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Biosynthetic studies of coronatine and polyoximic acid

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Rice University, 1993
RICE UNIVERSITY

BIOSYNTHETIC STUDIES OF CORONATINE AND POLYOXIMIC ACID

by

ALAN E. WALKER

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

APPROVED, THESIS COMMITTEE

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Houston, Texas

April, 1993
Abstract

Biosynthetic Studies of Coronatine and
Polyoximic Acid

by

Alan E. Walker

Coronatine (1) (Fig 1) is a novel, non-host specific phytotoxin elaborated in the liquid fermentation broths of several different Pseudomonas syringae pathogens. Infection of tomato plants by P. syringae pv. tomato,1 Italian ryegrass by P. syringae pv. atropurpurea,2,3 soybean by P. syringae pv. glycinea,3,4 and Prunus spp. by P. syringae pv. morsprunorum3 induces yellow chlorotic haloes, stunting, and plant tissue hypertrophy due to the production of coronatine.2,4 Coronatine presents two distinct biosynthetic problems, one being the origin of coronafacic acid (2), the other the origin of the novel amino acid coronamic acid (3) (Fig 1). The investigations
presented in this dissertation were directed towards an understanding of
the biosynthetic pathway to coronamic acid. Our results from the
administration of specifically labeled precursors to Pseudomonas
syringae pv. glycinea (PDCC # 4182) suggest that: 1) the retention of
the tritium label from [3-3H]-L-alloisoleucine rules out the intermediacy
of 3-methylene-norvaline on the biosynthetic pathway to coronamic
acid, 2) the incorporation of approximately half of the 15N label from a
mixture of (2-13C,15N)-DL-isoleucine and (2-13C,15N)-DL-
alloisoleucine indicates that the cyclisation reaction leading to the
formation of coronamic acid from L-alloisoleucine does not involve the
loss of nitrogen and 3) the incorporation of (4-2H1,5-2H1)-(±)-
coronamic acid provides some experimental evidence in support of the
hypothesis that coronamic acid and coronafacic acid are biosynthesised
separately and coupled by the formation of an amide bond to give
coronatine.

Polyoximic acid (8) (Fig 4) is the amino acid component of
polyoxins A, F, H, I, and K, a unique class of antifungal antibiotics
elaborated in the fermentation broths of Streptomyces cacaoi var.
asoensis.20,22,37 Our investigations presented in this dissertation on
the biosynthetic origin of polyoximic acid by the administration of
specifically labeled precursors to *Streptomyces cacaoi* var. *asoensis* (ATCC # 19094) suggest that: 1) polyoximic acid is biosynthetically derived from L-isoleucine, 2) the incorporation of the $^{13}$C label from (1-$^{13}$C)-L-alloisoleucine into C-1" of polyoximic acid requires that the formation of the exocyclic double bond of polyoximic acid be formed by a syn-elimination of hydrogen from C-3 and not an anti-elimination as previously reported$^{38}$ and 3) the retention of two deuterium atoms, at C-4" of polyoximic acid derived from (6-$^{13}$C,2H$_3$)-DL-isoleucine and (6-$^{13}$C,2H$_3$)-DL-alloisoleucine, indicates that the cyclisation reaction requires the loss of only one hydrogen atom from L-isoleucine and L-alloisoleucine.
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I would like to thank Dr.'s Alan Kook and Garry King for running some difficult NMR experiments, and my fellow coworkers in Dr. Parry's group, especially Sunil Mhaskar and Haridas Kochat.

Lastly I would like to thank my wife, Anjanika, who bore the brunt of my frustration when things didn't go as expected, and who was a constant source of motivation.
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INTRODUCTION

Presently, biosynthesis finds itself as the starting point for research activities bridging the traditional boundaries between chemistry and biology. Due to major advances in the fields of genetics and molecular biology, particularly those dealing with bacteria such as of \textit{Streptomyces, E. coli} and \textit{Pseudomonas}, many genes responsible for the construction of natural products have been identified and sequenced.

On the other hand modern nuclear magnetic resonance techniques are now capable of tracing molecules through biological systems. This will surely lead to a more thorough understanding of the significance, role and mode of action of numerous natural products having medicinal, agricultural or pharmaceutical applications.

Ultimately the goal of any biosynthetic investigation is the isolation of the gene products, the proteins and enzymes, which are responsible for the reactions leading to secondary metabolites of unique structure and function. Many of the questions, whose answers remain unclear from simple initial feeding experiments, will only find solutions when these enzymes have been isolated and characterised. In order to
reach that stage and, more importantly to have the best understanding possible will require the expertise, insight, and hard work not only of organic chemists, but also of biologists, microbiologists, plant physiologists and molecular biologists.

The aim of this dissertation is to present the research conducted towards an understanding of the biosynthetic pathways leading to coronatine, a novel phytotoxin, and polyoximic acid, an unusual amino acid which is found in several members of a group of antibiotics called the polyoxins.

A) Coronatine.

Coronatine (1) (Fig 1) is a novel, non-host specific phytotoxin elaborated in the liquid fermentation broths of several different *Pseudomonas syringae* pathovars. Infection of tomato plants by *P. syringae pv. tomato*,$^1$ Italian ryegrass by *P. syringae pv. atropurpurea*,$^2,3$ soybean by *P. syringae pv. glycinea*,$^3,4$ and *Prunus* spp. by *P. syringae pv morsprunorum* $^3$ induces yellow chlorotic haloes, stunting, and plant tissue hypertrophy due to the production of coronatine.$^2,4$ In high enough local concentrations, infection also produces necrotic lesions around the halo sites. Coronatine has been
Figure 1: The structures of coronatine (1), coronafacic acid (2), & coronamic acid (3).
isolated from such infected sites, but its detailed role and mechanism of action remain unclear. In a recent study, nontoxigenic Tn5 mutants of *P. syringae pv. tomato* (cor<sup>-</sup>) were compared with wild type (cor<sup>+</sup>) strains. The mutants were shown to infect tomato plants and to produce the same number of lesions as the wild type strain, but the lesions were smaller (0.2 mm compared to 0.6 mm), stopped expanding sooner, and had a lower bacterial population. These investigations suggest that coronatine production may play a crucial role in the initial stages of infection, leading to increased susceptibility, a more virulent infection and ultimately better survival chances for coronatine producers, when compared to non-producing strains.

To date, two independent research groups have undertaken efforts to identify the genes responsible for coronatine production in *P. syringae pv. tomato*. C. Bender and coworkers, utilising Tn5 generated mutants of *P. syringae pv. tomato* PT23.2, were able to isolate an indigenous plasmid pPT23A. When this plasmid was inserted into *P. syringae pv. syringae* PS61, a known non-producer of coronatine, transconjugate PS61(pPT23A) fermentation broths were shown to contain coronatine, indicating that pPT23A is involved in coronatine production in *P. syringae pv. tomato*. 6
R. A. Moore and coworkers, utilising Tn5 generated mutants of *P. syringae* pv. *tomato* DC3000, isolated a 19-kb region of genomic DNA, in the plasmid pEC18. Sequencing of part of this fragment showed it to have homology to DNA present only in known coronatine producers, *P. syringae* pv. *tomato, P. syringae* pv. *atropurpurea* and *P. syringae* pv. *glycinea*. However pEC18 did not confer coronatine producing ability on known non-producing *P. syringae* pathovars, suggesting that regions outside the cloned segment must also be important in toxin production.  

Coronatine (1) presents two distinct biosynthetic problems, one being the mode of construction of coronafacic acid (2), the other the origin of the novel amino acid, coronamic acid (3) (Fig 1, p 3).

Previous investigations in our laboratory, utilising specifically labeled forms of acetic acid and pyruvic acid, have shown that coronafacic acid (2) is a polyketide derived from the head to tail linkage of five acetate units and an additional pyruvate unit (Scheme IA). More recent experiments have shown that (1-13C)-butyrate is specifically incorporated into C-5 of coronafacic acid and it therefore appears to be
Scheme IA & B.

A: The assembly of coronafacic acid (2) from acetate and pyruvate

B: Correlation of the absolute stereochemistry of coronamic acid (3) with the absolute configuration of L-isoleucine (4) and L-alloisoleucine (5)
the source of carbons C-5 to C-9 of the coronafacate skeleton. A close examination of the structure of coronafacic acid shows that once a polyketide chain has been formed by the linking of acetate units, the incorporation of pyruvate would require C-C bond formation between three nucleophilic carbon atoms 3a, 7a, and 4 (Scheme IA).

Based on available evidence, four routes appear to be possible by which coronafacic acid could be constructed from acetate and pyruvate (Fig 2). The most conventional of these is A), which would involve acetate as a starter unit leading to the formation of a "normal" polyketide framework, followed by the introduction of pyruvate. The next possibility, B, postulates that pyruvate is a starter unit and condenses with either acetyl or malonyl CoA to give citramalyl CoA which would then be chain extended to give the coronafacate framework. The above two routes are somewhat conventional but two others, C and D can be considered. They are somewhat unconventional in the sense that they suggest that coronafacate is constructed from more than one polyketide chain. Route C would proceed by starting at C-9 and terminating at C-7, with pyruvate being introduced into the middle of the growing polyketide chain. Route D postulates that pyruvate acts as a starter unit and it is chain extended with two acetate units to yield a
Figure 2: Possible biosynthetic routes and precursors to coronafacic acid.
polyketide that then condenses with a second polyketide chain consisting of three acetate units (Fig 2).

An experiment in which ethyl-(3-^{13}C)-acetoacetate was administered to *P. syringae* showed a 27% enrichment at C-8 of methyl coronafacate, with no other positions showing any label, suggesting that acetoacetate is not broken down to (1-^{13}C)-acetate. In order to confirm that acetoacetate is incorporated intact into coronafacate, doubly labeled ethyl-(2-^{13}C,3-^{13}C)-acetoacetate was administered to *P. syringae*. A 6% intact incorporation was observed at C-6 and C-8 of methyl coronafacate, suggesting that acetoacetate is incorporated into the C-5,6,8,9 fragment of coronafacate (Fig 2, p 8) and also confirming that acetoacetate is not broken down to acetate prior to incorporation. This result would also appear to disfavor routes A and C which postulates that acetoacetate would give rise to carbons 1, 2, 7 and 7a.

The two routes, B and D, both postulate the formation of citramalate. The NAC ester of (5-^{13}C)-(S)-citramalate (Fig 2) was synthesised and tested as a precursor for coronafacate biosynthesis. No incorporation was observed in the isolated methyl coronafacate. Also no labelling was observed which could have been due to the breakdown of
the precursor. This result does not rule out either route B or D since (R)-citramalic acid (Fig 2) is a known naturally occurring compound and it may be that this enantiomer is the true precursor of coronafacic acid. The synthesis of this precursor is presently underway. In order to differentiate between routes B and D, the N-acetylcysteamine derivative of (2,3-$^{13}$C$_2$)-3-oxohexanoate (Fig 2) was synthesised and evaluated as a precursor to coronafacate. If hexanoate is really a precursor then it would be expected to give rise to the C-10,4,5,6,8,9 fragment of coronafacate. Administration of this precursor to Ps. syringae showed that the precursor had undergone significant breakdown to acetate prior to incorporation. Based on the work of Vederas$^{10,11}$ it is known that acetate derived polyketide thioesters undergo rapid breakdown via $\beta$-oxidation. It may therefore be necessary to minimize $\beta$-oxidation by the addition of inhibitors such as 4-thiastearic acid or 3-hydroxy-4-pentynoic acid$^{10,11}$ or by the use of a glucose rich medium. It is possible that trans-2-hexenoate, which would be derived in vivo from hexanoate may be less susceptible to breakdown by $\beta$-oxidation. Therefore, the NAC ester of (2,3-$^{13}$C$_2$)-trans-2-hexenoate (Fig 2) will also have to be synthesised and tested as a precursor. The above investigations are still underway. Once the results are known we should be able to limit the
number of possible mechanisms by which coronafacate is constructed in *Ps. syringae*.

Previous investigations in our laboratory have shown that coronamic acid is derived from isoleucine. An examination of the absolute configuration of coronamic acid suggests that it should be derived from L-isoleucine (4) via L-alloisoleucine (5) (Scheme IB, p6). Feeding experiments utilising a mixture of (1-\(^{13}\text{C}\))-DL-isoleucine and (1-\(^{13}\text{C}\))-DL-alloisoleucine and isolation of coronatine as its methyl ester showed incorporation specifically at C-1'. A similar experiment with a mixture of (6-\(^{13}\text{C}\))-DL-isoleucine and (6-\(^{13}\text{C}\))-DL-alloisoleucine showed a \(^{13}\text{C}\) enrichment at C-6' of coronatine, indicating that the CH\(_2\) bridge of coronamate is derived from the C-6 methyl group of isoleucine. Administration of commercially available (1-\(^{13}\text{C}\))-L-isoleucine and (1-\(^{13}\text{C}\))-L-alloisoleucine showed that both amino acids are incorporated, but that L-alloisoleucine is a much more efficient precursor than L-isoleucine (9% vs. 60% incorporation).\(^{13}\)

In an attempt to determine the number of hydrogen atoms lost from C-6 of isoleucine during coronamic acid biosynthesis, a mixture of (6-\(^{13}\text{C},6-\text{2H}_3\))-DL-isoleucine and (6-\(^{13}\text{C},6-\text{2H}_3\))-DL-alloisoleucine was synthesised\(^{14}\) and fed to the producing organism. \(^{13}\text{C}\) NMR
analysis while simultaneously decoupling $^1$H and $^2$H, so that the $^{13}$C peak will appear as a singlet, showed a peak at 23.55 ppm, shifted upfield by 0.59 ppm relative to the peak at 22.96 ppm corresponding to C-6' of coronatine methyl ester. The magnitude of the shift suggests that two deuterium atoms were retained on C-6'.\textsuperscript{15} The conversion of L-alloisoleucine to coronamate must therefore proceed by the loss of only one hydrogen atom from C-6. It is important to notice that in this experiment the incorporation of the doubly labeled amino acid was only 0.5% compared to 31% for the singly labeled species. This is most likely due to the operation of a substantial deuterium isotope effect. The loss of only one hydrogen atom also indicates that the highest oxidation state reached by C-6 during the biosynthesis is no higher than that of an alcohol. This result suggests that 6-hydroxy-L-alloisoleucine might be an intermediate in the cyclisation process. In order to evaluate this possibility, a mixture of (1-$^{13}$C)-6-hydroxy-DL-isoleucine and (1-$^{13}$C)-6-hydroxy-DL-alloisoleucine was synthesised and administered to \textit{P. syringae}. No incorporation of the $^{13}$C label was detected in the isolated coronatine methyl ester. The preceding results\textsuperscript{13} are summarised in Table 1.
Table 1: Incorporation of Labeled Precursors into Coronatine Methyl Ester.\textsuperscript{13}

<table>
<thead>
<tr>
<th>expt</th>
<th>precursor</th>
<th>% enrichment or incorporation</th>
<th>labeling pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(1-\textsuperscript{13}C)-L-isoleucine</td>
<td>9</td>
<td>C-1'</td>
</tr>
<tr>
<td>2</td>
<td>(1-\textsuperscript{13}C)-L-alloisoleucine</td>
<td>60</td>
<td>C-1'</td>
</tr>
<tr>
<td>3</td>
<td>(6-\textsuperscript{13}C)-DL-isoleucine + (6-\textsuperscript{13}C)-DL-alloisoleucine</td>
<td>31</td>
<td>C-6'</td>
</tr>
<tr>
<td>4</td>
<td>(6-\textsuperscript{13}C,6-\textsuperscript{2}H\textsubscript{3})-DL-isoleucine + (6-\textsuperscript{13}C,6-\textsuperscript{2}H\textsubscript{3})-DL-alloisoleucine</td>
<td>0.5</td>
<td>\textsuperscript{13}C and \textsuperscript{2}H\textsubscript{2} at C-6'</td>
</tr>
</tbody>
</table>
The above investigations provided some valuable insight into the biosynthetic pathway to coronamic acid. Nevertheless there are still a large number of questions to be answered. It was the aim of the investigations described in this dissertation to throw some additional light on the details of the biosynthetic pathway.

Another possible intermediate between L-alloisoleucine and coronamic acid is 3-methylene-L-norvaline (6), which would be formed by the removal of hydrogen from C-3 & C-4 of L-alloisoleucine (Scheme IIA). If such a process takes place, then administration of [3-3H]-L-alloisoleucine should yield coronatine methyl ester which shows complete loss of tritium. On the other hand, the complete retention of tritium would rule out the intermediacy of 3-methylene-norvaline.

The published investigations of coronamic acid biosynthesis indicated that C-1' of L-alloisoleucine is retained during the formation of coronamic acid. However, they do not provide any evidence as to whether the carbon-nitrogen bond of L-alloisoleucine remains intact or whether the nitrogen is lost as a result of the cyclisation process. The
Scheme II A & B.

A: Hypothetical intermediacy of 3-methylene norvaline (6) on the biosynthetic pathway to coronamic acid (3).

