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INVESTIGATION OF VALANIMYCIN BIOSYNTHESIS

by

WENYING LI

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE
DOCTOR OF PHILOSOPHY

APPROVED, THESIS COMMITTEE

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Houston, Texas
January, 1996
ABSTRACT

Investigation of Valanimycin Biosynthesis

by

Wenying Li

The antibiotic valanimycin is an azoxy compound produced by Streptomyces viridifaciens MG456-hF10. Precursor incorporation experiments with [4-\textsuperscript{13}C]-N-(isobutylamino)serine and [\textsuperscript{15}N\textsubscript{2}]-N-(isobutylamino)serine suggest that N-(isobutylamino)serine is a specific precursor of valanimycin.

Searching for enzymes related to the biosynthetic pathway of valanimycin led to the identification of valine decarboxylase and isobutylamine N-hydroxylase in crude extracts of S. viridifaciens MG456-hF10 cells. Isobutylamine N-hydroxylase requires FAD and NADH for activity. The enzyme was found to consist of two protein components. The A component of isobutylamine N-hydroxylase was partially purified and the B component was purified to homogeneity. The properties of isobutylamine N-hydroxylase were studied. Enzyme replacement experiments suggested that the A component catalyzes the reduction reaction of FAD or FMN with NADH to form FADH\textsubscript{2} or FMNH\textsubscript{2}, and that the B component catalyzes the hydroxylation reaction of isobutylamine with the aid of FADH\textsubscript{2} or FMNH\textsubscript{2}, and molecular oxygen.

Attempts to clone the B component of isobutylamine N-hydroxylase were made. A library of S. viridifaciens MG456-hF10 genomic DNA was constructed using a Lambda DASH II phage vector. The library was screened with a oligonucleotide probe derived from the N-terminal protein sequence of
the B component of isobutylamine N-hydroxylase. No gene fragment corresponding to the N-terminal sequence was found. Further screening will be necessary.
Acknowledgments

I would like to take this opportunity to express my gratitude to my advisor, Professor Ronald Parry, for his guidance and encouragement.

Special thanks to Dr. Jeffrey Hoyt, for teaching me techniques in enzymology and protein chemistry, and for being a source of advices.

I am grateful to Dr. Howard Cooper, for helping me with regard to library screening.

I would also like to thank all members of Dr. Parry's group.

Finally, I would like to thank Professors Paul Engel and Frederick Rudolph for serving on the thesis committee and for suggestions regarding the thesis.

This thesis is dedicated to my wife, Li Pan, and to my parents.
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List of Abbreviations

ATP  
adenosine-5'-triphosphate

BIS-TRIS  
bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane

BSA  
bovine serum albumin

CAPS  
3-cyclohexylamino-1-propanesulfonic acid

DEP  
diethyl pyrocarbonate

DIBAL  
diisobutylaluminum hydride

dATP  
deoxyadenosine-5'-triphosphate

DTNB  
5',5'-dithio-bis(2-nitrobenzoic acid)

DTT  
dithiothreitol

DNA  
deoxyribonucleic acid

E64  
trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane

E. coli  
Escherichia coli

EDTA  
ethylenediaminetetraacetic acid

FAD  
flavine-adenine dinucleotide (oxidized form)

FADH$_2$  
flavine-adenine dinucleotide (reduced form)

FMN  
flavine mononucleotide (oxidized form)

FMNH$_2$  
flavine mononucleotide (reduced form)

FPLC  
fast protein liquid chromatography

HMPA  
hexamethylphosphoramide

HPLC  
high pressure liquid chromatography

HRMS  
high resolution mass spectroscopy

IAM  
iodoacetamide

IBA  
isobutylamine
<table>
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<th>Full Form</th>
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<tr>
<td>IBHA</td>
<td>isobutylhydroxylamine</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropylthio-β-D-galactoside</td>
</tr>
<tr>
<td>IR</td>
<td>infrared spectroscopy</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>acetyl-Leu-Leu-Arg-al</td>
</tr>
<tr>
<td>MES</td>
<td>4-morpholineethanesulfonic acid</td>
</tr>
<tr>
<td>MPLC</td>
<td>medium pressure liquid chromatography</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectroscopy</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>nicotinamide-adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>reduced form of NAD⁺</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>nicotinamide-adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>reduced form of NADP⁺</td>
</tr>
<tr>
<td>N-B</td>
<td>N-terminal amino acid sequence of the B component of isobutylamine N-hydroxylase</td>
</tr>
<tr>
<td>NBD-Cl</td>
<td>4-chloro-7-nitrobenzo-2-oxa-1,3-diazole chloride</td>
</tr>
<tr>
<td>NOE</td>
<td>nuclear Overhauser effect</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>isovaleryl-Val-Val-Sta-Ala-Sta</td>
</tr>
<tr>
<td>pHMB</td>
<td>4-(hydroxymercuri)benzoic acid</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>PLP</td>
<td>pyridoxal-5'-phosphate</td>
</tr>
<tr>
<td>r.t.</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Sta</td>
<td>(3S,4S)-4-amino-3-hydroxy-6-methylheptanoic acid</td>
</tr>
<tr>
<td>Abbr.</td>
<td>Description</td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TES</td>
<td>2-[[tris-(hydroxymethyl)methyl]amino] ethane sulfonic acid</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TPP</td>
<td>thiamine pyrophosphate</td>
</tr>
<tr>
<td>TPTZ</td>
<td>2,4,6-tripyridyl-s-triazine</td>
</tr>
<tr>
<td>Tricine</td>
<td>N-[tris(hydroxymethyl)methyl]glycine</td>
</tr>
<tr>
<td>TRIS</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet spectroscopy</td>
</tr>
<tr>
<td>WLA</td>
<td>a DNA probe derived from the N-terminal amino acid sequence of the B component of isobutylamine N-hydroxylase</td>
</tr>
<tr>
<td>X-gal</td>
<td>3-bromo-4-chloro-3-indolyl-β-D-galactoside</td>
</tr>
</tbody>
</table>
Chapter One
Introduction

1.1 General introduction

In living organisms, chemical compounds are synthesized and degraded by a series of chemical reactions mediated by enzymes. These processes are known as metabolism. Primary metabolism involves those reactions and compounds which are essential for the survival and well-being of the organisms. Secondary metabolism, on the other hand, can be defined as those additional sets of reactions producing and utilizing compounds which usually have no apparent utility. The products of secondary metabolism (secondary metabolites or natural products) are processed by only certain species and occur only at specific times during the life cycle of those species.

Humans have used natural products, although as crude plant extracts, since ancient times. Since the nineteenth-century, many natural products have been isolated and characterized. The structures of many of these compounds have been elucidated. While their structural complexities represent a considerable challenge to the synthetic chemist, these natural products attract chemists and biochemists to study their biosynthetic processes, not only because many of these compounds have great value as antibiotics in the service of mankind, but also because the biosynthetic studies provide a pathway to understand life and nature.

Research on the biosynthesis of secondary metabolites involves radioactive tracer techniques, modern spectroscopic techniques (NMR, MS,
UV), isotopically labeled compound synthesis, molecular biological techniques, and enzyme purification and characterization. These studies provide an essential bridge between the physical and life sciences.

1.2 Naturally occurring azoxy compounds

1.2.1 Isolation

Compounds containing N-N bonds constitute a widespread and diverse group of natural products (LaRue, 1977). Naturally occurring azoxy compounds represent a growing class of compounds of this type. The toxic macrozamin, first isolated by Cooper from the Australian cycad *Macrozamia spiralis* (Cooper, 1940) and characterized as an azoxy compound in 1951 (Lythgoe, 1949; Langley, 1951a, 1951b, 1952), was the first reported azoxy natural product. Since then, several azoxy compounds have been isolated and characterized. Some of them are shown in Figure 1.1. The antibiotic elalomycin was isolated from the fermentation broth of *Streptomyces gelaticus* (Haskell, 1954; Stevens, 1956, 1958, 1959; Taylor, 1972). LL-BH872α is an antifungal agent produced by *Streptomyces hinnulinus* (McGahren, 1969, 1970, 1972). Valanimycin, the target compound of this thesis work, was isolated as antibiotic from the broth of *Streptomyces viridifaciens* MG456-hF10 (Yamato, 1986a). The antifungal antibiotics maniwamycins A and B were isolated from the culture broth of *Streptomyces prasinopilosus* (Nakayama, 1989; Takahashi, 1989). Jietacins A and B, nematocidal compounds were produced by a strain of *Streptomyces* (Imamura, 1989). Recently, azoxybacilin, a new azoxy antifungal agent, was found in the culture broth of *Bacillus cereus* (Fujiu, 1994).
Figure 1.1 Some naturally occurring azoxy compounds
1.2.2 Hypothesis for N-N bond formation

Most of N-N bond containing natural products are produced by pseudomonads, streptomycetes, bacilli, actinomycetes, higher fungi and plants. Since most of these organisms are not known to fix atmospheric nitrogen (LaRue, 1977), it is unlikely that N-N moiety in these compounds is derived from dinitrogen. Although many of these compounds have been isolated, relatively little appears to be known about the biosynthesis of the N-N bonds.

There are a few reports on the biotransformation of synthetic amines to N-N compounds. *Escherichia coli* in anaerobic culture will convert meso-diaminomuconic acid (m-DAS) to fumaric acid and hydrazine (Scheme 1.1) (Suzuki, 1952). Better known are the transformations by soil bacteria of aniline derivatives to the corresponding azobenzenes (Scheme 1.2) (Bordeleau, 1972; Miyadera, 1975). Herbicide 3,4-dichloropropionanilide (known as propanil) was converted to 2,4-dichloroaniline (DCA) and further converted to 3,3',4,4'-tetrachloroazobenzene (TCAB) by soil microorganisms. These reactions appear to involve peroxidases. The peroxidase-catalyzed conversion of DCA to TCAB was proposed to proceed by the way of 3,4-dichlorophenylhydroxylamine (DCHA) which was detected by formation of a red complex with trisodium pentacyanoaminoferroate. The resulting aryl hydroxylamine was capable of undergoing a non-enzymatic reaction with DCA to form 3,3',4,4'-tetrachlorohydrazobenzene (TCHAB). Although attempts to detect TCHAB failed, the intermediacy of this compound could be rationalized by indirect evidence: it rapidly gave rise to TCAB when incubated in soil. This information provided a potential clue to the mechanism of N-N bond formation (Scheme 1.3).
Scheme 1.1 Conversion of m-DASA to hydrazine by *E. coli* (Suzuki, 1952)

Scheme 1.2 Formation of TCAB in soil (Bordeleau, 1972)
Scheme 1.3  Proposed pathway of TCAB formation in soil
(Bordeleau, 1972)
1.2.3 Biosynthetic studies of elaionycin

Elaionycin was the first azoxy compound to be studied in detail. Precursor incorporation studies showed that C-5 to C-12 and the β-nitrogen atom of elaionycin are derived from n-octylamine, C-2 to C-4 and the α-nitrogen atom of the antibiotic are derived from serine, and C-1 is derived from C-2 of acetate. The O-methyl group was found to be derived from methionine (Figure 1.2) (Parry, 1982, 1984). These results showed that the two nitrogen atoms in elaionycin were derived from two amines, which was consistent with Bordeleau's hypothesis about N-N bond formation. Unfortunately, further studies of elaionycin were precluded due to insurmountable microbiological difficulties. Since that time, no other biosynthetic studies of naturally occurring azoxy compounds were attempted until Yamato first isolated valanimycin in 1986.

1.3 Valanimycin

During the course of prescreening of potential antitumor antibiotics using *E. coli* BE1121 as a test organism, an active substance was isolated from culture filtrates of a streptomycete (Yamato, 1986a). The active substance was named valanimycin on basis of its possible biosynthesis from valine and alanine. The producing organism was designated as *Streptomyces viridifaciens* MG456-hF10.

1.3.1 Biological activities (Yamato, 1986a)

Valanimycin is an antibiotic that is active against both Gram-positive and Gram-negative bacteria (MIC: 1 - 10 μg/ml), and it is especially active against *E. coli*. BE1121, a DNA-repair deficient mutant of *E. coli* K12 (MIC: 0.078 μg/ml). This suggests that the primary target of valanimycin is DNA. Besides
Figure 1.2 Incorporation of precursors into elamycin (Parry, 1982, 1984)
the antibacterial activity, valanimycin was toxic to *in vitro* cultures of cells of mouse leukemia L1210, P388/S, and P388/ADR with the IC$_{50}$ values of 0.79, 2.65 and 1.44 µg/ml, respectively. Valanimycin also prolonged the life span of mice inoculated with Ehrlich carcinoma or L1210 cells.

### 1.3.2 Chemical and physical properties

The properties of valanimycin are shown in Table 1.1 and 1.2 (Yamato, 1986a).

#### Table 1.1  Physico-chemical properties of valanimycin (Yamato, 1986a)

<table>
<thead>
<tr>
<th>Property</th>
<th>Value/Condition</th>
</tr>
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<tbody>
<tr>
<td>Appearance</td>
<td>Colorless oil</td>
</tr>
<tr>
<td>Stability</td>
<td>Unstable if dried especially in the absence of salt</td>
</tr>
<tr>
<td>Soluble in</td>
<td>H$_2$O, MeOH, EtOH, EtOAc</td>
</tr>
<tr>
<td>Insoluble in</td>
<td>CHCl$_3$, n-hexane</td>
</tr>
<tr>
<td>UV</td>
<td>230 nm (shoulder)</td>
</tr>
<tr>
<td>TLC (silica gel)</td>
<td>CHCl$_3$-MeOH-AcOH (200:40:1)</td>
</tr>
<tr>
<td></td>
<td>$R_f = 0.5$</td>
</tr>
<tr>
<td>pKa</td>
<td>ca. 4.7 (solvent extraction)</td>
</tr>
</tbody>
</table>
Table 1.2 NMR spectral data (δ^D_2O ppm) of valanimycin (Yamato, 1986a)

<table>
<thead>
<tr>
<th></th>
<th>1H (400 MHz)</th>
<th>13C (100 MHz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>6H, d, J = 7.3 Hz</td>
<td>20.3 (q)</td>
</tr>
<tr>
<td>2.38</td>
<td>1H, m</td>
<td>29.2 (d)</td>
</tr>
<tr>
<td>4.14</td>
<td>2H, d, J = 7.3 Hz</td>
<td>78.1 (t)</td>
</tr>
<tr>
<td>5.40</td>
<td>1H, s</td>
<td>115.7 (t)</td>
</tr>
<tr>
<td>5.72</td>
<td>1H, s</td>
<td>149.4 (s)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>170.5 (s)</td>
</tr>
</tbody>
</table>

Valanimycin was found to readily react with various amines to form amine adducts. The ammonia adduct was prepared by treating valanimycin with 2 N NH₄OH (Scheme 1.4). The valanimycin ammonia adduct is a white powder, which is stable at room temperature. This adduct was used in most of precursor incorporation experiments since it was easier to handle than valanimycin.

![Scheme 1.4](image_url)  
Valanimycin ammonia adduct

Scheme 1.4 Formation of valanimycin ammonia adduct.
1.4 **Biosynthetic studies on valanimycin**

Since the structure of valanimycin is relatively simple, it appears to be an ideal target for a detailed investigation of the azoxy compound biosynthesis, especially for the mechanism of N-N bond formation. Due to its unusual structure and biological activity, the biosynthesis of valanimycin has been studied since its first isolation.

1.4.1 **Yamato’s studies** (Yamato, 1986b)

Preliminary biosynthetic studies were carried out by Yamato in 1986. The results were: 1) Addition of either valine or alanine to the fermentation broth stimulated valanimycin production. 2) When [3,4(\(\text{H}\))-L-valine and \([U^{14}\text{C}]-L\)-alanine were simultaneously administered to a fermentation of *S. viridifaciens* and the valanimycin produced was then hydrogenated to give isobutylamine and alanine, \(^3\text{H}\) was detected only in the isobutylamine, while \(^{14}\text{C}\) was present in both the isobutylamine and alanine (experiment (I), Scheme 1.5). 3) Administration of \([U^{14}\text{C}]-L\)-valine gave 7.5% incorporation of \(^{14}\text{C}\) label into isobutylamine, whereas \([1^{14}\text{C}]-L\)-valine was not incorporated (experiment (II), Scheme 1.6).

These results suggested that valine was incorporated efficiently into the isobutyl moiety of valanimycin after decarboxylation at some stage, and that alanine was incorporated in part into the dehydroalanine moiety and in part into the isobutyl moiety.

1.4.2 **Previous studies in Professor Parry’s Laboratory**

Dr. Yan Li and Dr. Fwu-Lin Lii, who worked previously on this project in Professor Parry’s research group, had employed precursors labeled with
Scheme 1.5 Radioactive precursor feeding experiment (I) (Yamato, 1986b)
Scheme 1.6 Radioactive precursor feeding experiment (II)  
(Yamato, 1986b)
stable isotopes \(^{13}\text{C},^{15}\text{N}\) to obtain more precise information (Parry, 1992). Since valanimycin is unstable, the antibiotic produced in these experiments was isolated as the stable ammonia adduct. The \(^{13}\text{C}\) NMR spectrum of the valanimycin ammonia adduct was used to determine the labeling pattern and incorporation level of the precursors. In some cases, precursors were administered to washed cells of \textit{S. viridifaciens} MG456-hF10 to obtain more interpretable information.

\([1^{-13}\text{C},^{15}\text{N}]\)-isobutylamine was administered to fermentation broth of \textit{S. viridifaciens} MG456-hF10, and the valanimycin ammonia adduct was isolated. It exhibited \(^{15}\text{N}^{-^{13}\text{C}}\) coupling with 8.6 \% \(^{13}\text{C}\) incorporation at C-4 (Scheme 1.7). This suggests that isobutylamine is a specific precursor of the isobutyl moiety of valanimycin, and that the \(\alpha\)-nitrogen atom of valanimycin is derived from isobutylamine. In the similar experiment with \([1^{-13}\text{C},^{15}\text{N}]\)-isobutylhydroxylamine, the valanimycin ammonia adduct also exhibited \(^{15}\text{N}^{-^{13}\text{C}}\) coupling with higher (48\%) \(^{13}\text{C}\) incorporation at C-4 (Scheme 1.7). This suggests that isobutylhydroxylamine is a closer precursor to valanimycin on the biosynthetic pathway. These experiments supply the first evidence for the intermediacy of a hydroxylamine in the biosynthesis of an aliphatic azoxy compound.

Administration of \([1^{-13}\text{C}]-D,L\)-alanine and \([3^{-13}\text{C}]-D,L\)-alanine to washed cells of \textit{S. viridifaciens} gave 8.5\% and 23.5\% incorporation of \(^{13}\text{C}\) label into C-1 and C-3 of valanimycin, respectively, while some of the \(^{13}\text{C}\) in \([3^{-13}\text{C}]-D,L\)-alanine was also incorporated into C-6 (2.1\%) (Scheme 1.8). These results were consistent with Yamato’s observations. Administration of \([2^{-13}\text{C},^{15}\text{N}]\)-D,L-alanine into washed cells gave an unexpected result: no \(^{15}\text{N}^{-^{13}\text{C}}\)
Scheme 1.7 Incorporation of precursors into the isobutyl moiety of valanimycin (Parry, 1992)
Scheme 1.8 Incorporation of alanine into valanimycin (Parry, 1992)
coupling was observed in the $^{13}\text{C}$ NMR spectrum of the valanimycin ammonia adduct that was isolated, while $^{13}\text{C}$ enrichments were found at C-2 (6.2%) as well as C-4 (1.7% total) and C-5 (1.9% total) with $^{13}\text{C}-^{13}\text{C}$ coupling between C-4 and C-5 (Scheme 1.8). This suggested that alanine might not be a direct precursor of valanimycin, since according to elaiomycin biosynthetic studies, it was expected that the two nitrogen atoms of valanimycin would originate from the amine and amino acid building blocks of valanimycin.

An alternative origin for the dehydroalanine moiety of valanimycin might be serine. Serine has been shown to be the precursor of the dehydroalanine residues of berninamycin (Pearce, 1979). Indeed, administration of [1-$^{13}\text{C}$]-D,L-serine and [3-$^{13}\text{C}$]-D,L-serine to washed cells of S. viridifaciens gave valanimycin ammonia adduct that exhibited very high enrichment at C-1 (96%) and C-3 (77%) of valanimycin. Also when [2-$^{13}\text{C}$,$^{15}\text{N}$]-D,L-serine was fed to the washed cells, the valanimycin ammonia adduct showed $^{15}\text{N}$-$^{13}\text{C}$ coupling with 83% $^{13}\text{C}$ incorporation at C-2 (Scheme 1.9). These results suggest that serine is a more direct precursor of the dehydroalanine moiety of valanimycin and that the $\beta$-nitrogen atom of valanimycin is derived from serine.

1.4.3 Summary of previous work

Previous work on the biosynthesis of valanimycin indicates that valanimycin is derived from serine and valine: the dehydroalanine moiety and $\beta$-nitrogen atom are derived from serine, while the isobutyl moiety and $\alpha$-nitrogen atom are derived from valine via the intermediacy of isobutylamine and isobutylhydroxylamine. These results are summarized in Scheme 1.10.
Scheme 1.9 Incorporation of serine into the dehydroalanine moeity of valanimycin (Parry, 1992)
Scheme 1.10 Proposed biosynthetic pathway for valanimycin
(Parry, 1992)
1.5 The scope of this project

At the beginning of my project, the nature of the metabolic building blocks for valanimycin had been established, but the mechanism of N-N bond formation, the key step in valanimycin biosynthesis, was still unknown. The objectives of this project were: 1), to examine the possible intermediacy of a hydrazine intermediate, 2), to identify enzymes related to the valanimycin biosynthetic pathway, especially enzyme(s) involved in the N-N formation, and 3), to purify and characterize the enzyme(s) of interest.
Chapter Two
Preliminary Studies

2.1 Introduction

In previous work in this laboratory, several difficulties were encountered: 1), there was no efficient assay for valanimycin or the valanimycin ammonia adduct, 2), the original \textit{S. viridifaciens} MG456-hF10 culture contained low or non-producing organisms, and 3), there was no efficient method to isolate the valanimycin ammonia adduct. Occasionally, at the end of isolation no valanimycin ammonia adduct was obtained. It was necessary to solve these problems before any further studies could be undertaken.

2.2 Assay system for valanimycin

2.2.1 Bioassay

Yamato used the paper disk-agar diffusion method with \textit{E. coli} 1121 as a test organism to assay for valanimycin (Yamato, 1986a). Since \textit{E. coli} 1121 was not available in our laboratory, \textit{E. coli} DH1 which was used in previous work by Norman Yang was chosen as a test organism for valanimycin. Unfortunately, It was found that \textit{S. viridifaciens} MG456-hF10 produced another antibiotic which was responsible for the activity of the fermentation broth against \textit{E. coli} DH1 and that valanimycin showed no activity against \textit{E. coli} DH1. Therefore, it was decided that a chemical assay, rather than a bioassay, should be used to detect valanimycin.
2.2.2 HPLC assay

Valanimycin (in 50 mM sodium phosphate buffer pH 7.0) was isolated according to the method of Yamato (Yamato, 1988a) and its structure was confirmed by $^1$H NMR. Using this valanimycin buffer solution, an HPLC assay system was developed for valanimycin detection (see section 8.9.1). Separation of valanimycin from other materials in the fermentation broth was achieved with a C$_{18}$ reversed-phase column (4.6 mm ID x 23.5 cm) using isocratic elution with 15% (v/v) acetonitrile / water plus 0.05% (v/v) acetic acid at 1 ml / min. flow rate and monitoring at 230 nm. A typical HPLC trace of the fermentation broth is shown in Figure 2.1. The retention time of valanimycin was 16 minutes.

Since valanimycin was unstable, no salt-free valanimycin was isolated and no quantitative relation between the area of the valanimycin peak and the valanimycin concentration in the fermentation broth was established.

2.3 Assay system for valanimycin ammonia adduct

Since valanimycin is unstable, valanimycin ammonia adduct, instead of valanimycin, was isolated in precursor incorporation experiments by treatment of the fermentation broth with 2 N NH$_4$OH. In order to monitor the isolation, an HPLC assay system for valanimycin ammonia adduct was developed (see section 8.9.2). A C$_{18}$ reversed-phase column (4.6 mm ID x 23.5 mm) and an isocratic elution with water at 1 ml / min. flow rate were employed. The eluate was monitored at 254 nm and the retention time of valanimycin ammonia adduct was 10.4 min. A typical HPLC trace of the fermentation broth after reaction with 2 N NH$_4$OH is shown in Figure 2.2. The concentration
Figure 2.1 Detection of valanimycin --
HPLC trace of fermentation broth
Figure 2.2  Detection of the valanimycin ammonia adduct --
HPLC trace of fermentation broth treated with $\text{NH}_4\text{OH}$
of the valanimycin ammonia adduct was calculated from the peak area with the aid of a standard curve (Figure 2.3).

2.4 Time course of valanimycin production

By means of a bioassay, Yamato determined the time course of valanimycin production in liquid cultures (30°C, 220 rpm) of S. viridifaciens MG456-hF10 which were inoculated with spores from a slant culture. The concentration of valanimycin in the culture broth reached the maximum at about 35 hours of incubation under these conditions (Yamato, 1986a). In our laboratory, 100 ml of fermentation medium was inoculated by addition of 0.5 ml of frozen stock to give a seed culture. The seed culture was incubated at 30°C, 220 rpm for 48 hours. Five ml of the resulting seed culture was used to inoculate 100 ml of fermentation medium to give the producing culture. The producing culture was incubated at 30°C, 180 rpm, and the valanimycin ammonia adduct was isolated from 40 hour old producing culture.

