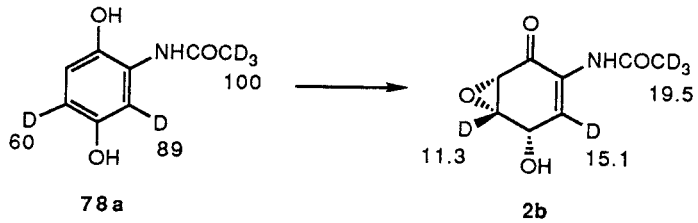


Figure 7. 400 MHz ^{1}H NMR Spectrum and 61.4 MHz ^{2}H NMR Spectra of **2**. A. ^{1}H NMR spectrum of **2** in methanol-d_4. B. ^{2}H NMR spectrum of **2b** in methanol with *t*-BuOH for chemical shift reference. C. ^{2}H NMR spectrum of **2c**. D. ^{2}H NMR spectrum of **2D**.

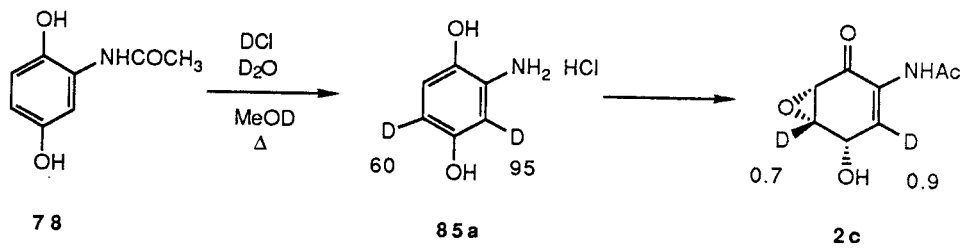
A portion of **78a** (100 mg) was fed to a 200-mL fermentation broth of *S.* LL-C10037 four days after inoculation of the production broth. After an additional day the fermentation was worked up and the product, **2b**, was analyzed by ^{2}H NMR spectroscopy (Figure 7B).[44] The resonances for deuterium at C-3, C-5 and the methyl group were readily observed, and the relative deuterium enrichments (indicated on the structure) reflected those of the material fed. Thus, the hydroquinone **78** was specifically and effectively (9.1%) incorporated into **2** (Scheme 15).



Scheme 15. Incorporation of **78** into **2**

2,5-Dihydroxyaniline

With the role of **78** established, it was necessary to determine the sequence of the first three reactions --- acetylation, decarboxylation and hydroxylation --- from **62**. When **78a** was hydrolyzed with aqueous HCl at reflux, 2,5-dihydroxyaniline, **85**, was obtained devoid of deuterium. Therefore, **78** was hydrolyzed in DCl/D_2O at reflux and yielded the hydrochloride salt [4,6-$^{2}H_2$]**85a**. Whereas the free base was very unstable,[45] the salt was relatively stable and was purified.

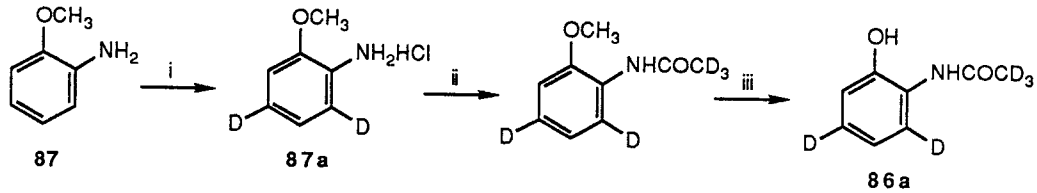


Scheme 16. Synthesis of **85a** and Its Incorporation into **2**

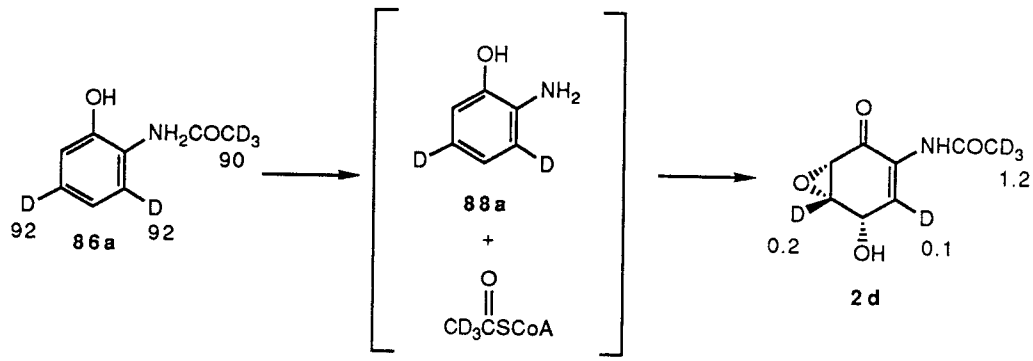
[4,6-$^2H_2$]**85a** was then fed to a 400-mL fermentation broth under the standard conditions (as those used for the feeding of **78a**), and the sample obtained, **2c**, was enriched in deuterium at C-3 and C-5 to the extent of 0.9% and 0.7%, respectively, as determined by ^2H NMR (Scheme 16 and Figure 7C). The incorporation of **85a** into **2c** was found to be 0.5%.

2-Hydroxyacetanilide

The specific incorporation of **85** suggested the acetylation as the last step among the three steps from **62** to **78**. However, while acetylation could occur at numerous points, one would have speculated that it would take place prior to the hydroxylation so that the reactivity of the benzene ring with three heteroatoms could be attenuated by the presence of an acetamide functionality. From this perspective, [2',2',2',4,6-$^2H_5$]-2-hydroxyacetanilide, **86a**, was next prepared as shown in Scheme 17. An 82% recovery of the exchanged **87a** was obtained from 2-methoxyaniline, **87**, and this was acetylated and demethylated to **86a** in 71% overall yield. After feeding 100 mg of **86a** to 400 mL fermentation broth under the standard conditions, the bulk of this material (89 mg) was recovered during the workup, which also yielded a normal quantity of **2d** (45 mg). Examination of the ^2H NMR spectrum of this sample (Figure 7D) revealed deuterium enrichments at all three sites; however, the relative enrichments did not reflect those of the material fed. Thus, enrichment of H-3 was approximately half that of H-5, whereas the methyl group was at least 10 times as enriched. The much higher labeling of the methyl group was taken to indicate that **86a** had not been incorporated intact but had first undergone deacetylation to [4,6-$^2H_2$]-2-hydroxyaniline, **88a**, and --- presumably --- [$^2H_3$]acetylCoA (Scheme 18). The latter was apparently then more efficiently incorporated into **2**. The fact that **86** was incorporated with hydrolysis of the acetyl moiety eliminated the possibility of acetylation prior to hydroxylation in the conversion from **62** to **78**, consistent with the feeding of **85a**. The lower enrichment at H-3 of **2** could be explained if it were associated with the hydroxylation of the aromatic ring, possibly involving an NIH shift.[15-17] On the basis of unrecovered **86a**, a 0.54% incorporation of the ring and a 6.4% incorporation of the acetyl residue was obtained.



Scheme 17. Synthesis of **86a** from **87**



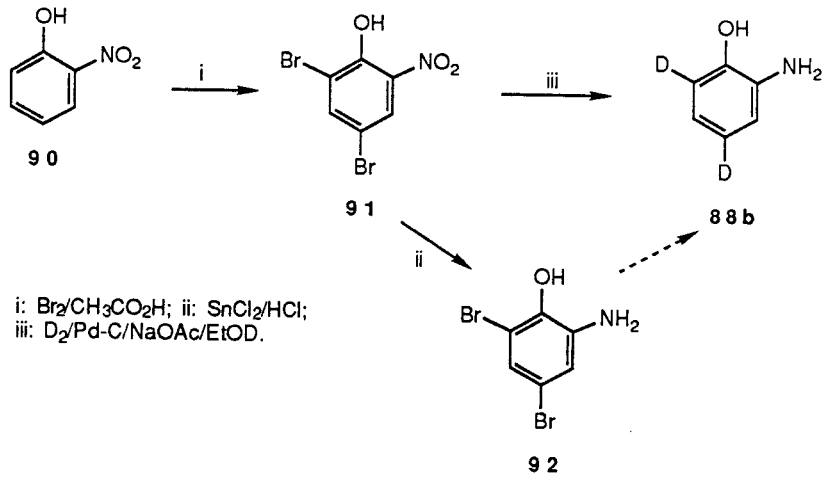
Scheme 18. Incorporation of **86** into **2** with Hydrolysis of the Acetyl Group

2-Hydroxyaniline

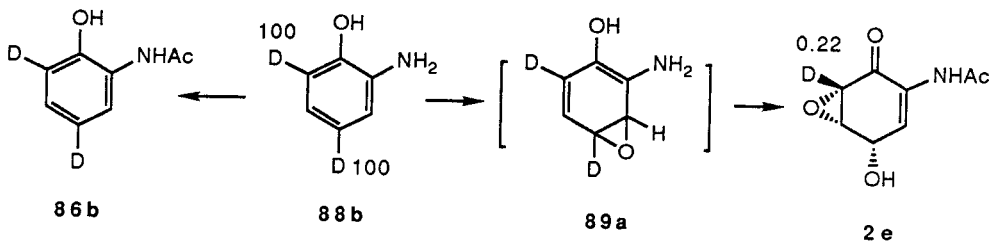
In order to test 2-hydroxyaniline, **88**, as an intermediate and to investigate the mechanism of the hydroxylation, presumably via an arene oxide **89** with an NIH shift, [3,5-$^2H_2$]**88b** was prepared as shown in Scheme 19. Since exchange of **87** had yielded deuterium at H-4 and H-6, catalytic dehalogenation[46-50] of a 3,5-dibromide was now used. Thus, 2-nitrophenol, **90**, was brominated to the dibromide **91** in 76% yield.[51] This was first reduced with stannous chloride[52] in HCl to give the aniline derivative **92** in 56% yield, with plans to then hydrogenolyze the bromines catalytically. However, it was found that direct reduction of **91**, using Pd-C as catalyst in the presence of sodium acetate, gave **88b** in 64% yield. Again, 100 mg of the substrate was fed to a 400-mL fermentation broth under the standard conditions. In this case, 27 mg of

the labeled **2e** was isolated. In addition, 98 mg of the acetamide **86b** (corresponding to 71 mg of **88b**) was isolated from this experiment!

A 0.22% enrichment at C-6 was obtained in **2e**, confirming **88** as an intermediate. On the basis of the 29 mg of **88b** not recovered, a 0.13% incorporation was obtained. However, the deuterium enrichment at C-3, which could result from the NIH shift via an arene oxide **89**, was ambiguous due to the extremely low incorporation of **88**. Although exceptions have been reported,[53,54] the second hydroxylation of an aromatic ring is usually accompanied with complete loss of the ring hydrogen[15-17] (Scheme 20).



Scheme 19. Synthesis of **88b** from **90**



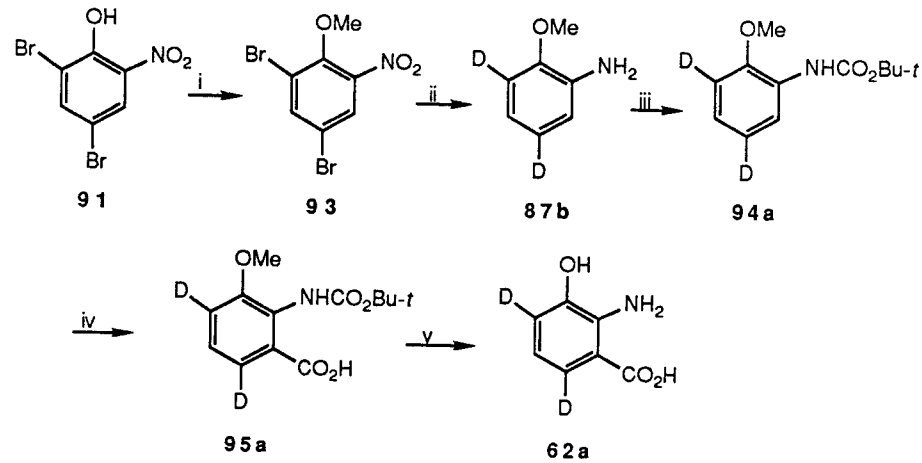
Scheme 20. Incorporation of **88b** into **2**

The results obtained with **86a** and **88b** are remarkable. Apparently **86** is not an intermediate and is not readily taken up by this organism. However, what does get into the cell is efficiently deacetylated to **88**, which is an

intermediate. When cells are presented with a high concentration of **88**, production of **2** seems to be substantially decreased and the bulk of the aniline is acetylated and excreted. This would be consistent with the aniline being toxic at high concentrations; numerous *Streptomyces* protect themselves from their own[55-61] and from others'[62-66] antibiotics by transacetylation. Other microorganisms also use transacetylation for antibiotic resistance.[67-69]

3-Hydroxyanthranilic Acid

The question of whether an NIH shift was involved in the hydroxylation of **88** remained unsolved. However, if it were possible to specifically label **62** in such a fashion that its incorporation would reveal mechanistic details of the conversion of **62** to **78**, it might be possible to establish the mechanism for the hydroxylation without suffering from poor incorporation found with **88b**, since **62** has been very effectively incorporated.[12] From this consideration, [4,6-$^2H_2$]**62a** was chosen as the probe, in which the deuterium at C-4 could be used as an internal standard to establish the level of incorporation while the deuterium at C-6 would function as a marker to indicate the NIH shift.

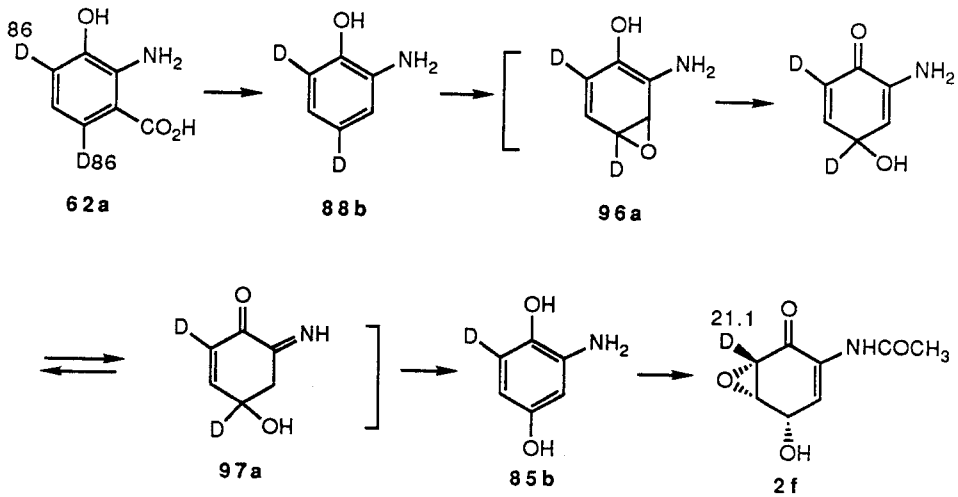


i. MeI/Acetone, ii. D_2/Pd-C, iii. $(t\text{-BuOCO})_2O$/THF, iv. t-BuLi/THF/CO_2, v. BBr_3/CCl_3

Scheme 21. Synthesis of **62a** from **91**

Scheme 21 shows the synthesis of **62a**. Thus, **91** was first protected as the methyl ether **93** (81% yield) and then reduced under an atmosphere of D_2

catalyzed by Pd-C to yield [4,6-$^2H_2$]-2-aminoanisol, **87b**, in 96% yield. The amino group of **87b** was protected as *t*-butyl carbamate, **94a**, in 85% yield. Directed by the amide nitrogen, **94a** was regioselectively lithiated and then reacted with CO_2 to yield protected 3-hydroxyanthranilic acid, **95a**, in 44-65% yield.[70-75] Demethylation with boron tribromide using an aqueous workup to hydrolyze the *t*-butoxylcarbonyl group, resulted in **95a** being converted to **62a** in 26-75% yield. The poor yield of the last step resulted mainly from the difficulty of the purification of **62**.



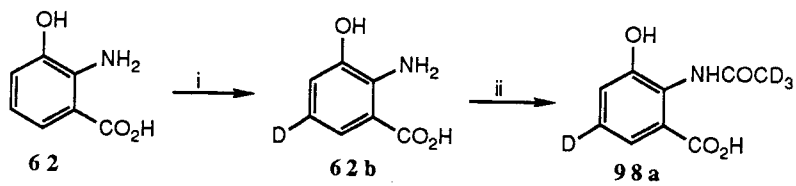
Scheme 22. Incorporation of **62a** into **2**

A portion of **62a** (35 mg) was fed to a 200-mL fermentation broth under standard conditions, and the sample obtained, **2f**, was analyzed by ^2H NMR spectroscopy. A 21.1% enrichment at C-6 clearly demonstrated the effective incorporation of **62a** (17.8% incorporation). No deuterium was observed at C-3, which was expected to be labeled if an NIH shift had occurred, but a small amount of deuterium was observed at C-5.[15-17] Therefore, it was concluded that no NIH shift was involved in the hydroxylation of **88**. The lower enrichment at H-3 of **2d**, derived from the feeding of **86a** could be explained by the formation of arene oxide **96**, which was converted to **85b** with total loss of D-4 as in the literature and partial loss of H-3 --- presumably via the formation of an imine intermediate **97a** (Scheme 22). The additional low deuterium enrichment observed at C-5 of **2f** was confusing at first glance. However, it was subsequently confirmed that deuterium scrambling occurred

during the synthesis of **62a** and the deuterium at C-5 of **2f** resulted directly from that at C-5 of **62a**.

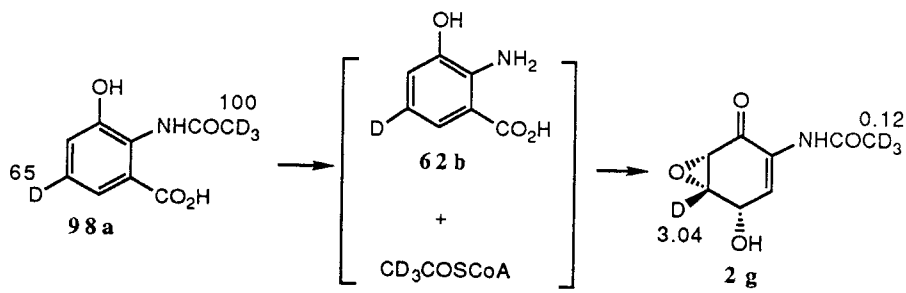
N-Acetyl-3-Hydroxyanthranilic Acid

The specific incorporation of **85a** has established the acetylation as the last step; the feeding data for **86a**, **88b** and **62a** led to the sequence prior to acetylation as decarboxylation --> hydroxylation. According to this sequence, N-acetyl-3-hydroxyanthranilic acid, **98**, resulted from direct acetylation of **62**, is not an intermediate. To test this deduction, [2',2',2',5-$^2H_4$]-N-acetyl-3-hydroxyanthranilic acid, **98a**, was next prepared as shown in Scheme 23. Thus, exchange of **62** with deuterated trifluoroacetic acid at 80 °C resulted in [5-^2H]-3-hydroxyanthranilic acid, **62b**, which was then acetylated with [$^2H_3$]acetic anhydride in the presence of K_2CO_3 to yield **98a** in a 79% overall yield.



i. TFA-*d1*, ii.$(CD_3CO)_2O/K_2CO_3$

Scheme 23. Synthesis of **98a** from **62**



Scheme 24. Incorporation of **98a** into **2**

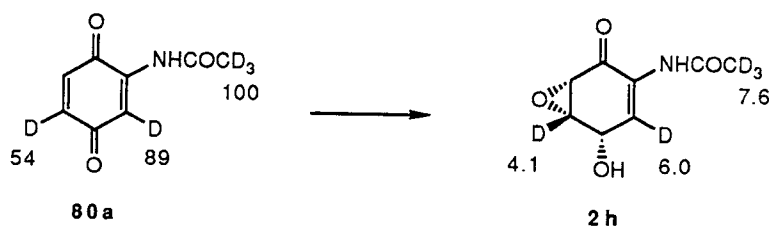
A portion of **98a** (100 mg) was fed to a 400-mL fermentation broth under standard conditions. In this case, 50 mg of the labeled **2g** was obtained. The

deuterium enrichments of 3.04% at C-5 and 0.12% at C-2' were easily observed by ^2H NMR spectroscopy. However, the relative enrichments at the two positions did not reflect those of the material fed, indicating that **98** was not incorporated intact into **2**. Thus, as shown in Scheme 24, **98** was first deacetylated *in situ*, to **62** which was then very effectively incorporated into **2**. The incorporations of the ring and the acetyl moiety had, therefore, been 2.5% and 0.1%, respectively. These feeding data confirmed that **98** was not an intermediate in the conversion of **62** to **78**, further supporting the established biosynthetic sequence: decarboxylation --> hydroxylation --> acetylation.

Conversion from 2,5-Dihydroxyacetanilide to LL-C10037α

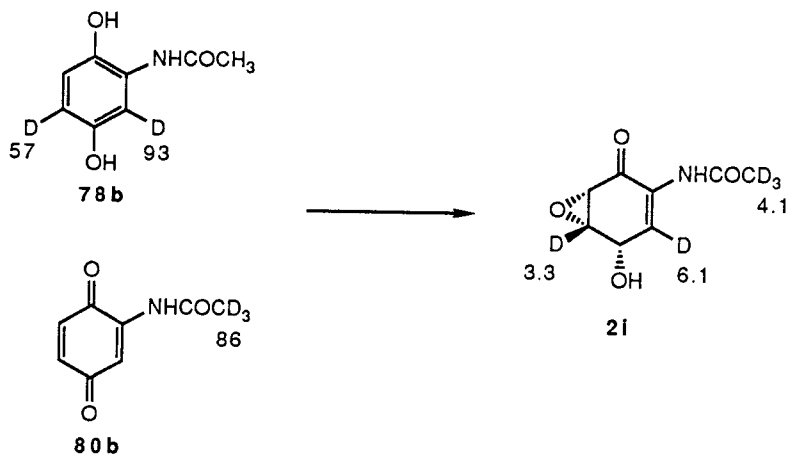
Acetamido-1,4-benzoquinone

[2',2',2',3,5-$^2H_5$]**80a** was prepared from the hydroquinone **78a** by oxidation with either lead dioxide[76] in 86% yield or $K_2Cr_2O_7/H_2SO_4$[77] in 94% yield (Scheme 13). A portion of **80a** (100 mg) was fed to a 400-mL fermentation broth under standard conditions and yielded **2h**. In this case, as with the sample derived from **78a**, H-3, H-5, and the methyl group were labeled with deuterium in proportion to the material fed, although the total incorporation was only 1.6% (Scheme 25). The different levels of incorporation for **78a** (9.1%) and **80a** (1.6%), which were obtained from separate experiments, could not be taken as an indication of their relative position in the pathway. It is very dangerous to assume that separate experiments of feeding to microorganism are carried out under identical conditions.



Scheme 25. Incorporation of **80a** into **2**

Since the incorporation of **80a** could have been due to the fortuitous action of an available dehydrogenase, a direct competition was established by feeding equimolar amounts of [4,6-$^2H_2$]**78b** and [2',2',2'-$^2H_3$]**80b** to the same 400-mL fermentation broth under the standard conditions. In this case, **2i**, actually representing two subpopulations of labeled **2**, was obtained with nearly equal enrichments at all three sites (10.2% incorporation of **78b** and 7.9% incorporation of **80b**) (Scheme 26). This established the ease of redox equilibration between **78** and **80** without, unfortunately, revealing **80** as an obligatory intermediate or its correct relationship to the pathway if it was on the pathway. Such experiments emphasize the variability in biological systems and the caution that must be exercised when analyzing incorporation figures from separate experiments. The incorporation data from separate experiments (9.1% for **78** and 1.6% for **80**) could very easily mislead one to the conclusion that **78** was more efficiently incorporated than **80**; the direct competition feeding demonstrated that **78** (10.2%) and **80** (7.9%) were almost equally effectively incorporated.

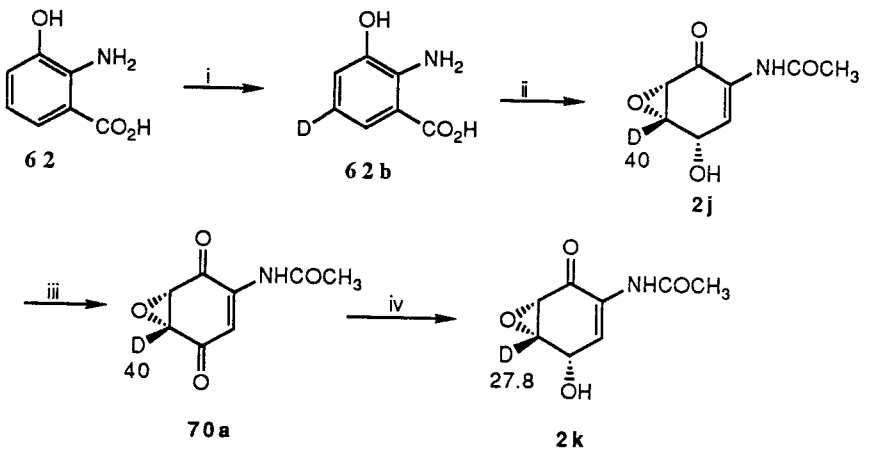


Scheme 26. Competitive Incorporation of **78b** and **80b** into **2**

2-Acetamido-5,6-epoxy-1,4-benzoquinone

As shown in Scheme 12, the conversion from **78** to **2** could either be a one-step process via a transitory species as **79**, or be a multistep process via **70** as an intermediate. In order to distinguish these possibilities, [5-^2H]**70a** was next prepared. The literature reports several methods for the epoxidation of

benzoquinones,[24,78,79] but in most cases the yield was low. On the other hand, it has been shown that **62** was very effectively incorporated into **2**[12] and **2** could be oxidized to **70**.[80] The synthesis of **70a**, therefore, was completed as shown in Scheme 27. Thus, a portion of **62b** (600 mg), obtained by deuterium exchange in deuterated trifluoroacetic acid, was fed to 4 x 400-mL fermentation broth under standard conditions and approximately 225 mg of **2j** was obtained with 40% deuterium enrichment at C-5. This was subsequently oxidized by PCC to give **70a** in 50% yield. A portion of **70a** (50 mg) was fed to a 200-mL fermentation broth under standard conditions and the sample, **2k**, was enriched at H-5 to the extent of 27.8%, as determined by ^2H NMR. Therefore, the incorporation of **70a** into **2k** had been 34.4%. Unless there were an active dehydrogenase representing a shunt pathway or a xenobiotic reaction, this experiment strongly suggested **70** as an immediate precursor of **2** and eliminated the one-step process of direct epoxidation of **78** to **2**.



i. TFA-d_1. ii. *S.* LL-C10037, iii. PCC/NaOAc/CH_2Cl_2, iv. *S.* LL-C10037

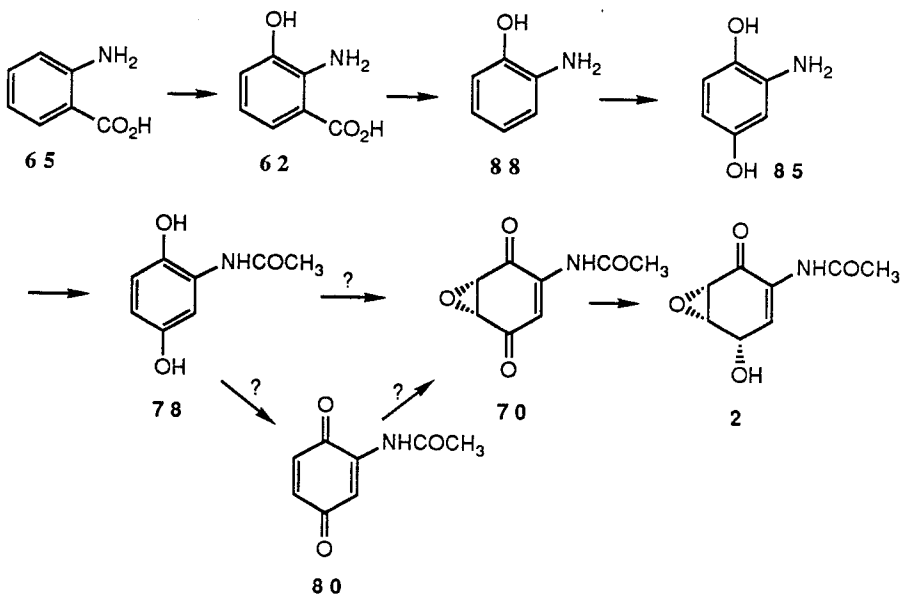
Scheme 27. Synthesis of **70a** from **62** and Its Incorporation into **2**

Biosynthesis of 2 from 65

The data resulting from the *in vivo* feeding studies can be summarized by the pathway shown in Scheme 28. An anthranilate 3-hydroxylase may be involved in the hydroxylation of **65** to **62**. Subsequent metabolism of **62** follows the biosequence of decarboxylation, hydroxylation and acetylation to yield **78**. Although it had not been distinguished yet whether **78** or **80** was

epoxidized --- presumably by an enzymatic process involving a monooxygenase --- **78** was further converted to **2** via **70** as intermediate.

The *in vivo* studies of the biosynthesis of **2** from **78** demonstrated the limitations of this approach. Although both **78** and **80** have been demonstrated to be taken up by the organism and incorporated into **2**, the two pathways leading to **70** as shown in Scheme 28 could not be differentiated at this stage. However, if the individual enzyme activity responsible for each conversion could be isolated, it would be possible to explain the relationship of **78** and **80** to **2** unambiguously and establish the substrate for the epoxidation.



Scheme 28. Biosynthesis of **2** from **65**

Experimental

General Procedures

^{1}H NMR spectra were taken on a Varian FT 80, a Bruker AC 300 or Bruker AM 400 spectrometer; ^{13}C NMR spectra were taken at 100.6 MHz and ^{2}H NMR spectra were obtained at 61.4 MHz on the Bruker AM 400 spectrometer. All ^{2}H NMR spectra were obtained with the following parameters: sweep width =

952 Hz, data point = 4K zero filled to 8K, pulse with = 90°, acquisition time = 2.15 s. All ^{13}C NMR spectra were broadband decoupled and ^{2}H NMR spectra were proton decoupled and run unlocked. Five-mm NMR tubes were used for all NMR measurements. ^{1}H and ^{13}C NMR samples were referenced with TMS, CH_3CN or *t*-BuOH. ^{2}H NMR samples were prepared in ^{2}H-depleted water or in methanol; with 25 µl of *t*-BuOH added as reference for chemical shift (1.28 ppm) and quantification (0.38 µmol ^{2}H in the methyl groups).

IR spectra were recorded on a Perkin Elmer 727B or Nicolet 5DXB FTIR spectrometer. UV spectra were taken on a IBM 9420 UV-Visible spectrophotometer. Low resolution mass spectra were taken on a Varian MAT CH-7 spectrometer.

Melting points were taken on a Büchi melting point apparatus and are uncorrected. Flash chromatography was carried out on silica gel (EM Reagents, Keiselgel 60, 230-400 mesh). Analytical thin layer chromatography (TLC) was carried out on precoated Keiselgel 60 F_{254} (either 0.2-mm aluminum sheets or 0.25-mm glass plates) and visualized by long and/or short wave UV.

Standard Culture Conditions

S. LL-C10037 was maintained at 4 °C as spores on sterile soil. A loopful of this material was used to inoculate 50 mL of seed medium containing 1.0% glucose, 2.0% soluble potato starch, 0.5% yeast, 0.5% N-Z Amine A 59027, and 0.1% $CaCO_3$ in glass distilled water, the whole adjusted to pH 7.2 with 2% KOH. The culture, contained in a 250-mL Erlenmeyer flask, was incubated for 3 days at 28 °C, 240 rpm in a rotary incubator. Production broths (200 mL in 1-L Erlenmeyer flasks), consisting of 1.0% glucose, 0.5% bactopeptone, 2.0% molasses (Grandma's famous light unsulfured), and 0.1% $CaCO_3$ in glass distilled water and adjusted to pH 7.2 with 10% HCl prior to sterilization, were subsequently inoculated 5% (v/v) with vegetative inoculum from seed broths. The production broths were incubated for 120 h.

Synthesis of Labeled Precursors

[5-^{2}H]Tryptophan (81a)

In a 50-mL flask was dissolved NaOAc (140 mg, 1.71 mmol) in D_2O (2 mL) and lyophilized. A mixture of **82** (220 mg, 0.78 mmol) and 10% Pd-C (22 mg) in 90% EtOD-D_2O (20 mL) was then added. The resulting suspension was stirred under an atmosphere of D_2 at room temperature for 1.5 h. The suspension was filtered though celite and the residue was washed with 0.2 N NaOD (20 mL). The filtrate was concentrated and adjusted to pH 4.2 with 0.5 N HCl. The solution was loaded onto an AG-50W-2X cation resin (H^+ form, 100-200 mesh) column (1 x 20 cm). The column was washed with water and eluted with 0.1 N NH_4OH. The fractions containing tryptophan were combined and lyophilized to give **81a** (170 mg) as a white fluffy solid in quantitative yield: ^{1}H NMR (D_2O-NaOD, 400 MHz) δ 3.04 (1H, dd, *J* = 14.4, 7.1 Hz), 3.20 (1H, dd, *J* = 14.3, 5.2 Hz), 3.59 (1H, m), 7.25 (1H, s), 7.26 (1H, d, *J* = 8.9 Hz), 7.52 (1H, d, *J* = 8.2 Hz), 7.75 (1H, s).

[4,6-$^2H_2$]-2,5-Dimethoxyaniline (83a)

2,5-Dimethoxyaniline **83** (600 mg, 3.92 mmol), MeOD (20 mL), D_2O (2.0 mL), and TFA-*d* (1.5 mL) in a flame-dried 50-mL round-bottomed flask were heated at reflux for 12 h. All solvents were removed by vacuum distillation. ^{1}H NMR analysis (methanol-*d4*, 80 MHz) of a portion of the residue (560 mg, 93%) indicated 90% exchange at H-4 (δ 6.20) and at H-6 (δ 6.25).

2,5-Dimethoxyacetanilide (84)

2,5-Dimethoxyaniline **83** (154 mg, 1 mmol) was dissolved in CH_2Cl_2 (5 mL), and Et_3N (168 μL, 122 mg, 1.2 mmol) and acetyl chloride (83 μL, 95 mg, 1.2 mmol) were added at 0 °C. After stirring at room temperature for 1 h, the mixture was diluted with CH_2Cl_2 (20 mL) and washed with H_2O (2 x 5 mL). The organic layer was dried over $MgSO_4$ and concentrated *in vacuo* to give **84** in quantitative yield as grey crystals: m.p. 91-92 °C (lit 91 °C[81]); ^{1}H NMR (acetone-d_6, 80 MHz) δ 8.80 (1H, bs), 8.00 (1H, d, *J* = 3.8 Hz), 6.82 (1H, d, *J* = 8.2 Hz), 6.48 (1H, dd, *J* = 8.2, 3.8 Hz), 3.81 (3H, s), 3.78 (3H, s), 2.10 (3H, s).

[4,6,2',2',2'-$^2H_5$]-2,5-Dimethoxyacetanilide (84a)

With use of the above procedure, **83a** (560 mg, 3.6 mmol), acetyl chloride-d_3 (332 μL, 381 mg, 4.7 mmol) and Et_3N (652 μL, 473 mg, 4.7 mmol) in CH_2Cl_2 (20 mL) yielded **84a** (672 mg, 92%). ^1H NMR analysis (acetone-d_6, 80 MHz) indicated 70% enrichment at H-4 (δ 6.48), 90% enrichment at H-6 (δ 8.00), and 100% enrichment in the methyl group (δ 2.10).

2,5-Dihydroxyacetanilide (78).

2,5-Dimethoxyacetanilide **84** (98 mg, 0.5 mmol) was dissolved in CH_2Cl_2 (25 mL) in a dried 100-mL round-bottomed flask which was then flushed with Ar, cooled to -78 °C, and treated with a 1 M BBr_3/CH_2Cl_2 solution (1.2 mL, 1.20 mmol). After 1 h, the stirred mixture was warmed to room temperature and stirred for an additional 6 h. The reaction mixture was quenched with water (20 mL) and extracted with EtOAc (5 x 20 mL), and then washed with saturated brine. The combined organic fractions were dried (Na_2SO_4) and concentrated *in vacuo* to give nearly pure **78** (83 mg, 100%) as white crystals. Recrystallization from EtOAc gave **78** (55 mg, 65%) as a grey powder: m.p. 165-168 °C (sealed capillary) (lit 100 °C[36]); ^1H NMR (acetone-d_6, 400 MHz) δ 9.10 (1H, bs, exch D_2O), 8.57 (1H, s, exch D_2O), 7.89 (1H, s, exch D_2O), 7.03 (1H, d, J = 2.6 Hz), 6.72 (1H, d, J = 8.7 Hz), 6.52 (1H, dd, J = 8.6, 2.7 Hz), 2.18 (3H, s); ^{13}C NMR (acetone-d_6, 100.6 MHz) δ 170.90, 151.24, 141.89, 127.94, 118.81, 112.96, 109.35, 29.85.

[4,6,2',2',2'-$^2H_5$]-2,5-Dihydroxyacetanilide (78a)

With use of the above procedure, **84a** (640 mg, 3.26 mmol) in CH_2Cl_2 (75 mL) was treated with a 1 M BBr_3 solution (7.83 mL, 7.83 mmol) in CH_2Cl_2. Workup gave a brown solid that was recrystallized from EtOAc-MeOH to yield **78a** (460 mg, 83%) as tan crystals (m.p. 171-172 °C). ^1H NMR analysis (acetone-d_6, 400 MHz) indicated 60% deuterium enrichment at H-4 (δ 6.5), 89% at H-6 (δ 7.0), and 100% at the methyl group (δ 2.2).