B: Postulated incorporation of \((2-^{13}\text{C},^{15}\text{N})\)-DL-isoleucine/
\((2-^{13}\text{C},^{15}\text{N})\)-DL-alloisoleucine into coronatine (1)
synthesis and administration of a mixture of (2-$^{13}$C, $^{15}$N)-DL-isoleucine and (2-$^{13}$C, $^{15}$N)-alloisoleucine followed by analysis of the isolated methyl coronatine by $^{13}$C NMR should reveal whether the nitrogen atom is retained or whether it is lost as a result of cyclopropane ring formation (Scheme IIIB). If the nitrogen label is retained, the carbon-$^{13}$ NMR should show a doublet for C-2′ (Scheme IIIB), due to an intact $^{13}$C-$^{15}$N coupling. On the other hand if the nitrogen label is lost, the carbon-$^{13}$ NMR should show only a $^{13}$C enriched peak due to C-2′.

Since the results of the incorporation experiment with (6-$^{13}$C,2$^3$H)-DL-isoleucine and (6-$^{13}$C,2$^3$H)-DL-alloisoleucine suggest that a large deuterium isotope effect accompanies the cyclisation to form the cyclopropane ring, it should be possible to obtain additional mechanistic information by means of an experiment using chiral methyl groups. L-alloisoleucine bearing three different hydrogen isotopes at C-6 could be synthesised and administered to P. syringae. Analysis of the chirality of the isotopic labels at the C-6′ bridge of coronamic acid should provide invaluable information on the stereochemical course of the conversion of L-alloisoleucine into coronamic acid.\textsuperscript{16,17} Without going to the trouble of carrying out a chiral methyl group experiment, some stereochemical information might be obtained by the administration
of (1-13C)-2-amino-3-ethyl-n-pentanoic acid (7) (Scheme IIIA, p18). Isolation of an analogue of the methyl ester of coronatine bearing an additional methyl group at C-6' would indicate that the enzyme can accommodate an ethyl group instead of the methyl group present in the natural substrate. Additional experiments could then be carried out to determine the chirality of the new chiral center in the methylated analogue and to determine the stereochemistry of hydrogen loss from the ethyl side chain.

In addition to the mode of cyclisation of L-alloisoleucine to coronamic acid, another important problem associated with the biosynthesis of coronatine concerns the mechanism and timing of amide bond formation between coronafacic acid and the amino acid component. Mitchell and co-workers have isolated and characterised a family of coronatine analogs, which arise by the formation of an amide bond between coronafacic acid and a number of naturally occurring amino acids\textsuperscript{18,19} (Fig 3). The structures of a number of these analogs have been verified by synthesis.\textsuperscript{19} It should be noted that coronatine is the most abundant of all the analogs. On the other hand, there is no clear evidence concerning the question of whether coronafacic acid couples to L-alloisoleucine (5) to give L-alloisoleucyl coronafacate which then
Scheme III A & B.

A: Postulated incorporation of 2-amino-3-ethyl-n-pentanoic acid into coronatine

B: Possible pathways for the formation of an amide bond between coronafacic acid (2) and either L-alloisoleucine (5) or coronamic acid (3)
Figure 3: The structure of coronatine (1), coronafacic acid (2) and two naturally occurring analogs.
undergoes a cyclisation reaction to give coronatine or whether coronafacic acid couples directly with coronamic acid (3) to give rise to coronatine (Scheme IIIB, p 18).

Due to the existence of the coronatine analogs shown in Fig 3, Mitchell appears to favor the cyclisation of L-alloisoleucine after it has coupled to coronafacic acid. In order to gain some insight into this problem, we planned to synthesise deuterated coronamic acid and administer this precursor to P. syringae. Deuterium NMR analysis of the isolated methyl coronatine should reveal whether coronamic acid could be biosynthesised separately from coronafacic acid and then coupled to coronafacic acid.

Previous biosynthetic work has established that the cyclopropane ring systems of natural products are generally derived from methionine via S-adenosyl methionine. Many prokaryotic systems have been shown to contain cyclopropane fatty acids where the additional methylene group is derived from the methyl group of S-adenosyl methionine and is introduced at the site of a double bond in the growing fatty acid chain\textsuperscript{70,71,72} D. Arigoni has shown that 1-aminocyclopropane-1-carboxylic acid, in plants, is formed from methionine via S-adenosyl
methionine and that this amino acid is an intermediate along the pathway to the plant hormone ethylene.74,75,76,77 L. J. Altman and coworkers have shown that the cyclopropane ring occurring in the plant terpenoid cycloartenol arises from the collapse of a methyl group onto a neighbouring carbocation. He also established, by utilizing precursors bearing chirally labeled methyl groups, that the ring closure occurs with overall retention of configuration of the stereochemistry around the methyl group.73 Our experimental observations which show that coronamic acid is derived from L-isoleucine and L-alloisoleucine suggest that the cyclopropane ring of coronamic acid may be formed by a different mechanism to those generally encountered in biological systems.

B) Polyoxinic acid.

Polyoxin A and B (Fig 4) were first isolated in 1965 by K. Isono and coworkers from the culture filtrates of Streptomyces cacaoi var asoensis in a systematic screening program for antibiotics active against several phytopathogenic fungi.20 One year later, seven other polyoxins, C, D, E, F, G, H, and I (Fig 5) were isolated from the same organism.21 The structures and physical properties of these components have been reported.22 The polyoxins have been shown to be competitive inhibitors of chitin synthetase.23,24,25,26,27 In a more recent screening
Figure 4: The structures of polyoxin A, polyoxin B & polyoximic acid (8).
Figure 5: The Polyoxins.
program, Isono has described the isolation and characterisation of a more potent group of chitin synthetase inhibitors, the neopolyoxins A, B, and C (Fig 6), which are elaborated in the culture filtrates of a newly isolated strain of *S. cacaoi var asoensis*. Neopolyoxins A, and B, contain a 4-formyl-2-oxoimidazole base in place of the uracil base in the polyoxins and have a 3-hydroxypyridine amino acid side chain instead of a 5-O-carbamoyl-2-amino-2-deoxy-L-xylonic acid (carbamoylpolyoxamic acid) residue (Fig 6). None of the neopolyoxins have been shown to contain a polyoximic acid (8) residue (Fig 4). A structurally related class of compounds, the nikkomycins, were isolated by Zaher and his coworkers from *Streptomyces tendae* and were shown to have both acaricidal and insecticidal activity (Fig 7). The structure, biological activity and biosynthesis of the known nucleoside antibiotics are the subject of a review article.

Biosynthetic studies conducted by Isono and his coworkers on the polyoxins have shown that the substituent present at C-5 of the uracil ring is derived from C-3 of serine and is introduced onto the uracil ring by the action of an enzyme system that is different from the normal thymidylate synthetase. The carbamoylpolyoxamic acid residue is derived from L-glutamic acid and 3-ethylidene-L-azetidine-2-carboxylic acid (polyoximic acid) (8) is derived from L-
Neopolyoxin A: \( R = -\text{CHO} \)

Neopolyoxin B: \( R = -\text{COOH} \)

Neopolyoxin C

Figure 6: The structure of the neopolyoxins A, B, and C.
Nikkomycin J

Nikkomycin I

Figure 7: The structure of nikkomycin J & I
Isoleucine. It has also been shown that structurally modified polyoxins are elaborated when uracil analogs, modified at C-5, are administered to the fermentation broth of *S. cacaoi var asoensis*. The administration of 5-fluorouracil in place of uracil led to the isolation of the 5-fluoropolyoxins C M and L (Fig 8). It is interesting to note that in this case the incorporation of a C-1 unit at C-5 of the uracil base, which would lead to the production of polyoxins A, F, and H, is completely inhibited. Incorporation experiments with $^{14}$C-labeled, glucose, allose, and glycerol have shown that the 5'-amino-5'deoxy-D-allofuranosyluronic acid moiety of the polyoxins is derived from glucose. Glucose is is initially converted to two three carbon trioses, followed by either 1) the oxidation of one of the trioses to a three carbon acid and subsequent condensation with another three carbon sugar to form the C-6 uronic acid or 2) there exists an 80:20 equilibrium mixture of the two trioses which condense to give a hexose, which is then oxidised to the C-6 uronic acid.

Polyoximic acid (8) (Fig 4 & 5) is the amino acid component of polyoxins A, F, H, I, and K and is a unique naturally occurring cyclic amino acid. Only two other azetidine derivatives have been reported to occur in nature. L-azetidine-2-carboxylic acid was isolated
5-fluoropolyoxin L : $R_2 = \text{OH}$

5-fluoropolyoxin M : $R_2 = \text{H}$

5-fluoropolyoxin C

Figure 8 : The structures of 5-fluoropolyoxins L, M, & C
in 1956 by Fowden from *Liliaceae* \(^{40}\) and nicotianamine was isolated from *Nicotiana* in 1971 by Noma et al.\(^{41}\) A synthesis of racemic polyoximimic acid has recently been published.\(^{42}\)

To date only one group has undertaken biosynthetic investigations of polyoximimic acid. Isono and coworkers have shown by precursor incorporation experiments utilising \([1-^{14}C]-L\)-isoleucine,\(^{37}\) that polyoximimic acid is derived from *L*-isoleucine. It has also been shown that the formation polyoximimic acid from *L*-isoleucine proceeds with loss of the 4 *pro R* hydrogen atom of *L*-isoleucine (Scheme IVA).\(^{38}\) Based on the results of Isono’s work, it is tempting to think that the cyclisation of *L*-isoleucine to polyoximimic acid (8) might be related to the biosynthesis of coronamic acid (3) from *L*-alloisoleucine (5). In order to reconfirm Isono’s results, we planned to administer \([1-^{13}C]-L\)-isoleucine to *Streptomyces cacaoi* var *asoensis* and isolate polyoxin A. If polyoximimic acid is truly derived from *L*-isoleucine, then \(^{13}C\) NMR analysis should show an enrichment at the carboxyl group of polyoximimic acid. If *L*-isoleucine (4) is incorporated intact then, by analogy to coronamic acid biosynthesis, *L*-alloisoleucine (5) might also be a precursor (Scheme V). Administration of \([1-^{13}C]-L\)-alloisoleucine to *S. cacaoi* var *asoensis* and isolation of polyoxin A should show
Scheme IV A & B: The formation of polyoxinic acid from L-Isoleucine (4) & L-Alloisoleucine (5).

Anti elimination of the pro-R hydrogen from C-4 of isoleucine (8).

Syn elimination of the pro-R hydrogen from C-4 of alloisoleucine (5).

A+: electron acceptor, B: electron donor.
Scheme V: Hypothetical biosynthetic route to polyoximic acid (8)
from L-isoleucine (4) & L-alloisoleucine (5).

3-ethyl-cis-azetidine-2-carboxylic acid

2-Amino-3-methyl-3-pentenoic acid

3-ethyl-trans-azetidine-2-carboxylic acid
whether this amino acid is a precursor. If L-alloisoleucine is incorporated, it would require that the exocyclic double bond in polyoximic acid be formed by syn-elimination of hydrogen and not an anti-elimination as suggested by Isono's results with (2RS,3S,4S)-[2-14C,4-3H]-isoleucine (Scheme IVA & B).38

The cyclisation process from L-isoleucine or L-alloisoleucine to polyoximic acid may proceed with the loss of only one hydrogen atom from the C-5 methyl group of L-isoleucine (Scheme VI). In order to evaluate this possibility, we planned to synthesise a mixture of (6-13C,2H3)-DL-isoleucine and (6-13C,2H3)-DL-alloisoleucine and administer this to the producing fermentation broth of S. cacaoi var asoensis. Carbon-13 NMR analysis of the isolated polyoxin A, while simultaneously decoupling 1H and 2H resonances should allow us to determine the number of deuterium atoms remaining at C-5 of polyoximic acid.

Previous biosynthetic work on the origin of naturally occurring azetidine compounds has shown that L-azetidine-2-carboxylic acid, produced by Convallaria majalis, is derived from both methionine and S-adenosyl methionine.78 The administration of labeled forms of 2,4-
Scheme VI: Cyclisation of Isoleucine/Alloisoleucine to polyoximic acid (8).

$\text{(6-^{13}\text{C},^2\text{H}_3)}$-DL-Isoleucine/

$\text{(6-^{13}\text{C},^2\text{H}_3)}$-DL-Alloisoleucine.

\[ \xrightarrow{S. \text{ cacaoi var. } \text{asoensis.}} \]
diaminobutanoic acid and 1-aminocyclopropane-1-carboxylic acid, both biological derivatives of methionine, to *Convallaria majalis* has shown that neither of these compounds is an intermediate on the pathway to L-azetidine-2-carboxylic acid. These observations suggest that L-azetidine-2-carboxylic acid is most probably formed by a direct cyclisation of S-adenosyl methionine.\(^7\) Isonos' observations, which show that polyoximic acid is derived from L-isoleucine rather than methionine or S-adenosyl methionine, suggest that polyoximic acid may be formed by a different mechanism to other azetidines.
RESULTS & DISCUSSION

A) Coronatine

Our initial investigations on the biosynthetic pathway to coronatine were focused on two objectives: 1) increasing the fermentation yields of both coronatine and coronafacic acid and 2) the development of an reproducible, accurate HPLC system which would allow us to monitor the fermentation broth for both coronatine and coronafacic acid production. Previous investigations in our laboratory using *Ps. syringae* pv. *glycinae* (PDCC # 4182) typically gave 3-4 mg of pure coronatine methyl ester and 5-7 mg of methyl coronafacate from a two liter fermentation. The use of standard strain improvement techniques, with *Ps . syringae* pv. *glycinae* (PDCC # 4182) proved to be unsuccessful giving no increase in the production of coronatine or coronafacic acid. Given that the natural host of *Ps. syringae* pv. *glycinae* are soybean plants, we attempted to revive our culture by inoculation and re-isolation of the organism from the chlorotic halo sites on the leaves of infected plants. Following the methods of Gnanamannickam, Starratt, and Ward soybean seeds were allowed to germinate in the dark at room temperature on moist filter paper for 4-5 days. The seeds were then planted in potting soil and grown at between 22-24°C, under fluorescent
lighting, in a controlled environmental chamber for seven days at which time unifoliate leaves had begun to develop. While the plants were growing, a culture of the \textit{Ps. syringae} pv. \textit{glycinae} (PDCC \# 4182) was prepared in King's B medium. Based on optical density measurements as an estimate of the bacterial population, 0.7 mL of the mature broth (corresponding to approximately $1.2 \times 10^6$ colony forming units/mL) was diluted to 500 mL with sterile water and administered to the underside of the unifoliate soybean leaves until wetting was induced. Chlorotic haloes started to appear after a further 5 days incubation at 22-24$^\circ$C. The infected leaves were then used to inoculate several freshly made King's B medium agar plates. After incubation at room temperature for 3 days, individual fluorescent colonies were visible. Single colonies were selected and used to set up small scale (200 mL) fermentations which were monitored for the production of coronatine and coronafacic acid by HPLC. None of the selected colonies showed any significant increase in the production of either coronatine or coronafacic acid. Several further attempts were made using the same protocol, none of which met with any success.

Ultimately we obtained a strain, \textit{Ps. syringae} pv. \textit{glycinae} (PDCC \# 4180), as a gift from C. Bender at Oklahoma State University
which produced in our hands 7-10 mg of coronatine methyl ester and 10-12 mg of methyl coronafactate from 2 litres of fermentation broth. Although this does not represent a massive increase in production, it proved to be adequate for our investigations. The \(^1\text{H}\) and \(^{13}\text{C}\) NMR of coronatine methyl ester isolated from \textit{Ps. syringae pv. glycinea} (PDCC \# 4180) are shown in figures (9) and (10).

The development of an HPLC system for coronatine production was considered to be important since it allows us to determine the time at which coronatine production started, when the production was at its maximum, and the best time to administer labeled precursors. Our first attempts were concentrated on developing a system for the methyl ester derivatives of coronatine and coronafacic acid. We found that using a 25 cm, \(C_{18}\) reverse phase column and running a solvent gradient from 100\% water to 100\% acetonitrile worked satisfactorily. However processing small samples of the fermentation to yield the methyl ester derivatives of coronatine and coronafacic acid proved to be quite a chore. Following the work of Cuppels,\(^7\) we were able to develop a system with which we could analyse our fermentation samples for free coronatine and
Figure 9: $^1$H NMR of Coronadine Methyl Ester at 250 MHz.
Figure 10: $^{13}$C NMR of Coronatine Methyl Ester at 75.45 MHz
coronafacic acid as opposed to their methyl ester derivatives. We found
the best solvent system to be 28% acetonitrile, 72% water and 0.05%
trifluoroacetic acid. Using this solvent system retention times of 12-13
min for coronatine and 7-8 min for coronafacic acid were obtained on a
25 cm, C₁₈ reverse phase column. The retention times of both coronatine
and coronafacic acid were found to be very sensitive to the amount of
acetonitrile in the mobile phase. Increasing the acetonitrile concentration
to greater than 30% gave retention times which were too short, whereas
concentrations of less than 25% acetonitrile gave retention times which
were too long. Our HPLC analysis also showed that coronatine and
coronafacic acid production started after 24 hours and gradually
increased to reach a maximum after 5 days at which time coronatine and
coronafacic acid could be isolated as their methyl ester derivatives. Based
on these results we chose to pulse administer our labeled precursors after
the fermentation had proceeded for 24, 48, and 72 hours.