With the aid of the valanimycin HPLC assay system, the valanimycin concentrations in the seed culture and the producing culture at different incubation time points were measured and determined from the area of the valanimycin peak (Figure 2.4). The time course for the seed culture was similar to Yamato's observation. In the producing culture, the organism started to produce valanimycin earlier, and the valanimycin concentration increased gradually and reached the maximum level at about 30 hours.

A surprising result from the proceeding experiments was that the maximum concentration of valanimycin in seed culture was found to be much
Figure 2.3  Standard curve for the valanimycin ammonia adduct
Figure 2.4  Time course of valanimycin production
higher than that in producing culture. Since the only difference between the two cultures was that one was inoculated from a spore suspension and the other from a liquid culture. It was thought that the size of the inoculum might affect valanimycin production. Additional studies (Figure 2.5) suggested that this was the case: among five different producing cultures, the culture with a 2% inoculum (100 ml fermentation medium was inoculated with 2 ml seed culture) gave the highest maximum concentration of valanimycin.

Based on these results, the inoculation procedure was changed to using 2 ml of seed culture per 100 ml of medium and the valanimycin ammonia adduct was isolated from 36 hour old producing cultures.

2.5 Strain selection

The original *S. viridifaciens* MG456-hF10 strain was obtained as lyophilized stock from the Institute of Microbial Chemistry, Tokyo, Japan. One lyophilized tube was opened and used to prepared frozen stock. The frozen stock was stored at -70°C and was used to inoculate fermentation medium. The frozen stock appeared to be unstable at -70°C, or to contain low or non-producing forms of the organism, since sometimes no valanimycin ammonia adduct could be isolated from the producing culture. It therefore became necessary to carry out strain selection (see section 8.3.4) to obtain a stable high producing form of the organism.

A dilutied sample of frozen stock was used to inoculate agar plates in order to grow single colonies. Sixteen single colonies were picked and frozen stocks were prepared from these single colonies. A 0.5 ml aliquot of each frozen stock was then used to inoculate 100 ml of fermentation medium which
Figure 2.5  Time course of valanimycin production in producing cultures with different inoculum (seed culture) volumes added to 100 ml fermentation medium
was incubated at 30°C, 220 rpm. The concentration of valanimycin (expressed as area of the valanimycin peak) in these cultures was determined by HPLC after 31 hours of incubation (Table 2.2).

Colony number 12 was selected due to its high valanimycin production. A large number of tubes of frozen stock was prepared from colony number 12. The new frozen stock was quite stable at -70°C and gave consistently high valanimycin production. Although this strain selection process did not result in a large increase in valanimycin production, it did appear to eliminate low or non-producing strain of the organism and it provided a stable strain for use in further investigations.

Table 2.1 Valanimycin production of strains derived from single colonies

<table>
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<tr>
<th>Colony number</th>
<th>Area of valanimycin peak (x10⁻⁵)</th>
<th>Colony number</th>
<th>Area of valanimycin peak (x10⁻⁵)</th>
</tr>
</thead>
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<tr>
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<td>9</td>
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<tr>
<td>8</td>
<td>58</td>
<td>16</td>
<td>48</td>
</tr>
</tbody>
</table>
2.6 Isolation of the valanimycin ammonia adduct

The valanimycin produced in cultures was isolated as the ammonia adduct. The procedure employed in previous work (Parry, 1992) was modified with the aid of the valanimycin ammonia adduct HPLC assay system. The improved procedure (Scheme 2.1) provided an efficient isolation of the valanimycin ammonia adduct.
Culture (1000ml)
   \[\text{Filter through Celite}\]
   \[\text{Filtrate}\]
   \[1) \text{Adjust to pH 3.0} \]
   \[2) \text{Extract with EtOAc (3 x 300ml)}\]
   \[\text{EtOAc extract}\]
   \[1) \text{Evaporate in vacuo at r.t.}\]
   \[2) \text{Add 100ml 2N NH}_4\text{OH}\]
   \[3) \text{Stir for 2 hours at r.t.}\]
   \[4) \text{Evaporate in vacuo}\]
   \[\text{Crude valanimycin ammonia adduct}\]
   \[1) \text{Load onto reverse-phase column}\]
   \[2) \text{Elute with water}\]
   \[3) \text{Lyophilize eluant}\]
   \[\text{The valanimycin ammonia adduct}\]
   \[\text{(white powder)}\]

Scheme 2.1 Isolation of the valanimycin ammonia adduct
Chapter Three
N-N Bond Formation

3.1 Introduction

Bordeleau suggested that the biological formation of N-N bond might involve the reaction of a hydroxylamine with an amine to yield a hydrazine. Previous work indicated that the dehydroalanine moiety and β-nitrogen atom of valanymycin were derived from serine, while the isobutyl moiety and α-nitrogen atom of valanymycin were derived from isobutylhydroxylamine. Therefore, N-N bond formation in valanymycin biosynthesis might proceed through a hydrazino acid intermediate --- N-(isobutylamino)serine (1) (Scheme 3.1). Feeding experiments with isotopically labeled compounds were used to examine this hypothesis.

3.2 Synthesis of N-(isobutylamino)serine

3.2.1 Unlabeled form

The unlabeled N-(isobutylamino)serine (1) was synthesized in racemic form from ethyl 2,3-dibromopropionate (Scheme 3.2). The chemistry used to transform ethyl 2,3-dibromopropionate (2) to O-benzyl-N-aminoserine (4) was based upon the syntheses of serine (Wood, 1940) and of α-hydrazino acids (Cram, 1960).

Hydrogenolysis was used to remove the benzyl group from O-benzyl-N-aminoserine (4). A possible side reaction in this deprotection step would be cleavage of the N-N bond to give serine, instead of N-aminoserine. Since the chemical shifts of serine and
Scheme 3.1 Proposed biosynthetic pathway for valanimycin
Scheme 3.2 Synthesis of N-(isobutylamino)serine 1
N-aminoserine were pH sensitive, it was difficult to distinguish between them by $^1$H NMR analysis. Mass spectrometry was therefore employed to differentiate between compound 5 and serine. N,O-Trifluoroacetyl-n-butyl esters of the two compounds were prepared by the method of Kaiser (Scheme 3.3) (Kaiser, 1974), and the derivatives were subjected to MS analysis. In the mass spectrum of the N,O-bis(trifluoroacetyl)-n-butyl ester of serine between m/e 200 and 400 (Figure 3.1), three intense peaks at m/e 252, 280 and 298 were found. These are rationalized as shown in Scheme 3.4. In the mass spectrum of the N,N',O-tris(trifluoroacetyl)-n-butyl ester of compound 5 between m/e 200 and 500 (Figure 3.2), a strong ion peak at m/e 363, which corresponded to the loss of the butyloxycarbonyl group from N-trifluoroacetyl-n-butyl ester of N-aminoserine (Scheme 3.5), was found. These results suggested that compound 5 was different from serine and was in fact N-aminoserine.

Compound 1 was then prepared by coupling N-aminoserine (5) with isobutyraldehyde followed by reduction with NaBH$_3$CN. The structure of 1 was confirmed by $^1$H NMR, $^{13}$C NMR, mass spectrometry and high resolution mass spectrometry analysis (section 8.4.4). $^1$H NMR and $^{13}$C NMR analysis of [4-13C]-N-(isobutylamino)serine (section 8.4.6), as well as $^{13}$C NMR and $^{15}$N NMR analysis of [$^{15}$N$_2$]-N-(isobutylamino)serine (section 8.4.12) further confirmed that compound 1 was N-(isobutylamino)serine.

It was found that the hydrazino acid 1 was unstable and decomposed slowly in aqueous solution. NMR analysis suggested that 1 was converted into
Scheme 3.3 Derivatization of N-aminoserine (5) and serine
Figure 3.1  Mass spectrum (m/e range: 200 - 400) of
N,O-bis(trifluoroacetyl)-n-butyl ester of serine
Scheme 3.4  Fragmentation of the N,O-bis(trifluoroacetyl)-n-butyl ester of serine
Figure 3.2  Mass spectrum (m/e range: 200 - 500) of N,N',O-tris(trifluoroacetyl)-n-butyl ester of compound 5
Scheme 3.5  Fragmentation of the N,N',O-tris(trifluoroacetyl)-n-butyl ester of N-aminoserine (5)
the hydrazone 7. This transformation could be the result of air oxidation of the
hydrazine group followed by decarboxylation (Scheme 3.6). To avoid air
oxidation, compound 1 was converted to its hydrochloride salt (8) by treatment
with excess 1 N HCl.

3.2.2 [4-\textsuperscript{13}C]-N-(Isobutylamino)serine hydrochloride

A \textsuperscript{13}C label was introduced by using \textsuperscript{13}C-isobutyraldehyde (Scheme
3.7). \textsuperscript{13}C-Isobutyronitrile was synthesized from K\textsuperscript{13}CN by the method of
Parry (Parry, 1992) and reduced with diisobutylaluminum hydride (DIBAL) to
give \textsuperscript{13}C-isobutyraldehyde in CH\textsubscript{2}Cl\textsubscript{2}. Since isobutyraldehyde was volatile,
the crude \textsuperscript{13}C-isobutyraldehyde was used directly in a condensation
reaction with N-aminoserine (5) in MeOH/H\textsubscript{2}O solution. The ratio of CH\textsubscript{2}Cl\textsubscript{2},
MeOH and H\textsubscript{2}O was carefully adjusted so that they formed a homogenous
solvent in which N-aminoserine remained in solution. [4-\textsuperscript{13}C]-N-
(Isobutylamino)-serine hydrochloride (10) was obtained after NaBH\textsubscript{3}CN
reduction of the coupling mixture and HCl treatment.

3.2.3 [\textsuperscript{15}N\textsubscript{2}]-N-(Isobutylamino)serine hydrochloride

A \textsuperscript{15}N double label was introduced by use of \textsuperscript{15}N\textsubscript{2}-hydrazine (Scheme
3.8). In the preparation of the unlabeled form, excess of 50% (v/v) aqueous
solution of hydrazine was used to convert 2-bromo-3-benzoxypropionic acid
(3) into O-benzyl-N-aminoserine (4). Since the \textsuperscript{15}N labeled hydrazine was
expensive, 1 equiv. of NaOH and 2 equiv. of \textsuperscript{15}N\textsubscript{2}-hydrazine was used in this
reaction. \textsuperscript{15}N\textsubscript{2}-Hydrazine sulfate is the only form of \textsuperscript{15}N labeled hydrazine
that is commercially available. To avoid diluting the labeled hydrazine, which
would give a poor yield for displacement reaction, \textsuperscript{15}N\textsubscript{2}-hydrazine was not
prepared by neutralizing \textsuperscript{15}N\textsubscript{2}-hydrazine sulfate in aqueous solution.
Scheme 3.6 Possible route for N-(isobutylamino)serine (1) decomposition
Scheme 3.7  Synthesis of [4-^{13}\text{C}]\text{-N-(isobutylamino)serine hydrochloride}
Scheme 3.8  Synthesis of $[^{15}\text{N}_2]$-N-(isobutylamino)serine hydrochloride
Instead, [\(^{15}\text{N}_2\)]-hydrazine and water were distilled from the mixture of [\(^{15}\text{N}_2\)]-hydrazine sulfate and NaOH pellets \textit{in vacuo}. Using this \(^{15}\text{N}\) labeled hydrazine solution, [\(^{15}\text{N}_2\)]-O-benzyl-N-aminosine (10) was synthesized and [\(^{15}\text{N}_2\)]-N-(isobutylamino)serine hydrochloride (12) was prepared from 10.

3.3 Precursor incorporation with [4-\(^{13}\text{C}\)]-N-(isobutylamino)-serine hydrochloride

[4-\(^{13}\text{C}\)]-N-(isobutylamino)serine hydrochloride (9) (150 mg) was dissolved in 10 ml of Milli Q water and administered to washed cells of \textit{S. viridifaciens} MG456-hF10. After work up, 60 mg of valanimycin ammonia adduct was isolated from the culture. If N-(isobutylamino)serine was the precursor of valanimycin, then C-4 of the valanimycin ammonia adduct was expected to be enriched with \(^{13}\text{C}\).

In order to obtain a \(^{13}\text{C}\) NMR spectrum in which the heights of the \(^{13}\text{C}\) resonances are proportional to the degree of the \(^{13}\text{C}\) enrichment, a water soluble paramagnetic relaxation reagent, diethylene triamine pentaacetic acid, iron(III) disodium salt dihydrate (Na\(_2\)[Fe(DTPA)]\(_2\)\(\cdot\)2H\(_2\)O) (Figure 3.3) (Wenzel, 1982), was employed. A gated proton-decoupling sequence was also used to eliminate the Nuclear Overhauser Effect (NOE) observed for carbons bonded to hydrogen. Addition of the paramagnetic relaxation reagent shortens the relaxation times of carbons not bonded to hydrogen and suppresses the NOE for carbons bonded to hydrogen. Under these conditions, an integratable \(^{13}\text{C}\) NMR spectrum of the valanimycin ammonia adduct was obtained (Figure 3.4). In this spectrum, the ratio of the integrated peaks was 1:1:1:2:1:2 for C-1:C-2: C-3:C-4:C-5:C-6. Because only C-6 has two identical carbons in the
Figure 3.3  Diethylenetriamine pentaacetic acid, iron(III) disodium salt dihydrate (Na₂[Fe(DTPA)]·2H₂O)
Figure 3.4 Quantitative $^{13}$C NMR spectrum of the valanimycin ammonia adduct from a washed cell culture fed with [4-$^{13}$C]-N-(isobutylamino)serine hydrochloride (9).
valanimycin ammonia adduct, it was clear that only C-4 was enriched with $^{13}$C. Since the natural abundance of $^{13}$C is 1.1%, the $^{13}$C incorporation at C-4 is 1.1%. This result suggested that N-(isobutylamino)serine was specifically incorporated into valanimycin.

3.4 Precursor incorporation with $[^{15}\text{N}_2]$-N-(isobutylamino)-serine hydrochloride

In the feeding experiment with [4-$^{13}$C]-N-(isobutyl-amino)serine, it was possible that N-(isobutylamino)serine might be degraded *in vivo* and the resulting fragments incorporated into valanimycin. There were three possible cleavage points in N-(isobutylamino)serine (Scheme 3.9): one at the N-N bond and two at the C-N bonds. The resulting fragments containing an N-N linkage must lose one nitrogen atom before being incorporated into valanimycin, since the two nitrogen atoms of valanimycin are derived from two different building blocks. This means that the N-N linkage would be cleaved during the incorporation if N-(isobutylamino)serine were not a direct precursor of valanimycin.

To examine these possibilities, 110 mg of $[^{15}\text{N}_2]$-N-(isobutylamino)serine hydrochloride (12) was administered to washed cells of *S. viridifaciens* MG456-hF10 and 30 mg the valanimycin ammonia adduct was isolated from the culture. $^{15}$N NMR spectroscopy of the valanimycin ammonia adduct in the presence of the paramagnetic relaxation reagent $\text{Na}_2[\text{Fe(DTPA})]\cdot2\text{H}_2\text{O}$ was performed. Two groups of peaks were found in the spectrum (Figure 3.5).

In group A, the peak at 345.55 ppm corresponded to the natural abundance of the $^{15}$N at the $\alpha$-position; the doublet at 345.39 ppm and 345.63
Scheme 3.9 Possible catabolism of N-(isobutylamino)serine when being incorporated into valanimycin
Figure 3.4 $^{15}$N NMR spectrum of the valaniycin ammonia adduct from a washed cell culture fed with $[^{15}$N$_2]$-N-(isobutylamino)serine hydrochloride (12)
ppm corresponded to $^{15}$N incorporated into the α-position, which was split by $^{15}$N at the β-position. The presence of $^{15}$N at the β-nitrogen also caused the doublet to be shifted upfield. Group B, which represented the β-nitrogen, had a similar pattern. The doublet due to the β-nitrogen atom was also shifted upfield due to the presence of $^{15}$N at the α-position. This resulted in an unsymmetric doublet. This labeling pattern indicated that the two $^{15}$N labels of the precursor were still connected to each other in the valanimycin ammonia adduct. Therefore $[^{15}\text{N}_2]$-N-(isobutyl-amino)serine was incorporated into valanimycin without cleavage of the N-N bond. The peak area of the doublet in group A was about double that for naturally existing $^{15}$N. Since the natural abundance of $^{15}$N was 0.36%, the $^{15}$N incorporation in this experiment was estimated to be 0.7%, which was consistent with the incorporation level of $[4\cdot^{13}\text{C}]$-N-(isobutylamino)serine.

### 3.5 Discussion

Feeding experiments with the $^{13}$C labeled form and the $^{15}$N doubly labeled form of N-(isobutylamino)serine suggested this compound was incorporated intact into valanimycin. This supplied the first evidence for the intermediacy of a hydrazine in the biosynthesis of an aliphatic azoxy compound and supported Bordeleau's hypothesis.

However, a question arose when the incorporation level (about 1%) of N-(isobutylamino)serine was compared with that of isobutylhydroxylamine (48%) and serine (83%). The fact that the N-(isobutylamino)serine was a poor precursor might be due to following factors: 1) the N-(isobutylamino)serine was fed in racemic form; 2) the N-(isobutylamino)serine was less efficiently
transported to the site of valanimycin biosynthesis; and 3) the N-(isobutylamino)serine partially decomposed in the culture medium during the incorporation process. A future goal would therefore be to establish a solid relationship between N-(isobutylamino)serine and valanimycin biosynthesis by studies at the enzymatic level.
Chapter Four
Studies at the Enzymatic Level (I) -- Valine Decarboxylase

4.1 Introduction

At this point, precursor incorporation experiments had established a partial pathway for valanimycin biosynthesis which included the N-N bond forming step (Scheme 4.1). For further investigations, studies at the enzymatic level were carried out. The most interesting step in the pathway is that involving N-N bond formation, which would be catalyzed by an unknown enzyme (III). However, several difficulties are associated with a detailed study of this reaction: 1) the enzymatic reaction product, hydrazino acid 1, is unstable under neutral pH conditions; 2) there is no UV chromophore in the hydrazino acid 1, and no suitable derivatization method had been developed for this compound; 3) the N-N bond forming reaction might involve more than one enzyme (for example, the hydroxyl group of isobutylhydroxylamine might need to be activated before the coupling reaction), and require several cofactors. Therefore, it was decided that other enzymes in the pathway would be studied first. If one of these enzymes was purified and cloned, the genes encoding the entire pathway might be identified. In this way, the formation of the N-N bond could be studied at both the genetic and enzymatic level.

In the early steps of valanimycin biosynthesis, it appears likely that two enzymes (Enz I and Enz II) would be involved. Enz II, an N-hydroxylase that would catalyze the conversion of isobutylamine to isobutylhydroxylamine, was
Scheme 4.1 Proposed pathway for valanomycin biosynthesis
chosen for detailed study in Chapter Five for the following reasons: 1) N-hydroxylases that utilize aliphatic amines as substrates are not common in other species, so that isobutylamine N-hydroxylase activity might be due to an enzyme required for valanimycin biosynthesis; 2) the enzyme itself would be novel; 3) the hydroxylation reaction would be the biosynthetic step immediately preceding the N-N bond forming step.

The first enzymatic reaction in the conversion of valine into valanimycin is likely to be valine decarboxylase which catalyzes the conversion of valine to isobutylamine. Valine decarboxylase (EC number 4.1.1.14) is a common enzyme that has been found in *Proteus vulgaris* (Sutton, 1962), *Bacillus sphaericus* (Bast, 1971) and marine red algae (Hartmann, 1972). The enzyme requires pyridoxal-5'-phosphate (PLP) (Figure 4.1) as a cofactor and a plausible mechanism for the reaction is shown in Scheme 4.2. The activity of valine decarboxylase in a cell free system of *S. viridifaciens* MG456-hF10 was examined to provide the evidence that isobutylamine, a putative substrate for isobutylamine N-hydroxylase, was able to be synthesized in these cells.

4.2 Detection of valine decarboxylase activity in the cell free extract of *S. viridifaciens* MG456-hF10

The enzyme sample (EVD) for detection of valine decarboxylase activity was prepared as shown in Scheme 4.3. *S. viridifaciens* MG456-hF10 cells were disrupted by sonication, and the cell debris was removed by centrifugation. After removal of nucleic acids by streptomycin sulfate precipitation, the enzyme solution was purified by ammonium sulfate
Figure 4.1 Pyridoxal-5'-phosphate
Scheme 4.2: The reaction mechanism for valine decarboxylase
Scheme 4.3 Preparation of enzyme sample (EVD) for detection of valine decarboxylase activity
fractionation. The partially purified enzyme solution was dialyzed to remove small molecules that might interfere with the valine decarboxylase assay.

Valine decarboxylase activity was detected using a radioactive assay (Boeker, 1983). The decarboxylation reaction was monitored by using [1-^{14}\text{C}]-L-valine which evolved \textsuperscript{14}CO\textsubscript{2} on decarboxylation. The radioactive CO\textsubscript{2} was then absorbed by a paper disk saturated with a solution of 2-aminoethanol and 2-methoxyethanol. The amount of absorbed CO\textsubscript{2} was measured by liquid scintillation counting (section 8.10.1) (Scheme 4.4).

Since the assay was based on measuring CO\textsubscript{2} released in the enzymatic reaction, it was possible that the activity detected was not due to valine decarboxylase, but due to two other enzymatic reactions, in which valine underwent transamination to give an \(\alpha\)-keto acid (catalyzed by an aminotransferase) and CO\textsubscript{2} was released from the \(\alpha\)-keto acid by an \(\alpha\)-keto acid decarboxylase (Scheme 4.5). The aminotransferase would be a PLP dependent enzyme (Scheme 4.6) (Voet, 1990). The \(\alpha\)-keto acid decarboxylase should require thiamine pyrophosphate (TPP) (Figure 4.2) for its activity (Scheme 4.7) (Voet, 1990). To determine whether the observed CO\textsubscript{2} releasing activity was related to valine decarboxylase or to a combination of an aminotransferase and an \(\alpha\)-keto acid decarboxylase, the cofactor requirements of the decarboxylation reaction were investigated (Table 4.1).

Experiment number 1 used a boiled enzyme (i.e. inactive enzyme) to serve as a negative control. In this experiment, the low level of counts suggested that the activity observed in the other tests was due to enzymatic activity. The activity shown in experiment number 2 in which no cofactors were
Scheme 4.4 Radioactive assay for valine decarboxylase

\[ \text{[1-}^{14}\text{C]-L-valine} \xrightarrow{\text{valine decarboxylase, PLP}} \text{isobutylamine} + {^{14}\text{CO}_2} \] 

\[ \text{paper disk} \xrightarrow{{^{14}\text{CO}_2}} \text{radioactive detector (scintillation counter)} \rightarrow \text{Counts (dpm)} \]
Scheme 4.5 The second route for releasing CO$_2$ from valine
Scheme 4.6 The mechanism of ω-keto acid formation by a PLP-dependent aminotransferase.
Figure 4.2 Thiamine pyrophosphate
Scheme 4.7 The reaction mechanism of an α-keto acid decarboxylase
added indicated the enzyme sample contained some of the cofactor(s) necessary for $^{14}\text{CO}_2$ production. In experiment number 3, the addition of TPP alone did not increase the activity, whereas in experiment number 4, the addition of PLP greatly stimulated the activity. When PLP and TPP were added simultaneously (experiment number 5), the activity was only slightly increased compared to that in experiment number 4. These results indicated that the activity observed was more dependent on PLP than on TTP.

Table 4.1  Tests for cofactor dependence of CO$_2$ releasing activity$^a$

<table>
<thead>
<tr>
<th>No.</th>
<th>Enzyme (280 μl)</th>
<th>Cofactor added (final concentration)</th>
<th>Counting Result (dpm)</th>
<th>Conversion of valine to CO$_2$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EVD (boiled before added)</td>
<td>1 mM PLP</td>
<td>1160</td>
<td>0.05</td>
</tr>
<tr>
<td>2</td>
<td>EVD</td>
<td>no cofactor added</td>
<td>17191</td>
<td>0.77</td>
</tr>
<tr>
<td>3</td>
<td>EVD</td>
<td>1 mM TPP</td>
<td>14412</td>
<td>0.65</td>
</tr>
<tr>
<td>4</td>
<td>EVD</td>
<td>1 mM PLP</td>
<td>98331</td>
<td>4.43</td>
</tr>
<tr>
<td>5</td>
<td>EVD</td>
<td>1 mM PLP + 1 mM TPP</td>
<td>111535</td>
<td>5.02</td>
</tr>
</tbody>
</table>

$^a$ The data represent two similar determinations.

4.3 Conclusion

Although no attempt was made to detect isobutylamine, the tests for cofactor dependence suggested that valine decarboxylase activity was present in the cell free extracts. This provides some evidence that isobutylamine can be formed enzymatically from valine in *S. viridifaciens* MG456-hF10 cells. These results provided a basis for the study of isobutylamine N-hydroxylase, which would utilize isobutylamine as a substrate.
Chapter Five
Studies at the Enzymatic Level (II) --
Isobutylamine N-Hydroxylase

5.1 Introduction

In the valanimycin biosynthetic pathway (Scheme 4.1), isobutylamine N-hydroxylase (IBA N-hydroxylase) catalyzes the hydroxylation reaction, in which isobutylamine (IBA) is converted to isobutylhydroxylamine (IBHA) (Scheme 5.1). IBA N-hydroxylase was identified in the cell-free extracts of

\[
\text{NH}_2 \xrightarrow{\text{isobutylamine N-hydroxylase}} \text{NHOH}
\]

IBA \qquad \text{IBHA}

Scheme 5.1 The reaction catalyzed by isobutylamine N-hydroxylase

\textit{S. viridifaciens} MG456-hF10 and found to be related to other external flavoprotein monooxygenases. The enzyme was then purified and characterized.