[4,6-$^2H_2$]-2,5-Dihydroxyaniline Hydrochloride (85a)

2,5-Dihydroxyacetanilide **84** (300 mg, 1.80 mmol), MeOD (10 mL), D_2O (5 mL), and 3 M DCl (5 mL) in a 50-mL round-bottomed flask were heated at reflux under N_2 atmosphere for 5 h. After cooling, solvents were removed *in vacuo* and the light green powder recrystallized from EtOAc-MeOH to give **85a** (260 mg, 90%): ^1H NMR (methanol-d_4, 400 MHz) δ 6.84 (0.4 H, d, J = 8.8 Hz), 6.77 (0.05 H, d, J = 2.4 Hz), 6.73 (1H, d, J = 8.8, 2.9 Hz).

[4,6-$^2H_2$]-2-Methoxyaniline Hydrochloride (87a)

2-Methoxyaniline **87** (500 mg, 4.06 mmol) and 4 M DCl (6 mL) were sealed in a heavy-walled 25-mL flask and heated at 100 °C for 30 min in an oil bath. After cooling, the solvent was removed *in vacuo* and the black solid was recrystallized from EtOH/EtOAc to give **87a** (538 mg, 82%) as beige needles: m.p. 232-233 °C; ^1H NMR analysis (methanol-d_4, 80 MHz) indicated >90% exchange at H-4 and H-6 (δ 6.7 and 6.5).

2-Methoxyacetanilide

2-Methoxyaniline **87** (493 mg, 4.0 mmol) was treated with acetyl chloride (379 mg, 4.8 mmol) and Et_3N (486 mg, 4.8 mmol) in CH_2Cl_2 (35 mL) for 2 h. Additional CH_2Cl_2 (20 mL) was added and the mixture washed with water (2 x 10 mL). After drying over $MgSO_4$ and filtration, the filtrate was concentrated *in vacuo* to give a white solid. Recrystallization from EtOH yielded 2-methoxyacetanilide (660 mg, 100%): m.p. 84-85 °C (lit. 84 °C[81]); ^1H NMR ($CDCl_3$, 80 MHz) δ 8.30 (1H, bdd, J = 8, 3 Hz), 7.70 (1H, bs), 6.90 (3H, m), 3.85 (3H, s), 2.17 (3H, s).

[4,6,2',2',2'-$^2H_5$]-2-Methoxyacetanilide

With use of the above procedure, a mixture of **87a** (360 mg, 2.3 mmol), acetyl chloride-d_3 (189 μL, 217 mg, 2.8 mmol), and Et_3N (747 μL, 542 mg, 5.6 mmol) in CH_2Cl_2 (30 mL) gave deuterated 2-methoxyacetanilide (280 mg, 74%).

2-Hydroxyacetanilide (86)

2-Methoxyacetanilide (41 mg, 0.25 mmol) in dry CH_2Cl_2 (15 mL) under an Ar atmosphere was treated at -78 °C with a 1 M BBr_3/CH_2Cl_2 solution (300 μL, 0.30 mmol). After 1 h the mixture was warmed to room temperature and after an additional 3 h was worked up as described for **78** to yield **86** (35 mg, 93%) after recrystallization from EtOH: m.p. 208-209 °C (lit 209 °C[82]); ^1H NMR (acetone-d_6, 400 MHz) δ 9.49 (1H, s, exch D_2O), 9.40 (1H, bs, exch D_2O), 7.39 (1H, dd, J = 8.1, 1.3 Hz), 7.03 (1H, m), 6.89 (1H, dd, J = 8.1, 1.3 Hz), 6.80 (1H, m), 2.21 (3H, s); ^{13}C NMR (acetone-d_6, 100.6 MHz) δ 171.22, 149.42, 127.72, 126.61, 122.84, 120.51, 118.96, 23.49.

[4,6,2',2',2'-$^2H_5$]-2-Hydroxyacetanilide (86a)

As described above, [4,6,2',2',2'-$^2H_5$]-2-methoxyacetanilide (280 mg, 1.65 mmol) was demethylated with a 1 M BBr_3/CH_2Cl_2 solution (2.0 mL, 2.0 mmol) to yield **86a** (247 mg, 96%) (m.p. 206-207). ^1H NMR analysis (acetone-d_6, 400 MHz) indicated 92% deuteration at H-4 (δ 7.03) and at H-6 (δ 7.39), and 90% deuteration at the methyl group (δ 2.18).

2-Nitro-4,6-dibromophenol (91)

2-Nitrophenol, **90**, (6.95 g, 0.05 mol) was dissolved in glacial HOAc (30 mL) and treated over 1 h with Br_2 (7.95 mL, 0.15 mol) in glacial HOAc (4 mL). The mixture was heated at *ca.* 60 °C for 24 h and then cooled and poured into water (100 mL). The resulting yellow precipitate was collected and recrystallized from EtOH to give **91** (1.35 g, 76%) as bright yellow needles: m.p. 116-119 °C (lit. 113-114 °C[51]); ^1H NMR ($CDCl_3$, 80 MHz) δ 10.68 (1H, bs), 7.89 (1H, d, J = 2.5 Hz), 7.62 (1H, d, J = 2.5 Hz).

[3,5-$^2H_2$]-2-Hydroxyaniline (88b)

Sodium acetate (410 mg, 4 mmol) was dissolved in D_2O (5 mL) in a 100-mL round-bottomed flask and then lyophilized. **91** (500 mg, 1.68 mmol), 10% Pd-C (100 mg), and EtOD (70 mL) were added to the flask and the resulting

suspension stirred under an atmosphere of D_2 at room temperature for 24 h. The suspension was filtered and the filtrate was concentrated *in vacuo*. The residue was extracted with EtOAc (50 mL) and this was washed with H_2O (2 x 3 mL) and then with saturated brine (5 mL). After drying ($MgSO_4$) and concentration *in vacuo*, the residue was chromatographed on silica gel (20 cm x 1.5 cm) by eluting with hexane-EtOAc (1:1) and the crude product was recrystallized from benzene to give **88b** (119 mg, 64%) as yellow-brown crystals: m.p. 172-176 °C (lit. 175-177 °C[82]); ^1H NMR ($CDCl_3$, 400 MHz) δ 6.73 (1H, s), 6.58 (1H, s).

2-Nitro-4,6-dibromoanisole (93)

2-Nitro-4,6-dibromophenol **91** (0.96 g, 3.23 mmol) was dissolved in dry acetone (60 mL). K_2CO_3 (1.78 g, 12.9 mmol) and MeI (3.66 g, 25.8 mmol) were then added to the flask. The resulting suspension was heated at 60 °C for 24 h. After removing as much acetone as possible on a water bath, the residue was acidified with 2 N H_2SO_4 with cooling. The resulting solution was diluted with water (20 mL) and extracted with $CHCl_3$ (4 x 30 mL). The combined $CHCl_3$ extract was washed with water (10 mL) and brine (10 mL) and dried over $MgSO_4$. After concentration *in vacuo*, the yellow crystals were recrystallized from 95% EtOH to give **8** (0.81 g, 81%) as yellow needles: m.p. 76-77 °C (lit. m.p. 76-77 °C[83]); ^1H NMR ($CDCl_3$, 300 MHz) δ 7.92 (1H, d, J = 2.3 Hz), 7.89 (1H, d, J = 2.3 Hz).

[3,5-$^2H_2$]-2-Methoxyaniline (87b)

2-Nitro-4,6-dibromoanisole, **93**, (1.98 g, 6 mmol), NaOAc (1.48 g, 8 mmol), 100% EtOH (50 mL) and 10% Pd-C (400 mg) were added to a 100-mL flask. The resulting suspension was stirred under an atmosphere of D_2 at room temperature for 50 h. The suspension was filtered and the filtrate was concentrated *in vacuo*. The residue was extracted with EtOAc (200 mL), and the extract was washed with water (2x10 mL) and brine (10 mL). After drying over Na_2SO_4, concentration *in vacuo* afforded **87b** (720 mg, 96%) as a brown liquid: ^1H NMR ($CDCl_3$, 300 MHz) δ 6.77 (1H, bs), 6.75 (1H, bs), 3.89 (2H, bs, exchangeable), 3.87 (3H, s).

2-Methoxy-N-(*t*-butoxycarbonyl)aniline (94)

To a dry 100-mL flask was dissolved 2-methoxyaniline **87** (720 mg, 5.76 mmol) and di-*t*-butyl dicarbonate (1.5 g, 6.91 mmol) in THF (40 mL). The resulting solution was heated to reflux for 2 h. The solvent was partially removed by vacuum distillation. The remaining solvent was diluted with EtOAc (100 mL) and washed with 1 M citric acid (10 mL), water (10 mL) and brine (10 mL). After drying over $MgSO_4$, concentration *in vacuo* yielded a brown liquid, which was further purified by Kugelrohr distillation (1.5 mmHg, 95-100 °C) to give **94** (1.10 g, 85%) as a slightly yellow liquid: ^1H NMR ($CDCl_3$, 300 MHz) δ 8.07 (1H, bd, J = 5.6 Hz), 7.10 (1H, s), 6.89-6.98 (2H, m), 6.81-6.85 (1H, m), 3.83 (3H, s), 1.52 (9H, s) (This compound has not been reported before).

[3,5-$^2H_2$]-2-Methoxy-N-(*t*-butoxycarbonyl)aniline (94a)

With use of the above procedure, a mixture of [3,5-$^2H_2$]-2-methoxyaniline **87b** (480 mg, 3.78 mmol) and di-*t*-butyl dicarbonate (906 mg, 4.1 mmol) in 40 mL THF yielded **94a** (738 mg, 87%): ^1H NMR ($CDCl_3$, 300 MHz) analysis δ 8.03 (1H, s), 7.05 (1H, s), 6.92 (1H, s), 3.82 (3H, s), 1.49 (9H, s), indicated 100% deuterium enrichments at H-3 and H-5, respectively.

3-Methoxy-N-(*t*-butoxycarbonyl)anthranilic Acid (95)

All glassware was well dried and the reaction was carried out under N_2. To a 50-mL flask was dissolved **94** (2.67 g, 12 mol) in THF (40 mL) and the solution was cooled to -78 °C. A 1.7 M *t*-BuLi solution (17.0 mL, 28.8 mol) was transferred with a double-tipped needle and the resulting yellow solution was kept at -20 °C for 2.5 h. This phenyllithium solution was transferred into a dry ice-THF slurry and the slurry was slowly warmed up to room temperature over 2 h. This solution was then partitioned between Et_2O and 5% $NaHCO_3$ (Et_2O/$NaHCO_3$=200/100). The organic layer was washed with 5% $NaHCO_3$ (2 x 40 mL), brine (2 x 5 mL), and dried over $MgSO_4$. After concentration, 810 mg of **94** was recovered from the organic layer. The combined aqueous solution was adjusted to acidic pH by adding solid citric acid and extracted with Et_2O (5 x 40 mL). The Et_2O extract was washed with brine (2 x 5 mL), dried over $MgSO_4$ and

concentrated *in vacuo* to give a yellow solid, which was recrystallized from EtOH-MeOH (MeOH less than 5%) to give **95** (980 mg, 44% corrected yield) as colorless prisms: m.p. 180-181 °C; ^{1}H NMR (acetone-d_6, 300 MHz) δ 11.19 (1H, bs, exchangable), 7.72 (1H, s, exchangable), 7.42 (1H, m), 7.16-7.25 (2H, m), 3.88 (3H, s), 1.44 (9H, s) (This compound has not been reported before).

[4,6-^{2}H$_2$]-3-Methoxy-N-(*t*-butoxycarbonyl)anthranilic Acid (95a)

With use of the above procedure, a solution of **94a** (321 mg, 1.43 mmol) in THF (7.0 mL) was subsequently treated with a 1.7 M *t*-BuLi solution (2.0 mL, 3.4 mmol) and a CO_2-THF slurry gave 74 mg of **95a** (62% corrected yield) with 220 mg of **94a** recovered. ^{1}H NMR (acetone-d_6, 300 MHz) δ 11.17 (1H, bs, exchangeable), 7.72 (1H, s, exchangeable),7.20 (1H, s), 3.88 (3H, s), 1.44 (9H, s).

[4,6-^{2}H$_2$]-3-Hydroxyanthranilic Acid (62a)

A solution of **95a** (197 mg, 0.876 mmol) was dissolved in dry $CHCl_3$ (80 mL, dry $CHCl_3$ was prepared by passing through an Al_2O_3 column) and cooled to -20 °C. A 1.0 M BBr_3 solution (5.25 mL, 6.26 mmol) was added slowly and the resulting solution was stirred at -20 °C, then allowed to warm to room temperature for 15 h. The yellow suspension was then poured into ice-water (200 mL) and stirred for 10 min. The organic layer was collected and the aqueous layer was extracted with Et_2O (5x100 mL). The combined Et_2O and $CHCl_3$ extract was washed with brine and dried over Na_2SO_4. Concentration *in vacuo* yielded 120 mg of crude product, which was further purified on a Silicar CC4 column (1.5x30 cm), eluted with MeOH/$CHCl_3$ (15/85) to give **62a** (35 mg, 26%) as a powder: ^{1}H NMR (methanol-d_4, 300 MHz) δ 7.35 (0.14 H, d J = 8.1 Hz), 6.80 (0.14 H, d, J = 7.6 Hz), 6.46 (1H, s).

[5-^{2}H]-3-Hydroxyanthranilic Acid (62b)

In a well-dried heavy-walled flask was dissolved **62** (350 mg, 2.29 mmol) in TFA-*d* (20 mL). The flask was flushed with Ar for a few minutes, sealed and heated in an oil bath at 80 °C for 45 h. The resulting reaction mixture was

concentrated *in vacuo* to yield 590 mg of the crude product, which was further purified on an AG 50W - 2X (in H^+ form, 100-200 mesh) cation exchange resin column to give **62b** (283 mg, 65.5%) as the hydrochloride salt: ^1H NMR (D_2O-NaOD, 300 MHz) δ 6.90 (1H, d, J = 1.8 Hz), 6.62 (1H, d, J = 1.8 Hz).

N-Acetyl-3-hydroxyanthranilic Acid (98)

To a solution (4 mL) of **62** (61 mg, 0.4 mmol) and K_2CO_3 (221 mg, 1.6 mmole) was added Ac_2O (49 mg, 0.44 mmol). The resulting solution was stirred at room temperature for 1 h. The solution was acidified with 1 N HCl and extracted with EtOAc. The EtOAc extract was dried (Na_2SO_4) and concentrated *in vacuo* to yield a crystalline brown solid **98**: m.p. 139-140 °C (lit. m.p. 143-144 °C),[84] ^1H NMR (methanol-d_4, 300 MHz) δ 7.50 (1H, dd, J = 1.8, 7.4 Hz), 7.14 (1H, t, J = 7.8 Hz), 7.09 (1H, dd, J = 1.9, 8.0), 2.20 (3H, s).

[2',2',2',5-$^2H_4$]-N-Acetyl-3-hydroxyanthranilic Acid (98a)

With use of the above procedure, a mixture of **62b** (153 mg, 1 mmol), K_2CO_3 (828 mg, 6 mmol) and [$^2H_6$]acetic anhydride (112 mg, 1.1 mmol) in water (10 mL) gave **98a** (157 mg, 79%). ^1H NMR analysis (methanol-d_4, 300 MHz) indicated 65 % deuterium enrichment at H-5 (δ 7.14) and 100 % deuterium enrichment at the acetyl methyl group (δ 2.20).

Acetamido-1,4-benzoquinone (80)

Method A: A portion of **78** (34 mg, 0.2 mmol) in Et_2O (25 mL) and CH_2Cl_2 (25 mL) was treated with PbO_2 (287 mg, 1.2 mmol) and the resulting black suspension stirred at room temperature for 12h. The mixture was filtered through a silica gel column (10 cm x 1.5 cm), eluted with EtOAc-hexane (1:1), and the yellow fractions were combined and concentrated to give a crystalline residue. Recrystallization from EtOAc then yielded **80** (28.1 mg, 85%) as orange crystals: m.p. 148-149 °C (dec, sealed capillary) (lit. m.p. 142 °C);[85] ^1H NMR (acetone-d_6, 400 MHz) δ 8.85 (1H, bs, exch D_2O), 7.42 (1H, d, J = 2.9 Hz), 6.78 (1H, d, J = 10.3 Hz), 6.64 (1H, dd, J = 10.2, 2.2 Hz), 2.28 (3H, s); ^{13}C NMR (acetone-d_6, 100.6 MHz) δ 189.21, 183.37, 171.62, 140.22, 118.16, 134.70, 114.67, 24.48.

Method B: Into a 150-mL beaker was dissolved **78** (1.0g, 6.0 mmol) in warm water (20 mL) and the resulting solution was then cooled in ice-water bath. A solution of $K_2Cr_2O_7$ (0.88g, 3.0 mmol) in 20% H_2SO_4 (5.0 mL) was added gradually to the cool solution with stirring. The reaction was completed in 45 minutes. The resulting yellowish green slurry was extracted with EtOAc (3 x 30 mL) and the EtOAc extract was washed with brine and dried over $MgSO_4$. Concentration *in vacuo* yielded a bright yellow residue, which was recrystallized from EtOAc to give **80** (930 mg, 94%) as orange crystals. (Method B would be preferred for large scale preparation).

[3,5,2',2',2'-$^2H_5$]-2-Acetamido-1,4-benzoquinone (80a)

By using the above procedure, **78a** (193 mg, 1.13 mmol) in Et_2O (100 mL) and CH_2Cl_2 (100 mL) was oxidized with PbO_2 (1.63 g), and the product was purified (silica gel column, 15 cm x 1.5 cm) to give **80a** (164 mg, 86%) that was 89.5% deuterated at H-3 (δ 7.42), 54% deuterated at H-5 (δ 6.64), and 100% deuterated in the methyl group (δ 2.25).

2-Acetamido-5,6-epoxy-1,4-benzoquinone (70)

To a solution of **2** (200 mg, 1.1 mmol) in CH_2Cl_2 (50 mL) was added NaOAc (90 mg, 1.1 mmol) and PCC (355 mg, 1.65 mmol). The resulting solution was stirred at room temperature for 1.5 hours. The brown reaction mixture was then filtered through a celite pad and the residue was washed with CH_2Cl_2. The combined CH_2Cl_2 filtrate was concentrated *in vacuo* to give a brown residue, which was further purified on a silica gel column (1.5 x 15 cm) eluted with CH_2Cl_2. The fractions containing the product were combined and concentrated *in vacuo* to provide a yellow solid, which was further recrystallized from EtOAc to yield **70** (100 mg, 50%) as bright yellow crystals: m.p. 135-136 °C (lit. 144-145 °C[80]); ^1H NMR ($CDCl_3$, 400 MHz) δ 7.89 (1H, bs), 7.51 (1H, d, J = 2.2 Hz), 3.91 (1H, d, J = 3.7 Hz), 3.83 (1H, dd, J = 3.7, 2.2 Hz), 2.22 (3H, s).

[5-^{2}H]-2-Acetamido-5,6-epoxy-1,4-benzoquinone (70a)

With use of the above procedure, a mixture of [5-^{2}H]-LL-C10037α, **2j**, (200 mg, 1.1 mmol), NaOAc (90 mg, 1.1 mmol), PCC (355 mg, 1.65 mmol) in CH_2Cl_2 gave **70a** (100 mg, 50%) as bright yellow crystals. ^{1}H NMR analysis ($CDCl_3$, 400 MHz) indicated 40% deuterium enrichment at H-5 (δ, 3.83).

Protocol for Feeding Experiments

Isolation

The cultures were filtered through cheesecloth and celite, and the filtrate was adjusted to pH 4.0-4.5 with solid KH_2PO_4. This was then saturated with $(NH_4)_2SO_4$ and extracted repeatedly with EtOAc (typically 8 times). After concentration *in vacuo* the residue was dissolved in a minimum volume of methanol and adsorbed onto a small quantity of silica gel. This was applied to the top of a column of flash grade silica gel (50 g/400 mL fermentation) prepared in 40% hexane/EtOAc. After low-polarity colored impurities had been eluted the solvent was changed to 20% hexane/EtOAc and elution yielded **2**, which was recrystallized from methanol.

General Procedure for Feeding

Sets of preliminary experiments are usually required to establish the best conditions under which the labeled compounds should be fed. At least, it has to be determined when and how much of the labeled compound will be fed to the culture. The timing of feeding and amount of material fed were adapted directly from those reported by Gould and Whittle.[12] Thus, labeled precursor was dissolved in 2-5 mL of an appropriate H_2O/ethanol mixture and added in a sterile manner through micropore filters to the production broth after *ca.* 96 h of fermentation. The fermentation was worked up after a total of 120 h incubation. Most of the feedings were carried out in single doses under the conditions described above; however, in the feeding of **81a**, pulse feeding (see Table 14) was applied, which, in general, would increase the efficiency of incorporation.

Specific incorporation relevant to this research was calculated according to the following equation:

$$\% \text{ Specific Incorporation} = \frac{\text{mole of } ^2H \text{ at a specific position in the compound enriched}}{\text{mole of } ^2H \text{ at a specific position in the compound fed}} \times 100$$

Feeding of Putative Precursors to Fermentations of *S.* LL-C10037

Deuterium enrichments of the compounds fed were determined by ^1H NMR. Deuterium enrichments of the enriched LL-C10037α, **2**, were determined by ^2H NMR and quantitated by comparison of the integrals with that from 25 μL of *t*-BuOH (0.38 μmol) added to each sample. The amount of **2** produced from each fermentation was estimated from the amount of **2** isolated by correcting for that lost during the isolation and purification (*ca.* 10% of the **2** isolated).

[5-^{2}H]Tryptophan (81a)

Table 14. No Incorporation of Tryptophan into **2**

Fermentation size	200 mL
Amount **81a** fed	5 mg at 96 h 5 mg at 102 h
Deuterium enrichment of **81a**	100% at H-5 (δ 7.25)
Amount **2a** produced	30.8 mg
Amount **2a** isolated	28.0 mg
Deuterium enrichment of **2a** (^2H NMR 12992 scans)	0
% incorporation	0
Fermentation size	200 mL
Amount **81a** fed	25 mg at 96 h 25 mg at 102 h
Deuterium enrichment of **81a**	100% at H-5 (δ 7.25)
Amount **2a** produced	35.2 mg
Amount **2a** isolated	32 mg
Deuterium enrichment of **2a** (^2H NMR 7256 scans)	0
% incorporation	0

[4,6,2',2',2'-$^2H_5$]-2,5-Dihydroxyacetanilide (78a)

Table 15. Incorporation of **78a** into **2b**

Fermentation size	400 mL
Amount **78a** fed	100 mg
Deuterium enrichment of **78a**	60% at H-4 (δ 6.5)
	89% at H-6 (δ 7.0)
	100% at H-2' (δ 2.2)
Deuterium distribution	H-4/H-6/H-2' = 6/8.9/10
Amount **2b** produced	59.0 mg
Amount **2b** isolated	53.4 mg
Deuterium enrichment of **2b**	11.3% at H-5 (δ 3.79)
(^2H NMR, 6976 scans)	15.1% at H-3 (δ 7.19)
	19.5% at H-2' (δ 2.08)
Deuterium distribution	H-5/H-3/H-2' = 5.8/7.7/10
% Incorporation	9.10

[4,6-$^2H_2$]-2,5-Dihydroxyaniline (85a)

Table 16. Incorporation of **85a** into **2c**

Fermentation size	400 mL
Amount **85a** fed	100 mg
Deuterium enrichment of **85a**	60% at H-4 (δ 6.84)
	95% at H-6 (δ 6.77)
Deuterium distribution	H-4/H-6 = 0.63/1.0
Amount **2c** produced	53.5 mg
Amount **2c** isolated	47.6 mg
Deuterium enrichment of **2b**	0.67% at H-5 (δ 3.79)
(^2H NMR, 16309 scans)	0.94% at H-3 (δ 7.19)
Deuterium distribution	H-5/H-3 = 0.71/1.0
% Incorporation	0.47

[4,6,2',2',2'-^2H]-2-Hydroxyacetanilide (86a)

Table 17. Incorporation of **86a** into **2d**

Fermentation size	400 mL
Amount **86a** fed	100 mg
Deuterium enrichment of **86a**	92% at H-4 (δ 7.03)
	92% at H-6 (δ 7.39)
	90% at H-2' (δ 2.18)

Table 17 continued:

Deuterium distribution	H-4/H-6/H-2'= 1.0/1.0/0.95
Amount **2b** produced	50.0 mg
Amount **2b** isolated	45.2 mg
Amount **86a** recovered	89.0 mg
Deuterium enrichment of **2d**	0.19% at H-5 (δ 3.79)
(^{2}H NMR, 15358 scans)	0.12% at H-3 (δ 7.19)
	1.21% at H-2' (δ 2.08)
Deuterium distribution	H-5/H-3/H-2'= 1.0/0.63/6.3
% Incorporation	0.54 (based on H-5)

[3,5-$^2H_2$]-2-Hydroxyaniline (88b)

Table 18. Incorporation of **88b** into **2e**

Fermentation size	400 mL
Amount **88b** fed	100 mg
Deuterium enrichment of **88b**	100% at H-3 (δ 6.73)
	100% at H-5 (δ 6.61)
Deuterium distribution	H-3/H-5 = 1.0/1.0
Amount **2e** produced	29.2 mg
Amount **2e** isolated	26.6 mg
Amount **86b** isolated	98.0 mg
Deuterium enrichment of **2e**	0.22% at H-6 (δ 3.50)
(^{2}H NMR, 39414 scans)	0.14% at H-3 (δ 7.19) (uncertain)
	0.09% at H-2' (δ 2.08)
% Incorporation	0.13 (based on H-6)

[4,6-$^2H_2$]-3-Hydroxyanthranilic acid (62a)

Table 19. Incorporation of **62a** into **2f**

Fermentation size	200 mL
Amount **62a** fed	35 mg
Deuterium enrichment of **62a**	86% at H-4 (δ 6.80)
	14% at H-5 (δ. 6.46)[a]
	86% at H-6 (δ 7.35)
Deuterium distribution	H-4/H-5/H-6 = 1.0/0.17/1.0
Amount **2f** produced	28.0 mg
Amount **2f** isolated	25.0 mg
Deuterium enrichment of **2f**	21.1% at H-6 (δ 3.79)

Table 19 continued:

(^{2}H NMR, 17724 scans)	3.2% at H-5 (δ 7.19)
Deuterium distribution	H-6/H-5 = 100/0.15
% Incorporation	17.8 (based on H-6)

a. ^{2}H NMR was done on **94a** to establish the ^{2}H enrichments at H-4, H-5 and H-6.

[5,2',2',2'-$^2H_4$]-N-Acetyl-3-Hydroxyanthranilic acid (98a)

Table 20. Incorporation of **98a** into **2g**

Fermentation size	400 mL
Amount **98a** fed	100 mg
Deuterium enrichment of **98a**	65% at H-5 (δ 7.14)
	100% at H-2' (δ 2.2)
Deuterium distribution	H-5/H-2' = 0.65/1.0
Amount **2g** produced	50.0 mg
Amount **2g** isolated	40.0 mg
Deuterium enrichment of **2g**	3.04% at H-5 (δ 3.79)
(^{2}H NMR, 7842 scans)	0.12% at H-2' (δ 2.08)
Deuterium distribution	H-5/H-2' = 25.3/1.0
% Incorporation	2.53 (based on H-5)

[3,5,2',2',2'-$^2H_5$]-2-Acetamido-1,4-benzoquinone (80a)

Table 21. Incorporation of **80a** into **2h**

Fermentation size	400 mL
Amount **80a** fed	100 mg
Deuterium enrichment of **80a**	89% at H-3 (δ 7.42)
	54% at H-6 (δ 6.64)
	100% at H-2' (δ 2.25)
Deuterium distribution	H-3/H-5/H-2' = 8.8/5.4/10
Amount **2h** produced	25.6 mg
Amount **2h** isolated	21.0 mg
Deuterium enrichment of **2h**	4.1% at H-5 (δ 3.79)
(^{2}H NMR, 6976 scans)	6.0% at H-3 (δ 7.19)
	7.6% at H-2' (δ 2.08)
Deuterium distribution	H-3/H-5/H-2' = 7.9/5.4/10
% Incorporation	1.6

[4,6-$^2H_2$]-2,5-Dihydroxyacetanilide (78b) and [2',2',2'-$^2H_3$]-2-Acetamido-1,4-benzoquinone (80b)

Table 22. Competitive Incorporation of **78b** and **80b** into **2b**

Fermentation size	400 mL
Amount **78b** fed	32.0 mg
Deuterium enrichment of **78b**	57% at H-4 (δ 6.5)
	93% at H-6 (δ 7.0)
Amount **80b** fed	32.0 mg
Deuterium enrichment of **80b**	86% at H-2' (δ 2.25)
Amount **2i** produced	58.6 mg
Amount **2i** isolated	53.0 mg
Deuterium enrichment of **2i**	3.27% at H-5 (δ 3.79)
(^2H NMR, 14983 scans)	6.07% at H-3 (δ 7.19)
	4.10% at H-2' (δ 2.08)
% Incorporation	10.2 for **78b**
	7.9 for **80b**

[5-^2H]-3-Hydroxyanthranilic acid (62b)

Table 23. Incorporation of **62b** into **2j**

Fermentation size	4 x 400 mL
Amount **62b** fed	600 mg
Deuterium enrichment of **62b**	100% at H-5 (δ 6.62)
Amount **2j** produced	250.0 mg
Amount **2j** isolated	225.0 mg
Deuterium enrichment of **2j**	40% at H-5 (δ 3.79)
(by ^1H NMR)	
% Incorporation	12.5

[5-^2H]-2-Acetamido-5,6-epoxy-1,4-benzoquinone (70a)

Table 24. Incorporation of **70a** into **2k**

Fermentation size	200 mL
Amount **70a** fed	50 mg
Deuterium enrichment of **70a**	40% at H-5 (δ 3.84)
Amount **2k** produced	25 mg
Amount **2k** isolated	22 mg
Deuterium enrichment of **2k**	27.8% at H-5 (δ 3.79)

Table 24 continued:

(^{2}H NMR 3255 scans)	
% Incorporation	34.4

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Chapter 4

Enzymological Studies on the Biosynthesis of LL-C10037α and MM 14201

Part of this work has been accepted for publication
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Methodology in *in vitro* Studies

In defining any biosynthetic pathway, sooner or later, one would wish to study the individual enzymatic transformations which characterize that metabolic sequence. Although only rarely has such study been done in secondary metabolism, progress was made in the past decade. It is at this level that the study of biosynthesis blends with traditional biochemistry. Having once demonstrated that a suspected transformation does indeed take place under enzyme catalysis, it is then possible to bring all the techniques of modern enzymology to bear on establishing the detailed mechanism of any given reaction. At this level of inquiry it is appropriate to ask what are the key ground state intermediates, if any, and what type of reactive species --- cations, anions, radicals --- are actually involved.

Until recently most cell-free biosynthetic studies of secondary metabolism have been carried out with crude cell-free extracts consisting of mixtures of many enzymes. The main advantage of such studies is the elimination of permeability barriers both to the incorporation of added substrates and to the detection and isolation of transitory products. In many cases all the relevant enzymes of the biosynthetic pathway will be present in the extract; in others only a few of the normal *in vivo* transformations will be possible. Obviously, all the isotopic labeling techniques and kinetic methods available for whole-cell studies are applicable to cell-free investigations. Although dilution by endogenous metabolites is usually less of a problem, it is essential that the same standards be applied to establish the position and distribution of isotopic labels in enzyme products as in "whole-cell" metabolites. Experiments designed to establish competence of putative intermediates are also less subject to uncertainties posed by permeability barriers.

The difficulties in isolating enzymes of secondary metabolic pathways and in establishing uniformly applicable conditions for the analysis of the complex biochemical systems have mainly resulted from the extremely low level of enzymes produced, the unstable and diverse nature of the enzymes, and their existence only during a narrow window in the organism's lifetime. Furthermore, these enzymes often must be isolated from cells containing large amounts of secondary metabolites which after disintegration of their sites of

storage can denature proteins and inactivate enzymes. Such difficulties fortunately are waning, for it is becoming increasingly clear that the use of the recombinant DNA method will overcome problems of this kind. Suitable techniques of gene cloning and overexpression are available, which could facilitate the purification of interested enzymes and have great promise for rapidly accelerating the pace at which new knowledge will be acquired for the studies of secondary metabolic pathways.[1]

Although it is safe to state that in no case have all the enzymes that are required for the biosynthesis of any secondary metabolite been isolated and purified to homogeneity, much progress has been made towards this goal in some cases. Yet this requirement has to be met (or, at least, methods have to be devised for the assay of each enzyme) if one expects to achieve complete understanding of a given biosynthetic pathway. Investigations with enzyme preparation are of importance in the elucidation of the details of secondary metabolic pathways. Each step can be examined independently from the metabolic grid of the producer cell without interference from other transformations of the administered precursor or the products formed. The reaction in question can be measured by following the transformation of the substrates as well as the synthesis of the products and the consumption of coenzymes, e.g., NADH or NADPH. With relatively pure enzyme systems, comparisons among several potential substrates can be reliably made by establishing the characteristic V_{max} and K_m values for each compound. The ratio V_{max}/K_m, which corresponds to the second order rate constant for catalysis, and which takes into account both catalytic rate and active site binding, provides one of the best means for differentiating among several potential substrates, assuming that V_{max}/K_m will be greatest for the natural substrate. On the other hand, the demonstration that a given transformation is enzyme-catalyzed is not equivalent to proof that the reaction is essential or even relevant to the intact, *in vivo*, biosynthetic pathway. Information from cell-free experiments must therefore be carefully evaluated in the light of whatever complementary evidence is available from isotope labeling, kinetic, and mutant investigation.

Biological Oxidation and Reduction

In light of the results from the *in vivo* feeding studies, it appeared that the formation of **2** involved an epoxidation of either **78** or **80**, presumably via an enzymatic process requiring a monooxygenase. On the other hand, the competitive incorporation of **78** and **80**, and the demonstration of **70** as the immediate precursor for **2** suggested potential oxidation and reduction between hydroquinone, semiquinone and quinone, presumably by dehydrogenases requiring nicotinamide coenzymes. Therefore, the following discussion will focus briefly upon these enzymes.

Monooxygenases

Monooxygenases react with molecular oxygen. They introduce one atom of an oxygen molecule into the substrate, while the second oxygen atom forms water with a reduced cofactor.[2,3] NADH, NADPH, L-ascorbic acid, and tetrahydrobiopterin are used as cofactors by the so-called external monooxygenases, which require externally added reducing reagents.

$S + O_2 + RH_2 \dashrightarrow SO + H_2O + R$

S: substrate; RH_2: reduced cofactor;

SO: oxygenated product; R: oxidized cofactor

The so-called internal monooxygenases refer to those that split hydrogen from the substrate itself.[2,3]

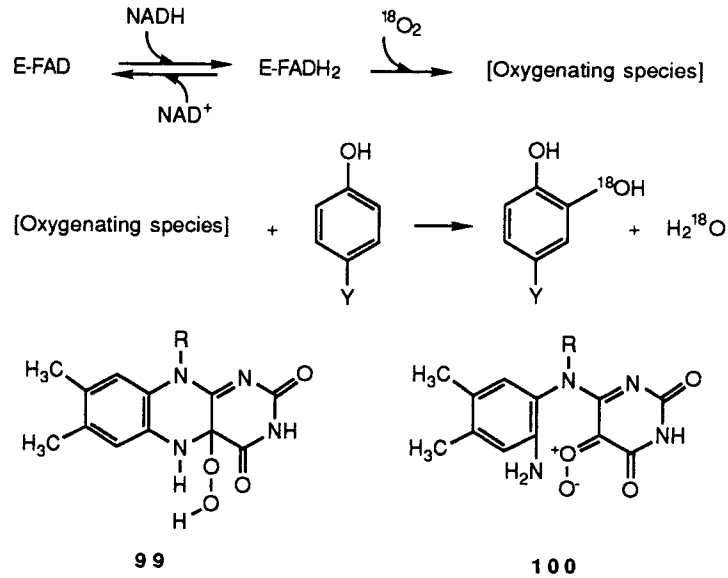
$SH_2 + O_2 \dashrightarrow SO + H_2O$

SH_2: substrate; SO: oxygenated product

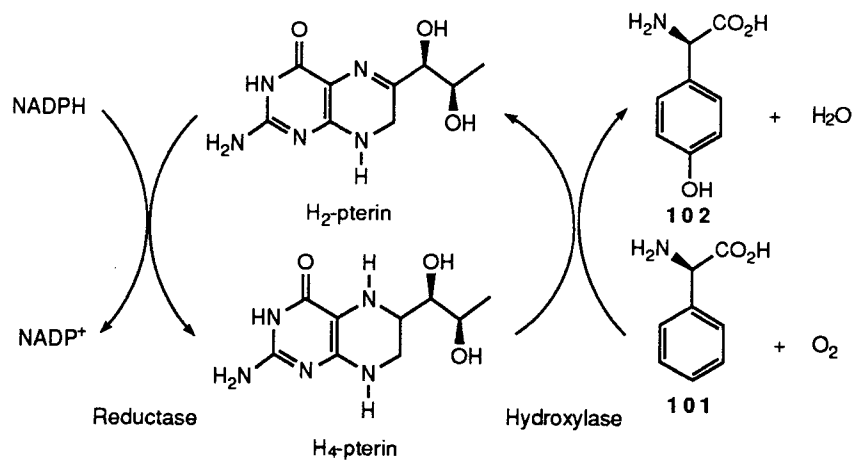
There is great variability in the way hydrogen that is cleaved from the cofactor is transferred to the oxygenating enzyme. In the simplest case, it is taken over by a flavin enzyme which also carries out the oxygenation. Enzymes of this type include, for instance, flavoprotein hydroxylases isolated from bacteria (Scheme 29). Both the flavin hydroperoxide, **99**, and the carbonyl oxide, **100**, have been proposed as the "hydroxylating species", although further experiments are required to determine the nature of the oxygen-transfer agent.[4]

Flavoprotein hydroxylases are restricted to bacteria and eukaryotic microorganisms. In animal cell metabolism, monooxygenase reactions do not

in general involve flavin enzymes in the actual hydroxylation step. Enzymes of this type are more complicated in the sense that they dehydrogenate the cofactor by a separate protein which itself interacts with the oxygenating enzyme. An example of this type is phenylalanine monooxygenase,[5] which catalyzes the hydroxylation of phenylalanine, **101**, to form tyrosine, **102**, requiring tetrahydrobiopterin as cofactor in the presence of NADPH and molecular oxygen (Scheme 30).



