National Library of Canada
Acquisitions and Bibliographic Services Branch
395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada
Direction des acquisitions et des services bibliographiques
395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Canada

Metabolites of *Streptomyces akiyoshiensis* and their Relationship to HON Biosynthesis

by

Kevin Craig Smith
M Sc., Acadia University, 1987

submitted in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

at

Dalhousie University,
Halifax, Nova Scotia
August, 1995

ISBN 0-612-08798-0

Name [redacted]

Dissertation Abstracts International is arranged by broad, general subject categories. Please select the one subject which most nearly describes the content of your dissertation. Enter the corresponding four-digit code in the spaces provided.

Organic Chemistry
SUBJECT TERM

0490
SUBJECT CODE

U·M·I

Subject Categories

THE HUMANITIES AND SOCIAL SCIENCES

COMMUNICATIONS AND THE ARTS
Architecture 0729
Art History 0377
Cinema 0900
Dance 0378
Fine Arts 0357
Information Science 0723
Journalism 0391
Library Science 0399
Mass Communications 0708
Music 0413
Speech Communication 0459
Theater 0465

EDUCATION
General 0515
Administration 0514
Adult and Continuing 0516
Agricultural 0517
Art 0273
Bilingual and Multicultural 0282
Business 0688
Community College 0275
Curriculum and Instruction 0727
Early Childhood 0518
Elementary 0524
Finance 0277
Guidance and Counseling 0519
Health 0680
Higher 0745
History of 0520
Home Economics 0278
Industrial 0521
Language and Literature 0279
Mathematics 0280
Music 0522
Philosophy of 0998
Physical 0523
Psychology 0525
Reading 0535
Religious 0527
Sciences 0714
Secondary 0533
Social Sciences 0534
Sociology of 0340
Special 0529
Teacher Training 0530
Technology 0710
Tests and Measurements 0288
Vocational 0747

LANGUAGE, LITERATURE AND LINGUISTICS
Language
- General 0679
- Ancient 0289
- Linguistics 0290
- Modern 0291

Literature
- General 0401
- Classical 0294
- Comparative 0295
- Medieval 0297
- Modern 0298
- African 0316
- American 0591
- Asian 0305
- Canadian (English) 0352
- Canadian (French) 0355
- English 0593
- Germanic 0311
- Latin American 0312
- Middle Eastern 0315
- Romance 0313
- Slavic and East European 0314

PHILOSOPHY, RELIGION AND THEOLOGY
Philosophy 0422
Religion
- General 0318
- Biblical Studies 0321
- Clergy 0319
- History of 0320
- Philosophy of 0322

Theology 0469

SOCIAL SCIENCES
American Studies 0323
Anthropology
- Archaeology 0324
- Cultural 0326
- Physical 0327

Business Administration
- General 0310
- Accounting 0272
- Banking 0770
- Management 0454
- Marketing 0338

Canadian Studies 0385
Economics
- General 0501
- Agricultural 0503
- Commerce Business 0505
- Finance 0508
- History 0509
- Labor 0510
- Theory 0511

Folklore 0358
Geography 0366
Gerontology 0351
History
- General 0578
- Ancient 0579
- Medieval 0581
- Modern 0582
- Black 0328
- African 0331
- Asia, Australia and Oceania 0332
- Canadian 0334
- European 0335
- Latin American 0336
- Middle Eastern 0333
- United States 0337

History of Science 0585
Law 0398
Political Science
- General 0615
- International Law and Relations 0616
- Public Administration 0617

Recreation 0814
Social Work 0452
Sociology
- General 0626
- Criminology and Penology 0627
- Demography 0938
- Ethnic and Racial Studies 0631
- Individual and Family Studies 0628
- Industrial and Labor Relations 0629
- Public and Social Welfare 0630
- Social Structure and Development 0700
- Theory and Methods 0344

Transportation 0709
Urban and Regional Planning 0999
Women's Studies 0453

THE SCIENCES AND ENGINEERING

BIOLOGICAL SCIENCES
Agriculture
- General 0473
- Agronomy 0285
- Animal Culture and Nutrition 0475
- Animal Pathology 0476
- Food Science and Technology 0359
- Forestry and Wildlife 0478
- Plant Culture 0479
- Plant Pathology 0480
- Plant Physiology 0817
- Range Management 0777
- Wood Technology 0746

Biology
- General 0306
- Anatomy 0287
- Biostatistics 0308
- Botany 0309
- Cell 0379
- Ecology 0329
- Entomology 0353
- Genetics 0369
- Limnology 0793
- Microbiology 0410
- Molecular 0307
- Neuroscience 0317
- Oceanography 0416
- Physiology 0433
- Radiation 0821
- Veterinary Science 0778
- Zoology 0472

Biophysics
- General 0786
- Medical 0760

EARTH SCIENCES
Biogeochemistry 0425
Geochemistry 0996
Geodesy 0370
Geology 0372
Geophysics 0373
Hydrology 0388
Mineralogy 0411
Paleobotany 0345
Paleoecology 0426
Paleontology 0418
Paleozoology 0985
Palynology 0427
Physical Geography 0368
Physical Oceanography 0415

HEALTH AND ENVIRONMENTAL SCIENCES
Environmental Sciences 0768
Health Sciences
- General 0566
- Audiology 0300
- Chemotherapy 0992
- Dentistry 0567
- Education 0350
- Hospital Management 0769
- Human Development 0758
- Immunology 0982
- Medicine and Surgery 0564
- Mental Health 0347
- Nursing 0569
- Nutrition 0570
- Obstetrics and Gynecology 0380
- Occupational Health and Therapy 0354
- Ophthalmology 0381
- Pathology 0571
- Pharmacology 0419
- Pharmacy 0572
- Physical Therapy 0382
- Public Health 0573
- Radiology 0574
- Recreation 0575
- Speech Pathology 0460
- Toxicology 0383

Home Economics 0386

PHYSICAL SCIENCES
Pure Sciences

Chemistry
- General 0485
- Agricultural 0749
- Analytical 0486
- Biochemistry 0487
- Inorganic 0488
- Nuclear 0738
- Organic 0490
- Pharmaceutical 0491
- Physical 0494
- Polymer 0495
- Radiation 0754

Mathematics 0405
Physics
- General 0605
- Acoustics 0986
- Astronomy and Astrophysics 0606
- Atmospheric Science 0608
- Atomic 0748
- Electronics and Electricity 0607
- Elementary Particles and High Energy 0798
- Fluid and Plasma 0759
- Molecular 0609
- Nuclear 0610
- Optics 0752
- Radiation 0756
- Solid State 0611

Statistics 0463

Applied Sciences

Applied Mechanics 0346
Computer Science 0984

Engineering
- General 0537
- Aerospace 0538
- Agricultural 0539
- Automotive 0540
- Biomedical 0541
- Chemical 0542
- Civil 0543
- Electronics and Electrical 0544
- Heat and Thermodynamics 0348
- Hydraulic 0545
- Industrial 0546
- Marine 0547
- Materials Science 0794
- Mechanical 0548
- Metallurgy 0743
- Mining 0551
- Nuclear 0552
- Packaging 0549
- Petroleum 0765
- Sanitary and Municipal 0554
- System Science 0790

Geotechnology 0428
Operations Research 0796
Plastics Technology 0795
Textile Technology 0994

PSYCHOLOGY
General 0621
Behavioral 0384
Clinical 0622
Developmental 0620
Experimental 0623
Industrial 0624
Personality 0625
Physiological 0989
Psychobiology 0349
Psychometrics 0632
Social 0451

TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES (continued)

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

ABSTRACT

5-Hydroxy-4-oxo-L-norvaline (HON) produced by *Streptomyces akiyoshiensis* is derived from the incorporation of aspartate and the methyl group of acetate. To further elucidate the HON biosynthetic pathway, the culture supernatant of HON$^-$ mutant L138, which stimulated the production of HON in mutant L127, was examined for the accumulation of biosynthetic intermediates. An assay based on the cosynthetic relationship between L127 and L138 was developed for directing the purification of substances responsible for transforming mutant L127 into a HON producer. During the attempted purification of the L138 metabolite, circumstantial evidence suggested that the L138 metabolite was not consistent with an accumulating intermediate. Cross-feeding experiments using [2-^{14}C]acetate as a supplement showed that the L138 metabolite was an initiator of *de novo* HON production in mutant L127. The nature of its binding to various chromatographic media and its extractability into organic solvents suggests that the metabolite is highly polar with a weakly acidic substituent, possibly a phenol. A compound having similar solubility and chromatographic properties to the L138 metabolite is the autoregulator cosynthetic factor 1.

An L138 metabolite initially identified as the stimulating substance was characterized by spectroscopic methods and found to be identical to a synthetic sample of *N*-acetyl-L-dopa. However, synthetic *N*-acetyl-L-dopa did not transform mutant L127 into a HON producer. Culture conditions were examined to optimize production of *N*-acetyl-L-dopa for biosynthetic investigations. Supplementing cultures with possible precursors L-tyrosine, *N*-acetyl-L-tyrosine or L-dopa greatly stimulated the production of *N*-acetyl-L-dopa but L-tyrosine stimulated at a lower level. This would be consistent with a larger number of steps required to convert L-tyrosine to *N*-acetyl-L-dopa. Although two possible pathways exist from L-tyrosine to *N*-acetyl-L-dopa, the presence of an acetylating enzyme in cell-free extracts that efficiently acetylates L-dopa but not L-tyrosine suggests that L-dopa is an intermediate of the pathway.

ABBREVIATIONS AND SYMBOLS

$\{\alpha\}_D$	optical rotation at the D line of sodium
ε	absorption coeficient
υ_{max}	wavenumbers at absorption maximum
λ_{max}	wavelength at an absorption maximum
asd	aspartate semialdehyde dehydrogenase gene
BCA	bicinchonic acid
c	concentration (g/mL)
C_{18}	octadecyl
ca.	circa (or about)
Ci	Curie
CN	cyanopropyl
(d)	doublet
(dd)	doublet of doublets
eims	electron impact mass spectroscopy
×g	×gravitational force
Hz	Hertz
HON	5-hydroxy-4-oxo-L-norvaline
HON$^-$	HON nonproducing phenotype
hp^{1}c	high pressure liquid chromatography
hsd	homoserine dehyrogenase gene
I.D.	inner diameter

ir	infrared
J	coupling constant
lys A	diaminopimelate decarboxylase gene
$\{M^+\}$	molecular ion
m/z	mass to charge ratio
NADP	nicotinamide adenine dinucleotide phosphate
nmr	nuclear magnetic resonance
NTG	*N*-methyl-*N'*-nitro-nitrosoguanidine
O.D.	outer diameter
opa	*o*-phthalaldehyde
(q)	quartet
R_f	retention factor
rel. int.	relative intensity
rpm	revolutions per minute
(s)	singlet
sh	shoulder
(t)	triplet
t_R	retention time
tlc	thin layer chromatography
uv	ultraviolet
v/v	volume/volume
w/v	weight/volume

ACKNOWLEDGEMENTS

I would like to thank Dr. Robert L. White for his time, patience, encouragement and for having trust in my abilities before and during my Ph.D. program. I am also forever in the debt of Dr. Leo C. Vining, whose wisdom will always amaze me Thank you to Yunzheng Le and Dr. Leo C. Vining for providing the HON$^-$ mutants used in this project. Also, thank you to the Walter C. Sumner Foundation, Dalhousie University and NSERC for their financial support, ARMRC for the use of their nmr facilities and Drs. J.-H. Kim and S. Grossert for providing mass spectra.

I express my graditude to all members, past and present, of the White research group. I reserve a special thank you for Danny Davis, who was invaluable for my maintaining some sense of sanity. Thank you to members of the *Strep.* discussion group (especially Drs. S. Ayer and J. Doull, Keqian, Peter, Roy and Yunzheng) whose useful discussions helped me look at the chemical world in a very different light.

I am forever indebted to Elizabeth Grant and David Nunn, Sheena and Danny Davis, and Nancy and Bob Gauthier for opening their homes and hearts.

Finally, I thank my family for many years of love, patience and support. This is particularly true for my mother Ida Mae, who provided me with life and was devoted to making it worth living. For that, I have more love and respect than you will ever know. Also, thank you to Keegan and Christina for your love and laughter, given to a dad who was too often not at home to tuck you in.

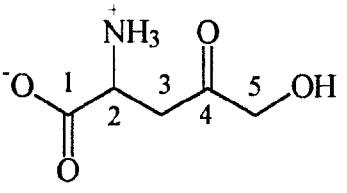
To Jacki, for your never ending patience and support, I dedicate this work and devote my life to you for being my light and my love.

Kev.

INTRODUCTION

It has been known for over half a century that actinomycetes produce an elaborate variety of secondary metabolites in the form of antibiotics, pigments and extracellular proteins which do not function as cellular building blocks (1). Over 60% of the compounds known to have antibiotic activity are produced by actinomycetes, particularly members of the genus *Streptomyces* which make up the richest source (2).

The secondary metabolite, (*S*)-2-amino-5-hydroxy-4-oxopentanoic acid, more commonly referred to as 5-hydroxy-4-oxo-L-norvaline or HON (1), is produced in large quantities by *Streptomyces akiyoshiensis* (3). *S. akiyoshiensis* (ATCC 13480) is a highly filamentous Gram-positive soil bacterium which produces red pigments around its colonies during periods of slow growth.