One possible intermediate between L-alloisoleucine (5) and
coronamic acid (3) is 3-methylene-norvaline, which would be formed by
the loss of hydrogen from C-3 and C-4 of L-alloisoleucine (Scheme II
A, p 15). In order to test this possibility, we synthesised a mixture of [1-
₁⁴C]-L-alloisoleucine and [3-³H]-L-alloisoleucine and administered this
mixture to *Ps. syringae* pv. *glycinae*. Both labeled amino acids were synthesised by a modification of Oppolzers' amino acid synthesis utilising (+)-10-dicyclohexylsulfamoyl-L-isoborneol as a chiral auxiliary to direct the stereochemistry at C-3 (Schemes VII & VIII). [3-^3\text{H}]-L-Alloisoleucine (3.9 mg, specific activity 11.50 microcuries/mg) and [1-^14\text{C}]-L-alloisoleucine (1.3 mg, specific activity 7.52 micro curies/mg) were mixed and recrystallised to constant activity to give a starting 3\text{H}/14\text{C} ratio of 3.89. The amino acid mixture was dissolved in 15.0 mL of water containing 1.0 eq of NaOH and administered to the fermentation broth, through a sterile filter, after 24, 48, and 72 hours. The fermentation was stopped after five days and coronatine harvested as its methyl ester derivative. We used a modified isolation procedure similar to that outlined by Ichihara and coworkers.\textsuperscript{45} The crude fermentation broth was centrifuged to remove the cells and the supernatant adsorbed on to a mixture of charcoal and Celite. The active components were eluted from the charcoal/Celite mixture with acetone (ca. 300 mL). The acetone eluant was adjusted to pH 2.5 with 2.5 M H\textsubscript{2}SO\textsubscript{4}, then concentrated five fold in vacuo and extracted vigorously with ethyl acetate. The combined ethyl acetate extracts, containing
Scheme VII: Synthesis of \( [3-^3\text{H}]\)-L-Alloisoleucine.

\[
\text{O} \quad \text{O} \quad \text{O} \quad \text{O} \quad \text{O} \\
\text{Bu} \quad \text{Bu} \quad \text{Bu} \quad \text{Bu} \quad \text{Bu} \\
\text{EtOH, 0°C-RT.} \quad \text{NEt}_3 \quad \text{DBU, DMF} \\
\text{[Cu(tBu)_4]} \quad \text{[COCl]}_2 \quad \text{p-TsOH} \\
\text{BF_3.OEt_2} \quad \text{AgCN} \quad \text{C_6H_5} \\
\text{-78°C, Et_2O} \quad \text{Heat} \\
\text{BuLi(Ph)_2NH} \quad \text{NaN_3} \\
\text{TMSCl} \quad \text{DMF, RT.} \\
\text{NBS, THF, -78°C.} \\
\text{Br} \quad \text{N_3} \\
\text{Yb} \quad \text{Yb} \\
\text{[3-^3\text{H}] L-Alloisoleucine.} \\
\text{OH} \quad \text{OH} \\
\text{NH_2} \quad \text{NH_2} \\
\text{O} \quad \text{O} \\
\text{Yb = (+)-10-Dicyclohexyl-sulfamoyl-L-isoborneol}
Scheme VIII: Synthesis of [1-\(^{14}\)C]-L-Alloisoleucine.

\[
\begin{align*}
\text{CH\(_3\)Cl} & \quad \xrightarrow{1) \text{Mg, I\(_2\), Et\(_2\)O.}} \quad \text{CH\(_3\)COOH} \\
& \quad \xrightarrow{2) \text{Ba\(^+\)CO\(_3\), H\(_2\)SO\(_4\)}} \quad \text{CH\(_3\)CO} \\
& \quad \xrightarrow{(\text{COCl})_2, Y\(_3\)H, AgCN} \quad \text{CH\(_3\)CO} \\
& \quad \xrightarrow{\text{BuLi, (iPr\(_2\)NH, TMSCl, NBS.}} \quad \text{CH\(_3\)CO} \\
& \quad \xrightarrow{\text{DBU, DMF, RT.}} \quad \text{CH\(_3\)CO} \\
\end{align*}
\]

[1-\(^{14}\)C]-L-alloisoleucine.

\(Yb = (+)-10\text{-Dicyclohexysufamoyl-L-isoborneol}\)
coronatine, were concentrated in vacuo, redissolved in a minimal amount of methanol and treated with excess ethereal diazomethane. The excess diazomethane was removed using a stream of nitrogen gas and the methyl esters finally purified by preparative thin layer chromatography. Authentic coronatine methyl ester (11.5 mg) was added as carrier prior to the final purification step by PTLC. This gave 4.8 mg of pure coronatine methyl ester having a final $^3$H/$^{14}$C ratio of 3.73. The starting and final $^3$H/$^{14}$C ratios indicate that, within experimental error, none of the tritium is lost from L-alloisoleucine. In order to confirm this result and to remove any lingering radiochemical impurities, the isolated coronatine methyl ester was further diluted with authentic coronatine methyl ester (8.1 mg) and reduced to dihydrocoronatine methyl ester (31) (Fig 11, p 46) with lithium tri-t-butoxy aluminum hydride in dry THF. Acidic work up gave the crude alcohol which was further purified by silica gel PTLC (elutant 10% iso-propanol/hexane $R_f$ = 0.162). This gave 5.5 mg of dihydrocoronatine methyl ester having a $^3$H/$^{14}$C ratio of 3.67, (Table 2, expt 1). The carbon-13 NMR of (31) shows a peak at 75.18 ppm due to C-1, a peak at 31.3 ppm due to C-2 and a peak at 42.4 ppm due to C-7a (Fig 11). A comparison of the starting and the isolated $^3$H/$^{14}$C ratios showed an overall 94.3% retention of tritium. This clearly indicates that
Table 2: Incorporation of Additional Labeled Precursors into Coronatine Methyl Ester.

<table>
<thead>
<tr>
<th>expt</th>
<th>precursor ((^{3}\text{H}^{14}\text{C}))</th>
<th>% enrichment or incorporation</th>
<th>labeling pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>([1,^{14}\text{C},3,^{3}\text{H}])-L-alloisoleucine (3.89)</td>
<td>1.8</td>
<td>(^{3}\text{H}^{14}\text{C} = 3.67) (94% (^{3}\text{H}) retention)</td>
</tr>
<tr>
<td>2</td>
<td>((1,^{13}\text{C}))-DL-2-amino-3-ethyl pentanoic acid</td>
<td>0.0</td>
<td>none</td>
</tr>
<tr>
<td>3</td>
<td>((2,^{13}\text{C},^{15}\text{N}))-DL-isoleucine + ((2,^{13}\text{C},^{15}\text{N}))-DL-alloisoleucine</td>
<td>5.4 ((^{13}\text{C})), 2.4 ((^{13}\text{C}).(^{15}\text{N}))</td>
<td>C-2' and N atom of amide bond</td>
</tr>
<tr>
<td>4</td>
<td>((5,^{2}\text{H}<em>{1},6,^{2}\text{H}</em>{1})-(\pm))-coronamic acid</td>
<td>32</td>
<td>C-4' and C-5'</td>
</tr>
</tbody>
</table>
Figure 11: $^{13}$C NMR of Dihydrocoronatine Methyl Ester at 75.45 MHz
coronamic acid cannot be formed by the loss of hydrogen from C-3 of L-alloisoleucine and therefore rules out 3-methylene-norvaline as an intermediate in coronamic acid biosynthesis.

This result, along with the earlier finding which showed that 6-hydroxyalloisoleucine is not an intermediate, further reduces the number of possible mechanisms by which coronamate can be formed from L-alloisoleucine. It is tempting to think that coronamic acid might be formed by a mechanism which represents a diversion from an enzymic hydroxylation to an oxidative cyclisation (Scheme IX, p 48). Such pathways have been encountered in the biosynthesis of clavaminic acid,\textsuperscript{46} isopenicillin N,\textsuperscript{47} and desacetoxycephalosporin C.\textsuperscript{47} The mechanism of coronamic acid formation might also be related to the sulfur insertion reactions involved in biotin and lipoic acid biosynthesis.\textsuperscript{48} Further experiments, in which L-alloisoleucine bearing a chirally labeled C-6 methyl group is administered to \textit{Ps. syringae}. pv. \textit{glycinae}, followed by the isolation, degradation and analysis of the stereochemistry of the C-6' CH\textsubscript{2} group of coronamic acid (Fig 1, p3) should provide an experimental test of this speculation. If the stereochemistry of the methylene group is random, it may be that the randomisation is due to a radical process, whereas a specific
Scheme IX: The iron (II) dependent oxidative cyclisation reaction involved in isopenicillin-N biosynthesis & an analogous cyclisation leading to the formation of coronamic acid (3) from L-alloisoleucine
stereochemistry of the methylene group would suggest a different mechanism.

In order to gain some stereochemical information about the formation of coronamic acid, without having to synthesise L-alloisoleucine bearing a chiral methyl group, we synthesised (1-\(^{13}\)C)-DL-2-amino-3-ethyl pentanoic acid (Scheme X). If the enzyme which is responsible for the cyclisation of L-alloisoleucine (5) to coronamic acid (3) is able to accommodate an ethyl group rather than a methyl group in the precursor then we should be able to isolate an analog of methyl coronatine having an additional methyl group at C-6'. If (1-\(^{13}\)C)-DL-2-amino-3-ethyl pentanoic acid is incorporated, the isolated analog should show an enrichment at C-1' in its \(^{13}\)C NMR. We assumed that the analogue of methyl coronatine had almost identical retention times on HPLC and R\(_f\) values on silica PTLC as authentic methyl coronatine.

(1-\(^{13}\)C)-DL-2-Amino-3-ethyl-n-pentanoic acid (324 mg) was dissolved in 30 mL of water containing 1eq of NaOH and was administered to \(P. \text{ syringae pv. glycinae}\) (PDCC # 4180) after 24, 48, and 72 hours. The fermentation was stopped after five days and coronatine isolated as its methyl ester derivative. Carbon-13 NMR analysis showed no enrichment at C-1'(Fig 12), (Table 2, expt 2, p45).
Scheme X: Synthesis of (2-$^{13}$C, $^{15}$N)-DL-isoleucine, (2-$^{13}$C, $^{15}$N)-DL-alloisoleucine & (1-$^{13}$C)-DL-2-amino-3-ethyl-n-pentanoic acid.

1. $\text{Br} \xrightarrow{\text{NaCN, HMPA, RT}} \text{CN}$
2. $\xrightarrow{\text{DIBAL-H}} \text{H}$
3. $\xrightarrow{\text{NaHSO}_3, \text{H}_2\text{O}, \text{EtOH, KCN}}$
4. $\xrightarrow{\text{Ba(OH)}_2, 8\text{H}_2\text{O}} \text{NH}_2$
5. $\xrightarrow{\text{H}_2\text{O, reflux}}$
6. $\xrightarrow{\text{NH}_2\text{OH}}$
7. $\text{CN}$

(2-$^{13}$C, $^{15}$N)-DL-Isoleucine.

(1-$^{13}$C)-DL-2-Amino-3-ethyl-n-pentanoic acid.
Figure 12: 13C NMR of Coronamine Methyl Ester Derived from (2-13C)-DL-2-Amino-3-ethyl pentanoic acid at 62.89 MHz.
Figure 13: $^{13}$C DEPT NMR of Coronatine Methyl Ester Derived from (1-$^{13}$C)-DL-2-Amino-3-ethyl pentanoic acid at 75.45 MHz
A carbon-13 DEPT NMR analysis confirmed that our isolated material did not exhibit an additional methyl group at C-6' (Fig 13). One possible reason for the apparent lack of conversion of this amino acid by \textit{Ps. syringae} into a analog of coronatine methyl ester may well be that it was missed in the isolation procedure. A more sensitive experiment would have been to administer a radioactive form of the amino acid. On the other hand if our result is correct, then it suggests that the enzyme may well have a stereochemically restricted active site. Mitchell had shown earlier that the fermentation broths of the coronatine producing strain, \textit{Ps. atropurpurea} (4328) contained N-coronafacoylvaline and N-coronafacoylisoleucine\textsuperscript{18} (Fig 3, p19), which suggests that coronafacic acid can couple to a variety of different amino acids. It may well be that the additional methyl group in 2-amino-3-ethyl-n-pentanoic acid, when compared to isoleucine and valine, adds too much steric bulk to the substrate such that it cannot be accommodated in the active site of the enzyme.

An examination of the absolute configuration of coronamic acid\textsuperscript{12} (3) suggests that it is formed from L-isoleucine (4) via L-alloisoleucine (5) (Scheme IB, p 6). This would require that L-isoleucine (4) undergo an epimerisation at C-3 giving rise to L-alloisoleucine (5). Our published
results\textsuperscript{13} show that both L-isoleucine (4) and L-alloisoleucine (5) are incorporated into methyl coronatine, but that L-alloisoleucine is a much more efficient precursor than L-isoleucine (60\% vs 9\% incorporation) (Table 1, expt 1 and 2, p13). Our previous investigations had not shown us whether the nitrogen atom of coronamic acid is derived from L-alloisoleucine or whether it is lost in the cyclisation reaction to form coronamic acid. In order to test this possibility a mixture of (2-\textsuperscript{13}C, \textsuperscript{15}N)-DL-isoleucine and (2-\textsuperscript{13}C, \textsuperscript{15}N)-DL-alloisoleucine was synthesised by a modification of the Strecker amino acid synthesis (Scheme X, p 50). The amino acid mixture was dissolved in water and pulse administered to \textit{Ps. syringae pv. glycin}ae after 24, 48, and 72 hours. Carbon-13 NMR analysis of the isolated coronatine methyl ester showed a \textsuperscript{13}C enriched peak at 38.26 ppm representing a 5.4\% specific incorporation of the \textsuperscript{13}C label into C-2' of coronatine methyl ester (Fig 14). This peak was further split into a doublet at 38.20 ppm and 38.36 ppm (coupling constant = 12.2 Hz) representing a 2.4\% intact incorporation of the \textsuperscript{13}C-\textsuperscript{15}N label (Fig 15) and (Scheme II B, p 15), (Table 2, expt 3). A \textsuperscript{13}C DEPT analysis confirmed that our \textsuperscript{13}C enriched peak was a quaternary carbon atom, C-2' (Fig 16). Our results also show that approximately half of the \textsuperscript{15}N label was lost from the precursor. We speculate that this might be the
Figure 14: $^{13}$C NMR of Coronatine Methyl Ester Derived from

$(2^{-13}C,^{15}N)$-DL-Isoleucine/$(2^{-13}C,^{15}N)$-DL-Alloisoleucine

at 75.45 MHz
Figure 15: Expanded $^{13}$C NMR of Coronatine Methyl Ester Derived from (2-$^{13}$C, $^{15}$N)-DL-Isoleucine/
(2-$^{13}$C, $^{15}$N)-DL-Alloisoleucine at 75.45 MHz
Figure 16: $^{13}$C DEPT NMR of Coronatine Methyl Ester Derived from (2-$^{13}$C, $^{15}$N)-DL-Isoleucine/$^{15}$N)-DL-Alloisoleucine at 75.45 MHz
result of an epimerisation reaction which interconverts L-isoleucine (4) to L-alloisoleucine (5). This would lead to L-alloisoleucine bearing a $^{13}$C label at C-2 but having no $^{15}$N label. This would explain the $^{13}$C enriched peak at 38.26 ppm. The intact $^{13}$C-$^{15}$N incorporation would then be due to the direct cyclisation of the already present (2-$^{13}$C-$^{15}$N)-L-alloisoleucine to coronamic acid without loss of nitrogen. If we postulate that L-isoleucine loses its nitrogen atom via the formation of an $\alpha$-keto acid, mediated by a pyridoxal phosphate dependent enzyme, we would expect to lose approximately half of our $^{15}$N label from a 50/50 mixture of (2-$^{13}$C,$^{15}$N)-DL-isoleucine and (2-$^{13}$C,$^{15}$N)-DL-alloisoleucine (Scheme XI). Pyridoxal phosphate is a well understood coenzyme responsible for many biochemical reactions involving amino acids. A recent paper indicates that the free $\alpha$-keto acid, derived from L-isoleucine, does not undergo epimerisation at C-3 at biological pH. This suggests that loss of the $^{15}$N label may be due to the transamination of L-isoleucine by a process which is unrelated to the epimerisation reaction at C-3. Experimental evidence obtained in our laboratory has shown that when a known pyridoxal phosphate inhibitor, aminooxy acetic acid, is added to the fermentation broth of *Ps. syringae* (PDCC # 4180) coronatine production is halted. This suggests that one of the two steps, either the epimerisation of L-isoleucine to L-
Scheme XI: The formation of an α-keto acid from L-isoleucine (4).