With the aid of either FAD or FMN (Figure 5.1), flavoprotein monooxygenases incorporate one atom of an O\textsubscript{2} molecule into the substrate, while the other oxygen atom is reduced to H\textsubscript{2}O\textsubscript{2}. The external flavoprotein monooxygenases are a class of flavoprotein monooxygenases which require an external reductant such as NADH or NADPH (Figure 5.2) for catalytic activity. The external flavoprotein monooxygenases are also called flavoprotein hydroxylases since they catalyze hydroxylation reactions. The
Figure 5.1 FAD and FMN
Figure 5.2. The structures of NAD⁺, NADH, NADP⁺, and NADPH.
external flavoprotein monooxygenases require four substrates: NAD(P)H to reduce the flavin, the substrate to be hydroxylated, FAD or FMN, and molecular oxygen. Most of the external flavoprotein monooxygenases hydroxylate the substrate at carbon atoms while some hydroxylate at nitrogen atoms. Many external flavoprotein monooxygenases have been characterized, and the mechanism of the reaction has been intensively studied and reviewed (Massey, 1975, 1994, 1995; Entsch, 1995). Two external flavoprotein monooxygenases will be discussed below since they share some unusual features with isobutylamine N-hydroxylase.

## 5.1.1 Lysine N⁶-hydroxylase

Lysine N⁶-hydroxylase from *E. coli* catalyzes the hydroxylation of the terminal amino group of L-lysine which is the first step in the biosynthesis of aerobactin (Scheme 5.2) (Plattner, 1989; Macheroux, 1993).

\[
\begin{align*}
\text{NH}_3^+ & \quad \text{NADPH} & \quad \text{NADP}^+ \\
\text{OOC} & \text{CH}-(\text{CH}_2)_4 & \text{NH}_2 \\
& \quad \text{FAD} & \quad \text{O}_2 & \quad \text{H}_2\text{O} \\
& \quad \text{NH}_3^+ & \quad \text{OOC} & \text{CH}-(\text{CH}_2)_4 & \text{NHOH}
\end{align*}
\]

**Scheme 5.2** The reaction catalyzed by lysine N⁶-hydroxylase

The enzyme consists of four identical subunits, each about 50 kDa in molecular weight. Unlike other external flavoprotein monooxygenases which normally have tightly bound FAD or FMN, the binding of FAD by lysine N⁶-hydroxylase (0.79 FAD / subunit) is rather weak with a *K*ₐ of 30 μM at 4°C, and the enzyme loses FAD easily during dialysis. The reaction mechanism proposed for the enzyme was similar to that of other external flavoprotein monooxygenases (Scheme 5.3). In the absence of L-lysine, the enzyme
Scheme 5.3 The reaction mechanism proposed for lysine N^6-hydroxylase
slowly oxidizes NADPH to generate hydrogen peroxide. In the presence of L-lysine, the rate of NADPH oxidation is enhanced by a factor of 10-12 and the generation of hydrogen peroxide is depressed, while L-lysine is hydroxylated.

5.1.2 p-Hydroxyphenylacetate-3-hydroxylase

p-Hydroxyphenylacetate-3-hydroxylase catalyzes the introduction of a second hydroxyl group into the benzene nucleus of p-hydroxyphenylacetate at a position ortho to the existing hydroxyl group (Scheme 5.4).

![Scheme 5.4 The reaction catalyzed by p-hydroxyphenylacetate-3-hydroxylase](image)

In contrast to most of the other external flavoprotein monooxygenases, which consist of a single protein component, p-hydroxyphenylacetate-3-hydroxylase purified from *Pseudomonas putida* requires two protein components for complete activity: a flavoprotein, and a colorless protein (coupling protein) (Arunachalam, 1992, 1994a, 1994b). The flavoprotein exists as a dimer of two identical or similar subunits, each with a molecular weight of 30.8 kDa. The coupling protein has a subunit molecular weight of 38.5 kDa. The native molecular weight of the coupling protein could not be determined since the protein aggregates.
In the absence of the coupling protein, the flavoprotein can only catalyze the oxidation of NADH to form hydrogen peroxide without substrate hydroxylation and the NADH oxidation can be stimulated nonspecifically by the presence of a variety of substrate analogs referred to as effectors. On the other hand, the reduction of the substrate-free flavoprotein by NADH is slow and not altered by the presence of the coupling protein. The coupling protein and the flavoprotein together catalyze the hydroxylation reaction with high substrate specificity for p-hydroxyphenylacetate. The role of the coupling protein is probably not to act as a hydroxylating agent itself, but rather as a regulatory protein which forms a complex with the flavoprotein component in order to switch the flavoprotein from an NADH oxidase to a hydroxylase, and to restrict the hydroxylation specificity to p-hydroxyphenylacetate. These results are summarized in Scheme 5.5.

Scheme 5.5  Reactions catalyzed by the flavoprotein in the presence and absence of the coupling protein.
5.2 Assay development for isobutylamine N-hydroxylase

To begin our studies, methods were developed for detecting IBA N-hydroxylase. A discontinuous method was used in which the enzyme reaction was stopped by acidification at certain time points and the amount of product (IBHA) was then measured. The enzyme activity was expressed as nmol IBHA / min produced in the assay mixture (200 μl).

5.2.1 Spectrophotometric assay

An assay method had been developed to quantify primary and secondary arylalkyl and aliphatic hydroxylamine compounds by Belanger, et. al. (Belanger, 1981). This method was based on the reduction of ferric ion by the hydroxylamine moiety with the resulting ferrous ion being quantified by complexion with 2,4,6-tripyridyl-s-triazine (TPTZ) to form a blue complex which had an absorbance maximum at 594 nm (Scheme 5.6) (Day, 1979).

A modified procedure which used less enzyme was developed to measure the concentration of IBHA in a 200 μl enzyme assay solution (Scheme 5.7). An absorption coefficient of ε = 2.12 mM⁻¹ cm⁻¹ was obtained from a standard curve with IBHA standard solution (Figure 5.3). The practical detection limit of the assay was 5 μM in the assay mixture (200 μl). This assay was simple, rapid and could analyze many samples in a short of time. This assay was used unless otherwise indicated.
Scheme 5.6 Spectrophotometric assay for hydroxylamines

RNHOH + Fe$^{3+}$ → [RNO] + Fe$^{2+}$

Fe$^{2+}$ + TPTZ → Blue complex ($\lambda_{m}$ at 594 nm)
Assay mixture (200 µl)
(including enzyme, IBA and cofactors)

1) 400 µl 10%(w/v) TCA
2) Centrifugation

Supernatant (400 µl)

1) 1 ml Color reagent
2) Incubated for 30 min. at r.t.

Measure absorbance at 594 nm

Color reagent: 10 ml 4 M sodium acetate buffer pH 5.9 plus
50 µl 48 mM TPTZ and 40 µl 200 mM FeCl₃

Scheme 5.7 Spectrophotometric assay for IBA N-hydroxylase
Figure 5.3  Standard curve for spectrophotometric assay of IBHA
5.2.2 HPLC assay

Although the spectrophotometric assay was convenient, it had several inherent disadvantages: 1) this assay was not a specific assay for IBHA; 2) any compound which was able to reduce ferric ion would interfere with the assay; 3) the practical detection limit was high due to interference. Therefore a HPLC analysis which employed derivatization with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole chloride (NBD-Cl) (Scheme 5.8) was developed to provide an assay that was specific for IBHA and had a lower detection limit.

NBD-Cl derivatization has been used for amino acid analysis (Scheme 5.9) (Ahnoff, 1981; Yoshida, 1982). NBD-Cl reacts with both primary and secondary amino groups to form derivatives at 60°C in MeOH-borate buffer, pH 9.0. Under these conditions, secondary amino groups react faster than primary amino groups. The hydrolysis product NBD-OH is also formed in the reaction.

The conditions initially employed to derivatize IBHA utilized 6.67 mM NBD-Cl, 33% (v/v) MeOH in 133 mM NaBO₃ buffer, pH 9.0, at 80°C for 3 minutes. NBD-derivatives of IBHA, IBA and valine were prepared and separated with HPLC system (1) (Table 5.1). The result is shown in Table 5.2.
Scheme 5.8  NBD-Cl derivatization of IBHA

#  Hypothetical structure of NBD-IBHA. The actual structure is unknown.
Scheme 5.9 NBD-Cl derivatization of amino acids
Table 5.1  
HPLC system (1) for the detection of IBHA

<table>
<thead>
<tr>
<th>Column</th>
<th>Whatman C$_{18}$ column (4.6 mm ID x 110 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monitor at</td>
<td>450 nm</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1 ml/min</td>
</tr>
<tr>
<td>Solvent A</td>
<td>MeOH / THF / 100 mM sodium phosphate buffer, pH 6.0, (23: 1.27: 75.73) (v:v:v)</td>
</tr>
<tr>
<td>Solvent B</td>
<td>MeOH / H$_2$O (4:6) (v:v)</td>
</tr>
<tr>
<td>Gradient elution</td>
<td>Time (min.)</td>
</tr>
<tr>
<td></td>
<td>Solvent A%</td>
</tr>
<tr>
<td></td>
<td>Solvent B%</td>
</tr>
</tbody>
</table>

Table 5.2  
Retention time of NBD-derivatives with HPLC system (1)

<table>
<thead>
<tr>
<th>NBD-derivative</th>
<th>NBD-OH</th>
<th>NBD-IBHA</th>
<th>NBD-valine</th>
<th>NBD-IBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time (min.)</td>
<td>2.9</td>
<td>6.1</td>
<td>8.6</td>
<td>19.2</td>
</tr>
</tbody>
</table>

Clearly, IBA, a substrate of the enzyme, would not interfere with the detection of IBHA in this system. However some unknown compounds in the enzyme sample gave peaks with retention times around 6.1 minutes.

Additional investigations showed that IBHA could react with NBD-Cl even at pH 7.0 and 60°C, whereas most amino acids and IBA did not react at all under these conditions. Therefore, a pH of 7.0 and a temperature of 60°C were adopted for derivatization. However, NADH, a cofactor of the enzyme, still interfered with the detection using HPLC system (1). To eliminate this
interference, a new HPLC system, system (2), was devised (Table 5.3), and the assay mixture was incubated with TCA at room temperature for one hour to completely destroy NADH before derivatization. IBHA was found to be stable under these conditions.

With the HPLC system (2), the retention times for NBD-OH and NBD-IBHA were 4.4 and 9.5 minutes, respectively. The NADH decomposition peak appeared at 7.7 minutes. No other peak was found between 6 to 12 minutes. Therefore an HPLC method for detection of IBHA in the enzyme assay mixture was established (Scheme 5.10). A standard curve of NBD-IBHA peak area vs. IBHA concentration in the assay mixture was obtained with the IBHA standard solution (Figure 5.4). The practical detection limit of the assay was 5 μM. This assay was used only in kinetic studies.

<table>
<thead>
<tr>
<th>Table 5.3</th>
<th>HPLC system (2) for the detection of IBHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Whatman C_{18} column (4.6 mm ID x 235 mm)</td>
</tr>
<tr>
<td>Monitor at</td>
<td>450 nm</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1 ml / min.</td>
</tr>
<tr>
<td>Isocratic elution with</td>
<td>MeOH / 25 mM sodium phosphate buffer pH 7.0 (3 : 7) (v:v)</td>
</tr>
</tbody>
</table>
Enzyme assay mixture (200 µl)
(Including enzyme, IBA and cofactors)

1) 20 µl 50% (w/v) TCA
2) Centrifugation

Supernatant (25 µl)

1) Incubated for 1 hour at r.t.
2) Added 2.3 µl 3N NaOH
3) Added 5 µl 0.5 M sodium phosphate buffer pH 7.0
4) 16 µl 20 mM NBD-Cl methanol solution

20 µl of resulting mixture was analyzed with HPLC system (2)

Scheme 5.10 HPLC assay for IBA N-hydroxylase
Figure 5.4  Standard curve for HPLC assay of IBHA

\[ y = 2.8775 + 1.2017x \quad R = 0.99998 \]
5.3  Identifying IBA N-hydroxylase in the cell-free extract of *S. viridifaciens* MG456-hF10

Using the spectrophotometric assay, we searched for IBA N-hydroxylase activity in the cell-free extract of *S. viridifaciens* MG456-hF10. Since IBA N-hydroxylase might be a flavoprotein hydroxylase and require NADH or NADPH for the activity, a NADPH regenerating system with D-glucose-6-phosphate dehydrogenase (Belanger, 1981) was included in the enzyme assay mixture in the initial work to avoid direct addition of NADH or NADPH which might interfere with the spectrophotometric assay and prevent low enzyme activity from being detected.

At this point, it was not known whether the enzyme required any other cofactors. To avoid removing any possible cofactors, cells were disrupted in Buffer A without pre-washing. Three portions were collected after centrifugation: 1) the supernatant was treated with polyethyleneimine to remove nucleic acids and marked as CFS; 2) the cell debris was washed with Buffer A plus 4% (w/v) Triton x 100, and the proteins soluble in detergent were obtained after removal of cell debris by centrifugation; this fraction was CFPS; 3) the washed cell debris was suspended in Buffer A and marked as CFPP. CFS showed very low activity, while CFPS and CFPP showed no activity. This suggested the enzyme was soluble in the buffer.

Since the activity of CFS was so low, a concentrated protein solution was tested. The protein solution was concentrated by adding ammonium sulfate to CFS to 70% saturation and dissolving the resulting precipitate in a minimal amount of Buffer A. This concentrated enzyme solution gave higher activity in
the activity assay and confirmed that the observed activity with CFS was real. Thus, the story of IBA N-hydroxylase began.

5.3.1 Is it an IBHA forming activity?

Since the spectrophotometric assay is not specific for IBHA, it was necessary to show that the absorption at 594 nm was due to IBHA formation. High resolution mass spectrometry analysis was employed (section 8.7.2 C). The enzyme assay mixture obtained from a concentrated enzyme solution gave a strong peak corresponding to 89.0842 (the expected precise mass of isobutylhydroxyl-amine is 89.084055), whereas the control (assay mixture without IBA) did not give the same peak. Also, the enzyme assay mixture gave a peak which had the same retention time (9.5 min.) as that of NBD-IBHA standard in the HPLC assay, but the control gave no such peak. These results indicated that the activity observed with the spectrophotometric assay was an IBHA forming activity.

5.3.2 Is it an enzymatic activity?

When the ammonium sulfate concentrated enzyme (AM) was filtered through a CentriCon 10 (10,000 MW cutoff) to remove protein, no activity was observed in the filtrate. This suggested that the observed activity is indeed an enzymatic activity.

5.3.3 Tests for cofactor dependence

It was found that if the cells were washed with Buffer A before disruption by sonication, the enzyme preparation did not show activity unless FAD was added to the enzyme assay mixture. Tests for cofactor dependence (Table 5.4) suggested that the enzyme requires an external reductant (NADH or NADPH)
as well as FAD or FMN. At this stage of the enzyme purification, NADH was a better substrate than NADPH and FMN was found to be slightly better than FAD for stimulating enzyme activity. Therefore, NADH (2mM) and FAD (5µM) were included in the enzyme assay mixture. The binding of FAD to the enzyme is weak since the FAD could be removed from the enzyme by dialysis.

The fact that IBA N-hydroxylase required FAD and NADH suggested that the enzyme is a external flavoprotein monoxygenase and is similar to lysine N⁶-hydroxylase since both enzymes hydroxylate the substrate at a nitrogen atom and have weak FAD binding.

<table>
<thead>
<tr>
<th>Test No.</th>
<th>Cofactor(s) added and final concentration</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 µM FAD</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2 mM NADPH</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>5 µM FMN</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>2 mM NADH</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>5 µM FMN + 2 mM NADPH</td>
<td>1.1</td>
</tr>
<tr>
<td>6</td>
<td>5 µM FAD + 2 mM NADPH</td>
<td>1.0</td>
</tr>
<tr>
<td>7</td>
<td>5 µM FMN + 2 mM NADH</td>
<td>4.6</td>
</tr>
<tr>
<td>8</td>
<td>5 µM FAD + 2 mM NADH</td>
<td>4.3</td>
</tr>
<tr>
<td>9</td>
<td>10 µM FAD + 2 mM NADH</td>
<td>4.2</td>
</tr>
</tbody>
</table>

a Cofactor(s) was added to assay mixture containing 0.37 mg protein per assay) and 10 mM IBA, and the incubation was carried out in 100 mM sodium phosphate buffer (pH 7.6) at 30°C for three hours.
5.3.4 Is the enzyme related to valanimycin biosynthesis?

Since the focus of the project was valanimycin biosynthesis, substrate analog tests were carried out with phenyl agarose purified enzyme to determine whether the enzyme activity is related to valanimycin biosynthesis. The ammonium sulfate concentrated enzyme solution containing 1 M ammonium sulfate was loaded onto a phenyl agarose column pre-equilibrated with 0.5 M ammonium sulfate in Buffer B (pH 7.0) and the column was then washed with 0.5 M ammonium sulfate in Buffer B (pH 7.0). The phenyl agarose purified enzyme was then eluted with Buffer B and concentrated by use of a stirred pressure cell with a PM 30 membrane. The results of the substrate analog tests (Table 5.5) indicated that isobutylamine is the best substrate of those examined. This suggested that the enzyme is likely to be associated with valanimycin biosynthesis.

5.3.5 Two-protein-component system

Attempts to purify further the phenyl agarose purified enzyme encountered great difficulty: no activity was found after chromatography of the enzyme on DEAE agarose (weak anion-exchange chromatography) or propyl agarose. Efforts were therefore made to fine tune the phenyl agarose step in order to enhance the purification of the enzyme.

The ammonium sulfate concentrated enzyme solution containing 1 M ammonium sulfate was loaded onto a phenyl agarose column pre-equilibrated with 0.5 M ammonium sulfate in Buffer B (pH 7.0). The column was washed with 0.5 M ammonium sulfate in buffer B (pH 7.0), and then eluted with 0.25 M ammonium sulfate in Buffer B (pH 7.0) to give fraction A followed by elution
<table>
<thead>
<tr>
<th>Test No.</th>
<th>Substrate</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CH$_3$NH$_2$</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>CH$_3$CH$_2$NH$_2$</td>
<td>0.20</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0.33</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0.89</td>
</tr>
<tr>
<td>5</td>
<td>NH$_2$ (IBA)</td>
<td>1.0</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>0.75</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>0.70</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>0.46</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>0.44</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>0.37</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>0.33</td>
</tr>
</tbody>
</table>

* The relative activities represent three similar determinations (two hour incubation at 30°C in 100 mM sodium phosphate buffer, pH 7.6, with protein concentration of 1.2 μg/μl).
with Buffer B to give fraction B. No enzyme activity was detected in fraction A or fraction B. However, combining these two fractions restored the enzyme activity. This indicated that IBA N-hydroxylase consists of two protein components, both of which are required for the hydroxylation activity. The fractions A and B were called the A component and the B component, respectively.

5.3.6 Validity of the assay for IBA N-hydroxylase

To compare the enzyme activities, several terms are used. The activity of IBA N-hydroxylase is defined as amount (nmol) of IBA formed in 200 μl of assay mixture in a certain time period (min) under defined conditions and its units are nmol/min. The specific activity of IBA N-hydroxylase is defined as the amount (nmol) of IBA formed in 200 μl assay mixture in a certain time period (min) with certain amount (mg) of total protein under defined conditions and its units are nmol/min/mg. When kinetic data are analyzed, the term "reaction velocity" is used. Velocity is defined as d[P]/dt or -d[S]/dt, where [P] is concentration of product and [S] is concentration of substrate. The units of velocity are μM/min. Thus, velocity times assay volume (200 μl) would be equal to activity.

A. Time course of the enzyme reaction

Since a discontinuous method was used for the assay of IBA N-hydroxylase, the average velocity (v) of the enzyme reaction in a certain period is used to represent the true initial velocity (v₀). In order to keep this assumption valid, the rate of the product (IBHA) formation should be constant over the entire time interval of the assay. Figure 5.5 shows the progress of the
Figure 5.5  Time course of IBA N-hydroxylase reaction

(Reaction was in 100 mM BIs-Tris-HCl buffer, pH 7.5
     at 30°C, with protein concentration of 0.8 μg/μl)
IBA N-hydroxylase reaction. The concentration of IBHA formed during the reaction appears to be linearly proportional to the reaction time up to 4 hours. Therefore a time interval between one to three hours was used for the assays.

B. Enzyme activity vs. total protein

When the enzyme is present in catalytic amount, the initial velocity ($v_0$) of the enzyme reaction is linearly proportional to the enzyme concentration, and this fact can be used to quantify the concentration of enzyme in any preparation. Since the activity of IBA N-hydroxylase requires the presence of the two components, an equilibrium between the active form $A_mB_n$ and two components can be assumed. The $v_0$ should be linearly proportional to the concentration of the active form $[A_mB_n]$.

When the two components were mixed to give an active enzyme solution, no linear relationship between the enzyme activity (represented by $v_0$) and the amount of the testing enzyme $[A]_t+[B]_t$ was obtained (Figure 5.6), where $[A]_t$ or $[B]_t$ is the total concentration of $A$ or $B$. The curve appears to be composed of three regions: a sigmoidal region at low $[A]_t+[B]_t$, a linear region and a slightly concave region at high $[A]_t+[B]_t$. This relationship could be explained as follows.

There is an equilibrium between the active form $(A_mB_m)$ and the two components:

\[ mA + nB \rightleftharpoons A_mB_n \]  \hspace{1cm} (1)

\[ K_a = [A_mB_n]_e / [A]^m[B]^n \]  \hspace{1cm} (2)
Figure 5.6 Activity of IBA N-hydroxylase vs. protein concentration in the assay mixture with a fixed ratio between the A and B component. Incubation was for one hour at 30°C in 100 mM Bis-Tris HCl buffer, pH 7.5.
where $K_a$ is the association constant of $A_mB_n$ and $[A_mB_n]_e$ is the concentration of $A_mB_n$ when equilibrium is reached.

If $[A]_t$ and $[B]_t$ are the total concentration of $A$ and $B$, respectively, then

$$K_a = \frac{[A_mB_n]_e}{([A]_t - [A_mB_n]_e)^n ([B]_t - [A_mB_n]_e)^m}$$  \hspace{1cm} (3)

Supposed there are two enzyme solutions (Solution 1 and 2): $[A]_t$ and $[B]_t$ in Solution 1 are $r$ fold of those in Solution 2 (i.e. $[A]_{t2} = r [A]_{t1}$, $[B]_{t2} = r [B]_{t1}$, $r > 1$). Thus, the concentration of $A_mB_n$ in Solution 2, $[A_mB_n]_{t2}$, should also be higher than that in Solution 1. If $[A_mB_n]_{t2} = r [A_mB_n]_{t1}$, where $[A_mB_n]_{t1}$ is the concentration of $A_mB_n$ when equilibrium is reached in Solution 1, then

$$\frac{[A_mB_n]_{t2}}{[A]_{t2}^m [B]_{t2}^n} = \frac{[A_mB_n]_{t2}}{([A]_{t2} - [A_mB_n]_{t2})^n ([B]_{t2} - [A_mB_n]_{t2})^m}$$

$$= \frac{r [A_mB_n]_{t1}}{(r[A]_{t1} - r[A_mB_n]_{t1})^n (r[B]_{t1} - r[A_mB_n]_{t1})^m}$$

$$= \frac{r^{1-n-m} [A_mB_n]_{t1}}{([A]_{t1} - [A_mB_n]_{t1})^m ([B]_{t1} - [A_mB_n]_{t1})^n}$$

$$= r^{1-n-m} K_a$$  \hspace{1cm} (4)

Obviously $m, n >= 1$, and $r > 1$, thus

$$\frac{[A_mB_n]_{t2}}{[A]_{t2}^m [B]_{t2}^n} < K_a$$  \hspace{1cm} (5)

so more $A_mB_n$ would be formed to reach the equilibrium in Solution 2, thus
\[ [A_m B_n]_{e2} > r [A_m B_n]_{e1} \]  \tag{6}

where \([A_m B_n]_{e2}\) is the concentration of \(A_m B_n\) when equilibrium is reached in Solution 2. So,

\[
1 < \frac{[A]_{l2}}{[A]_{l1}} = \frac{[B]_{l2}}{[B]_{l1}} < \frac{[A_m B_n]_{e2}}{[A_m B_n]_{e1}} \tag{7}
\]

Therefore, increasing \([A]_l + [B]_l\) by a given factor will increase \([A_m B_n]_{e}\) by a greater factor. This effect is bigger at low \([A]_l + [B]_l\) (compared to \(1/K_a\)) and decrease as \([A]_l + [B]_l\) increases.

Since \(v_o\) is linearly proportional to the \([A_m B_n]_{e}\) at low enzyme concentration, \(v_o\) vs. \([A]_l + [B]_l\) would be a curve in this region. As \([A]_l + [B]_l\) increases, \(v_o\) is no longer linearly proportional to the \([A_m B_n]_{e}\); this compensates for and finally overcomes the effect of equation (7) and gives a linear line then a slightly concave curve at high \([A]_l + [B]_l\) region.

C. Assays for individual components

Since both components of IBA N-hydroxylase are essential for the hydroxylation activity, the assay for the individual components (tested component) requires the presence of the other component (helper component). Figure 5.7 shows that when the amount of the A component is fixed, the observed activity increases as the amount of the B component increases, but there is no linear relationship.

Thus, if the B component is considered as the tested component, the observed activity is not directly proportional to the amount of the B even when
Figure 5.7  Activity of IBA N-hydroxylase vs. amount of the B component

Amount of the A component was fixed at 88 µg per assay,
incubation was for one hour at 30°C in 100 mM Bis-Tris•HCl
buffer, pH 7.5.
the amount of the helper component (A) is fixed. Similarly, if the A component is considered as the tested component, the observed activity of A will change in a non-linear way as the amount of the helper component (B) is varied. Clearly, the observed activity of the tested component will be controlled by two factors: the amount of the tested component and the amount of helping component. Furthermore, the specific activity of helping component might change due to enzyme denaturation. These facts make it very difficult to measure the activity of the individual components accurately and to compare the data from different assays.