HON, **1**

HON is referred to as a nonprotein amino acid since it is not found in protein either due to the lack of a specific transfer RNA or because it does not arise from protein amino acids as a result of post-translational modification (4). The origins of nonprotein amino acids are diverse but most are the result of secondary metabolism. Some are intermediates of metabolic pathways or come about through the metabolism or detoxification of foreign compounds. The majority of these compounds are produced by members of the plant world or by microorganisms. The latter excrete a large variety

of compounds into the surrounding culture medium

At the time of its structural elucidation (5, 6), HON was the first natural source nonprotein amino acid found to contain a nonconjugated carbonyl in a relatively simple aliphatic structure. A number of structurally similar amino acids such as 2-amino-4-oxohexanoic acid (7), 2-amino-4-oxopentanoic acid (8) and 2-amino-3-methyl-4-oxopentanoic acid (9) have since been isolated from different bacterial sources.

Early screening for antibiotic activity (10) showed HON to be an inhibitor of pathogenic tubercle bacilli This antibiotic activity was shown (11) to be different from the action of other antitubercle agents such as isonicotinic acid hydrazide (INAH) and streptomycin since it showed little cross-resistance in strains resistant to these common agents In this same study, structure-activity relationships using 16 structural homologs indicated that all structural features of HON were required for antibiotic activity HON has been reported (10) to show little inhibitory action on common Gram-positive and Gram-negative bacteria and fungi but recently, HON (rediscovered as the antibiotic RI-331) has been shown to act as an antifungal agent (12, 13) and may represent a new candidate for use in antifungal chemotherapy The antifungal activity of HON has been confirmed by Le (14)

A recent report (15) has attributed the growth inhibition of *Candida albicans* by HON to perturbations in the development of parts of the cell walls associated with bud development On the other hand, growth inhibition in *Saccharomyces cerevisiae* has been associated with a decrease in protein synthesis due to a depletion of several amino acids from cellular pools (16). The depletion is the result of a decrease in the rate of

de novo biosynthesis of the aspartate family of amino acids, *i.e.*, threonine (**6**), isoleucine (**7**) and methionine (**10**), due to the inhibition of homoserine dehydrogenase (17, 18) Homoserine dehydrogenase is responsible for the NADPH dependent reduction of aspartic β-semialdehyde (**4**) to homoserine (**5**, Figure 1), and a recent investigation (19) of the kinetics of this inhibition has shown that HON acts as a competitive inhibitor of homoserine dehydrogenase. The degree of inhibition increases with increasing NADP concentration, suggesting that HON binds to the enzyme-NADP binary complex forming an inactive enzyme-NADP-HON ternary complex.

Inhibition of this enzymatic step in prototrophic organisms results in the depletion of threonine (**6**), isoleucine (**7**) and methionine (**10**). Since mammals lack this pathway, these amino acids are nutritionally essential, and no major toxic effects from HON have been observed in mammals (16).

Methods of analysis

A high performance liquid chromatography (hplc) method for the detection of HON in the fermentation broths of *S. akiyoshiensis* has been developed by White, DeMarco and Smith (20). This method involves the use of *o*-phthalaldehyde (**11**) which reacts with primary amines in the presence of a thiol, such as β-mercaptoethanol (**12**), to produce highly fluorogenic 1-alkylthiol-2-alkyl substituted isoindoles (**13**, Figure 2) that can be separated by hplc on a C_{18} reversed phase column. This opa-hplc method is fast with a run time of only five minutes and is sensitive to picomole quantities of HON.

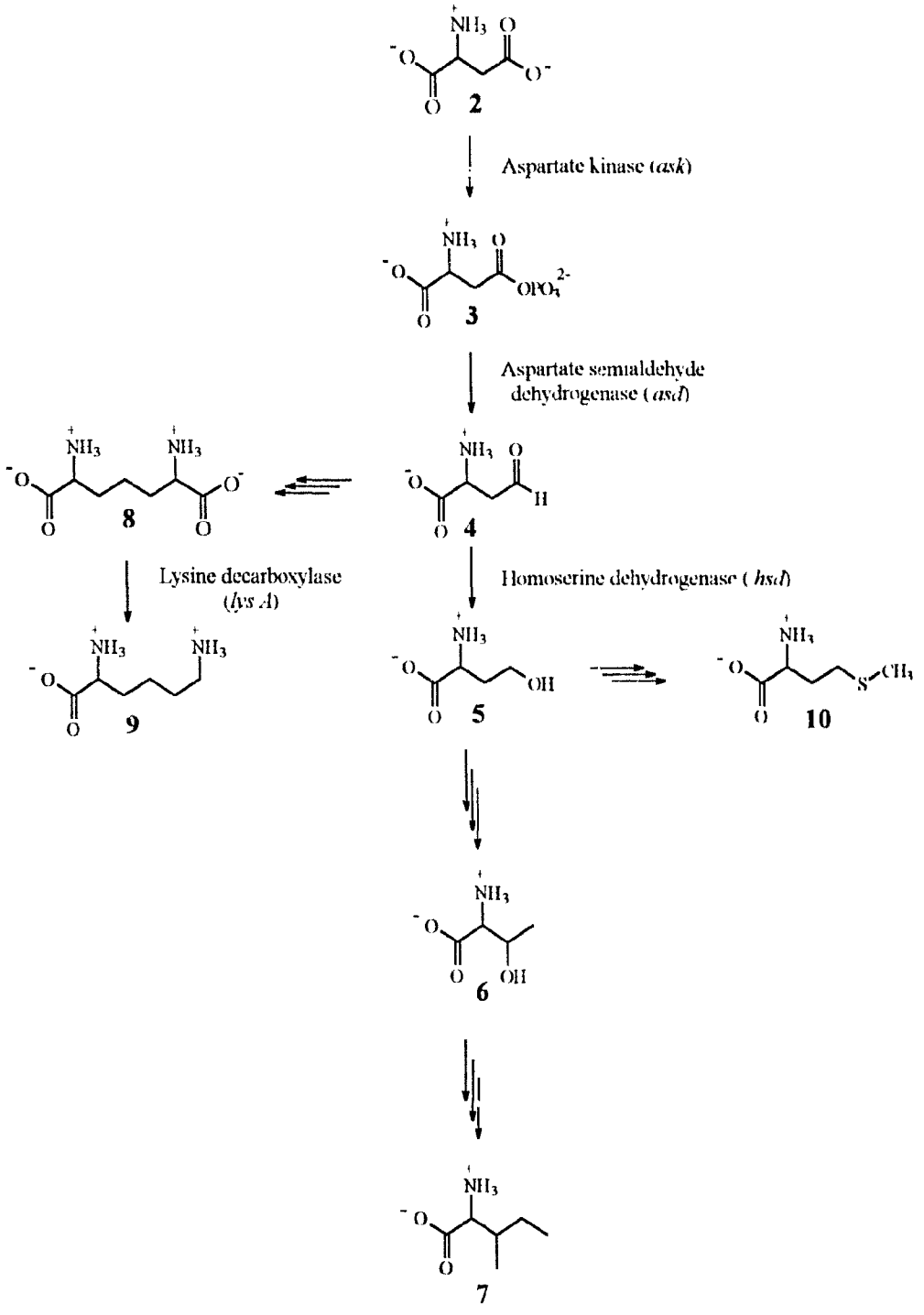


Figure 1. Biosynthesis of aspartate family amino acids

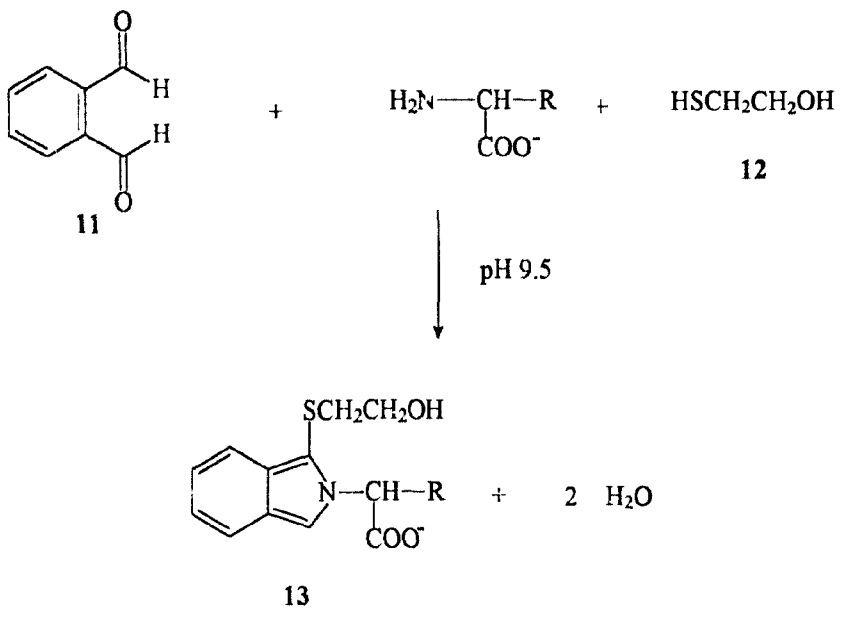


Figure 2. Opa reaction used in the analysis of amino acids

A bioassay utilizing the sensitivity of fungi, specifically yeast, to small quantities of HON has also been developed (14). When a test sample is placed on yeast containing agar, HON can be detected by the retention of a clear zone where yeast growth is inhibited. The quantitative aspects of this bioassay have not been investigated and its use has been limited to screening for HON nonproducing mutants.

Biosynthetic investigations

The biosynthesis of HON has been studied by feeding isotopically labelled compounds to cultures of *S. akiyoshiensis* (21, 22) with isotope enrichments being estimated by ^{13}C nmr spectroscopy. Results from these feeding experiments are

summarized in Figure 3. Feeding experiments using sodium [1-^{13}C]acetate showed incorporation of labels at the C-1 and C-4 positions of HON whereas sodium [2-^{13}C]acetate was incorporated into all carbons, but mainly into C-2, C-3 and C-5 The proton decoupled ^{13}C nmr spectrum of the γ-lactone of 4,5-dihydroxynorvaline, obtained from the $NaBH_4$ reduction of HON, showed enhanced doublets when [1,2-$^{13}C_2$]acetate was added to cultures, indicating the incorporation of two intact acetate units into HON. This pattern of labelling suggested that C-1 through C-4 of HON derives from a four carbon intermediate of the citric acid cycle or aspartic acid (**2**), an amino acid formed by transamination of oxaloacetate.

To investigate the nature of the four carbon intermediate, cultures were supplemented with DL-[4-^{13}C]aspartic acid. Labelling was observed at C-4, as expected

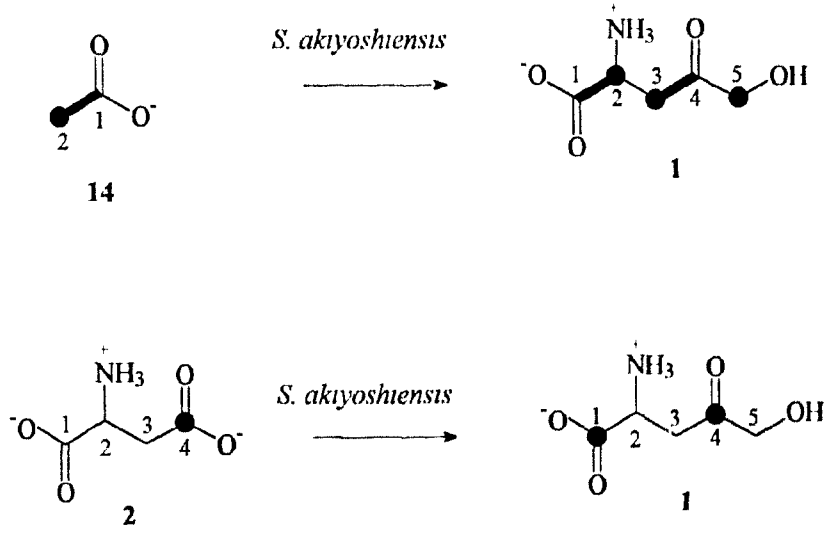


Figure 3. Pattern of isotopic incorporation in feeding studies

from a direct incorporation into HON. However, a smaller enrichment was found at C-1. Deamination of aspartate to oxaloacetate and conversion to symmetrical intermediates of the citric acid cycle (*e.g.*, fumarate or succinate) would distribute label between these two positions. To distinguish between a citric acid cycle intermediate and aspartate as the source of the four carbon precursor incorporated into HON, DL-[2-^{13}C,^{15}N]aspartic acid was used in feeding studies. It was incorporated with maintenance of the carbon-nitrogen bond, indicating that aspartate is the precursor that supplies C-1 through C-4.