PLP = Pyridoxal phosphate
alloisoleucine or the cyclisation reaction to form coronamic acid is mediated by a pyridoxal phosphate dependent enzyme. The fact that we observe some intact incorporation of the $^{15}\text{N}$ label suggests that the cyclisation reaction does not require the loss of an $\text{NH}_2$ group. Final confirmation that it is the interconversion of L-isoleucine and L-alloisoleucine, and not the cyclisation reaction that results in loss of nitrogen will require that $(2,^{13}\text{C},^{15}\text{N})$-L-isoleucine be synthesised and administered to $\textit{Ps. syringae}$.

Another important question in the biosynthesis of coronatine is the mechanism and timing of amide bond formation between coronafacic acid and the amino acid component. At present there is no clear evidence to show whether coronafacic acid (2) couples to L-alloisoleucine (5) to give L-alloisoleucyl coronafacate which then cyclises to coronatine or whether coronafacic acid couples directly with coronamic acid (3) to give rise to coronatine (Scheme IIIB, p18). To gain some insight into this problem we synthesised $(5-2\text{H},6-2\text{H})(\pm)$-coronamic acid (Scheme XII) and pulse administered this precursor to $\textit{Ps. syringae pv.glycinae}$ after 24, 48, and 72 hours. The fermentation was stopped after five days and coronatine was isolated as its methyl ester derivative. This gave twenty-five milligrams of pure coronatine methyl ester after
Scheme XII: Synthesis of $(5^2\text{H}_2, 6^2\text{H}_1)$-Coronamic acid.

1) NEt(iPr)$_3$, EtOCOCl,
2) NaN$_3$, H$_2$O, 0°C,
3) C$_6$H$_6$ reflux,
4) 6N HCl, reflux,

\[ \text{(5}^2\text{H}_2, 6^2\text{H}_1)\text{-Coronamic acid.} \]
puriﬁcation by PTLC. An earlier experimental observation, which had shown that the administration of unlabeled coronamic acid to \textit{P.s. syringae pv. glycinea}e stimulated the production of coronatine, had led us to expect a higher yield of coronatine (25 mg vs 5-7 mg) when we fed our labeled precursor. Carbon-13 NMR analysis of the coronatine methyl ester isolated after deuterium-labeled coronamic acid was administered to the fermentation showed two \textsuperscript{13}C triplets at 13.03 ppm and at 20.01 ppm, due to C-5' and C-4' of coronatine methyl ester, respectively (Fig 17). An expansion of the \textsuperscript{13}C spectrum shows that each triplet is shifted from the normal carbon resonances by approximately 0.25 ppm, indicating that C-4' and C-5' each carry one deuterium atom (Fig 18), (Table 2, expt 4, p45). Each peak appeared as a triplet as a result of an intact \textsuperscript{13}C-\textsuperscript{2}H coupling of 20 Hz for C-5' and 18 Hz for C-4'. Analysis of the same compound by \textsuperscript{2}H NMR in benzene, showed two peaks at 1.00 ppm and 1.65 ppm relative to the benzene peak at 7.25 ppm (Fig 19) and conﬁrmed that deuterium atoms were present at two sites in the coronatine methyl ester isolated. The integration for these two peaks, after correcting for the natural deuterium abundance of 0.02\%, corresponded to a 32\% \textsuperscript{2}H enrichment at both C-4' and C-5' of coronatine methyl ester.
Figure 17: $^{13}$C NMR of Coronatine Methyl Ester Derived from
(5'-H$_3$,6'-H$_3$)-Coronamic acid at 75.45 MHz

\[
\begin{align*}
\text{CDCl}_3
\end{align*}
\]
Figure 18: Expanded $^{13}$C NMR of Coronatine Methyl Ester Derived from (5-$^3$H$_1$,6-$^3$H$_1$)-(+)-Coronamic acid at 75.45 MHz
Figure 19: $^2$H NMR of Coronatine Methyl Ester Derived from (5-$^2$H$_5$,6-$^2$H$_4$)-\((\pm)\)-Coronamic acid

at 46.07 MHz
The intact incorporation of (5-\textsuperscript{2}H\textsubscript{1},6-\textsuperscript{2}H\textsubscript{1})-coronamic acid, along with our observation that coronamic acid stimulates coronatine production, provides some experimental evidence for the hypothesis that coronafacic acid and coronamic acid are biosynthesised separately followed by the formation of an amide bond to give coronatine. Ultimately this could be confirmed using a cell free system of \textit{Ps. syringae} pv. \textit{glycinae} with an assay designed to detect the enzyme responsible for the amide bond formation.

B) Polyoxin Project

Our initial investigations into the biosynthetic pathway to polyoximic acid (Fig 4, p 22) were focused on 1) the development of an accurate analytical HPLC system for polyoxin A production, which would allow us to carry out strain improvement studies using \textit{Streptomyces. cacaoi} var \textit{asoensis} (ATCC \# 19094) and 2) the development of a suitable method for the isolation and purification of polyoxin A from fermentation broths of \textit{S. cacaoi} var \textit{asoensis} (ATCC \# 19094).

Using the methods outlined in Snyder's, Glajch's, and Kirkland's book\textsuperscript{50} on 'Practical HPLC Method Development,' we found that using a 25 cm, Whatman Partisphere C\textsubscript{18} reverse phase column and a mobile
phase consisting of 10% acetonitrile, 90% water, and 1% acetic acid worked well. Using this system polyoxin A had a retention time of 7.0 minutes, which was identical to that of an authentic sample of polyoxin A, which we had obtained as a gift from K. Isono.\textsuperscript{51} Using our HPLC system we were able to carry out strain improvement studies on \textit{S. cacaoi var asoensis}. \textit{S. cacaoi var asoensis} (ATCC # 19094), was used to inoculate a culture tube containing 4 mL of seed medium. The culture was grown at 28°C, 210 rpm for 24 hours before being used to inoculate a batch of freshly made ISP 2 slants. After being allowed to grow at 30°C for 4-5 days one slant was selected and used to make a spore suspension and a batch of serial dilutions.\textsuperscript{43} Each serial dilution was then used to inoculate freshly made ISP 2 agar plates. The plates were incubated at 30°C for 4-5 days, at which time the organism had started to sporulate. Ten individual colonies were selected and used to set up small scale fermentations of \textit{S.cacaoi var asoensis}. Each fermentation was monitored for polyoxin A production at 24 hour intervals, using our reverse phase HPLC system. Our analysis showed that polyoxin A production started after 24 hours and had reached a maximum after 72 hours. Two out of the ten individual colonies we had selected showed an enhanced production of polyoxin A. These two colonies were preserved
by lyophilisation and stored at 4°C prior to being used to set up fermentations for the administration of labeled precursors.

Before labeled precursors were administered to *S. cacaoi* var *asoensis* we checked our strains for the levels of polyoxin A production. One lyophile was opened and used to inoculate a culture tube containing 4 mL of seed medium. This tube was incubated at 28°C, 210 rpm for 24 hours and then used to inoculate 100 mL of seed culture. The seed culture was grown at 28°C, 210 rpm for a further 24 hours and then used to inoculate fifteen 1.0 L flasks each containing 120 mL of production medium. The fermentation was grown at 28°C, 210 rpm and monitored for polyoxin A production by HPLC. The fermentation was stopped after it had proceeded for 72 hours and polyoxin A isolated. The fermentation broth was centrifuged at 9000 rpm for 40 minutes to remove the cellular growth and the supernatant filtered through glass wool and adjusted to pH 2.0 with 1 N HCl. The active ingredients were adsorbed onto a mixture of charcoal and Celite by stirring at room temperature for one hour. This solution was loaded into a column and washed with water (100 mL). The active ingredients were then eluted with acetone (350 mL) and concentrated in vacuo to give typically 1.2 g of crude polyoxin complex as a brown oil. Analytical TLC (cellulose, BAW 4:1:2) gave a UV and ninhydrin positive spot at $R_f = 0.27$ due to
polyoxin A. The sample was further purified by MPLC (Merck LiChroprep RP-18 silica, eluant 10% acetonitrile, 90% water, and 1% acetic acid) and the collected samples analysed for polyoxin A by HPLC. The polyoxin A rich samples were pooled and concentrated in vacuo. This gave, on average, 25-30 mg of polyoxin A. Any remaining impurities were removed by repeated cellulose column chromatography (eluant BAW; 4:1:2) followed by preparative HPLC on a 10 mm ID, 25 cm long, Altex ODS C₁₈ column (eluant 10% acetonitrile/90% water/1% acetic acid). This gave 4-7 mg of polyoxin A, which had identical chromatographic and spectrophotometric properties as our authentic sample. The ¹H and ¹³C NMR spectra of our isolated polyoxin A are shown in figure 20 and 21. A partial assignment of the carbon-13 NMR spectrum is shown in Table 4.

Isono and his coworkers had reported earlier that polyoximic acid (8) (Fig 4, p 22) is biosynthetically derived from L-isoleucine. In order to confirm this observation we chose to administer commercially available (1-¹³C)-L-isoleucine to S. cacaoi var. asoensis (ATCC # 19094) and isolate polyoxin A. An intact incorporation of this precursor should show a ¹³C enriched peak due to the carboxylic acid group of the polyoximic acid residue. (1-¹³C)-L-isoleucine (200 mg)
Figure 20: $^1$H NMR of Polyoxin A at 500 MHz
Figure 21: $^{13}$C NMR of Polyoxin A at 62.89 MHz
Table 4: Partial $^{13}$C assignments for Polyoxin A (see Fig 4).

<table>
<thead>
<tr>
<th>$^{13}$C NMR shift, ppm</th>
<th>Tentative assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.9</td>
<td>C-6&quot; polyoximic acid</td>
</tr>
<tr>
<td>56.2</td>
<td>C-4&quot; polyoximic acid</td>
</tr>
<tr>
<td>56.5</td>
<td>C-7 uracil base</td>
</tr>
<tr>
<td>56.8</td>
<td>C-5'</td>
</tr>
<tr>
<td>59.1</td>
<td>C-4&quot; polyoximic acid</td>
</tr>
<tr>
<td>83.4</td>
<td>C-4' ribose ring</td>
</tr>
<tr>
<td>91.4</td>
<td>C-1' ribose ring</td>
</tr>
<tr>
<td>114.1</td>
<td>C-5 uracil base</td>
</tr>
<tr>
<td>120.7</td>
<td>C-5&quot; polyoximic acid</td>
</tr>
<tr>
<td>125.4</td>
<td>C-3&quot; polyoximic acid</td>
</tr>
<tr>
<td>140.6</td>
<td>C-6 uracil base</td>
</tr>
<tr>
<td>151.6</td>
<td>C-2 uracil base</td>
</tr>
<tr>
<td>159.2</td>
<td>C-6&quot; polyoxamic acid</td>
</tr>
<tr>
<td>165.0</td>
<td>C-6'</td>
</tr>
<tr>
<td>168.1</td>
<td>C-1*</td>
</tr>
<tr>
<td>168.7</td>
<td>C-4 uracil base</td>
</tr>
<tr>
<td>174.0</td>
<td>C-1&quot; polyoximic acid</td>
</tr>
<tr>
<td>174.4</td>
<td>C-1&quot; polyoximic acid</td>
</tr>
</tbody>
</table>
was dissolved in water (45 mL) and pulse administered to the fermentation broth of *S. cacaoi var asoensis* after 24, 36 and 48 hours. After the fermentation had proceeded for a further 24 hours, it was stopped and polyoxin A isolated. The final purification step by preparative HPLC gave 6 mg of pure polyoxin A. Carbon-13 NMR analysis of our isolated material showed two $^{13}$C enriched peaks at 174.02 and 174.43 ppm (Fig 22 and 23) (Table 3, expt 1). A comparison with our $^{13}$C NMR spectrum of authentic polyoxin A allowed us to calculate the $^{13}$C enrichments due to each peak. The peak at 174.02 ppm corresponded to a $^{13}$C enrichment of 3.5% whereas the peak at 174.43 ppm corresponded to a $^{13}$C enrichment of 0.36%.(Table 3, expt 1). Both peaks appear at the correct position for the C-1' carboxylic acid group of the polyoximic acid residue (Fig 4, p22), which confirmed that L-isoleucine is the source of polyoximic acid. However it was unclear why we observed two enriched peaks.

In order to confirm our observations, we synthesised a mixture of (6-$^{13}$C)-DL-isoleucine and (6-$^{13}$C)-DL-alloisoleucine (Scheme XIII). Incorporation of the $^{13}$C label should lead to a $^{13}$C enriched peak due to C-4 of polyoximic acid (8) (Fig 4). (6-$^{13}$C)-DL-Isoleucine and (6-$^{13}$C)-DL-alloisoleucine (169 mg) was dissolved in water (45 mL) and pulse administered to *S. cacaoi var asoensis* after 24, 36, and 48
Figure 22: $^{13}$C NMR of Polyoxin A Derived from (1-$^{13}$C)-L-Isoleucine at 62.89 MHz

MeOH
Figure 23: Expansion of the $^{13}$C NMR of Polyoxin A Derived from (L-$^{13}$C)-L-Isoleucine at 62.89 MHz
Table 3: Incorporation of Labeled Precursors into Polyoxin A (see Fig 4).

<table>
<thead>
<tr>
<th>expt</th>
<th>precursors</th>
<th>% enrichment</th>
<th>labeling pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(1-\textsuperscript{13}C)-L-isoleucine</td>
<td>3.5, 0.36</td>
<td>C-1&quot; carboxyl group of polyoximic acid</td>
</tr>
<tr>
<td></td>
<td>(6-\textsuperscript{13}C)-DL-Isoleucine &amp; (6-\textsuperscript{13}C)-DL-Alloisoleucine</td>
<td>3.9, 7.6</td>
<td>C-4&quot; CH\textsubscript{2} group of polyoximic acid</td>
</tr>
<tr>
<td>2</td>
<td>(1-\textsuperscript{13}C)-L-Alloisoleucine</td>
<td>4.9, 5.9</td>
<td>C-1&quot; carboxyl group of polyoximic acid</td>
</tr>
<tr>
<td>3</td>
<td>(6-\textsuperscript{13}C,\textsuperscript{2}H\textsubscript{3})-DL-Isoleucine &amp; (6-\textsuperscript{13}C,\textsuperscript{2}H\textsubscript{3})-DL-Alloisoleucine</td>
<td>undetermined</td>
<td>C-4&quot; CH\textsubscript{2} group of polyoximic acid</td>
</tr>
</tbody>
</table>
Scheme XIII: Synthesis of (6-$^{13}$C)-DL-Isoleucine/(6-$^{13}$C)-DL-Alloisoleucine.

\[
\begin{align*}
\text{CN} & \quad \text{LDA} \quad \text{CH}_3\text{I} \quad \text{THF, -78°C} \quad \text{CN} \quad \text{DIBAL-H} \quad \text{CH}_2\text{Cl}_2 \quad \text{RT} \quad \text{CN} \quad \text{NaHSO}_3 \quad \text{KCN, H}_2\text{O} \quad \text{EtOH, RT} \quad \text{OH} \quad \text{CN} \\
& \quad 32 \quad 33 \quad 34 \\
\text{NH}_4\text{OH, H}_2\text{O, EtOH, RT} & \quad \text{NH}_2 \quad \text{NH}_2 \quad \text{NH}_2 \quad \text{NH}_2 \quad \text{CO}_2\text{H} \\
& \quad 35 \quad 36 \quad (6-$^{13}$C)-DL-Isoleucine \quad (6-$^{13}$C)-DL-Alloisoleucine
\end{align*}
\]
hours from the start of the fermentation. The fermentation was stopped after it had proceeded for 72 hours and polyoxin A isolated. After purification by preparative HPLC, 5.1 mg of pure polyoxin A was isolated. To our surprise carbon-13 analysis showed two $^{13}$C enriched peaks at 56.19 ppm and 59.10 ppm (Fig 24). A carbon-13 DEPT experiment confirmed that both of these peaks were methylene (CH$_2$) groups (Fig 25) and they both appeared at a chemical shift reasonable for C-4" of a polyoximic acid (Fig 4, p22) residue. The peak at 56.19 ppm exhibited a $^{13}$C enrichment of 3.9%, whereas the peak at 59.10 ppm exhibited a $^{13}$C enrichment of 7.6% (Table 3, expt 2, p76) This result confirmed our observation that polyoximic acid is derived from isoleucine, but did not throw any light on why we observed two enriched peaks.