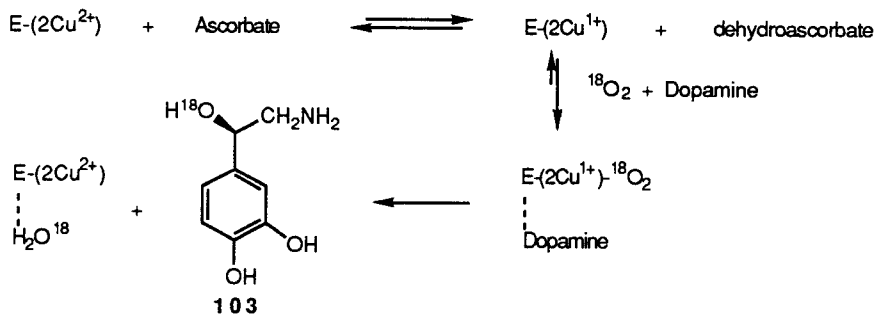
Scheme 29. Flavin-dependent Monooxygenase



Scheme 30. Pterin-dependent Monooxygenase

Unlike the tightly bound flavin enzyme, the pterin cofactors dissociate from the enzyme at the end of each catalytic cycle and are regenerated by action of the dihydropteridine reductase. It is the consecutive actions of the hydroxylase/reductase couple that complete the overall hydroxylation of pterin-dependent monooxygenase.

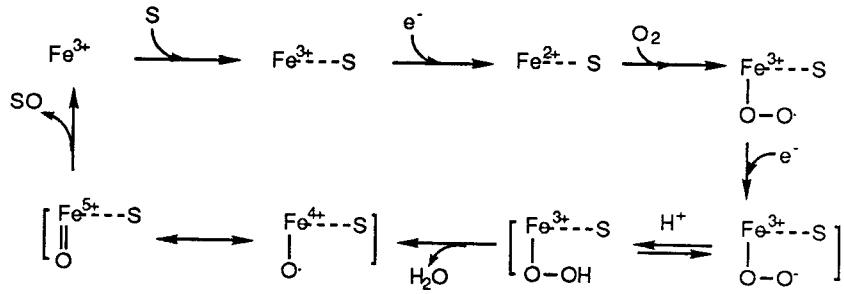
As in the reaction catalyzed by flavin- or pterin-dependent monooxygenases, which require an external reducing agent, NADH or NADPH, ascorbate is specifically required as the reducing agent in some of the copper-containing monooxygenases. For example, it has been shown that in dopamine-β-hydroxylase,[6] ascorbate binds to the Cu^{2+}-enzyme and reduces it to Cu^{1+}-enzyme, which presumably interacts with O_2 to activate it for subsequent hydroxylation. Scheme 31 shows one of the proposed catalytic cycles with two copper atoms in electric contact in the hydroxylation of dopamine to from norepinephrine, **103**.



Scheme 31. Ascorbate-dependent Copper-containing Monooxygenase

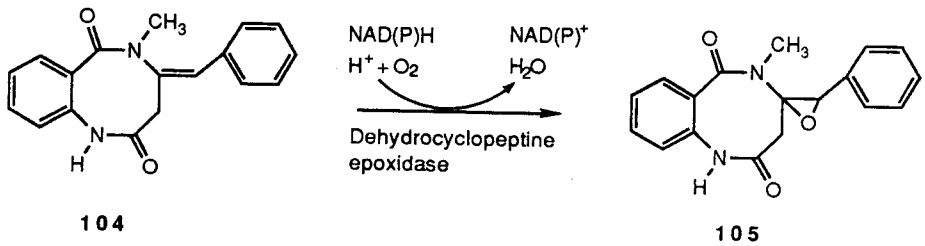
The most complex systems are the cytochrome P_{450}-dependent monooxygenases.[2,7-9] These complexes possess special electron transport chains with cytochrome P_{450} at the terminal site. Its catalytic cycle has been deduced largely from a study of the camphor hydroxylase of *P. putida*.[10,11] As shown in Scheme 32, it begins with binding of the substrate (S) to the ferric enzyme. The substrate-bound ferric enzyme accepts one electron from the flavoprotein NADPH-cytochrome P_{450} reductase to yield the corresponding substrate-bound ferrous enzyme, which binds molecular oxygen rapidly to form an oxygenated complex. This oxygenated complex can accept a second electron from the flavoprotein, iron-sulphur protein (ferredoxins) and/or cytochrome b_5, depending upon the source of the enzyme, to generate an

activated iron-oxygen complex. It has been suggested that this complex is a perferryl oxygen, formally $(Fe=O)^+$, and is the ultimate oxidizing species. Oxygen is then added to substrate and the oxygenated substrate (SO) dissociates to leave the iron in the original ferric state.



Scheme 32. Cytochrome P_{450}-dependent Monooxygenase

As discussed above, the most extensively studied monooxygenase-catalyzed reactions are those of various hydroxylations, particularly hydroxylation at unactivated carbons and aromatic rings. Monooxygenases-catalyzed epoxidations, for instance those which catalyze various epoxidation reactions of essential fatty acids and fatty acid derivatives[12-15] and those involved in the biosynthesis of steroids,[8,14,16,17] have attracted significant attention of both chemists and biochemists.[8,9] Mechanistically, the two types of oxygen insertion are very similar, and in some cases it has been suggested that these oxygenation reactions are likely to arise from the same type of oxygen species, presumably with the oxidation state of peroxide.[18]



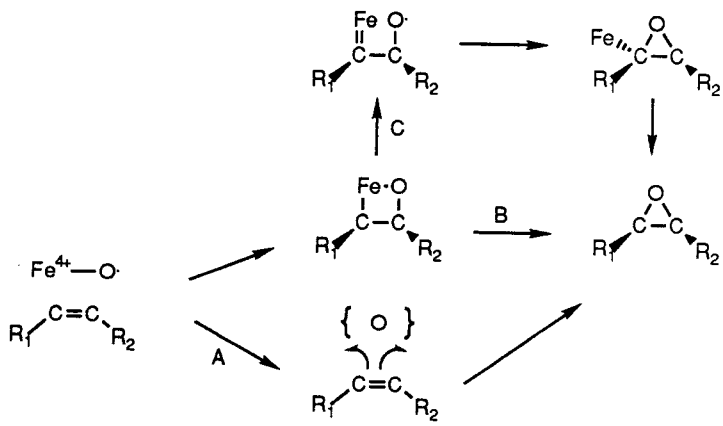
104 105

Scheme 33. Epoxidation Catalyzed by a Flavin-dependent Monooxygenase

As an example of a flavin protein, Voigt and Luckner isolated the dehydrocyclopeptine epoxidase[19] from *Penicillium*, which catalyzed the

epoxidation of dehydrocyclopeptine, **104**, to form cyclopepenin, **105**. The epoxidation required molecular oxygen and use NAD(P)H, ascorbate or 6-methyl-5,6,7,8-tetrahydropteridine as cofactors (Scheme 33).

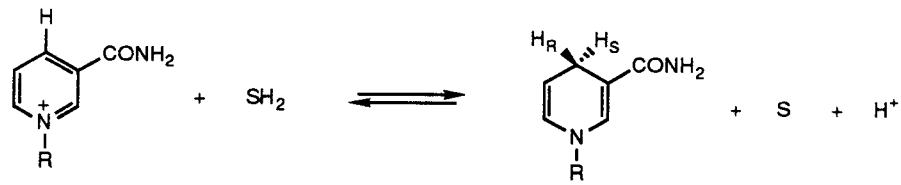
On the other hand, mechanisms of epoxidations catalyzed by iron-containing monooxygenases are very complex and less well understood. For instance, like the P_{450} monooxygenase-mediated hydroxylation, the same perferryl oxygen complex has been generally accepted as the active oxygen-transferring species in P_{450} monooxygenase-mediated epoxidations,[8,9,14,16-17,20-26] such as the epoxidation of steroids,[17] 1,7-octadiene,[20] retinoic acid[22] and 18-hydroxy-9-octadecenoic acid.[25] At least three routes for the delivery of the oxygen to the double bond have been proposed (Scheme 34).[9,17,27,28] The first route involves a concerted insertion of the six-electron oxenoid species into the double bond (path A).[17] The second and third routes are both stepwise mechanisms including the electrophilic addition of the perferryl oxygen complex to the double bond to form an oxometallocyclic species, which could collapse to yield the epoxide directly (path B)[17,27,28] or via an iron carbene species (path C).[27,28] However, deuterium labelling experiments gave conflicting results,[27,28] which in one case supported path C[27] and in another case supported path B.[28]



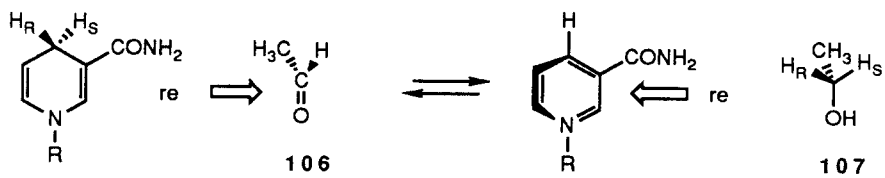
Scheme 34. Epoxidation Catalyzed by a Cytochrome P_{450}-dependent Monooxygenase

Dehydrogenases

Dehydrogenases catalyze the addition or the removal of hydrogen or electrons. The following discussion is limited to those dehydrogenases in which the hydrogen of the substrate is transferred to the pyridine nucleotide NAD^+ or $NADP^+$. The reactions catalyzed by nicotinamide nucleotide-dependent dehydrogenases in most cases are reversible.[29,30]



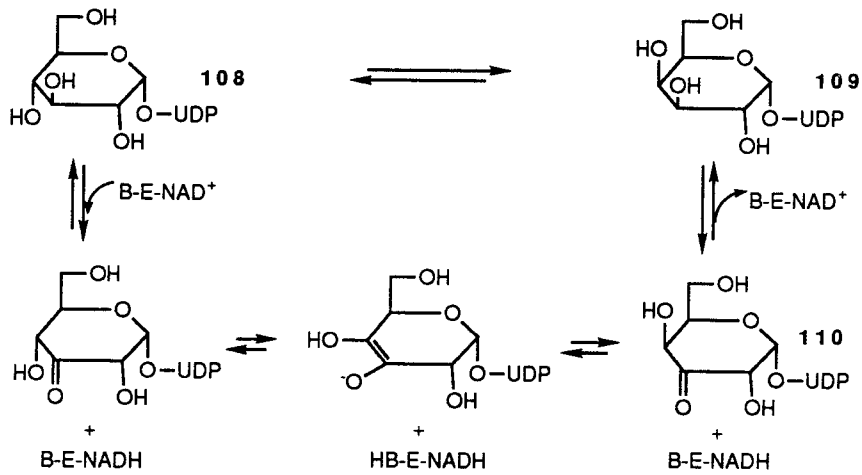
Enzymes which specifically transfer either H_S or H_R from either NADH or NADPH have been reported. For instance, in alcohol dehydrogenase, H_R is transferred from C-4 of NADH to the *re* face of acetaldehyde, **106**, introducing the H_R in the ethanol product. In the direction of ethanol, **107**, oxidation, H_R attack must proceed at the *re* face of the bound NAD^+ (Scheme 35).[31]



Scheme 35. Mechanism for the Stereochemistry of Alcohol Dehydrogenase

As in most cases of dehydrogenases, either NADH or NADPH is an external cofactor as reducing power and an apparent hydride transfer is observed. However, there are exceptions where the redox function is not so apparent in the stoichiometry of the transformation. In these examples, the oxidized coenzyme, which is tightly bound as a 1:1 complex at the outset, remains tightly bound, and functions catalytically. The oxidized form is regenerated at the end of each catalytic turnover cycle, so this role of $NAD(P)^+$/NAD(P)H is distinct from the external role of $NAD(P)^+$/NAD(P)H discussed above. A classical example would be UDP-glucose epimerase.[32] As proposed in Scheme 36, this reaction is an epimerization of UDP-glucose, **108**,

at C-4 to form UDP-galactose, **109**, without predicting any requirement for NAD^+ or $NADP^+$. However, mechanistic studies demonstrated the masked redox chemistry. Thus, the enzyme-bound NAD^+ was first reduced to NADH by abstracting a hydride from **108**; the NADH formed then delivered the necessary reducing power to the 3'-keto-galactose species, **110**, to form **109** with commitment to regenerate NAD^+ for the next cycle.



Scheme 36. Proposed Mechanism for the Epimerization of **108**

Acetamido-1,4-benzoquinone (ABQ) Dehydrogenase from *S.* LL-C10037

The competitive incorporation of **78** and **80** suggested the existence of an active dehydrogenase.[33] This had motivated the search for the ABQ dehydrogenase, which would be responsible for the fast equilibrium between **78** and **80**.

Preparation

It has been generally accepted that the production of secondary metabolic enzymes has a parallel relationship with the production of the metabolites. The growth of *S.* LL-C10037 and the production of **2**, therefore, were monitored over time, and the results are presented in Figure 8. On this

basis, cells were harvested at 96 h by centrifugation at 4 °C. They were then washed with 1 M KCl, then with 0.8 M NaCl to remove any surface proteases, suspended in 10 mM potassium phosphate buffer, pH 7.0, and sonicated with cooling. Centrifugation then yielded a crude cell-free extract, in which the ABQ dehydrogenase activity was readily detected.

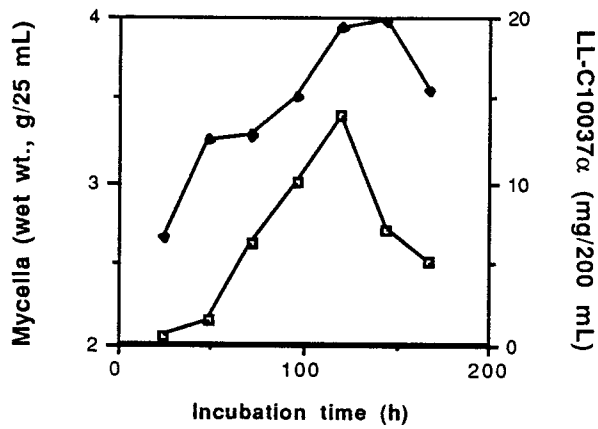


Figure 8. Growth of *S.* LL-C10037 and the Production of **2**

Table 25. Purification of ABQ Dehydrogenase from *S.* LL-C10037

Step	sp U (μmole/min.mg)	Fold
CFE	2.5	1
PS precipitation	5.0	2
AS precipitation	28.6	11
Sephacryl S-200	185.5	74

PS: protamine sulfate; AS: ammonium sulfate. Neither the total activity nor total protein was quantitatively determined.

The following steps of purification included precipitation with polyvinylpolypyrrolidone (PVPP) to remove endogenous phenolic metabolites and with protamine sulfate to remove nucleic acids; the latter was especially important due to the high content of nucleic acid in the crude cell-free extract of *Streptomyces* sp.[34] Ammoniun sulfate precipitation at 0-80% saturation then yielded a pellet which was subsequently purified on a size exclusion column packed with Sephacryl S-200. The profile of the ABQ dehydrogenase on the Sephacryl S-200 column is shown in Figure 9 and the part showing the

enzyme activity is shaded. Because the fractionation range of proteins on Sephacryl S-200 is 5K to 250K, the ABQ dehydrogenase appeares to be smaller than 250K. Fractions containing the ABQ dehydrogenase activity were pooled and used as the source of enzyme for the following studies. The purification from this procedure was 74-fold (Table 25).

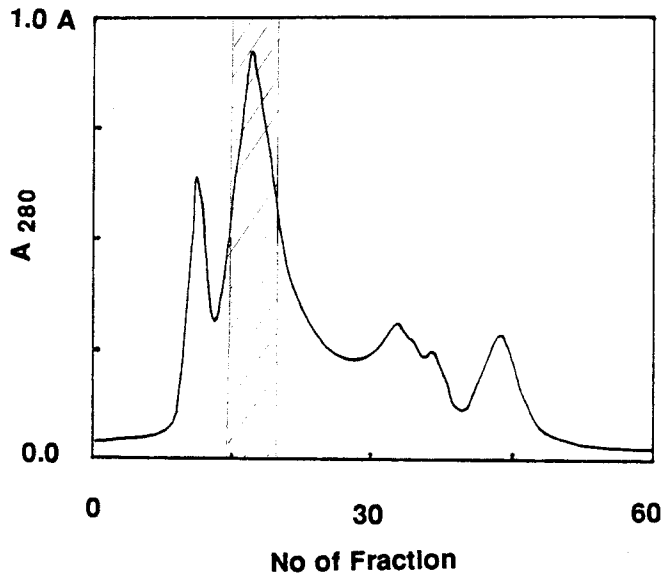


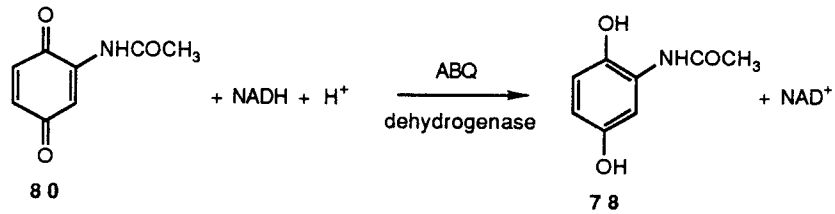
Figure 9. Chromatogram of ABQ Dehydrogenase on a Sephacryl S-200 Column

Enzymatic Formation of 78 from 80

An assay mixture containing the partially purified ABQ dehydrogenase, 0.2 mM NADH, and 0.05 mM **80** in 0.1 M potassium phosphate buffer, pH 7.0, was incubated at 23 °C for 15 min and the reaction was terminated by extraction with EtOAc. The EtOAc extract was subjected to HPLC and TLC analysis with authentic **78** as reference. The product detected had the same retention time on HPLC and R_f on TLC as **78**, and therefore, was assigned as **78** (Scheme 37). However, under the same reaction conditions studied, **80** was not detected when the enzyme preparation was supplied with **78** and NAD^+.

The cofactor requirement of the ABQ dehydrogenase was then investigated. A total of 1.0 mL assay solution containing 0.05 mM **80** and 0.2 mM NADH or NADPH in the presence of the ABQ dehydrogenase in 0.1 M potassium phosphate buffer, pH 6.5, was incubated at 23 °C. The UV absorption at 340 nm

was monitored over a period of 6 minutes. Because the substrates **80** (ε_{340nm} = 1140) and NAD(P)H (ε_{340nm} = 6220) had strong absorption at 340 nm while that of the products **78** (ε_{340nm} = 530) and $NAD(P)^+$ (ε_{340nm} = 0) were very weak, the ΔA_{340nm} was very sensitive to the consumption of **80** and NAD(P)H. Therefore, an UV assay was developed for the assay of the ABQ dehydrogenase and the ΔA_{340nm} measured was proportional to the conversion of **80**. Table 26 summarizes all the results.



Scheme 37. Enzymatic Formation of **78** from **80**

Table 26. ABQ Dehydrogenase Catalyzed Reduction of **80** to **78**

Entry	Assay components	$\Delta A_{340\ nm}$[a]
1	**80** + NADH	0.092
2	**80** + NADPH	0.115
3	E + NADH	0.036
4	E + NADPH	0.048
5	**80** + E + NADH	0.495
6	**80** + E + NADPH	0.127
7	**80** + boiled E + NADH	0.072

a. The absorption coefficients are : NADH ε_{340nm} = 6220, NAD^+ ε_{340nm}= 0, **80** ε_{340nm} = 1140, **78** ε_{340nm} = 530. It has to be mentioned that upon completion of the reduction, the observed total ΔA_{340nm} was approximately twice as large as that calculated according to the absorption coefficients. This can not be explained so far but it appeared to be associated with the enzymatic reduction since the same assay method demonstrated that equimolar **80** and NADH were required for the chemical reduction.

Entry 1 and 2 showed that **80** can be reduced by either NADH or NADPH chemically under the assay conditions, consistent with the difference of the redox potentials between **80** + NADH and **78** + NAD^+, which favors the reduction of **80** thermodynamically.[35] Entry 3 and 4 showed a small consumption of NAD(P)H without the presence of **80**, presumably resulting from contamination of NAD(P)H dehydrogenase in the enzyme preparation.

When NADPH was added to the assay solution, the ΔA_{340nm} (entry 6, 0.127) matched that of the total from the chemical reduction and NADPH oxidation (entry 2 and 4, 0.163). However, when NADH was supplied the ΔA_{340nm} observed (entry 5, 0.495) was four times that of the total from the chemical reduction and NADH oxidation (entry 1 and 3, 0.128), clearly indicating an enzymatic reduction of **80**. Finally, as expected, with boiled enzyme, the ΔA_{340nm} observed (entry 7, 0.072) was no more than that from the chemical reduction and NADH oxidation. Therefore, it was concluded that the ABQ dehydrogenase catalyzed the reduction of **80**, requiring NADH exclusively as cofactor.

pH Dependence

pH dependence of the ABQ dehydrogenase is shown in Figure 10. A total of 1.0 mL assay solution containing 0.05 mM **80** and 0.2 mM NADH in the presence of the ABQ dehydrogenase in 0.1 M potassium phosphate buffer, pH 5.5 to 7.5, was incubated at 23 °C. The UV absorption at 340 nm was monitored for a period of 6 minutes. The ABQ dehydrogenase displayed the maximum activity at pH 6.5. The quinone **80** was unstable in base and started to decompose above pH 7.5.

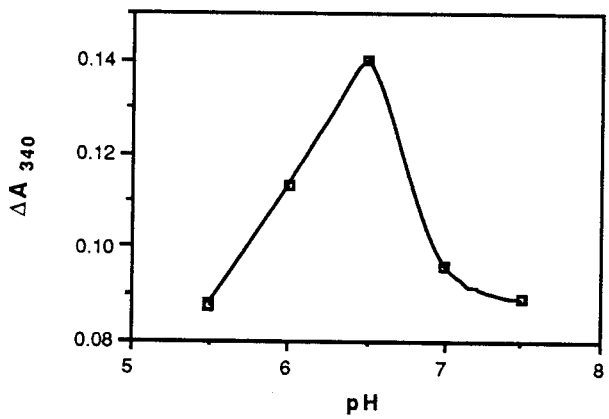


Figure 10. pH Dependence of ABQ Dehydrogenase
ΔA_{340nm} shown here was the net change resulting from the enzymatic reduction. ΔA_{340nm} from both the chemical reduction of **78** and the NADH oxidation have been corrected by sets of controls.

Thermostability

The ABQ dehydrogenase displayed a tremendous thermostability. When an enzyme solution in 0.1 M potassium phosphate buffer, pH 6.0, was pre-heated for 10 minutes, and then was assayed under the same conditions as above, more than 80% of the enzyme activity remained even with the temperature as high as 85 °C. This kind of thermostability is very rare, but has been reported.[36] However, the enzyme activity was totally lost upon boiling because of heat denaturation (Figure 11).

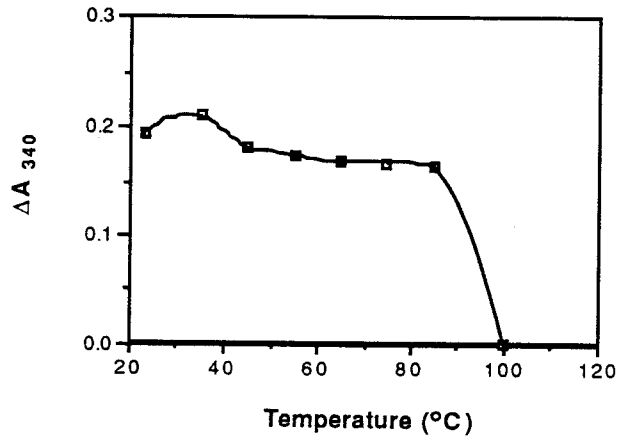


Figure 11. Thermostability of the ABQ Dehydrogenase
As in Figure 10, ΔA_{340nm} shown here was the net change resulting from the enzymatic reduction.

ABQ Dehydrogenase and Biosynthesis of 2

ABQ dehydrogenase isolated from *S.* LL-C10037 catalyzed the conversion of **80** to **78**, requiring NADH as coenzyme exclusively. The enzyme had an optimal pH of 6.5 and showed a tremendous thermostability. The detection of this dehydrogenase was consistent with the *in vivo* studies of the biosynthesis of **2**. The competitive feeding experiment demonstrated that both **80** (7.9% incorporation) and **78** (10.2% incorporation) were almost equally effectively incorporated, suggesting the ease of the redox equilibration between them. However, enzymatic conversion *in vitro* indicated that the conversion between **80** and **78** was unidirectional, eliminating the possibility of **78** as the immediate precursor of **80** on the pathway to biosynthesis of **2**. These results

further suggested that **80** was not an obligatory intermediate and its incorporation into **2** resulted from the easy availability of this fortuitous ABQ dehydrogenase. Therefore, the substrate for the epoxidation to form **70** should be **78**.

The mechanism of enzymatic quinone reduction has been studied and different processes for the electron transfer between the coenzyme and the substrate have been proposed.[37] For instance, the mitochondrial NADH dehydrogenase catalyzed a one-electron reduction of quinone and the semiquinone anion radical thus formed was detected.[37a,c] On the other hand, either two-electron-transfer or mixed-type mechanisms have been proposed in some other cases.[37b,c]

2,5-Dihydroxyacetanilide (DHA) Epoxidase from *S.* LL-C10037

The isolation of ABQ dehydrogenase confirmed the rapid conversion of **80** to **78**, consistent with the *in vivo* studies. However, in order to establish unambiguously whether **80** or **78** was epoxidized, it became necessary to isolate the epoxidase activity, which presumably would catalyze only the epoxidation of **78**, if the incorporation of **80** indeed resulted from the fortuitous existence of the ABQ dehydrogenase.

Preparation

As in the preparation of the ABQ dehydrogenase, the washed cells were used but in this case, they were suspended in 50 mM potassium phosphate buffer containing 20% glycerol and 0.2 mM EDTA. The resulting cell suspension was treated with PVPP (3 mg/mL) and 1 mM phenylmethylsulfonyl fluoride (PMSF),[38] sonicated with cooling, and centrifuged to afford a crude cell-free extract (CFE).

Enzymatic Formation of 70 from 78

When either 0.1 mM **78** or 0.1 mM **80** in 0.5 M potassium phosphate buffer, pH 6.5, was initially incubated with either 0.2 mM NADH or 0.2 mM NADPH, respectively, in the presence of CFE for 5 h at 30 °C, **70** was detected by

TLC of the EtOAc extracts. For clear identification of the product, a 100-ml solution containing 0.13 mmole of **78** and 0.069 mmole of NADH in 0.5 M potassium phosphate buffer, pH 6.5, was incubated in the presence of 50 mL of CFE at 28 °C and 250 rpm in a rotary incubator for 13 h. Extractive workup and preparative TLC yielded quantities of two products that were confirmed to be **80** and **70** by ^{1}H NMR spectroscopy. The isolation of **80** from the assay mixture presumably resulted from air oxidation of **78**; a stirred aqueous solution of **78** will be oxidized to **80** over such a period of time. However, the formation of **70** clearly must have been enzymatic.

Although the isolation of the ABQ dehydrogenase suggested that **78** would be more likely to be epoxidized, unfortunately at that stage, the quinone **80** was mistaken as the substrate for the epoxidase. This was mainly concluded from reasoning by analogy to the NNM-A monooxygenase reported by Omura and coworkers[39], which catalyzed the epoxidation of **47** (a quinone) to form **48** in the presence of either NADH or NADPH. Consequently, the epoxidase was misnamed as "ABQ epoxidase" and the cofactor requirements of the "ABQ epoxidase" were investigated with **80** as the substrate. The results are presented in Table 27.

Table 27. Epoxidation of **78** by a CFE Preparation from *S.* LL-C10037

Entry	Assay components[a]	Products by TLC			%**70**[b]
	Aerobic				
1	**80** + NADH + CFE	**78**		**70**	3.8
2	**80** + NADPH + CFE	**78**		**70**	3.8
3	**80** + NADH	**78**	**80**		
4	**80** + NADPH	**78**	**80**		
5	**80** + CFE	**78**		**70**	11.6
6	**80** + **78** + NADH + CFE	**78**	**80**	**70**	10.1
	Anaerobic[c]				
7	**80** + NADH + CFE	**78**			
8	**80** + NADPH + CFE	**78**			

a. 10 mL assay solution containing 5 mL of CFE, 0.1 mM **80**, 1.0 mM NADH or NADPH, in 0.1 M potassium phosphate buffer, pH 5.5, was incubated at 30 °C for 2 h.
b. Determined by extraction with EtOAc after saturation with NaCl, preparative TLC of the extract, and UV quantitation ($\varepsilon_{310\ nm} = 7.2 \times 10^3$, MeOH).
c. Run under N_2 atmosphere.

From entries 1 and 2 it is clear that O_2 and either NADH or NADPH can support the epoxidation of **80** and from entries 3 and 4 that the reaction is clearly enzymatic. However, in all four cases, **78** was always produced, the formation of which can be explained from either enzymatic reduction by ABQ dehydrogenase or chemical reduction by NAD(P)H. This reduction complicated the observation that **70** could result from **78** instead of from **80** directly. The former was further supported by the observation that substantially more **70** was produced when **78** was provided in addition to **80** (entry 6). The formation of **70** in entry 5 could then be explained by the assumption that sufficient NAD(P)H was present in the CFE to reduce **80**. Finally, as expected, under anaerobic conditions only **78** was produced from **80** (entries 7 and 8).

From the data presented above, it became necessary to remove the ABQ dehydrogenase and the endogenous NAD(P)H from the CFE in order to unambiguously determine whether **78** or **80** was the substrate for the epoxidase. Thus, the CFE was subjected to several steps of fractionation and a partially purified DHA epoxidase preparation (after the Phenyl-5 PW column, for detail see Chapter 5) was used to re-investigate its cofactor requirement. The results are presented in Table 28.

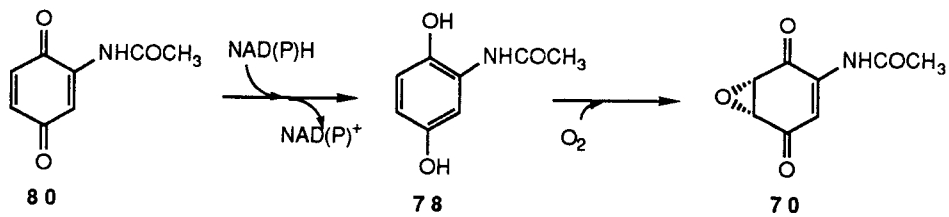
Table 28. DHA Epoxidase Catalyzed Epoxidation of **78**

Entry	Assay components[a]	Incubation time (min)	Product (% mmole)[b] **78**	**80**	**70**
1	**78** + NADH + NAD^+	5	95	5	0
2	**80** + NADH + NAD^+ + E	5	45.2	41.3	13.4
3	**78** + NADH + NAD^+ + E	5	66.4	0	33.6
4	**80** + E	5	11.7	88.3	0
5	**80** + E	10	17.5	79.8	2.7
6	**80** + E	20	18.1	75.7	6.2
7	**80** + E	30	19.1	70.9	10.1
8	**78** + E	5	68.2	2.1	29.7
9	**78** + E	10	46.4	1.2	52.4
10	**78** + E	20	22.5	0	77.5
11	**78** + E	30	12.8	0	87.2

a. A 0.5-mL assay solution containing 0.1 mM **78** or **80**, 0.1 mM NADH and NAD^+, and 150 μL enzyme solution in 0.1 M potassium phosphate buffer, pH 7.0, was incubated at 30 °C for a period of 5 to 30 minutes.

b. The assay mixture was directly analyzed on Radi-Pak C_{18} cartridge column in a mobile phase of H_2O/CH_3CN/TFA = 88/12/0.1 and quantitated by calibration of the column with authentic samples.

Entry 1 confirmed that under the assay conditions **78** cannot be chemically oxidized back to **80**. Entries 2 and 3 clearly showed that in the presence of NADH, **78** (33.6% **70** formed) was a better substrate for the epoxidation than **80** (13.4 % **70** formed). Both **78** and **80** were subsequently incubated in the absence of any added cofactor. Surprisingly, **78** was very efficiently epoxidized to yield **70** in almost 90% conversion in 30 minutes (entries 8 to 11), while **80** was barely epoxidized (entries 4 to 7). Therefore, it was finally concluded that **78** was the actual substrate for the epoxidation and the "ABQ epoxidase" actually catalyzes the epoxidation of **78** in the presence of molecular oxygen to form **70**, requiring no added cofactor. Although NADH and **80** can formally support the epoxidation as well, neither NADH nor **80** was necessary for the epoxidation and their presence served only to generate **78** *in situ*, which was subsequently epoxidized (Scheme 38). At that moment, the enzyme was re-named correctly as the 2,5-dihydroxyacetanilide (DHA) epoxidase. It has to be mentioned that a noticeable amount of **78** was observed in the assay mixture of **80** without the presence of NADH. This is difficult to explain, although some **78** can always be detected in the methanol-H_2O solution of **80**.



Scheme 38. Enzymatic Formation of **70** from **78**

DHA Epoxidase and the Biosynthesis of 2

DHA epoxidase, isolated from *S.* LL-C10037, catalyzed the epoxidation of **78** to yield **70** in the absence of any added coenzyme. Molecular oxygen is required for this process and this enzyme is a monooxygenase. Consistent with the *in vivo* studies, the isolation of the DHA epoxidase unambiguously established **78** as the substrate for the epoxidation and determined the relative position of **78** on the pathway for the biosynthesis of **2**. Thus, it was **78** that was epoxidized to form **70**, which was subsequently reduced to **2**. Such

experiments emphasize the effectiveness of the applications of both *in vivo* and *in vitro* studies to establish biosynthetic pathways. The complementary results from the *in vivo* and *in vitro* studies, as in this case, led to the isolation of the DHA epoxidase, which in turn confirmed **78** as an obligatory intermediate for the biosynthesis of **2**.

Omura and coworkers reported an enzyme activity in the study of the biosynthesis of **48** in *S. rosa* var. *notoensis*.[39] The enzyme catalyzed the epoxidation of quinone **47** in the presence of O_2 and either NADH or NADPH, and was named as NNM-A monooxygenase in which a quinone was the substrate for the epoxidation. In the study of the biosynthesis of **52** in *P. patulum*,[40a,b] Light and Priest reported an enzyme preparation which catalyzed the epoxidation of **45** and **54** (both are hydroquinones) to form the corresponding epoxyquinones in the absence of any added coenzyme. Their preliminary studies suggested that a metal ion and possibly a sulfhydryl group might be involved in the reaction. The third related enzyme activity would be the vitamin K epoxidase,[41,42] detected in microsomal preparations from rat livers. Although at first glance either NADH or NADPH supported the epoxidation of the quinone **56**, Suttie and coworkers subsequently established that the hydroquinone **57** was the actual substrate for the epoxidation. Vitamin K epoxidase catalyzed the epoxidation of **57** in the presence of molecular oxygen, requiring no added cofactor. Although very little was known about the nature of this epoxidase, it was suggested that the epoxidation of **56** was not mediated by cytochrome P_{450}.[41] In terms of its choice of substrate and requirements of coenzyme, the DHA epoxidase from *S.* LL-C10037 appeared to be more similar to the epoxidase from *P. patulum* and to the vitamin K epoxidase. Unfortunately, in all the three cases discussed above, only the crude or partially purified homogenate preparations were used, which limited further studies to clarify the subtle differences between these enzymes and their mechanism of epoxidations.

2-Acetamido-5,6-epoxy-1,4-benzoquinine (AEBQ) Dehydrogenase from *S.* LL-C10037

The *in vivo* studies have suggested **78**, **80** and **70** as intermediates in the pathway for the biosynthesis of **2**. The *in vitro* studies, on the other hand,

have excluded **80** as an obligatory intermediate and established the late biosynthetic sequence as **78** --> **70** --> **2**. The 2-acetamido-5,6-epoxy-1,4-benzoquinine (AEBQ) dehydrogenase, which should catalyze the reduction of **74** to **2**, was the next to be researched.

Preparation

By using the same protocol as that for the preparation of the DHA epoxidase, the washed cells were suspended in 50 mM potassium phosphate buffer, pH 7.0, containing PVPP (3 mg/mL), 20% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.2 mM EDTA, disrupted by sonication and partially purified by sequential treatment with protamine sulfate and ammonium sulfate. The AEBQ dehydrogenase activity was located in the pellet obtained by 41-62% saturation with ammonium sulfate (Table 29).