The results from feeding experiments led White *et al.* (21, 22) to propose a plausible route for the biosynthesis of HON in *S. akiyoshiensis* (Figure 4). In this pathway, the condensation of aspartate (**2**) with the methyl group of an additional acetate unit (**14**), which becomes C-5 of HON, is catalyzed by a hypothetical "aspartate:acetyl coenzyme A ligase". To facilitate this Claisen-type condensation, activation of both acetate and aspartate are required. The most likely candidates for the activated form of the acetate are acetyl CoA (**15**) or malonyl CoA, the thiolesters known to act as nucleophiles in enzymatic condensation reactions in polyketide and fatty acid biosynthesis. The activated aspartate species functioning as an electrophilic substrate may be an acyl β-phosphate (**3**), adenylate, or thiolester derivative (23) or aspartate β-semialdehyde (**4**). This condensation would form either 2-amino-4-oxoadipate thiolester (**16**) or 2-amino-4-hydroxyadipate thiolester, if the semialdehyde was the substrate. Hydrolysis of the thiolester (**16**) would lead to the β-keto acid (**17**), which would provide HON (**1**) by decarboxylation to 4-oxonorvaline (**18**) and subsequent

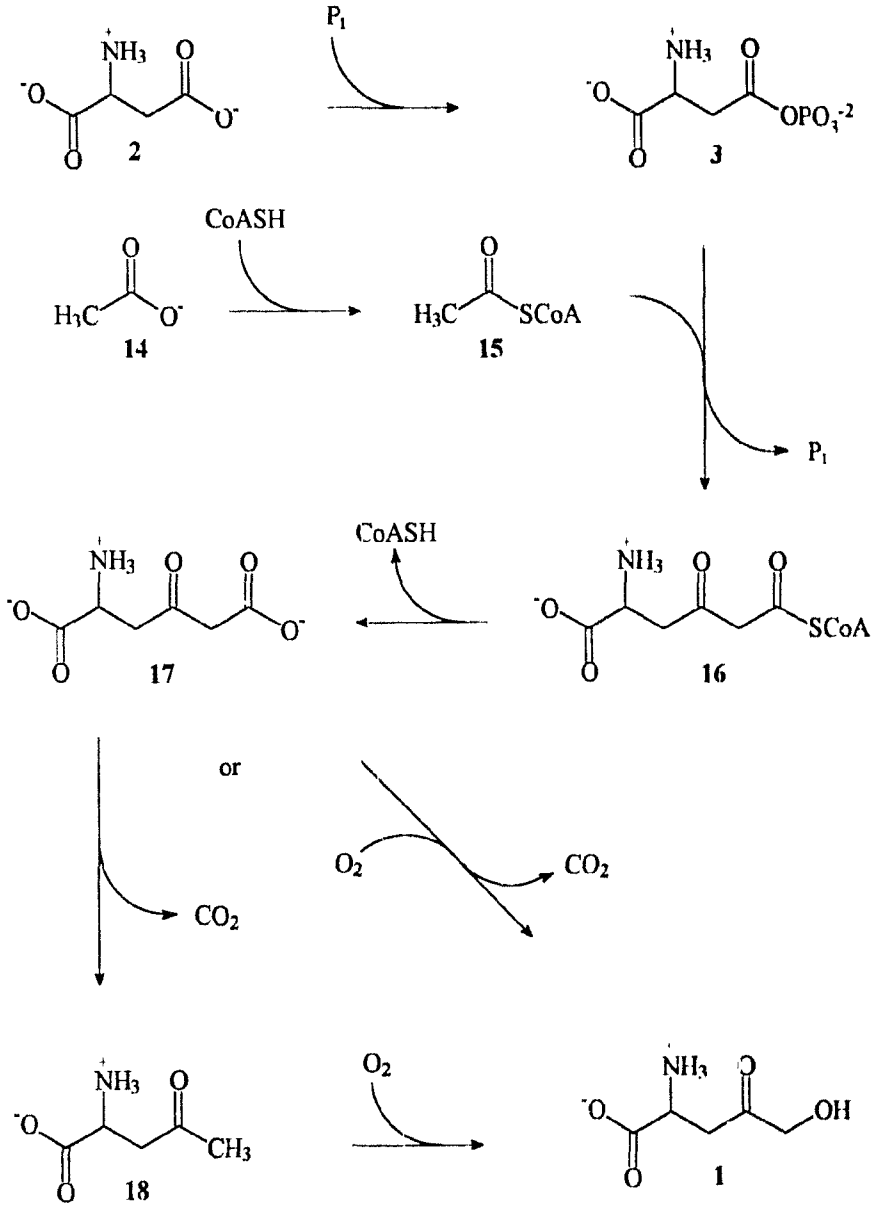


Figure 4. Hypothetical biosynthetic pathway for HON (21, 22)

hydroxylation. Alternatively, direct oxidative decarboxylation to afford HON (**1**) in a single step from the β-keto acid is possible.

Although isotopic feeding studies showed that C-1 to C-4 of HON (**1**) originate from aspartate (**2**), the HON biosynthetic pathway may branch from the aspartate pathway at aspartate (**2**), aspartyl phosphate (**3**), or aspartyl semialdehyde (**4**). Recent work by Le and coworkers (14, 24) attempted to locate this branchpoint by studying the effect of genetic lesions in mutants on HON production. One group of auxotrophic mutants produced by chemical mutagenesis with *N*-methyl-*N*'-nitro-nitrosoguanidine (NTG) grew when the medium was supplemented with lysine (**9**) but not with a mixture of LL- and *meso*-diaminopimelate (DAP, **8**), indicating a lesion in *lys A*, the gene coding for diaminopimelate decarboxylase (Figure 1). These mutants produced HON at levels near those observed in the wild-type, implying that the branchpoint occurs before the final step in lysine biosynthesis. Another auxotrophic mutant, found to require threonine (**6**) or methionine (**7**) for growth, produced about 20% more HON than its revertant or the wild-type strain after 144 h of incubation. This mutant had aspartate kinase activity comparable to that observed in both the revertant and the wild-type organisms. However, homoserine dehydrogenase activity was significantly reduced, suggesting this mutant had a lesion in *hsd*, the gene coding for this enzyme. Since this mutant was not blocked in HON biosynthesis, the branchpoint was concluded to occur before homoserine dehydrogenase.

Attempts by Le to obtain *asd*-specific mutants by chemical mutagenesis and molecular cloning techniques were unsuccessful (14, 24). This has been attributed to

an inability to compensate for the *asd* lesion in the auxotrophic mutants since diaminopimelate (DAP, 8), a component required for the building of cell walls in Streptomycetes, is not easily taken into the cell Efforts to construct an *asd* mutant are still in progress

During the biosynthetic investigations described above, a series of prototrophic HON nonproducing (HON) mutants were produced by NTG mutagenesis and isolated (14, 24, 25) Combinations of these mutants grown on casein-starch liquid medium were able to overcome their biosynthetic deficiencies to produce HON An interpretation of this phenomenon suggested by Le (14, 24) was that these mutants were blocked in different steps of HON biosynthesis One blocked mutant produces and excretes an intermediate of the HON biosynthetic pathway that can be converted by the second, or convertor, mutant to HON Producer-convertor relationships were established by exchanging the mycelia and culture supernatants from 48-h mutant cultures and assaying for HON after an additional 24 h of incubation Three groups of mutants that produced HON under these conditions were identified This led Le (14) to propose that there were at least three distinct steps for the biosynthesis of HON (Figure 5) A fourth group of HON$^-$ mutants, unable to cosynthesize HON when mixed in combinations with other HON$^-$ mutants, was denoted as a group of regulatory mutants and was not further characterized.

An alternative explanation for the observed co-synthesis phenomenon is that these nonproducing mutants are blocked in the biosynthetic pathway of a required substance, such as a coenzyme or regulator. Since these nonproducing mutants are

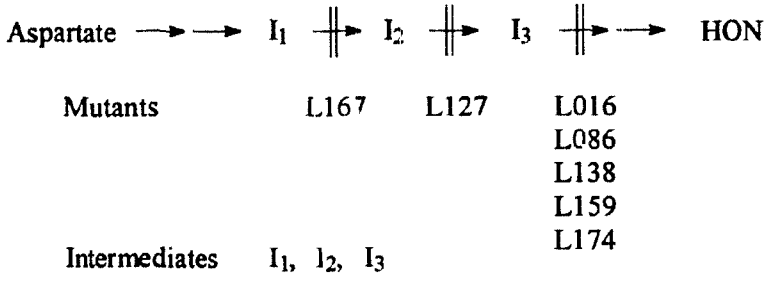


Figure 5. HON$^-$ mutants of *S. akiyoshiensis*

nonauxotrophic, a missing coenzyme must be specific to HON biosynthesis. Also, it would be expected that a deficiency in an excreted coenzyme or regulator would be overcome by supplementing mutant cultures with the supernatant from cultures of wild-type *S. akiyoshiensis*. HON production by mutant L138 was not stimulated by supplementing cultures with wild-type supernatant (14), suggesting that the mutant was blocked in the HON pathway. However, a large amount of HON was present in the wild-type supernatant, and a small additional production of HON may not have been apparent.

The objective of this project was to increase our knowledge of the metabolic processes of *S. akiyoshiensis* by 1) further investigating the biosynthesis of HON and 2) isolating and characterizing metabolites produced by *S. akiyoshiensis*. To accomplish these objectives, efforts were focused on the isolation of metabolites from HON$^-$ mutant L138 that stimulate HON biosynthesis in the HON$^-$ mutant L127. The interpretation (14) that pairs of mutants able to co-synthesis HON are blocked at different steps in HON biosynthesis suggests that one blocked mutant (*e.g.*, L138) must excrete an

intermediate into the culture supernatant that is converted by the second mutant (*e.g.*, L127) into HON. The amount of HON produced in mixed cultures of mutants L127 and L138 was similar to that produced in wild-type cultures (6-8 mmol/L after 96-h) indicating that relatively large amounts of an intermediate would be present.

Since mutant L138 appears to be blocked in a later step in the biosynthetic pathway, the availability of an L138-produced intermediate could provide a substrate for the investigation of biosynthetic enzymes. Although HON can be detected by opa-hplc methods, no suspected intermediate thus far tested has provided HON production in cell-free extracts (14, 26).

Literature biosynthetic analogies

An analogy has been pointed out (21) between the proposed aspartate:acetate coenzyme A ligase of the HON pathway and the initial enzyme proposed to be involved in the biosynthesis of the carbapenem antibiotic, thienamycin (27) (**19**, Figure 6) in *Streptomyces cattleya.* The first step in the carbapenem hypothesis is the condensation of γ-glutamyl phosphate (**20**) or glutamate γ-semialdehyde with acetyl CoA (**15**), a step that could be catalyzed by a "glutamate:acetyl coenzyme A ligase". The carbapenem antibiotics are a relatively new class of β-lactam antibiotics which contain the 1-azobicyclo-[3.2.0]hept-2-ene ring system (28). This class of β-lactams is structurally distinct from the more common penicillins and cephalosporins in that they do not possess a sulfur atom in the bicyclic nucleus. The carbapenem family antibiotics possess an extremely wide spectrum of activity against both Gram-positive and negative

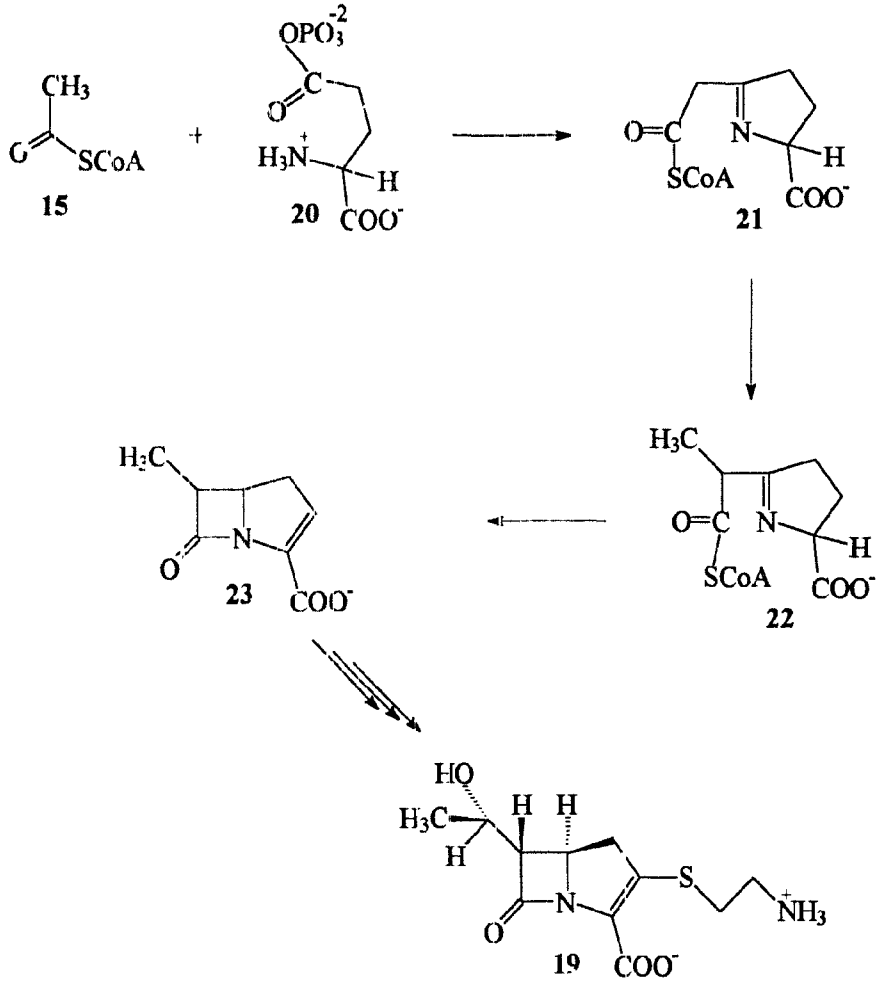
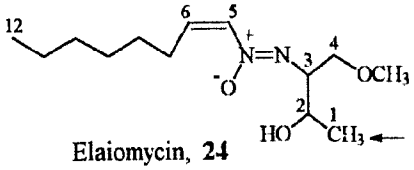


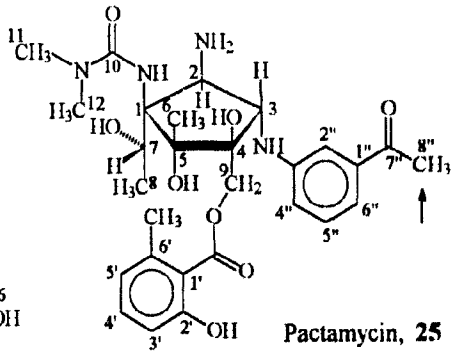
Figure 6. Hypothetical biosynthetic pathway for thienamycin (27)

bacteria that is undiminished against strains with β-lactamase-induced resistance to penicillins and cephalosporins.