A close examination of the 500 MHz proton NMR spectrum of our isolated polyoxin A shows there are two peaks corresponding to the vinyl proton at C-6 of the uracil base of polyoxin A, at 7.68 ppm (1H) and 7.65 ppm (1H) (Fig 20, p70). This suggested that we may have isolated a mixture of two polyoxins. A comparison of the integrals of the above two peaks with the integral of the doublet at 1.6 ppm (6H), which is assigned to the allylic methyl group protons on C-6" of the
Figure 25: $^{13}$C DEPT NMR of Polyoxin A Derived from (6-$^{13}$C)-DL-Isoleucine & (6-$^{13}$C)-DL-Allolsoleucine at 62.89 MHz
polyoximic acid residue (Fig 4 & Fig 20), suggested that if there are two polyoxins present then they both must contain a polyoximic acid residue. Our initial speculation was that along with polyoxin A, we had isolated another polyoxin which carried a different substituent at C-5 of the uracil base. Of the known polyoxins (Fig 5, p23), polyoxin F, which has a carboxyl group at C-5 in place of a CH2OH group in polyoxin A, seemed the most likely contaminant. Isono's work\textsuperscript{21,22} on the characterisation of the different polyoxins reported that polyoxin F and polyoxin A had essentially the same $R_f$ values by TLC, 0.16 and 0.15 respectively, on silica, eluant BAW 4:1:2. We were able to rule out polyoxin H, which has a methyl group at C-5, due to the fact that we did not see any additional methyl group resonance in our proton NMR spectrum (Fig 20, p70). In order to confirm our speculation, we decided to carry out a three bond, 2-dimensional $^{13}$C-$^1$H heteronuclear correlation NMR experiment. If we really have isolated polyoxins A and F we would expect one of the proton peaks at 7.65 and 7.68 ppm to correlate to C-1', C-2, C-4, and a CH$_2$ group, C-7 of polyoxin A, and the other one to correlate to C-1', C-2, C-4, and a CO$_2$H group of polyoxin F (Fig 26). The result of the 3 bond, 2-dimensional $^{13}$C-$^1$H correlation experiment is shown in figure 27. Our result shows that we observe the expected correlation to four different carbon atoms at 58.8
Figure 26: Expected 3 bond, $^{13}$C-H* couplings for Polyoxin A and Polyoxin F
Figure 27: Two Dimensional, $^1$H-$^1$C Heteronuclear Correlation NMR spectrum of Polyoxin A.

C-7, 58 ppm
C-1, 99 ppm
C-2, 159 ppm
C-4, 169 ppm
Figure 28: Expansion of the $^{13}$C-$^1$H Heteronuclear Correlation NMR spectrum of Polyoxin A
ppm due to the CH$_2$ at C-7 of polyoxin A, at 93.7 ppm due to C-1', at 153.7 ppm due to C-2, and at 169.1 due to C-4 (Fig 27). An expansion of the regular spectrum (Fig 28) shows that both the proton at 7.65 ppm and the proton at 7.68 ppm correlate to the same carbon atoms. This clearly showed that we only have one type of uracil base, that carrying a CH$_2$OH group, and that there is no polyoxin F in our isolated material. Given that our isolated material seemed to be only one compound, our initial thought was that we have two isomers of polyoxin A which differ in the orientation of the uracil base (Fig 29). If this is the case then we should be able to make the two rotational isomers equivalent if we record $^1$H NMR spectra at varying temperatures while observing the two proton peaks at 7.65 and 7.68 ppm. These two peaks would be expected to merge if our speculation is true. The result of the variable temperature NMR analysis at 25$^\circ$C, 40$^\circ$C, and 70$^\circ$C is shown in figures 30 and 31. This result clearly shows that the two peaks remain separate indicating that we do not have two rotational isomers. Two other isomers are still possible which might be able to explain why we see two enriched peaks from our feeding experiments. One is that S. cacaoi var asoensis makes both enantiomers of polyoximic acid to give the polyoxins shown in figure 32. Another possibility is that we have E and Z isomers of the double bond in polyoximic acid (Fig 33). It remains to
Figure 29: Possible rotational isomers of polyoxin A
**Figure 31**: Variable Temperature $^1$H NMR of Polyoxin A at 300 MHz.
Figure 32: Two possible isomers of polyoxin A differing in the stereochemistry of the polyoximic acid carboxyl group.
Figure 33: Two possible isomers of Polyoxin A differing in the orientation of the exocyclic double bond of polyoximic acid.
be proved by further experiments if either of these possibilities is correct.

Given that L-isoleucine is incorporated into polyoximic acid, it is tempting to think that the cyclisation reaction to form polyoximic acid might be related to the cyclisation of L-alloisoleucine to give coronamic acid. If this is the case then we would expect L-alloisoleucine to be a precursor on the biosynthetic pathway leading to polyoximic acid. To confirm this hypothesis we pulse administered commercially available (1-\textsuperscript{13}C)-L-alloisoleucine (200 mg in 45 mL water) to S. cacaoi var asoensis after 24, 36, and 48 hours. The fermentation was stopped after 72 hours and polyoxin A isolated. Purification of our sample by preparative HPLC gave 4.8 mg of polyoxin A. Carbon-13 NMR analysis showed two enriched carbonyl peaks at 174.01 ppm and 174.42 ppm corresponding to the C-1" carboxyl group of the polyoximic acid residue (8) (Fig 4, p22 and Fig 34). The peak at 174.01 exhibited a \textsuperscript{13}C enrichment of 5.9\%, whereas the peak at 174.42 exhibited a \textsuperscript{13}C enrichment of 4.9\% (Table 3, expt 3, p76). This result confirms that L-alloisoleucine can act as a precursor on the biosynthetic pathway to polyoximic acid. It also requires a reinterpretation of Isono's results\textsuperscript{38} which showed that the exocyclic bond of polyoximic acid is formed by a
Figure 34: $^{13}$C NMR of Polyoxin A Derived from (1-13C)-L-Alloisoleucine
at 62.89 MHz
anti-elimination of hydrogen from L-isoleucine. The fact that L-alloisoleucine is incorporated into polyoximic acid requires that the double bond be formed by an syn-elimination of hydrogen (Scheme IV A & B, p30). Our result also shows that L-alloisoleucine is a better precursor than L-isoleucine (5.9% vs 3.5%) (Table 3, expt 2 and 3, p76).

The cyclisation reaction from L-isoleucine and L-alloisoleucine to give polyoximic acid may proceed with the loss of only one hydrogen atom from the C-6 methyl group of each amino acid (Scheme VI, p 33). To test this possibility we synthesised a mixture of \((6-^{13}C,^{2}H_3)\)-DL-isoleucine and \((6-^{13}C,^{2}H_3)\)-DL-allioisoleucine (Scheme XIV). Incorporation of this amino acid mixture should yield polyoxin A which shows a \(^{13}C\) and \(^2H\) enrichment at C-4" of polyoximic acid (Fig 4, p22). The amino acid mixture (189 mg) was dissolved in water (45 mL) and pulse administrated to \(S.~cacaoi\) var \(asoensis\) after 24, 36, and 48 hours. The fermentation was stopped after 72 hours and polyoxin A isolated. Purification by preparative HPLC gave 6.7 mg of polyoxin A. Carbon-13 NMR analysis of the isolated polyoxin A showed two broad multiplets at approximately 55.7 ppm and 58.6 ppm corresponding to an incorporation of both \(^{13}C\) and \(^2H\) into C-4" of polyoximic acid (Fig 4, Fig 35 & Fig 36). Both peaks are shifted upfield by approximately
Scheme XIV: Synthesis of (6-\textsuperscript{13}C, \textsuperscript{2}H\textsubscript{3})-DL-isoleucine / (6-\textsuperscript{13}C, \textsuperscript{2}H\textsubscript{3})-DL-alloisoleucine.

\[
\text{BuLi, (iPr)}_2\text{NH,} \quad 13\text{CD}_3\text{I, THF, } -78^\circ\text{C.} \quad \text{(36)}
\]

\[
\text{DIBAL-H} \quad \text{CH}_2\text{Cl}_2, \text{ RT.} \quad \text{(37)}
\]

\[
\text{NaHSO}_3, \text{ H}_2\text{O,} \quad \text{EtOH, KCN.} \quad \text{(38)}
\]

\[
\text{NH}_4\text{OH,} \quad \text{EtOH, 18hrs.} \quad \text{(39)}
\]

\[
\text{Ba(OH)}_2\cdot8\text{H}_2\text{O} \quad \text{H}_2\text{O, reflux.}
\]

(6-\textsuperscript{13}C, \textsuperscript{2}H\textsubscript{3})-DL-isoleucine.

(6-\textsuperscript{13}C, \textsuperscript{2}H\textsubscript{3})-DL-alloisoleucine.
Figure 36: Expansion of the $^{13}$C NMR of Polyoxin A Derived from
(6-$^{13}$C$_2$H$_2$)-DL-Isoleucine & (6-$^{13}$C$_2$H$_2$)-DL-Alloisoleucine
at 75.45 MHz
0.5 ppm from the normal $^{13}$C resonances for these two peaks at 56.2 ppm and 58.6 ppm respectively (Fig 36) (Table 3, expt 4, p 76). The magnitude of the $^{13}$C shift$^{15}$ suggests that two $^2$H atoms are retained on C-5 and that the cyclisation reaction to give polyoximic acid results in the loss of only one hydrogen atom from C-5 of L-isoleucine and L-alloisoleucine. This result needs to be confirmed by carrying out a $^{13}$C NMR experiment while simultaneously decoupling the $^1$H and $^2$H resonances. This will result in the collapse of the two multiplets to two singlets and allow a more accurate measure of the upfield shift caused by the deuterium substitution.

Scheme V (p 31) outlines the possible biosynthetic pathways by which polyoximic acid might be biosynthesised from L-isoleucine (4) and L-alloisoleucine (5). L-isoleucine or L-alloisoleucine may first be dehydrogenated to give (E)-(S)-2-amino-3-methyl-3-pentenoic acid (Route B) followed by cyclisation to give polyoximic acid. An alternative would be that L-isoleucine (4) first undergo a cyclisation reaction to give (2S, 3R)-ethyl-cis-azetidine-2-carboxylic acid followed by dehydrogenation to give polyoximic acid (Route A). If L-alloisoleucine (5) is a precursor then a similar cyclisation reaction would give rise to 3-ethyl-trans-azetidine-2-carboxylic acid (Route C). It is possible that the
cyclisation reaction might proceed by the hydroxylation and phosphorylation of the C-6 methyl group of L-isoleucine. The intact incorporation of a labeled form of 2-amino-3-methyl-3-pentenoic acid into polyoximic acid would suggest that the dehydrogenation reaction precedes the cyclisation reaction.

At this stage it is tempting to speculate that polyoximic acid might be formed by a similar cyclisation process to that which we have speculated to lead to coronamic acid, that is an oxidative cyclisation involving an iron-oxo species (Scheme IX, p 48). At this stage this is only speculation, but the administration of L-alloisoleucine which bears a chirally labeled C-6 methyl group,\textsuperscript{16,17} to both \textit{Ps. syringae} pv. \textit{glycinae} and \textit{S.cacaoi} var \textit{asoensis}, followed by degradation and analysis of the stereochemistry at the C-6' CH\textsubscript{2} bridge of coronamic acid (Fig 1, p3) and the stereochemistry at the C-4 CH\textsubscript{2} group of polyoximic acid (Fig 4, p22) will hopefully show whether these cyclisation reactions are radical based reactions. A randomisation of the stereochemistry at the methylene groups of both coronamic acid and polyoximic acid would suggest that a radical intermediate is involved in the cyclisation, whereas a specific stereochemistry at the methylene groups would suggest an alternative mechanism.
EXPERIMENTAL

General methods

$^1$H NMR spectra were obtained using a Jeol FX-90 (90 MHz), Bruker AC-250 (250 MHz) and a IBM AF-300 (300 MHz) spectrometers. $^{13}$C NMR spectra were recorded at 75.45 MHz on the AF-300 and at 62.89 MHz on the AC-250. Chemical shifts are reported in ppm on the $\delta$ scale from internal TMS, CDCl$_3$, or D$_2$O standards. Deuterium NMR spectra were taken on the IBM AF300 at 46.07 MHz. Benzene was added as a standard for the calculation of $^2$H enrichment. Mass spectra were recorded on a Finnigan 3300 (low resolution) mass spectrometer and a CEC-111021-110B (high resolution) mass spectrometer. Melting points were determined using a Fischer-Johns apparatus and are uncorrected. Flash chromatography was performed as outlined by Still and coworkers. Preparative silica gel TLC was performed using 0.75 mm plates made from Merck silica gel, type 60, PF-254. Analytical TLC was performed using 0.25 mm commercially available Whatman silica gel plates, type 60A, K6F and 0.1 mm, precoated Merck Cellulose F (Art 5718) plates. Visualisation was by short wavelength UV, iodine, phosphomolybdate or ninhydrin dip reagent followed by heat. Cellulose chromatographic columns were run using Merck microcrystalline cellulose. Samples for chromatography were concentrated using a Savant
Speedvac prior to analysis. High-performance-liquid-chromatography was performed using a Spectra-Physics SP8700 pump, SP8750 organiser, an Isco UA-5 monitor and an Isco type 6 optical unit. All samples were run on a 25 cm, reverse-phase (C18), Partisphere column. All solvents were spectroscopic grade and purchased from EM-reagents. Gas chromatography was performed on a Hewlett-Packard 5710A gas chromatograph using a 10%SE-30 glass column (6 ft, 2mm i.d.), equipped with a flame ionisation detector, connected to a Houston Instruments 4500 Microscribe strip chart recorder. Microbiological media for slants, agar plates and fermentation broths were either purchased from Difco or prepared from the appropriate reagents. Microbiological manipulations were carried out in a Becton Dickinson BBL Biohazard Cabinet. Equipment and broths were sterilized using a Barnstead Laboratory Sterilizer. Fermentations were performed using a New Brunswick G-26R rotary shaker. This was connected to a Forma Scientific CH/P 2067 circulating water bath in order to carry out fermentations of Ps. syringae at 18°C. Fermentations of S. cacaoi var asoensis were carried out in the same shaker at 28°C. Fermentation samples for HPLC were centrifuged using a Beckman CPR benchtop centrifuge. Whole fermentations were harvested using a Dupont Instruments Sorvall RC-5B refrigerated superspeed centrifuge. Slants
and agar plates were either grown at room temperature or incubated at 30°C in a American Scientific Incubator model IC-62. Lyophiles were prepared on a FTS Systems Maxi Dry Lyophilizer. Reagents and compounds were weighed on a Mettler H51AR, Mettler P1200 or a Perkin-Elmer AD-2 Autobalance (for radiolabeled compounds). Measurements of radioactivity were carried out with a Beckman LS-3801 liquid scintillation counter.

Materials

_Pseudomonas syringae_ pv _glycinea_ (PDDCC # 4182) was obtained as a gift from Prof. Carol Bender of Oklahoma State University. _Streptomyces cacaoi_ subsp _asoensis_ (ATCC # 19093 & 19094) was obtained from the American Type Culture Collection. All reagents and compounds were purchased from Aldrich Chemical Co, Fluka, ICN, Signa Chemical Co and were purified, where necessary, by standard techniques. Radiolabeled compounds were purchased from Amersham. Stable isotopically labelled compounds were purchased from Cambridge Isotope Laboratories. Unless otherwise stated all reactions were performed under a dry nitrogen atmosphere.
Synthesis of $[3\text{-}^3\text{H}]$-L-alloisoleucine (17)$^{53}$ (Scheme VII) $[3\text{-}^3\text{H}]$-t-Butyl 3-Hydroxybutyrate (9).

Unlabeled sodium borohydride (9 mg) was added to a stirred solution of t-butyl acetoacetate (600mg, 3.8 mmol) in dry ethanol (2.72 mL) at 0°C. The solution was stirred at 0°C for 15 minutes and $[^3\text{H}]$-sodium borohydride (100mCi, specific activity 500 mCi/mmol) added and the reaction mixture stirred at 0°C for 30 minutes. The ice bath was removed and the mixture stirred at room temperature overnight. The reaction mixture was then cooled to 0°C, a further 65 mg of unlabeled sodium borohydride was added and the reaction mixture was stirred at 0°C for 15 minutes and then at room temperature for 2 hours. The ethanol was removed in vacuo, water was added, and the pH of the solution was adjusted to 5 with glacial acetic acid. The aqueous layer was extracted with ethyl acetate and the combined organic extracts were dried over anhydrous MgSO$_4$, filtered and concentrated in vacuo. The clear residual oil was further purified by Kugelrohr distillation in vacuo (0.1 Torr). This gave 0.439 g, (72.2%) of $[3\text{-}^3\text{H}]$-t-butyl 3-hydroxybutyrate (9): bp 60-65°C at 0.1 Torr, radiochemical yield, 11.1%, specific activity, 0.0253 mCi/mg, $^1\text{H}$ NMR (250 MHz, CDCl$_3$) $\delta$ 1.2 (d, 3H), 1.4 (s, 9H), 2.4 (s, 3H), 3.1 (s, -OH), 3.9-4.2 (m, 2H), HRMS, calculated for
C₈H₁₆O₃, 160.1099, found (M⁺) 160.1095, calculated for
¹³C₁C⁷H₁₆O₃, 161.1132, found (M⁺,¹³C) 161.1129.

[3-³H]-t-Butyl 3-mesyloxybutyrate (10).