To simplify this problem, a fixed and excess amount (judged by activity) of the helping component was included in the assay mixture to assay the tested component during the purification process. Therefore the activity data of the individual components should be interpreted with caution.

5.3.7 Summary

Isobutylamine N-hydroxylase was identified in cell-free extracts of S. viridifaciens MG456-hF10. The evidence suggests that the enzyme is probably a component of the valanimycin biosynthetic pathway. The enzyme requires NADH, FAD (FMN) and two protein components for the hydroxylation activity, and the binding of FAD is very weak. Therefore, it appears that the enzyme might be related to other external flavoprotein monooxygenases, especially to the enzymes lysine N6-hydroxylase and p-hydroxyphenylacetate-3-hydroxylase. Assay and purification of this enzyme system proved to be difficult due to its two component nature.
5.4 Purification of the B component of IBA N-hydroxylase

A typical purification sequence for the B component is presented in Table 5.6. The progress of the purification to homogeneous enzyme is demonstrated by SDS-polyacrylamide gel electrophoresis (Figure 5.8).

Table 5.6 Purification of the B component of IBA N-hydroxylase\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Protein (mg)</th>
<th>Activity (nmol/min)</th>
<th>Specific activity (nmol/min/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyethyleneimine</td>
<td>246</td>
<td>203</td>
<td>0.83</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate (0-70%)</td>
<td>135</td>
<td>520</td>
<td>3.8</td>
<td>4.6</td>
<td>256</td>
</tr>
<tr>
<td>Phenyl agarose</td>
<td>13.4</td>
<td>237</td>
<td>17.1</td>
<td>21</td>
<td>117</td>
</tr>
<tr>
<td>Phenyl superose</td>
<td>3.1</td>
<td>169</td>
<td>54</td>
<td>65</td>
<td>83</td>
</tr>
<tr>
<td>Resource Q (anion exchange)</td>
<td>0.63</td>
<td>142</td>
<td>224</td>
<td>270</td>
<td>70</td>
</tr>
<tr>
<td>Blue 3G-A agarose</td>
<td>0.48</td>
<td>68</td>
<td>142</td>
<td>171</td>
<td>33</td>
</tr>
</tbody>
</table>

\(^a\) The purification uses cells from four 100 ml cultures of \textit{S. viridifaciens}. The activity assay was carried out in 100 mM sodium phosphate buffer, pH 7.6 and incubation was for three hours at 30 °C.

The Resource Q step yields an enzyme preparation that displays two bands on an SDS-PAGE gel. The Blue 3G-A step removes the minor contamination band, but the specific activity is actually decreased. This might be due to denaturation of the enzyme in this step. The purified protein displays only one band on an SDS-PAGE gel.
Figure 5.8 SDS-PAGE of pooled active fractions during the purification of the B component of IBA N-hydroxylase. (Protein bands visualized by silver stain.)

Lane 1 and 8, molecular weight markers: bovine serum albumin (66,200), hen egg white ovalbumin (42,699), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and hen egg white lysozyme (14,400).

Lane 2, polyethyleneimine precipitation (1 μg).
Lane 3, ammonium sulfate precipitation (1 μg).
Lane 4, phenyl agarose (1.1 μg).
Lane 5, phenyl superose (0.8 μg).
Lane 6, Resource Q (0.6 μg).
Lane 7, Blue 3G-A (0.3 μg).
5.5 Properties of the B component of IBA N-hydroxylase

5.5.1 Molecular weight and isoelectric point

The subunit molecular weight of the B component is estimated to be 40 kDa by the SDS-PAGE gel (Figure 5.9).

The attempt to measure the native molecular weight of the B component on a calibrated FPLC Superose 6 gel filtration column (with 25 mM sodium phosphate buffer, pH 7.0) failed since the position of the protein peak shifted from run to run. A nondenaturing x SDS two-dimensional gel electrophoresis of the purified B component showed several bands that had the same subunit molecular weight at 40 kDa. This suggested that the B component might aggregate under these conditions. A nondenaturing x non-denaturing 2D gel electrophoresis of the B component was then carried out to confirm this hypothesis. The result (Figure 5.10) showed that the band corresponding to MW of 40 kDa on the first nondenaturing gel gave several bands corresponding to MW of 80 kDa or higher in the second nondenaturing gel, while the band corresponding to MW of about 80 kDa on the first nondenaturing gel gave a band corresponding to MW of 80 kDa as well as a band corresponding to MW of about 120 kDa. These results indicated that the B component existed as monomer, dimer, trimer as well as higher molecular weight aggregates and that the distributions among these forms varied from time to time. Interestingly, when 0.5% of Triton x 100 was included in the nondenaturing-PAGE gel, the B component appeared to exist mainly in high aggregated polymers and showed one major band corresponding to MW of about 160 kDa in the gel.
Figure 5.9  Determination of the subunit molecular weight of the B component of IBA N-hydroxylase by SDS-PAGE gel electrophoresis (12% T, 2.7 C%) (Figure 5.7)
Figure 5.10  Two-dimensional gel electrophoresis of the purified B component of iBA N-hydroxylase. (Protein bands visualized by silver stain.)

Lane $M_1$ (the first dimension) and $M_2$ (the second dimension), molecular weight markers: bovine serum albumin (66,000, 132,000).

Lane $B_1$ (the first dimension) and $B_2$ (the second dimension), the purified B component (0.5 µg).
The B component also displayed several bands on an isoelectric focusing gel, therefore the isoelectric point of the B component was not easily measurable.

### 5.5.2 UV absorption spectrum and half-reaction

The B component (44 μM in 50 mM sodium phosphate buffer, pH 7.0) exhibited a weak and featureless absorption in the range between 300 to 800 nm.

No NADH oxidation activity was observed with the B component by monitoring the absorbance decrease at 340 nm of a mixture containing 10 mM IBA, 0.5 mM NADH, 5 μM FAD and the B component. This suggests that the B component might be similar to the coupling protein of p-hydroxyphenylacetate-3-hydroxylase.

### 5.5.3 N-terminal amino acid sequence

About 4 μg of the purified B component (100 pmole) was blotted to a PVDF membrane by western transfer. The protein band corresponding to MW of 40 kDa was cut out and subjected to the N-terminal protein sequencing. The result is showed in Table 5.7.
Table 5.7 The N-terminal amino acid sequence of the B component of IBA N-Hydroxylase

<p>| | | | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Met</td>
<td>2</td>
<td>Arg</td>
<td>3</td>
<td>Ser</td>
<td>4</td>
<td>Leu</td>
<td>5</td>
<td>Asp</td>
<td>6</td>
</tr>
<tr>
<td>11</td>
<td>Cys</td>
<td>12</td>
<td>Glu</td>
<td>13</td>
<td>Arg</td>
<td>14</td>
<td>Leu</td>
<td>15</td>
<td>His</td>
<td>16</td>
</tr>
<tr>
<td>21</td>
<td>Ala</td>
<td>22</td>
<td>Leu</td>
<td>23</td>
<td>Glu</td>
<td>24</td>
<td>Glu</td>
<td>25</td>
<td>Leu</td>
<td>26</td>
</tr>
<tr>
<td>31</td>
<td>His</td>
<td>32</td>
<td>Ala</td>
<td>33</td>
<td>Glu</td>
<td>34</td>
<td>Gly</td>
<td>35</td>
<td>Ser</td>
<td>36</td>
</tr>
<tr>
<td>41</td>
<td>Phe</td>
<td>42</td>
<td>Arg</td>
<td>43</td>
<td>Ala</td>
<td>44</td>
<td>His</td>
<td>45</td>
<td>Gly</td>
<td></td>
</tr>
</tbody>
</table>

No similar sequence was found in a database (SwissProt.) search with the N-terminal protein sequence using the Wisconsin GCG Package, version 8.1-Unix.
5.6 Purification of the A component of IBA N-hydroxylase

As indicated in section 5.3.5, fraction A was separated from fraction B (the B component) by phenyl agarose chromatography. However, the A component was detected not only in the fraction A but also in the wash fraction, and the specific activities of these two fractions were the same. Therefore, in the phenyl agarose chromatography step (section 8.9.4) the A component was obtained without an increase of its specific activity.

It was found that a lot of protein (including the B component) would be precipitated, while the A component stayed in the solution if the final concentration of polyethyleneimine rose from 0.15% to 0.3% in the polyethyleneimine precipitation step. Although the B component was lost by this procedure, this step was utilized for the purification of the A component to replace normal polyethyleneimine precipitation (0.15%), ammonium sulfate precipitation and phenyl agarose chromatography steps, since it could achieve the same specific activity in less time and there was a higher recovery of activity.

A typical purification sequence for the A component is presented in Table 5.8. After the Superose 6 step, the enzyme preparation still displays several bands on an SDS-PAGE gel and it was not possible to identify the A component.
Table 5.8  Purification of the A component of IBA N-hydroxylase$^a$

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein</th>
<th>Activity</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>nmol/min</td>
<td>nmol/min/mg</td>
</tr>
<tr>
<td>Crude extract</td>
<td>305</td>
<td>542</td>
<td>1.8</td>
</tr>
<tr>
<td>Polyethyleneimine</td>
<td>182</td>
<td>944</td>
<td>5.0</td>
</tr>
<tr>
<td>Green A agarose</td>
<td>50</td>
<td>1093</td>
<td>22</td>
</tr>
<tr>
<td>ω-Aminobutyl agarose</td>
<td>17</td>
<td>703</td>
<td>41</td>
</tr>
<tr>
<td>Resource Q (anion exchange)</td>
<td>2.7</td>
<td>282</td>
<td>104</td>
</tr>
<tr>
<td>Superose 6 (gel filtration)</td>
<td>0.58</td>
<td>98</td>
<td>182</td>
</tr>
</tbody>
</table>

$^a$ The purification uses cells from four 100 ml of cultures of S. viridifaciens. The activity assay was conducted in 100 mM sodium phosphate buffer, pH 7.6 and the incubation was for three hours at 30 °C.

Attempts to further purify the A component encountered great difficulties. These included: 1) enzyme instability; 2) in some cases, either the enzyme and most of the other proteins did not bind to the column, or no active fraction could be eluted from the column; 3) in some cases, the enzyme was bound to the column and could be eluted, but the activity was in all fractions and the specific activity did not increase. Methods that were tried are listed in Table 5.9.
Table 5.9  Unsuccessful methods for purification of the A component

<table>
<thead>
<tr>
<th>Method</th>
<th>Change in specific activity</th>
<th>Activity recovery</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>phenyl superose</td>
<td>none</td>
<td>20%</td>
<td>spread in many fractions</td>
</tr>
<tr>
<td>FAD agarose</td>
<td>2-10 fold increase</td>
<td>4 - 8%</td>
<td>the matrices degraded easily, low reproducibility</td>
</tr>
<tr>
<td>FMN agarose</td>
<td>decrease</td>
<td>30%</td>
<td>no binding</td>
</tr>
<tr>
<td>ADP agarose (linked by ribose hydroxyls)</td>
<td>none</td>
<td>60%</td>
<td>no binding</td>
</tr>
<tr>
<td>NAD agarose (linked at N⁶)</td>
<td>decrease</td>
<td>66%</td>
<td>no binding</td>
</tr>
<tr>
<td>NAD agarose (linked at C⁸)</td>
<td>decrease</td>
<td>63%</td>
<td>no binding</td>
</tr>
<tr>
<td>Chelating Superose (charged with Zn²⁺)</td>
<td>decrease</td>
<td>10%</td>
<td>no binding, lost activity</td>
</tr>
<tr>
<td>Hydroxyapatite agarose</td>
<td>decrease</td>
<td>47%</td>
<td>no binding</td>
</tr>
<tr>
<td>Resource S (cation exchange)</td>
<td>decrease</td>
<td>42%</td>
<td>no binding</td>
</tr>
<tr>
<td>Rotofor (preparative IEF)</td>
<td>decrease</td>
<td>0%</td>
<td>completely lost activity</td>
</tr>
<tr>
<td>Mono P (chromatofocusing)</td>
<td>1-2 fold increase</td>
<td>10 - 45%</td>
<td>lost activity, hard to remove polybuffer, low reproducibility</td>
</tr>
<tr>
<td>preparative nondenaturing-PAGE</td>
<td>none or decrease</td>
<td>10%</td>
<td>lost activity</td>
</tr>
</tbody>
</table>

The native molecular weight of the A component was estimated as 35 - 45 kDa from gel filtration and preparative nondenaturing-PAGE. The isoelectric
point of the A component was estimated as about 4.5 from chromatofocusing. Attempts to examine NADH oxidation activity by monitoring the absorbance decrease at 340 nm of a mixture containing 10 mM IBA, 0.5 mM NADH, 5 μM FAD and the partially purified A component failed, since some contaminating proteins also oxidized NADH and gave a very high background.

Purification studies on the A component of IBA hydroxylase suggested that it might be impossible to purify this component to homogeneity through conventional methods. Since genes for antibiotic biosynthesis are generally clustered, it was hoped that cloning of the B component might provide access to the gene coding for A. The A component could then be obtained by overexpression. In this way, the A component might be obtained in homogenous form and studied.

5.7 Characterization of IBA N-hydroxylase

Since the A component has not been fully purified, studies of IBA N-hydroxylase were carried out with the partially purified enzymes.

5.7.1 Kinetic studies

The enzyme might utilize four substrates: NADH, IBA, FAD and molecular oxygen. The requirement of molecular oxygen by the enzyme has not been examined since some contaminating protein might utilize molecular oxygen as well. The $K_M$ values for NADH and IBA were determined. Since addition of FAD is required for activity, the effect of FAD on the enzyme reaction was examined. The oxygen concentration in the assay mixture was assumed to be constant during the incubation. The concentration of oxygen in an air-
saturated aqueous solution was reported to be about 0.25 mM at 27°C (Hickman, 1978).

A. $K_M$ for NADH

Preliminary studies suggested that the $K_M$ values for NADH and IBA were lower than 0.1 mM. The concentration of IBA was fixed at 10 mM to maintain a saturating concentration and NADH concentrations were varied between 50 to 500 μM. Concentrations of product (IBHA) of about 6 to 12 μM were reached at the end of the incubations. The HPLC assay was used to quantify IBHA and a Hanes-Woolf plot, $[S]/v_o$ vs. [S], was used to analyze the data. From the Hanes-Woolf equation (8):

$$\frac{[S]}{v_o} = \frac{1}{V_{max}} [S] + \frac{K_M}{V_{max}} \quad (8)$$

the slope is $1/V_{max}$, and the intercept on the $[S]/v_o$ axis gives $K_M/V_{max}$. Thus

$$K_M = \frac{\text{intercept on } [S]/v_o \text{ axis}}{\text{slope}} = \text{intercept on } [S] \text{ axis} \quad (9)$$

Plot A of Figure 5.11 is a plot of the initial velocity $v_o$ versus the NADH concentration. The data were then replotted as a Hanes-Woolf plot (Plot B of Figure 5.11). The apparent $K_M$ for NADH under these conditions is 68.4 μM. No substrate inhibition was observed at NADH concentration up to 2 mM.
Figure 5.11  Kinetic constants for NADH, determined with the HPLC assay
(Incubation was for one hour at 30°C in 100 mM sodium phosphate buffer, pH 7.6, with protein concentration of 0.18 µg/µl. The data represent one determination.)
Plot A: $v_0$ vs. [NADH]
Plot B: Hanes-Woolf plot of $[\text{NADH}]/v_0$ vs. [NADH]
B. $K_M$ for IBA

In this study, the concentration of NADH was fixed at 2 mM ($29 \times K_M$) to maintain a saturating concentration. The HPLC assay was used to quantify IBHA production. Since the detection limit of the HPLC assay is 5 μM, IBA concentrations between 20 to 200 μM were used and product (IBHA) concentrations of about 8 to 12 μM were reached at the end of incubations. The extent of IBA conversion in these reactions reached 40%, and so the average velocity ($v$) can no longer be used to represent the true initial velocity ($v_0$). Therefore, a integrated form of the Michaelis-Menten equation (10) (Segel, 1975) was used. This equation is valid over the entire course of the reaction as long as the decrease in velocity with time results only from decreasing saturation of the enzyme.

$$V_{\text{max}} t = K_M \ln \left( \frac{[S]_0}{[S]} \right) + ([S]_0 - [S])$$

(10)

where $[S]_0$ is initial concentration of substrate and $[S]$ is concentration of substrate by time $t$. If the concentration of product produced by time $t$ is $[P]$, then

$$[P] = [S]_0 - [S]$$

(11)

and the average velocity

$$v = \frac{[P]}{t}$$

(12)

Thus (10) becomes

$$\frac{V_{\text{max}}}{v} = \frac{K_M \ln \left( \frac{[S]_0}{[S]} \right)}{[P]} + 1$$

(13)
A new term $[S]_a$ is then defined as in equation (14)

$$[S]_a = \frac{[P]}{\ln \frac{[S]_o}{[S]}}$$  \hspace{1cm} (14)

Then (13) can be rearranged to give a Michaelis-Menten like equation:

$$v = \frac{V_{max} [S]_a}{K_M + [S]_a}$$  \hspace{1cm} (15)

where $[S]_a$ may be considered as the "average" substrate concentration corresponding to the average velocity ($v$).

A Hanes-Woolf like equation can also be obtained:

$$\frac{[S]_a}{v} = \frac{1}{V_{max}} [S]_a + \frac{K_M}{V_{max}}$$  \hspace{1cm} (16)

In this study, IBHA is product, P, and IBA] is substrate, S. Then $\{IBA\}_a$, that is $[S]_a$, could be obtained from the initial IBA concentration, $[IBA]_o$, and $[IBHA]$ with equations (11) and (14). Figure 5.12 shows a plot of $v$ vs. $[IBHA]_a$ (Plot A) and a plot of $[IBHA]_a/v$ vs. $[IBHA]_a$ (Plot B). The apparent $K_M$ for IBA under these conditions is 6.3 μM. No substrate inhibition was observed at IBA concentrations up to 20 mM in a separate experiment.
Figure 5.12 Kinetic constants for IBA, determined with the HPLC assay
(Incubation was for one hour at 30°C in 100 mM sodium phosphate buffer, pH 7.6, with protein concentration of 0.18 μg/μl. The data represent one determination.)

Plot A: V vs. [IBA]_a

Plot B: Hanes-Woolf plot of [IBA]_a/V vs. [IBA]_a
C. Effects of FAD

The studies were carried out with 10 mM of IBA (158 x $K_M$) and 2 mM of NADH (29 x $K_M$). FAD concentrations between 2.5 to 25 µM were used and the spectrophotometric assay was employed. Figure 5.13 shows a plot of $v_o$ vs. [FAD] (plot A) and a double reciprocal plots of $1/v_o$ vs. $1/[FAD]$ (Plot B). The apparent $K_M$ for FAD is 8.0 µM. FAD exhibited substrate inhibition at FAD concentration higher than 15 µM under these conditions. In another experiment (Figure 5.14), the apparent $K_M$ for FAD is 8.6 µM, while FAD exhibited substrate inhibition at FAD concentration higher than 5 µM. These results suggested that the FAD concentration at which FAD inhibition began to be obvious was dependent on enzyme concentration or on the ratio of A to B. The average of apparent $K_M$ values for FAD from these two experiments was 8.3 µM.
Figure 5.13  Kinetic constants for FAD (I)

(Incubation was for one hour at 30°C in 100 mM Bis-Tris•HCl buffer, pH 7.5, with protein concentration of 0.35 μg/μl.

The data represent one determination).

Plot A: $v_o$ vs. [FAD]

Plot B: a double reciprocal plot of $1/v_o$ vs. $1/[FAD]$

$v_{max} = 1/0.2217 = 4.51 \mu\text{M} / \text{min}$

$K_M = 1.7774/0.2217 = 8.0 \mu\text{M}$
Figure 5.14 Kinetic constants for FAD (II)

(In incubation was for one hour at 30°C in 100 mM Bis-Tris-HCl buffer, pH 7.5, with protein concentration of 1.65 µg/µl.

The data represent one determination.)

Plot A: $v_0$ vs. [FAD]

Plot B: a double reciprocal plot of $1/v_0$ vs. $1/[FAD]$

Dashed lines represent the extrapolated fit of data points for [FAD] less than 5 µM.

$V_{max} = 1/0.044776 = 22.3 \mu M/min$

$K_M = 0.38646/0.044776 = 8.6 \mu M$
5.7.2 Effect of pH

Figure 5.15 shows a plot of relative activity versus pH for IBA N-hydroxylase with different buffers. Surprisingly, it appears to give two curves which divide the buffers into two groups. The first group includes only sodium phosphate buffer in which enzyme activity is lower than that in the other buffers (Na•MES, Bis-Tris•HCl, Na•TES and Tris•HCl) at the same pH, while the other buffers are in the second group. IBA N-hydroxylase activity reaches a maximum at about pH 7.5 in both buffer groups. The optimal pH for IBA N-hydroxylase is similar to that for lysine N⁶-hydroxylase (pH 8.0). Bis-Tris•HCl buffer, pH 7.5 was chosen as the assay buffer since the pKₐ of Bis-Tris is independent of temperature (Stoll, 1990).

The low enzyme activity in sodium phosphate buffer seems due to the presence of phosphate ion, since Na•MES and Na•TES buffers also contain sodium ion.
Figure 5.15  pH-activity profile for IBA N-hydroxylase.

(Incubation was for one hour at 30°C in 100 mM buffer, with protein concentration of 1.65 μg/μl. The relative activities represent two similar determinations).
5.7.3 Effect of temperature

Most chemical reactions proceed at a faster velocity as the reaction temperature is raised. On the other hand, high temperature might disturb the enzyme structure and denature the enzyme. Therefore, as the reaction temperature increases, enzyme activity will reach a maximum, then decrease. The optimum temperature would depend on reaction time, enzyme thermostability, and the activation energy of the enzyme reaction. Figure 5.16 and Figure 5.17 display plots of IBA N-hydroxylase activity vs. temperature in 100 mM sodium phosphate buffer (pH 7.6) and 100 mM Bis-Tris•HCl buffer (pH 7.5), respectively. The optimum temperature for one hour reaction in sodium phosphate buffer is about 40°C, while that in Bis-Tris•HCl buffer is 30°C. These results suggest that IBA N-hydroxylase has better thermostability in sodium phosphate than in Bis-Tris•HCl buffer.
Figure 5.16  Temperature-activity profile of IBA N-hydroxylase in 100 mM sodium phosphate buffer, pH 7.6 (1 hour incubation, with protein concentration of 2.0 μg/μl).

The profile represents two similar determinations.
Figure 5.17  Temperature-activity profile of IBA N-hydroxylase in 100 mM Bis-Tris•HCl buffer, pH 7.5 (1 hour incubation, with protein concentration of 1.65 µg/µl).

The profile represents two determinations.
**5.7.4 Effect of monovalent cations**

To examine the effect of monovalent cations, the enzyme was desalted and buffer exchanged using a PD-10 column (G-25M) equilibrated with 20 mM Bis-Tris•HCl buffer, pH 7.0, plus 15% (v/v) glycerol. The assay was performed in 100 mM Bis-Tris•HCl buffer pH 7.5 with NADH as its Tris salt and FAD in the form of the free acid. Mمونvalent cations were introduced as their chloride salts. When 100 mM of a monovalent cation (Li⁺, Na⁺, K⁺, Rb⁺, Cs⁺ or NH₄⁺) was included in the assay mixture (Test I) (Table 5.9), the enzyme exhibited higher activity relative to the control (assay number 1). The activity increase with monovalent cations became more apparent as the incubation was prolonged.

<table>
<thead>
<tr>
<th>No.</th>
<th>Salt that was added to the assay mixture</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>Bis-Tris•HCl (pH 7.5)</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>LiCl</td>
<td>1.4</td>
</tr>
<tr>
<td>4</td>
<td>NaCl</td>
<td>1.2</td>
</tr>
<tr>
<td>5</td>
<td>KCl</td>
<td>1.3</td>
</tr>
<tr>
<td>6</td>
<td>RbCl</td>
<td>1.3</td>
</tr>
<tr>
<td>7</td>
<td>CsCl</td>
<td>1.2</td>
</tr>
<tr>
<td>8</td>
<td>NH₄Cl (pH 7.5)</td>
<td>1.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>30 min. incubation</th>
<th>60 min. incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>2.1</td>
<td>1.8</td>
</tr>
<tr>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>1.9</td>
<td>1.9</td>
</tr>
</tbody>
</table>

**Table 5.10 Monovalent cations test (I)**

a Assay was carried out at 30°C in 100 mM Bis-Tris•HCl buffer pH7.5.

b The final concentration of Bis-Tris•HCl is 200 mM in this assay.
from 30 minutes to 60 minutes. When additional 100 mM Bis-Tris•HCl (pH 7.5) was added to the assay mixture to reach a final concentration of 200 mM of Bis-Tris•HCl (assay number 2), the enzyme gave the same activity as the control. This suggests that the effect of monovalent cations is not due to a change of ionic strength. The results of these experiments suggest that the role of the monovalent cations here is to stabilize the enzyme.