Table 29. Preparation of AEBQ Dehydrogenase from *S.* LL-C10037

Enzyme prep.[a]	% HPLC Integration counts[b]	
	70	**2**
CFE	86.8	13.2
PS supernatant	87.4	12.6
AS pellet solution		
20.5-41.1%	100	0
41.1-61.6%	59.6	40.4
61.6-82.2 %	100	0

a. A 500-μL assay solution containing 0.1 mM **70**, 0.2 mM NADPH, 40-100 μL of enzyme preparation in 0.1 M potassium phosphate buffer, pH 7.0, was incubated at 30 °C for 20 minutes.

b. The assay mixture was directly analyzed on a Radi-Pak C_{18} cartridge column in a mobile phase of H_2O/CH_3CN/TFA = 88/12/0.1.

Further purification of this enzyme by various conventional chromatographic techniques and by FPLC techniques has also been explored. The profiles of the AEBQ dehydrogenase on each column are shown in Figures 12, 13 and 14, and the parts showing enzyme activity are shaded. Again, the AEBQ dehydrogenase can be fractionated on the Sephacryl S-200 column, indicating that it is smaller than 250K (Figure 12).

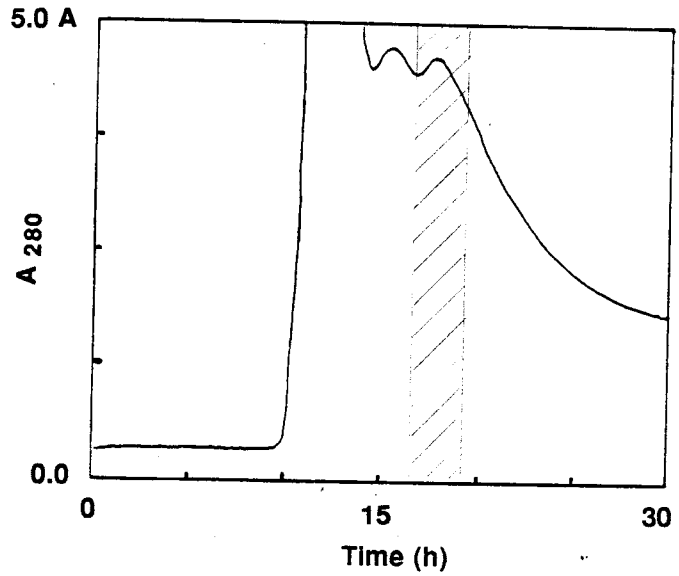


Figure 12. Chromatogram of AEBQ Dehydrogenase on a Sephacryl S-200 Column

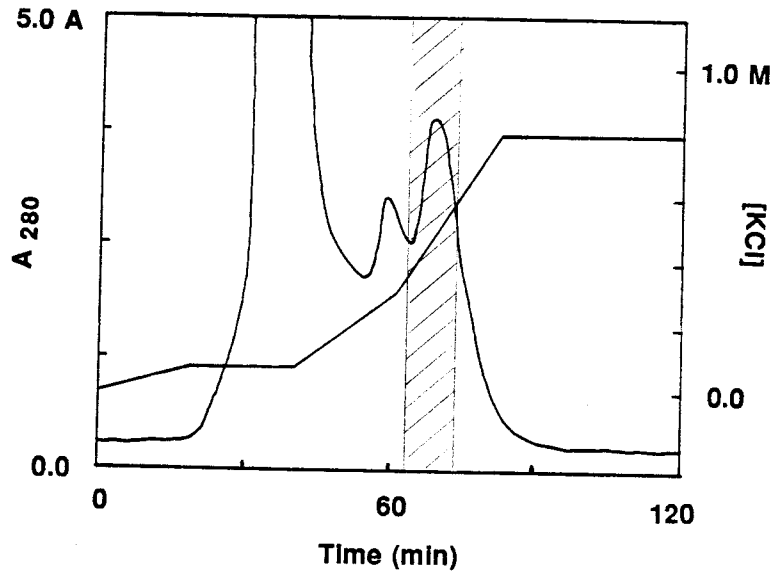


Figure 13. Chromatogram of AEBQ Dehydrogenase on an Accell QMA Column

When the combined active fraction was loaded on an anion exchange column (Accell QMA), the enzyme activity bound to the column under the given conditions. However, it was selectively eluted by applying a KCl gradient (Figure 13). By a totally different mechanism of separation, the combined active fractions from the anion exchange column were next loaded on a hydrophobic column. As shown in Figure 14, the enzyme was eluted in buffer containing very low concentration of $(NH_4)_2SO_4$, indicating that the AEBQ

dehydrogenase might be hydrophobic in nature. Although no quantitative data were collected, the combined application of these columns appeared to be promising for further purification of the AEBQ dehydrogenase.

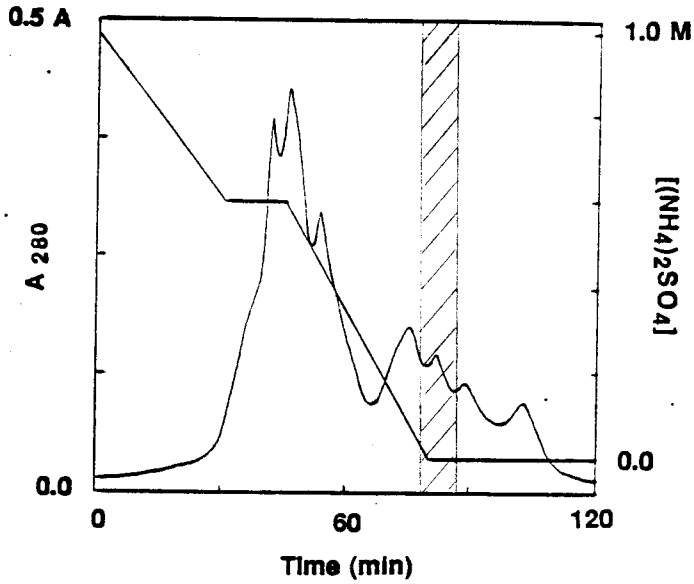


Figure 14. FPLC of AEBQ Dehydrogenase on an HIC (Phenyl 5 PW) Column

Enzymatic Formation of 2 from 70

Initially, a 2.0-mL assay solution containing 0.1 mM **70**, 0.5 mM NADH or NADPH, and 1.0 mL of CFE in 0.1 M potassium phosphate buffer, pH 6.5, was incubated at 28 °C for 18 h. The assay solution was saturated with solid $(NH_4)_2SO_4$ and extracted with EtOAc. Concentration of the EtOAc extract afforded a residue which was analyzed by TLC (CH_3Cl/CH_3OH/Hexane = 5/1/1). By comparision with authentic **2**, it was observed that the major product was **2** if NADPH was supplied, while an unknown compound was produced if NADH was supplied. In order to confirm the enzymatic production of **2**, a 40-mL assay solution containing 1.0 mM **70**, 1.0 mM NADPH, and 20 mL of CFE in 0.1 M potassium phosphate buffer, pH 6.5, was incubated at 28 °C, 225 rpm in a rotary incubator for 2.5 h. Extractive workup and preparative TLC yielded quantities of a product that was confirmed to be **2** by ^1H NMR spectroscopy. Unfortunately, when a large scale incubation (40 mL) with NADH as coenzyme was repeated under the same conditions, **70** was basically unchanged.

Although the production of the unknown was detected by TLC, its structure has not been determined due to the failure of its isolation.

Table 30. Enzymatic Formation of **2** from **70**

Entry	Assay composition[a]	HPLC Integration counts%[b] **70**	**2**
1	**70** + NADH	100	0
2	**70** + NADPH	100	0
3	**70** + NDAH + CFE	>95[c]	0
4	**70** + NDAPH + CFE	16	84
5	**70** + NADPH + CFE (denatured)[d]	100	0

a. A 500 μL assay solution containing 0.1 mM **70**, 0.2 mM NADH or NADPH, and 200 μL of CFE in 0.1 M potassium phosphate buffer, pH 6.5, was incubated at 30 °C for 90 minutes.
b. The assay mixture was directly analyzed on Radi-Pak C_{18} cartridge column in a mobile phase of $H_2O/CH_3CN/TFA$ = 88/12/0.1.
c. This value was estimated on the result of TLC analysis. No **2** was detected and a small amount of **70** was converted to an unknown compound.
d. The enzyme solution was heated in boiling water for 5 minutes.

The cofactor requirements of the reaction were then investigated. As shown in Table 30, entries 1 and 2 indicated that either NADH or NADPH was inert to **70** chemically. Although a small amount of **70** was consumed when it was incubated with CFE and NADH, no **2** was detected (entry 3). On the contrary, when **70** was incubated with CFE and NADPH, more than 80% of **70** was converted to **2** (entry 4), clearly demonstrating the requirement of NADPH for the enzymatic reduction. Finally, entry 5 showed that the reaction was enzymatic and that the AEBQ dehydrogenase activity was totally lost upon heat denaturation. Furthermore, **2** can be enzymatically oxidized back to **70** if the AEBQ dehydrogenase and $NADP^+$ were provided. Therefore, the AEBQ dehydrogenase catalyzed a reversible conversion between **70** and **2**.

Molecular Weight Determination

The AEBQ dehydrogenase has not yet been purified to homogeneity. The molecular weight (MW) of the enzyme was therefore estimated by the gel filtration method using a Protein-Pak 300 SW column (8x300 mm, Waters Assoc.). Fractions from the size exclusion column were assayed and the

retention time of the fraction showing the maximum activity was assigned as that of the AEBQ dehydrogenase. As shown in Figure 15, the AEBQ dehydrogenase was assigned a molecular weight of 100K ± 30K by comparison with standards of known MW.[43] Although a very good linear correlation of the standards was obtained (R = 0.984), it has to be pointed out that the MW determined by this manner is only an approximate value; this is particularly true for large proteins because of their relatively small differences of retention times. As evidence for this statement, β-amylase, which is three times larger than bovine serum albumin (BSA), was eluted only 3.0 min earlier than BSA. A half minute difference of retention time could lead to 20K-25K difference in the MW determined; therefore, care has to be exercised when analyzing these data.

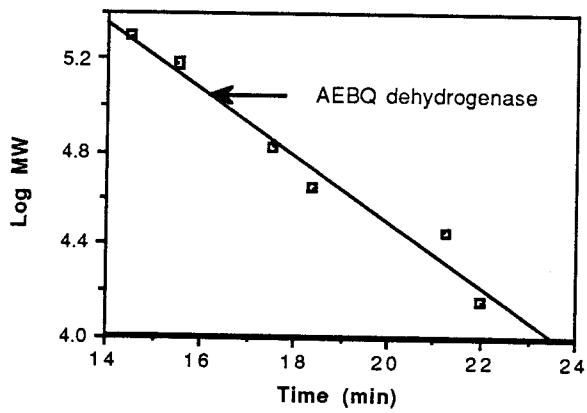


Figure 15. MW Determination of AEBQ Dehydrogenase on a Protein-Pak 300 SW Column MW standards: α-lactalbumin, 14,200; carbonic anhydrase, 29,000; chicken egg albumin, 45,000; bovine serum albumin, 66,000; alcohol dehydrogenase, 150,000; β-amylase, 200,000.

pH Dependence

Figure 16 shows the pH dependence of the AEBQ dehydrogenase. For the reduction of **70** in the presence of NADPH in 0.1 M potassium phosphate buffer, the AEBQ dehydrogenase displayed a maximum activity at pH 7.0. On the other hand, when the oxidation of **2** to **70** was assayed in the presence of $NADP^+$ in 0.1 M potassium phosphate or Tris-HCl buffer, a steady increase of the activity was observed from pH 6.5 to 8.0. It has to be pointed that **70** was not

stable under basic conditions and significant decomposition occurred with pHs above 7.5.

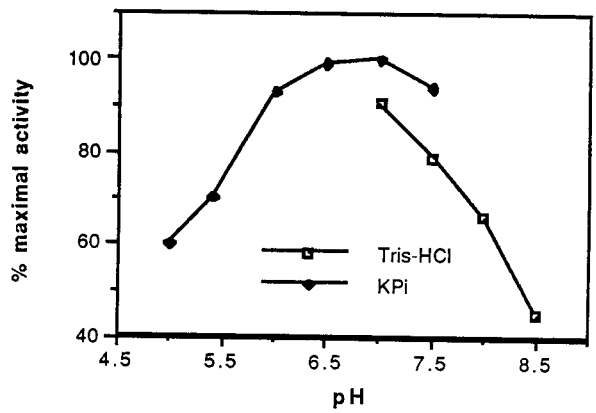


Figure 16. pH Dependence of AEBQ Dehydrogenase

Kinetics

Partially purified AEBQ dehydrogenase was used for the kinetic study. Thus, the CFE was first treated sequentially with protamine sulfate and ammonium sulfate. The active ammonium sulfate pellet was then further purified on a size exclusion column (Sephacryl S-200), an anion exchange column (Accell QMA) and an HIC (phenyl 5 PW) column.

Steady-state approach and pseudo-1st order treatment[44-46] have been applied to perform the kinetic study for the reduction of **70**. The kinetic study was done in 0.1 M potassium phosphate buffer, pH 7.0. Because **70** and NADPH have relatively strong absorption at 340 nm (**70** ε_{340nm} 2600 and NADPH $\varepsilon_{340\ nm}$ 6220) and **2** and $NADP^+$ have almost no absorption at all at the same wave length (**2** ε_{340nm} 83 and $NADP^+$ 0), an ideal spectroscopic assay was developed by monitoring continuously the change of A_{340nm}. Over a given time period, ΔA_{340nm} measured was proportional to the conversion of **70** and Figure 17 and 18 show the dependence of the initial velocity of the reduction on either **70** or NADPH concentration, respectively. Lineweaver-Burk plots of the results (R = 0.994 for NADPH and R = 0.988 for **70**) yielded an apparent K_m of 45 ± 3 μM for NADPH, and 8.2 ± 0.5 μM for **70**.

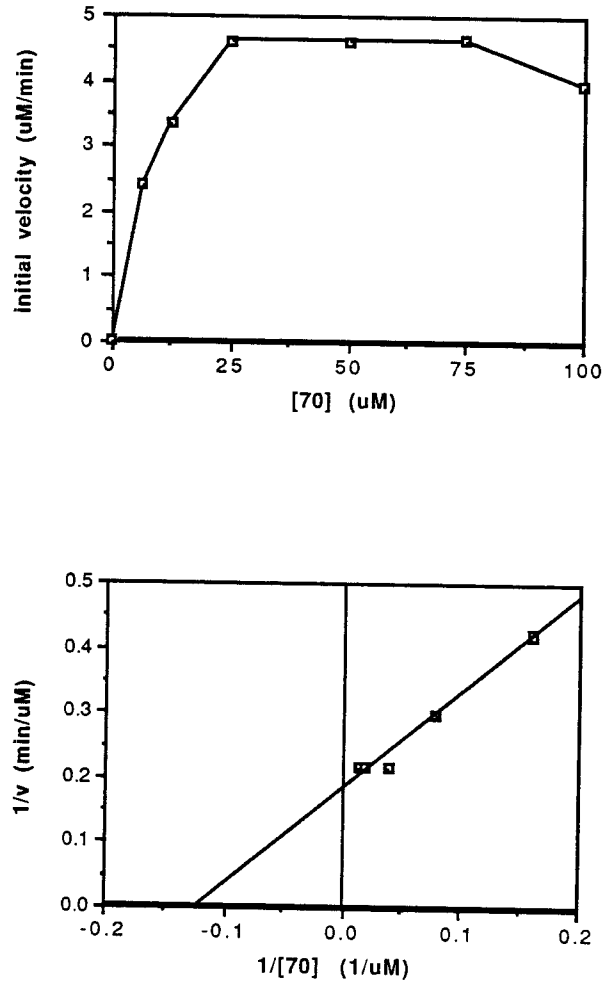


Figure 17. Effect of **70** Concentration on AEBQ Dehydrogenase Activity. A 1.0 mL assay solution containing 0.5 mM NADPH, indicated concentration of **70** (6.25 μM to 100 μM), and 50 μL of the AEBQ dehydrogenase in 0.1 M potassium phosphate buffer, pH 7.0, was incubated at 22 ^{o}C. The initial velocity was determined by ΔA_{340nm} measured over the initial period of 7.5 minutes.

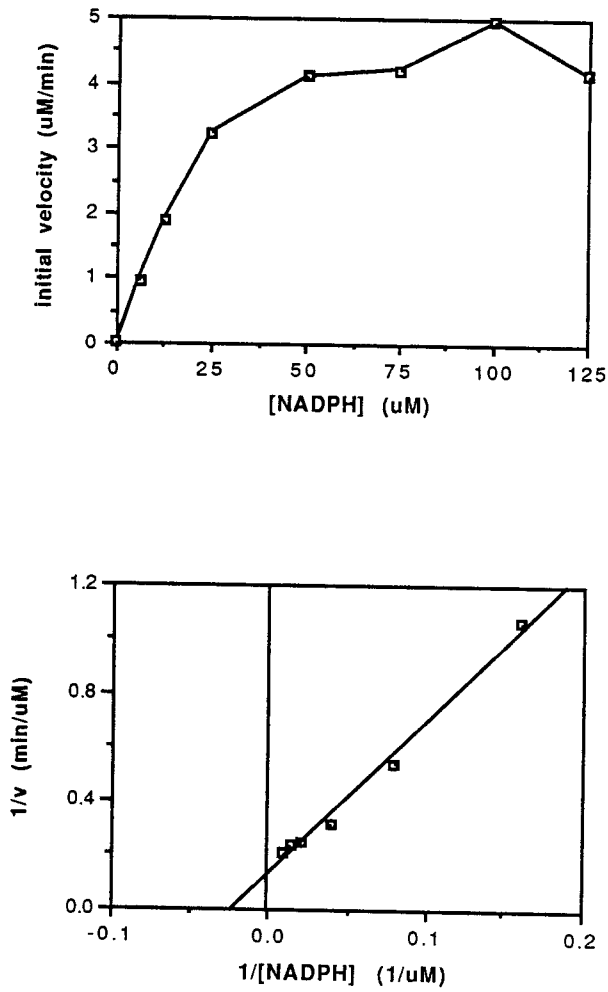
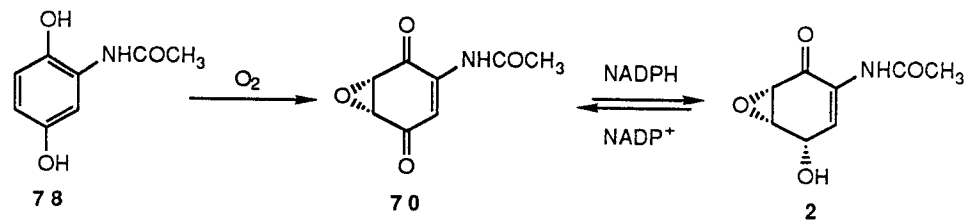


Figure 18. Effect of NADPH Concentration on AEBQ Dehydrogenase Activity. Assays were run under the same conditions as described in Figure 9 except they contained 0.5 mM **70** and the indicated concentration of NADPH (6.25 μM to 125 μM). The initial velocity was determined by ΔA_{340nm} measured over the initial period of 7.5 minutes.

AEBQ Dehydrogenase and the Biosynthesis of 2

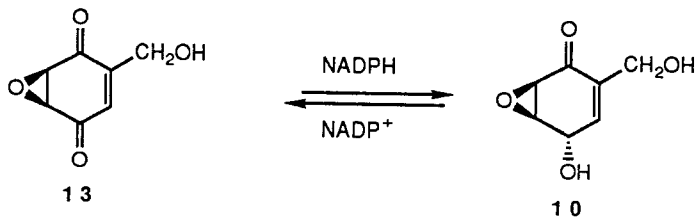
AEBQ dehydrogenase, isolated from *S.* LL-C10037, catalyzed the reduction of **70** to **2**, requiring NADPH as the exclusive coenzyme. This enzyme has a molecular weight of 100K ± 30K and an optimal pH of 7.0. The apparent K_m's for **74** and NADPH are 8.2 μM and 45.3 μM, respectively. Consistent with the *in vivo* studies, the isolation of AEBQ dehydrogenase resolves the last step of the biosynthesis of **2**. Thus the late stage of the biosynthesis of **2** can be revised as shown in Scheme 39.



Scheme 39. Biosynthesis of **2** in *S.* LL-C10037

Although numerous epoxysemiquinones have been reported,[47-78] and in some cases, the corresponding epoxyquinones were found as co-metabolites, relatively little has been explored in the biosynthesis of the semiquinone functionality at the enzyme level. In the biosynthesis of **52**, Sekiguchi and Gaucher reported a cell-free extract prepared from *P. urticae*,[79] which catalyzed the oxidation of **10** to **13** (Scheme 40). This enzyme, or isoepoxydon dehydrogenase as it was called, required $NADP^+$ as the coenzyme. The reversible interconversion of **13** to **10** occurred optimally at pH 5.8 and was completely inhibited by 1 mM *p*-chloromercuribenzoic acid (PCMBA). This enzyme activity displayed interesting substrate specificity: epoxydon, **9**, an epimer of **10**, was not utilized. While a *trans* stereochemistry of the reduced semiquinone, relative to the oxirane, was preferred in the isoepoxydon dehydrogenase, the AEBQ dehydrogenase, isolated from *S.* LL-C10037, represents a new kind of enzyme for the formation of epoxysemiquinones in the sense that NADPH is required exclusively as coenzyme and *cis* stereochemistry of the semiquinone relative to the oxirane is preferred. It would be interesting to study the reaction mechanism of the reduction,

particularly to understand how the *cis* and the *trans* stereochemistry are controlled.



Scheme 40. Enzymatic Interconversion of **10** and **13**

2.5-Dihydroxyacetanilide (DHA) Epoxidase from *S.* MPP 3051

Results from both *in vivo* and *in vitro* studies have led to the complete establishment of the pathway for the biosynthesis of **2**. Several enzymes related to the pathway have been isolated. One of them, DHA epoxidase, catalyzes the epoxidation of **78** to yield **70**, generating the absolute stereochemistry for this series of metabolites from a planar substrate. The structure and the absolute stereochemistry of **2** and **70** have been established.[81] Having the same gross structure and relative stereochemistry, **4**, produced by *S.* MPP 3051,[82] has been assigned as the desacetyl enantiomer of **2** by comparing the specific rotations.[81,82] Therefore, it had been subsequently postulated that the biosynthesis of **4** could either be totally different from that of **2** or more simply, be the same (exclusive of the stereochemistry) with addition of a deacetylation as the last step.[34,83] According to the latter, it could be further postulated that the stereochemistry of **4** and its related intermediates would be generated in the same fashion as that in *S.* LL-C10037 by the epoxidation of **78**. But in the case of *S.* MPP 3051, the DHA epoxidase would have an opposite facial specificity to lead to the enantiomeric series of metabolites. From this perspective, the search for the DHA epoxidase from *S.* MPP 3051 was initiated.

Preparation

When the Beecham seed medium[82] was initially used for the fermentation of *S.* MPP 3051, no sign of growth was observed under the given conditions. After several variations, the Beecham seed medium was given up and the Lederle seed medium[80] was adopted for the growth of *S.* MPP 3051. For the production stage of fermentation, either the Beecham production medium[82] or the Lederle production medium[80] was used. *S.* MPP 3051 grew in both media but the production appeared better in the Lederle medium. Therefore, the Lederle medium has been utilized through this study.

As in the preparation of the DHA epoxidase from *S.* LL-C10037, the same protocol was followed for the preparation of the DHA epoxidase from *S.* MPP 3051. Thus, cells were harvested from a fermentation of *S.* MPP 3051 at 22.5 h[84] by centrifugation at 4 °C and washed with 1 M KCl and 0.8 M NaCl to remove any surface proteases. The washed cells were suspended in 50 mM potassium phosphate buffer, pH 7.0, containing 20% glycerol, 1 mM PMSF, 3 mg/mL PVPP and 0.2 mM EDTA, and disrupted by sonication. Centrifugation then yielded a crude cell-free extract (CFE).

Enzymatic Formation of 77 from 78

A total of 500-μL assay solution containing 0.14 mM **80**, 0.25 mM either NADH or NADPH, and 300 mL of CFE was incubated at 30 °C. The assay mixture was analyzed by HPLC and Table 31 summarizes all the results.

Table 31. Formation of **77** in a Cell-free System from *S.* MPP 3051

Entry	Assay components[a]	Incub. time (min)	%77[b]
1	**80** + NADH + CFE	7.5	100
2		15	55
3		30	0
4		90	0
5	**80** + NADPH + CFE	30	0

a. Prior to recognizing the correct substrates, **80** and either NADH or NADPH, both of which are capable of generating **78**, were used for the enzyme incubation.
b. The assay mixture was directly analyzed on Radi-Pak C_{18} cartridge column in a mobile phase of H_2O/CH_3CN/TFA = 85/15/0.1.

At that time, it had not been realized yet that **78** was the actual substrate for the epoxidase. As that experienced by the DHA epoxidase from *S.* LL-C10037, the quinone **80** was mistaken as the substrate to be epoxidized in the presence of either NADH or NADPH with the analogy to the NNM-A monooxygenase.[39] When **80** was initially incubated with either NADH or NADPH in the presence of CFE for 30 min or 90 min, HPLC analysis of the assay mixtures showed a total disappearance of **80** with no detection of any recognizable products (entries 3, 4 and 5), suggesting that some other activities in the CFE could very efficiently consume **80**. However, when the incubation time was cut down first to 15 min, **77** was detected as the major product (entry 2) and subsequently to 7.5 min, **77** was virtually the only product observed (entry 1). Entry 2 also showed the efficiency of metabolism of **77** by other activities in the CFE and almost 50% of the **77** formed was further metabolized in an additional 7.5 minutes. Provided that most of the enzymes related to the biosynthesis of **4** were present in the CFE, the transitory accumulation of **77** in the assay solution and the unstable nature of the final metabolites would be consistent with the observation of fermentations of *S.* MPP 3051. It has been reported that **4** decomposed readily upon concentration.[82] Our efforts to isolate **4** and **3** as well as other related metabolites have also proven to be fruitless.[84]

For a clear identification, it became necessary to remove the enzyme activities which catalyzed further conversions of **77**, so that **77** could be produced in quantity for spectroscopic analysis. Therefore, the CFE was partially fractionated by sequential treatments with protamine sulfate and ammonium sulfate. An ammonium sulfate pellet at 62-92% saturation was used as the DHA epoxidase for the *in vitro* preparation of **77**. Thus a 12-mL solution of the DHA epoxidase was prepared from mycelia from 300-mL fermentation broth. An assay solution (24 mL total) containing 1.0 mM **80**, 1.0 mM NADH, and 12 mL of the DHA epoxidase in 125 mM potassium phosphate buffer, pH 7.0, was incubated for 10 min at 30 °C. The reaction was terminated by EtOAc extraction and the EtOAc extract was concentrated *in vacuo* to afford a residue. Chromatography of this residue on a silica gel column afforded a crude product, which was recrystallized from MeOH-EtOAc to give 1.3 mg (30%) of **77**, whose ^{1}H NMR spectrum was identical to that of the authentic **70**. As expected while **70** from the *S.* LL-C10037 pathway has a specific rotation of $[\alpha]^{20}_D$ + 115.6° (c 0.5, MeOH),[80,81] **77** obtained with the DHA epoxidase from *S.* MPP 3051

had a specific rotation of $[\alpha]^{20}_D$ - 112.2° (c 0.1, MeOH).[82] Clearly, **77** and **70** form a pair of enantiomers and the two DHA epoxidases display an opposite facial specificity in the epoxidation of the planar **78**.

As in the DHA epoxidase from *S.* LL-C10037, the initial effort to determine the substrate and the coenzyme requirement was misled by the fact that **80** can be reduced to **78** either chemically by NAD(P)H or enzymatically by ABQ dehydrogenase and NADH. Once that confusion was clarified and **78** was established as the substrate for the DHA epoxidase isolated from *S.* LL-C10037, the cofactor requirement of the DHA epoxidase was re-investigated. As shown in Table 32, under the same incubation conditions, 50.4% of **77** was formed from **78** (entry 4), while very little **77** was formed from **80** (entry 1 and 2). Therefore it was concluded that, as the enzyme from *S.* LL-C10037, the DHA epoxidase from *S.* MPP 3051 catalyzed the epoxidation of **78** to yield **77**, requiring no added cofactor.

Table 32. Epoxidation of **78** Catalyzed by DHA Epoxidase from *S.* MPP 3051

Entry	Assay components[a]	Incub. time (min)	% mmol by HPLC[b] **77**
1	**80** + E	5	1.7
2		10	2.9
3	**78** + E	5	27.5
4		10	50.4

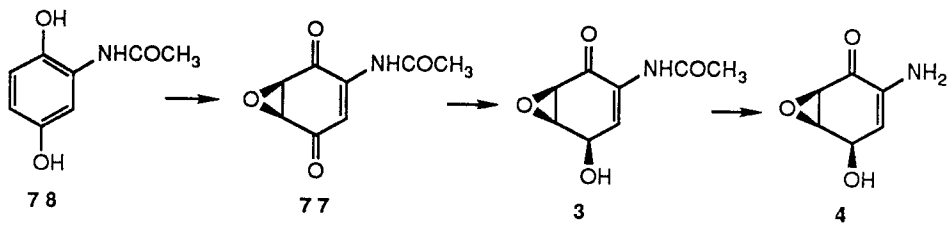
a. A 500-μL assay solution containing 0.1 mM **80** or **78** and DHA epoxidase was incubated at 30 °C for 5 or 10 minutes.

b. The assay mixture was directly analyzed on Radi-Pak C_{18} cartridge column in a mobile phase of H_2O/CH_3CN/TFA = 88/12/0.1.

DHA Epoxidases and the Biosynthesis of 2 and 4

Various efforts have been devoted to the production and the isolation of **4** from *S.* MPP 3051[84] with plans to establish the pathway for its biosynthesis *in vivo*. However, no progress has been made towards this direction, so far. Therefore, no complementary *in vivo* data are available to confirm the hypothesis that **4** could be derived by the parallel pathway of **2**. Nevertheless, the isolation of the DHA epoxidase from *S.* MPP 3051 strongly supports this

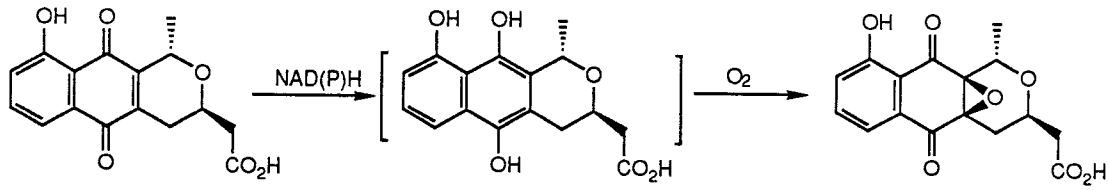
hypothesis, and this enzyme may play a key role in the biosynthesis of **4**, generating the stereochemistry for that series of metabolites in *S.* MPP 3051 (Scheme 41).



Scheme 41. Proposed Pathway for the Biosynthesis **4** in *S.* MPP 3051

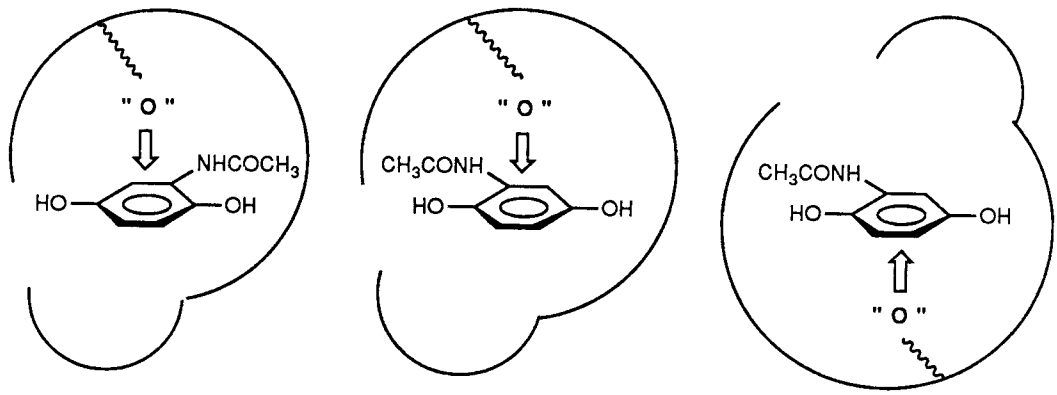
The similar nature of the two epoxidases from *Streptomyces* sp. and the vitamin K epoxidase[41,42] from rat livers, as well as the one from *P. patulum*,[40] which all epoxidize a hydroquinone to yield an epoxyquinone in the absence of any added coenzyme, implies the generality of this type of chemistry in the biosynthesis of epoxyquinones. This could clarify many conflicting reports in the literature in which the mechanism for the epoxidation was speculated on the basis of either a hydroquinone or a quinone as the substrate.

It would be proper at this time to make a few comments about the NNM-A monooxygenase. As reported by Omura and coworkers, the NNM-A monooxygenase catalyzed the epoxidation of **47** to form **48** in the presence of either NADH or NADPH.[39] This could be an exception from the above generality of the hydroquinone epoxidation for the biosynthesis of epoxyquinones. However, with the four epoxidases isolated from *Streptomyces*[83] and *Penicillium*[40] as well as from mammalian sources,[41,42] all of which catalyze the epoxidation of a hydroquinone in the absence of any added coenzyme, it would be more likely that the actual substrate for the NNM-A monooxygenase had been the hydroquinone of **47**, **111**. As shown in Scheme 42, **47** could be first reduced to **111**, which could then be epoxidized to form **48**. The reduction of **47** to **111** could be either a chemical or an enzymatic process. This proposal would be consistent with the observations from the DHA epoxidases and the vitamin K epoxidase, all of which could formally catalyze the epoxidation of a quinone in the presence of NAD(P)H.



47 111 48

Scheme 42. Proposed Mechanism for the NNM-A Monooxygenase



112 113 114

Figure 19. Proposed Mechanism for the Enantiomeric Specificity of DHA Epoxidases

Enantiomeric natural products, although not very common, have been reported. Their biosyntheses have only rarely been studied. In a few cases, it has been demonstrated that the two enantiomers were derived from the same precursor by a process in which two complementary enzymes displayed an opposite enantiomeric specificity. To our knowledge there have been four pairs of such enzymes reported.[85-92] However, very little study has been done with regards to the enantiomeric specificity of such enzymes. The two epoxidases from *Streptomyces* sp. are then the new additions to this family of enzymes. The opposite facial specificities of the two epoxidases could result from controlling the orientation of the substrate with the activated oxygenating species delivered from the same side (Figure 19, **112** and **113**). Alternatively, the two enzymes could deliver the activated oxygenating species from the opposite side with the substrate bound in the same orientation (Figure 19, **113** and **114**). Studies on the reaction mechanism and

determination of the three-dimensional features of the active sites that control the opposite facial specificities of the two epoxidases, therefore, may be of general significance, not only leading to the understanding of the biosynthesis of many other epoxyquinones but also providing information for the biosynthesis of enantiomeric natural products.

Experimental

General Procedures

^{1}H NMR spectra were taken on either a Bruker AM 400 or Bruker AC 300 spectrometer. Five-mm NMR tubes were used for all NMR measurements and TMS was used as reference. UV spectra were recorded on a IBM 9420 UV-Visible spectrophotmeter. Optical rotations with sodium light were measured on a Perkin-Elmer model 243 polarimeter. HPLC analysis was performed on a Waters 600E HPLC instrument with a Kratos Spectroflow 757 UV detector or a Waters 6000A HPLC instrument with a Linear UVIS 200 detector. An HP 3396A integrator was used with each system. The Radi-Pak C_{18} (Novapak, 4 μ, 8x100 mm) column was purchased from Waters Assoc and the Versapack C_{18} column (10 μ, 4.1x250 mm) was purchased from Alltech Assoc. Flash chromatography was carried out on silica gel (EM Reagents, Keiselgel 60, 230-400 mesh). Analytical thin layer chromatography (TLC) was carried out on precoated Kieselgel 60 F_{254} (either 0.2-mm aluminum sheets or 0.25-mm glass plates) and visualized by long and/or short wave UV.

All cell-free and enzyme work was carried out at 4 °C. An IEC B-20A refrigerated centrifuge was used. Cell disruption was performed on a Sonicator Model W-225R made by Heat Systems-Ultrasonic, Inc. For open columns, the flow rate was controlled by a Peristaltic Pump P-3 (Pharmacia) and the fractions were monitored by a set of Dual Path Optical-Control UV-2 units (Pharmacia). All FPLC columns were purchased from Waters Assoc. The Accell QMA anion exchange resin was purchased from Waters Assoc and Sephacryl S-200 was from Pharmacia. A Waters 650E FPLC system was used for the enzyme purification, with a Lambda-Max Model 481 LC spectrophotometer as detector. Incubations were performed in an IBM 9550 Heating/cooling Fluid Circulator (± 0.1 °C). PVPP and protamine sulfate were purchased from Sigma and PMSF

was purchased from Aldrich. Molecular weight standards for gel filtration were purchased from Sigma. Enzyme grade ammonium sulfate was used.

Streptomyces LL-C10037

Standard Culture Conditions

S. LL-C10037 was maintained at 4 °C as spores on sterile soil. A loopful of this material was used to inoculate 50 mL of seed medium containing 1.0% glucose, 2.0% soluble potato starch, 0.5% yeast, 0.5% N-Z Amine A 59027, and 0.1% $CaCO_3$ in glass distilled water, all adjusted to pH 7.2 with 2% KOH. The seed inoculum contained in a 250-mL Erlenmeyer flask, was incubated for 3 days at 28 °C, 240 rpm. Production broths (200 mL in 1-L Erlenmeyer flasks), consisting of 1.0% glucose, 0.5% bactopeptone, 2.0% molasses (Grandma's famous light unsulfured), and 0.1% $CaCO_3$ in glass distilled water and adjusted to pH 7.2 with 10% HCl prior to sterilization, were subsequently inoculated 5% (v/v) with vegetative inoculum from seed broths. The production broths were incubated for 96 h for the harvest of cells and for 120 h for the isolation of **2**.