Triethylamine (338 mg, 3.34 mmol) was added to a stirred solution of
[3-³H]-t-butyl 3-hydroxybutyrate (9) (0.435 g, 2.71 mmol) in 16 mL
dry CH₂Cl₂ at room temperature. The solution was stirred at room
temperature for 10 min, cooled in an ice bath to 0 °C, and
methanesulfonyl chloride (0.382 g, 3.34 mmol) added dropwise. After
stirring at room temperature for a further 1 hour, the reaction mixture
was carefully poured into 1 M NaHCO₃ and the aqueous and organic
layers separated. The aqueous layer was further extracted with CH₂Cl₂
and the combined organic extracts washed once with water, dried over
anhydrous MgSO₄, filtered and concentrated. The residual oil was
further purified by Kugelrohr distillation. This gave [3-³H]-t-butyl 3-
mesyloxybutyrate (10), 0.622 g, (96.5%), ¹H NMR (250 MHz,
CDCl₃) δ 1.4 (s, 9H), 1.5 (s, 3H), 2.5-2.7 (m, 2H), 3.0 (s, 3H), 5.0-
5.2 (m, 1H), HRMS, calculated for C₈H₁₅O₂, 143.0690; found, (M⁺,-
MsO) 143.0694, calculated for ¹³C₁C⁷H₁₅O₂, 144.0724; found
(M⁺,¹³C,-MsO) 144.0718.
[3-\textsuperscript{3H}]-Crotonic acid (11).

1,8-Diazabicyclo-[5.4.0]-undec-7-ene (DBU) (796 mg, 5.23 mmol) was added dropwise to a stirred solution of [3-\textsuperscript{3H}]-t-butyl 3-mesyloxybutyrate (10) (0.622 mg, 2.6 mmol) in 25 mL dry CH\textsubscript{2}Cl\textsubscript{2}. The reaction mixture was stirred at room temperature for 24 hours before being quenched by the addition of 20 mL of 1 M acetic acid. The aqueous layer was extracted with CH\textsubscript{2}Cl\textsubscript{2} and the combined organic extracts washed with water, dried over anhydrous MgSO\textsubscript{4}, filtered and concentrated in vacuo. The residual oil was purified by Kugelrohr distillation. This gave 0.37 g, (2.6 mmol) of [3-\textsuperscript{3H}]-t-butyl crotonate, \textsuperscript{1}H NMR (90 MHz, CDCl\textsubscript{3}) (trans isomer) \textdelta 1.5 (s, 9H), 1.82 (dd, \textsuperscript{3}J\textsubscript{HH}=7.7 Hz, \textsuperscript{4}J\textsubscript{HH}=2.5 Hz, 3H), 5.74 (dd, \textsuperscript{3}J\textsubscript{HH}=16.7 Hz, \textsuperscript{4}J\textsubscript{HH}=2.5 Hz, 1H), 6.7-7.0 (m, 1H), HRMS, calculated for C\textsubscript{8}H\textsubscript{14}O\textsubscript{2}, 142.0993; found (M\textsuperscript{+}) 142.0994, calculated for C\textsubscript{13}C\textsubscript{1}C\textsubscript{7}H\textsubscript{14}O\textsubscript{2}, 143.1027; found (M\textsuperscript{+}, C\textsubscript{13}) 143.1028.

The tritiated t-butyl crotonate was dissolved in 25 mL of freshly distilled benzene and p-toluenesulfonic acid (84 mg, 0.44 mmol) added. The flask was fitted with a Dean-Stark trap and condenser and the whole warmed at reflux for 1 hour. The reaction mixture was cooled to room temperature and the benzene removed in vacuo. The white, crystalline solid was further purified by passage through a CC-4 silica gel column
(15 g, eluant 5%-20% ethyl acetate/hexane). This gave 0.189 g, (84%),
[3-3H]-crotonic acid (11) as a white solid, 1H NMR (90 MHz, CDCl3)
(trans isomer) δ 1.90 (dd, 3J_HH=7.7 Hz, 4J_HH=2.6 Hz, 3H), 5.82
(dd, 3J_HH=16.7 Hz, 4J_HH=2.6 Hz, 1H), 6.7-7.1 (m, 1H).

[3-3H]-(+)-10-Dicyclohexylsulfamoyl-L-isobornyl crotonate
(12).54

Oxalyl chloride (290 mg, 2.29 mmol) was added dropwise to a stirred
solution of [3-3H]-crotonic acid (11) (189.7 mg, 2.20 mmol) in 10 mL
dry benzene at room temperature. The solution was stirred at room
temperature for 24 hours and then at 40 °C for 1 hr. Silver cyanide (294
mg, 2.20 mmol) and (+)-10-dicyclohexyl-sulfamoyl-L-isoborneol (665
mg, 1.67 mmol) were added, the flask fitted with a reflux condenser,
and warmed at reflux for 72 h. After 72 h no chiral auxillary remained as
shown by TLC (15% ethyl acetate/hexane). The reaction mixture was
cooled to room temperature, filtered through Celite and the benzene
removed in vacuo. The residue was purified by flash chromatography
(3.0 cm column, eluant 5%-20% ethyl acetate/hexane). This gave 232
mg, (29.8%) of [3-3H]-(+)-10-dicyclohexylsulfamoyl-L-isobornyl
crotonate (12), 1H NMR (250 MHz, CDCl3) (trans isomer) δ 0.85 (s,
3H), 1.0 (s, 3H), 1.1-2.0 (m, 25H), 2.59-2.7 (d, J=14 Hz, 2H), 3.1-
3.5 (m, 4H), 4.9 (d, J=3.8 Hz, 1H), 5.1 (d, J=5.1 Hz, 1H) 5.82 (dd, 3\text{J}_{HH}=16.7 \text{ Hz}, 4\text{J}_{HH}=2.6 \text{ Hz}, 1H), 6.8-7.1 (m, 1H).

[3-\text{H}]-(+)-10-Dicyclohexylsulfamoyl-L-isobornyl-3-methylpentanoate (13).\text{55,56,57}

Ethyllithium (1.09 mmol) was added dropwise to a stirred solution of tetra-(tri-n-butylphosphine) copper (I) iodide (424 mg, 0.27 mmol) in 7 mL dry ether at -78 °C. The solution was stirred at -78 °C for 30 min before boron trifluoride etherate (154 mg, 1.09 mmol) was added. After stirring at -78 °C for a further 30 min, the tritiated crotonate (12) (225 mg, 0.49 mmol) was added as a solution in 2 mL ether/1mL THF and the reaction mixture stirred for 2 h at -78 °C. After 2 hrs the reaction mixture was quenched by the addition of 7 mL NH₄Cl (aq) and the aqueous layer extracted with ether. m-Chloroperoxybenzoic acid (245 mg, 1.42 mmol) was added to the combined ether extracts and the solution stirred for 10 min, filtered through Celite and washed with 1 M NaOH until colorless. The ether extracts were further dried over anhydrous MgSO₄, filtered, and concentrated in vacuo. The residue was purified by flash chromatography (3.0 cm column, elutant 5%-20% ethyl acetate/hexane). This gave [3-\text{H}]-(+)-10-dicyclohexylsulfamoyl-L-isobornyl-3-methylpentanoate (13), 173 mg, (71.5%) as a white solid, ¹H NMR (250 MHz, CDCl₃) δ 0.85 (s,3H), 0.95 (s, 3H), 1.1-2.4 (m,
34H), 2.55-2.7 (d, J=14 Hz, 2H), 3.1-3.4 (m, 4H), 4.85-4.95 (dd, J=5.1 Hz, J=3.8 Hz, 1H).

**Ethyllithium solution in ether.**

A solution of ethyl bromide (11 g, 0.1 mol) in 20 mL of ether was added dropwise to a solution of lithium (1.72 g, 0.24 mol) in 30 mL ether cooled to below 10 °C. After the addition was complete the reaction mixture was stirred at 0 °C for 3h and stored at 4 °C. Titration against diphenylacetic acid in THF with 1,10-phenanthroline as an indicator gave an average molarity of 1.365.

[3-^3H]-2-Bromo-(-)-10-dicyclohexylsulfamoyl-L-isobornyl-3-methyl pentanoate (14).\(^{53}\)

Butyllithium (0.595 mmol) was added dropwise to a stirred solution of di-isopropylamine (60 mg, 0.595 mmol) in 6 mL dry THF between 0 °C and -10 °C. The solution was stirred for 30 min before being cooled to -78 °C. A solution of (13) in 4 mL of dry THF was added and the whole stirred at -78 °C for 1 h. Trimethylsilyl chloride (105.8 mg, 0.974 mmol) was added and the reaction mixture stirred for 45 min before recrystallised and dried N-bromosuccinimide (105.9 mg, 0.595 mmol) was added. The reaction mixture was stirred at -78 °C for 5 h. After 5h the reaction was quenched by the addition of NH\(_4\)Cl (aq). The aqueous layer was extracted with ether, the combined extracts were washed with
water, sodium chloride, dried over anhydrous MgSO₄, filtered and concentrated in vacuo. The residue was purified by flash chromatography (2.0 cm column, eluant 4%-20% ethyl acetate/hexane). This gave 118 mg of the tritiated bromide (14) as a white solid, ¹H NMR (250 MHz, CDCl₃) δ 1.9 (s, 3H), 1.0 (s, 3H), 1.1-2.2 (m, 31H), 2.55-2.72 (d, J=14 Hz, 2H), 3.2-3.4 (m, 4H), 4.1-4.2 (d, J=7.6 Hz, 2H), 4.9-5.05 dd, J=5.1 Hz, J=3.8 Hz, 1H).

[3-³H]-2-Azo-(+)-10-dicyclohexylsulfamoyl-L-isobornyl-3-methyl pentanoate (15).yükle

A mixture of sodium azide (30.7 mg, 0.472 mmol) and the tritiated bromo ester (14) (90.3 mg, 0.157 mmol) in 3 mL DMF was stirred at room temperature for 3 days and periodically monitored by TLC (10% ethyl acetate/hexane). After 3 days the reaction mixture was quenched with water and extracted with ether. The combined ether extracts were washed with water, and saturated sodium chloride. They were then dried over anhydrous MgSO₄, filtered and concentrated in vacuo. The residue was purified by flash chromatography (2.0 cm column, eluant 5%-15% ethyl acetate/hexane). This gave 72 mg, (85%) of the tritiated azide.(15), ¹H NMR (250 MHz, CDCl₃) δ 0.9 (s, 3H), 0.95 (s, 3H), 1.1 (s, 3H), 1.15-2.2 (m, 28H), 2.6-2.75 (d, J=14 Hz, 2H), 3.1-3.45 (m, 4H), 3.8-3.9 (d, J=7.6 Hz, 2H), 5.0-5.2 (dd, J=3.8 Hz, J=5.1 Hz, 1H).
[3-3H]-2-Amino-(+)-10-dicyclohexylsulfamoyl-L-isobornyl-3-methyl pentanoate (16).  

A mixture of the tritiated azide (15) (72.1 mg, 0.133 mmol) and 5% palladium/barium sulfate catalyst (50.4 mg) in 5 mL absolute ethanol was stirred at room temperature for 24 h under a hydrogen atmosphere (balloon pressure). After 24 h the reaction mixture was filtered through Celite to remove the catalyst and the residue washed with CHCl₃. The filtrate was concentrated in vacuo and the residue purified by flash chromatography (2.0 cm column, 5%-50% ethyl acetate/hexane). This gave 53 mg, (78%) of [3-3H]-2-amino-(+)-dicyclohexylsulfomanyl-L-isobornyl-3-methylpentanoate (16), ¹H NMR (250 MHz, CDCl₃)  δ 0.9 (s, 3H), 0.95 (s, 3H), 1.0 (s, 3H), 1.1 (s, 3H), 1.15-2.2 (m, 25H), 2.5-2.75 (dd, J=14 Hz, 2H), 2.95-3.15 (d, J=6.4 Hz, 2H), 3.2-3.35 (m, 4H), 4.7-4.9 (dd, J=7.6 Hz, J=3.8 Hz, 1H).

[3-3H]-L-Alloisoleucine (17).

A mixture of the tritiated amino ester (16) (53.3 mg, 0.103 mmol) and barium hydroxide (57.5 mg, 0.18 mmol) in 4 mL water/2 mL ethanol was heated at reflux for 24 h. After 24 h the reaction mixture was cooled to room temperature, filtered through Celite and concentrated in vacuo. The residue was redissolved in 10 mL water and treated with 8 mg (NH₄)₂CO₃. The milky white solution was filtered and concentrated
before being redissolved in water and treated with a second batch of 
(NH4)2CO3. The solution was filtered one more time to remove any 
remaining BaCO3 and then stirred in a water bath at 60-65 °C for 1 hr. 
After 1 h the water was removed in vacuo and the white residual solid 
purified by flash chromatography on cellulose (2.0 cm column, eluant 
butanol:acetic acid:water, BAW, 12:3:5). This gave 16 mg of [3-3H]-L-
alloisoleucine (17), whose specific activity was 11 uCi/mg, 1H NMR 
(250 MHz, D2O) δ 1.0 (m, 6H), 1.3-1.5 (m, 2H), 2.0 (m, 1H), 3.6 (d, 
J=0.36 Hz, 1H), 4.9 (s, D2O), 13C NMR (75.46 MHz, D2O, 1 drop 
methanol as standard) δ 11.17, 13.43, 25.65, 35.78, 48.9 (MeOH), 
58.58, 174.89.

**Synthesis of (1-13C)-2-Amino-3-ethyl-n-pentanoic acid (20)** 
(Scheme X).

(1-13C)-2-Hydroxy-3-ethylvaleronitrile (18). 
Absolute ethanol was added to a solution of 2-ethylbutyraldehyde 
(0.73g, 7.3 mmol) and sodium hydrogen sulfite (0.889g, 8.5 mmol) in 
water (6 mL) until the solution became homogenous and clear. After 
stirring for 1 hour at room temperature (13C)-potassium cyanide was 
added and the reaction mixture was stirred at room temperature for 3 h. 
The ethanol was removed in vacuo and the organics extracted with ether.
The combined ether extracts were washed with water, and saturated NaCl. They were then dried over Na2SO4 and concentrated in vacuo. This gave 1.14 g of the crude cyanohydrin (18) as a clear oil.

(1-13C)-2-Amino-3-ethylvaleronitrile (19).

Crude (1-13C)-2-hydroxy-3-ethylvaleronitrile (18) was dissolved in concentrated ammonium hydroxide (16.8 mL) and absolute ethanol added until the solution became homogenous and clear. The reaction mixture was stirred at room temperature overnight, the ethanol was removed in vacuo, and the organics taken up in ether. The combined organic extracts were washed with water, and saturated NaCl. They were then dried over Na2SO4 and concentrated in vacuo. This gave 0.79 g of the crude aminonitrile (19) as a clear oil.

(1-13C)-2-Amino-3-ethyl-n-pentanoic acid (20).

A mixture of 0.79 g of (19) in water (48 mL) and barium hydroxide (7.98 g, 25.2 mmol) was heated at reflux for 18 h. The solution was cooled to room temperature and concentrated in vacuo. The residue was redissolved in water and filtered through Celite. The filtrate was treated twice with solid (NH4)2CO3 to remove any excess barium hydroxide as insoluble barium carbonate. The filtrate was then stirred in a water bath at 60-65 °C for 1 h. Concentration in vacuo gave a residue which was purified by cellulose chromatography to give 336 mg (26%) of (1-13C)-
2-amino-3-ethyl-n-pentanoic acid (20) as a white solid, Rf (BAW 12:3:5) =0.58, \textsuperscript{1}H NMR (250 MHz, D\textsubscript{2}O) \( \delta \) 0.7-0.8 (t, 6H), 1.0-1.3 (m, 4H), 1.3-1.5 (m, 1H), 1.6-1.75 (m, 2H, NH\textsubscript{2}), 3.6 (d, J=1.2 Hz, 1H), 4.68 (s, D\textsubscript{2}O), \textsuperscript{13}C NMR (250 MHz, D\textsubscript{2}O, 1 drop MeOH as standard) \( \delta \) 11.1, 11.4, 21.9, 22.3, 22.4, 42.7, 49.0 (MeOH), 56.2, 56.9, 174.8 (enriched).