Test (II) was carried out to examine this hypothesis (Table 5.10). The monovalent cation (50 mM final concentration) was added to the enzyme solution in 20 mM Bis-Tris•HCl buffer, pH 7.0, plus 15% (v/v) glycerol. After pre-incubation at 4°C for 16 hours, the resulting mixture was then assayed for enzyme activity. Since a monovalent cation in enzyme solution was present in the assay mixture and this might cause a difference in the assay, LiCl (100 mM) was included in the assay mixture to overcome the effect of the monovalent cation on the assay and ensure that enzyme activity differences among assays were only due to the effects of monovalent cations on the enzyme during the pre-incubation. The control (entry 1) lost most of the activity, and addition of more Bis-Tris•HCl (entry 2) did not help. In contrast, enzyme pre-incubated with 50 mM of a monovalent cation retained most of its activity. Clearly, the enzyme is stabilized by monovalent cations.
Table 5.11  
Monovalent cations test (II)\textsuperscript{a}

<table>
<thead>
<tr>
<th>Entry No.</th>
<th>Salt that enzyme was pre-incubated with\textsuperscript{b} (50 mM final concentration)</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>0.17</td>
</tr>
<tr>
<td>2</td>
<td>Bis-Tris$\cdot$HCl (pH 7.5)$\textsuperscript{c}$</td>
<td>0.17</td>
</tr>
<tr>
<td>3</td>
<td>LiCl</td>
<td>0.97</td>
</tr>
<tr>
<td>4</td>
<td>NaCl</td>
<td>0.95</td>
</tr>
<tr>
<td>5</td>
<td>KCl</td>
<td>1.0</td>
</tr>
<tr>
<td>6</td>
<td>RbCl</td>
<td>0.95</td>
</tr>
<tr>
<td>7</td>
<td>CsCl</td>
<td>0.96</td>
</tr>
<tr>
<td>8</td>
<td>NH$_4$Cl (pH 7.5)</td>
<td>0.96</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Assay was carried out at 30\textdegree C for one hour in 100 mM Bis-Tris$\cdot$HCl buffer, pH 7.5 plus 100 mM LiCl.

\textsuperscript{b} Enzyme (0.33 mg per assay) in 20 mM Bis-Tris$\cdot$HCl buffer, pH 7.5, containing 15% (v/v) glycerol and monovalent cation salt was pre-incubated at 4\textdegree C for 16 hours before assay.

\textsuperscript{c} Final concentration of Bis-Tris$\cdot$HCl in the enzyme solution is 70 mM.

In Test (III) (Table 5.11), monovalent cations (50 mM final concentration) were added to an inactive enzyme solution that had been incubated in 20 mM Bis-Tris$\cdot$HCl buffer, pH 7.0, plus 15% (v/v) glycerol. The mixture was pre-incubated at 4\textdegree C for about 20 hours and then subjected to activity assay. The results of these experiments suggest that the addition of monovalent cations can restore activity to the enzyme.
Table 5.12  Monovalent cations test (III)\(^{a}\)

<table>
<thead>
<tr>
<th>Entry No.</th>
<th>Salt that enzyme was pre-incubated with(^{b}) (50 mM final concentration)</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>0.11</td>
</tr>
<tr>
<td>2</td>
<td>Bis-Tris•HCl (pH 7.5)(^{c})</td>
<td>0.10</td>
</tr>
<tr>
<td>3</td>
<td>LiCl</td>
<td>0.98</td>
</tr>
<tr>
<td>4</td>
<td>NaCl</td>
<td>0.95</td>
</tr>
<tr>
<td>5</td>
<td>KCl</td>
<td>1.0</td>
</tr>
<tr>
<td>6</td>
<td>RbCl</td>
<td>1.0</td>
</tr>
<tr>
<td>7</td>
<td>CsCl</td>
<td>0.99</td>
</tr>
<tr>
<td>8</td>
<td>NH(_4)Cl (pH 7.5)</td>
<td>0.94</td>
</tr>
</tbody>
</table>

\(^{a}\) Assay was carried out at 30\(^{\circ}\)C for one hour in 100 mM Bis-Tris•HCl buffer, pH7.5, containing 100 mM LiCl.

\(^{b}\) Inactive enzyme (0.33 mg per assay) in 20 mM Bis-Tris•HCl buffer, pH 7.5, containing 15% (v/v) glycerol and monovalent cation salt was pre-incubated at 4\(^{\circ}\)C for 20 hours before assay.

\(^{c}\) Final concentration of Bis-Tris•HCl in the enzyme solution is 70 mM.

These results suggest that IBA N-hydroxylase requires a monovalent cation (Li\(^{+}\), Na\(^{+}\), K\(^{+}\), Rb\(^{+}\), Cs\(^{+}\) or NH\(_4\)\(^{+}\)) to stabilize its structure. Without a monovalent cation, the enzyme may gradually lose its activity. This might result from a disruption of the tertiary structure. The monovalent cation could possibly help the denatured enzyme reassume its active structure.
5.7.5 Effects of amino acid modification

Four amino acid modification agents were used in these studies. DTNB, pHMB and IAM are known to modify sulphydryl groups specifically (Voet, 1990) (Scheme 5.11). DEPC has been shown to react specifically with histidyl residues in proteins under neutral conditions (Miles, 1977) (Scheme 5.12). A variety of nucleophilic residues in proteins, including sulphydryl, tyrosyl and lysyl groups, also react with DEPC at a much lower rate than histidyl residues.

All modification reactions were performed in Buffer B (pH 7.0). In Test (I) (Table 5.12), the A and B components and the substrate were mixed before the modification reaction. The modified enzyme solution was assayed without removal of the modification reagent.

Under these conditions, both IAM and pHMB inhibit the enzyme and no substrate protection was observed. This suggests that cysteine and/or methionine residues are required for activity and that these residues might not be at the substrate binding sites. No inhibition by DTNB was observed, which might be ascribed to the greater steric bulk of this reagent.

DEPC also inhibits the enzyme activity. IBA, FAD and NADH showed some degree of protection against DEPC modification. These results suggest that histidine residues are required for enzyme activity and that some of these histidine residues might be present at the substrate binding sites.
Scheme 5.11 Modification of sulfhydryl groups
Scheme 5.12 Modification of histidyl groups by DEPC
Table 5.13  Effect of amino acid modification on enzyme activity (I)\textsuperscript{a}

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>pHMB</th>
<th>DTNB</th>
<th>IAM</th>
<th>DEPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final concentration (mM)</td>
<td>0</td>
<td>0.91</td>
<td>0.91</td>
<td>22.7</td>
<td>1.7</td>
</tr>
<tr>
<td>Treatment time</td>
<td>—</td>
<td>15 min</td>
<td>15 min</td>
<td>16 hr</td>
<td>15 min</td>
</tr>
<tr>
<td>Residual activity (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Without protection: 100 2 100 18 11
- Protected with 0.1 mM IBA: — 1 — 22 23
- Protected with 5 \mu M FAD: — 2 — 18 33
- Protected with 0.5 mM NADH: — 4 — 15 42

\textsuperscript{a} The modification reaction was performed in the presence of both A and B components. The data represent two similar determinations.
Since IBA N-hydroxylase consists of two components, Test (II) was carried out to examine the effect of amino acid modification on individual components (Table 5.13). The A (or B) component was treated with the modification reagent alone. After the treatment, the reagent was removed by dialysis and the treated enzyme solution was assayed. The results show that the A component is less liable than B to attack by pHMB, while the B component is less liable than A to attacks by DTNB and DEPC.

Table 5.14 Effect of amino acid modification on enzyme activity (II)\textsuperscript{a}

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>pHMB</th>
<th>IAM</th>
<th>DEPC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Final concentration (mM)</strong></td>
<td>0</td>
<td>0.91</td>
<td>22.7</td>
<td>1.7</td>
</tr>
<tr>
<td><strong>Treatment time</strong></td>
<td>—</td>
<td>15 min</td>
<td>16 hr</td>
<td>15 min</td>
</tr>
<tr>
<td><strong>Residual activity (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>modifying A component</td>
<td>1.0</td>
<td>25</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>modifying B component</td>
<td>1.0</td>
<td>3</td>
<td>73</td>
<td>44</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The modification reaction was performed with only one component. The data represent one determination.
5.8 Summary

Isobutylamine N-hydroxylase was identified in the cell-free extracts of *S. viridifaciens* MG456-hF10. The enzyme appears to catalyze a reaction in valanimycin biosynthesis, and to be an external flavoprotein monooxygenase since it requires NADH and FAD for activity. The apparent $K_M$ values of NADH, IBA and FAD are 63.4 μM, 6.3 μM and 8.3 μM, respectively.

The optimal pH for enzyme activity is 7.5 and the optimal temperature for activity is 30°C in 100 mM Bis-Tris•HCl buffer, pH 7.5. Monovalent cations stabilize the enzyme. Cysteine (or methionine) and histidine residues are required for the activity.

The enzyme consists of two protein components, both of which are required for the hydroxylation activity. The A component was purified 101 fold. The B component was purified to homogeneity. The subunit molecular weight of B is 40 kDa. The native molecular weight and isoelectric point of B could not be determined since B aggregated under the experimental conditions required to obtain these measurements. No NADH oxidation activity was observed with B.

The N-terminal amino acid sequence of B was obtained. This sequence should allow the design of oligonucleotide probes to search for the DNA that encodes the B component of IBA N-hydroxylase. This could, in turn, provide access to the cluster of genes that encode the entire valanimycin biosynthetic pathway.
Chapter Six
Construction and Probing of a Gene Library from
*Streptomyces viridifaciens* MG456-hF10

6.1 Introduction

An important reason to purify isobutylamine N-hydroxylase is to obtain amino acid sequence information for the enzyme in order to clone the gene encoding the enzyme. This chapter describes attempts to find the gene encoding the B component of IBA hydroxylase. The strategy used was to screen a library of *S. viridifaciens* MG456-hF10 genomic DNA with a probe derived from the N-terminal amino acid sequence of B and to sequence the DNA fragments that strongly hybridize with this probe.

6.2 Construction of a library of *S. viridifaciens* MG456-hF10 genomic DNA with bacteriophage $\lambda$

Use of a bacteriophage $\lambda$ library has several advantages over a plasmid library: 1) Lambda can be used to clone longer DNA inserts (9 kb - 23 kb), which would reduce the effort needed to obtain a given gene segment from the bacteria genome. 2) Lambda offers a greater cloning efficiency and a large amount of DNA insert can be purified easily. 3) Lambda vectors provide greater ease in library screening, amplification and storage. 4) Primary genomic libraries constructed in lambda vectors are generally more complete and can be used with different probes. Therefore, Lambda DASH II phage vector (from Stratagene) was chosen to construct the library. This engineered form of lambda contains 41.9 kb of DNA. The central third of the phage DNA is not required for phage infection and can be replaced by foreign DNA.
The Lambda DASH II / BamH I vector kit obtained from Stratagene contains the left arm (20 kb) and right arm (9.1 kb) of Lambda DASH II (cut at BamH I sites), with the central part (12.8 kb) removed. The DNA insert can be ligated to the left and right arms and packaged into phage heads. The recombinant DNA is packaged into λ phage heads only if its final length is between 75% and 105% of the 48.5 kb wild type lambda genome (Voet, 1990). Thus, a DNA insert with a length of about 9 kb to 18 kb could be cloned into the phage. There is an inherent positive selection for recombinant phages, since ligation of the left and right arms to each other will not produce phage as the overall length of the DNA is too small.

The DNA genome of S. viridifaciens MG456-hF10 was fragmented by partial digestion using the restriction enzyme Sau3A I, a frequent cutter which would yield cohesive ends (5'-GATC-3') compatible with the BamH I ends (3'-CCTAG-5') of the lambda vector arms. The digested DNA was then size-fractionated by sucrose gradient centrifugation. The fraction containing DNA fragments with 9 - 20 kb length was ligated to the left and right arms of Lambda DASH II. Packaging the recombinant phage DNA into phage heads yielded an lambda phage library of S. viridifaciens MG456-hF10 genomic DNA, which was labeled as λWLα. The concentration of the lambda phage library λWLα was about 2x10^6 plaque-forming units per milliliter (pfu/ml).
6.3 The screening of the lambda phage library of *S. viridifaciens* MG456-hF10

6.3.1 DNA probe for the screening

To find the gene encoding the B component of IBA N-hydroxylase, a probe derived from the N-terminal amino acid sequence of B (Table 5.7) was used to screen the genomic library. Using the standard genetic code (Voet, 1990) and the fact that *Streptomyces* DNA is high in G and C, a degenerate oligonucleotide probe WLA (35 mer) was designed from a region of the N-terminal amino acid sequence of B (Asp-5 to Pro-16) (Table 6.1).

**Table 6.1 DNA probe derived from the N-terminal amino acid sequence of B**

<table>
<thead>
<tr>
<th>AA sequence</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
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<tr>
<td>Possible</td>
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<td>GCC</td>
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<td>GAC</td>
<td>ACC</td>
<td>TGC</td>
<td>GAG</td>
<td>CGC</td>
<td>CTG</td>
<td>CAC</td>
</tr>
<tr>
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<td>GCC</td>
<td>GCC</td>
<td>CGC</td>
<td>CGC</td>
<td>GAT</td>
<td>ACG</td>
<td>TGT</td>
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<td>CGG</td>
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<tr>
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<td>GCT</td>
<td>CGT</td>
<td>ACG</td>
<td>TGT</td>
<td>GAA</td>
<td>CGG</td>
<td>CTT</td>
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<td>CGA</td>
<td>CTA</td>
<td>CCA</td>
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<td>GCA</td>
<td>CGA</td>
<td>ACT</td>
<td></td>
<td>AGG</td>
<td></td>
<td>AGG</td>
<td></td>
<td>AGA</td>
<td>TTA</td>
<td></td>
</tr>
</tbody>
</table>

**WLA 5’- GAC GCS GCS MGS GAC ACS TGC GAG MGS YTS CAC CC**

(where S = G or C; M = A or C; Y = C or T)

The probe WLA was synthesized by Integrated DNA Technologies, Inc. as a single stranded DNA without the 5'-phosphate group, and was then radioactively labeled by introducing $[^32P]$-phosphate group into the 5' end of the probe with $[^32P]$-ATP.
6.3.2 Colony hybridization with WLA

The library λWLα was used to infect the host cells (E. coli XL1-Blue MRA) and plated on 100-mm NZY plates at about 2000 pfu/plate. The phage plaques were then transfer to Hybond-N nylon membranes. The phage DNA was released and denatured with NaOH treatment, and was then fixed to the membranes with UV light. The membranes were preincubated in 6 X SSC containing Denhardt's reagent at the hybridization temperature for 2 hours, before hybridization. The Denhardt's reagent contains Ficoll 400, polyvinylpyrrolidone, and bovine serum albumin which would block nonspecific binding of the probe to the membrane surface. Hybridization was started with the addition of the radioactive probe. During the hybridization, the plaques which contain the sought-after genes would bind to the radioactive probe and give a positive signal when the membrane was exposed to X-ray film.

The library λWLα was screened with the radiolabelled probe WLA at a hybridization temperature of 35°C in 6 x SSC. Five strongly hybridizing phage plaques were selected from eight library plates. The corresponded phage colonies were isolated and plated for a secondary screening. Only two of these phage colones, λA3 and λA1, gave positive singals in the secondary screening. Phage DNA was prepared from each of the two hybridizing colonies.

6.3.3 Screening the digested phage DNA with WLA

The DNA isolated from λA3 and λA1 was digested with Sal I, since there is only one Sal I site in each arm of lambda DASH II and the Sal I sites are very
close to the clone sites (BamH I site). The digested DNA was separated using an agarose electrophoresis gel and transferred to a Hybond-N nylon membrane by Southern transfer. The membrane was then probed with the radiolabelled probe WLA at a hybridization temperature of 50°C in 6 x SSC. A 5 kb Sal I fragment from λA3 was found to hybridize strongly to WLA. This fragment, named as Sal-1, was isolated from an agarose gel and was cloned into Sal I site of pBluescript plasmid vector.

The recombinant plasmid, pBSal-1, containing Sal-1 fragment, was amplified in E. coli DH5α cells. The recombinant plasmid pBSal-1 was isolated and digested with three different restriction enzymes, Pst I, Xho I and BamH I, since there is one Pst I site, one Xho I site and one BamH I site in the multiple cloning site of the pBluescript plasmid. The digested pBSal-1 was subjected to agarose gel electrophoresis and transferred to a Hybond-N nylon membrane by Southern transfer. The membrane was then probed with the radiolabelled probe WLA at a hybridization temperature of 50°C in 6 x SSC. The resulting autoradiogram and the gel are shown in Figure 6.1. The pBSal-1 digested with BamH I gave four bands (5 kb, 1.8 kb, 1 kb and 0.5 kb) on the gel. The 5 kb fragment strongly hybridized with the WLA probe. Since the pBluescript plasmid vector is 3 kb in length, this 5 kb BamH I fragment (named pBBam-1) should contain the vector and a 2 kb insert (named Bam-1). The pBSal-1 digested with Pst I and Xho I did not give shorter fragments which hybridized with the WLA probe. The pBSal-1 was also digested with Sal I to provide a control. The vector (3 kb band) did not hybridize with the WLA probe while the insert (Sal-1, 5 kb band) did. The pBBam-1 was then religated at BamH I site to give the circular plasmid form and amplified in E. coli DH5α cells.
Figure 6.1 Screening of pBSal-1 with WLA. Part A: 0.6 % agarose gel; Part B: autoradiogram

Lane 1, DNA marker, Hind III digest of λ DNA:
- 23130 bp, 9416 bp, 6557 bp, 4361 bp, 2322 bp, 2027 bp, and 564 bp.

Lane 2, BamH I digest of pBSal-1.
Lane 3, Xho I digest of pBSal-1.
Lane 4, Pst I digest of pBSal-1.
Lane 5, Sal I digest of pBSal-1.
6.3.4 Analysis of Bam-1 fragment

Using T3 and T7 primers, and a "gene walking" process, the sequence of Bam-1 fragment was obtained from pBBam-1 (Table 6.1). This sequence starts from the SalI end of the insert.

Table 6.2 DNA sequence of Bam-1a

<table>
<thead>
<tr>
<th>Bam-1</th>
<th>Length: 1858</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TCGACGGGCG GATGGGTTG TCCTGGGTG GACGGGTCAG CGAGCGGCTC</td>
</tr>
<tr>
<td>51</td>
<td>GCGACCGGCC TCCGGTTGGC GCCGCGGGAA CTGCCACGAC GGGAGCGGCC</td>
</tr>
<tr>
<td>101</td>
<td>CCAGTGATGC GCACGACGGTA TCACCACTC GGCAAGGACG GTACGCCTTC</td>
</tr>
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<td>151</td>
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<tr>
<td>851</td>
<td>CCTCCCGGGG CGCGANGATCG TCGGCCAGCG CGCCCTCCGAC CNCCCTGCCAC</td>
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Continued on next page
<table>
<thead>
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<th>DNA sequence of Bam-1 (continued)(^a)</th>
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\(^a\) N = G or C or T or A.

Comparison between Bam-1 and probe WLA with the best-fit program in the GCW Wisconsin Package showed that there is no sequence region in Bam-1 identical to WLA in both directions. Some regions of Bam-1 are similar
to WLA (Table 6.3), which might lead to hybridization between Bam-1 and WLA during the screening.

Table 6.3  
Comparison between Bam-1 and WLA

<table>
<thead>
<tr>
<th>WLA x Bam-1 :</th>
</tr>
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<tbody>
<tr>
<td><strong>WLA</strong></td>
</tr>
<tr>
<td>3 CGCSGCSMGSGACACSTGCGAGMGSYTGSCACCC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>WLA x Bam-1 (reverse) :</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WLA</strong></td>
</tr>
<tr>
<td>2 ACGCSGCSMGSGACACSTGC</td>
</tr>
</tbody>
</table>

(S = G or C; M = A or C; Y = C or T; N = A or T or G or C)

Peptide sequences (Bam1.p to Bam6.p) were translated from all six reading frames of the DNA sequence of Bam-1 using the standard codes. These peptide sequences were then compared with the N-terminal amino acid sequence of the B component (N-B) using the best-fit program. No match was found in these comparisons (Table 6.4).
Table 6.4 Comparisons between N-B and peptide sequences of Bam-1

<table>
<thead>
<tr>
<th>N-terminal amino acid sequence of B, N-B, Length: 41</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 MRSLDAARDT CERLHFGLIK ALEELPALER HAEGSPVLDI F 41</td>
</tr>
</tbody>
</table>

1) N-B x Bam1.p (translated from 1 to 1858 of Bam-1)

<table>
<thead>
<tr>
<th>2 RSL......DARDCERLHFGLIKALEELPALE...RHAEGSPVLD 39</th>
</tr>
</thead>
</table>

2) N-B x Bam2.p (translated from 2 to 1858 of Bam-1)

<table>
<thead>
<tr>
<th>3 SLDAARDCERLHFGLIK...ALEELPALER HAEGSPVLDIF 41</th>
</tr>
</thead>
<tbody>
<tr>
<td>346 GRAVRRGRADRFDPALGARGVEGGDRFFLLGGGEAVERARDARGLGDVF 394</td>
</tr>
</tbody>
</table>

3) N-B x Bam3.p (translated from 3 to 1858 of Bam-1)

<table>
<thead>
<tr>
<th>1 MRSLDAARDCERLHFGLIKALEELPALER HAEGSPV 37</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 RMAYVPGGVSEPLATGVRFAPRELPTGERRRVSAV 39</td>
</tr>
</tbody>
</table>

4) N-B x Bam4.p (translated from 1857 to 1 of Bam-1)

| 5 REARPAAGAEFAGPRHGVRGGLTDSAVAERTATTFCARRV 44 |

5) N-B x Bam5.p (translated from 1857 to 1 of Bam-1)

| 482 LRLGSPRSLXXPAQAVTQPYALHFQVRRVHRELRLHRQHQRQXHLGV 532 |

6) N-B x Bam6.p (translated from 1856 to 1 of Bam-1)

| 422 WKAASNQTPGEPAGLXVRNLARLDFGWEDDKDDRAGSDAQMSPL 471 |

..
A database search with the DNA sequence of Bam-1 and the peptide sequences translated from six frames of the DNA sequence did not yield any positive match related to flavoprotein monooxygenases or related proteins. These results suggest that Bam-1 does not encode the B component.

6.4 Conclusion

A genomic library of *S. viridifaciens* MG456-hF10 was constructed using the Lambda DASH II / *BamH* I vector. The library was screened with a probe derived from the N-terminal amino acid sequence of the B component of IBA N-hydroxylase. No gene fragment related to the N-terminal sequence was found. Further screening will be necessary to find the gene encoding the B component of IBA N-hydroxylase.
Chapter Seven
Summary of Work

This work is mainly composed of three parts: isotopically labeled precursor incorporation experiments, enzymatic studies and DNA library screening.

Successful precursor incorporation experiments with $[4^{-13}\text{C}]-\text{N-}$\-(isobutylamino)serine and $[^{15}\text{N}_2]-\text{N-}$\-(isobutylamino)serine provided evidence for the intermediacy of N-\-(isobutylamino)serine in the valanimycin biosynthetic pathway.

Two enzymes related to valanimycin biosynthesis, valine decarboxylase and isobutylamine N-hydroxylase, were found in cell-free extracts of S. \textit{viridifaciens} MG456-hF10. Valine decarboxylase catalyzes a reaction which synthesizes isobutylamine from valine. Isobutylamine N-hydroxylase converts isobutylamine to isobutylhydroxylamine, which is a possible substrate for an unknown N-N bond forming enzyme(s).

Isobutylamine N-hydroxylase appears to be an external flavoprotein monooxygenase since it requires NADH and FAD for activity. The enzyme was found to consist of two protein components. Both components are required for the hydroxylation activity. The A component was partially purified and the B component was purified to homogeneity. The properties of the enzyme were studied, and the N-terminal amino acid sequence of B was obtained.
A DNA library of *S. viridifaciens* MG456-hF10 was constructed using a lambda vector. The library was screened with a DNA probe derived from the N-terminal amino acid sequence of the B component of isobutylamine N-hydroxylase. Although no gene fragment corresponding to the N-terminal sequence was found, further library screening should find the gene encoding the B component.

In order to screen the library more efficiently, multiple probes may be necessary. Therefore, additional amino acid sequence information of the B component should be obtained. Internal amino acid sequencing and amino acid analysis of the B component are in progress.
Chapter Eight
Materials and Methods

8.1 Materials

8.1.1 Chemicals

Electrophoresis reagents were purchased from Bio-Rad Laboratories, Inc. Isobutylhydroxylamine was synthesized by Dr. Yan Li in this laboratory (Parry, 1992). All other chemicals were purchased from Aldrich Chemical Co. and Sigma Chemical Co.

\[^{32}\text{P}]\text{-Adenosine-5\text{'-triphosphate (>5000Ci / mmol) and }[^{35}\text{S}]\text{-}
\text{deoxyadenosine-5\text{'-triphosphate (>1000Ci / mmole) were purchased from ICN Biomedicals, Inc. Other stable and radioactive isotopically labeled compounds were purchased from Cambridge Isotopes Laboratories, Inc.}

Water was purified by a Millipore Milli Q water purification system and was used in all solutions and protocols with the exception of fermentation medium. The water in this case was taken directly from the main water supply.

8.1.2 Bacterial strains, plasmids and viruses

BamH I predigested Lambda DASH II arms and the host strain Escherichia coli XL1-Blue MRA were obtained as a LAMBDA DASH II/BamH I VECTOR KIT from Stratagene Cloning Systems.

pBluescript II SK (+/-) phagemid was obtained from Stratagene. E. coli DH5α component cells were purchased from Life Technologies, Inc.
8.1.3 Enzymes

Restriction endonucleases, T4 DNA ligase and T4 polynucleotide kinase were purchased from Promega. DNase I, RNase A and proteinase K were obtained from Sigma.

8.1.4 Oligonucleotides

Custom oligonucleotides were prepared by Integrated DNA Technologies, Inc.