Production Curve

From a 400-mL fermentation flask, aliquots of 25 mL were taken at different times during the incubation. These samples were centrifuged (2000g, 10 min, r.t.) to separate the supernatant from the cells. The wet cells were weighed and the supernatant was extracted with EtOAc (2 x 25 mL). The EtOAc extract was concentrated and the residue was analyzed by HPLC on a C_{18} column with H_2O/CH_3CN = 60/40 as the mobile phase. Authentic **2** of known concentration was used to calibrate the HPLC system for quantitative analysis.

Buffers

Buffer I: 10 mM potassium phosphate, pH 7.0.

Buffer II: 50 mM potassium phosphate, pH 7.0.

Buffer III: 50 mM potassium phosphate, pH 7.0, 10% glycerol.

Buffer IV: 50 mM potassium phosphate, pH 7.0, 20% glycerol, 0.2 mM EDTA.

Buffer V: 1.0 M KCl + Buffer IV.
Buffer VI: 1.0 M $(NH_4)_2SO_4$ + Buffer IV.

ABQ Dehydrogenase

CFE. Cells (25 g, wet wt.) from 700-mL fermentation of *S.* LL-C10037, incubated for 96 h, were collected by centrifugation (4 °C, 38400g, 30 min) and washed successively with 100 mL of H_2O, 1.0 M KCl, 0.8 M NaCl and 50 mL of Buffer I. The washed cells was made into approximately 50 mL of slurry with Buffer I and 150 mg of polyvinylpolypyrrolidone (PVPP) was then added. The resulting cell slurry was disrupted by sonication with cooling in an ice-water bath (maximum power, 50% duty, pulse for 10 min) and centrifugation (4 °C, 26700g, 30 min) then yielded 50 mL of the crude cell-free extract (CFE).

Protamine sulfate precipitation. To 45 mL of the CFE was added slowly with stirring a 2% protamine sulfate aqueous solution to a final concentration of 0.2%. The resulting preparation was stirred at 4 °C for 0.5 h and centrifuged (4 °C, 26700g, 30 min) to give approximately 50 mL of clear supernatant.

Ammonium sulfate precipitation. To 34 mL of the protamine sulfate supernatant, which has been dialyzed against Buffer I, was added 18.3 g of solid ammonium sulfate (enzyme grade) to approximately 80% saturation. The mixture was stirred very gently at 4 °C overnight; the protein was collected by centrifugation (4 °C, 38400g, 20 min) as a pellet and this pellet was redissolved in the minimum amount of Buffer I.

Dialysis. Dialysis was carried out in tubing that had been boiled 6-8 times in fresh portions of doubly-distilled (dd) H_2O and stored in 10 mM EDTA. Dialysis was carried out overnight against two changes of 1 to 2 L each of Buffer I.

Sephacryl S-200. 5 mL of the 80% ammonium sulfate pellet solution after dialysis was applied to a Sephacryl S-200 column (3.0 x 30 cm) equilibrated with Buffer II. The column was eluted with the same buffer at the

flow rate of 2.0 mL/min and fractions in the size of 6 mL were collected. The ABQ dehydrogenase was found mainly in fraction 10 to fraction 16 (Figure 9).

Protein concentration. At this stage, the protein concentration was determined by Warburg and Christian's method,[93] by which protein (mg/mL) can be calculated according to the following equation by measuring A_{280nm} and A_{260nm}.

Protein (mg/mL) = 1.55 A_{280nm} - 0.76 A_{260nm}

HPLC analysis. The HPLC analysis was performed on a Radi-Pak C_{18} cartridge column in a mobile phase of $H_2O/CH_3CN/TFA$ = 85/15/0.1. At flow rate of 1.0 mL/min, **78** and **2** have retention times of 5.0 min and 9.2 min, respectively. The fractions were monitored by a UV detector at 225 nm and the UV detector was connected to an integrator for quantification.

DHA Epoxidase

CFE. Cells from 1.6 L of fermentation of *S.* LL-C10037 incubated for 96 h were collected by centrifugation (4 °C, 13800g, 30 min) and washed successively with 200 mL of H_2O, 1.0 M KCl, 0.8 M NaCl and Buffer I to afford 50 g of cells (wet wt.). The washed cells were then suspended in an additional 200 mL of Buffer I and disrupted by sonication with cooling in an ice-water bath (maximum power, 50% duty, pulse for 4 min). Centrifugation (4 °C, 26700g, 20 min) then yielded 130 mL of the crude cell-free extract (CFE).

Enzymatic formation of 70. An assay solution (100 mL) containing acetone (4 mL), **78** (20 mg) and CFE (50 mL) in 0.5 M potassium phosphate buffer, pH 6.5, was incubated at 28 °C and 250 rpm for 13 h. Extractive workup and preparative TLC purification yielded 6 mg of **80** and 6 mg of **70**, whose structures were confirmed by ^1H NMR spectroscopy.

HPLC analysis. The HPLC assay of the DHA epoxidase was performed on either a Radi-Pak C_{18} cartridge column in a mobile phase of $H_2O/CH_3CN/TFA$ = 85/15/0.1 or a Versapak C_{18} column in a mobile phase of H_2O/CH_3CN = 85/15. In the former at flow rate of 1.0 mL/min, **78**, **70** and **80** have retention times of

5.0, 8.0 and 9.2 min, respectively. In the latter at flow rate of 1.0 mL/min, **78**, **70** and **80** have retention times of 4.2, 7.3 and 8.3 min, respectively. The fractions were monitored by a UV detector at 225 nm and at this wavelength the relative mole responses for **78**, **80** and **70** were 1.09, 0.68 and 1.0, respectively. The UV detector was connected to an integrator and quantitative analysis was performed by correction of the integration area with the corresponding mole response.

AEBQ Dehydrogenase

Enzymatic formation of 2. Cells from 400 mL of fermentation of *S.* LL-C10037 incubated for 96 h were collected by centrifugation (4 °C, 13800g, 30 min) and washed successively with 100 mL of H_2O, 1.0 M KCl, 0.8 M NaCl and Buffer I to afford 18 g of cells (wet wt.). The washed cells were brought to 50 mL of cell suspension with Buffer III. PVPP (150 mg) was added, and the cells were disrupted by sonication with cooling (maximum power, 50% duty, pulse for 4 min). Centrifugation (4 °C, 26700g, 20 min) then yielded 42 mL of the crude cell-free extract (CFE).

An assay solution (40 mL) containing **70** (7.2 mg) and CFE (20 mL) in 0.1 M potassium phosphate buffer, pH 6.5, was incubated at 28 °C and 225 rpm for 2.5 h. Extractive workup and preparative TLC purification yielded 2 mg of **2**, whose structure was confirmed by ^1H NMR spectroscopy.

CFE. Cells from 6.0 L of fermentation of *S.* LL-C10037 incubated for 96 h were collected by centrifugation (4 °C, 13800g, 10 min) and washed successively with 1.2 L of H_2O, 1.0 M KCl, 0.8 M NaCl and Buffer I to afford 220 g of cells (wet wt.). The washed cells were brought to 600 mL of cell suspension with Buffer IV. To this was added PVPP (1.8 g) and a solution (600 μL) of 1.0 M PMSF in acetone to a final concentration of 1.0 mM. The resulting cell suspension was equally dispensed into six 150-mL beakers and each part was disrupted by sonication with cooling in an ice-water bath (maximum power, 50% duty, pulse for 4 x 30 sec). Centrifugation (4 °C, 13800g, 10 min) then yielded 440 mL of the crude cell-free extract (CFE).

Protamine sulfate precipitation. The above CFE was brought to 0.01% with protamine sulfate by dropwise addition of a 2.0% aqueous solution. The resulting solution was stirred for 0.5 h and centrifuged (4 °C, 38400g, 20 min) to give 440 mL of supernatant.

Ammonium sulfate precipitation. The above protamine sulfate supernatant was brought to 41.1% saturation by addition of solid ammonium sulfate (enzyme grade). The suspension was stirred for 1 h and the precipitate was removed by centrifugation (4 °C, 13,800g, 10 min). The resulted supernatant was brought to 61.6% saturation with solid ammonium sulfate and stirred for 1 h. The active pellet was collected by centrifugation (4 °C, 38400g, 10 min).

Sephacryl S-200. The 41-61% pellet was dissolved in minimum amount of Buffer IV and applied to a Sephacryl S-200 column (2.4 x 110 cm) (for better resolution no more than 10 mL of the pellet solution was loaded to the column per run). The column was eluted with Buffer IV and fractions of 7.5 mL were collected at a flow rate of 7.5 mL per 30 minutes. Active fractions (52 mL) (Figure 12) were pooled for the next step.

Accell QMA. The pooled fractions (52 mL) from the Sephacryl S-200 column were divided into two parts and each part was applied to an Accell QMA column (0.8 x 15 cm). The column was first washed with Buffer IV to remove all the unbound proteins (until the $A_{280\ nm}$ returned to baseline) and then eluted with a gradient of Buffer IV - Buffer V at the flow rate of 1.0 mL/min. The column profile and the KCl gradient are shown in Figure 13. Active fractions (16 mL) were pooled for the next step.

HIC (Phenyl 5 PW). 4 mL of the active fractions from the Accell QMA column was brought to 1.0 M ammonium sulfate by addition of solid ammonium sulfate and this solution was loaded on an HIC (Phenyl 5 PW) column (8 x 75 mm). The column was first washed with Buffer VI to remove all the unbound proteins (until the $A_{280\ nm}$ returned to baseline) and then eluted with a gradient of Buffer VI - Buffer IV at a flow rate of 0.8 mL/min. The column

profile and the $(NH_4)_2SO_4$ gradient are shown in Figure 14. Active fractions were pooled for the kinetic study.

HPLC analysis. The HPLC assay of the AEBQ dehydrogenase was performed on a Radi-Pak C_{18} cartridge column in a mobile phase of H_2O/CH_3CN/TFA = 88/12/0.1. At flow rate of 1.0 mL/min, **70** and **2** have retention times of 8.5 min and 4.0 min, respectively. The fractions were monitored by a UV detector at 225 nm and the UV detector was connected to an integrator for quantitative analysis.

MW estimation. A Protein-Pak 300 SW column (8 x 300 mm) was calibrated with MW standards. Eluted with Buffer IV at a flow rate of 0.5 mL/min, the retention times of these standards and the AEBQ dehydrogenase are listed in Table 33.

Table 33. FPLC of AEBQ Dehydrogenase on a Protein-Pak 300 SW Column

Protein	MW	Retention time (min)
β-Amylase	200,000	14.5
Alcohol dehydrogenase	150,000	15.5
AEBQ dehydrogenase		16.0
Albumin, Bovine serum	66,000	17.5
Albumin, Chicken egg	45,000	18.4
Carbonic anhydrase	29,000	21.2
α-Lactalbumin	14,200	22.0

pH Dependenc. 500-μL Assay solutions containing 0.07 mM **70**, 0.2 mM NADPH and 50 μL of enzyme solution in 0.1 M potassium phosphate buffer, pH from 5.0 to 7.5, were incubated at 30 °C for 10 minutes. The reactions were terminated by addition of 100 μL of CH_3CN/20% TFA (2/1, v/v); the mixtures were centrifuged to remove the precipitated protein and the supernatants were analyzed by HPLC.

Kinetic study. Assay solutions (1.0 mL) containing 0.5 mM NADPH, **70** (6.25 μM to 100 μM) and 50 μL of the AEBQ dehydrogenase from the HIC (Phenyl 5 PW) column in 0.1 M potassium phosphate buffer, pH 7.0, was incubated in a UV cuvette at 22 °C. The reaction was continuously monitored at 340 nm and the

initial velocity was determined by $\Delta A_{340\ nm}$ measured over the initial period of 7.5 minutes. The molar absorption coefficients (ε) at 340 nm are 2600 (**70**), 6200 (NADPH), 83 (**2**) and 0 ($NADP^+$). According to the following equation, ΔC_t (substrate consumed at time t) could be calculated from the measurements of ΔA_t (UV absorption change at 340 nm at time t).

$$\Delta C_t = \frac{\Delta A_t}{\Delta \varepsilon \times l} = \frac{\Delta A_t}{(2600 + 6200 - 83 - 0) \times 1.0} = 0.115 \times \Delta A_t \ (mM)$$

Assay solutions (1.0 mL) containing 0.5 mM **70**, NADPH (6.25 μM to 125 μM) and 50 μL of the AEBQ dehydrogenase from the HIC (Phenyl 5 PW) column in 0.1 M potassium phosphate buffer, pH 7.0, were incubated and the data were analyzed in the same manner as above to determine the K_m for **70**.

Streptomyces MPP 3051

Preparation of Sterile Soils

Method 1: Sterile soil prepared in this method[94] has been used for the preservation of *Streptomyces* LL-C10037. Sandy loam soil was dried by hot air (in oven at 150 °C) and sieved through a fine or medium sieve to get uniform soil. It was then transferred to 13 x 125-mm test tubes (about 0.75 inch depth) and the test tubes were plugged with cotton plugs covered with cheesecloth. Sterilization in autoclave for 1 h each day on three consecutive days (total 3 x 1 h autoclaving) afforded the sterile soil. A spore suspension of *S.* LL-C10037 was prepared from a 14-day-old slant (20 x 150 mm slant tube) by addition of 10 mL dd H_2O. 1.0 mL of this spore suspension was dispensed per tube of sterile soil and the inoculated soil culture was kept at room temperature for 3-4 weeks until dry. They were then moved to 4 °C for long term preservation.

Method 2: Sterile soil prepared in this method[95] has been used for the preservation of *Streptomyces* MPP 3051. Presifted potting soil (100 g) was spread in a Pyrex baking dish and sterilized for 1 h. The soil was then incubated at 37 °C overnight and sterilized for additional 1 h. $CaCO_3$ (1% w/w) was added to the soil, which was then sterilized for another 1 h (total 3 x 1 h

autoclaving). The sterile soil was distributed to 13 x 100-mm tubes (1 g per tube) and plugged with cotton plugs covered with cheesecloth. A seed culture of *S.* MPP 3051 in YME medium (4 g of yeast extract, 10 g of malt extract and 4 g of glucose in 1.0 L dd H_2O, adjusted to pH 7.2 with 2% KOH before sterilization) gave good growth. Each tube was inoculated with 0.1 mL of this seed broth and incubated at 37 °C until good sporulation was visible (3-4 weeks). They were then kept at room temperature.

Medium

Lederle Seed		**Beecham seed**	
Glucose	1.0%	Glucose	3.0%
Starch	2.0%	Oxoid Bacteriological peptone	1.0%
Yeast extract	0.5%	K_2HPO_4-$3H_2O$	0.2%
N-Z amine	0.5%	NaCl	0.1%
$CaCO_3$	0.1%		
adjusted to pH 7.2 prior to sterilization		adjusted to pH 7.0 prior to sterilization	
Lederle production		**Beecham production**	
Glucose	1.0%	Glucose	1.0%
Bacto peptone	0.5%	$NaNO_3$	1.0%
Molasses	2.0%	Pharmamedia	0.1%
$CaCO_3$	0.1%	$CaCO_3$	0.5%
adjusted to pH 7.2 prior to sterilization		adjusted to pH 7.0 prior to sterilization	

Standard Culture Conditions

S. MPP 3051 was maintained at room temperature (20 - 25 °C) as spores on sterile soil. A loopful of this material was used to inoculate 50 mL of Lederle seed medium and the seed inoculum, contained in a 250-mL Erlenmeyer flask, was incubated for 48 h at 28 °C, 250 rpm. Production broths (200 mL in 1-L Erlenmeyer flasks), consisting of either the Beecham medium or the Lederle medium, were subsequently inoculated 5% (v/v) with vegetative inoculum from seed broths. The production broths were incubated for 22.5 h for the harvest of cells and for 24 h for attempts to isolate **3** and **4**.

DHA Epoxidase

CFE. The cells (3.6 g, wet wt) from 400 mL of 22.5 h fermentations (Lederle production medium) were collected by centrifugation (4 °C, 13,800g, 5 min) and washed successively with 100 mL of Buffer I, 1.0 M KCl, 0.8 M NaCl and Buffer I. After each wash the cells were centrifuged as above. The cells were then suspended in 30 mL of Buffer IV, treated with PMSF by adding a 1 M acetone solution of PMSF to a final concentration of 1 mM, and then PVPP powder was added to 3 mg/mL. Compared with that from *S.* LL-C10037, cells from *S.* MPP 3051 were softer, smaller in size and easier to be disrupted. Therefore this cell suspension was sonicated for a relatively short time (4 °C, maximum power, 90% duty cycle, pulsed for 4 x 15 sec) cooling in an ice-water bath. Centrifugation (4 °C, 23200g, 10 min) yielded 35 mL of the CFE.

Enzymatic formation of 77. The CFE was brought to 0.01% with protamine sulfate by addition of a 2.0% solution, stirred at 4 °C for 0.5 h, and centrifuged (4 °C, 23,200g, 20 min). Part of the supernatant (25 mL) was treated with solid $(NH_4)_2SO_4$ at 4 °C with a gentle stirring. The resulting 62-92% pellet was collected by centrifugation (4 °C, 23,200g, 10 min) and brought to 12.0 mL with Buffer IV. One milliliter of this was added to each of 12 tubes to a final volume of 2.0 mL assay solution, containing 0.125 M potassium phosphate buffer, pH 7.0, 1.0 mM **80** and 1.0 mM NADH. After 10 min incubation at 30 °C, the assay solutions were combined, saturated with solid NaCl and extracted with EtOAc (3 x 36 mL). The combined EtOAc extract was dried over $MgSO_4$ and concentrated *in vacuo* to afford a brown residue. This residue was loaded on a silica column (1x20 cm) and eluted with $CHCl_3$/MeOH/hexane (5/1/1). The fractions containing **77** were pooled and concentrated to yield brown crystals, which were recrystallized from MeOH-EtOAc to afford 1.3 mg (30%) of **77**. Its structure was confirmed by ^1H NMR and its specific rotation was measured in methanol solution ($[\alpha]^{20}_D$ - 112.2°, c 0.1 in MeOH).

HPLC analysis. The HPLC assay of the DHA epoxidase was performed on either a Radi-Pak C_{18} cartridge column in a mobile phase of H_2O/CH_3CN/TFA = 85/15/0.1 or a Versopack C_{18} column in a mobile phase of H_2O/CH_3CN = 85/15. In the former at the flow rate of 1.0 mL/min, **78**, **77** and **80** have retention

times of 5.0, 8.0 and 9.2, respectively. In the latter at the flow rate of 1.0 mL, **78**, **77** and **80** have retention times of 4.2, 7.3 and 8.3, respectively. The fractions were monitored by a UV detector at 225 nm and at this wavelength the relative mole responses for **78**, **80** and **77** were 1.09, 0.68 and 1.0, respectively. The UV detector was connected to an integrator and quantitative analysis was performed by correction of the integration area with the corresponding mole response.

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Chapter 5

2,5-Dihydroxyacetanilide Epoxidases from *Streptomyces* LL-C10037 and *S.* MPP 3051

Optimizations and Clarification of the Cell-Free Extract

Initially, as described in Chapter 4, the CFE from *S.* LL-C10037 was prepared in 10 mM potassium phosphate buffer, pH 7.0, and the DHA epoxidase activity was completely lost at 4 °C in 24 h. Two approaches have been adopted to optimize the conditions for preparation of the CFE.[1] One was to develop a method which would give maximum DHA epoxidase activity in the CFE and the other was to search for conditions under which the DHA epoxidase could be stabilized.

Preparation of CFE

There are many methods for cellular disintegration, since there are many types of cells. Most cells have particular characteristics which need special attention during disintegration. Bacteria vary from fairly fragile organisms that can be broken up by digestive enzymes or osmotic shock to more resilient species with thick cell walls, needing vigorous mechanical treatment for disintegration. It is generally not advisable to use a disruption treatment more vigorous than necessary, since labile enzymes may be inactivated once liberated into solution.

Three methods for disruption of cells from *S.* LL-C10037 have been tried and compared. They are the vigorous techniques of French press, ultrasonication and the bead mill. Thus, 50 mL of cell suspension (15 g cell from 400 mL fermentation) was subjected to ultrasonication (4 °C, 50% duty, pulse for 4 min), French press (4 °C, 15000-20000 psi), and bead mill (with 50% volume of glass beads, 0.5 mm, for 2 min), respectively. Cell debris was removed by centrifugation (4 °C, 26700g, 20 min) and the supernatants were assayed for the DHA epoxidase activity. Comparable total activity was obtained from the three methods and for convenience and good recovery the ultrasonication method was prefered.

Initially, the cells were sonicated for 12 min (4 °C, 50% duty, pulse fashion) but very little DHA epoxidase was detected in this CFE, presumably due to inactivation from over-sonication. The sonication time was subsequently investigated. 25 mL of cell suspension (10 g of cell) was sonicated for 0.5, 1.0, 2.0, 4.0 and 8.0 minutes (4 °C, 50% duty, pulse fashion), respectively. By

comparing the specific activity of DHA epoxidase in the CFE, it was concluded that 1 to 2 min sonication (4 °C, 50% duty, pulse fashion) was sufficient for releasing most of the DHA epoxidase.

Clarification of the CFE was subsequently investigated before any attempt at the purification of the DHA epoxidase was made. Microorganisms cause a variety of problems with their extracts. First, there is the large amount of nucleic acid material extracted from rapidly proliferating cells. Second, during cell disruption the cell wall material may either be finely dispersed to give a turbid extract, or partially solubilized so that as well as protein and nucleic acids there is a large amount of gumlike polysaccharides in solution. Bacterial extracts made by ultrasonication are viscous due to DNA and contain much ribosomal material. Nucleic acids can generally be precipitated by forming an aggregate with a polycationic macromolecule such as protamine sulfate or polyethyleneimine. The total amount needed should be tested; it may be as much as 5 mg per gram of raw material, however, unnecessary excess will result in significant loss of enzyme activity by precipitating proteins as well. If it proves unnecessary or undesirable to use protamine or polyethyleneimine, DNA viscosity can be reduced by inclusion of DNase when making the extract. Ribosomes can be precipitated using streptomycin; although the amount of nucleic acid material removed by streptomycin is much less than if using protamine. Little protein is lost by adsorption to streptomycin precipitates. From these considerations, several pre-treatments have been applied to prepare the CFE in order to maximize the DHA epoxidase activity. The washed cells were disrupted by sonication (4 °C, 90% duty, pulse for 80 sec) (90% duty was used to shorten the total sonication time). The CFE was treated with protamine sulfate and streptomycin sulfate, respectively, with the intention to remove nucleic acids or ribosomes. When the CFE was added a 2.0% aqueous protamine sulfate solution to give a final concentration of 0.1%, a clean supernatant was afforded after centrifugation. However enzyme activity was not recovered in either the supernatant or the pellet. By addition of protamine sulfate to a final concentration of 0.01%, the CFE was clarified without significant loss of enzyme activity. When streptomycin sulfate was added to the CFE up to a final concentration of 2.0%, no significant pellet was formed after centrifugation at 18,000 g for 40 min. PVPP, which was

reported to be used in the removal of phenolic metabolites from the CFE,[2] was also included to maximize the extraction of the enzyme activity.

Stabilization of DHA Epoxidase

The CFE prepared showed DHA epoxidase activity but this activity was very unstable and was completely lost in 24 h at 4 °C. The general reason for the loss of enzyme activity is that enzymes are often sensitive protein molecules which, when released from their natural protective environment, are subjected to a variety of stresses they may not be able to resist. The enzymes are exposed to three types of threat which may lead to loss of activity: 1. denaturation, 2. inactivation of the catalytic site, 3. proteolysis.

Denaturation can be minimized if the principal causes are avoided. These are extremes of pH, temperature, and denaturants such as organic solvents. The natural pH inside a cell would normally be in the range of 6 - 8, so that buffers within this range, or as close as possible to the actual pH in the tissue concerned, should protect against pH denaturations. Therefore, 10 mM potassium phosphate buffer, pH 7.0, 50 mM potassium phosphate buffer, pH 7.0 and 50 mM Tris buffer pH 7.0, have been tried, respectively. No significant differences among them have been observed.

It was thought that enzyme purification had to be carried out in the cold because enzymes are so labile; reducing the temperature by 20 °C decreases the rate of most processes by a factor of 3 - 5, so low temperature should be beneficial. Therefore, all the preparations and purifications were carried out at 4 °C.

The inactivation of enzymes due to a specific effect on the catalytic site is more difficult to avoid. Loss of cofactors can be prevented (if they are known) by replacing them or including them in the buffer used. A more serious problem is covalent modifications of the active site due to exposure to the more hostile environment. The most troublesome is cysteine; sulfhydryl residues at the active site may be in the ionized form, which is very prone to oxidation. Normally inside the cell the reducing atmosphere and the presence of other sulfhydryl-containing molecules protect these groups. Exposed to a higher oxygen tension, several fates for a sulfhydryl are possible. Formation of a disulfide bond requires another sulfhydryl to be in the vicinity and an

oxidation process to occur. Disulfide formation is greatly accelerated by the presence of divalent ions which can activate the oxygen molecule and complex with the sulfhydryls. Two protective actions can be taken: 1. removing all traces of (heavy) metal ions using a complexing agent such as EDTA; 2. including a sulfhydryl-containing reagent in the solution such as β-mercaptoethanol, dithiothreitol (DTT) or dithioerythritol. A routinely successful procedure is to use β-mercaptoethanol diluted 1 part in 1000 (12 mM) during the preparation (if it is required at all), and for long term storage dithiothreitol at 1 - 5 mM. Ethylenediaminetetraacetic acid disodium salt (EDTA) is usually present in all buffers at a concentration of 0.1-0.2 mM. From these considerations, buffers containing 10 mM DTT or 0.2 mM EDTA have been prepared and tested. Because the epoxidation product **70** was very reactive to a nucleophile and the presence of DTT interfered with the assay of DHA epoxidase, it was found that buffers containing DTT could not be used. Compared with the control (74% activity retained after 48 h at 4 °C in 50 mM potassium phosphate buffer, pH 7.0, 20% glycerol), EDTA did stabilize the enzyme activity (84% activity retained in the same buffer with addition of 0.2 mM EDTA).

In microorganisms, hydrolytic enzymes are often to be found between the plasma membrane and the cell walls, largely for the purpose of digesting extracellular macromolecules. The process for obtaining an enzyme extract inevitably destroys this delicate balance and allows these enzymes to mix with the cell contents, leading to the hydrolysis of the proteins. Unfortunately, there are many different classes of proteolytic enzymes and not all of them can be successfully inhibited. The principal attacks on the proteolytic enzyme problem have been directed at the serine proteases. PMSF is the most commonly used inhibitor. Although not very soluble, PMSF can be dissolved in acetone before dispensing into the extract to a final concentration of 0.5 - 1 mM. PMSF also inhibits some thiol proteases and some carboxypeptidases; once inhibited, the enzymes are dead, and no more PMSF is needed. The class of metalloproteinases, which require divalent metals for activity, can be inhibited simply by complexing metal ions with EDTA. From these considerations, 1 mM PMSF was used in the preparation of the CFE. Compared with the control (26% activity retained after kept at 4 °C for 48 h in 50 mM potassium phosphate buffer, pH 7.0, 10% glycerol), PMSF significantly

stabilized the DHA epoxidase (44% activity was retained in the same buffer from the CFE treated with PMSF). Comprehensive protease inhibitor mixtures have been reported such as the so-called protease inhibitor cocktail of Santi[3,4] consisting of 1.0 mM *o*-phenanthroline, 1.0 mM benzamidine, 50.0 μM PMSF, 20.0 μg/mL leupeptin, 50.0 μg/mL soybean trypsin inhibitor, and 50.0 μg/mL aprotinin. When the CFE was treated either with Santi's cocktail or with 1.0 mM PMSF alone, the latter showed much better stabilization (87% activity retained after storage at 4 °C for 48 h in 50 mM potassium phosphate buffer, pH 7.0, 20% glycerol, 0.2 mM EDTA) than the former (48% activity retained under the same conditions), regardless of whether other protease inhibitors were present.

Aqueous 1-2 % solutions of proteins are not in a comparable state to the natural environment of the proteins. Consequently, stabilization (against denaturation and other more subtle conformational changes that lead to inactivation) can be accomplished by attemping to mimic the conditions of relatively low water activity. The most widely used method is to include glycerol in the buffer solution. Glycerol forms strong hydrogen bonds with water, effectively slowing down the motion of the water molecules and so reducing the water activity. Glycerol up to 50% (wt/vol) is used, although the highest concentrations are usually reserved for storage, since the viscosity of such a solution is too high to allow any manipulations other than electrophoresis. 20 - 30% (wt/vol) glycerol mixtures do not have a large viscosity so most procedures can be carried out in buffers containing this amount of glycerol. As an alternative to glycerol, sugar or sugar alcohol solutions (glucose, sucrose, fructose, sorbitol) have been used successfully. Stabilization of DHA epoxidase in 50 mM potassium phosphate buffer, pH 7.0, in the presence of glycerol and sucrose was investigated and the data are listed in Table 34. Apparently, addition of both glycerol and sucrose to the buffer can stabilize the DHA epoxidase however glycerol had a more favorable effect than sucrose.

In conclusion, CFE, prepared in the presence of PVPP (3 mg/ mL) and 1.0 mM PMSF in 50 mM potassium phosphate buffer pH 7.0, containing 0.2 mM EDTA and 20 % glycerol and treated with 0.01% protamine sulfate, provided the preparation of crude DHA epoxidase with maximum enzyme activity.

Table 34. Stabilization of DHA Epoxidase by Glycerol or Sucrose

(%) Glycerol or sucrose		Rel act. (%)[a]
control[c]	0	7
glycerol:[b]	10	44
	20	74
sucrose:[c]	8	16

a. CFE was kept at 4 °C for 48 h and the DHA epoxidase activity was compared with CFE at time 0.
b. In 50 mM potassium phosphate buffer, pH 7.0.
c. In 10 mM potassium phosphate buffer, pH 7.0.

DHA Epoxidase from S. LL-C10037

The pathway for the biosynthesis of **2** has been established by whole-cell and cell-free studies[5-8] and several enzymes on the pathway have been isolated.[7,8] The most intriguing discovery was the isolation of the DHA epoxidase, which catalyzed the epoxidation of **78** to yield **70**. Furthermore, **4** has been proven to be the desacetyl enantiomer of **2**.[9] The same gross structure and relative stereochemistry led to speculation that **4** could be derived by the same pathway as **2** (exclusive of stereochemistry) with addition of a deacetylation as the last step. This has resulted in the isolation of the DHA epoxidase from *S.* MPP 3051, which indeed catalyzed the epoxidation of **78** to yield epoxyquinone **77**, whose specific rotation confirmed it as the enantiomer of **70**.[7] In order to gain some insight into the mechanism of the epoxidation and to explore the opposite facial specificity of the two epoxidases, presumably by comparing their characteristics, it became necessary to purify and to characterize the DHA epoxidase from *S.* LL-C10037.

Purification of DHA Epoxidase.

Ammonium Sulfate Precipitation: The purification of the DHA epoxidase was started with $(NH_4)_2SO_4$ fractional precipitation. As shown in Table 35, more than 90% of the DHA epoxidase activity could be precipitated at 52%-66% saturation of $(NH_4)_2SO_4$.

Sephacryl S-200 Column: The pellet from ammonium sulfate precipitation could be dissolved in minimum amount of buffer and such a small volume would ideal for a size exclusion column; small sample volume was essential for a successful size exclusion column and high salt sample could be applied directly without desalting. Therefore, a Sephacryl S-200 column had been placed as the first column for the purification; the choice of Sephacryl S-200 versus other size exclusion materials was made upon its properties of high flow rate, good recoveries and proper fractionation range. Figure 20 shows the column profile and the DHA epoxidase was eluted in approximately 1.5 void volumes (the active fraction is shaded). Because the exclusion limit for Sephacryl S-200 is 250 K for proteins, the molecular weight of DHA epoxidase was, therefore, estimated to be smaller than 250 K.

Table 35. Ammonium Sulfate Fractional Precipitation of DHA Epoxidase

% Saturation	% DHA epoxidase
0 - 41.1	0
41.1 - 46.2	0
46.2 - 51.3	0
51.3 - 56.5	12.7
56.5 - 61.6	53.2
61.6 - 66.8	28.6
66.8 - 71.7	3.4
71.8 up	0

Accell QMA Column: Based on a different mechanism of separation, the purification of DHE epoxidase was next tried on ion exchange columns. The pI of the enzyme was estimated to be 6.5 as described next. Thus, the enzyme preparation was added to a set of test tubes in which ion exchanger has been equilibrated in buffers with different pHs. If the pH of the buffer is above its pI, the enzyme would bind to the ion exchanger (for anion resins) so that no enzyme activity would be detected in the supernatants. This estimated pI of 6.5 determined the pH of buffers used for the anion exchange columns. The choice of Accell QMA versus other materials was made upon its rigid structure, which allowed FPLC application, and large capacity. Figure 21 shows the column profile and the active fraction is shaded. If one recalled the profile of the AEBQ

dehydrogenase (Figure 13), under the same conditions the DHA epoxidase was completely separated from the AEBQ dehydrogenase on the Accell QMA column. Purification of the DHA epoxidase on a cation exchange column (Protein-Pak SP 5 PW, 8 x 78 mm from Waters Assoc.) was investigated as well. However, no satisfactory result was obtained, and under all the conditions tested (pH as low as 4.8 and ionic strength as low as 10 mM), the DHA epoxidase, along with most of other proteins loaded, passed the column without any retention.

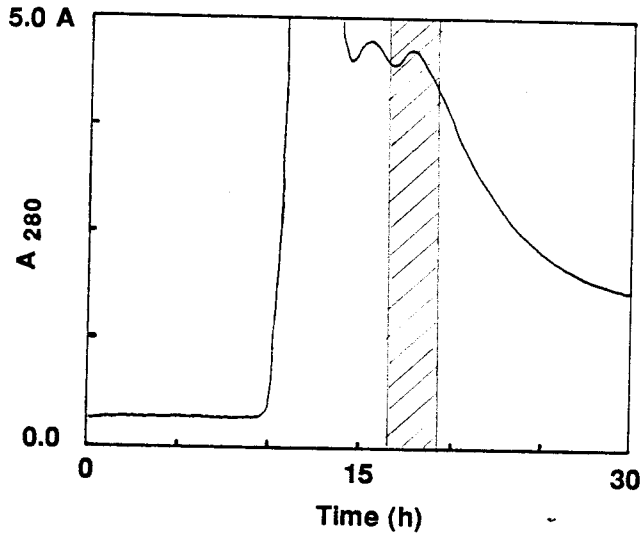


Figure 20. Chromatogram of DHA Epoxidase on a Sephacryl S-200 Column

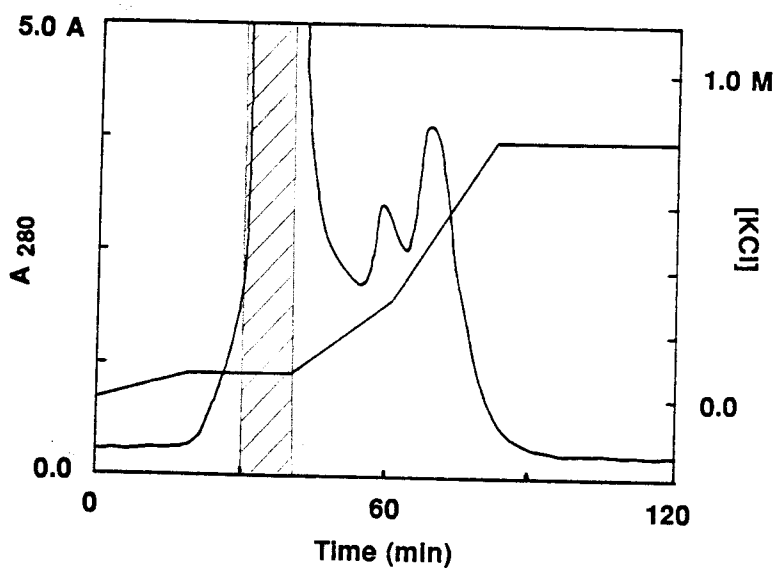


Figure 21. FPLC of DHA Epoxidase on an Accell QMA Column

HIC (Phenyl 5 PW) Column: Hydrophobic interaction was chosen as the mechanism for the next step of purification. The choice of an HIC column at this stage was proper because the active fraction, which was in high KCl concentration from the Accell QMA column, could be brought to a $(NH_4)_2SO_4$ solution without considering the removal of KCl. As shown in Figure 22, the DHA epoxidase was selectively bound to the column at high concentration of $(NH_4)_2SO_4$. After washing out all the unbound proteins, the enzyme was eluted by decreasing the concentration of $(NH_4)_2SO_4$ in buffer (active fraction is shaded). The major disadvantage of this step resulted from the low capacity of the column. Unfortunately, when a large column (2.4 x 50 cm), packed with Phenyl Sepharose CL-4B (Pharmacia), was run under the same conditions with plan to scale the purification, no satisfactory result was obtained.