Synthesis of (2-\textsuperscript{13}C,\textsuperscript{15}N)-DL-Isoleucine/(2-\textsuperscript{13}C,\textsuperscript{15}N)-DL-allo-isoleucine (25) (Scheme X)

(1-\textsuperscript{13}C)-2-Methylbutyronitrile (21).\textsuperscript{59}

(\textsuperscript{13}C)-Sodium cyanide (2.0 g, 40.8 mmol) was added to a solution of 2-bromobutane (3.72 g, 27.2 mmol) in 50 mL HMPA at room temperature. The reaction mixture was stirred at room temperature for 48 h and then connected via a U-tube, cooled in liquid nitrogen, to a vacuum pump and distilled at room temperature. This gave 0.913 g, (40.0%) of (1-\textsuperscript{13}C)-2-methylbutyronitrile (21) as a colorless liquid, \textsuperscript{1}H NMR (250 MHz, CDCl\textsubscript{3}) \( \delta \) 1.05 (s, 3H), 1.3 (s, 3H), 1.55 (m, 2H), 2.5-2.65 (m, 1H), \textsuperscript{13}C NMR (62.89 MHz, CDCl\textsubscript{3}) \( \delta \) 11.35, 11.39, 26.59, 27.24, 27.27, 27.46, 122.91 (enriched).
(1-\textsuperscript{13}C)-2-Methylbutyraldehyde (22).\textsuperscript{60}

DIBAL (13.43 mL, 13.4 mmol) was added dropwise to a stirred solution of (1-\textsuperscript{13}C)-2-methylbutyronitrile (21) (0.913 g, 11 mmol) in dry CH\textsubscript{2}Cl\textsubscript{2} (25 mL) at room temperature. After 1.5 h the reaction mixture was cautiously poured into 5\% H\textsubscript{2}SO\textsubscript{4} (50 mL) and the aqueous and organic layers separated. The aqueous layer was further extracted with dichloromethane and the combined organic extracts were dried over anhydrous MgSO\textsubscript{4}, filtered, and concentrated by distillation at atmospheric pressure. The crude (1-\textsuperscript{13}C)-2-methylbutyraldehyde (22) distilled at 90-92\(^{\circ}\)C, \textsuperscript{1}H NMR (250 MHz, CDCl\textsubscript{3}) \(\delta 0.9-1.0\) (t, 3H), 1.1-1.15 (t, 3H), 1.3-1.8 (m, 2H), 2.2-2.35 (m, 1H), 9.25-9.95 (d, \textsuperscript{1}J\textsubscript{CH}=40.9 Hz, 1H).

(2-\textsuperscript{13}C)-2-Hydroxy-3-methylvaleronitrile (23).

Ethanol was added to a stirred solution of (1-\textsuperscript{13}C)-2-methylbutyraldehyde (22) (0.946g, 11mmol) and sodium hydrogen sulfite (1.3g, 12.5 mmol) in water (10mL) until the solution became homogenous and clear. After stirring at room temperature for 1 h, potassium cyanide (0.73g, 11.2 mmol) was added and the reaction mixture stirred at room temperature for 3 hours. The ethanol was removed in vacuo and the aqueous layer extracted with ether. The combined ether extracts were washed with water, and saturated NaCl.
They were then dried over MgSO₄, filtered, and concentrated in vacuo. This gave 0.76g, (61%) of crude (2-¹³C)-2-hydroxy-3-methylvaleronitrile (23).

(2-¹³C, ¹⁵N)-2-Amino-3-methylvaleronitrile (24).

Ethanol was added to a solution of (2-¹³C)-2-hydroxy-3-methylvaleronitrile (23) (0.76g, 6.72 mmol) in (¹⁵N)-ammonium hydroxide (4.08 mL of a 3.3N solution) until the solution became homogenous and clear. The reaction mixture was stirred at room temperature for 18 h, the ethanol removed in vacuo and the aqueous layer extracted with ether. The combined ether extracts were washed with water, saturated NaCl, dried over MgSO₄, filtered and concentrated. This gave 0.721g of crude (2-¹³C, ¹⁵N)-2-amino-3-methylvaleronitrile (24).


Barium hydroxide (8.22g, 26 mmol) was added to a solution of (2-¹³C, ¹⁵N)-2-amino-3-methylvaleronitrile (24) (0.721g, 6.32 mmol) in water (50 mL). The flask was fitted with a reflux condenser and the mixture refluxed for 18 h. The reaction mixture was then cooled to room temperature, filtered through Celite and the filtrate treated twice with (NH₄)₂CO₃ to remove barium as insoluble barium carbonate. The resulting clear filtrate was stirred at 60-65°C in a water bath for 1h and finally concentrated in vacuo. Chromatography of the residue on a
cellulose column (2.0 cm column, eluant BAW 12:3:5) gave, 0.354g, (42%) of doubly-labeled amino acid (25) as a white solid, $^1$H NMR (250 MHz, CDCl$_3$) δ 0.6-0.75 (m, 6H), 1.0-1.25 (m, 2H), 1.65-1.85 (m, 1H), 3.4 (ddd, $^1$J$_{CH}$=162 Hz, $^3$J$_{HH}$=25 Hz, $^2$J$_{NH}$=6.4 Hz, 1H), $^{13}$C NMR (62.89 MHz, CDCl$_3$) δ 11.23, 11.29, 13.46, 14.82, 22.83, 24.61, 25.63, 35.99, 49.00 (MeOH), 58.50, 58.59, 59.58, 59.67 ($^{13}$C, $^{15}$N, $^1$J$_{CN}$=68 Hz), 180.62.

Synthesis of (5-2H$_1$, 6-2H$_1$)-Coronamic acid (30) (Scheme XII).

(Z)-Dicarbomethoxybut-2-ene-1,4-diol (26).$^6$1

Pyridine (4.01 mL, 50 mmol) was added to a stirred solution of cis-but-2-ene diol (2.0 g, 23 mmol) in anhydrous ether (20 mL) at -3°C. After stirring for 15 minutes at -3°C, methyl chloroformate (3.6 mL, 46 mmol) was added and the reaction mixture allowed to warm to room temperature over 2h and 45 minutes. The reaction mixture was poured into water and the aqueous layer extracted with ether. The combined ether extracts were washed with 1N HCl, water, and saturated NaCl. The organic layer was then dried over Na$_2$SO$_4$, filtered and concentrated in vacuo. This gave 3.5g, (74%) of (Z)-dicarbomethoxybut-2-ene-1,4-diol (26) as a clear liquid, which crystallised on storing at 4°C.
overnight. $^1$H NMR (250 MHz, CDCl$_3$) $\delta$ 3.8 (s, 6H), 4.75 (d, $J$=7.5 Hz, 4H), 5.75-5.85 (m, 2H).

1,1-Dicarbomethoxy-2-vinylcyclopropane (27).\textsuperscript{62} A flame dried and cooled 500 mL round bottomed flask was charged with (Z)-dicarbomethoxybut-2-ene-1,4-diol (26) (6.0 g, 29.4 mmol) and dimethyl malonate (3.36 mL, 29.4 mmol) in 200 mL freshly distilled THF under an argon atmosphere. The solution was frozen at -198°C using a liquid nitrogen bath and tetrakis(triphenylphosphine)-palladium (0) (676 mg, 2 mol %) quickly added against a flow of argon gas. The reaction mixture was allowed to thaw and then stirred at room temperature overnight. The THF was removed in vacuo and the residue purified by flash chromatography, (4.0 cm column, eluant 5%-15% ethyl acetate/hexane). This gave 1.52 g, (28%) of 1,1-dicarbomethoxy-2-vinylcyclopropane (27) as a clear liquid, R$_f$ 0.6 (10% ethyl acetate/hexane, silica). $^1$H NMR (250 MHz, CDCl$_3$) $\delta$ 1.60 (dd, $J$=8 Hz, 7Hz, 1H), 1.72 (dd, $J$=8Hz, 7Hz, 1H), 2.60 (dd, $J$=14 Hz, 12 Hz, 1H), 3.74 (s, 6H), 5.14 (dd, $J$=8Hz, 7Hz, 1H), 5.25-5.45 (m, 2H).

(6-$^2$H$_1$,7-$^2$H$_1$)-1,1-Dicarbomethoxy-2-ethylcyclopropane (28).\textsuperscript{63,64,65} A flame dried and cooled 1000 mL three-necked round bottomed flask, fitted with a reflux condenser and dropping funnel, was charged with
1,1-dicarboxemethoxy-2-vinylcyclopropane (27) (1.0 g, 5.43 mmol) and dipotassium azodicarboxylate (68.9 g, 355 mmol) in 100 mL methanol-O-d. Acetic acid-O-d (30.1 mL) was added dropwise. The original deep yellow solution turned pale yellow and the methanol began to reflux. The acetic acid was added at such a rate as to maintain reflux. Once the addition was complete the reaction mixture was stirred at room temperature. After 2.5 h the pale yellow solution had become white. After stirring for 5.5 h the reaction mixture was transferred to a 500 mL round bottomed flask and the methanol removed in vacuo. The residue was dissolved in water (50 mL) and extracted with ether. The combined ether extracts were washed with 1N NaHCO₃, water, saturated NaCl, dried over anhydrous MgSO₄, filtered and concentrated in vacuo. The residue was purified by flash chromatography on silica (4.0 cm column, eluant 5%-15% ethyl acetate/hexane). This gave 0.8 g, (80%) of (6-²H₁, 7-²H₁)-1,1-dicarboxemethoxy-2-ethylcyclopropane (28), as a clear liquid, ¹H NMR (250 MHz, CDCl₃) δ 0.9 (m, 2H), 1.2-1.3 (m, 1H), 1.3-1.5 (m, 2H), 1.8-2.0 (m, 1H), 3.7 (s, 3H), 3.8 (s, 3H).

(6-²H₁,7-²H₁)-1-Carboxy-1-carboxemethoxy-2-ethylcyclopropane (29). Potassium hydroxide (0.25 g, 4.4 mmol) was added to a stirred solution of (6-²H₁,7-²H₁)-1,1-dicarboxemethoxy-2-ethylcyclopropane (28) in
methanol (18 mL) at room temperature. The reaction mixture was stirred at room temperature for 4 days. The methanol was removed in vacuo and the residue dissolved in water (20 mL). The aqueous layer was extracted with ethyl acetate and the combined ethyl acetate extracts washed with saturated NaCl, filtered, and concentrated in vacuo. This gave 0.41 g, (85%) of (6-²H₁, 7-²H₁)-1-carboxy-1-carbomethoxy-2-ethylcyclopropane (29) as a clear liquid, ¹H NMR (250 MHz, CDCl₃) δ 0.95 (m, 2H), 1.4-1.7 (m, 1H), 1.7-1.8 (m, 1H), 1.9-2.15 (m, 2H), 3.7 (s, 3H).

(5-²H₁, 6-²H₁)-Coronamic acid (30).¹⁷

Ethyl chloroformate (0.68 mL, 7.1 mmol) was added dropwise to a stirred solution of (6-²H₁,7-²H₁)-1-carboxy-1-carboxymethyl-2-ethyl cyclopropane (29) (0.41 g, 2.35 mmol) and N-ethyl-di-isopropylamine (0.82 mL, 4.72 mmol) in acetone (5 mL) at 0°C. The solution was stirred for at 0°C for 30 minutes, sodium azide (0.76 g, 11.82 mmol), as a solution in water (5 mL) was added, and the reaction mixture was stirred at 0°C for 2 h. The acetone was removed in vacuo and the aqueous layer extracted with ethyl acetate. The combined ethyl acetate extracts were washed with water, saturated NaCl, dried over MgSO₄, filtered and concentrated in vacuo. This gave 0.45 g of the crude acyl azide as a pale yellow oil. The crude acyl azide was dissolved in toluene
(6 mL) and warmed at reflux for 30 minutes. The reaction mixture was cooled to room temperature and the toluene removed in vacuo. The brown residual oil was dissolved in 6N HCl (6 mL) and warmed at reflux for 6 h. The solution was cooled to room temperature and the acid removed in vacuo. The residue was redissolved in water (7 mL) and loaded onto a strongly acidic ion-exchange resin (Dowex 50W-X8, H+-form, 15 mL). The amino acid fraction was eluted with 2N NH4OH (60 mL) and concentrated in vacuo. This gave 0.250 g, (40%) of (5.2H1, 6-2H1)-coronamic acid (30) as a pale yellow solid. TLC (BAW, 4:1:2, cellulose, Rf = 0.69), ^1H NMR (300 MHz, D2O) δ 0.75 (m, 2H), 1.15 (d, ^3J_HH=1.2 Hz, 2H), 1.3-1.4 (m, 1H), 1.4-1.5 (m, 1H), ^13C NMR (62.89 MHz, D2O) δ 12.13 12.44 12.73 (t), 17.05, 19.26 19.57 19.91 (t), 27.61, 39.85, 48.93 (MeOH), 174.11.

Maintenance of *Pseudomonas syringae* pv. *glycinae* (PDDCC # 4182).

*Pseudomonas syringae* pv. *glycinae* (PDDCC # 4182) was obtained in lyophilised form as a gift from Prof. Carol Bender of Oklahoma State University. The lyophile was suspended in 1.0 mL of sterile water and used to inoculate freshly made and autoclaved King's B medium. To prepare King's B medium Difco proteose peptone # 3 (0.4 g), glycerol
(0.2 g), anhydrous K₂HPO₄ (30 mg), and MgSO₄·7H₂O (30 mg) were dissolved in 20 mL water and the pH adjusted from 7.3 to 7.2 with 1N HCl. Five mL of this broth was pipetted into each culture tube. The inoculated tubes were incubated at 25°C, 250 rpm for 24 h. After 24 h the medium was used either to inoculate a batch of freshly made mannitol-glutamate-yeast-agar slants or seed medium in preparation for a fermentation. The composition of mannitol-glutamate-yeast-agar slants was as follows: mannitol, (10 g), L-glutamic acid, sodium salt, (2.0 g), KH₂PO₄, (0.5 g), NaCl, (0.2 g), MgSO₄·7H₂O, (0.2 g), yeast extract, (0.25 g), and agar, (15.0 g, 1.5%) were dissolved in 1.0 L of water and the pH adjusted to 7.0. After heating to dissolve the agar, the solution was pipetted into culture tubes and sterilised. After inoculation, the slants were incubated at room temperature for 3 days before being stored at 4°C prior to making lyophiles or setting up a fermentation.

Lyophilisation of *Pseudomonas syringae* pv. *glycinae* (PDDCC # 4182).⁶⁹

Two mL of freshly made mist. desiccans was added to a slant having significant bacterial growth. A sterile loop was used to detach the organism from the agar surface and 0.3 mL samples were pipetted into six sterile lyophilisation ampoules. The ampoules were attached to the
lyophilisation apparatus, freeze-dried overnight, sealed in vacuo, and stored at 4°C. Mist desiccans was composed of two solutions: A) glucose, (1.0 g), Nutrient broth, (43 mg), and water (3.3 mL). This solution was sterilized by filtration. B) Sterile horse serum, reconstituted by adding 10 mL of sterile water to one ampoule of lyophilised horse serum. A) and B) were mixed together and the solution of mist desiccans stored at 4°C prior to use.

**Fermentation of *P. syringae pv glycinae* (PDDCC # 4182).**

*Ps. syringae* growing on one mannitol slant was suspended in 1.0 mL of sterile water and the suspension used to inoculate 100 mL of seed medium, which consisted of: yeast extract, (500 mg), glucose, (500 mg), MgSO4.7H2O, (20 mg), KH2PO4, (410 mg), K2HPO4, (360 mg), dissolved in 100 mL water and the pH adjusted to 6.8. The inoculated seed medium was incubated at 25°C, 250 rpm for 24 h. After 24 h, mature seed medium broth was used to inoculate (10 mL per flask) 10, 1 L flasks each containing 200 mL of production medium. Production medium consisted of two solutions: A) glucose, (20.0 g), MgSO4.7H2O, (0.4 g), KH2PO4, (8.2 g), K2HPO4, (7.2 g), dissolved in 2.0 L of water, and adjusted to pH 6.8 with 1N NaOH. This solution was sterilised by autoclaving at 121°C for 20 mins. After
this solution had cooled to room temperature one mL of solution B) consisting of: NH4Cl, (2.0 g), biotin 2.0 mg, dissolved in 10 mL water was added to each production medium flask through a sterile filter. The inoculated fermentation flasks were incubated at 18 °C, 200 rpm and monitored for coronatine production by HPLC. HPLC traces showed that coronatine production starts on day 1 and reaches a maximum on day 5. After 5 days the cells were removed by centrifugation at 8000 rpm for 40 min. The supernatant broth was filtered through glass wool, and stirred over a mixture of activated charcoal (20 g) and Celite (20 g) for 30 min before being loaded onto a column and washed with 400 mL of distilled water. The column was then eluted with ca 300 mL of acetone. The acetone elutant was concentrated five fold, adjusted to pH 2.5 with 2.5 M H2SO4, and vigorously extracted three times with ethyl acetate. The combined ethyl acetate extracts were dried over anhydrous MgSO4, filtered through Celite and concentrated in vacuo. The acidic residue was dissolved in 2.0 mL methanol and treated with excess diazomethane. The excess diazomethane was blown off in the hood under a stream of nitrogen and the methanol removed in vacuo. The residue was further purified by preparative thin layer chromatography (0.75 mm non-commercial silica gel plates, eluant 10 % isopropanol/hexane). In order to obtain an adequate purification it was necessary to develop the plate 2
or 3 times. This gave, on average, 10 mg of coronatine as its methyl ester derivative.

Conversion of coronatine methyl ester to dihydrocoronatine methyl ester (31).