8.1.5 Filters and membranes

Hybond-N nylon membrane for DNA hybridization was purchased as 82 mm diameter filters or sheet membrane from Amersham Life Science. Trans-Blot PVDF (polyvinylidene fluoride) membrane was purchased from Bio-Rad.

Pressure dialysis membranes, CentriCon concentrators and Microcon-100 concentrators were purchased from Amicon, Inc.

8.1.6 Chromatography media

Gel matrices were purchased from Sigma, Amicon and Prometic BioSciences (USA). Prepacked FPLC columns were purchased from Pharmacia Biotech Inc.
8.1.7 Buffers and solutions

Buffer A
50 mM Sodium phosphate, pH 7.0
1 mM EDTA•Na₂
1 mM Benzamidine•HCl
15% (v/v) Glycerol
1 mM PMSF
0.5 μg / ml E64
1 μg / ml Pepstatin A
1 μg / ml Leupeptin

Buffer B
50 mM Sodium phosphate, pH 7.0
1 mM EDTA•Na₂
1 mM Benzamidine•HCl
15% (v/v) Glycerol

Buffer C
50 mM Sodium phosphate, pH 7.0
1 mM EDTA•Na₂
15% (v/v) Glycerol

S M
50 mM Tris•HCl, pH 7.5
100 mM NaCl
8.1 mM MgSO₄
0.001% (w/v) Gelatin

20 x SSC
3 M NaCl
0.3 M Na₃citrate
pH 7.0

TE
10 mM Tris•HCl, pH 8.0
0.1 mM EDTA•Na₂

CM
10 mM CaCl₂
10 mM MgCl₂

5 x Denhardt's
1% (w/v) Ficoll 400
1% (w/v) Polyvinylpyrrolidone
(Av. Mol. Wt. 360 kDa)
1% (w/v) Bovine serum albumin

TNE
10 mM Tris-HCl, pH 7.5
0.1 mM EDTA•Na₂
0.1 M NaCl
5 x TBE
54 g Tris base
27.5g Boric acid
20 ml 0.5 M EDTA•Na₂ (pH 8.0)
Milli Q water to 1000 ml

50 x TEA
240 g Tris base
57.1 ml glacial acetic acid
100 ml 0.5 M EDTA•Na₂ (pH 8.0)
Milli Q water to 1000 ml

TM
10 mM Tris•HCl, pH 7.5
10 mM MgCl₂

8.1.8 Growth media

Fermentation Medium
0.1% (w/v) MgSO₄•7H₂O
0.3% (w/v) NaCl
2% (w/v) Maltose
0.5% (w/v) Bacto peptone
0.3% (w/v) Yeast extract
0.5% (w/v) Beef extract
1000 ml Tap water
1 N NaOH to pH 7.2

Washed Cell Medium
50 mM Sodium phosphate, pH 7.0
0.1% (w/v) MgSO₄•7H₂O
0.3% (w/v) NaCl
0.1% (w/v) L-Valine
NZY Medium

Difco casein hydrolysate 10 g
Yeast extract 5 g
NaCl 5 g
MgSO₄•7H₂O 2 g
pH (with 1N NaOH) 7.5
Milli Q water to 1000 ml

CHYCM

Difco casein hydrolysate 10 g
Yeast extract 5 g
NaCl 5 g
Casamino acids 1 g
MgSO₄•7H₂O 2 g
Milli Q water to 1000 ml

NZY Medium Plate

Difico agar 15 g
NZY Medium 1000 ml
8.2 General methods

8.2.1 Analysis

Proton nuclear magnetic resonance (NMR) spectra were performed on an IBM AF300 at 300 MHz or a Bruker AC250 at 250 MHz. Chemical shifts in D₂O are referenced with the water signal (4.80 ppm). Carbon-13 NMR spectra were recorded on the same two instruments at 75.45 MHz and 62.89 MHz, respectively. Methanol (49.00 ppm) was used as internal reference when D₂O was the solvent. Nitrogen-15 spectra were performed on either an IBM AF300 at 30.41 MHz or a Bruker AC500 at 50.68 MHz. ¹⁵N-L-Glycine (31.50 ppm) was used as external reference. The following abbreviations were used for spin multiplicity: b = broad, s = singlet, d = doublet, t = triplet, q = quartet, and m = multiplet.

Mass spectra were recorded on Finnigan 3300 and CEC 111021-110B mass spectrometers. Ultraviolet-visible (UV-Vis) spectra were performed on a Hewlett-Packard HP 8452A Diode Array Spectrophotometer. Infrared (IR) spectra were recorded on a Nicolet 205 FT-IR spectrophotometer.

pH was measured with an Orion 611 Research Digital pH/millivolt Meter equipped with a combination electrode.

Melting points were taken on a Fisher-Johns Melting Point Apparatus and were uncorrected.

Analytical TLC was done on glass plates precoated with Whatman Cellulose K2 or Silica Gel K6F. Cellulose plates were visualized with short wavelength ultraviolet light or by spraying with a 2% solution of ninhydrin in
ethanol and heating. Silica gel plates were visualized with short wavelength ultraviolet light or by spraying with a 5% solution of phosphomolybdic acid in ethanol and heating.

High pressure liquid chromatography (HPLC) was performed using a Whatman Partisphere C$_{18}$ reversed-phase column (4.6 mm ID x 235 mm, 5 μm) with a Spectra-physics SP8800 ternary HPLC pump, a Spectra Chrom programmable wavelength detector or a Spectra 100 variable wavelength detector, and a ChromJet integrator or a Rainin Dynamax MacIntegrator.

8.2.2 Separation

Flash column chromatography was performed using Merck silica gel, type 60A, 230-400 mesh as the stationary phase. Cellulose column chromatography utilized Merck Microcrystalline cellulose.

Medium pressure liquid chromatography (MPLC) was carried out on a column (11 mm ID x 40 cm) of Baker reversed-phase octadecylsilane bonded to silica gel (average particle diameter of 40 μm). Solvent was delivered with a Rainin Model B-100-S Eldex pump. The column output was monitored with an ISCO Model UA-5 absorbance-fluorescence detector with an ISCO type 6 optical unit (2 mm flow cell, wavelength 254 nm) and a built-in recorder. Fraction were collected with an ISCO Model 273 fraction collector.

Protein chromatographic separations were performed with a Pharmacia Fast Protein Liquid Chromatography system (FPLC) or a BioRad Econo system. Column elution was monitored at 280 nm.
8.2.3 Sterilization of media

Media and other solution were sterilized by heating at 121°C, 16 psi for 20 minutes in a Barnstead laboratory sterilizer. Antibiotics and other heat labile solutions were sterilized by filtering through a sterile 0.2 μm filter (Millipore). Sterile operations were carried out in a BBL Class II Biohazard Cabinet.

8.3 Methods related to valanimycin production

8.3.1 HPLC assay for valanimycin

A 0.5 ml aliquot of the fermentation broth of *S. viridifaciens* MG456-hF10 was transferred from the flask to a microcentrifuge tube. After centrifugation at maximum speed in an Eppendorf 5415C centrifuge for 3 minutes, the supernatant was filtered through a 0.2 μm pore size filter. A aliquot of the filtrate (5 μl) was injected onto a Whatman Partisphere C18 reversed-phase column (4.6 mm ID x 235 mm, 5 μm) and elution was carried out with a mixture of 15% acetonitrile and 0.05% acetic acid in water at a flow rate of 1 ml / min. The eluation was monitored at 230 nm, and the retention time of valanimycin was 16 min.

8.3.2 HPLC assay for the valanimycin ammonia adduct

A 0.5 ml aliquot of the fermentation broth of *S. viridifaciens* MG456-hF10 was transferred from the flask to a microcentrifuge tube. After centrifugation at maximum speed in an Eppendorf 5415C centrifuge for 3 minutes, 0.2 ml of supernatant was mixed with 0.4 ml of 2 M aqueous ammonium hydroxide solution in a 10 ml flask. The mixture was stirred at room temperature for 1 hour, then evaporated to dryness. Water (0.2 ml) was added to dissolve the residue. A 5 μl aliquot of the solution was then injected onto a Whatman
Partisphere C$_{18}$ reversed-phase column (4.6 mm ID x 235 mm, 5 μm) and elution was carried out with water. The elution was monitored at 254 nm, and the retention time of the valanimycin ammonia adduct was 10.4 min.

8.3.3 Maintenance of *Streptomyces viridifaciens* MG456-hF10

A. Frozen stock

The original *S. viridifaciens* MG456-hF10 strain was obtained from the Institute of Microbial Chemistry, Tokyo, Japan as lyophilized stock. One lyophilized tube was opened and 2 ml of sterile 20% (v/v) glycerol in water was added. A 0.5 ml aliquot of the resulting mixture was added to 100 ml of fermentation medium. After incubation at 30°C, 220 rpm for 48 hours, the fermentation medium was used to inoculate Difco ISP-2 slants (0.1 ml per slant). The slants were incubated at 30°C for 8 days, then 4 ml of sterile 20% (v/v) glycerol in water was added to each slant, the spores were suspended with a sterile pipette and vortexed briefly. The resulting green spore suspension was placed in sterile Falcon polypropylene tubes (12 x 75 mm or 17 x 100 mm) and stored at -70°C, marked as *S. viridifaciens* MG456-hF10 frozen stock. The frozen stock was used to inoculate fermentation medium for precursors administration studies and enzyme purification work.

B. Lyophilized Stock

The spores from three colonies from the strain selection process (see section 8.9.4) were lyophilized for long term storage. A 3.3 ml aliquot of lyophilizing solution (10.0 g of D-glucose and 0.43 g of Difco dehydrated nutrient broth in 33 ml of water) was mixed with a solution of 1 bottle of Difco desiccated horse serum in 10 ml of water and filtered through a sterile filter
A 3 ml aliquot of the filtrate was added to each well-sporulated slant of *S. viridifaciens* MG456-hF10. The spores were suspended with a sterile pipette and vortexed. The spore suspension (3 ml) was added to a sterile ampoule and lyophilized at 100 mtorr for 24 hours. The ampoules were then sealed with an oxygen-natural gas torch while still under vacuum and stored at 4°C. They were marked as *S. viridifaciens* MG456-hF10 lyophilized stock.

### 8.3.4 Strain selection of *Streptomyces viridifaciens* MG456-hF10

Frozen stock (1 ml) (in 20% (v/v) glycerol aqueous solution) was mixed with 2 ml sterile water and filtered through sterile cotton wool with centrifugation at 3000 rpm for 1 minute in a Beckman CS-6R centrifuge. To 2 ml of the filtrate, 2 ml of sterile 20% (v/v) glycerol in water was added and the resulting solution was diluted successively with sterile 20% glycerol / water by factors of ten to a final dilution of 10^{10}.

Each dilution (0.1 ml) was spread on a Difco ISP-2 Bacto yeast malt extract agar in a Petri dish, with a sterile glass rod. The agar plates were incubated at 30°C for about 9 days to allow the bacteria to sporulate. Single colonies were well separated on the agar plates inoculated with 10^3 and 10^4-fold dilutions. A total of 16 single colonies (marked No. 1 to No. 16) were picked from these two plates and each colony was used to inoculate two ISP-2 slants with a sterile loop. After incubation at 30°C for 7 days, one slant derived from each colony was washed with 4 ml of sterile 20% (v/v) glycerol in water to obtain spores for a frozen spore suspension stock, while the duplicate slants were kept at 30°C.
A 0.5 ml aliquot of each frozen stock was used to inoculate 100 ml of fermentation medium contained in a 500 ml Erlenmeyer flask. After incubation at 30°C, 220 rpm for 31 hours, the contents of each flask were sampled and analyzed by HPLC for valanimycin production. Culture number 12 was selected due to its high valanimycin production. The spores on the corresponding duplicate slants were then preserved by lyophilization. This selection process eliminated low or non-producing organisms from stock cultures.

8.3.5 Growth of *Streptomyces viridifaciens* MG456-hF10 for enzyme purification

Fermentation medium (100 ml) in a 500 ml wide-mouth Erlenmeyer flask was inoculated by the addition of 0.5 ml of frozen stock, and then incubated at 30°C, 200 rpm, for 24 hours. The mycelia were harvested by centrifugation at 7000xg for 15 minutes at 4°C. The pellet was used immediately.

8.3.6 Growth of *Streptomyces viridifaciens* MG456-hF10 for precursor administration (washed cells)

Fermentation medium (100 ml) in a 500 ml wide-mouth Erlenmeyer flask was inoculated by the addition of 0.5 ml of frozen stock, and then incubated at 30°C, 200 rpm for 48 hours. A 5 ml portion of this culture was used to inoculated 100 ml of fermentation medium in each of ten 500 ml wide-mouth Erlenmeyer flasks. The cultures were incubated at 30°C, 200 rpm, for 20 hours and the mycelia were then harvested by centrifugation at 7000xg for 15 minutes at 4°C. The mycelial pellets were washed by suspension in 500 ml of
sterile 0.85% aqueous NaCl and centrifuged at 7000xg for 15 minutes at 4°C. This washing was repeated once.

The washed pellets were distributed equally in ten 500 ml wide-mouth Erlenmeyer flasks containing 100 ml of washed cell medium. The aqueous solution of the precursor was then added to each flask through a sterile 0.2 μm filter. The mixture was incubated at 30°C, 200 rpm, for 24 hours. The resulting broth was called the "washed cell broth".

8.3.7 Isolation of the valanimycin ammonia adduct

The "washed cell broth" (1000 ml) (section 8.9.6) was filtered through Celite, the filtrate was adjusted to pH 3.0 and it was then extracted with ethyl acetate (3 x 300 ml). The ethyl acetate extract was evaporated and the residue was stirred with 100 ml of 2 N NH₄OH for 2 hours at room temperature. The orange mixture was concentrated in vacuo to about 4 ml and loaded on a column packing with Baker reversed-phase octadecylsilane packing (10 mm ID x 45 cm) MPLC system. The column was eluted with water at a 2 ml/ min. flow rate and the elution was monitored at 254 nm. The appropriate fractions were examined by HPLC. The fractions containing the valanimycin ammonia adduct were pooled and lyophilized to give 70 mg of pure valanimycin ammonia adduct as a white powder.
NMR characterization of the valanimycin ammonia adduct:

$^1$H NMR (D$_2$O, 250 MHz): δ 1.0 [m, 6H, (CH$_3$)$_2$CH],
2.4 [m, 1H, (CH$_3$)$_2$CHCH$_2$],
3.5 [d, 2H, J = 6 Hz, CH$_2$NH$_2$],
4.2 [d, 2H, J = 7 Hz, CHCH$_2$N(O)N],
4.8 [t, 1H, J = 6 Hz, (O)NNCH].

$^{13}$C NMR (D$_2$O, 75 MHz): δ18.0 [(CH$_3$)$_2$CH], 26.6 [(CH$_3$)$_2$CH],
39.2 [CHCH$_2$NH$_2$], 63.1 [(O)NNCH],
76.0 [CHCH$_2$N(O)N], 171.3 [COOH].

To obtain a $^{13}$C NMR spectrum that could be integrated, 4 mg of Na$_2$[Fe(DTPA)]$\cdot$2H$_2$O per 50 mg of the valanimycin ammonia adduct was added to the NMR tube, and spectrum was recorded with gated decoupling (D1 = 3 seconds) to quench the NOE. Under these condition, the spectrum was as follows:

δ 18.0 [2C, (CH$_3$)$_2$CH], 26.6 [1C, (CH$_3$)$_2$CH], 39.2 [1C, CHCH$_2$NH$_2$],
63.1 [1C, (O)NNCH], 76.0 [1C, CHCH$_2$N(O)N], 171.3 [1C, COOH].

$^{15}$N NMR

To about 50 mg of pure valanimycin ammonia adduct in 0.5 ml D$_2$O in an NMR tube (5 mm ID), 4 mg of Na$_2$[Fe(DTPA)]$\cdot$2H$_2$O was added. The $^{15}$N spectrum was obtained using a Bruker AC500 operating at 50.68 MHz. [$^{15}$N]-L-Glycine (31.50 ppm) was used as external reference. Two peaks were shown in the spectrum: δ 345.8 ppm [CH$_2$N(O)NCH], and δ 348.14 [CH$_2$(O)NNCH].
The peaks were assigned according to $^{15}\text{N}$ NMR spectrum of the valanimycin ammonia adduct isolated from the culture that was fed with $[1-^{13}\text{C},^{15}\text{N}]$-isobutylhydroxylamine (Y. Li, unpublished data). In the spectrum, only one doublet peak at $\delta$ 345.8 ppm was found. Since $^{15}\text{N}$ labeled of isobutylamine would be incorporated into the $\alpha$-nitrogen [$\text{CH}_2\text{N(O)NCH}$] of valanimycin (Scheme 1.7), the peak at $\delta$ 345.8 ppm was due to the $\alpha$-nitrogen [$\text{CH}_2\text{N(O)NCH}$] of the valanimycin ammonia adduct. The $^{15}\text{N}$ chemical shifts of primary aliphatic amines are between 1 to 60 ppm (Levy, 1979). Although there was one primary aliphatic amine group in the valanimycin ammonia adduct, no peak was found between 1 to 100 ppm in the $^{15}\text{N}$ NMR spectrum due to deuterium - proton exchange on the amino group. The peak at $\delta$ 348.14 ppm was due to the $\beta$-nitrogen [$\text{CH}_2\text{N(O)NCH}$] of the valanimycin ammonia adduct.
8.4 Chemical syntheses

8.4.1 O-Benzyl-N-aminoserine (4)

This synthesis was modified from the methods of Wood and Carmi (Wood, 1940; Carmi, 1960). To ethyl 2,3-dibromopropionate (2.6 g, 10 mmol) in an ice bath, a solution of sodium (0.24 g, 10.5 mmol) in 6 ml benzyl alcohol was added slowly with vigorously stirring. At the end of the addition, the ice bath was removed and the mixture was allowed to warm to room temperature. After 3 ml water and 20 ml ether were added, the mixture was acidified with 1 N HCl. The ether layer was separated and the aqueous layer was reextracted with ether (2 x 10 ml). The combined ether extracts were washed with water and dried with Na₂SO₄. The ether was evaporated to give a colorless oil. The oil was cooled in an ice bath, and 2 ml each of water and 5 N NaOH were added. After being stirred at room temperature for 30 minutes, the solution was acidified with 4 ml of 2.5 N H₂SO₄, then the mixture was extracted with ether (3 x 20 ml). The ether extracts were washed with water and dried with Na₂SO₄. The ether was removed by evaporation and the resulting colorless oil (crude 2-bromo-3-benzoxypropionic acid) was added to 10 ml of 50 % (v/v) aqueous hydrazine. The mixture was stirred at room temperature for 2 days, at the end of which time the excess hydrazine was removed in vacuo. The residue was dissolved in 20 ml water and the solution was extracted with ether (2 x 30 ml). The aqueous layer was then evaporated to dryness. Ethanol (20 ml) was added to the resulting white solid and the mixture was filtered. The solid was washed with cold ethanol and ether, then dried in vacuo to give the crude product. The crude product was purified by chromatography on a cellulose column with MeOH / H₂O / pyridine (20 : 5 : 1) and recrystallized from water to give pure 4 (0.88 g, yield: 42 %) as colorless crystals.
Characterization of 4:

mp: 153-155°

TLC (cellulose): MeOH / H₂O / pyridine (20 : 5 : 1), Rᵢ = 0.59.

¹H NMR (D₂O, 250 MHz): δ 7.42 [s, 5H, ArH], 4.80 [s, 2H, OCH₂Ph],
3.94 [dd, 1H, J₁ = 10.9 Hz, J₂ = 4.0 Hz, OCHHC],
3.88 [dd, 1H, J₁ = 10.9 Hz, J₂ = 4.6 Hz, OCHHC],
3.81 [dd, 1H, J₁ = 4.0 Hz, J₂ = 4.6 Hz, CHCO₂H].

¹³C NMR (D₂O, 63 MHz): δ 171.9, 136.3, 127.9, 127.4, 72.2, 66.6, 62.6.

El-MS (70 eV): m/z 210 [M⁺], 179, 165.

8.4.2  N-Aminoserine (5)

Compound 4 (210 mg, 1 mmole) was dissolved in 10 ml water. To this solution, 0.5 ml of 1 N HCl and 21 mg of 10% Pd/C were added. After 7 hours of hydrogenation under 1 atm of H₂ at room temperature, the reaction mixture was filtered. The filtrate was evaporated to dryness and purified by chromatography on a cellulose column with MeOH / H₂O / pyridine (20 : 5 : 1) to give 5 (92 mg, yield: 76%) as colorless crystals.

Characterization of 5:
mp: 130-133 °C, decomp.
TLC (cellulose): MeOH / H₂O / pyridine (20 : 5 : 1), Rᵣ = 0.30.

¹H NMR (D₂O, 250 MHz): δ 3.98 [dd, H, J₁ = 12.2 Hz, J₂ = 3.8 Hz, HOCH₂C₆H₄₁]
3.90 [dd, H, J₁ = 12.2 Hz, J₂ = 5.2 Hz, HOCH₂C₆H₄₁],
3.68 [dd, 1H, J₁ = 3.8 Hz, J₂ = 5.2 Hz, CHCO₂H].

¹³C NMR (D₂O, 63 MHz): δ 172.6, 64.8, 58.8.

HRMS: Calcd: 120.05347. Found: 120.05341.

L-N-Aminoserine has been synthesized by another route (Niederer, 1993), the NMR data (¹H and ¹³C NMR) of L-N-aminoserine are virtually identical to those of compound 5.
8.4.3 Derivatization of N-aminoserine (5) with n-butanol and trifluoroacetic anhydride

The derivatization procedure was modified from the method of Kaiser (Kaiser, 1974): compound 5 (2 mg) was placed in a Pyrex culture tube (13 mm ID x 100 mm). Under N$_2$ purging, the tube was left in a 55 - 60 °C sand bath for 3 minutes, a solution of 3 ml of 3 N HCl in n-butanol was then added. After purging with N$_2$, the tube was sealed with a screw cap and sonicated for 1 minute in an ultrasonic bath. It was then incubated at 100°C for 25 minutes with agitation by vortexing every 5 minutes. After esterification, the solvent was removed by evaporation in a 55 - 60 °C sand bath under N$_2$ purging. Methylene chloride (1 ml) was added and removed. The next stage of the reaction was then carried out by the addition of 0.5 ml CH$_2$Cl$_2$ and 0.4 ml of trifluoroacetic anhydride (TFAA). After purging with N$_2$, the tube was sealed and incubated at 150°C for 6 minutes. The tube was then cooled to room temperature and the solvent was removed by purging with N$_2$ at room temperature. The resulting residue, a yellow syrup consisting of N,N',O-tri-trifluoroacetyl-n-butyl ester of N-aminoserine (5), was subjected to mass spectral analysis.

Characterization of N,N',O-tri-trifluoroacetyl-n-butyl ester of N-aminoserine:
El-MS (20 eV): m/z 465 [(M+H)$^+$], 409, 393, 363.
8.4.4  N-(Isobutylamino)serine (1)

To a solution of compound 5 (70 mg, 0.58 mmole) in 2 ml water and 3 ml methanol, isobutyraldehyde (55 µl, 0.58 mmole) was added and the mixture was stirred for 2 minutes. Then NaBH₃CN (37 mg, 0.58 mmole) was added and pH of the solution was adjusted to about 3 with 1 N HCl. After being stirred for 10 minutes, the reaction mixture was acidified with 3 ml 1 N HCl and the resulting solution was evaporated to dryness. The solid was suspended in 20 ml ethanol and filtered. The filtrate was concentrated and purified by chromatography on a silica gel column with 1-propanol / H₂O (90 : 1) to give 1 (65 mg, yield: 64%) as a colorless film.

Characterization of 1:
TLC (silica gel): 1-propanol / H₂O (90 : 1), Rₚ = 0.29.

¹H NMR (D₂O, 250 MHz): δ 3.84 [m, 2H, NCHCH₂OH],
3.56 [m, 1H, NCHCH₂OH],
2.82 [d, 2H, J = 7.0 Hz, CHCH₂N],
1.93 [m, 1H, CHCH₂N],
0.95 [d, 6H, J = 6.7 Hz, (CH₃)₂CH].

¹³C NMR (D₂O, 63 MHz): δ 175.8, 162.7, 60.7, 55.7, 24.0, 18.6, 18.4.

EI-MS (70 eV): m/z 177 [(M+H)+], 176 [M⁺], 133, 127.

8.4.5  N-(Isobutylamino)serine hydrochloride (8)

To pure 1 in water, excess 0.5 N HCl was added and the solution was evaporated to dryness to give 8.

Characterization of 8:

$^1$H NMR (D$_2$O, 250 MHz): δ 3.97 [d, 2H, J = 4.2 Hz, NCHCH$_2$OH],

3.91 [m, 1H, NCHCH$_2$OH],

3.10 [d, 2H, J = 7.3 Hz, CHCH$_2$N],

2.12 [m, 1H, CHCH$_2$N],

1.01 [d, 6H, J = 6.8 Hz, (CH$_3$)$_2$CH].

$^{13}$C NMR (D$_2$O, 63 MHz): δ 172.4, 60.3, 60.1, 55.9, 23.1, 18.3, 18.2.

8.4.6  [4-$^{13}$C]-N-(Isobutylamino)serine hydrochloride (9)

The isobutynitrile was prepared by the method of Parry (Parry, 1992). [$^{13}$C]-Potassium cyanide (0.43 g, 6.5 mmole) was stirred with 2-bromopropane (670 µl, 7.1 mmole), 18-crown-ether (0.58 g, 2.2 mmole) and 5.2 ml of HMPA at room temperature for 3 days. The reaction mixture was then distilled in vacuo at 40° C. A U shape trap in a liquid nitrogen bath was employed to collect 0.59 g of colorless liquid. From $^1$H NMR analysis, the amount of [1-$^{13}$C]-isobutynitrile was estimated as 0.22 g (3.2 mmole, yield: 49%).