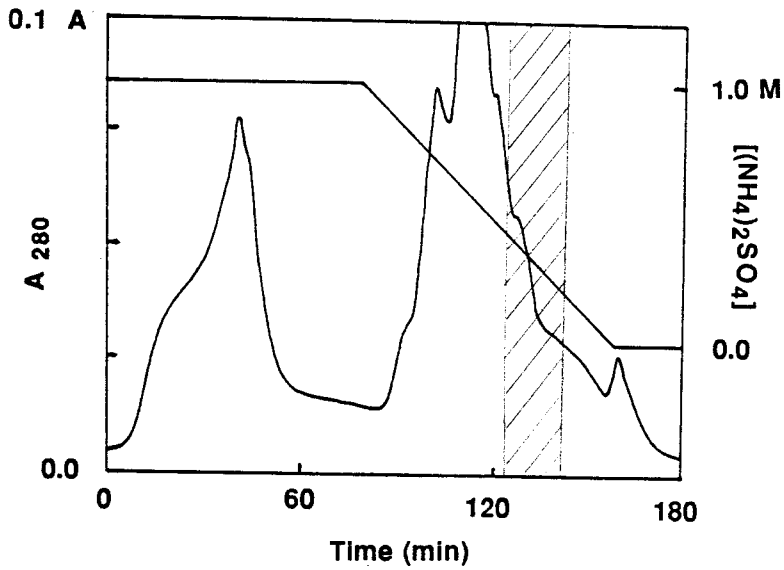


Figure 22. FPLC of DHA Epoxidase on an HIC (Phenyl 5 PW) Column

DEAE 5 PW Column: Although the mechanism is very similar in nature with the Accell QMA, the DEAE 5 PW column was applied next. A different resolution from the Accell QMA was expected because the DEAE 5 PW column was an FPLC column, packed with smaller size materials to ensure a high resolution. Figure 23 shows the column profile and the active fraction is shaded. Similar to the Accell QMA column, the DHA epoxidase was eluted in the front of a KCl gradient; however, some resolution was achieved.

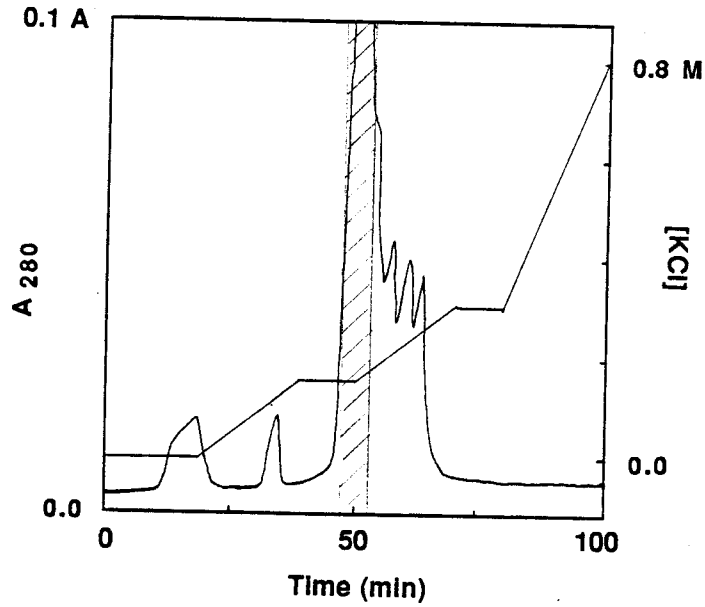


Figure 23. FPLC of DHA Epoxidase on a DEAE 5 PW Column

300 SW Column: After the steps of purification described above, it became practicable to run the FPLC size exclusion column, which presumably should give better resolution. Unfortunately, as shown in Figure 24, only one peak was observed, indicating that the sizes of all the proteins were very similar. Careful assay of the fractions under the peak, however, did show that the DHA epoxidase was located in the front part of the peak (active fraction is shaded).

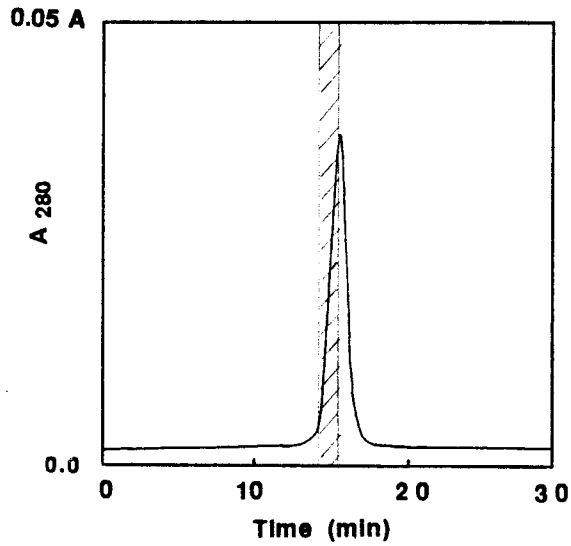


Figure 24. FPLC of DHA Epoxidase on a 300 SW Column

DEAE 5 PW Column: By change of the counterion, an ion exchange column could display different resolution. This mechanism has been proved to be successful in enzyme purification.[45] From this consideration the DEAE 5 PW was chosen as the last column for the purification. However, the column was eluted this time with a Tris buffer. Figure 25 shows the column profile and the active fraction is shaded. A symmetrical peak was obtained, which had the DHA epoxidase activity.

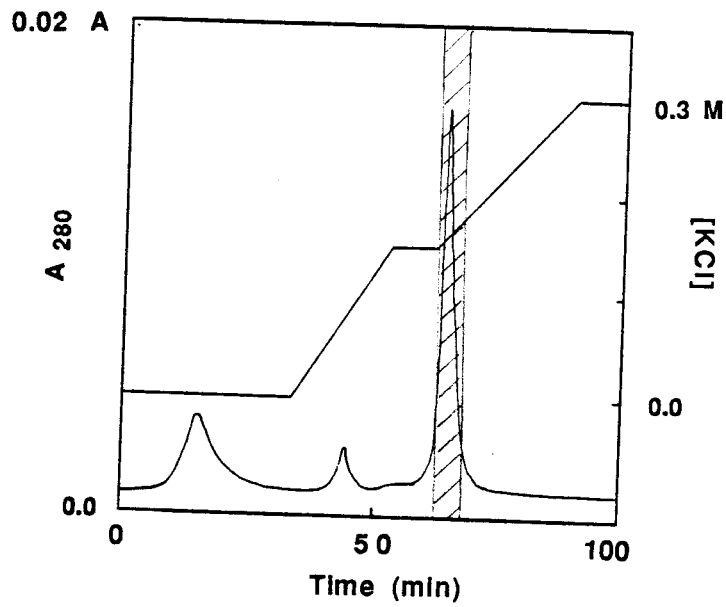


Figure 25. FPLC of DHA Epoxidase on a DEAE 5 PW Column

In conclusion, this purification scheme is summarized in Table 36. The enzyme was purified with a overall factor of 640.

The homogeneity of the purified DHA epoxidase was examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). The gels were stained with Coomassie brilliant blue R. As shown in Figure 26, DHA epoxidase preparations of the CFE and the $(NH_4)_2SO_4$ pellet solution had many proteins (Lane 3 and 4). However, the purified DHA epoxidase from the last step displayed virtually one band, indicating that it has been purified to homogeneity (Lane 7 and 8). Compared with the standards of known molecular weight, the subunit of the DHA epoxidase was estimated to be 22.3K daltons.

Table 36. Purification of DHA Epoxidase from *S. LL-C10037*

Step	Vol. (mL)	Protein[a] (mg)	U[b]	U%	sp U[c]	Purifn x-fold
CFE	565	867	10.8	100	0.0124	1
PS precip.	565	654	10.0	92.6	0.0153	1.2
$(NH_4)_2SO_4$ precip.	11.5	298	6.2	57.2	0.021	1.7
Sephacryl S-200	42	93.6	4.4	41.0	0.047	3.8
Accell QMA	35	23.3	3.7	34.4	0.16	12.8
HIC (phenyl-5 PW)	40	4.6	2.5	23.2	0.54	43.8
DEAE 5-PW (Kp_i)	4	0.83	1.7	15.6	2.05	165
300 SW	9	0.22	1.2	10.7	5.45	440
DEAE 5-PW (Tris)	4	0.112	0.86	7.9	7.68	640

a. In the early stages of the purification, total protein was determined by Lowry's method. In the late stages of the purification, total protein was determined by Bradford's method (Bio-Rad protein assay kit).

b. One unit of enzyme activity (U) is defined as the consumption of 1 mmol of **78** min^{-1} or the production of 1 mmol of **70** min^{-1}.

c. sp U was defined as 1 U per mg of protein.

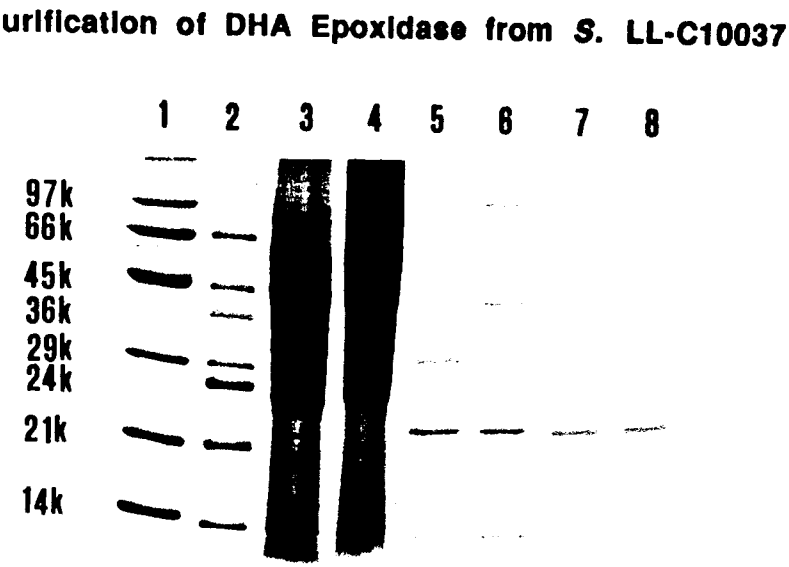


Figure 26. SDS-PAGE of DHA Epoxidase from *S.* LL-C10037
Lane 1: Bio-Rad Low MW standards; Lane 2: Sigma MW Standards; Lane 3: sample after step 1; Lane 4: sample after step 3; Lane 5 and 6: two active fractions after step 9; Lane 7 and 8: two active fractions after step 9 (samples for Lane 5, 6 and Lane 7, 8 were from two different runs).

Although numerous epoxyquinones and epoxysemiquinones were reported (in the 1,4-benzoquinone family alone more than 25 have been isolated),[10-42] very little was known about the formation of the epoxyquinone functionality. Priest and Light reported a particulate preparation from *P. patulum*,[43] which catalyzed the epoxidation of **54** and **45**. Attempts to purify this epoxidase failed due to the particulate nature of the enzyme. In the study of the biosynthesis of nanaomycins, Omura and coworkers[44] described a cell-free preparation from *S. rosa* var. *notoensis*, which catalyzed the epoxidation of **47**. No attempt was ever made to purify this epoxidase activity. Therefore, the DHA epoxidase from *S.* LL-C10037 was the first of this kind of enzyme to be purified.

It has to be pointed out that, very recently, Furie and coworkers[46] purified the vitamin K-dependent carboxylase to homogeneity. It had been suspected on the basis of the analysis of crude and partially purified vitamin K-dependent carboxylase and vitamin K epoxidase activities, that the carboxylation of glutamic acid residures and the epoxidation of vitamin K are reactions that are coupled.[47] With the pure enzyme Furie and coworkers[46] demonstrated unambiguously that the vitamin K-dependent carboxylation and vitatime K epoxide formation were catalyzed by the same enzyme.

Molecular Weight Determination

The MW of the DHA epoxidase was estimated on the Protein-Pak 300 SW. The column was calibrated with standards of known MW. As discussed in the MW determination of the AEBQ dehydrogenase, although a very good linear correlation of the standards was obtained (R = 0.984), care has to be exercised in analyzing data. Nevertheless, as shown in Figure 27, the purified DHA epoxidase had been eluted in 16 minutes on the 300 SW column and, therefore, its MW was estimated to be 117K ± 10K. Because only a single band with MW of 22.3K was shown on the SDS-PAGE, on the assumption that this band resulted from the enzyme, the DHA epoxidase was likely to be a pentamer or hexamer, having a subunit MW of 22.3K.

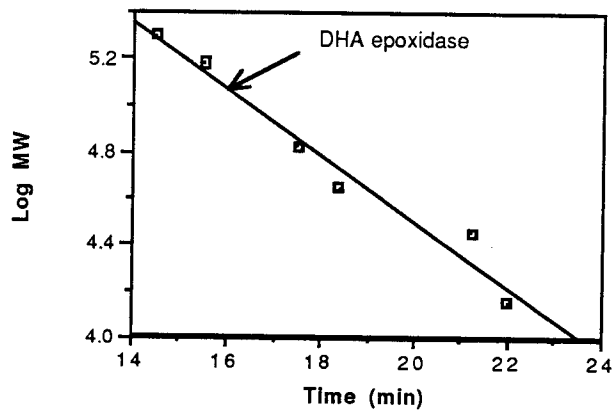


Figure 27. MW Determination of DHA Epoxidase on a Protein-Pak 300 SW Column
MW standards: α-lactalbumin, 14,200; carbonic anhydrase, 29,000; chicken egg albumin, 45,000; bovine serum albumin, 66,000; alcohol dehydrogenase, 150,000; β-amylase, 200,000.

pH Dependence

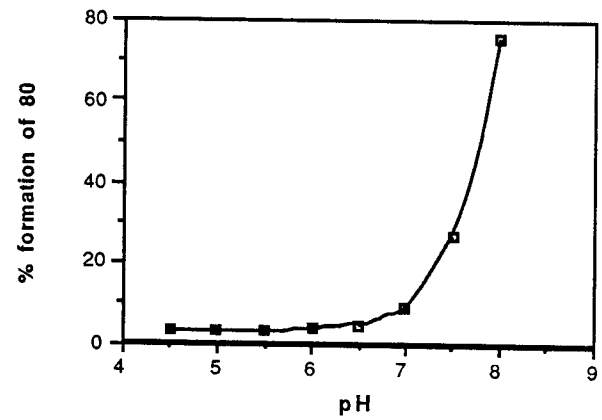


Figure 28. Air Oxidation of **78** in 0.1 M Potassium Phosphate
0.1 mM **78** in 0.1 M potassium phosphate buffer was incubated at 30 °C for 10 min and the mixture was analyzed by HPLC.

The epoxidation product **70** starts to decompose significantly in basic media (> pH 7.5). The substrate **78** was prone to air oxidation and as shown in Figure 28 this oxidation became predominant as the pH increased (>25% of **78** was converted to the corresponding quinone **80** at pH 7.5). Therefore, when the enzymatic epoxidation was carried out in buffer above pH 7.5, competitions

between enzymatic epoxidation and air oxidation of **78**, and decomposition of **70** had to be taken into consideration. Nevertheless, under the given conditions the DHA epoxidase displays an optimal pH of 6.5 in 0.1 M potassium phosphate buffer (Figure 29).

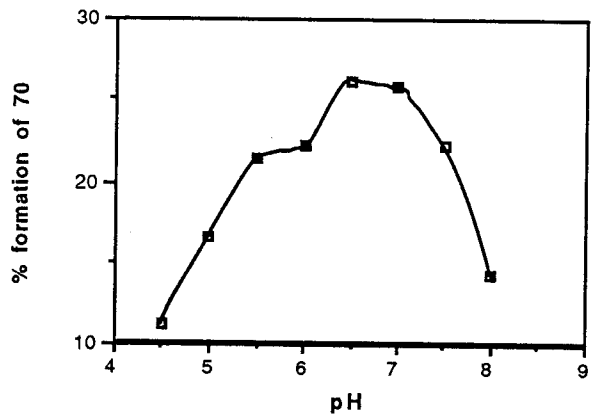


Figure 29. pH Dependence of DHA Epoxidase in 0.1 M Potassium Phosphate

Kinetics

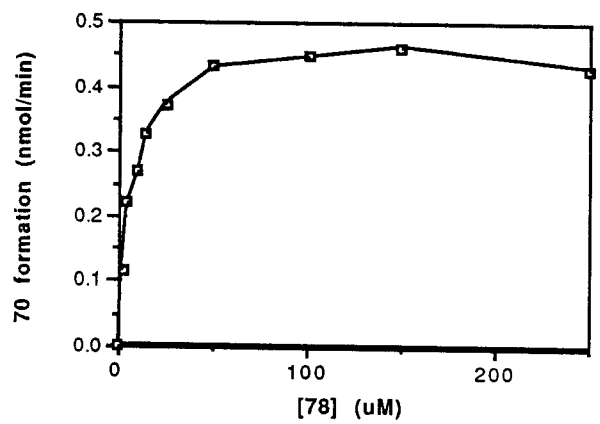


Figure 30. Effect of the Concentration of **78** on the DHA Epoxidase Activity

On the assumption that the O_2 concentration is constant in the assay solution, *pesudo*-1st order treatment and steady-state approach were applied for the kinetic analysis. The effect of the initial concentration of **78** on the formation of **70** is shown in Figure 30 and the DHA epoxidase follows classical

Michaelis-Menten kinetics. Substrate inhibition was observed at concentrations above 150 μM. From the Lineweaver-Burk plot, as shown in Figure 31, a very good linear correlation was obtained (R = 0.992). The apparent K_m and V_{max} were estimated to be 8.4 ± 0.5 μM and 3.7 ± 0.2 μmol min^{-1} mg^{-1} (or 430 ± 20 min^{-1}, if 117K is taken as the molecular weight for the DHA epoxidase). This V_{max} value is typical of other monooxygenases, such as 550 min^{-1} (*p*-hydroxybenzoate hydroxylase),[48a] and 1300 min^{-1} (3-hydroxybenzoate 6-hydroxylase),[48b]

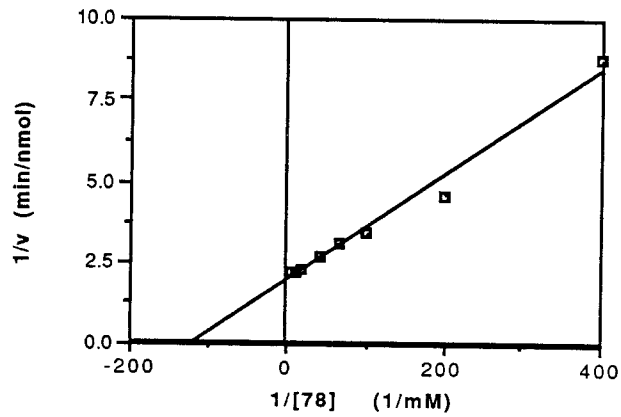


Figure 31. Lineweaver-Burk Plot of **78**

Cofactor Requirement

The preliminary account of the DHA epoxidase has demonstrated that **78** was epoxidized in the absence of any added cofactor, requiring molecular oxygen.[7] To extend this study, potential cofactors for monooxygenases were added to the assay solution to investigate their effects on the epoxidase. As shown in Table 37, the reaction apparently did not involve any loosely bound cofactors. Addition of NADH, NADPH, NAD^+, $NAD(P)^+$, FAD or FMN neither stimulated nor inhibited the DHA epoxidase. As demonstrated before, **80** and NADH can also support the epoxidation (entry 3), but this epoxidation resulted from **78**, which was generated by the chemical reduction of **80** with NADH[7] and no epoxidation was observed when **80** was used alone as the substrate (entry 2). The enzyme appeared to be very fragile and approximately 30% of the activity was lost simply by degassing and flushing air through the assay

solution (entry 11). Removing O_2 by exchange with nitrogen inhibited the epoxidation completely (entry 12).

Table 37. Effect of Potential Cofactors of Monooxygenase on DHA Epoxidase

Entry	Assay component[a]	Addition	(μM)	Rel act.(%)[b]
1	**78** + E	none		100
2	**80** + E	none		0
3	**80** + E	NADH	(100)	40
4	**78** + E (heat denatured)	none		0
5	**78** + E	NADH	(100)	101
6	**78** + E	NADPH	(100)	100
7	**78** + E	NAD^+	(100)	98
8	**78** + E	$NADP^+$	(100)	96
9	**78** + E	FMN	(10)	94
10	**78** + E	FAD	(10)	96
11	**78** + E	air[c]		67
12	**78** + E	N_2		0

a. Assay composition and conditions are described under Experimental.
b. The control (entry 1) corresponds to 26% formation of **70** (6.5 nmole).
c. The assay tube was degassed and flushed with the desired gas several time in 5 min and then assayed as usual. For N_2 the assay tube was sealed after the 5 min pretreatment and then assayed as usual.

The origin of the oxygen of the oxirane functionality of epoxyquinones and epoxysemiquinones has been a key issue in the investigation of the biosynthesis of this class of metabolites. Although the oxygen in all cases studied comes from molecular oxygen,[5,44,49-50] presumably via an enzymatic process involving a monooxygenase,[43,44,51-53] the nature of the epoxidation remained a matter of speculation. Either a quinone[6,44,49a,50] or a hydroquinone[7,43,49a,51-54] was postulated as the direct precursor for the epoxyquinones/epoxysemiquinones. The purification of the DHA epoxidase from *S.* LL-C10037 and the subsequent study of its cofactor requirement unambiguously established the stoichiometry of the biosynthesis of the epoxyquinone. Hydroquinone can be epoxidized directly to form epoxyquinone and molecular oxygen is required for this process. Unlike most of the monooxygenases which require a reduced cofactor such as NAD(P)H to provide the reducing power, the DHA epoxidase catalyzed the epoxidation of a hydroquinone in the absence of any added cofactor. The substrate hydroquinone itself may serve as the reducing equivalent as in the case of

amine oxidases; this type of enzyme has been named an internal monooxygenase. Thus, consistent with the vitamin K epoxidase,[51] the particulate preparation from *P. patulum*[43] and the DHA epoxidase from *S.* MPP 3051,[7] as well as the NNM-A monooxygenase,[44] in which the hydroquinone of NNM-A, **111**, might be the actual substrate (see Scheme 42), the DHA epoxidase-catalyzed epoxidation elucidated here may be of general significance, leading to biosynthesis of other epoxyquinones and epoxysemiquinones.

Inhibition

A great variety of mechanisms have been proposed for the activation of molecular oxygen by monooxygenases. As in some of the flavoprotein hydroxylases,[55,56] it was suggested that these oxygenation reactions were likely to arise from an oxygen species, presumably at the oxidation state of peroxide. In other cases metal ions are required and iron and copper are the predominant metal ions to be found.[57] From these considerations chelating reagents, catalase and P_{450} inhibitors were added to the assay solution. Table 38 summarizes all the results. Addition of catalase, which will act as hydrogen peroxide scavenger, did not inhibit the epoxidation (entries 2 and 3), thus ruling out hydrogen peroxide as a free intermediate in the enzymatic reaction. Although both CO and CN^- have been used as diagnostic inhibitors for a P_{450} system, significant inhibition resulted from addition of CN^- (entry 4), while CO had no more effect than did air (entries 11 and 12). This would be inconsistent with the cytochrome P_{450} system. It was interesting to note the difference between the addition of EDTA and 1,10-phenanthroline. While as high a concentration of EDTA as 5.0 mM had no significant effect (entries 7 and 8), the reaction was completely inhibited by addition of as little as 0.2 mM 1,10-phenanthroline (entry 9 and 10). This result suggested an involvement of a metal ion in the DHA epoxidase which may selectively form a very tight complex with 1,10-phenanthroline, but not with EDTA. However, the failure to observe inhibition with a particular chelating agent need not be absolute evidence to argue which metal may be involved. As reported by Vallee and Galdes,[58] EDTA did not inhibit yeast alcohol dehydrogenase in contrast to its effect on liver alcohol dehydrogenase, but 1,10-phenanthroline inhibited both. The inhibition by 1,10-phenanthroline could be reversed by addition of

Cu^{2+}, Cu^{1+} or Fe^{2+} (entries 13 - 15). Thus, the enzyme was first treated with 1,10-phenanthroline, leading to complete loss of activity. To this inhibited solution were added indicated amounts of Cu^{2+}, Cu^{1+} or Fe^{2+}, and the resulting solution was re-assayed for DHA epoxidase activity. As shown in entries 13 to 15, most of the enzyme activity was recovered. This data further supported the requirement of a metal ion in the epoxidase. It could be speculated that the enzyme activity was reconstituted by either Cu^{2+}, Cu^{1+} or Fe^{2+} directly or by the natural metal ion(s), which had been freed from its 1,10-phenanthroline complex upon addition of Cu^{2+}, Cu^{1+} or Fe^{2+}. The latter implied that Cu^{2+}, Cu^{1+} or Fe^{2+} formed tighter complexes than did the natural metal ion. Finally, the epoxidation was significantly inhibited by addition of the sulfhydryl reagent PCMBA (entry 5 and 6). Therefore, it was concluded that the DHA epoxidase appeared to require a metal ion and a sulfhydryl group, and a P_{450} system was probably not involved.

Table 38. Effect of Potential Inhibitor of Monooxygenase on DHA Epoxidase

Entry	Addition[a]	Concentration	Rel act.(%)[b]
1	none		100
2	catalase	10 units	90
3	catalase	50 units	91
4	KCN	1.0 mM	44
5	PCMBA	0.5 mM	60
6	PCMBA	1.0 mM	46
7	EDTA	1.0 mM	93
8	EDTA	5.0 mM	92
9	1,10-phenanthroline	1.0 mM	0
10	1,10-phenanthroline	0.2 mM	0
11	air		67[c]
12	CO		70[c]
13	1,10-phenanthroline	0.2 mM	
	$CuSO_4$	0.4 mM	61[d]
14	1,10-phenanthroline	0.2 mM	
	CuCl	0.4 mM	67[d]
15	1,10-phenanthroline	0.2 mM	
	$FeSO_4$	1.0 mM	65

a. Assay composition and conditions are described under Experimental.
b. The control (entry 1) corresponds to 26% formation of **70** (6.5 nmole).
c. The assay tube was degassed and flushed with air or CO several time in 5 min and then assayed as usual.
d. In the presence of these metal ions the air oxidation of **78** become significant even at pH 6.5.

Metal Ion Effect

The above inhibition study suggested that the DHA epoxidase requires a metal ion. Therefore, metal ions were added to the assay solution and their effect on the enzymatic epoxidation was examined. Table 39 presents the data.

Table 39. Effect of Metal Ions on DHA Epoxidase

Entry	Addition[a]	Conc. (mM)	Rel act.(%)[b]
1	none		100
2	$FeSO_4$	1.0	71
3	$CuSO_4$	0.2	63[c]
4	CuCl	0.2	79[c]
5	$HgCl_2$	0.2	41[c]
6	$MgSO_4$	0.2	94
7	$ZnSO_4$	0.2	89
8	$CaCl_2$	0.2	95
9	$MnCl_2$	0.2	108[c]
10	$MnCl_2$	0.04	246
11	$NiCl_2$	0.2	266
12	$CoSO_4$	0.2	498

a. Assay composition and conditions are described under Experimental.
b. The control (entry 1) corresponds to 26% formation of **70** (6.5 nmole).
c. In the presence of these metal ions the air oxidation of **78** became significant even at pH 6.5.

As demonstrated in Figure 28, the substrate **78** was prone to air oxidation and this process was accelerated in the presence of Cu^{2+}, Cu^{1+}, Hg^{2+} and Mn^{2+}. The relative reactivities in these cases were probably a bit low since substrate concentration was depleted during the reaction by air oxidation to form **80**. This was demonstrated in the case of Mn^{2+}. When 0.2 mM was added, < 5.0% of **78** was left at the end of the incubation period and **78** was converted either to **80** or to **70** with a relative reactivity of 108% (entry 9). When the concentration of Mn^{2+} was decreased to 0.04 mM, however, <5% of **80** was formed and substantially more **70** was produced with a relative activity of 246% (entry 10). Therefore, if competition between the air oxidation and enzymatic epoxidation was corrected, it seemed that the addition of 0.2 mM Cu^{1+}, Cu^{2+}, Hg^{2+}, Mg^{2+}, Zn^{2+}, Ca^{2+} and 1.0 mM Fe^{2+} had neither activated nor inhibited the epoxidation (entries 2-8), although Cu^{2+}, Cu^{1+} or Fe^{2+} can

reconstruct the enzyme activity from a 1,10-phenanthroline inhibited preparation. Surprisingly, however, Co^{2+} and Ni^{2+} as well as Mn^{2+} accelerated the epoxidation as much as 5-fold (entries 10-12).

Reconstitution of DHA Epoxidase from an Apoenzyme

Monooxygenases requiring metal ions are well-known, and iron and copper are the predominant metal ions involved. However, the above metal ion study showed no effect of these metal ions. Instead, Mn^{2+}, Ni^{2+} and Co^{2+} each had a tremendous activation effect on the DHA epoxidase. This is of great significance and motivated an extension of the study of metal ion activation on the DHA epoxidase.

Enzymes that bind metal ions have been divided into two classes: metal-activated enzymes, which associate metal ions loosely (K_d = 10^{-3} to 10^{-8} M) and metalloenzymes, which bind metal ions tightly (K_d < 10^{-8} M).[59a] Metals in metal-activated enzymes could be very easily lost or removed, such as on size exclusion or ion exchange columns during the purification process. However, removal of metal ions from metalloenzymes requires specific procedures. Methods routinely employed could be categorized into three groups: those performed at neutral pH, acidic pH, and alkaline pH. The first of these is the most commonly employed, as most metalloproteins denature at extremes of pH. Most established procedures are variations of the use of a metal-chelating agent to remove metal ion(s) from the enzyme followed by the removal of chelated metal ions and excess chelating agent.[59a] 1,10-phenanthroline has been the most routinely used chelating agent for preparing apoenzymes at neutral pH. Others, such as dipicolinic acid or 8-hydroxyquinoline have also been employed.

Removal of metal ions from enzymes occurs by one of two mechanisms. Either the free metal ion concentration is depleted by the chelating agent to promote dissociation of metal ion from the enzyme or the chelating agent forms a transient ternary complex with the metal and enzyme with subsequent removal of the metal ion from the protein. The former method generally is a time-dependent process, while the latter is usually rapid.[59a,b,c]

As mentioned earier, 1,10-phenanthroline is the most routinely used chelating agent. Nevertheless, a large number of chelating agents have been

synthesized, and studies of their capacity to complex metals have provided a vast body of knowledge for extensive experimentation in metalloenzymology.[59d,e,f] The apoenzyme of the DHA epoxidase was prepared[59a,60,61] by 1,10-phenanthroline treatment as described below and showed complete loss of the epoxidase activity. On the assumption that the loss of the enzyme activity resulted from removing the natural metal of the DHA epoxidase and that the metal extraction process was reversible, the enzyme activity could be reconstructed if the right metal ion could be inserted back into the apoenzyme. Table 40 summarizes the results.

Table 40. Effect of Metal Ions on the Apoenzyme of DHA Epoxidase

Entry	Addition[a]	Conc. (mM)	Rel act.(%)[b]
1	holoenzyme		100
2	apoenzyme		0
3	$FeSO_4$	0.2	0
4	$CuSO_4$	0.2	0
5	CuCl	0.2	0
6	$HgCl_2$	0.2	0
7	$MgSO_4$	0.2	0
7	$ZnSO_4$	0.2	0
8	$CaCl_2$	0.2	0
9	$MnCl_2$	0.2	53[c]
10	$NiCl_2$	0.2	484
11	$CoSO_4$	0.2	518

a. Assay composition and conditions are described under Experimental.
b. The control (entry 1) corresponded to 18% formation of **70** (5.5 nmole).
c. Most of **78** was air oxidized to **80** (14.8 nmole, 59%).

Remarkably, the DHA epoxidase was reconstituted only by addition of Co^{2+}, Ni^{2+} and Mn^{2+} and the reconstructed enzyme showed even substantially better activity (entries 9-11). The recovery of the enzyme activity also demonstrated that the metal ion of the enzyme can be reversibly removed or inserted under the conditions developed. On the other hand, no epoxidation was observed at all upon addition of 0.2 mM Cu^{1+}, Cu^{2+}, Hg^{2+}, Mg^{2+}, Zn^{2+}, Ca^{2+} or Fe^{2+} (entries 3-8). This was particularly interesting in the cases of Cu^{2+}, Cu^{1+} or Fe^{2+}, which did reconstitut the epoxidase in the presence of the 1,10-phenanthroline complex (entries 13-15, Table 38). This could be explained only by postulating that, without removal of the chelated metal ion, the enzyme

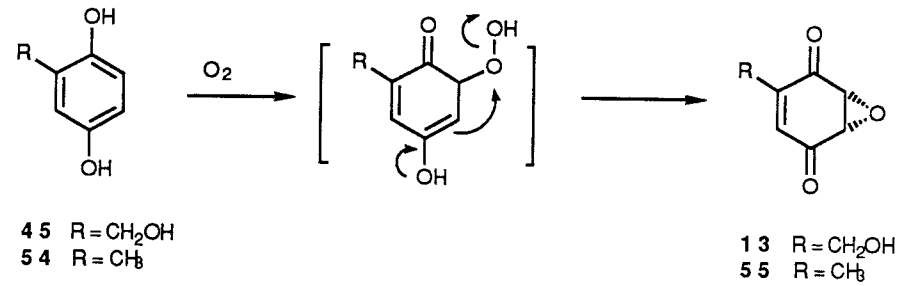
activity was reconstituted by Cu^{2+}, Cu^{1+} or Fe^{2+}, which regenerated the natural metal ion from its 1,10-phenanthroline complex.

These findings are striking. The DHA epoxidase from *S.* LL-C10037 apparently belongs to none of the defined groups of monooxygenases. The often found cofactors such as NADH, NADPH, NAD^+, $NADP^+$, FAD or FMN neither stimulate nor inhibit the epoxidation. The epoxidase activity was substantially inhibited by PCMBA, suggesting an involvement of a sulfhydryl group. Although EDTA has no effect, partial inhibition by CN^- and complete inhibition of the DHA epoxidase by addition of 0.2 mM 1,10-phenanthroline clearly indicated a requirement for a metal ion. These inhibition studies also demonstrated the clear difference between the DHA epoxidase and the particulate preparation from *P. patulum*. The latter was partially inhibited by either EDTA or 1,10-phenanthroline but was not affected at all by either CO or CN^-,[43] in contrast to the above-described behavior of the *S.* LL-C10037 enzyme. Furthermore, while either iron or copper, both of which are metal ions for common monooxygenases, cannot reconstitut the enzyme activity from the apoenzyme, the DHA epoxidase was reconstituted by additions of Mn^{2+}, Ni^{2+} or Co^{2+}.

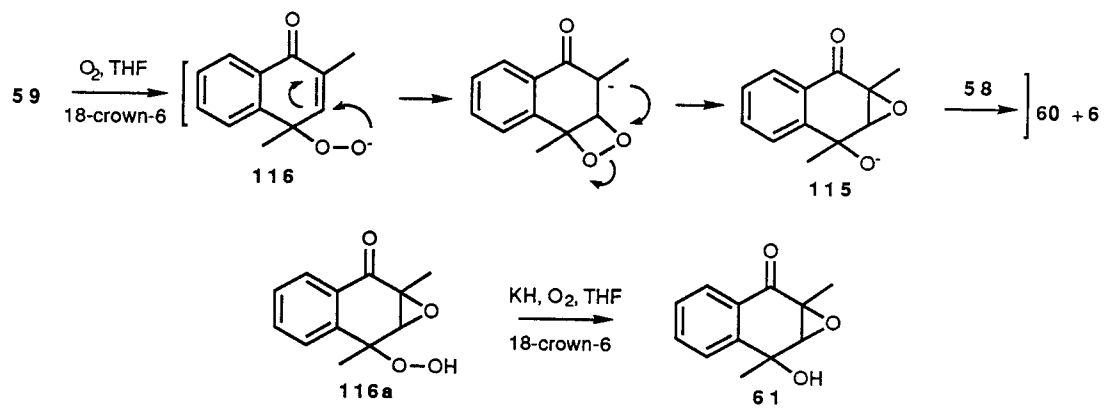
The natural metal ion of the DHA epoxidase was not yet been identified. Although 1,10-phenanthroline has very high stability constants[59d,e,f] toward Cu^{2+}, Fe^{2+} or Fe^{3+}, no attempt has been made to determine whether the apoenzyme was indeed free from any adventitious metals. By the chelating method with EDTA, McCord and Fridovich successfully prepared an apoenzyme of superoxide dismutase, in which two Cu^{2+} and one Zn^{2+} per subunit (two subunits for superoxide dismutase) had been removed.[59g] On the other hand, to complete removal of Fe^{3+} from adrenodoxin to prepare the apoadrenodoxin, it was necessary to dialyze the protein against a 5% trichloroacetic acid solution for 48 h at 5 °C.[59h] Therefore, one must be very cautious in analyzing results from apoenzymes, unless it has been demonstrated that the apoenzyme is indeed metal free. On the assumption that 1,10-phenanthroline removed all metal ion(s), the DHA epoxidase could be an enzyme which requires Mn^{2+}, Ni^{2+} or Co^{2+}, representing a new type of monooxygenase. Alternatively, the DHA epoxidase could contain tightly bound Cu^{2+}, Fe^{2+} or Fe^{3+} for catalysis, and requires some other metal ion(s), not bound to the enzyme as tightly to activate its epoxidase activity. These metal ion(s) were removed by formation

of a complex with 1,10-phenanthroline. Reconstitution of the epoxidase from the apoenzyme was only successful upon addition of Mn^{2+}, Ni^{2+} or Co^{2+} suggesting at least one of these may be native to the enzyme. The apoenzyme was quite stable, and this could explain the activation of DHA epoxidase by addition of Mn^{2+}, Ni^{2+} or Co^{2+}, possibly replacing equivalents lost during the purification. It is also possible, but less likely, that neither Mn^{2+}, Ni^{2+} nor Co^{2+} is the natural metal ion, but they could substitute for the natural one to reconstitute the enzyme activity. With both carboxypeptidase A[59i,j] and thermolysin,[59k] zinc could be replaced by various metals, including Mn^{2+}, Ni^{2+}, Co^{2+}. In fact, Co^{2+}-substituted carboxypeptidase A showed better activity (215%) than its natural enzyme,[59i,j] and so did the Co^{2+}-substituted thermolysin (200%).[59k] However, neither Zn^{2+}, nor any of six other metals could be re-activated DHA epoxidase apoenzyme.