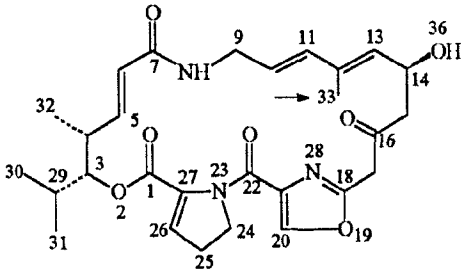
A number of other literature examples exist where isotopic feeding studies have supported acetate as a methyl group donor in the biosynthesis of secondary metabolites. Investigations of the biosynthesis of the antibiotic elaiomycin (**24**) have shown that the C-1 methyl is derived from C-2 of acetate. Parry and Mueller (29) proposed that the initial step in acetate incorporation may involve a Claisen-type condensation between an activated serine and malonyl CoA, resulting in a β-keto ester which is then hydrolyzed, decarboxylated and reduced. Evidence supporting acetate as the methyl group donor of C-8" of the Streptomycete-produced antibiotic, pactamycin (**25**), has also been documented (30), although no suggestions regarding the biosynthetic incorporation have been proposed. The C-33 methyl group of the macrocyclic antibiotic, virginiamycin M_1 (**26**), has been suggested (31) to originate through a unique aldol-type



Elaiomycin, **24**



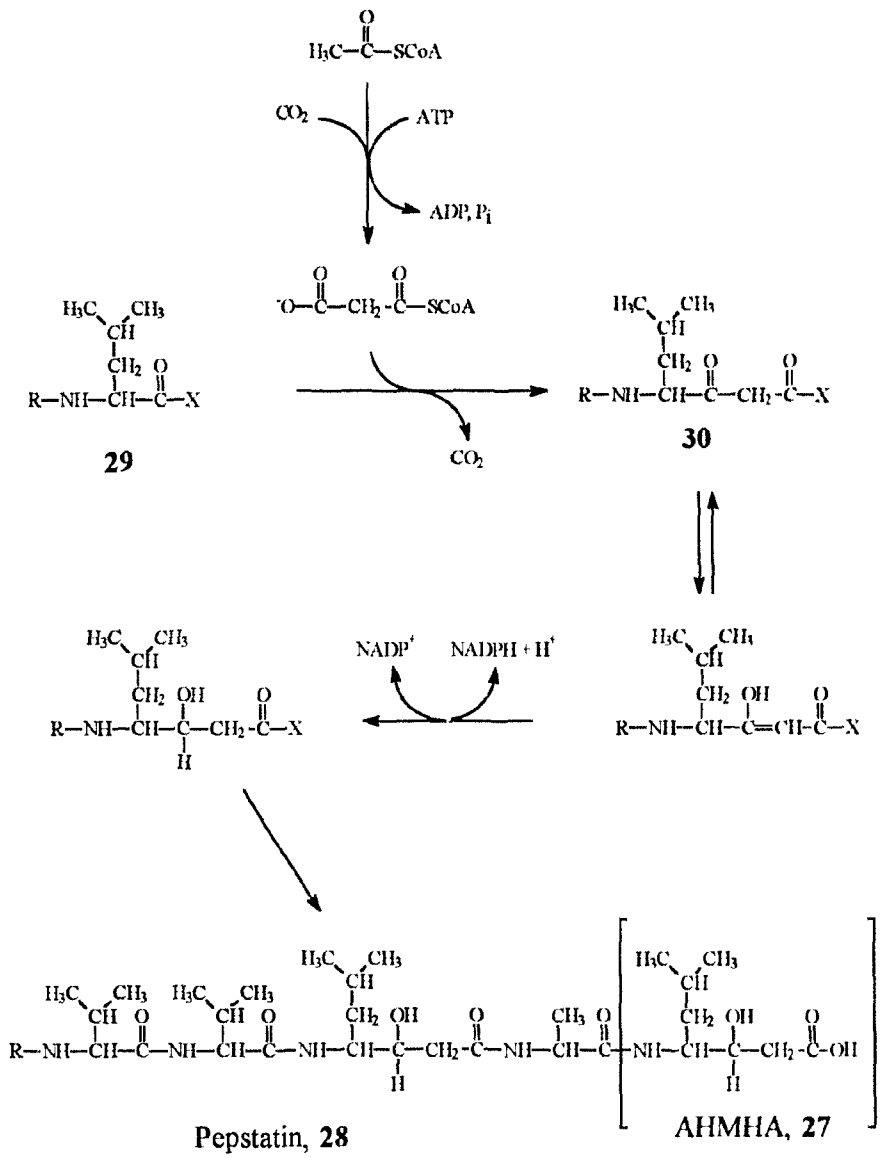
Pactamycin, **25**



Virginiamycin M 1, **26**

condensation between an acetate unit, probably in the form of malonyl CoA, and a preformed polyketide chain to yield a β-hydroxy ester Further processing in the way of hydrolysis, decarboxylation and dehydration would result in the desired metabolite. Although the utilization of acetate as a methyl donor is not routine in primary metabolism, the occurrence within secondary metabolic pathways may not be quite so rare

A Claisen-type condensation has also been proposed in the biosynthesis of the 4-amino-3-hydroxy-6-methylheptanoic acid (AHMHA, **27**, Figure 7) moiety of the pepsin inhibitor, pepstatin (**28**), produced by various Streptomycetes (32) Pepstatins are a group of pentapeptides containing two moles of AHMHA in their structure and in which the *N*-termini are acylated with a variety of fatty acids Isotopic feeding studies suggest that the AHMHA carbon backbone results from the condensation of the α-carboxyl of L-leucine and C-2 of a malonate unit. However, feeding [U-^{14}C]AHMHA to the producer organism *Streptomyces testaceus* showed that no radioactivity was incorporated into pepstatin Also, competition experiments in which [U-^{14}C]leucine diluted with 100 equivalents of cold AHMHA was fed to *S. tetstaceus* showed no dilution of radioactive label when compared to the feeding of [U-^{14}C]leucine alone. This suggested that AHMHA was not incorporated into the peptide as an intact unit but may be assembled in a stepwise fashion as the peptide is formed Therefore, the proposed biosynthetic pathway involves the condensation of a leucine peptide (**29**) with acetate, probably as malonyl CoA The product (**30**) of this ligation then undergoes reduction to the alcohol function of the AHMHA moiety (32)



R = growing pepstatin peptide

X = activating group (*e.g.*, S-CoA or S-enzyme)

Figure 7. Proposed biosynthesis of Pepstatin (32)

Peptide antibiotics are usually biosynthesized on multienzyme systems similar to those involved in fatty acid and polyketide antibiotic formation. These multienzyme complexes may contain a wide range of cofactors and are capable of modifying amino acids prior to or after assembling them into peptides (33).

Mutants and amino acid biosynthesis

The biosynthesis of amino acids, the vital building blocks of proteins, has been of significant interest since only about half can be synthesized by mammals. The remainder are considered essential and must be obtained from dietary sources (34). However, bacteria have much broader synthetic powers and produce all amino acids from simple carbon and nitogen sources. During the 1950's and 60's, an increased understanding of the biosynthetic pathways operating in the production of protein amino acids was facilitated by the utilization of auxotrophic mutants of fungi and bacteria (35, 36). When blocked in biosynthetic pathways, mutants often accumulate substantial quantities of intermediates and identification of these compounds has provided the key to elucidating the steps and processes involved in amino acid biosynthesis. The terminology, methods and difficulties associated with blocked mutants in biosynthetic investigations has been well documented (37-39) and their use in studying the biosynthesis of secondary metabolites and natural products is now common.

An example of a biosynthetic investigation which extensively employed mutants to elucidate individual steps in the pathway is that of the nonprotein amino acid phosphinothricin (PT, **31**). Phosphinothricin, which has a unique phosphino group, is

found as the *N*-terminal amino acid in the peptides, bialaphos (BA, PT-L-ala-L-ala, **32**) (40), phosalacin (PT-L-ala-L-leu) (41) and trialaphos (PT-L-ala-L-ala-L-ala) (42) The demonstrated antimicrobial and herbicidal activities of these peptides is due to the inhibition of glutamine synthetase (43) by the glutamic acid analogue, phosphinothricin, which is released from the peptide by the action of nonspecific proteases

Extensive biosynthetic investigations, resulting from an interest in the unusual structure of bialaphos as well as its commercial utility, include isotopic feeding studies, the use of metabolic inhibitors, enzyme isolation and the preparation of BA mutants The mutants were essential for the isolation and characterization of genes The 35-kb bialaphos production (*bap*) cluster (43), which contains genes for at least 10 steps of bialaphos biosynthetic pathway (Figure 8), codes for a combination of enzymes with functions unique to this secondary metabolic pathway, as well as primary enzymes that are widespread in nature The *bap* gene cluster has been extensively studied in both known bialaphos producers, *Streptomyces hygroscopicus* and *S viridochromogenes*, and although considerable nucleotide sequence divergence has occurred, the complex biochemical and genetic organization of the bialaphos pathway is conserved (44)

Although the $-CH_2CH_2-P$ unit of phosphinothricin was believed to originate *via* an intramolecular rearrangement of phosphoenolpyruvate as had been proposed for the other known C-P containing metabolites 2-aminoethylphosphonic acid (45), fosfomycin (46) and FR33289 (47), efforts to detect C-P bond forming activity in the producing organisms were unsuccessful (48). Through the use of mutants blocked in bialaphos production, several intermediates were isolated that allowed this unique process to be

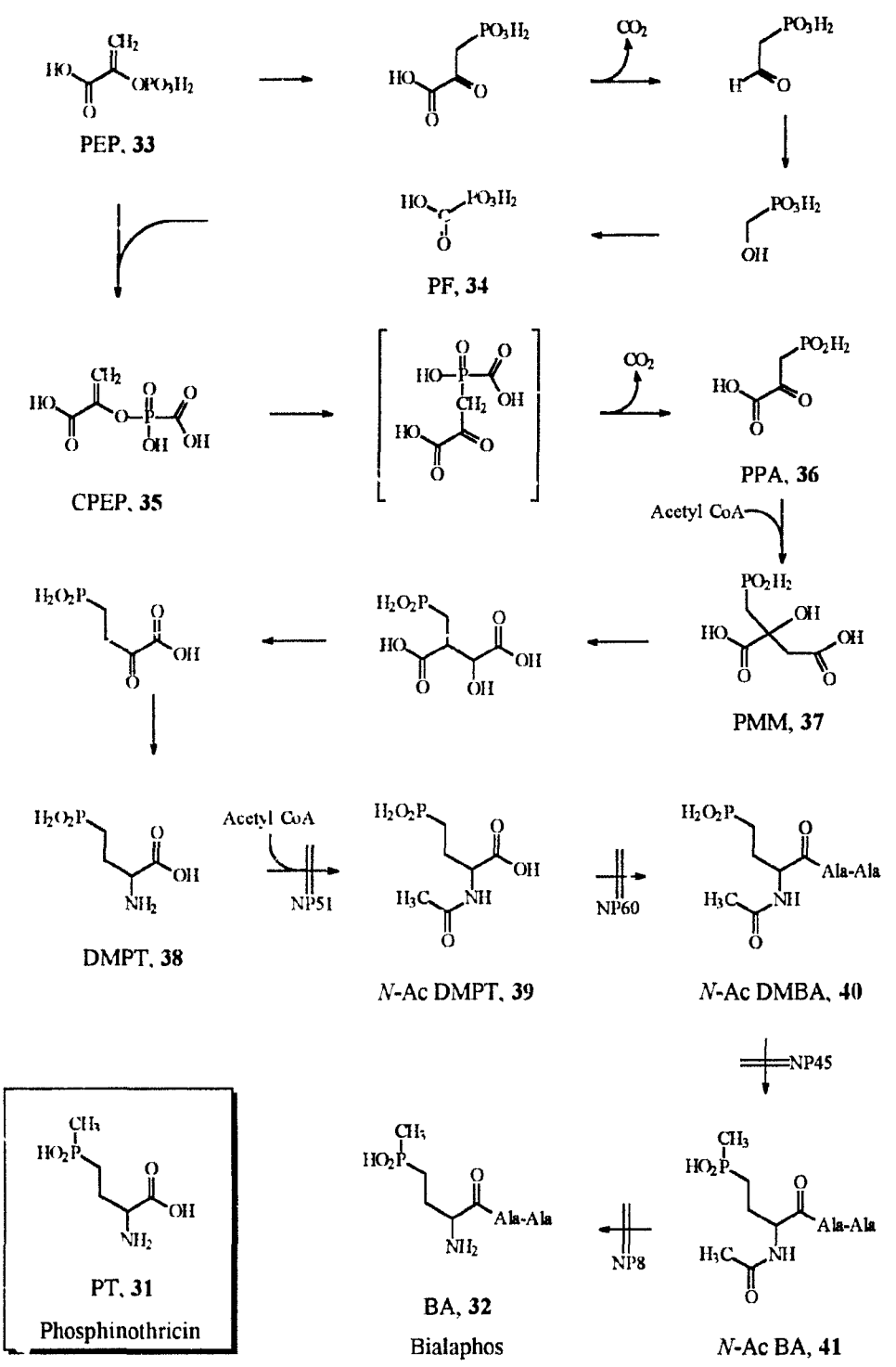


Figure 8. Biosynthesis of Bialaphos (48)

elucidated. The first 4 steps in the bialphos pathway transform phosphoenolpyruvate (PEP, **33**) into phosphonoformic acid (PF, **34**), which is regarded as masked phosphorous acid (48). Condensation of PF with a second molecule of PEP releases a molecule of phosphoric acid and generates carboxyphosphonoenolpyruvic acid (CPEP, **35**). CPEP phosphonomutase catalyzes the intramolecular rearrangement which generates the C-P bond found in bialaphos, and spontaneous decarboxylation yields phosphinopyruvic acid (PPA, **36**) that contains the reduced phosphino group.