To the crude [1-$^{13}$C]-isobutynitrile, 4.8 ml CH$_2$Cl$_2$ was added. The solution was transferred to a round bottom flask in an water-ice bath, and 4.8 ml of 1N DIBAL (diisobutylaluminum hydride) in CH$_2$Cl$_2$ was added with stirring. After being stirred at 40° C for 1 hour, 10 ml 1 N HCl was added and the
mixture was stirred for 20 min. at room temperature. The CH₂Cl₂ layer (about 9 ml) which contained [1-¹³C]-isobutyraldehyde was separated.

To the CH₂Cl₂ layer, 5 ml of methanol was added, then a solution of compound 5 (350 mg, 2.9 mmole) in 12 ml water and 40 ml methanol was added and the mixture was stirred at room temperature for 2 minutes. Then NaBH₃CN (200 mg, 3.3 mmole) was added and pH of the mixture was adjusted to about 3 with 1N HCl. After being stirred for 10 minutes, the reaction mixture was acidified with 15 ml 1 N HCl and the resulting solution was evaporated to dryness. The resulting solid was suspended in 50 ml ethanol and filtered. The filtrate was concentrated and purified by chromatography on a silica gel column with 1-propanol / H₂O (90 : 1), the fractions containing the product were pooled and 20 ml 0.5 N HCl was added. The resulting solution was evaporated to dryness to give 9 (210 mg, yield: 13% from K¹³CN).

Characterization of 9:

¹H NMR (D₂O, 250 MHz): δ 3.96 [d, 2H, J = 4.2 Hz, NCHCH₂OH],
3.91 [m, 1H, NCHCH₂OH],
3.10 [dd, 2H, JCH = 143.3 Hz, JHH = 7.3 Hz, CH₂N],
2.12 [m, 1H, CHCH₂N],
1.01 [d, 6H, J = 6.8 Hz, CH₃)₂CH].

¹³C NMR (D₂O, 63 MHz): δ 172.4, 60.3, 60.1, 55.9 [enriched],
23.1 [d, JCC = 34.6 Hz], 18.3, 18.2.
8.4.7 \([^{15}N_2]\)-O-Benzyl-N-aminoserine (10)

3 was synthesized from \([^{15}N_2]\)-hydrazine sulfate. \([^{15}N_2]\)-Hydrazine sulfate (1.0 g, 8.2 mmole) was mixed with 4 g of NaOH pellets in a round bottom flask which was connected to a vacuum pump through a glass tube. The flask was placed in a 100°C oven in a way that the glass tube was positioned horizontally in a 1-propanol-dry ice bath. The flask was shaken and the pressure was adjusted to 50 mm of Hg. After a 1 hour reaction, the glass tube was detached from the system, and the liquid was collected and weighed. The amount of \([^{15}N_2]\)-hydrazine was estimated from the refractive index of the distillate. To the \([^{15}N_2]\)-hydrazine solution, 220 µl of 12 N NaOH and 0.7 g crude 2-bromo-3-benzoxylopropionic acid were added. Compound 10 (0.4 g) was obtained in the same manner as described for the preparation of the unlabeled compound 4. The yield was 23% from \([^{15}N_2]\)-hydrazine sulfate.

Characterization of 10:
\(^{13}\)C NMR (D\(_2\)O, 63 MHz): \(\delta\) 171.9, 136.3, 127.9, 127.4, 72.2, 66.6,

\[62.6 \text{ [d, } J_{CN} = 4.8 \text{ Hz}.\]

\(^{15}\)N NMR (10% D\(_2\)O in H\(_2\)O, 30 MHz): \(\delta\) 71.6 \([d, J_{NN} = 4.9 \text{ Hz}\],

\[65.9 \text{ [d, } J_{NN} = 4.9 \text{ Hz}.\]
8.4.8 \( [^{15}\text{N}_2] \)-N-Aminoserine (11)

The procedure was the same as that described for the preparation of 5, except that the labeled compound 10 was used as the starting material. The yield was 64%.

Characterization of 11:
\(^{13}\text{C} \) NMR (D\(_2\)O, 63 MHz): \( \delta \) 172.6, 64.8 [d, J\(_{CN}\) = 3.8 Hz], 58.8.
\(^{15}\text{N} \) NMR (D\(_2\)O-H\(_2\)O(1:9), 30 MHz): \( \delta \) 70.4 [d, J\(_{NN}\) = 4.6 Hz], 66.1 [d, J\(_{NN}\) = 4.6 Hz].

8.4.9 \( [^{15}\text{N}_2] \)-N-(Isobutylamino)serine hydrochloride (12)

The procedure was the same as that described for the preparation of 1 and 8, except that the labeled compound 11 was used as the starting material. The yield was 76%.

Characterization of 12:
\(^1\text{H} \) NMR (D\(_2\)O, 250 MHz): \( \delta \) 3.97 [d, 2H, J = 4.2 Hz, NCHCH\(_2\)OH],
3.91 [m, 1H, NCHCH\(_2\)OH],
3.10 [d, 2H, J = 7.3 Hz, CHCH\(_2\)N],
2.12 [m, 1H, CHCH\(_2\)N],
1.01 [d, 6H, J = 6.8 Hz, (CH\(_3\))\(_2\)CH]
\(^{13}\text{C} \) NMR (D\(_2\)O, 63 MHz): \( \delta \) 172.5, 60.2 [m], 55.9 [dd, \(^1\text{J}_{CN}\) = 4.2 Hz, \(^2\text{J}_{CN}\) = 1.9 Hz], 23.1 [d, J\(_{CN}\) = 0.96 Hz], 18.3 [d, J\(_{CN}\) = 1.3 Hz],
18.2 [d, J\(_{CN}\) = 1.1Hz].
\(^{15}\text{N} \) NMR (10%D\(_2\)O in H\(_2\)O, 30 MHz): \( \delta \) 89.9 [d, J\(_{NN}\) = 5.6 Hz],
74.7 [d, J\(_{NN}\) = 5.6 Hz].
8.5 Protein determination

Protein concentrations were determined with the Bradford assay (Bradford, 1976). The assay solution was prepared as following: 100 mg Coomassie Brilliant Blue G-250 (Sigma) was dissolved in 50 ml of 95% ethanol, 100 ml of 85%(w/v) phosphoric acid was added, the solution then was diluted to 1000 ml with distilled water. Standard curves were obtained with bovine serum albumin (BSA) as the protein standard. Two procedures were used for different concentration ranges.

8.5.1 Standard assay procedure

To 100 µl of dilute protein sample containing 7.5 - 70 µg of protein (use sample diluent as the "blank"), 5 ml of assay solution was added. The solution was mixed well and left at room temperature for 10 minutes. The absorbance at 596 nm was measured against the "blank" and the amount of protein in the sample was determined from a standard curve.

8.5.2 Micro assay procedure

To 1.0 ml of dilute protein sample containing 1 - 7 µg of protein (use sample diluent as the "blank"), 1.0 ml of assay solution was added. The solution was mixed well and the absorbance at 596 nm was measured against a "blank" (no incubation was necessary). The amount of protein in the sample was determined from a standard curve.
8.6 Electrophoresis

All electrophoretic procedures were performed with the Bio-Rad Mini Protein II system, unless otherwise indicated.

8.6.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli (Laemmli, 1970). Using a Tris-glycine discontinuous buffer system, the stacking gel was cast as 4% T, 2.7% C (4% total acrylamide containing 2.7% bis-acrylamide), while the resolving gel was cast as 12% T, 2.7% C. Normally the gels were 0.75 mm thick, and the electrophoresis was performed at 200 V constant voltage at room temperature for about 50 minutes.

8.6.2 Nondenaturing-polyacrylamide gel electrophoresis

Nondenaturing-PAGE (resolving gel: 10% T, 2.7% C; stacking gel: 2.8% T, 2.7% C; gel thick: 0.75 mm) was performed with the Laemmli system without SDS. The electrophoresis was performed at 150 V constant voltage at room temperature for about 80 minutes.

8.6.3 Isoelectric focusing (IEF)

IEF was performed according to the method of Robertson (Robertson, 1987). The 0.75 mm thick gel was cast with 5% T, 2.7% C and 2% ampholytes (pH range 3-10). Isoelectric focusing was performed at 200 V constant voltage for 2 hours at 4°C, then at 400 V constant voltage for 2 hours at 4°C.
8.6.4 Two-dimensional-polyacrylamide gel electrophoresis

Three kinds of two-dimensional (2-D) electrophoresis were employed: IEF X SDS PAGE, nondenaturing PAGE X SDS PAGE and nondenaturing PAGE X nondenaturing PAGE.

The IEF X SDS 2-D gel electrophoresis was performed according to the method of O'Farrell (O'Farrell, 1975, 1977).

The nondenaturing X SDS 2-D gel electrophoresis was performed in a similar manner, except that a gel slice from a nondenaturing electrophoresis gel was used.

The nondenaturing X nondenaturing 2-D gel electrophoresis was performed in a similar manner procedure to the nondenaturing X SDS 2-D gel electrophoresis, except that the gel slice from the first nondenaturing electrophoresis was pre-equilibrated with nondenaturing electrophoresis loading buffer before being placed on the second nondenaturing electrophoresis gel.

8.6.5 Gel staining and drying

A. Coomassie blue staining

After electrophoresis, gels were immersed in staining solution [0.25% (w/v) Coomassie Brilliant Blue R250 in methanol - distilled water - acetic acid (4.5:4.5:1.0)] and gently agitated for 10 minutes. The gel was then destained with destaining solution (staining solution minus Coomassie Brilliant Blue R250) until bands showed clearly.
B. Silver staining

Silver Staining was performed according to Morrissey's method (Morrissey, 1981).

C. Gel drying

After being stained, gels were air dried with the BioDesign GelWrap and drying frame from BioDesign Inc. of New York.

8.6.6 Preparative electrophoresis

Nondenaturing preparative electrophoresis was performed with either the Bio-Rad Mini-Protein II Cell, or the Bio-Rad Model 491 Prep Cell.

A. Buffer system and sample preparation

A discontinuous gel buffer system modified from the MZE 3328.1V buffer system (Jovin, 1973; Moos, 1988) was employed for preparative electrophoresis. The upper buffer was prepared by dissolving 10.07 g of TES free acid and 23.66 g of Bis-Tris base in water to a final volume of 1000 ml (44 mM TES, 113 mM Bis-Tris, pH 7.25 at 25°C). The lower buffer was 63 mM Bis-Tris+HCl (pH 5.9). A 10% acrylamide (containing 2.7% bis-acrylamide) resolving gel was cast with 10% (v/v) glycerol in 123 mM Bis-Tris HCl buffer pH 6.61. A 3.6% acrylamide (containing 2.7% bis-acrylamide) stacking gel was cast with 10% (v/v) glycerol in 123 mM Bis-Tris+HCl buffer, pH 6.61. The gel was pre-run for 30 minutes at 30-40 mA in 0.1 mM thioglycoate, 123 mM Bis-Tris+HCl, pH 6.61 before sample loading. One tenth volume of 0.05% (w/v) bromophenol blue in 50% (v/v) glycerol was added to the protein sample in
Buffer B before loading onto the gel. In the case of the Prep Cell, protein fractions were eluted with 113 mM Bis-Tris HCl containing 10% (v/v) glycerol.

B. Prep Cell 491

The Prep Cell was run according to the manufacturer's instruction. The resolving gel (2.8 mm ID x 10 cm) and stacking gel (28 mm ID x 2 cm) were cast at 4°C 1 day in advance and stored at room temperature. Electrophoresis was performed at 10 W constant power in the cold room (4°C) for a total of about 6 hours. The dye front reached the end of the gel tube in about 3 hours. During the electrophoresis, the elution buffer (113 mM Bis-Tris HCl containing 10% (v/v) glycerol) was pumped through the end of the gel tube at 1 ml/min. flow rate. The fractions eluted were monitored (at 280 nm) and collected with a Bio-Rad Econo System.

C. Mini-Protein II Cell

A 1.5 mm thick gel was used and run at 100V constant voltage at 4°C. After electrophoresis, the gel strips were cut out and the proteins were eluted by electroelution.

D. Electroelution

Electroelution was performed with the Little Blue Tank (ISCO, Inc.). In order to control pH, the optional membrane holders were used. The outer cathode chamber and the outer anode chamber were filled with 200 mM sodium phosphate, pH 7.0. The inner cathode chamber buffer was 50 mM sodium phosphate, pH 7.0, containing 5% (v/v) glycerol. The inner anode chamber buffer was 20 mM Bis-Tris, 56 mM Tricine (pH 7.0 at 25°C) containing 5% (v/v) glycerol. The sample trap buffer was 5% (v/v) glycerol, 5 mM Bis-Tris,
14 mM Tricine (pH 7.0 at 25°C). Electroelution was performed at 150 V constant voltage at 4°C for 10 hours.

8.6.7 Western transfer

Protein samples were heated with an equal volume of 2 x treatment buffer (0.125 mM Tris•HCl pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol) at 40°C for 15 minutes before being loaded onto a 0.75 mm thick SDS gel (resolving gel: 12%T, 2.7%C) which was cast 1 day in advance. The gel was subjected to electrophoresis as usual. Western transfer was performed with a Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell and Bio-Rad’s Trans-Blot PVDF membrane. The transfer of protein from the SDS gel to the PVDF membrane was performed at 50V for 1 hour at 4°C in transfer buffer (10 mM Na•CAPS pH 10.4, 15% (v/v) MeOH, 0.5 mg/ml dithiothreitol). After transfer, the membrane was stained with Coomassie Blue (stained with 0.25% (w/v) Coomassie Brilliant Blue R250, 40% (v/v) methanol in water for 5 minutes, then destained with 50% (v/v) methanol in water). The protein bands of interest were cut out for N-terminal protein sequencing.

The membrane prepared for internal protein sequencing and protein amino acid analysis was stained with Ponceau S (0.5%(w/v) 3-hydroxy-4-[2-sulfo-4-(4-sulfo-phenylazo)phenylazo]-2,7-naphthalenedisulfonic acid, sodium salt and 1%(v/v) acetic acid in water) and destained with water. The protein bands of interest were cut out and placed in a microcentrifuge tube, the membrane pieces were washed with water three times in the tube. After water was drained the tube was sealed and stored at -20°C before sequencing.
8.6.8 Protein sequencing

N-terminal protein sequencing was performed at the Biochemistry Core Facility, Baylor College of Medicine. Internal protein sequencing and protein amino acid analysis were performed at the Harvard Microchemistry Facility.

8.6.9 Submarine agarose gel electrophoresis of DNA

Electrophoresis of DNA was carried out in a horizontal submarine slab gel apparatus. The gel contained between 0.4-1.2% (w/v) agarose and 0.5 μg/ml ethidium bromide in 1 x TEA buffer. A 1/6 volume of gel loading buffer (0.25% (w/v) bromophenol blue, 0.25% (v/v) xylene cyanol FF, 40% (w/v) sucrose in water) was added to DNA samples before being loaded on the gel. Electrophoresis was performed with 1 x TEA buffer at 100 V for about 2 hours. The DNA was visualized on a UV transilluminator (wavelength 366 nm) and photographed with a Polaroid camera using type 667 film.
8.7 Enzyme assays

8.7.1 Assay for valine decarboxylase

This assay was modified from Boeker's method (Boeker, 1983). To a polystyrene tube (17 x 100 mm) equipped with a paper disk containing 50 µl of 2-aminoethanol / 2-methoxyethanol (1 / 1), 170 µl of 10 mM potassium phosphate buffer, pH 7.0, 40 µl of 250 mM L-valine with 1 µCi of [1-14C]-L-valine, 10 µl of cofactor (50 mM PLP), and 280 µl of enzyme solution were added. After incubation for 1 hour at 37°C, 200 µl of 2.5 M H2SO4 was added, then the mixture was incubated for another 30 minutes at 37°C. The paper disk was transferred to a 20 ml scintillation vial. About 18 ml of ECONFLUOR pre-mixed scintillation solution was added and the amount of 14CO2 produced in the reaction was measured with the Beckman LS-100 liquid scintillation counter (2σ%: 1.50). The assay background was made by adding H2SO4 to the assay tube before adding the enzyme sample.

8.7.2 Assay for isobutylamine N-hydroxylase

Assay was carried out in 100 mM of sodium phosphate buffer pH 7.6 in a 1.7 ml microcentrifuge tube. The enzyme assay mixture contained 5 µM FAD, 2 mM NADH, and 10 mM isobutylamine (IBA) and enzyme. The reaction was started by adding 50 µl or less of enzyme solution, containing both the A and B components, to give a final volume of 200 µl. Incubation was for 1 to 3 hours at 30°C. Alternatively, the assay was performed in 100 mM Bis-Tris-HCl buffer pH 7.5, containing 5 µM FAD, 2 mM NADH, 100 mM NaCl and 500 µM IBA. Enzyme activity was calculated according to the amount of isobutylhydroxylamine (IBHA) produced, which was determined as follows.
A. Spectrophotometric assay

This assay was a modification of Belanger’s method (Belanger, 1981). The enzyme assay mixture (200 μl) was mixed with 400 μl of 10% (w/v) trichloroacetic acid at the end of incubation time (1-3 hours), and the suspension was centrifuged at maximum speed with the Eppendorf 5415C centrifuge for 8 minutes. A portion of supernatant (400 μl) was transferred to a glass test tube (12 x 70 mm) and mixed with 1 ml of "Color Reagent " (4M NaOAc buffer pH 5.9 with 240 nM TPTZ (2,4,6-tripyridyl-s-triazine) and 800 nM FeCl₃). After incubation at room temperature for 30 minutes, the absorbance at 594 nm was measured against a control (assay mixture without isobutylamine). An absorption coefficient of $\varepsilon = 2.12$ mM⁻¹ cm⁻¹, which was obtained from an IBHA standard solution, was used to calculate IBHA concentration in the enzyme assay mixture.

B. HPLC assay

A 50% (w/v) aqueous solution of trichloroacetic acid (20 μl) was added to the enzyme assay mixture (200 μl) at the end of incubation time, the suspension was centrifuged at maximum speed with the Eppendorf 5415C centrifuge for 8 minutes and supernatant was transferred to another tube. After incubation at room temperature for 1 hour to allow NADH to decompose, 25 μl of supernatant was mixed with 2.3 μl of 3 N NaOH, 5 μl of 0.5 M sodium phosphate buffer pH 7.0 and 16 μl of a 20 mM methanolic solution of NBD-Cl (4-chloro-7-nitrobenzo-2-oxa-1,3-diazole chloride). The mixture was incubated for 3 minutes in a 60°C water bath, then cooled in an ice-water bath. A 20 μl aliquot of this solution was injected onto a Whatman Partisphere C₁₈ reversed-phase column (4.6 mm ID x 235 mm, 5 μm) and eluted with a methanol, 25 mM
sodium phosphate buffer, pH 7.0 (3 : 7) at a 1 ml / min. flow rate. The elution was monitored at 450 nm, and the retention time of NBD-IBHA was 9.5 min. IBHA concentration in the enzyme assay mixture was calculated from the peak area with a standard curve of peak area vs. IBHA concentration.

C. High resolution mass spectrometry analysis

At the end of incubation time, the enzyme assay mixture was filtered through CentriCon 10 (10,000 MW cutoff) to remove protein. To the deproteinized mixture, an equal volume of 1 N HCl was added. The acidified solution was evaporated to small volume (volume of glycerol in the enzyme assay mixture) and was subjected to high resolution mass spectrometry analysis.

8.8 Preparation of enzyme sample for valine decarboxylase activity assays

8.8.1 Extraction of cells

Cells from 100 ml of fermentation broth of S. viridifaciens MG456-hF10 were washed by suspension in 100 ml of a 0.85% aqueous NaCl solution and centrifuged at 7000xg for 15 minutes at 4°C. This washing was repeated once. The washed mycelia were suspended in 10 ml of 10 mM potassium phosphate buffer, pH 7.0, containing 0.1 mM EDTA-Na$_2$, 0.5 mM dithiothreitol, 20%(v/v) glycerol and 0.5 mM PMSF and were sonicated for 9 x 20 seconds at 70% constant output with a 50% duty cycle. The sonicated mixture was centrifuged at 25,000xg for 1 hour. To the supernatant streptomycin sulfate was added to reach a final concentration of 2.5%, while the pH of mixture was maintained at 7.0 with 1 N HCl. The resulting mixture was centrifuged at 9200xg for 20 minutes to give the supernatant as a crude extract.
8.8.2 Ammonium sulfate fractionation

Solid ammonium sulfate was added to the crude extract at 4°C with constant stirring to 40% saturation (22.6 g / 100 ml) while the pH of the mixture was maintained at 7.0 with 1 N NaOH. The mixture was centrifuged at 9200xg for 20 minutes. To the supernatant solid ammonium sulfate was added to 80% saturation (51.6 g / 100 ml), while the pH of the mixture was maintained at 7.0 with 1 N NaOH. The yellow precipitate obtained by centrifugation at 9200xg for 20 minutes was suspended in 3 ml of 10 mM potassium phosphate buffer, pH 7.0, containing 0.1 mM EDTA•Na$_2$, 0.5 mM dithiothreitol and 20%(v/v) glycerol and dialyzed against 500 ml of the same buffer overnight using 25,000 molecular weight cutoff dialysis tubing. The resulting protein solution (4.5 ml, 7.2 mg protein / ml) was labeled as EVD and was used in the tests for cofactor requirement.
8.9  **Purification of isobutylamine N-hydroxylase**

This enzyme contained two protein components (A and B), and they were separated at the phenyl agarose chromatography step. FPLC chromatography was done at room temperature, all other procedures were carried out at 4°C.

8.9.1  **Extraction of cells**

Cells for enzyme purification, obtained as described in section 8.3.5, were isolated from 400 ml of fermentation broth of *S. viridifaciens* MG456-hF10 and were washed by suspending the mycelia in 200 ml of Buffer A. The cell suspension was centrifuged at 7000xg for 15 minutes at 4°C. This washing was repeated once. The washed cells were then suspended in 100 ml of Buffer A and sonicated 3 x 5 minutes at 70% constant output. After centrifugation at 15,600xg for 1 hour, about 110 ml of supernatant was obtained as crude cell free extract (CFE).

8.9.2  **Polyethyleneimine precipitation**

A solution of 7.5% of polyethyleneimine was added to crude cell free extract with stirring to reach final a concentration of 0.1%, while the pH of this mixture was maintained at 7.0 with 1 N HCl. After stirring for 30 minutes, the precipitate was removed by centrifugation at 15,600xg for 30 minutes to give the polyethyleneimine supernatant (PS).

8.9.3  **Ammonium sulfate precipitation**

Solid ammonium sulfate was added to the polyethyleneimine supernatant with constant stirring to 70% saturation (43.6 g / 100 ml), while the pH of the
mixture was maintained at 7.0 with 1 N NaOH. The mixture was stirred for 40 minutes and then centrifuged at 13,000 rpm for 20 minutes. The yellow precipitate was suspended in 10 ml of Buffer A and dialyzed against 1000 ml of Buffer B overnight using 10000 molecular weight cutoff dialysis tubing. The resulting suspension was centrifuged at 15600xg for 20 minutes to give the ammonium sulfate fraction (ASF) as a clear brown solution.

8.9.4 Separation of component A and B with phenyl agarose chromatography

The ammonium sulfate fraction was mixed with 0.4 volume of saturated cold ammonium sulfate solution (about 4 M) in 50 mM potassium phosphate (pH 7.0) to give a final ammonium sulfate concentration of about 1 M. The mixture was centrifuged at 13,000 rpm for 10 minutes to remove any precipitate. The supernatant was then loaded on a phenyl agarose column (100 ml bed volume, 50 mm ID) equilibrated with 0.25 M (NH₄)₂SO₄ in Buffer B (pH 7.0) and eluted with 300 ml of equilibration buffer. This fraction was concentrated by use of a stirred pressure cell with a PM 30 membrane to about 4 ml and labeled as EB (component B). Component A was eluted with 300 ml of Buffer B and concentrated with a PM 30 membrane to about 5 ml, and marked as EA.

8.9.5 Further purification of the B component
A. Phenyl superose chromatography

EB was mixed with 0.4 volume of saturated cold ammonium sulfate solution (about 4 M) in 50 mM potassium phosphate (pH 7.0) to give a final ammonium sulfate concentration of about 1 M. The mixture was filtered
through a 0.2 μm filter. The filtrate was divided into several portions (about 5 mg protein per portion). Each portion was loaded onto a Phenyl Superose HR 5/5 FPLC column (1 ml bed volume) equilibrated with 1M (NH₄)₂SO₄ in Buffer B (pH 7) and the enzyme was eluted by a (NH₄)₂SO₄ gradient of 1 - 0 M at a 0.4 ml / min. flow rate. The gradient program was as following: 100% A (i.e. 0% B), 10 min.; 0-40% B, 13 min.; 40-60% B, 40 min.; 60% B, 10 min.; 60-100% B, 5 min. (A was 1M (NH₄)₂SO₄ in Buffer B (pH 7) and B was Buffer B). Fractions eluting between 45-60% B were pooled and concentrated by use of a stirred pressure cell with a YM10 membrane. The resulting solution was called BPH.