Very little was known about the mechanism of this novel enzymatic epoxidation of a hydroquinone. As shown in Scheme 43, Priest and Light proposed[43] a mechanism for epoxidation of the hydroquinone involving the formation of a hydrogen peroxide intermediate. However, catalase had no effect on the enzymatic epoxidation, hence ruling out the possibility of free hydrogen peroxide as an intermediate. On the other hand, glutathione peroxidase showed an inhibitory effect on the vitamin K-dependent microsomal carboxylase.[52] Since it was believed that the vitamin K epoxidase was directly coupled in some obligatory manner to the vitamin K-dependent carboxylation[46,47,51,53,62,63] and that the purified carboxylase catalyzed both the carboxylation and epoxidation,[45] this inhibitory effect by glutathione peroxidase may indirectly suggest an involvement of a peroxide intermediate, presumably at the stage of vitamin K epoxidation. In fact, a nonenzymic model for the action of the vitamin K-dependent carboxylase was recently proposed.[64] As shown in Scheme 44, Ham and Dowd reported in their model that the formation of an epoxy alkoxide intermediate, **115**, was essential as a strong base to effect the condensation (internal carboxylation) of **58**. Although no explanation was given for the activation of molecular oxygen, a peroxide intermediate, **116**, was suggested; a stirred solution of **116a** in the presence of a base and 18-crown-6 also resulted in the formation of **61**. This model is interesting and no metal ion is required to activate molecular oxygen.

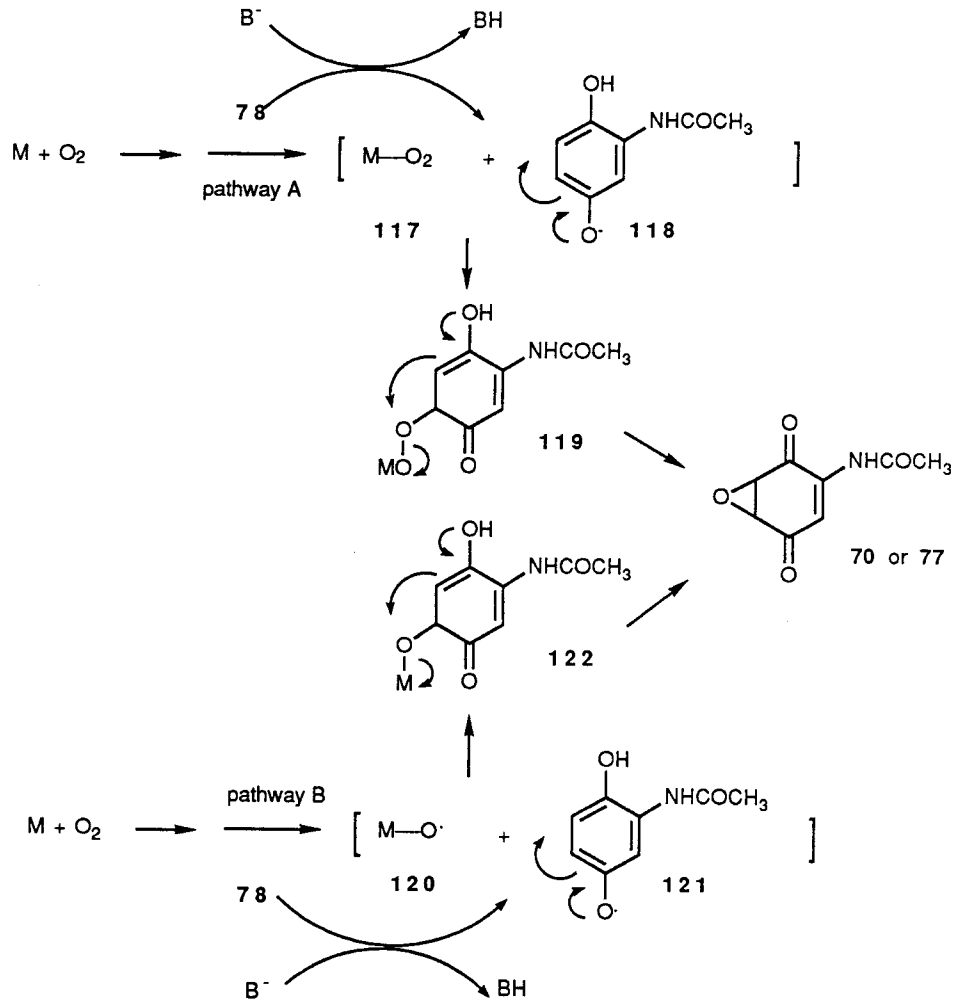


Scheme 43. Proposed Mechanism for the Enzymatic Epoxidation of **45** and **54**



Scheme 44. Nonenzymatic Model for Epoxidation by the Vitamin K-dependent Carboxylase

No inhibitory effect was observed upon addition of catalase to the DHA epoxidase, clearly eliminating the involvement of a free hydrogen peroxide intermediate. While, in Ham and Dowd's model, no metal ion was required, metal ion is essential to the DHA epoxidase. Like those proposed in hydroxylations or epoxidations catalyzed by the P_{450} or other metal-containing monooxygenases,[57,58,65-74] the same type of enzyme-bound metal-oxygen species should be preferred for the activation of molecular oxygen and the epoxidation of the hydroquinone by the DHA epoxidase. Thus, as proposed in Scheme 45, the enzymatic epoxidation could either be ionic (pathway a) or radical (pathway b) in nature. A base or bases in the active site may assist with hydrogen abstraction.



Scheme 45. Proposed Mechanisms for the DHA Epoxidase Catalyzed Epoxidation

According to pathway A, molecular oxygen could be activated as oxometallic species **117**, which could electrophilically attack the hydroquinone anion **118** to yield an enzyme-bound peroxide intermediate **119**. Alternatively, as in pathway B, molecular oxygen could be activated as a radical species **120** by accepting an electron from the hydroquinone which itself was partially reduced to form the hydroquinone radical **121**. Coupling of **120** and **121** could yield the oxygenated intermediate **122**. Finally both **119** and **122** could be converted to the epoxyquinone **70** or **77**.

A number of mechanisms have been proposed for the activation of molecular oxygen by monooxygenases. These include simple flavoproteins[55,56] or enzymes having complicated electron transfer

mechanisms from molecular oxygen to the oxygenated substrates. Among these, the iron-containing monooxygenases, either heme or non-heme systems, are those being most intensively studied.[57,58] Model studies using metals other than iron to activate molecular oxygen have been developed.[75-94] However, to our knowledge, metal ions other than iron and copper have rarely been found in enzymes activating molecular oxygen. There were only three precedents in which Mn was reported to be involved in activation of molecular oxygen.[95-97] The DHA epoxidase is the first example in which Ni and Co are believed to be associated with the activation of molecular oxygen. Since the metabolism of molecular oxygen plays such a vital role in biological systems, further study of the mechanism of the DHA epoxidase epoxidation may lead not only to the detailed elucidation of the biosynthesis of epoxyquinones but may also provide fundamental information for the understanding of molecular oxygen activation in other biological systems.

DHA Epoxidase from *S.* MPP 3051

With the DHA epoxidase from *S.* LL-C10037 having been purified, it was tempting to purify and characterize the DHA epoxidase from *S.* MPP 3051. It was expected that subtle differences or similarities between the two epoxidases could be revealed by comparing the purified enzymes, which may lead to an understanding of how the two enzymes control the facial specificity for the epoxidation of a planar substrate.

Purification of DHA Epoxidase

S. MPP 3051 (22.5 h fermentation for cell harvest) grows much faster than *S.* LL-C10037 (96 h fermentation for cell harvest). Although the two organisms grew in the same medium, they behaved quite differently. The cells harvested from *S.* MPP 3051 are very light, soft with fine mycelia. About 45 g (wet wt) of cells could be harvested from a 5-L fermentation of *S.* MPP 3051, while as many as 179 g (wet wt) of cells could be harvested from a 5-L fermentation of *S.* LL-C10037. Moreover, the yield of protein from *S.* MPP 3051 was higher than that from *S.* LL-C10037; 32 mg protein per g of cells was

obtained from *S.* MPP 3051 and only 4.8 mg of protein per g of cells was obtained from *S.* LL-C10037.

Ammonium Sulfate Precipitation and Sephacryl S-200 Column: The same protocol developed for purification of the DHA epoxidase from *S.* LL-C10037 was followed initially, and some modifications were subsequently found to be necessary. The same procedure was adopted directly from *S.* LL-C10037 for the preparation of CFE. However, the DHA epoxidase from *S.* MPP 3051 was difficult to precipitate by $(NH_4)_2SO_4$. As shown in Table 41, the DHA epoxidase was distributed in most of the fractions of $(NH_4)_2SO_4$ precipitation. Because very little protein was precipitated at 41% saturation of $(NH_4)_2SO_4$, this step was omitted from the purification scheme of the DHA epoxidase.

Table 41. Ammonium Sulfate Fractional Precipitation of DHA Epoxidase

% Saturation	% DHA epoxidase
0 - 41.1	2
41.1 - 51.3	3
51.3 - 61.6	18.3
61.6 - 71.9	23.5
71.9 - 82.2	33.0
82.2 - 92.4	21

Although the step of $(NH_4)_2SO_4$ precipitation was eventually omitted, the pellets from this step were initially loaded on the Sephacryl S-200 column identically to that from *S.* LL-C10037. The DHA epoxidase was eluted in approximately 2 void volumes, indicating that its molecular weight was lower than that of the DHA epoxidase from *S.* LL-C10037. However, because the $(NH_4)_2SO_4$ precipitation step was omitted, it was impossibly to apply such a large volume of the supernatant from protamine sulfate directly on a size exclusion column without a step of concentration. Therefore, this step was left out from the purification scheme as well.

Accell QMA Column: Usually an ion exchange column would be the choice of columns placed early in a purification scheme. However, the enzyme did not bind to the cation exchange resin (Protein-Pak SP 5 PW) under any

conditions tried. However, the Accell QMA (an anion exchanger) column, eluted by applying a KCl gradient in the potassium phosphate buffer, gave satisfactory results. Figure 32 shows the column profile and the active fractions are shaded.

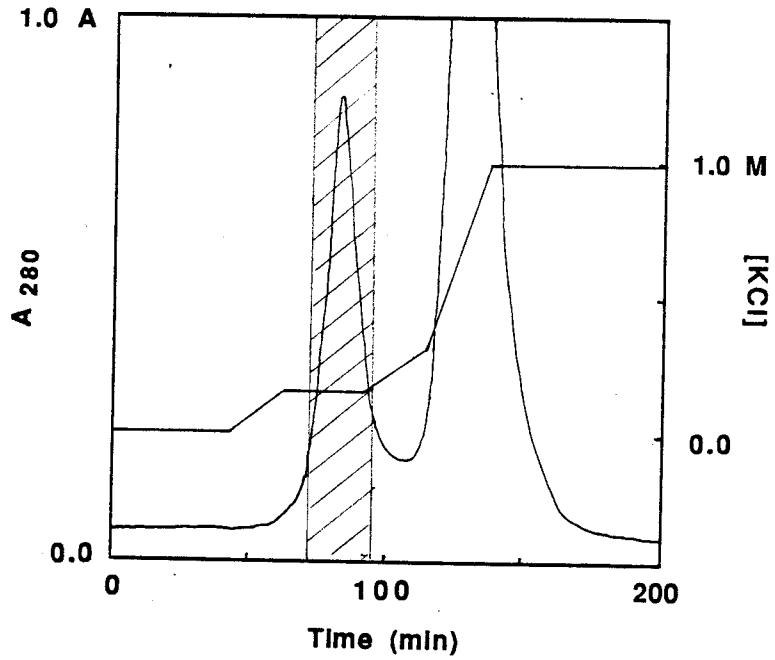


Figure 32. FPLC of DHA Epoxidase on an Accell QMA Column

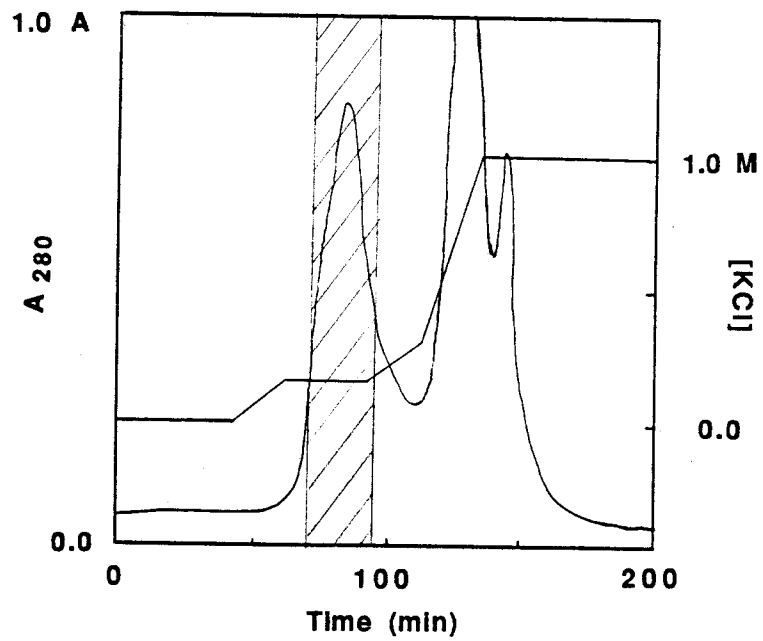


Figure 33. FPLC of DHA Epoxidase on an Accell QMA Column

The strategy of applying different buffers on the same column to achieve better resolution was applied next. The active fraction from above was dialysed to remove the KCl and applied to the same Accell QMA column. This time, however, the column was eulted by applying a KCl gradient in a Tris buffer and the proteins were further resolved as shown in Figure 33.

HIC (Phenyl 5 PW) Column: Hydrophobic interaction was chosen as the mechanism for the next step of purification. The active fraction from above was brought to 1.6 M $(NH_4)_2SO_4$; the enzyme can bind to the HIC (Phenyl 5 PW) column in buffer containing such a high concentration of $(NH_4)_2SO_4$. Figure 34 shows the column profile and the active fraction is shaded. The epoxidase was selectively eluted by decreasing $(NH_4)_2SO_4$ concentration in the buffer.

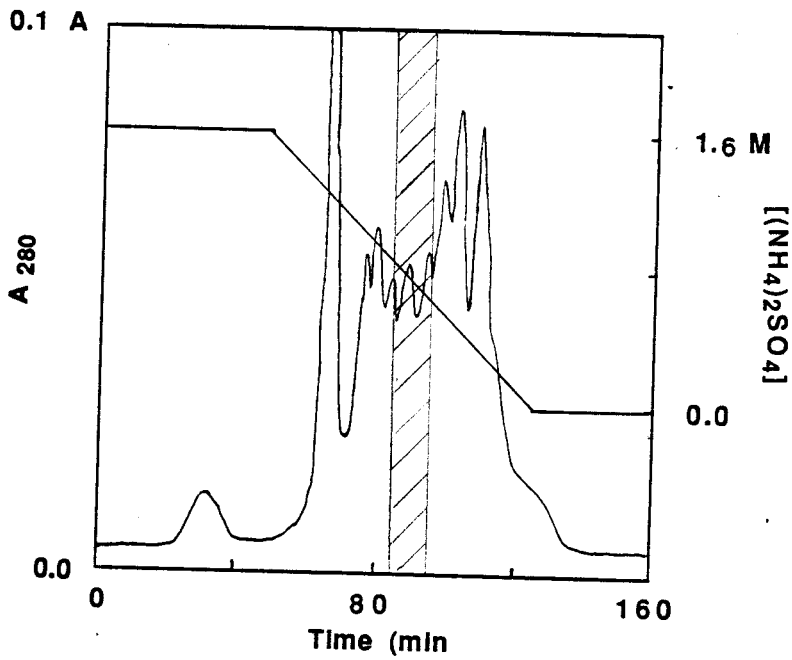


Figure 34. FPLC of DHA Epoxidase on a HIC (Phenyl 5 PW) Column

DEAE 5 PW Column: The DEAE column was next applied. It was expected that better resolution would be obtained on this FPLC anion exchange column. Figure 35 shows the column profile and the active fraction is shaded. Behaving similarly to the Accell QMA column, the enzyme activity was selectively eluted by applying a KCl gradient.

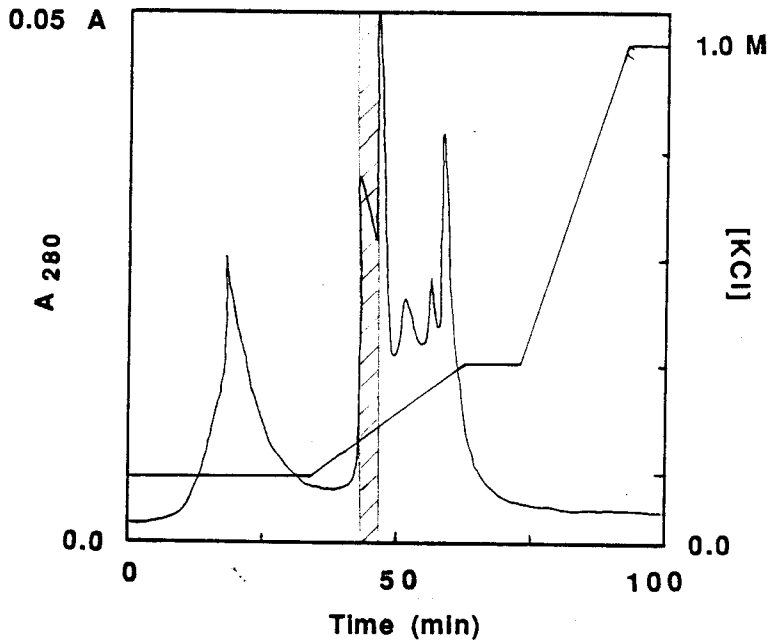


Figure 35. FPLC of DHA Epoxidase on a DEAE 5 PW Column

300 SW Column: At this stage of purification, it could be beneficial to include a size exclusion column in the purification scheme. Therefore, the Protein-Pak 300 SW column was chosen as the next step. The active fraction from above was first concnetrated to a very small volume; this was essential for good resolution on a size exclusion column. Figure 36 shows the column profile and the enzyme activity is shaded. As expected, the epoxidase was eluted later than that observed with the *S.* LL-C10037 enzyme. Careful assay of each fraction showed that the epoxidase was located in the tail part of the broad peak.

DEAE 5 PW Column: The Protein-Pak DEAE 5 PW was applied again as the last column for the purification of the DHA epoxidase. However, unlike in the enzyme from *S.* LL-C10037, the epoxidase here could not bind to the column in 50 mM Tris buffer. Consequently, 25 mM Tris buffer was used. As shown in Figure 37, the proteins were well separated by applying a KCl gradient and the DHA epoxidase was found under a symmetrical peak (active fraction is shaded).

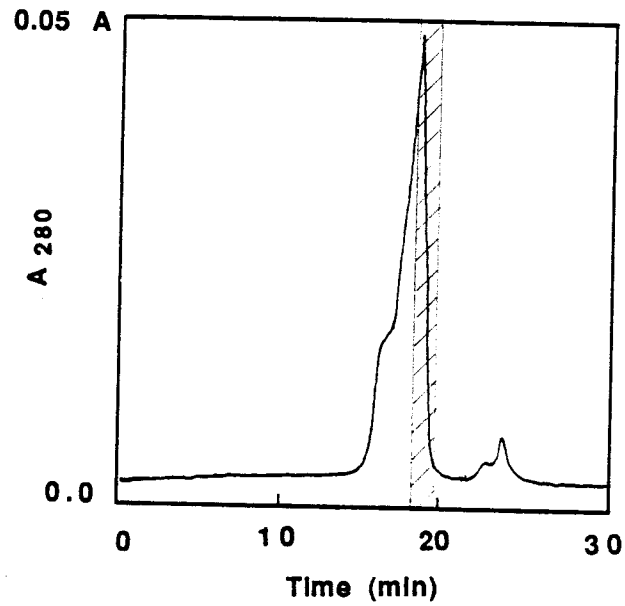


Figure 36. FPLC of DHA Epoxidase on a 300 SW Column

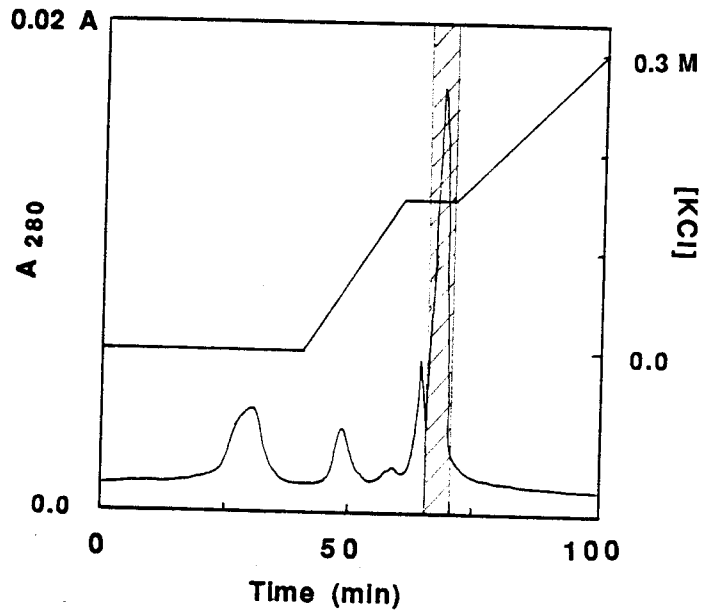


Figure 37. FPLC of DHA Epoxidase on a DEAE 5 PW Column

In conclusion, this purification scheme is summarized in Table 42. The enzyme was purified with an overall purification factor of about 1489.

Table 42. Purification of DHA Epoxidase from *S. MPP 3051*

Step	Vol. (mL)	Protein[a] (mg)	U[b]	U%	sp U[c]	Purifn x-fold
CFE	335	1072	5.04	100	0.0047	1
PS precip.	335	854	4.44	88	0.0052	1.2
Accell QMA(KPi)	360	148	3.58	71	0.0242	5.4
Accell QMA (Tris)	45	68.5	3.38	67	0.0493	10.5
HIC (phenyl-5 PW)	44	12.6	3.08	61	0.244	52
DEAE 5-PW (kp_i)	14	1.76	2.31	46	1..31	279
300 SW	7	0.365	0.84	17	2.3	490
DEAE 5-PW (Tris)	5	0.070	0.49	10	7.0	1489

a. In the early stages of the purification, total protein was determined by Lowry's method. In the late stages of the purification, total protein was determined by Bradford's method (Bio-Rad protein assay kit).

b. One unit of enzyme activity (U) is defined as the consumption of 1 mmol of 78 min^{-1} or the production of 1 mmol of 77 min^{-1}.

c. sp U was defined as 1 U per mg of protein.

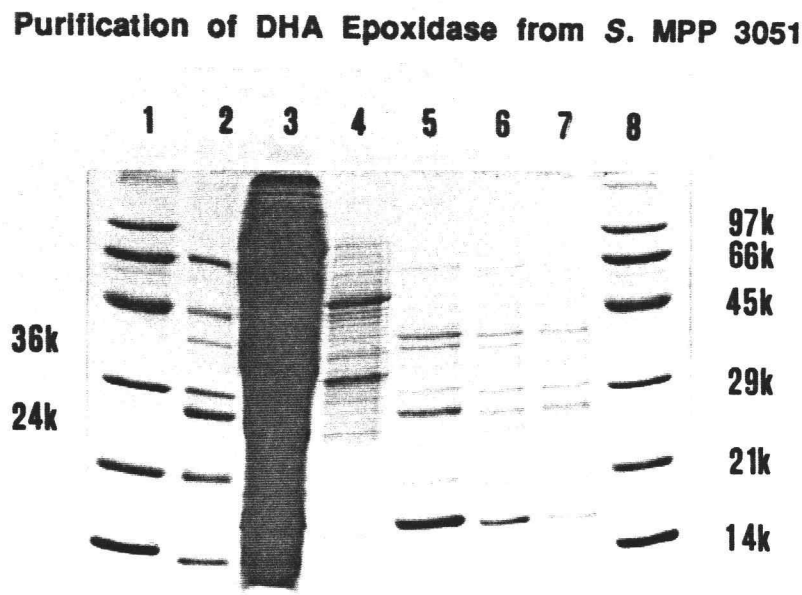


Figure 38. SDS-PAGE of DHA Epoxidase from *S.* MPP 3051
Lane 1 and 8: Bio-Rad Low MW standards; Lane 2: Sigma MW Standards; Lane 3: sample after step 1; Lane 4: sample after step 5; Lane 5, 6 and 7: active fractions after step 8.

The homogeneity of the purified DHA epoxidase was examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). The gels were stained with Coomassie brilliant blue R. As shown in Figure 38, DHA epoxidase preparations of the CFE had many proteins (Lane 3).

However, significant purification was achieved after step 5 (Lane 4). The purified DHA epoxidase from the last step (step 8) displayed one major band with additional minor bands, indicating that it has been purified to near homogeneity (Lanes 5 - 7). Because the native enzyme had a MW of 33K ± 2K (discussed late), the major band was believed to result from the DHA epoxidase. Therefore, the MW of the subunit was estimated to be 16K by comparision with the standards of known molecular weight.

Molecular Weight Determination

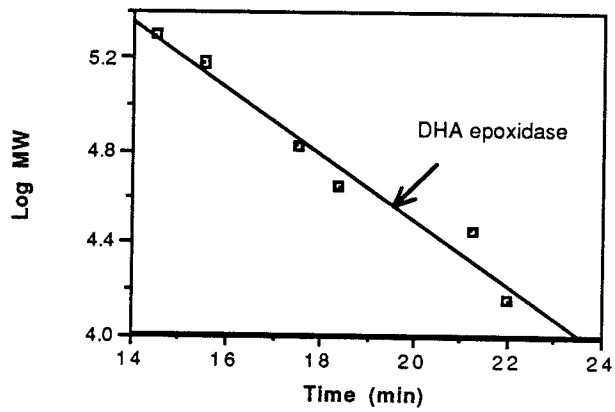


Figure 39. MW Determination of DHA Epoxidase on a Protein-Pak 300 SW Column
MW standards: α-lactalbumin, 14,200; carbonic anhydrase, 29,000; chicken egg albumin, 45,000; bovine serum albumin, 66,000; alcohol dehydrogenase, 150,000; β-amylase, 200,000.

The MW of the DHA epoxidase was again estimated on the Protein-Pak 300 SW. The column was calibrated with standards of known MW. As discussed in the MW determination of the AEBQ dehydrogenase, although a very good linear correlation of the standards was obtained (R = 0.984), care must be exercised in analyzing data. However, the accuracy of the method increases as the proteins get smaller, because of longer retention times relative to those larger proteins. Therefore, in this MW range, a half minute error in retention time results in only 2.5K difference in the MW determined. As shown in Figure 39, the purified DHA epoxidase has been eluted in 19.5 min on the 300 SW column so that its MW was estimated to be 33K ± 2K. Therefore, unlike that

from *S.* LL-C10037, the DHA epoxidase from *S.* MPP 3051 was much smaller and was found to be a dimer having a subunit Mr 16K (Figure 38).

pH Dependence

It has been pointed out that under basic conditions the epoxidation product **77** starts to decompose significantly (> pH 7.5) and the substrate **78** was prone to air oxidation (>25% of **78** was converted to **80** at pH 7.5). Therefore, when the enzymatic epoxidation was carried out in buffer above pH 7.5, competitions between enzymatic epoxidation and air oxidation of **78**, and decompositiom of **77** had to be taken into consideration. While the enzyme from *S.* LL-C10037 showed an optimal pH of 6.5, the DHA epoxidase from *S.* MPP 3051 displayed an optimal pH of 5.5 in 0.1 M potassium phosphate buffer (Figure 40).

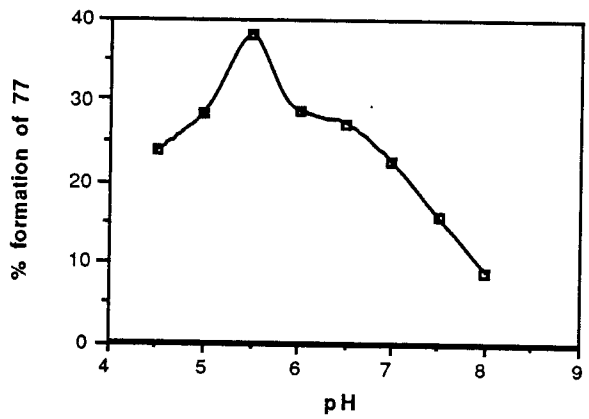


Figure 40. pH Dependence of DHA Epoxidase in 0.1 M Potassium Phosphate

Kinetics

Like that from *S.* LL-C10037, the DHA epoxidase also follows classical Michaelis-Menten kinetics. The effect of the initial concentration of **78** on the formation of **77** is shown in Figure 41. Substrate inhibition was observed at concentrations above 100 μM. From the Lineweaver-Burk plot, as shown in

Figure 42, a very good linear correlation was obtained (R = 0.996). The apparent K_m was estimated to be 7.2 ± 0.4 μM.

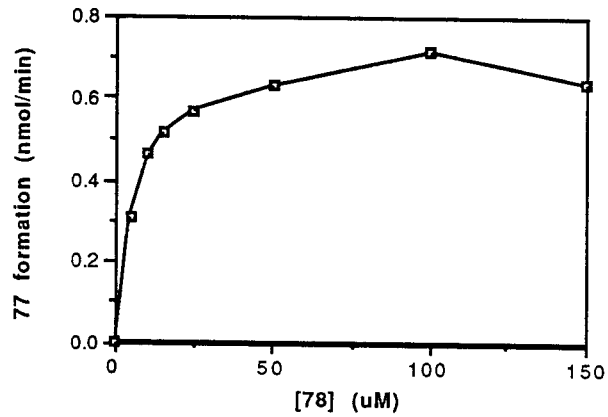


Figure 41. Effect of the Concentration of **78** on the DHA Epoxidase Activity

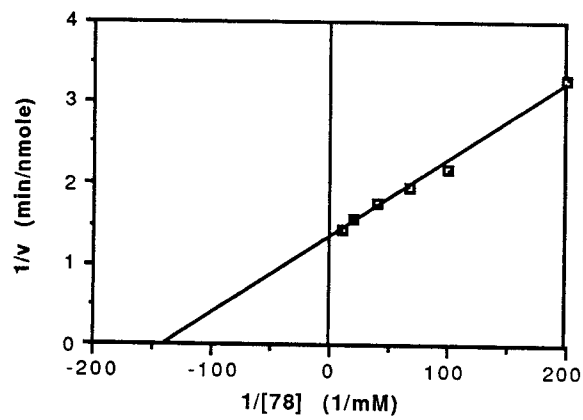


Figure 42. Lineweaver-Burk Plot of **78**

Cofactor Requirement

Similar to that from *S.* LL-C10037, the preliminary analysis of the DHA epoxidase had demonstrated that **78** was epoxidized in the absence of any added cofactor, requiring only molecular oxygen. To extend this study, potential cofactors for monooxygenases were added to the assay solution to investigate their effects on the epoxidase.

Table 43. Effect of Potential Cofactors of Monooxygenase on DHA Epoxidase

Entry	Assay component[a]	Addition	(μM)	rel act.(%)[b]
1	**78** + E	none		100
2	**78** + E (heat denatured)	none		0
3	**80** + E	none		0
4	**78** + E	NADH	(100)	102
5	**78** + E	NADPH	(100)	106
6	**78** + E	NAD^+	(100)	104
7	**78** + E	$NADP^+$	(100)	93
8	**78** + E	FMN	(10)	57
9	**78** + E	FAD	(10)	87
10	**78** + E	air		69[c]
11	**78** + E	N_2		0[c]

a. Assay composition and conditions are described under Experimental.
b. The control (entry 1) corresponds to 22% formation of **77** (5.5 nmole).
c. The assay tube was degassed and flushed with the desired gas several times in 5 min. For air the assay was done as above; for N_2 the assay tube was sealed after the 5 min pretreatment and then assayed as above.

As shown in Table 43, the reaction apparently did not involve any loosely bound cofactors. Addition of 0.1 mM NADH, NADPH, NAD^+, or $NAD(P)^+$ neither stimulated nor inhibited the DHA epoxidase (entries 4-7). In fact, slight inhibition was observed in the presence of FMN and FAD (entries 8 and 9). As demonstrated before, **80** and NADH can also support the epoxidation; but this epoxidation resulted from **78** which was generated by the chemical reduction of **80** by NADH, and non-epoxidation was observed when **80** was used alone as substrate (entry 3). The enzyme appeared to be very fragile, and approximately 30% of the activity was lost simply by the pretreatment used for the gas study (entry 10). Finally, as expected, the enzyme activity was totally lost upon heat denaturation (entry 2), and O_2 was required for the epoxidation (entry 11).

Inhibition

Encouraged by the results from the inhibition study on the DHA epoxidase from *S.* LL-C10037, effects of chelating reagents, catalase and P_{450} inhibitors on the DHA epoxidase from *S.* MPP 3051 were also examined. Table 44 summarizes all the results. Not surprisingly, almost the same type of effect as

that on the DHA epoxidase from *S.* LL-C10037 was observed on the DHA epoxidase from *S.* MPP 3051. Thus, addition of catalase had no effect on epoxidation, ruling out hydrogen peroxide as a free intermediate (entries 2 and 3). PCMBA substantially inhibited the reaction (entries 5 and 6), indicating a possible involvement of a sulfhydryl group. Although EDTA had no effect (entries 7 and 8), the epoxidase was completely inhibited on addition of 1,10-phenanthroline (entries 9 and 10), clearly indicative of a metal ion. However, unlike that from *S.* LL-C10037, the DHA epoxidase from *S.* MPP 3051 was partially inhibited by either CO or CN^- (entries 4 and 12).

Table 44. Effect of Potential Inhibitors of Monooxygenase on DHA Epoxidase

Entry	Addition[a]	Concentration	Rel act.(%)[b]
1	none		100
2	catalase	(10 units)	87
3	catalase	(50 units)	89
4	KCN	(1.0 mM)	81
5	PCMBA	(0.5 mM)	82
6	PCMBA	(1.0 mM)	29
7	EDTA	(1.0 mM)	108
8	EDTA	(5.0 mM)	106
9	1,10-phenanthroline	(1.0 mM)	0
10	1,10-phenanthroline	(0.2 mM)	0
11	air		69[c]
12	CO		43[c]

a. Assay composition and conditions are described in Material & Methods.
b. The control (entry 1) corresponded to 22% formation of 77 (5.5 nmole).
c. The assay tube was degassed and flushed with the desired gas several times in 5 min and then assayed as usual.

Metal Ion Effect

The above inhibition study suggested that the DHA epoxidase may require a metal ion, as in the case of the DHA epoxidase from *S.* LL-C10037. To extend this investigation, metal ions were added to the assay solution and their effects on the epoxidase were examined. Table 45 is a summary of all the results. Remarkably, no activation of the DHA epoxidase was observed by addition of any of these metal ions tested, in contrast to the enzyme with DHA epoxidase from *S.* LL-C10037. The latter was significantly activated by the presence of Mn^{2+}, Ni^{2+} or Co^{2+}. Unfortunately, no epoxidase activity could be

reconstituted from the corresponding preparation of the apoenzyme. This could be explained if the natural metal ion had not been provided, or the apoenzyme was unstable after removal of the metal ion so that it had been denatured. The latter would be consistent with the observation that none of the metal ions tested activated the DHA epoxidase. If metal ion was lost during the purification, the apoenzyme resulting would be denatured immediately. Consequently, no enzyme activity could be reconstituted even though metal ion was supplied subsequently. It is also possible that the metal ion simply cannot be reversibly inserted back into the apoenzyme. However, without removing the chelated metal ion, the epoxidase activity can be reconstituted by addition of Fe^{2+}, Cu^{2+} or Cu^{1+} (entries 13-15). These data suggested again that 1,10-phenanthroline may form a transient ternary complex with metal ion and enzyme, but this could be dissociated upon addition of external metals. In any case, these data at least supported an involvement of a metal ion in the epoxidase, although the actual metal ion remains unknown.

Table 45. Effect of Metal Ions on DHA Epoxidase

Entry	Addition[a]	Conc. (mM)	Rel act.(%)[b]
1	none		100
2	$FeSO_4$	1.0	71[c]
3	$CuSO_4$	0.2	40[c]
4	CuCl	0.2	60[c]
5	$HgCl_2$	0.2	54[c]
6	$MgSO_4$	0.2	101
7	$ZnSO_4$	0.2	105
8	$CaCl_2$	0.2	88
9	$MnCl_2$	0.2	60[c]
10	$NiCl_2$	0.2	100
11	$CoSO_4$	0.2	95
12	1,10-phenanthroline	0.2	0
13	1,10-phenanthroline	0.2	
	$CuSO_4$	0.4	65[c]
14	1,10-phenanthroline	0.2	
	CuCl	0.4	74[c]
15	1,10-phenanthroline	0.2	
	$FeSO_4$	1.0	80

a. Assay composition and conditions are described under Experimental.
b. The control (entry 1) corresponded to 22% formation of **77** (5.5 nmole).
c. In the presence of these metal ions the air oxidation of **78** became significant even at pH 6.5.