Attempts to isolate mutants involved in the chain elongation of PPA to demethylphosphinothricin (DMPT, **38**) were not successful (48). However, isotopic feeding studies had suggested that the two additional carbons were derived from an intact acetate unit. By comparing the structures of PPA and DMPT with oxaloacetate and glutamic acid, an analogy was proposed between the enzymatic steps of the tricarboxylic acid cycle (TCA) and the reactions involved in the transformation of PPA to DMPT. The addition of monofluoroacetic acid, an inhibitor of the TCA enzyme aconitase, resulted in the accumulation of phosphinomethylmalic acid (PMM, **37**) (49) This PPA:acetyl CoA condensation is analogous to that catalyzed by citrate synthase and porcine heart citrate synthase used PPA as a substrate, although at a slow rate (50). Although this suggested that citrate synthase from primary metabolism may play a role in bialaphos biosynthesis, the citrate synthase-produced PMM had the opposite configuration at the generated stereocentre with respect to the natural PMM, and could not be transformed into bialaphos (51, 52). The isolation of citrate synthase from the bialaphos producing organism revealed that PMM and citrate synthases had different

amino acid compositions and *N*-terminal amino acid sequences (52).

Investigations to elucidate the steps involved in the transformation of PMM to DMPT revealed that the transformations were catalyzed by a number of ubiquitous enzymes involved in the tricarboxylic acid cycle. It is not clear if these enzymes are solely responsible for these biosynthetic transformations, but this provides an explanation for fact that researchers failed to generate nonauxotrophic mutants blocked in these steps (48). Acetylation of the amino group of DMPT resulting in *N*-acetyldemethylphosphonothricin (*N*-Ac DMPT, **39**) is the next step in bialaphos biosynthesis. The acetyltransferase, which is the product of the *bar* gene, catalyzes this acetyl CoA dependant acetylation and confers resistance of the producing organism to phosophinothricin (43, 44), which is easily liberated from bialaphos by nonspecific protease activity (48). *N*-Acetyl derivatives are reported as intermediates in the biosynthesis of other antibiotics including kanamycin (47), leupeptin (53) and puromycin (54), and both *N*- and *O*-acetylation are well known as antibiotic resistance mechanisms used in producing (55-59) and target organisms (60-63). In addition to *N*-acetyl intermediates in these secondary metabolic pathways, *N*-acetyl amino acids such as *N*-acetyldiaminopimelate and *N*-acetylglutamate, are well known in primary processes as intermediates in the biosynthesis of L-lysine (64) and L-arginine (65), respectively.

The order of the remaining steps in the formation of bialphos: L-ala-L-ala peptide formation, methylation, and hydrolysis of the *N*-acetyl group, has been elucidated by the isolation of intermediates excreted by nonproducing mutants. Nonproducing mutant NP8 accumulated *N*-acetylbialaphos (*N*-Ac BA, **41**) which was converted to bialaphos by

other nonproducing mutants including mutants NP51, NP60 and NP45, suggesting that NP8 lacked the ability to remove the acetyl group from *N*-Ac BA (66) Mutant NP60 accumulated *N*-Ac DMPT (39) indicating that this mutant could not catalyze peptide bond formation, and NP45 was not capable of *P*-methylating *N*-Ac DMPT and *N*-acetyldemethylbialaphos (*N*-Ac DMBA, 40), indicating a lesion in the gene coding for the *P*-methylating enzyme (66). This suggests that peptide formation must precede *P*-metnylation, which is then followed by removal of the acetyl group to generate bialaphos Site-specific mutagenesis (67) and gene replacement techniques (68) have been used to locate a gene which is believed to encode a multifunctional enzyme responsible for assembling the *N*-Ac DMPT-ala-ala tripeptide (*N*-Ac LMBA), suggesting that bialaphos is assembled in a fashion similar to other known peptide antibiotics

The origin of the methyl group that forms the final C-P bond has been investigated, feeding studies have shown the methyl group of CD_3-methionine is incorporated into bialaphos (69). However, when *S. hygroscopicus* is grown in the absence of Co^{2+}, only DMPT and DMBA accumulate in culture supernatants (70) These compounds were also observed in the culture supernatant of vitamin B_{12} auxotrophic mutants, suggesting that *P*-methylation is mediated by an enzyme utilizing vitamin B_{12} as a cofactor. Investigation of methyl donors capable of transferring a methyl group to N-Ac DMBA (40) showed that only methylcobalamin was effective whereas other common methyl donors such as *S*-adenosylmethionine, betaine and 5-methylfolate were inactive (71) Cell extracts capable of *P*-methylating *N*-acetyl DMPT and *N*-acetyl DMBA failed to methylate the free amino acids, DMPT and DMBA,

indicating that the *N*-acetyl group is a substrate requirement (71).

In addition to the structural genes of *bap* gene cluster, a gene located on open reading frame ORF3 encodes a hydrophobic product with 448 amino acids (M_r 47203), which shows structural motifs found in a large family of integral membrane proteins that transport solutes across membranes. This suggests that ORF3 encodes a BA export protein, although it could encode a permease which allows the uptake of carbon source substrates (43).

Bialaphos production, like that of most secondary metabolites (72), occurs during periods of growth limitation (48). In *S. hygroscopicus*, transcriptional activation of the *bap* genes occurs *via* a regulatory gene *brpA*, which encodes a protein BrpA belonging to a family of transcriptional activator proteins. Therefore, BrpA is believed to exert its regulatory effect by binding to the promoters of the *bap* genes. Since BrpA from *S. viridochromogenes* activates transcription in *S. hygroscopicus*, it is believed that the *bap* promoter sequences are conserved (44).

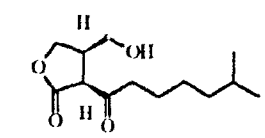
Effect of autoregulators in secondary metabolic processes

In wild-type Streptomycetes, during periods of high growth, the production of antibiotics is suppressed, but during periods of nutritional limitation (phosphate, carbon or nitrogen source depletion) or environmental stress (heat shock), the organism alters its development and progresses through a period of cytodifferentiation in which processes such as sporulation and aerial mycelium formation occur. During this period, mycelial growth slows and the processes of secondary metabolism including the

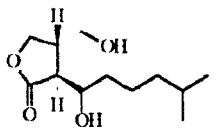
production of antibiotics and pigments are turned on (73)

Although the regulatory processes in Streptomycetes are still not fully understood, it is clear that the coordination of individual control mechanisms through many genes in a relay to form a global regulatory network is extremely complex During chemical mutagenesis (*e.g* , using NTG) to generate mutants blocked in specific biosynthetic pathways, it is quite common to obtain nonauxotrophic mutants having a significantly different phenotype from the wild-type organism These mutants may be unable to control cytodifferentation processes such as sporulation and aerial mycelium formation, and be unable to produce various secondary metabolites. Often these mutants are blocked in the production of substances referred to as autoregulators Autoregulators appear to act as "microbial hormones" similar to hormones in eukaryotic cells and are believed to be the "triggers" that ultimately control a significant number of cellular functions. The first autoregulator discovered was 2-(6'-methylheptanoyl)-3*R*-hydroxymethyl-4-butanolide (**42**, Figure 9), commonly refered to as A-factor, which was isolated from the culture supernatant of wild-type *Streptomyces griseus* A-factor, at a concentration of 10^{-9} mol/L, was capable of restoring aerial mycelium formation and the production of both a yellow pigment and streptomycin in *S griseus* mutants selected for a lack of streptomycin production after mutagenesis. These mutants, grown in the absence of A-factor, failed to produce at least ten proteins found in the wild-type mycelium. Mutant cultures supplemented with A-factor at the time of inoculation produced streptomycin a day earlier than unsupplemented wild-type cultures (74).

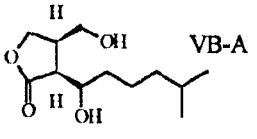
Since the discovery of A-factor, a number of similar γ-butyrolactone



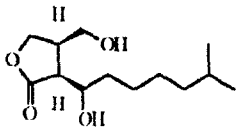
A-factor, **42** from *S. griseus*

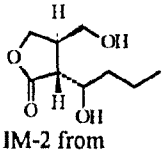


Factor I, *S. viridochromogenes*

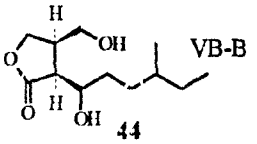


43

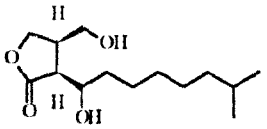




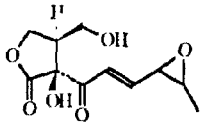
IM-2 from
Streptomyces sp. FRI-5



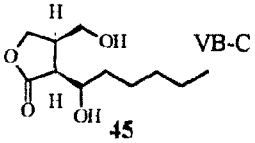
44



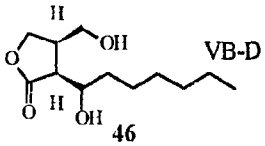
Factors from *S. bikiniensis*
and *S. cyaneofuscatus*



Butalactin from
Streptomyces sp. Y-86,36923

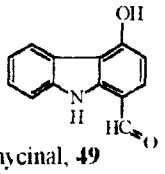


45

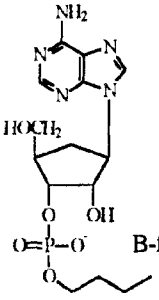


46

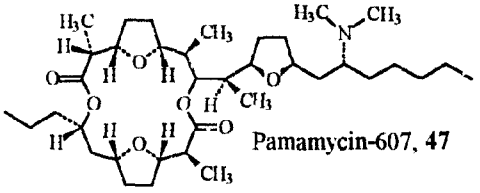
Virginiae butanolides from
Streptomyces virginiae



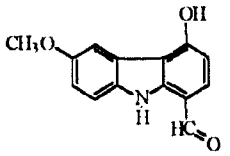
Carbazomycinal, **49**



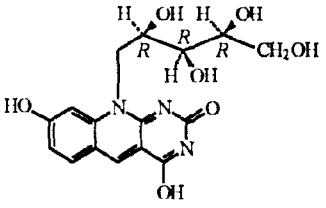
B-factor, **48**



Pamamycin-607, **47**



6-Methoxycarbazomycinal, **50**



CF-1 (cosynthetic factor 1, **51**)

Figure 9. Substances with autoregulatory activity

autoregulators, including the virginiae butanolides (75) (**43 - 46**), have been isolated from Streptomycetes. Examination of 203 actinomycetes revealed that 30 produced an A-factor-like substance (76), suggesting that γ-butyrolactones may play a general role in the regulation of secondary metabolism and cell differentiation

The ease with which A-factor mutants can be generated suggest that the genes that encode enzymes involved in A-factor biosynthesis in *S. griseus* are located on an unstable extrachromosomal element (77). A single gene, *afsA*, found to complement the A-factor deficiency encodes a single enzyme, "A-factor synthase", which is thought to catalyze a key step in A-factor biosynthesis. Although no regulatory gene controlling *afsA* expression has been found in *S. griseus*, two genes *afsR* and *afsK* (formerly known as *afsB* and *afsC*, respectively) have been characterized from another A-factor producer *S. coelicolor* A3(2) (78, 79). These two regulatory genes are believed to encode proteins that form an "orthodox" two-component signalling system (80).

The "orthodox" two-component signalling system consists of a pair of proteins that vary widely in both size and function. A transmembrane protein has an extracytoplasmic sensor domain and a cytoplasmic transmitter domain while the fully cytoplasmic protein has a receiver and a regulator domain. Activation of the sensor domain by a change in a specific extracelluar factor results in a protein conformational change causing a signalling response (*via* phosphorylation) between the transmitter-receiver regions of the proteins. This process results in a conformational change in regulator domain which in turn activates an enzyme or a DNA-binding region, the latter of which acts as a transcriptional activator (73).

Control of streptomycin biosynthesis by A-factor is thought to occur *via* a complex regulatory cascade. In *S. griseus*, A-factor binds to a single 26-kDa cytoplasmic protein with a dissociation constant (K_d) of 0.7×10^{-9} mol/L (81), which is consistent with the very low effective concentration required for A-factor activity. This protein, which acts as a repressor by binding to a yet unknown gene, is released from the promoter by the binding of A-factor. The product from this unknown gene is an activator for the transcription of the *strR* gene which encodes StrR, a protein believed to function as an antiterminator in the transcription of the first biosynthetic gene, *strB* 1, associated with streptomycin production (74).