B. Anion exchange chromatography

BPH was desalted and the buffer was exchanged using a prepacked Pharmacia PD-10 column (Sephadex G-25M) equilibrated with 25 mM sodium phosphate buffer, pH 7.0. The desalted protein solution was loaded onto a FPLC Resource Q column (1 ml bed volume) equilibrated with 25 mM sodium phosphate buffer, pH 7.0. The enzyme was eluted by a (NH₄)₂SO₄ gradient of 0 - 200 mM at a 1 ml / min. flow rate. The gradient program was as following: 100% A (i.e. 0% B), 4 min.; 0-25% B, 6 min.; 25-65% B, 23 min.; 65-100% B, 2 min. (A was 25 mM sodium phosphate buffer, pH 7.0 and B was 25 mM sodium phosphate buffer, pH 7.0, containing 200 mM (NH₄)₂SO₄). A single peak between 55%-60% B was collected and 0.4 volume of 50% glycerol in Buffer C was added. The mixture was then concentrated with a CentriCon-10 concentrator to give fraction BRQ.
C. Blue column chromatography

BRQ was diluted 10 fold with Buffer C and loaded onto a Cibacron Blue 3G-A agarose type 3000-CL-L (Sigma) column (10 ml bed volume, 25 mm ID) equilibrated with Buffer C. After extensively washing the column with Buffer B, the pure enzyme was eluted with 0.5 M (NH₄)₂SO₄ in Buffer C and concentrated by use of a stirred pressure cell with a YM10 membrane to give fraction BB. At this stage, the enzyme preparation displays only one band on an SDS-polyacrylamide gel after silver staining.

8.9.6 Further purification of the A component

The A component could be either purified from EA (section 8.9.4) or from crude cell free extract (CFE) (section 8.9.1). The procedure for purification from CFE was more efficient but the B component would be discarded during the purification. These two routes joined at the green column chromatography step.

A. Polyethyleneimine precipitation

A solution of 7.5% of polyethyleneimine was added to CFE with stirring to reach final a concentration of 0.3%, while the pH of the mixture was maintained at 7.0 with 1 N HCl. After stirring for 30 minutes, the precipitate was removed by centrifugation at 15,600xg for 30 minutes to give the polyethyleneimine supernatant (APS).
B. Green A column chromatography

a) From APS

APS was loaded onto a Amicon Green A agarose column (100 ml bed volume, 50 mm ID) equilibrated with Buffer B and the enzyme was eluted with 300 ml Buffer B. The eluent was concentrated by use of a stirred pressure cell with a YM30 membrane to about 100 ml. To the concentrated eluent, solid ammonium sulfate was added with constant stirring to 70% saturation (43.6 g / 100 ml), while the pH of the mixture was maintained at 7.0 with 1 N NaOH. The mixture was stirred for 40 minutes and centrifuged at 13,000 rpm for 20 minutes. The precipitate was suspended in 10 ml of Buffer B and the solution was desalted using a prepacked Pharmacia PD-10 column (Sephadex G-25M) equilibrated with Buffer B to give fraction AGP.

b) From EA

EA was desalted using a prepacked Pharmacia PD-10 column (Sephadex G-25M) equilibrated with Buffer B. The desalted enzyme solution was loaded onto a Green A agarose column (100 ml bed volume, 50 mm ID) equilibrated with Buffer B and the enzyme was eluted with 300 ml Buffer B. The eluant was concentrated by use of a stirred pressure cell with a YM30 membrane to give fraction AGP.

C. ω-aminobutyl agarose chromatography

AGP was loaded onto an ω-aminobutyl agarose column (100 ml bed volume, 50 mm ID) equilibrated with Buffer B and the enzyme was eluted with 300 ml Buffer B. The eluent was concentrated by use of a stirred pressure cell with a YM30 membrane. The resulting solution was labeled as AWP.
D. Anion exchange chromatography

AWP was desalted and buffer exchanged using a prepacked Pharmacia PD-10 column (Sephadex G-25M) equilibrated with 20 mM Bis-Tris•HCl buffer, pH 7.0, containing 10 μM FAD. The desalted protein solution was loaded to a FPLC Resource Q column (1 ml bed volume) equilibrated with 20 mM Bis-Tris•HCl buffer, pH 7.0, containing 10 μM FAD. The enzyme was eluted by a NaCl gradient of 0 - 500 mM at a 1 ml/min. flow rate. The gradient program was as following: 100% A (i.e. 0% B), 4 min.; 0-20% B, 4 min.; 20-70% B, 20 min.; 70-100% B, 4 min. (A was 20 mM Bis-Tris•HCl buffer, pH 7.0, containing 10 μM FAD, and B was 20 mM Bis-Tris•HCl buffer, pH 7.0, containing 10 μM FAD and 500 mM NaCl). Fractions (2 ml/fraction) were collected and 0.4 volume of 50% glycerol in Buffer C was added. The active fractions determined by the enzyme activity assay were pooled and concentrated with CentriCon-30 concentrators to give fraction ARF.

E. Gel filtration chromatography

The active fraction from Resource Q, ARF, was loaded onto a FPLC Superose 6 HR10/30 column (25 ml bed volume) equilibrated with 50 mM sodium phosphate buffer, pH 7.0. The enzyme was eluted with the equilibrium buffer at a 0.5 ml/min. flow rate. Fractions (1 ml/fraction) were collected and 0.4 ml of 50% glycerol in Buffer C was added. The active fractions determined by the enzyme activity assay were pooled and concentrated with CentriCon-30 concentrators to give fraction ASP.
8.10 Amino acid modification studies of IBA-N-hydroxylase

8.10.1 Sulfhydryl group modification reagents

The following procedures were used in the studies with pHMB (4-(hydroxymercuri)benzoic acid), DTNB (5',5'-dithio-bis(2-nitrobenzoic acid)) and IAM (iodoacetamide).

A. Studies with both protein components

To mixture of the A and B components in Buffer B (20 μl), a protection compound (0.4 μl) was added. After incubation at room temperature for 6 minutes, a 2 μl aliquot of aqueous solution (pH 7.0) of the modification reagent was added to the resulting mixture. After incubation at 30°C for 15 minutes or 16 hours, the resulting enzyme solution was subjected to activity assay. Water was used to replace the modifying reagent solution as control.

B. Studies with either the A component or the B component

To the A (or B) component in Buffer B (10 μl), a 1 μl aliquot of aqueous solution (pH 7.0) of the modification reagent was added. After incubation at 30°C for 15 minutes or 16 hours, the resulting enzyme solution was desalted with a CentriCon 30 concentrator. To the desalted enzyme solution 10 μl of enzyme solution containing another component (component B or A) was added and the mixture was subjected to activity assay. Water was used to replace the modification reagent solution as control.
8.10.2 Diethyl pyrocarbonate

The following procedures were used in the studies with diethyl pyrocarbonate (DEPC).

A. Studies with both protein components

To mixture of the A and B components in Buffer B (20 μl), a protection compound (0.4 μl) was added. After incubation at room temperature for 6 minutes, a 0.5 μl aliquot of a 71 mM ethanolic solution of DEPC was added to the resulting mixture. After incubation at 32°C for 10 minutes, a 4 μl aliquot of a 20 mM aqueous solution of N-acetyl histidine was added to the DEPC treated enzyme solution to quench the DEPC. The resulting mixture was assayed for enzyme activity. Two controls were employed: in control 1 ethanol was used to replaced DEPC; in control 2, N-acetyl histidine solution was added to the DEPC solution and the mixture was left in room temperature for 5 minutes before addition to the enzyme solution.

B. Studies with either the A component or the B component

To the A (or B) component in Buffer B (10 μl), a 0.5 μl aliquot of a 71 mM ethanolic solution of DEPC was added. After incubation at 32°C for 10 minutes, a 4 μl aliquot of a 20 mM aqueous solution of N-acetyl histidine was added to the DEPC treated enzyme solution to quench the DEPC. To the resulting mixture, 10 μl of enzyme solution containing another component (component B or A) was added and the mixture was assayed for enzyme activity.
8.11 Methods related to library screening
8.11.1 Preparation of *Streptomyces viridifaciens* MG456-hF10 chromosomal DNA

Chromosomal DNA was prepared by the method of Sambrook *et al.* (Sambrook, 1989). Mycelia (5 g) (without washing) from 100 ml of *S. viridifaciens* MG456-hF10 fermentation broth were ground in liquid nitrogen to a fine white powder. This was transferred to a sterile 250 ml Erlenmeyer flask and 50 ml of lysis solution (500 mM sterile EDTA•Na$_2$ (pH 8.0), 100 μg/ml proteinase K, 0.5% (w/v) N-lauryl sarcosine) was added. The solution was incubated at 50°C for three hours with occasional stirring and then gently extracted three times with equal volumes of phenol saturated with TE. The solution was treated with RNase A solution (100 μg/ml) at 37°C for three hours, extracted three times with phenol as before, then dialyzed overnight against TE buffer. A total of 80 ml of DNA solution was obtained. A 20 ml aliquot of this solution was divided equally into twenty-five 1.7 ml microcentrifuge tubes, and 0.1 volume of 3 M sodium acetate and 0.6 volume of 2-propanol were added to each followed by gentle mixing. The DNA was pelleted by centrifugation for 15 minutes at the maximum speed in an Eppendorf centrifuge. The pellets were air dried and then suspended in 100 μl of TE buffer, giving a final total volume of 2.5 ml. This concentrated genomic DNA was stored at 4°C and used for library construction.
8.11.2 Construction of a *Streptomyces viridifaciens* MG456-hF10 genomic library

A 2 ml aliquot of *S. viridifaciens* MG456-hF10 concentrated genomic DNA solution was divided equally into ten 1.7 ml microcentrifuge tubes (200 μl/tube). A 40 μl aliquot of 10 x buffer (provided with the restriction enzyme) and 160 μl of TE buffer were added to each tube. The digestion was started with the addition of 0.5 μl of *Sau* 3A I (0.8 unit/μl) and the mixture was incubated in a 37°C water bath. A 100 μl aliquot of digestion mixture was removed at 1 minute, 1.5 minutes, 2 minutes and 2.5 minutes from each tube and added to 20 μl of cold 0.5 M EDTA•Na₂ (pH 8.0) to stop the digestion. The contents in these tubes were pooled to give partially digested DNA.

The partially digested DNA was size-fractionated by means of sucrose gradient centrifugation. A 15%-40% sucrose gradient (in TE buffer) was formed as following: 4 ml of sterile 40% (w/v) sucrose in TE buffer was added to a centrifuge tube, 4 ml of sterile 15% (w/v) sucrose in TE buffer then was added carefully to the top of 40% sucrose layer. The tube was sealed with a rubber stop and placed on its side on the laboratory bench for 2 hours, then moved back to a vertical position. The partially digested DNA (about 4.2 ml) was concentrated with Microcon-100 concentrators (2500 rpm, 20 min., Eppendorf centrifuge) to about 200 μl before being loaded on the top of the gradient. The tube containing the digested DNA was centrifuged in a SW 41 ultracentrifuge rotor at 200,000xg at 4°C for 20 hours. The sized-fractionated DNA was harvested by carefully withdrawing 1 ml fractions from the top of the centrifuge tube with a pipette. DNA fractions were analyzed by electrophoresis on a 0.6%
agarose gel. Fraction 7, which contained DNA fragments in the appropriate size range (9-20 kb), was mixed with 0.1 volume of 3 M sodium acetate and 0.6 volume of 2-propanol. The precipitated DNA was pelleted by centrifugation for 15 minutes at the maximum speed of an Eppendorf centrifuge. The pellet was air dried and resuspended in 10 µl of TE buffer. To 2.8 µl of this DNA suspension, 0.5 µl 10 x ligation buffer, 1.0 µl of BamH I cut Lambda arms (DASH II / BamH I DNA,1 µg/µl, from Stratagene) and 0.7 µl T4 DNA ligase (3 unit/µl) were added and ligation was performed at 4°C overnight.

The phage library, labeled as λWLα, was constructed by packaging the recombinant phage DNA into phage heads in vitro using the Gigapack II Gold Packaging kit supplied by Stratagene. The manufacturer's protocol was used without modification. 1, 2 and 20 fold dilutions of the phage library (in SM buffer) were used to infect E. coli XL1-Blue MRA cells which were subsequently plated out on NZY medium plates to estimate the number of viable phage. The phage library was stored at 4°C.

8.11.3 Hybridization

A. Transfer of phage DNA to membranes

Recombinant bacteriophage containing the Streptomyces virdifaciens MG456-hF10 genomic library was used to infect E. coli XL1-Blue MRA cells which were subsequently plated out on NZY medium plates (d 100 mm) to give about 2000 plaques per plate. The plaques were transferred to a Hybond-N nylon membrane as described by the manufacturer.

After transfer, the membrane was treated in a modified version of the
manufacturer's protocol as described below (Cooper, 1992). The membrane was placed, plaque-side up, on a Whatman 3MM filter soaked in 0.5 M NaOH, 1.5 M NaCl for 5 minutes, then placed on a filter soaked in 0.5 M Tris•HCl (pH 7.5), 1.5 M NaCl for 5 minutes and on a fresh filter soaked in 0.5 M Tris•HCl (pH 7.5), 1.5 M NaCl for 3 minutes. The membrane was briefly washed by immersion into a solution of 2 x SSC before being allowed to air dry. The dried membrane was exposed to UV light (50 mJoule) in a Bio-Rad GS Gene Linker™ UV chamber to fix the DNA to the membrane.

B. Southern transfer

After electrophoresis of DNA in an agarose gel, the DNA was transferred to a Hybond-N nylon membrane according to method of Southern, as described by Hopwood (Hopwood, 1985). The membrane was air dried and exposed to UV light (50 mJoule) in a Bio-Rad GS Gene Linker™ UV chamber to fix the DNA to the surface of the membrane.

C. Radiolabeling of DNA

Oligonucleotides (without the 5'-phosphate group), which were derived from the N-terminal protein sequence of the B component of isobutylamine N-hydroxylase and prepared by Integrated DNA Technologies Inc., were labeled with [γ-32P]-ATP using the Promega DNA 5'-End Labeling System. The manufacturer's protocol was used without modification. The labeled oligonucleotides were purified by "spin-column" chromatography using Sephadex G-50M in a 1 ml syringe. The labeled oligonucleotides was spin eluted in 200 µl of TNE buffer at 1000 rpm in Beckman CS-6R centrifuge.
D. Hybridization

The Hybond-N nylon membrane with fixed DNA was pre-hybridized for 2 hours at a desired hybridization temperature in 10 ml of hybridization solution (6 x SSC, 5 x Denhardt's) in a hybridization bottle. The labeled oligonucleotide probe was added to the tube. Hybridization was for about 16 hours. The membrane was then washed twice with 200 ml of 6 x SSC for 1 hour each at the hybridization temperature. After being sandwiched between plastic wrap, the membrane was exposed to Kodak X-OMAT XAR-5 X-ray film at -70°C for 4-16 hours and the film was developed in a Konica QX-70 automatic developer.

E. Isolation of phage

Once clear signal was observed on the developed X-ray film, the plaques which strongly hybridize with the probe were selected. The autoradiogram was then aligned with the plate and a plug of agar corresponding to the site of hybridization was removed with the wide end or the tip of a sterile pasteur pipette. The agar plug was soaked in 300 µl SM buffer at 4°C overnight to obtain a phage suspension. This phage was then plated out for a secondary screening or for large scale phage DNA preparation.

8.11.4 Large scale phage DNA preparation

Large scale preparation of recombinant bacteriophage λ was performed according to the method of Yamamoto et al. (Yamamoto, 1970). SM buffer (4 ml) was added to the top of a phage plate in a Petri dish. The plate was gently agitated at 4°C for 4 hours. The resulting phage suspension was transferred to
a sterile Falcon tube. Two drops of chloroform were added to the tube and the phage titre was determined by dilution plating.

An overnight culture of *E. coli* XL1-Blue MRA (5 ml) was used to inoculate 100 ml of LB broth containing 0.4% (w/v) maltose. When the $A_{600}$ reading of the culture reached 1.2, the culture was centrifuged to harvest the cells. The cells were resuspended in cold CM buffer to a concentration of $1.6 \times 10^9$ cells/ml ($8 \times 10^8$ cells/ml was equivalent to $A_{600} = 1$). Twenty ml of the cell suspension were infected with the recombinant Lambda DASH II phage at an multiplicity of infection of 0.01 (1 phage per 100 cells) at $37^\circ$C for 15 minutes. The infected cells were then used to inoculate 400 ml of CHYCM medium in a 2000 ml flask and grown at $37^\circ$C until lysis occurred (about 6 hours).

The culture was centrifuged at $3600 \times g$, for 5 minutes and the supernatant was treated with a mixture of DNase I and RNase A (final concentration: 10 $\mu$g/ml each) for 1 hour at $37^\circ$C. The resulting mixture was centrifuged again. To the supernatant, PEG 6000 and NaCl were added to a final concentration of 10%(w/v) and 1M, respectively. After the solids were dissolved, the mixture was incubated at $4^\circ$C for 1 hour before centrifuging at $3600 \times g$ for 10 minutes. The phage pellet was resuspended in 5 ml of TM buffer.

The phage was purified by cesium chloride density equilibrium centrifugation (1.35 g/ml to 1.7 g/ml, 90,000xg, 2 hours at $4^\circ$C). The blue phage band was withdrawn from the centrifuge tube with a syringe. After dialysis against TM buffer (3 x 1000 ml), the phage solution was mixed with 0.5 M EDTA•Na$_2$ (pH 8.0) to 25 mM, SDS to 1%(w/v), proteinase K to 200 $\mu$g/ml
and incubated at 37°C for 1 hour. The mixture was extracted twice with phenol saturated with 50 mM Tris-HCl (pH 8.0); 10 mM EDTA-Na2; 10 mM NaCl and once with chloroform. The phage DNA was precipitated with 0.1 volume of 3M sodium acetate and 2.2 volume of cold ethanol, and dissolved in 500 µl of TE buffer. and stored at -20°C.

8.11.5 Restriction digestion of DNA

Restriction digests of DNA were performed at 37°C for 2 hours, in buffers recommended by the suppliers of the enzymes. In the case of double digests, a compromise buffer was chose to give both enzymes reasonable activity. After digestion, the samples were loaded directly onto gels.

8.11.6 Recovery of DNA fragments

After fractionating DNA on a agarose gel containing ethidium bromide, DNA bands were visualized by UV and the slices of the gel containing the DNA bands of interest were cut out. The DNA was extracted and purified with QIAquick Gel Extraction Kit purchased from QIAGEN Inc. The manufacturer's protocol was used without modification.

8.11.7 Ligation of DNA

Ligation was performed as described by Cooper (Cooper, 1992). The DNA fragments were ligated to previously cut pBluescript in 1 mM ATP, 10 mM DTT, 10 mM MgCl2 and 50mM Tris-HCl (pH 7.6) with T4 DNA ligase (10 units) overnight at 16°C and was used directly to transform competent E. coli cells. Usually several reactions were set up with different ratios of vector and insert DNA to obtain optimum results.
8.11.8 Transformation of *E. coli*

*E. coli* DH5α competent cells from Life Technologies were used in the transformation and the manufacturer's protocol was used without modification. Cells containing recombinant plasmids were identified by blue/white color selection.

8.11.9 Recombinant clone selection and maintenance

After transformation, the *E. coli* cells were plated on a LB agar plate containing 100 μg/ml ampicillin, 2.5 μM IPTG and 20 μg/ml X-gal. Only cells containing plasmids would grow. Cells without plasmids did not grow under these conditions because they lacked the ampicillin resistant gene.

Cells containing the original plasmid (pBluescript) gave blue colonies because pBluescript was capable of α-complementation of the host-cells and gave the β-galactosidase color reaction in the presence of X-gal and IPTG (Horwitz, 1964). Insertion of foreign DNA into the polycloning site of the plasmid disrupted the α-complementation, therefore cells containing recombinant plasmids formed white colonies.

LB broth (4 ml) containing 100 μg/ml ampicillin was inoculated with each white colony and incubated overnight at 37°C, 300 rpm. Frozen stock was made by mixing the broth with equal volume of sterile 50% glycerol and storing the mixture at -70°C.
8.141.10 **Plasmid preparation**

LB broth (4 ml) containing 100 µg/ml ampicillin was inoculated with 5 µl of frozen stock and incubated overnight at 37°C, 300 rpm. Plasmid was prepared from the overnight culture with QIAprep Spin Plasmid Kit from QIAGEN, according to the supplier's instructions. The resulting plasmids were analyzed by digestion with restriction enzymes and gel electrophoresis.

8.11.11 **DNA sequencing**

Manual DNA sequencing was performed with Promega TaqTrack Sequencing System using direct incorporation with [α-^32^S]-dATP. DNA sequencing reactions were resolved in 6% polyacrylamide gels (acylamide : bis-acrylamide, 19:1) containing 8 M urea in a buffer gradient of 0.5 - 5.0 x TBE buffer as described by Cooper (Cooper, 1992).

Automated DNA sequencing was performed at the Molecular Genetics Core Facility, University of Texas Houston Medical School.

8.11.12 **Sequence analysis**

Sequence analysis was performed with the Wisconsin GCG Package, version 8.1-UNIX, and Sequencer™, Macintosh version 3.0. Databases searched are:

- GenBank Release 89.0 (6/95)
- EMBL (Abridged) Release 42.0 (3/95)
- PIR-Protein Release 44.0 (3/95)
- SWISS-PROT Release 31.0 (3/95)
- PROSITE Release 12.2 (3/95)
- Restriction Enzymes (REBASE) (6/95)
Addendum

A.1 Functions of the individual components of IBA N-hydroxylase

In this addendum, I would like to present new experimental results about the function of the individual components of IBA N-hydroxylase which were obtained during submission of this thesis.

In section 5.5.2, no NADH oxidase activity was observed with the B component, which suggests that the B component might be similar to the coupling protein of p-hydroxyphenyl-acetate-3-hydroxylase and function as a regulatory protein. However, B binds to an ω-aminobutyl agarose column while most of A passes through the column under the same conditions. Since n-butylamine is a fair substrate of IBA N-hydroxylase (Table 5.5), the ω-aminobutyl agarose column could be considered to be acting as an affinity column. Therefore, A might not be able to bind the amine substrate (n-butylamine or IBA), while B might carry a binding site for IBA. This is not consistent to the two-component model of p-hydroxyphenyl-acetate-3-hydroxylase.

An alternative two-component model was provided by a recent report concerning a two-enzyme system that catalyzed the oxidation of the D-proline residue of pristinamycin II_B (P II_B) (Scheme A.1) (Thibaut, 1995). The oxidation of P II_B to P II_A is a complex process requiring NADH, FMN and molecular oxygen.
Scheme A.1  The oxidation of the D-proline residue of pristinamycin II$_B$

Two enzymes were shown to be necessary to catalyze this reaction. One is a NADH:FMN oxidoreductase which provides reduced FMN (FMNH$_2$). The second enzyme (P II$_A$ synthase) was believed to catalyze a hydroxylation of P II$_B$ by molecular oxygen with the aid of FMNH$_2$ immediately followed by a dehydration leading to P II$_A$.

Experiments (Table A.1) were carried out to examine whether IBA N-hydroxylase consists of a similar system to that described above. A commercial NAD(P)H:FMN oxidoreductase from Photobacterium fischeri (Boehringer Mannheim Co.) was used to replace the components of IBA N-hydroxylase. The B component and the NAD(P)H:FMN oxidoreductase together showed IBA hydroxylation activity in the presence of NADH and FMN, while the A component and the NAD(P)H:FMN oxidoreductase together showed no activity in the presence of the same cofactors.
Table A.1  Enzyme replacement experiments\textsuperscript{a}

<table>
<thead>
<tr>
<th>No.</th>
<th>Enzymes that were added to the assay mixture</th>
<th>NADH oxidation activity\textsuperscript{b} (nmol/min)</th>
<th>IBHA formation activity\textsuperscript{c} (nmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50 µl FR</td>
<td>4.6</td>
<td>0.00</td>
</tr>
<tr>
<td>2</td>
<td>50 µl FR + 13.6 µg B</td>
<td>4.7</td>
<td>0.48</td>
</tr>
<tr>
<td>3</td>
<td>50 µl FR + 0.32 mg A</td>
<td>—</td>
<td>0.01</td>
</tr>
<tr>
<td>4</td>
<td>13.6 µg B</td>
<td>0.0</td>
<td>0.06</td>
</tr>
<tr>
<td>5</td>
<td>0.32 mg A</td>
<td>—</td>
<td>0.00</td>
</tr>
<tr>
<td>6</td>
<td>13.6 µg B + 0.32 mg A</td>
<td>—</td>
<td>0.51</td>
</tr>
</tbody>
</table>

\textsuperscript{a} FR: NAD(P)H:FMN oxidoreductase from \textit{Photobacterium fischeri}, 0.02 unit / µl (1 unit = 1 µmol /min).

\textsuperscript{b} A, B: the A or B component of IBA N-hydroxylase

\textsuperscript{c} The activity was assayed by measuring the initial rate of NADH oxidation at 340 nm ($\varepsilon = 6.22$ mM\textsuperscript{-1}cm\textsuperscript{-1}) at 25 °C. The assay was carried out in 100 mM Bis-Tris-HCl buffer, pH 7.5, containing 0.4 mM NADH, 10 µM FMN, 10 mM IBA and 100 mM NaCl.

These results suggest that the A component is an FAD (FMN) reductase which produces FADH\textsubscript{2} or FMNH\textsubscript{2} while the B component utilizes FADH\textsubscript{2} or FMNH\textsubscript{2} and molecular oxygen to hydroxylate isobutylamine (Scheme A.2).
Scheme A.2 Proposed reaction model for IBA N-hydroxylase

The NAD(P)H:FMN oxidoreductases (EC 1.6.8.1) have been divided into several groups, which display no significant homology to one another (Blanc, 1995). In the cell free extracts of S. viridisfaciens, there might be more than one FAD (FMN) reductase which could couple with the B component. This might partially account for the difficulties encountered during the purification of the A component.

The fact that the A component can be replaced by a commercial NAD(P)H:FMN oxidoreductase will enable us to study the B component under clearly defined conditions.
References


Bast, E., Hartmann, T., & Steiner, M. (1971) Arch. Mikrobiol. 73, 12.