Lithium tri-tert-butoxyaluminum hydride (24.33 mg, 0.095 mmol as a solution in 1.2 mL THF) was added dropwise to a stirred solution of coronatine methyl ester (14.6 mg, 0.43 mmol) in 1.2 mL dry THF, at room temperature. The reaction mixture was stirred at room temperature while being monitored by TLC (10% iPrOH/Hexane). After 4 hours TLC showed that no starting material was left. The reaction was quenched by the addition of 1M acetic acid, the THF was removed in vacuo and the aqueous layer extracted with ethyl acetate. The combined organic extracts were dried over Na2SO4, filtered through Celite and concentrated in vacuo. This gave 7.2 mg, (49.7%) of dihydrocoronatine methyl ester (31) as a yellow oil, 13C NMR (75.45 MHz, CDCl3) δ 11.71, 13.7, 20.7, 20.9, 23.3, 24.4, 28.1, 28.8, 31.4, 33.3, 36.4, 37.7, 42.4, 52.6, 75.2, 136.5, 137.9, 169.2, 171.5.
Administration of labeled precursors to \textit{P syringae} pv \textit{glycinae} (PDDCC \# 4182).

Precursors were pulse fed to the producing organism after 24, 48, and 72 hours had elapsed since the start of the fermentation. Both stable and radiolabeled precursors were dissolved in a suitable volume of Milli-Q water and administrated to the fermentation flasks through a Millipore filter (pore size 0.22 \textmu m). Typically, for precursors labeled with stable isotopes, 100 mg were fed each day. Coronatine was isolated after the fermentation had proceeded for five days, converted to its methyl ester and purified by preparative thin layer silica chromatography.

Polyoxin Project.

Fermentation of \textit{Streptomyces cacaoi} var \textit{asoensis} (ATCC \# 19094).\textsuperscript{37}

One homemade lyophile of \textit{S. cacaoi} var \textit{asoensis} (ATCC \# 19094) was used to inoculate a culture tube containing 4 mL of freshly made seed medium, consisting of soluble starch 1\%, D-glucose 1\%, soybean meal 2\%, yeast extract 1\%, sodium nitrate 0.2\%, and potassium phosphate (dibasic) 0.2\%. This tube was incubated at 28\textdegree C, 210 rpm, for 24 hours and then used to inoculate a 500 mL flask containing 100 mL of seed medium. The freshly inoculated seed medium was incubated
at 28°C, 210 rpm, for 24 hours before being used to inoculate (5 mL per flask) fifteen 1.0 L flasks each containing 120 mL of production medium. Production medium consisted of: soluble starch 9%, D-glucose 1%, soybean meal 2%, yeast extract 4%, sodium nitrate 0.2%, and potassium phosphate (dibasic) 0.2%. The above ingredients were dissolved in 2000 mL of Milli-Q water and the pH adjusted to 7.0 with 1N NaOH. The inoculated production medium flasks were incubated at 28°C, 210 rpm and monitored for polyoxin A production by HPLC (25.0 cm Partisphere C-18 reverse phase column, eluant 10% acetonitrile, 90% water, and 1% acetic acid, flow rate = 1.0 mL/min). Using this system polyoxin A had a retention time 7.0 min. Our HPLC analysis showed that polyoxin A production started after 24 hours and reached a maximum after 72 hours. The fermentation was stopped after it had proceeded for 72 hours and polyoxin A isolated.

Isolation of polyoxin A from S. cacaoi var asoensis (ATCC #19094).37

Seventy two hour old fermentation broth was centifuged at 8000 rpm, for 40 minutes at 5°C to remove the cellular growth. The pH of the collected supernatant was adjusted to pH 2.0 with 1N HCl before being adsorbed onto a mixture of activated charcoal and Celite (50g/40g). The
mixture was stirred at room temperature for approximately one hour before being loaded into a chromatography column and washed with water (100 mL). The crude polyoxin complex was eluted from the charcoal/Celite mixture with 300 mL of acetone to give a yellow solution. The acetone elutant was concentrated in vacuo to give, on average, 1.2 g of the crude polyoxin complex. Further purification was achieved by reverse phase medium pressure liquid chromatography, on a column packed with Merck LiChroprep RP-18 silica (eluant 10% acetonitrile, 90% water, and 1% acetic acid). The column fractions were analysed for polyoxin A by HPLC. The appropriate fractions were collected to give 26-30 mg of crude polyoxin A. Any remaining impurities were removed by repeated cellulose column chromatography (eluant butanol:acetic acid:water; 4:1:2) followed by preparative HPLC on a 10 mm ID, 25 cm long, Altex ODS C-18 column (eluant 10% acetonitrile, 90% water, and 1% acetic acid, flow rate = 3.0 mL/min). This gave 7-15 mg of polyoxin A, TLC, cellulose, BAW 4:1:2, \( R_f = 0.27 \), visualised by UV light and ninhydrin dip reagent (yellow spot).

**Maintenance of *S. cacaoi var asoensis* (ATCC # 19094).**

Mature seed culture was used to inoculate freshly made Difco ISP 2 slants. The slants were incubated at 30°C for 4-5 days, at which time
there was significant growth and the organism had started to sporulate. The mature slants were used either to set up a fermentation or to prepare lyophilisation tubes. One slant was used to make six lyophilisation tubes by an identical method, using mist. desiccans, to that used for *P. syringae pv. glycinea*. The lyophilised tubes were stored at $4^\circ$C before use.

**Administration of labeled precursors to *S. cacaoi* var *asoensis* (ATCC # 19094).**

Precursors were pulse fed to the producing organism after 24, 36, and 48 hours had elapsed since the start of the fermentation. The compounds, labeled with stable isotopes were dissolved in a suitable volume of Milli-Q filtered water and administered to the fermentation flasks through a Millipore filter (pore size 0.22 um). Polyoxin A was isolated after the fermentation had proceeded for 3 days.

**Synthesis of (6-13C)-DL-Isoleucine/(6-13C)-DL-alloisoleucine (Scheme XIII). (5-13C)-2-Methylbutyronitrile (32).**

Butyronitrile (2.0 g, 28.93 mmol) was added dropwise to a stirred solution of commercially available lithium di-isopropylamide (3.71 g,
34.68 mmol) in 50 mL of dry THF at -78°C. The resulting yellow solution was stirred at -78°C for 30 minutes before (13C) methyl iodide (4.13 g, 28.93 mmol) was added. The reaction mixture was stirred at -78°C for a further 1 hour and then allowed to warm to 0°C over 3.5 hours. The reaction mixture was quenched at 0°C by the addition of saturated aqueous NH4Cl (30 mL). The THF was removed in vacuo and the residue extracted with CH2Cl2. The combined organic extracts were washed with water, saturated NaCl, dried over MgSO4, filtered, and concentrated at atmospheric pressure. This gave 2.5 g of crude (5.13C)-2-methylbutyronitrile (32) as a pale yellow liquid, 1H NMR (250 MHz, CDCl3) δ 1.0-1.1 (m, 3H), 1.35 (dd, JCH=125 Hz, JHH=7.9 Hz, 3H), 1.65-1.8 (m, 2H), 3.5-3.65 (m, 1H), 13C NMR (62.89 MHz, CDCl3) δ 11.35, 17.58 (enriched), 26.58, 27.56, 77.0 (CDCl3), 122.91.

(5-13C)-2-Methylbutyraldehyde (33).

Di-isobutyl aluminum hydride (37.2 mL, 37.2 mmol) was added dropwise to a stirred solution of (5-13C)-2-methylbutyronitrile (32) (2.5 g, 29.8 mmol) in dry CH2Cl2 (50 mL) at room temperature. The reaction mixture was stirred at room temperature for 1 hour and quenched by cautiously adding 5% aqueous H2SO4. The aqueous and organic layers were separated and the aqueous layer extracted with
CH₂Cl₂. The combined organic extracts were dried over MgSO₄, filtered through Celite and concentrated at atmospheric pressure. This gave 2 g of crude (5-¹³C)-2-methyl butyraldehyde (33).

(6-¹³C)-2-Hydroxy-3-methylvaleronitrile (34).

Ethanol (50 mL) was added to a solution of (33) and sodium bisulfite (3.53 g, 34.6 mmol) in water (50 mL), until the solution became homogenous and clear. The solution was stirred at room temperature for 1 hour before potassium cyanide (1.99 g, 30.6 mmol) was added and the reaction mixture stirred at room temperature for 3 hours. The ethanol was then removed in vacuo and the aqueous residue extracted with ether. The combined ether extracts were washed with water, saturated NaCl, dried over MgSO₄, filtered and concentrated in vacuo. This gave 2.3 g of crude (6-¹³C)-2-hydroxy-3-methylvaleronitrile (34).

(6-¹³C)-2-Amino-3-methylvaleronitrile (35).

The crude cyanohydrin (34) was dissolved in concentrated NH₄OH (34.6 mL) and ethanol (30 mL) added until the solution became homogeneous and clear. The reaction mixture was then stirred at room temperature for 18 hours. The ethanol removed in vacuo and the aqueous residue extracted with ether. The combined ether extracts were washed with water, saturated NaCl, dried over MgSO₄, filtered and concentrated in vacuo. This gave 1.7 g of the crude aminonitrile (35).

The crude aminonitrile (35) was dissolved in water (100 mL) and barium hydroxide (19.3 g, 61 mmol) added. The reaction mixture was then warmed at reflux for 18 hours. After cooling to room temperature the water was removed in vacuo and the solid white residue redissolved in water (50 mL). This solution was treated twice with solid (NH₄)₂CO₃ to remove any remaining barium as insoluble barium carbonate. The filtrate was stirred in a water bath at 60-65°C for 1 hour before being finally concentrated in vacuo. This gave 0.5 g of the crude amino acid mixture as a white solid. The remaining impurities were removed by repeated cellulose column chromatography (eluant BAW, 4:1:2). This gave 169 mg of the pure amino acid mixture as a flakey white solid, ¹H NMR (250 MHz, D₂O) (1:1 mixture of isoleucine and alloisoleucine) (one diastereomer, iso or alloiso) δ 0.85 (dd, ³JHH=6.9 Hz, ¹JCH=125.3 Hz, 3H), 1.00 (t, ³JHH=6.25 Hz, 3H), 1.36-1.5 (m, 2H), 2.04-2.12 (m, 1H), 3.69 (dd, ³JHH=3.95 Hz, ³JCH=3.92 Hz, 1H), (other diastereomer, iso or alloiso) δ 1.00 (t, ³JHH=6.25 Hz, 3H), 1.25 (dd, ³JHH=7.5 Hz, ¹JCH=125.9 Hz, 3H), 1.36-1.5 (m, 2H), 2.04-2.12 (m, 1H), 3.76 (dd, ³JHH=5.92 Hz, ³JCH=5.90 Hz, 1H), ¹³C NMR (62.89 MHz, D₂O, 1 drop methanol as standard) δ
11.14, 11.21, 13.42 (enriched), 14.79 (enriched), 24.56, 25.61, 35.70, 35.99, 48.98 (methanol), 58.58, 59.66, 174.3, 175.2.

Synthesis of (6-\textsuperscript{13}C,\textsuperscript{2}H\textsubscript{3})-DL-Isoleucine/(6-\textsuperscript{13}C,\textsuperscript{2}H\textsubscript{3})-DL-alloisoleucine (Scheme XIV).

\textsuperscript{(5.13}C,\textsuperscript{2}H\textsubscript{3})-2-methylbutyronitrile (36).\textsuperscript{67}

Butyronitrile (2.36 g, 34.25 mmol) was added dropwise to a stirred solution of commercially available lithium di-isopropylamide (4.39 g, 41 mmol) in dry THF (60 mL) at -78\textdegree C. This solution was stirred at -78\textdegree C for 30 minutes and (\textsuperscript{13}C,\textsuperscript{2}H\textsubscript{3})-iodomethane added. After stirring for a further 1 hour at -78\textdegree C the reaction mixture was allowed to warm to 0\textdegree C over 3.5 hours and quenched by the addition of saturated aqueous NH\textsubscript{4}Cl (40 mL). The THF was removed in vacuo and the aqueous layer extracted with CH\textsubscript{2}Cl\textsubscript{2}. The combined CH\textsubscript{2}Cl\textsubscript{2} extracts were washed with water, saturated NaCl, dried over MgSO\textsubscript{4}, filtered, and concentrated at atmospheric pressure. This gave (5-\textsuperscript{13}C,\textsuperscript{2}H\textsubscript{3})-2-methylbutyronitrile (36), 2.8 g, as a pale yellow liquid, \textsuperscript{1}H NMR (250 MHz, CDCl\textsubscript{3}) \textsuperscript{6} 1.0-1.1 (t, \textsuperscript{3}J\textsubscript{HH}=6.2 Hz, 3H), 1.5-1.8 (m, 2H), 2.5-2.65 (m, 1H), \textsuperscript{13}C NMR (62.89 MHz, CDCl\textsubscript{3}) \textsuperscript{6} 11.35, 17.58 (enriched septet, \textsuperscript{13}C,\textsuperscript{2}H\textsubscript{3}), 26.58, 27.56, 77.0 (CDCl\textsubscript{3}), 122.9.
(5-$^{13}$C, $^{2}$H$_{3}$)-2-Methylbutyraldehyde (37). 60

Di-isobutyl aluminum hydride (40 mL, 42.81 mmol) was added dropwise to a stirred solution of (5-$^{13}$C, $^{2}$H$_{3}$)-2-methylbutyronitrile (36) in dry CH$_{2}$Cl$_{2}$ (60 mL) at room temperature. The reaction mixture was stirred at room temperature for 1 hour and then quenched by cautiously adding 5% aqueous H$_{2}$SO$_{4}$ (50 mL). The aqueous and organic layers were separated and the aqueous layer further extracted with CH$_{2}$Cl$_{2}$. The combined organic extracts were washed with water, saturated NaCl, dried over MgSO$_{4}$, filtered and concentrated at atmospheric pressure. This gave crude (5-$^{13}$C, $^{2}$H$_{3}$)-2-methylbutyraldehyde (37), 2.75 g, as a yellow liquid.

(6-$^{13}$C, $^{2}$H$_{3}$)-2-Hydroxy-3-methylvaleronitrile (38).

Ethanol (60 mL) was added to a stirred solution of (5-$^{13}$C, $^{2}$H$_{3}$)-2-methyl-butyraldehyde (37) (2.75 g, 30.5 mmol) and sodium hydrogen sulfite (4.34 g, 41.7 mmol) in water until the solution became homogeneous and clear. This solution was stirred at room temperature for 1 hour and solid potassium cyanide (2.45 g, 37.6 mmol) added. The reaction mixture was then stirred at room temperature for a further 3 hours and the ethanol removed in vacuo. The aqueous layer was extracted with ether and the combined organic extracts washed with water, saturated NaCl. They were then dried over MgSO$_{4}$, filtered
through Celite and concentrated in vacuo. This gave 1.77 g of crude (6-13C,2H3)-2-hydroxy-3-methylvaleronitrile (38) as a yellow liquid.

(6-13C,2H3)-2-Amino-3-methylvaleronitrile (39).

Crude (6-13C,2H3)-2-hydroxy-3-methylvaleronitrile (38) (1.77 g, 15.1 mmol) was dissolved in concentrated NH4OH (25.8 mL) and ethanol (28 mL) added until the solution became homogeneous and clear. The reaction mixture was then stirred at room temperature for 18 hours and the ethanol removed in vacuo. The aqueous layer was extracted with ether and the combined organic extracts washed with water and saturated NaCl. They were then dried over MgSO4, filtered through Celite, and concentrated in vacuo. This gave 1.8 g of crude (6-13C,2H3)-2-amino-3-methylvaleronitrile (39) as a yellow oil.


The crude (6-13C,2H3)-2-amino-3-methylvaleronitrile was dissolved in water (95 mL) and barium hydroxide (18.0 g, 57 mmol) added. The reaction mixture was then heated at reflux for 18 hours. After cooling to room temperature the water was removed in vacuo and the residue redissolved in water (50 mL). This solution was treated twice with solid (NH4)2CO3 and filtered through Celite to remove any remaining barium as insoluble barium carbonate. The resulting filtrate was stirred for 1
hour at 60-65°C in a water bath before being finally concentrated in vacuo. This gave the crude amino acid mixture as a white solid. The solid was further purified by cellulose column chromatography (eluant BAW, 4:1:2). This gave 185 mg of (6-13C,2H3)-DL-isoleucine/(6-13C,2H3)-DL-alloisoleucine as a flakey white solid, 1H NMR (250 MHz, D2O) (1:1 mixture of isoleucine and alloisoleucine) δ 0.9-1.1 (m, 6H), 1.2-1.6 (m, 4H), 2.0-2.2 (m, 2H), 3.69 (dd, 3J_HH=3.95 Hz, 3J_CH=3.92 Hz, 1H), 3.76 (dd, 3J_HH=5.92 Hz, 3J_CH=5.90 Hz, 1H), 13C NMR (62.89 MHz, D2O, 1 drop methanol as standard) δ 11.14, 11.21, 13.42 (enriched septet, 13C,2H3), 14.79 (enriched septet, 13C,2H3), 24.56, 25.61, 35.70, 35.99, 49.0 (methanol), 58.58, 59.66, 174.3, 175.2.
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