In addition to the autoregulator γ-butyrolactone factors discussed above, a number of other autoregulator factors having quite different structures have been isolated from a number of sources (Figure 9). Pamamycin (**47**) isolated from *Sreptomyces alboniger* (82) was later found to be a mixture of at least eight homologues, all containing a 16-membered macrodiolide ring and having molecular weights of 593 to 691. Another regulator, B-factor (**48**), was isolated from a commercial yeast extract and found to restore rifamycin production in *Nocardia* sp. KB-993 at very low concentrations (83). Two structurally related autoregulators, carbazomycinal (**49**) and 6-methoxy-carbazomycinal (**50**), have been isolated from a *Streptoverticillium* species during a search for substances that induce differentiation (84).

In addition to the autoregulatory factors discussed above, a number of effector substances found to play crucial roles in antibiotic biosynthesis have been isolated. A substance, described as "substance X", was found to stimulate production of

oxytetracycline in blocked mutants of *Streptomyces rimosus* Further investigations revealed that a late step in tetracycline biosynthesis required trace amounts of this "cosynthetic factor 1" or (CF-1, **51**) Elucidation of the CF-1 structure (7,8-didemethyl-8-hydroxy-5-deazariboflavin (85)) revealed it to be identical to fragment F0 from coenzyme F_{420} (86).

Investigations into the biosynthesis of thienamycin by *Streptomyces cattleya* have failed to produce mutants blocked in the biosynthetic pathway (87) However, a pair of mutants were isolated which showed cross-feeding consistent with biosynthetically blocked mutants, but a substance isolated from one mutant did not incorporate radioactivity when produced in cultures supplemented with radioactive precursors of thienamycin. Two known autoregulators as well as a large number of cofactors were added to mutant cultures but no effect was observed When the substance was added to mutant cultures above a threshold concentration, no increase in thienamycin was observed. The authors have suggested that the substance is an activator of transcription although work continues to resolve the question.

RESULTS

The investigation described in this thesis focuses on the isolation and structure determination of substances produced and excreted by the HON$^-$ mutant L138. The culture supernatant of mutant L138 is capable of stimulating HON production by HON$^-$ mutant L127 and could contain an intermediate of the HON biosynthetic pathway. An intermediate accumulated by mutant L138 would be expected to resemble the final biosynthetic product HON, since the position of the biosynthetic block is late in the pathway (14) To obtain further information on this potential intermediate, a bioassay-directed purification was pursued

Development of an analytical method to quantify the L138 metabolite

The availability of a sensitive and rapid assay for the detection of a metabolite is required to guide its isolation and to monitor the effectiveness of each purification step. The amino acids proposed as intermediates of the HON biosynthetic pathway (Figure 4) do not absorb visible and ultraviolet light strongly. HON (**1**) has only a weak absorption at 271 nm (ε 24) (5). Therefore, direct spectroscopic detection in a complex and highly coloured culture broth was not feasible, and no prominent chromatographic peaks were detected in the L138 culture supernatants by the opa-hplc method previously developed by White, DeMarco and Smith (20). This methodology relies on the hplc separation and fluorescence detection of compounds containing primary amines, such as amino acids, as their 1-alkylthiol-2-alkyl isoindole derivatives (**13**) formed on reaction with *o*-phthalaldehyde (**11**) and β-mercaptoethanol (**12**, Figure 2).

The lack of structural information precluded the development of other chemical-based assays. However, an assay method based on the ability of the HON mutant L127 to produce HON when supplemented with L138 metabolite can be employed. This indirect approach requires two distinct steps: 1) stimulation of HON production by mutant L127 and 2) detection and quantification of HON by the opa-hplc method. Since a biosynthetic intermediate accumulated by mutant L138 may not be completely converted into HON by the L127 convertor organism, this assay provides a minimum estimate of the amount of an intermediate produced by mutant L138.

Sample preparation

A stable preparation of the L138 metabolite, needed to systematically investigate the parameters of the L127 assay, was obtained by lyophilization of 48-h L138 culture supernatants. The dry residues maintained activity when stored at -20°C, and could be reconstituted in as little as 10% of the original volume of the solution lyophilized. The concentrated solutions permitted additions of L138 metabolite to be made without substantially increasing the mutant L127 culture volume. Also, the availability of concentrated solutions of the L138 metabolite allowed the metabolite concentration to be varied during the development of the assay.

However, the high level of solids in the L138 culture supernatant (approx 15 g/L) limited the concentration to 10-fold and produced viscous mixtures containing lumps of semi-hydrated solids. The high viscosity slowed the diffusion of these solutions into agar. To overcome these limitations, lyophilized L138 culture supernatant

(approx 10-fold concentration) was dialysed against water, and the dialysate (small molecule fraction) was concentrated by rotary evaporation Dialysate at a 20-fold concentration could be stored at -20°C without significant change, and concentrations up to 75-fold were considerably less viscous than reconstituted lyophilized culture supernatant In this thesis, the fold concentration used to describe lyophilized culture supernatants and dialysates is referenced to the volume of the original culture supernatant

Liquid culture assay

Although the presence of metabolite in culture supernatants was demonstrated in the cross-feeding experiments of Le (14), this approach was not suitable for the analysis of purified samples. Therefore, the addition of metabolite samples to L127 liquid cultures was investigated as an assay procedure.

Variation of addition and sampling times

A routine and uncomplicated assay requires a defined incubation period between sample addition and opa-hplc analysis Since secondary metabolic processes are often only active at high rates for brief periods during an organism's life cycle (37), the influence of the age of the L127 culture on HON production was investigated by adding lyophilized L138 culture supernatant (5-fold concentration) to 10-mL L127 liquid cultures at 36, 48 and 60 h (Figure 10) Initial HON production was greatest when metabolite was added at 36 h, but maximum HON production was obtained with the

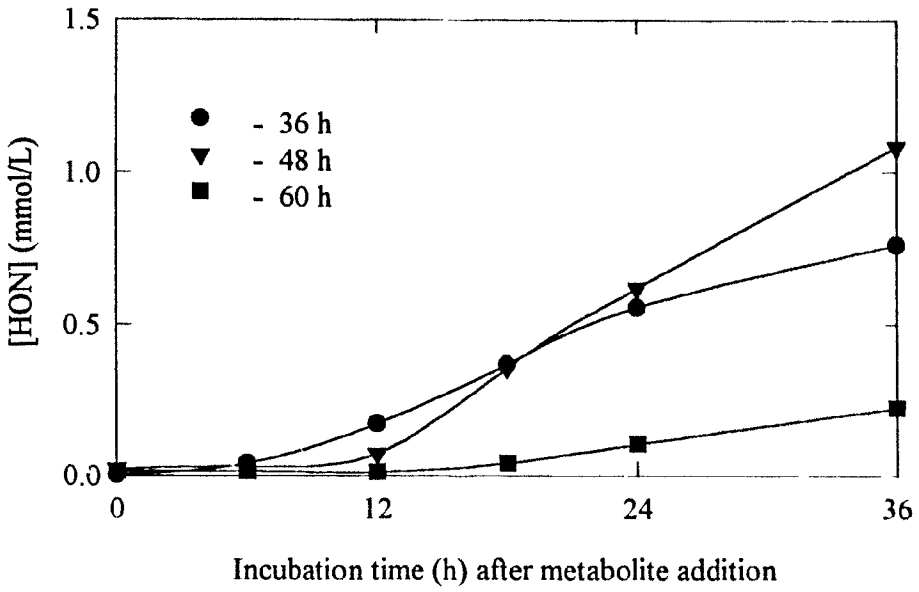


Figure 10. L127 Culture assay: addition of L138 lyophilized culture supernatant (5-fold concentration) at 36, 48 and 60 h.

48-h addition. Significantly smaller levels of HON were measured in L127 cultures supplemented at 60 h. Similar trends were observed in cultures supplemented with a more concentrated lyophilized culture supernatant (*e.g.*, 10-fold), but cultures supplemented with lower concentrations of metabolite, either fresh culture supernatant or lyophilized culture supernatant (1-fold), provided maximum HON production when supplemented at 36 h (Figure 11). From Figures 10 and 11, it is apparent that HON production did not reach a maximum level within 36 h. When HON production was monitored over a longer time period (Figure 12), maximum HON levels were reached between 96 and 120 h.

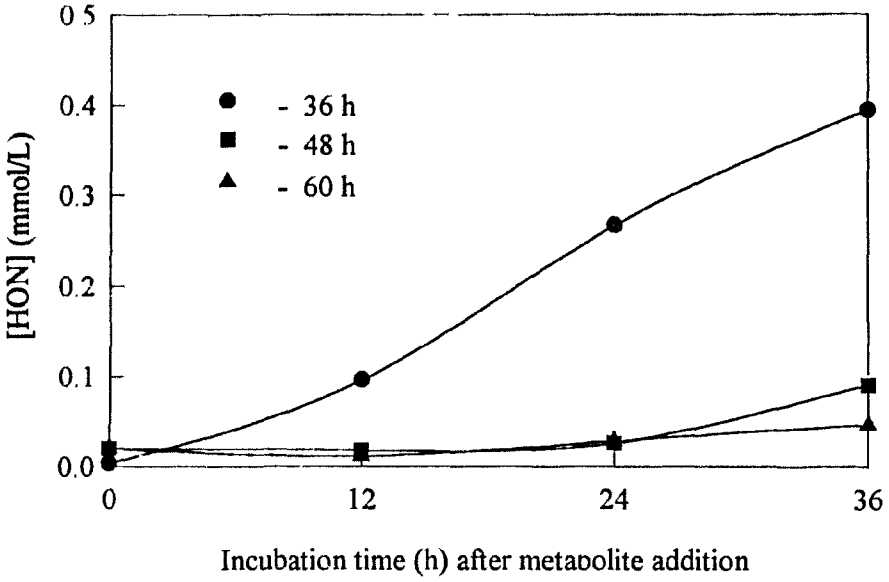


Figure 11. L127 Liquid culture assay: addition of fresh L138 culture supernatant at 36, 48 and 60 h.

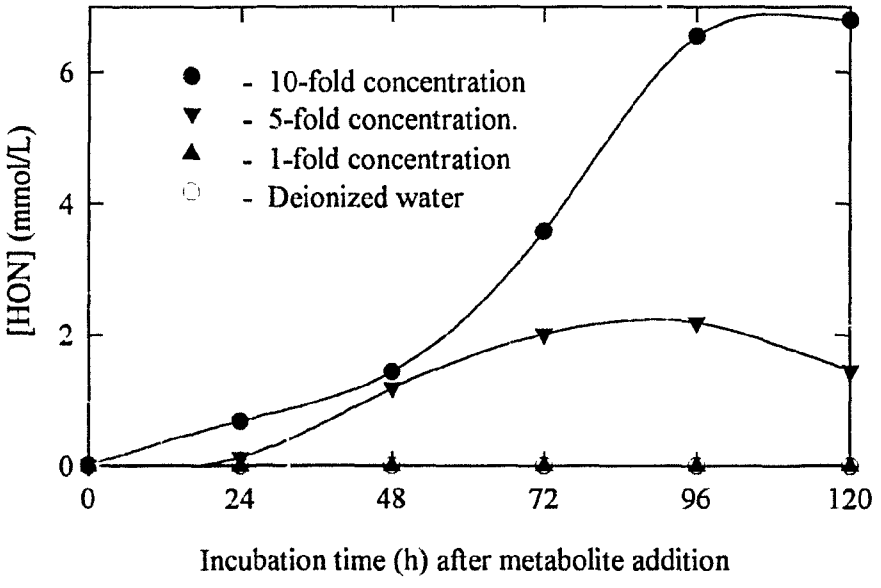


Figure 12. L127 Liquid culture assay: 36-h addition of L138 lyophilized culture supernatant at three concentrations

Volume of the liquid culture

In order to minimize the amount of L138 metabolite required to obtain a response in the L127 assay, smaller liquid culture sizes were examined Cultures containing 3 and 5 mL of media in 25- and 50-mL Erlenmeyer flasks, respectfully, were supplemented at 48 h with L138 lyophilized culture supernatant (10-fold concentration). Compared to the standard culture volume (10 mL per 125-mL flask), the 5-mL culture produced less HON and the 3-mL culture provided a 15% higher HON concentration at 36 h (Figure 13). The latter result may be attributed to concentration of the culture broth by evaporation.

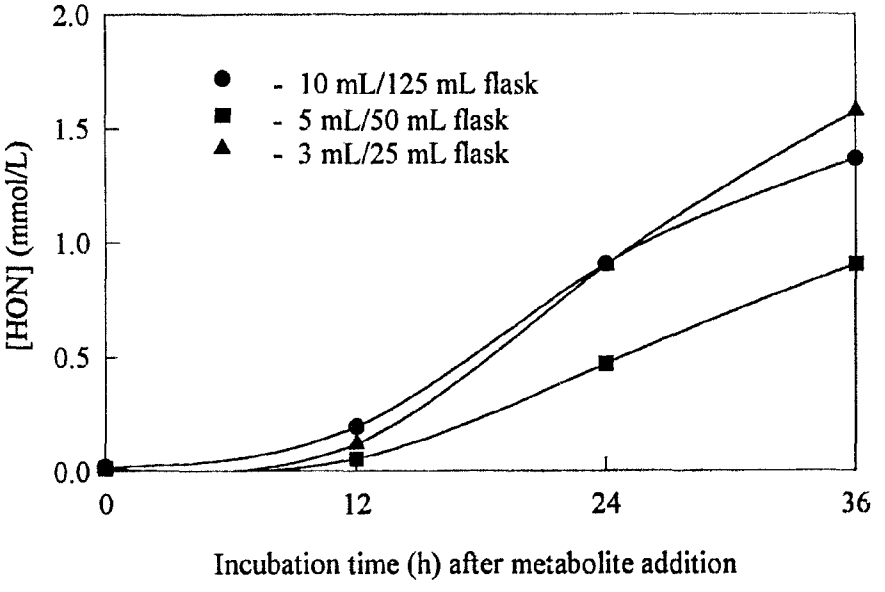


Figure 13. L127 Liquid culture assay: effect of culture volume

Non-shaking assay cultures

The significant evaporation noted in the small cultures would be minimized if the cultures were not shaken. Cultures of wild-type *S. akiyoshiensis* do not produce HON without shaking during incubation (88), but the enzymatic steps required to convert an intermediate to HON by mutant L127 may not require aeration. When a culture of mutant L127 was supplemented at 48 h with L138 lyophilized culture supernatant (10-fold concentration) and incubated without shaking, significant amounts of mycelia grew, but the culture produced less than 3% of the HON observed in shaken flasks. Therefore, standing cultures of mutant L127 are not suitable to assay the L138 metabolite.