Comparison of the Two DHA Epoxidases

Two DHA epoxidases were isolated from *S.* LL-C10037 and *S.* MPP 3051, respectively. Table 46 lists all the characteristics of the two enzymes.

Table 46. Characteristics of DHA Epoxidases from *S.* LL-C10037 and *S.* MPP 3051

Organism	*S.* LL-C10037	*S.* MPP 3051
substrate	**78** + O_2	**78** + O_2
product	**7 0**	**7 7**
molecular weight	117K ± 10K	33K ± 2K
subunit	22K	16K
number of subunits	5 or 6	2
apoenzyme	stable	unstable
optimal pH	6.5	5.5
K_m for **78**	8.4 μM	7.2 μM
substrate inhibition (**78**)	150 μM	100 μM
V_{max}	3.7 μmol min^{-1} mg^{-1}	undetermined
cofactor	none	none
PCMBA	inhibited	inhibited
1,10-phenanthroline	inhibited	inhibited
KCN	inhibited	inhibited
CO	no	inhibited
Mn^{2+}	activated	no
Ni^{2+}	activated	no
Co^{2+}	activated	no

Complementary enzymes which catalyze the formation of a pair of enantiomers from the same substrate are very rare.[98-105] Very little study has been done in regards to their enantiomeric specificity. The two epoxidases from *Streptomyces* sp. are the first example of this kind of enzyme being purified to this extent. As one would expect, the two enzymes have many similarities because they both catalyze the same reaction (exclusive of the stereochemistry). However, they are substantially different and these differences presumably are responsible for their opposite facial specificity towards the same planar substrate. As proposed in Figure 16, the two DHA epoxidases, different in size and number of subunits, could either bind the substrate **78** in opposite orientations or deliver the activated oxygenating species to **78** from opposite sides. However, although more is now known about the two epoxidases than before, it is still premature to make any detailed speculations on how the enzymes control their facial specificity. This

probably can be answered only by determining the three-dimensional features of the active sites. Nevertheless, it is clear that the two epoxidases are metalloenzymes, but they apparently do not belong to any known group of monooxygenases in the literature.

Experimental

General Procedures

UV spectra were recorded on an IBM 9420 UV-Visible spectrophotometer. HPLC analysis was performed on a Waters 600E HPLC instrument with a Kratos Spectroflow 757 UV detector or a Waters 6000A HPLC instrument with a Linear UVIS 200 detector. An HP 3396A integrator was used. The Radi-Pak C_{18} (Novapak, 4 μ, 8x100 mm) column was purchased from Waters Assoc and the Versopack C_{18} column (10 μ, 4.1x250 mm) was purchase from Alltech Assoc.

Cell-free preparations and enzyme purifications were carried out at 4 °C. An IEC B-20A refrigerated centrifuge was used. Cell disruption was performed on a Sonicator Model W-225R made by Heat Systems-Ultrasonic, Inc. For open columns, the flow rate was controlled by a Peristaltic Pump P-3 (Pharmacia) and the fractions were monitored by a set of Dual Path Optical-Control UV-2 units (Pharmacia). All FPLC columns were purchased from Waters Assoc. The Accell QMA anion exchange resin was purchased from Waters Assoc and Sephacryl S-200 was from Pharmacia. A Waters 650E FPLC system was used for the enzyme purification, with a Lambda-Max Model 481 LC spectrophotometer as detector. Incubations were performed in an IBM 9550 Heating/cooling Fluid Circulator (± 0.1 °C). PVPP and protamine sulfate were purchased from Sigma, and PMSF was purchased from Aldrich. Molecular weight standards for gel filtration were purchased from Sigma. Enzyme grade ammonium sulfate was used. Molecular weight standards for SDS-PAGE were purchased from Sigma and Bio-Rad. Reagents for SDS-PAGE were purchased from Bio-Rad. Mini-protein II slab cell (Bio-Rad) was used.

Protein Determination

Protein concentrations were determined by either the method of Lowry[106] or the method of Bradford,[107] using bovine serum albumin as the calibration standard.

Polyacrylamide Gel Electrophoresis

Denaturing gels were run according to the Laemmli procedure.[108] The separating gel and the stacking gel were 15% and 3.5% polyacrylamide, respectively.

Molecular Weight Determination

The MW of the native enzyme was determined by gel filtration on the Protein-Pak 300 SW column (8 x 300 mm). The column was eluted with 50 mM potassium phosphate buffer, pH 7.0, containing 20% glycerol and 0.2 mM EDTA at a flow rate of 0.5 mL/min. The following standards (purchased from Sigma) were used: β-amylase (200,000), alcohol dehydrogenase (150,000), bovine serum albumin (66,000), chicken egg albumin (45,000), carbonic anhydrase (29,000), α-lactalbumin (14,000). The subunit MW was determined by SDS-polyacrylamide gel electrophoresis using the low MW standards from both Sigma: bovine serum albumin (66,200), chicken egg albumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (21,000), α-lactalbumin (14,000) and from Bio-Rad: phosphorylase B (97,400), bovine serum albumin (66,200), chicken egg albumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), lysozyme (14,400).

Streptomyces LL-C10037

Standard Culture Conditions

S. LL-C10037 was maintained at 4 °C as spores on sterile soil. A loopful of this material was used to inoculate 50 mL of seed medium containing 1.0%

glucose, 2.0% soluble potato starch, 0.5% yeast, 0.5% N-Z Amine A 59027, and 0.1% $CaCO_3$ in Milli-Q water, all adjusted to pH 7.2 with 2% KOH. The seed inoculum, contained in a 250-mL Erlenmeyer flask, was incubated for 3 days at 28 °C, 240 rpm in a rotary incubator. Production broths (400 mL in 2-L Erlenmeyer flasks), consisting of 1.0% glucose, 0.5% bactopeptone, 2.0% molasses (Grandma's famous light unsulfured), and 0.1% $CaCO_3$ in Milli-Q water and adjusted to pH 7.2 with 10% HCl prior to sterilization, were subsequently inoculated 5% (v/v) with vegetative inoculum from seed broths. The production broths were incubated under the same conditions.

Enzyme Assay

The DHA epoxidase was assayed by following the consumption of **78** and the production of **70** simultaneously by high performance liquid chromatography (HPLC) on a Waters 6000A instrument. Typically, 500 µL of assay solution, consisting of 0.1 mM **78** in 0.1 M potassium phosphate buffer in the presence of the DHA epoxidase, was incubated at 30 °C. The assay was initiated by addition of the DHA epoxidase to the assay solution. The enzyme reaction was terminated by addition of 100 µL of CH_3CN/H_2O/TFA (66/27/7, v/v). The reaction mixtrue was then centrifuged to remove the protein and the supernatant was analyzed for **78** and **70** by HPLC either on a Waters Radi-Pak C_{18} column (H_2O/CH_3CN/TFA = 85/15/0.1%, flow rate 1.0 mL/min) or on Alltech Versopack C_{18} column (H_2O/CH_3CN = 85/15, flow rate 1.0 mL/min). A UV detector at 225 nm was connected to the HPLC system and the whole system was calibrated with known quantities of **78** and **70** (under the given conditions, **78** and **70** showed relative responses of 1.09 and 1.0 on a molar basis, respectively).

For the pH dependence study, complete assay solutions as above in 0.1 M potassium phosphate buffer, pH 4.5 to 8.0, were incubated at 30 °C for 5 min. The assay mixtures were then subjected to HPLC ananlysis.

For determination of kinetic parameters, 500 µL of assay solutions containing 2.5 µM to 250 µM **78** and 0.14 µg of DHA epoxidase (the purest sample) in 0.1 M potassium phosphate buffer, pH 6.5, were incubated at 30 °C for 5 min. The terminated assay mixtures were then analyzed by HPLC.

For inhibition and activation studies, unless otherwise specified, 250 μL of assay solutions containing 0.1 mM **78**, 0.5 μg of DHA epoxidase (the purest sample) and the indicated amount of either inhibitors or activators were incubated at 30 °C for 10 min. The terminated assay mixtures were then analyzed by HPLC.

Purification of DHA Epoxidase

Buffers:

I: 10 mM potassium phosphate, pH 7.0.

II: 50 mM potassium phosphate, pH 7.0, 20% glycerol, 0.2 mM EDTA.

III: 1.0 M KCl in buffer II.

IV: 1.0 M $(NH_4)_2SO_4$ in buffer II.

V. 50 mM Tris-HCl, pH 7.5, 20% glycerol, 0.2 mM EDTA.

VI. 1.0 M KCl in buffer V.

Step 1: Preparation of Cell-Free Extract: Cells from 5.0 L of 96-h fermentation of *S.* LL-C10037 were harvested by centrifugation (4 °C, 13800g, 10 min) and washed sequentially with buffer I, 1.0 M KCl, 0.8 M NaCl and buffer I. After each wash the cells were centrifuged as above. The washed cells (179 g, wet wt.) were then suspended in buffer II (550 mL); the cell suspension was subsequently brought to 3 mg/mL with powdered PVPP and 1.0 mM with 1 M PMSF solution in acetone. It was then equally distributed into six beakers and each cell suspension was disrupted by sonication (maximum power, 90% duty, pulse for 4 x 20 sec.). Cell debris was removed by centrifugation (4 °C, 13800g, 20 min) and the supernatants were combined to afford a crude cell-free extract (CFE, 565 mL).

Step 2: Protamine Sulfate Precipitate: The CFE was brought to 0.01 % with protamine sulfate by dropwise addition of a 2.0 % aqueous solution. The resulting solution was stirred for 0.5 h and centrifugation (4 °C, 38400g, 20 min) yielded 565 mL of supernatant.

Step 3: $(NH_4)_2SO_4$ Precipitation: The protamine sulfate supernatant (565 mL) was brought to 51.3% saturation by addition of solid ammonium

sulfate. The suspension was stirred for 1 h and the precipitate was removed by centrifugation (4 °C, 13800g, 20 min). The resulting supernatant was brought to 71.9% saturation with solid ammonium sulfate and was stirred for an additional hour. The active pellet was collected by centrifugation (4 °C, 38400g, 20 min).

Step 4: Sephacryl S-200 Column: The 51.3% - 71.9% pellet was dissolved in 11.5 mL of buffer II and divided equally into two parts. Each part was applied to a Sephacryl S-200 column (2.4 x 110 cm) equilibrated in Buffer II, respectively. The column was eluted with the same buffer (6.8 mL/30min, 6.8-mL fractions). The column profile is shown in Figure 20. The active fractions (90 mL) from two runs were pooled and concentrated to 42 mL (Amicon, Centriprep 30). Centriprep 30 was first washed with buffer II. Sample was added and centrifuged (4 °C, 2000g). Because of the high viscosity of the glycerol buffer, it took about 30 min to concentrate 15 mL to about 2 mL. Centriprep 30 was reused until any leakage was detected. Centriprep 30 has a MW cutoff of 30000.

Step 5: Accell QMA Column: The concentrated pooled fractions (42 mL) were divided equally into two parts and each part was applied to an Accell QMA column (1.8 x 50 mm) equilibrated in Buffer II. The column was first washed with buffer II until the A_{280nm} returned to baseline and then eluted with a gradient of buffer II - buffer III (2.5 mL/min, 7.5-mL fractions) (the detailed gradient and column profile are shown in Figure 21). The active fractions from two runs (80 mL) were pooled and concentrated to 35 mL (Amicon, Centriprep 30).

Step 6: Protein-Pak HIC (Phenyl-5 PW) Column: The concentrated pooled fractions (35 mL) were brought to a 1.0 M ammonium sulfate concentration by adding solid ammonium sulfate, and it was then divided equally into three parts. Each part was applied to an HIC (Phenyl-5 PW) column (8 x 75 mm) equilibrated in Buffer IV. The column was first washed with buffer IV until the A_{280nm} returned to baseline and then eluted with a gradient of buffer IV - buffer V (0.7 mL/min, 2.8-mL fractions) (the detailed gradient and column profile are shown in Figure 22). The active fractions

from three runs (about 100 mL) were pooled and concentrated to 40 mL (Amicon, Centriprep 30).

Step 7: Protein-PaK DEAE 5 PW Column: The concentrated pooled fractions (40 mL) from the HIC column were dialyzed against buffer II overnight and divided equally into two parts. Each part was applied to a DEAE 5 PW column (8 x 78 mm) equilibrated in Buffer II. The column was first washed with buffer II until the A_{280nm} returned to baseline and then eluted with a gradient of buffer II - buffer III (1.0 mL/min, 2.0-mL fractions) (the detailed gradient and column profile are shown in Figure 23). The active fractions (20 mL) from two runs were pooled and concentrated to 0.6 mL (Amicon, Centriprep 30).

Step 8: Protein-Pak 300 SW Column: The concentrated preparation (0.6 mL) was applied to a 300 SW column (8 x 300 mm) equilibrated in Buffer II (only 100 µL of the sample could be loaded per run if a good resolution was desired). The column was eluted with buffer II (0.5 mL/min, 0.5-mL fractions) (Figure 24). The active fractions from each run were pooled (9.0 mL).

Step 9: Protein-PaK DEAE 5 PW Column: The active fractions (9 mL) were applied to a DEAE-5 PW column (8 x 78 mm) equilibrated in Buffer V. The column was first washed with buffer V until the A_{280nm} returned to baseline and then eluted with a gradient of buffer V - buffer VI (1.0 mL/min, 1.0-mL fractions) (the detailed gradient and column profile are shown in Figure 25). The final preparation of 4.0 mL enzyme solution was collected and stored at - 80 °C without change of buffer.

Preparation of the Apoenzyme of DHA Epoxidase

Apoenzyme of DHA epoxidase was prepared by addition of a chelating reagent to the holoenzyme followed by removal of the chelated metal(s) and excess chelating reagent.[60,61] The enzyme preparation used here was less pure and in addition to the major band, some minor bands were shown on the SDS-PAGE. Thus, 500 µL of DHA epoxidase (16.6 µg) in Buffer II was brought to 0.2 mM 1,10-phenanthroline and the resulting solution was kept at ice

temperature for 10 min. It was then passed through a Sephadex G-25 column (1 x 5 cm) equilibrated in Buffer II by centrifugation (3000g, 5 min) to remove the chelated metal ion and excess 1,10-phenanthroline. The final volume of apoenzyme was 700 μL. This method of removing small molecules from large proteins has been proven to be effective if the sample volume is kept below 20% of the bed volume of Sephadex G-25.[1,60]

For reconstitution of the epoxidase activity, 250 μL of assay solution containing the apoenzyme (1.3 μg) and the indicated amount of metal ions were preincubated at room temperature (25 ± 5 °C) for 2 min. It was then assayed for DHA epoxidase activity by addition of **78** (0.1 mM); the resulting solution was incubated at 30 °C for 10 min and the terminated reaction mixture was analyzed by HPLC.

Streptomyces MPP 3051

Standard Culture Conditions

S. MPP 3051 was maintained at 20-25 °C as spores on sterile soil. A loopful of this material was used to inoculate 50 mL of seed medium containing 1.0% glucose, 2.0% soluble potato starch, 0.5% yeast, 0.5% N-Z Amine A 59027, and 0.1% $CaCO_3$ in Milli-Q water, all adjusted to pH 7.2 with 2% KOH. The seed inoculum, contained in a 250-mL Erlenmeyer flask, was incubated for 2 days at 28 °C, 250 rpm. Production broths (400 mL in 2-L Erlenmeyer flasks) were subsequently inoculated 5% (v/v) with vegetative inoculum from seed broths. Two kinds of production medium have been used. Medium L is made of 1.0% glucose, 0.5% bactopeptone, 2.0% molasses (Grandma's famous light unsulfured), and 0.1% $CaCO_3$ in Milli-Q water and is adjusted to pH 7.2 with 10% HCl prior to sterilization; medium B is made of 1.0% glucose, 1.0% $NaNO_3$, 0.1% Pharmamedia, and 0.5% $CaCO_3$ and is adjusted to pH 7.0 with 10% HCl prior to sterilization. The production broths were incubated under the same conditions.

Enzyme Assay

The DHA epoxidase was assayed by following the consumption of **78** and the production of **77** simultaneously by HPLC. The same assay method developed for the DHA epoxidase from *S.* LL-C10037 was adopted directly.

For the pH dependence study, assay solutions containing 0.1 mM **78** and DHA epoxidase in 0.1 M potassium phosphate buffer, pH 4.5 to 8.0, were incubated at 30 °C for 5 min. The assay mixtures were then subjected to HPLC analysis.

For determination of kinetic parameters, 500 μL of assay solutions containing 5.0 μM to 500 μM **78** and 0.05 μg of DHA epoxidase in 0.1 M potassium phosphate buffer, pH 5.5, were incubated at 30 °C for 5 min. The terminated assay mixtures were then analyzed by HPLC.

For inhibition and activation studies, unless otherwise specified, 250 μL of assay solutions containing 0.1 mM **78**, 0.6 μg of DHA epoxidase and the indicated amount of either inhibitors or activators in 0.1 M potassium phosphate buffer, pH 5.5, were incubated at 30 °C for 5 min. The terminated assay mixtures were then analyzed by HPLC.

Purification of DHA Epoxidase

Buffers:

I: 10 mM potassium phosphate, pH 7.0.

II: 50 mM potassium phosphate, pH 7.0, 20% glycerol, 0.2 mM EDTA.

III: 1.0 M KCl in buffer II.

IV. 50 mM Tris-HCl, pH 7.5, 20% glycerol, 0.2 mM EDTA.

V. 1.0 M KCl in buffer IV.

VI: 1.6 M $(NH_4)_2SO_4$ in buffer II.

VII. 25 mM Tris-HCl, pH 7.5, 20% glycerol, 0.2 mM EDTA.

VIII. 1.0 M KCl in Buffer VII.

Step 1: Preparation of Cell-Free Extract: Cells from 3.3 L of 22.5 h fermentations of *S.* MPP 3051 were harvested by centrifugation (4 °C, 13800g, 10 min) and washed sequentially with (1.0 L) buffer I, 1.0 M KCl, 0.8 M NaCl and buffer I. After each wash the cells were centrifuged as above. The washed cells

(30 g, wet wt.) were then suspended in buffer II (340 mL); the cell suspension was subsequently brought to 3 mg/mL with PVPP and 1.0 mM with a solution of 1.0 M PMSF in acetone. It was then equally distributed into four beakers and each cell suspension was disrupted by sonication (maximum power, 90% duty, pulse for 4 x 15 sec.). Cell debris was removed by centrifugation (4 °C, 13800g, 20 min) and the supernatants were combined to afford a crude cell-free extract (CFE, 335 mL).

Step 2: Protamine Sulfate Precipitate: The CFE from step 1 was brought to 0.01 % with protamine sulfate by dropwise addition of a 2.0 % aqueous solution. The resulting solution was stirred for 0.5 h and centrifugation (4 °C, 38400g, 20 min) yielded 335 mL of supernatant.

Step 3: Accell QMA Column: The supernatant (335 mL) from step 2 was divided equally into four parts and each part was applied to an Accell QMA column (1.8 x 50 mm) equilibrated in Buffer II. The column was first washed with buffer II until the A_{280nm} returned to baseline and then eluted with a gradient of buffer II - buffer III (3.0 mL/min, 12.0-mL fractions) (the detailed gradient and column profile are shown in Figure 32). The active fractions from four runs (360 mL) were pooled and dialyzed against 6.0 L of Buffer II overnight.

Step 4: Accell QMA Column: The dialyzed fractions (360 mL) from step 3 were applied to an Accell QMA column (1.8 x 50 mm); this time the column was equilibrated in Buffer IV. The column was first washed with buffer IV until the A_{280nm} returned to baseline and then eluted with a gradient of buffer IV - buffer V (3.0 mL/min, 12.0-mL fractions) (the detailed gradient and column profile are shown in Figure 33). The active fractions (108 mL) were pooled and concentrated to 45 mL (Amicon, Centriprep 30). Initially, Centriprep 10 was used, which has a MW cutoff of 10000. However, the concentrating speed was much slower with Centriprep 10 than with Centriprep 30. It was subsequently determined that the DHA epoxidase from *S.* MPP 3051 was larger than 30K; Centriprep 30 was then used.

Step 5: Protein-Pak HIC (Phenyl-5 PW) Column: The concentrated pooled fractions (40 mL) from step 3 were brought to 1.6 M ammonium sulfate by adding solid ammonium sulfate. It was then equally divided into four parts and each part was applied to an HIC (Phenyl-5 PW) column (8 x 75 mm) equilibrated in Buffer V. The column was first washed with buffer V until the A $_{280nm}$ returned to baseline and then eluted with a gradient of buffer V - buffer II (0.8 mL/min, 2.4-mL fractions) (the detailed gradient and column profile are shown in Figure 34). The active fractions from four runs were pooled and dialyzed against 4.0 L of Buffer II overnight.

Step 6: Protein-Pak DEAE 5 PW Column: The dialyzed fractions (44 mL) from step 5 were divided equally into three parts and each part was applied to a DEAE 5 PW column (8 x 78 mm) equilibrated in Buffer II. The column was first washed with buffer II until the A $_{280nm}$ returned to baseline and then eluted with a gradient of buffer II - buffer III (1.0 mL/min, 2.0-mL fgractions) (the detailed gradient and column profile are shown in Figure 35). The active fractions (14 mL) were pooled and concentrated to 1.0 mL (Amicon, Centriprep 30).

Step 7: Protein-Pak 300 SW Column: The concentrated preparation (1.0 mL) from step 6 was applied to a 300 SW column (8 x 300 mm) equilibrated in Buffer II (only 100 µL of the sample could be loaded per run if a good resolution was desired). The column was eluted with buffer II (0.5 mL/min, 0.5-mL fractions) (Figure 36). The active fractions from each run were pooled (7.0 mL).

Step 8: Protein-Pak DEAE 5 PW Column:

The active fractions (7.0 mL) from step 7 were diluted four-fold with a solution containing 20% glycerol and 0.2 mM EDTA and applied to a DEAE 5 PW column (8 x 78 mm) equilibrated in buffer VII. The column was first washed with buffer VII until the A $_{280nm}$ returned to baseline and then eluted with a gradient of buffer VII - buffer VIII (1.0 mL/min, 1.0-mL fractions) (the detailed gradient and column profile are shown in Figure 37). The final preparation of 5.0 mL enzyme solution was collected and stored at - 80 ^{o}C.

Preparation of the Apoenzyme of DHA Epoxidase

Apoenzyme of DHA epoxidase was prepared by addition of a chelating reagent to the holoenzyme followed by removal of the chelated metal ion(s) and excess 1,10-phenanthroline. Thus, 500 μL of DHA epoxidase (20 μg) in Buffer II was brought to 0.2 mM 1,10-phenanthroline and the resulting solution was kept at ice temperature for 10 min. Assay for DHA epoxidase showed total loss of the enzyme activity. It was then passed through a Sephadex G-25 column (1 x 5 cm) equilibrated in Buffer II by centrifugation (3000g, 5 min). However, 17% of the epoxidase was detected in the filtrate. 600 μL of DHA epoxidase (24 μg) was carried again though this process but 0.4 mM 1,10-phenanthroline was used. Again 10% of the epoxidase was detected after passing the Sephadex G-25 column. Finally, 600 μL of DHA epoxidase (24 μg) was brought to 4.0 mM 1,10-phenanthroline. No enzyme activity was found either before or after the Sephadex G-25 column. It could be possible that at such a high concentration of 1,10-phenanthroline, the DHA epoxidase was denatured by actions other than chelating the metal ion.

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Chapter 6

Conclusions: Structure, Biosynthesis and Enzymology of Two Epoxysemiquinones from *Streptomyces*

When this project was started almost four years ago, the objectives were to determine the absolute stereochemistry of **2**, to establish the pathway for the biosynthesis of **2**, and to explore some enzymology of the biosynthesis of epoxyquinone metabolites. Although during the progress of this work the project has developed in a variety of directions, the main objectives remained the same. From the data and discussion presented above, it appears proper to make these concluding remarks and comments.

What Has This Work Accomplished?

Structure

Antibiotic LL-C10037α has now been shown to have the epoxysemiquinone structure **2**, with 4*S*, 5*S*, 6*S* stereochemistry. Two additional metabolites of *Streptomyces* LL-C10037 were isolated and characterized as **66** and **68**, which were obviously biogenetically related to **2**.

Antibiotic MM 14201, **4**, has been established as the desacetyl enantiomer of **2**, having 4*R*, 5*R*, 6*R* stereochemistry. The enantiomeric relationship between the two epoxysemiquinones (**2** and **3**) and the two epoxyquinones (**70** and **77**) was established spectroscopically and confirmed by the biosynthetic studies.

Biosynthesis

The pathway for the biosynthesis of **2** has been established by combined applications of *in vivo* and *in vitro* studies. Thus, it is shown that **65** is first hydroxylated to form **62**, presumably via an anthranilate 3-hydroxylase, then decarboxylated to form **88**, and followed by a second hydroxylation to form **85**. N-acetylation of **85** affords **78**, which is subsequently epoxidized to form **70**. Reduction of **70** finally yields **2**. The epoxidation of **78** is catalyzed by a DHA epoxidase in the absence of any added cofactor and the reduction of **70** is catalyzed by an AEBQ dehydrogenase requiring NADPH as cofactor.

Speculation on the biosynthesis of **4** was based on its structural similarity with **2**, and it was postulated that **4** could simply be derived via the same pathway as **2** (exclusive of stereochemistry) with the addition of a

deacetylation as the last step. This hypothesis was given strong support by the isolation of the DHA epoxidase from *S.* MPP 3051, which catalyzed the epoxidation of **78** to form **77** in the absence of any added cofactor.

Enzymology

Four enzymes have been isolated from the cultures of *S.* LL-C10037 and *S.* MPP 3051.

ABQ dehydrogenase from *S.* LL-C10037 catalyzes the reduction of **80** to form **78** in the presence of NADH. The reduction is unidirectional under the conditions studied. The enzyme has an optimal pH of 6.5 and displays a remarkable thermostability.

AEBQ dehydrogenase from *S.* LL-C10037 catalyzes the reduction of **70** to form **2** in the presence of NADPH. The reaction is reversible. The enzyme has an optimal pH of 7.0 for the reduction of **70**. The molecular weight of the enzyme is estimated to be 100K ± 30K. The enzyme follows classical Michaelis-Menten kinetics and the apparent K_m for **70** and NADPH are 8.2 ± 0.5 μM and 45 ± 3 μM, respectively.

DHA epoxidase from *S.* LL-C10037 has been purified to apparent homogeneity, with an overall purification factor of about 640. The enzyme catalyzes the epoxidation of **78** to form **70**, requiring molecular oxygen but no added cofactors. The enzyme is a pentamer or a hexamer of molecular weight 117K ± 10K. The molecular weight of the subunit is 22.3K. The enzyme has an optimal pH of 6.5. It follows classical Michaelis-Menten kinetics and the apparent V_{max} and K_m are 3.7 ± 0.2 mmol min^{-1} mg^{-1} (or 430 ± 20 min^{-1}, if 117K is taken as the molecular weight for the DHA epoxidase) and 8.4 ± 0.5 μM, respectively, with **78** showing inhibition at 150 μM. Although the specific metal ion has not been identified yet, the enzyme is a metalloprotein requiring metal ion(s) for the epoxidation activity. While NADH, NADPH, NAD^+, $NADP^+$, FAD, FMN, CO, catalase, or EDTA neither stimulates nor inhibits the reaction, it is inhibited by CN^-, PCMBA and 1,10-phenanthroline. The enzyme is activated by Co^{2+}, Mn^{2+}, and Ni^{2+}. The apoenzyme of the DHA epoxidase has been prepared and appears to be relatively stable. Enzyme activity is totally reconstituted from its apoenzyme by addition of Co^{2+}, Mn^{2+} or Ni^{2+}; no other metals tested were effective.

DHA epoxidase from *S.* MPP 3051 has been purified to near homogeneity, with an overall purification factor of about 1489. The enzyme catalyzes the epoxidation of **78** to form **77**, requiring molecular oxygen but no added cofactors. The enzyme is a dimer of molecular weight 33K ± 2K. The molecular weight of the subunit is 16K. The enzyme has an optimal pH of 5.5. It follows classical Michaelis-Menten kinetics and the apparent K_m for **78** is 7.2 ± 0.4 μM, with **78** showing inhibition at 100 μM. While NADH, NADPH, NAD^+, $NADP^+$, FAD, FMN, catalase, or EDTA neither stimulates nor inhibits the reaction, it is inhibited by CN^-, CO, PCMBA and 1,10-phenanthroline. The enzyme is a metalloenzyme and its inhibition by 1,10-phenanthroline can be reversed by addition of Fe^{2+}, Cu^{2+} or Cu^{1+}. All the characteristics of the two epoxidases appear to be very similar. However, neither activation of the enzyme nor reconstruction of the epoxidase from the apoenzyme is observed by addition of any of the metal ions tested on the DHA epoxidase from *S.* MPP 3051. These differences have been explained by their different nature of apoenzymes. The apoenzyme of DHA epoxidase from *S.* MPP 3051 may be very unstable and could be denatured immediately upon removal of the metal ion. Consequently, the epoxidase activity could not be reconstituted regardless of the presence of metals. In addition, if some of the metal ion was lost during the purification and if the apoenzyme was denatured immediately, one would also not expect to gain any enzyme activity back by providing the metal ion.

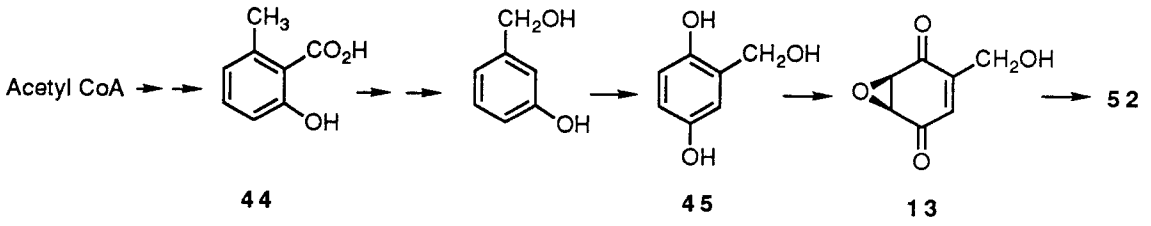
The two DHA epoxidases catalyze the epoxidation of **78** to yield the enantiomers **70** and **77**, respectively. Therefore, the two DHA epoxidases catalyze reactions of the same stoichiometry but display opposite facial specificity towards the planar substrate **78**.

What Are the Significant Features of This Research?

The significant features of this research are many fold. First, while numerous other naturally occurring epoxyquinones and epoxysemiquinones have been reported in the past thirty years, until recently,[1] while our work was in progress, in no case had the absolute stereochemistry of any of these been established by an unambiguous, nonempirical analysis. Absolute stereochemistry for the epoxide carbons had only been inferred from empirical correlation of the signs of Cotton effects in the circular dichroism

spectra (inverse quadrant rule). In our study as well as Scheuer's,[1] application of the inverse quadrant rule to the CD spectra of **2** yielded the correct absolute configuration for C-5/C-6. Thus, this rule may now be more reliably invoked.

Second, the biosynthesis of **2** represents a novel aromatic amino acid metabolism. This work established the detailed pathway for the biosynthesis of **2** from **65**. Having the pathway for the biosynthesis of **2** established, it would be interesting to re-examine the biosynthesis of **52**, one of the most well studied of all secondary metabolites.[2] Taking the biosynthesis of **2** as precedence and combining the results of the epoxidase reported by Priest and Light,[3] the pathway for the biosynthesis of **52** now could be revised as shown in Scheme 46. According to this pathway, gentisyl aldehyde, **53**, isoepoxydon, **10**, and formyl-1,4-benzoquinone, all of which have been proposed as intermediates on the pathway for the biosynthesis of **52**,[2] may actually be shunt metabolites.



Scheme 46. Pathway for the Biosynthesis of **52**

The successful applications of both *in vivo* and *in vitro* methods in this work demonstrated the effectiveness of the modern methodologies of biosynthesis. The biosynthesis of **2** could be used as a model to achieve complete understanding of the biosynthesis of a secondary metabolite. The bioorganic chemistry learned from the biosynthesis of **2** may be of general significance to other epoxyquinones or epoxysemiquinones. Consistent with the vitamin K epoxidase[4] and the particulate preparation from *P. patulum*,[3] the isolation of DHA epoxidases from both *S.* LL-C10037 and *S.* MPP 3051 catalyze the epoxidation of a hydroquinone to form the epoxyquinone functionality. The fact that that same type of chemistry is observed in all four cases is very tempting and leads one deduce that this type of chemistry may occur in the formation of other epoxyquinones as well. The NNM-A monooxygenase, which

catalyzes the epoxidation of a quinone requiring NAD(P)H as cofactors,[5] may actually catalyze the epoxidation of the NNM-A hydroquinone **111**; NAD(P)H may be required merely for the generation of **111** from **47** (Scheme 42). With the two epoxidases from *Streptomyces*, the stereochemistry of the oxiranes is determined by the facial specificity of the individual epoxidase.

Third, very little was known about the mechanism of the epoxidation. Only in three other cases have the epoxidase activity been reported;[3,4,5] the two epoxidases from *Streptomyces* as well as the vitamin K-dependent carboxylase[3] are now purified to homogeneity or near homogeneity. It is the first time that a hydroquinone has been unambiguously established as the substrate for an epoxidase with a purified enzyme. Only with the purified enzyme has it been possible to establish that this enzyme is a metalloenzyme, and iron or copper --- the predominant metal ions found in monooxygenases --- are unlikely to be involved. Although the nature of the enzymes was not established in the cases of vitamin K epoxidase, epoxidase from *P. patulum* and the NNM-A monooxygenase, preliminary data suggested that both the vitamin K epoxidase and the epoxidase from *P. patulum* were inhibited by chelating reagents and were not mediated by cytochrome P_{450}. Together with these epoxidases, the two epoxidases from *Streptomyces* may represent a new group of monooxygenases, whose metal ion requirements have not been fully understood. Iron or copper is not likely to be involved and Co^{2+}, Mn^{2+} or Ni^{2+} may be the metal of choice.

Fourth, although the natural metal ion of the DHA epoxidases has not been identified, the observed activation and reconstitution of the epoxidase from an apoenzyme by addition of Co^{2+}, Mn^{2+} or Ni^{2+} are very intriguing. Although manganese[6-15], cobalt[16-23] and nickel[24,25], together with some other transition metals, have been used in enzyme models to mimic enzymes associated with molecular oxygen metabolism, only in three previous reports has manganese been reported as the metal of an oxygenase.[26-28] Neither Co^{2+} nor Ni^{2+} has been found in any molecular oxygen-activating enzymes. Because the metabolisms of molecular oxygen play such an important role, the potential involvement of Co^{2+}, Mn^{2+} or Ni^{2+} in the DHA epoxidase is of special significance. If the epoxidases were indeed Co^{2+}-, Mn^{2+}- or Ni^{2+}-containing enzymes, together with the other three manganese-containing enzymes, they

may provide us a rare opportunity to explore some new chemistry of molecular oxygen metabolism.

Finally, the comparative biosynthesis of enantiomeric natural products has only rarely been studied and in a few cases it has been demonstrated that the two enantiomers were derived from the same precursor by a process in which two complementary enzymes displayed an enantiomeric specificity.[29-35] Little study has been done with regard to the enantiomeric specificity of such enzymes. The two epoxidases from *Streptomyces* are the only two enzymes of this kind to be purified to this extent. Although the mechanism of the enantiomeric specificity has not been elucidated, it is the first time that progress has been made towards this direction. The two epoxidases have been purified to near homogeneity and the two enzymes have been compared side by side. The two enzymes have a lot in common because they catalyze the same chemical reaction, yet they are at least distinctive in size and numbers of subunits. These differences may be sufficient for them to achieve the enantiomeric specifity by either binding the substrate **78** in opposite orientations or delivering the activated oxygenating species to **78** from opposite side. Therefore, these two epoxidases could be used as models to understand how Nature can control stereochemical selectivity. This work has made such a study very promising.

What Are the Areas for Future Study?

Although the pathway for the biosynthesis of **2** has been established with identification of all the intermediates and isolation of several related enzymes, further investigations are still required in order to achieve complete understanding of the pathway. Either the hydroxylation of **65** or the hydroxylation of **88** could be very interesting. The former, as shown by the whole-cell feeding experiment, may be a direct hydroxylation of **65** by an anthranilate 3-hydroxylase, which had been reported in only very few cases.[36-41] The latter is a hydroxylation of an activated aromatic compound. The whole-cell feeding experiments gave conflicting results, suggesting a possible involvement of an NIH shift during the hydroxylation. Therefore, isolation of both hydroxylases could provide further information for the understanding of this novel aromatic amino acid metabolism.

Among all possibilities, the first priority should be given to the establishment of the metal ion requirement of the DHA epoxidases. Once the enzyme has been purified in quantity, this can be established by atomic absorption spectrometry. If cobalt, nickel or manganese is indeed involved in the epoxidase reaction, not only the mechanism for epoxidation but also the mechanism of molecular oxygen activation would be very interesting.

The amount of the purified enzymes is limited from the wild type organism. In order to obtain sufficient amount of pure enzymes, alternative methods have to be developed. The most promising one would be the gene-cloning method.[42-45] With larger quantities of the purified enzyme in hand, it could then become possible to determine the three-dimensional features of the active sites, to locate the metal ion(s) and to investigate the opposite facial specificities of the two enzymes.

The mechanism of this enzymatic epoxidation is very intriguing. Although some mechanistic pictures have been proposed[4,46] and a nonenzymic model has been developed,[47] very few discriminating data are available. With the DHA epoxidases having been purified, it would be possible to carry further mechanistic studies, and to uncover this novel chemistry of hydroquinone epoxidation would be of general significance.

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