Table 1. Effect of L138 supernatant concentration on HON produced in 48-h L127 liquid cultures.

Addition (1 mL)	[HON] (mmol/L)		
	0 h	24 h	Difference (24 h - 0 h)
10-fold	0.009	1.09	1.08
5-fold	0.015	0.21	0.20
1-fold	0.017	0.015	-0.002
Control	0.019	0.008	-0.011

Difficulties and limitations

During several experiments (*e.g.*, Figure 12 and Table 1), it was observed that when L127 liquid cultures were supplemented with lower concentrations of metabolite, either fresh culture supernatant or lyophilized culture supernatant (1-fold concentration), no HON was produced. In general, L127 liquid cultures supplemented with lyophilized L138 culture supernatant at 5- and 10-fold concentrations reliably produced HON, but variable results were obtained with 1-, 2- and 3-fold concentrations. Because of the insensitivity of the liquid culture assay, large quantities of lyophilized L138 powder were required to obtain reliable responses to indicate the presence of the active metabolite. A linear relationship between the concentrations of L138 metabolite added and the amount of HON produced in the L127 liquid cultures was not observed. When 5- and 10-fold concentrations of L138 culture supernatant were added, often a 3- or 5-fold increase in HON production was measured, *e.g.*, Figure 12 and Table 1, instead of the expected 2-fold difference.

When smaller culture volumes were employed, mutant L127 grew as mycelial clumps which were larger (2 - 4 mm) and more varied in size than in the standard culture (approx. 0.5 mm). For example, a 3-mL culture produced only seven mycelial clumps with diameters ranging from approx. 0.5 to 4 mm. Variable growth morphology in cultures of *S. akiyoshiensis* has been previously studied by Glazebrook *et al.* (89), and clumping significantly affects HON production.

The variable responses described above and the relatively large quantities of metabolite needed to stimulate HON production suggested that the L127 liquid culture

assay was not suited to directing the isolation of the L138 metabolite. As an alternative, the addition of metabolite to surface cultures of mutant L127 growing on semi-solid agar was examined

Agar plug assay

HON produced by mycelium cultured on an agar surface must be extracted from the semi-solid medium into aqueous solution prior to analysis by the opa-hplc method. The diffusion of HON from agar into water was investigated using agar plates inoculated with wild-type *S. akiyoshiensis* and grown for 72 h. Five circular disks (12 mm diameter and 4 mm thick) were cut from the mycelial lawn and allowed to stand in water At various times, samples were removed for opa-hplc analysis of HON. HON diffused from the agar and reached 90% of its maximum level in the aqueous phase after 4 h (Figure 14). The maximum HON concentration was observed at 13 h, and a constant level was maintained over the next 26 h. Thus HON diffused from the agar plugs over a reasonable time.

Application method

Three ways of delivering metabolite containing sample to L127 mycelium on casein-starch agar disks (12 mm O.D.) were investigated: 1) sample was applied to the well (6 mm) of a donut-shaped plug (well method); 2) sample was placed into a short piece of drinking straw (5 mm diameter) pushed about 1 mm into the plugs' surface (straw method); and 3) sample was applied to a small disk (5 mm) of filter paper placed

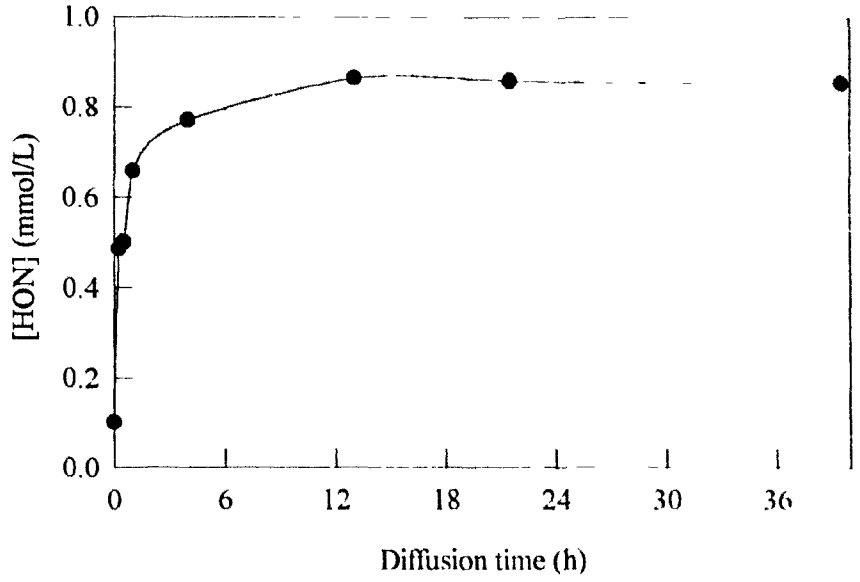


Figure 14. Diffusion time course for HON produced by wild-type *S. akiyoshiensis* grown on casein-starch agar

on the surface of the plug (disk method). For each concentration examined, the highest levels of HON were produced using the well method (Table 2). In both the straw and disk methods, the quantity of sample absorbed into the agar plug varied within replicate plugs, and the amount of unabsorbed sample solution was largest in the most concentrated culture supernatant. For these reasons, the well method was adopted as the standard for sample delivery. The nonlinear response noticed in the liquid culture assays was also apparent for each application method (Table 2).

L127 culture age and incubation period in casein- and nitrate-starch agar media

Casein-starch agar contains opaque blotches of gelatinous casein embedded in

Table 2. Effect of the sample application method.

Application method	Average [HON] (mmol/L)			
	[Culture supernatant]			
	5-fold	3-fold	1-fold	Control
Well	0.38	0.075	0.048	0.028
Straw	0.062	0.062	0.047	0.039
Disk	0.20	0.048	0.037	0.042

Sample (120 µL) was applied to 3 circular plugs (12 mm O.D.) cut from 72-h casein-starch cultures, and HON was measured 24 h later.

the medium. Although there was no evidence indicating that this non-homogeneous medium contributes to assay variability, a nitrate-starch medium provides a homogeneous and transparent agar and supports the production of HON in liquid culture (88).

Since the time of the addition of metabolite to liquid cultures had a profound affect on the production of HON by liquid cultures of mutant L127 (Figures 10 and 11), this was investigated using surface cultures grown on casein- and nitrate-starch media Lyophilized L138 culture supernatant (5-fold concentration) was applied to agar plugs at 48, 60, 72 and 89 h after inoculation. HON was extracted from the plugs after 24, 48 or 72 h of incubation with metabolite and measured by opa-hplc.

HON was produced in each assay, but a maximum level was not reached within 72 h (Table 3). Generally, HON production was greater and more rapid when metabolite was added to mutant L127 incubated on agar medium for longer times, and

Table 3. Effect of mycelium age and incubation period on HON production.

Mycelium age (h)	[HON] (mmol/L)[a] Incubation period (h)		
	24	48	72
Casein			
48	2.0	4 6	4 8
60	0 77	1 8	6 6
72	1.2	3.9	9 2 (60 h)
89	1.5	4 1	---
KNO_3			
48	0 20	3.8	7 6
60	0 36	2 9	8 0
72	2 3	7 0	13 4 (60h)
89	4 8	11 0	---

Sample (120 µL, 5-fold concentrate) was added to plugs (12 mm O D , 6 mm I D. and 4 mm thick).

an optimum time of 89 h or longer was indicated. The results obtained by adding metabolite to the 48-h casein-starch culture did not fit this trend, but a similar effect for young mycelium grown on nitrate-starch medium was not observed.

The rate of HON production in the second and third 24-h periods examined was usually greater than the production in the initial 24-h period, particularly for the additions of metabolite to older mycelium. For example, addition of L138 metabolite to a 72-h nitrate-starch culture stimulated 0.36, 2.54 and 5.1 mmol/L production of HON in three consecutive 24-h intervals. Overall, the nitrate-starch medium provides more rapid and higher levels of HON production.

To further investigate the incubation time of mutant L127 grown on nitrate-starch medium, plugs were cut from an 80.5-h culture and supplemented with dialysate (20-fold concentration). After various periods of incubation, plugs were harvested, and the amount of HON was determined by opa-hplc. The level of HON increased to a maximum at 58.5 h of incubation and decreased thereafter (Figure 15). Thus the 60- and 72-h data in Table 3 correspond to maximum levels of HON production.

Alternative carbon sources

Agar medium containing starch from various sources, individual sugars (*e.g.*, glucose and maltose), or mixtures of starch and glucose were inoculated with mutant L127. After 96 h, plugs were supplemented with 4-, 10- and 20-fold concentrations of L138 dialysate (Table 4). For all carbon sources, very little or no HON was produced at the lowest L138 metabolite concentration (4-fold). Although the medium containing

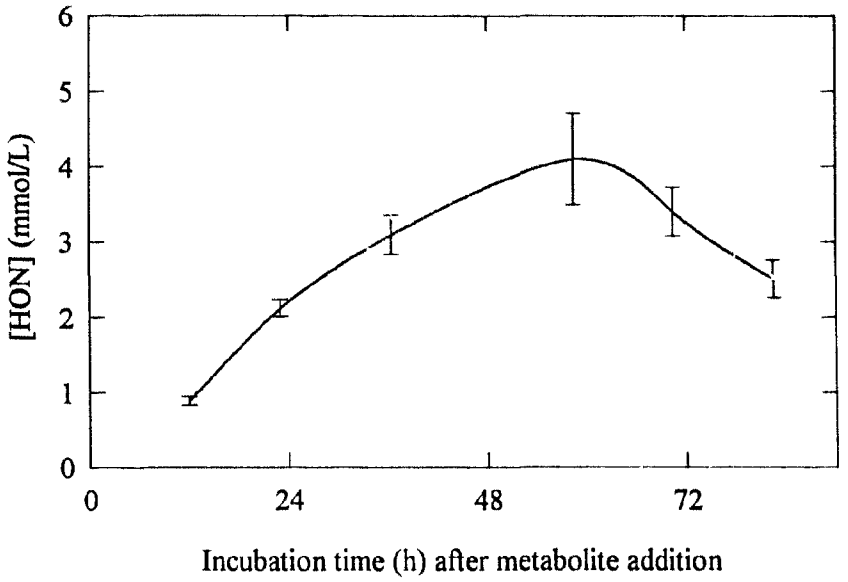


Figure 15. Time course for agar plug assay

glucose as the sole carbohydrate grew extremely poorly resulting in a patchy lawn, it provided the highest HON production (9.6 mmol/L) for the 20-fold dialysate sample; the 10-fold dialysate sample produced only 25% as much HON. The other media do not appear significantly different from one another and therefore the soluble starch was maintained as the carbon source in the agar culture medium.

Diffusion rate from whole, quartered or minced assay plugs

The effect of increasing the area of exposed agar surface on the diffusion of HON from nitrate-starch agar assay plugs was compared (Table 5). The initial diffusion of HON from intact agar plugs was slower than that from quartered or minced plugs, but although the initial rates differ significantly, the difference is negligible at 3.5 h

Table 4. Alternative carbon sources.

	[HON] (mmol/L)		
	L138 dialysate concentration		
Carbon source	20-fold	10-fold	4-fold
Soluble starch (2%)	5.6	4.5	0.12
Soluble starch (1 8%) + glucose (0.2%)	7.0	3.8	0.10
Soluble starch (1.0%) + glucose (1.0%)	4.2	2.9	0.17
Glucose (2.0%)	9.6	2.6	0.01
Maltose (2%)	4.1	1.7	0.00
Corn starch (2%)	5.6	4.2	0.01
Potato starch (2%)	4.9	1.1	0.00

Sample (120 µL) was added to plugs (12 mm O.D., 6 mm I.D. and 4 mm thick) cut from 96-h cultures and after an additional 72 h incubation, plugs were extracted for 3.5 h.

Table 5. Diffusion time course for HON from whole, quartered and minced plugs.

Diffusion time (h)	[HON] (mmol/L)		
	Plug treatment		
	Whole	Quartered	Minced
0	2.6	3.0	4.3
0.33	4.8	5.9	6.0
1	6.0	6.4	6.9
2	7.2	8.1	7.7
3.5	8.1	8.0	8.1
7	5.3	5.6	5.2

Sample (120 µL) was added to plugs (12 mm O.D., 6 mm I.D. and 4 mm thick) cut from 96-h nitrate-starch cultures and after an additional 45-h incubation, plugs were extracted for 6 h.

when the maximum HON concentration is reached. In contrast to the diffusion of HON from plugs inoculated with wild-type *S. akiyoshiensis* (Figure 14), the level of HON decreased by about 35% on standing for several hours. The difference in the rate of HON diffusion from the agar plugs can possibly be attributed to the reduced surface area of the disk-shaped plugs on which the wild-type *S. akiyoshiensis* was inoculated compared to the donut-shaped plug of the L127 culture and to the change of growth medium (casein-starch used with the wild-type *versus* nitrate-starch used in the L127 mutant culture) rather than a difference in the organism. Therefore, a 3.5 h diffusion

time is close to the optimum.

Effect of pH on HON production by mutant L127

Nitrate-starch medium was prepared over the pH range 5.0 - 7.0. Plugs were cut from mycelial lawns of 81-h L127 cultures and supplemented with L138 culture supernatant. Variation of the pH resulted in only slight differences in HON production at five different concentrations of L138 metabolite with an indication of higher levels produced at pH 6.0 (Table 6). Therefore, the pH was maintained at 6.0, the value used for all preliminary investigations.

Table 6. Effect of pH on HON production by mutant L127 grown on nitrate-starch medium

[L138 supernatant]	[HON] (mmol/L)				
	pH 5.0	pH 5.5	pH 6.0	pH 6.5	pH 7.0
5-fold	1.7	1.7	1.9	1.8	1.7
4-fold	2.2	1.8	2.1	1.8	1.8
3-fold	1.4	1.8	2.2	1.1	1.5
2-fold	1.3	0.93	1.3	0.85	0.81
1-fold	0.66	0.41	0.55	0.51	0.35

Sample (120 µL) was added to plugs (12 mm O.D., 6 mm I.D. and 2 mm thick) cut from 81-h cultures and after an additional 24 h incubation, plugs were extracted for 10 h.

Plug dimensions

Casein-starch agar plugs of varying thickness and diameter were supplemented with L138 culture supernatant (5-fold concentration). The production of HON decreased with increasing plug diameter (Table 7). However, an increase in the diameter increases both the surface area and the volume of the agar plug. The latter was investigated by varying the thickness of the agar and maintaining a constant surface area (Table 8) HON production was large when the volume of the plug was small When plug thicknesses between 2 and 4 mm are considered, a proportional relationship between the volume of the plug and HON production is observed The first entries in Tables 7 and 8 show plugs of approximately equal volume producing approximately equal quantities of HON. Two other entries of approximately equal volume (0 62 and 0 68 cm^3) indicate that a large plug diameter is detrimental to HON production.

It should be noted that although the results discussed above indicate that the smallest possible plug volume provides the highest production of HON, there were serious experimental difficulties when plugs with outer diameters ≤ 9 mm or thicknesses < 4 mm were used. About 60 - 70% of the 9-mm plugs were not usable due to tears produced in cutting. Plugs with a thickness of less than 4 mm showed significant drying and softening during the development of the mycelial lawn, making these plugs quite difficult to remove from the culture lawn after cutting. Increasing the concentration of agar from 1.5 to 2.0% (w/v) significantly increased the rigidity of 2 mm plugs, although still 35 - 40% of plugs were damaged beyond use during the transfer, no significant effect in HON production was observed in the assays with the increased agar content.

Table 7. Effect of plug surface area.

Outer diameter (mm)	Surface area (mm^2)	Volume (cm^3)	[HON] (mmol/L)
9.0	35	0.25	2.6
12	85	0.45	1.2
14	130	0.62	0.30
16	170	0.80	0.070

Sample (120 µL) was added to plugs (6 mm I.D. and 4 mm thick) cut from 72- h casein-starch cultures and after an additional 24 h incubation, plugs were extracted for 10 h.

Table 8. Effect of plug volume.

Thickness (mm)	Surface area (mm^2)	Volume (cm^3)	[HON] (mmol/L)
2.0	85	0.23	2.6
3.0	85	0.34	1.9
4.0	85	0.45	1.2
6.0	85	0.68	1.3

Sample (120 µL) was added to plugs (12 mm O.D. and 6 mm I.D.) cut from 72- h casein-starch cultures and after an additional 24 h incubation, plugs were extracted for 10 h.

Therefore, the plug dimensions chosen for future work were 12, 6 and 4 mm for O D , I D. and thickness, respectively.

Total L138 metabolite versus metabolite concentration

The increase in HON production observed with decreasing plug volume may be a result of metabolite concentration in the agar plug .ince the effective concentration of L138 metabolite to the organism is higher at small plug volumes and varying the volume of applied sample should produce no significant effect Agar plugs were supplemented with L138 metabolite at a range of concentrations (1- to 5-fold) at a constant volume (120 µL). In a second set of assays, equivalent amounts of metabolite were applied by varying the volume of L138 supernatant (5-fold concentration) If the production of HON depends on the concentration of L138 me:abolite in the solution applied, there should be no difference in HON production when different volumes of 5-fold L138 metabolite are used. However, if HON production depends on the concentration of L138 metabolite in the agar plug, similar effects should be observed by varying the volume or the concentration of the applied solution. Within the variability of the assay, there was no significant difference between the two methods of varying the metabolite concentration in the sample applied (Table 9) Therefore, HON production in the agar plug assay depends on the total amount of L138 metabolite added, regardless of the volume of the sample applied. The metabolite concentration in the agar plug is proportional to the total amount added

Table 9. Effect of L138 supernatant concentration on HON production in casein-starch agar medium.

Constant volume (120 µL)		Constant concentration (5-fold)	
[L138 supernatant]	[HON] (mmol/L)	Volume (µL)	[HON] (mmol/L)
5-fold	0.98	120	0.98
4-fold	0.87	96	0.53
3-fold	0.39	72	0.59
2-fold	0.21	48	0.16
1-fold	0.052	24	0.058

Sample (120 µL) was added to plugs (12 mm O.D., 6 mm I.D. and 2 mm thick) cut from 77-h casein-starch cultures and after an additional 24 h incubation, plugs were extracted for 10 h.

Threshold and leveling effects

One difficulty noted in the liquid culture assay was the lack of a linear relationship between the amount of metabolite added to an assay culture and the amount of HON formed. The agar plug assay has a similar limitation although there is a range of metabolite concentrations which provide a linear response (Figure 16). However, at concentrations below a minimum or threshold concentration, no HON is produced (Figure 12 and Tables 1, 2 and 4). When concentrations of metabolite are above this

range, the production of HON levels off at a maximum. The concentrations providing the linear response varied from one L127 agar culture to another, and a standard curve was used to define the linear portion of each assay.

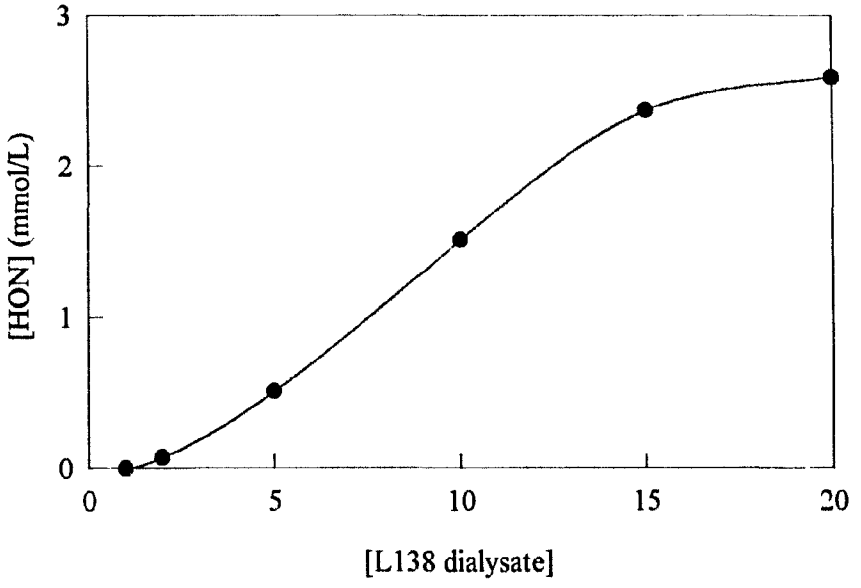


Figure 16. Agar plug assay: threshold effect

Carbohydrate effect

Several significant complications have arisen in the agar plug assay during attempted purifications of the L138 metabolite. Discrepancies were noticed in the levels of HON produced from dialysate and retentate samples (see *Dialysis* p. 65), and an indication of the origin of this effect was provided in the experiment using gel filtration. Agar plug assays performed on fractions collected during Sephadex G-10

chromatography (see *Gel Filtration* p 68) indicated a low recovery of the L138 metabolite applied to the column. The loss could not be accounted for by binding to the Sephadex resin or to poor metabolite stability However, combinations of Sephadex G-10 fractions produced larger amounts of HON than the individual fractions, indicating a synergistic relationship between separated components of the L138 culture supernatant. L138 cultures grown to produce metabolite for purification studies were harvested at 48 h of incubation. At this point, the cultures contained significant levels of starch (88) and the addition of carbohydrate to purified metabolite samples was examined.

Supplementation of L138 dialysate (5-, 10- and 20-fold concentration) with glucose (100 mmol/L) followed by a 42-h incubation in agar plug assays (78 h) resulted in an upward shift in the standard assay curve (Figure 17). In a second experiment, agar plugs were supplemented with dialysate (4- to 20-fold concentration) containing various concentrations of glucose, and HON was analyzed after 48-h of incubation. Low levels of glucose (50 or 100 mmol/L) only stimulate HON production at high concentrations of L138 metabolite, but 250 and 500 mmol/L glucose concentrations significantly increase HON production over the full range of L138 metabolite concentration (Figure 18). For both 250 and 500 mmol/L glucose, HON production in the agar plug assay appears to be linearly dependent on the concentration of L138 metabolite added to the plug

Variability of agar plug assay

A group of ten casein-starch plugs were supplemented with L138 culture

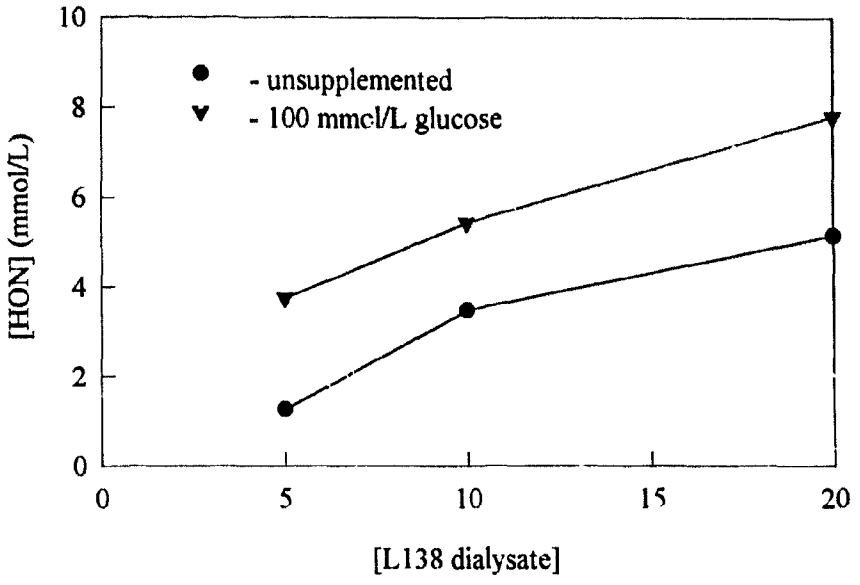


Figure 17. Agar plug assay: effect of 100 mmol/L glucose supplement

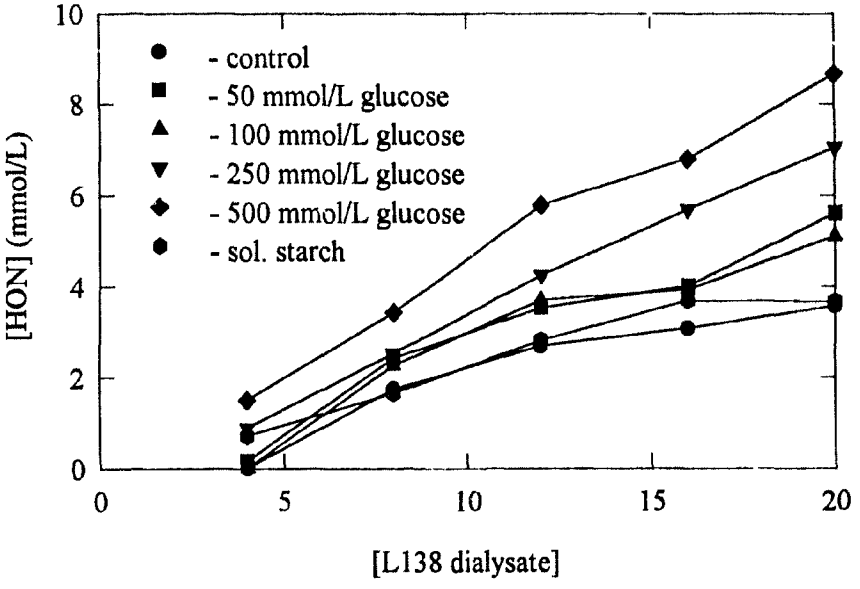


Figure 18. Agar plug assay: effect of carbohydrate supplement

supernatant (120 µL, 5-fold concentration). After a 24-h incubation period, each plug was suspended in water (1 mL) for 10 h, and the aqueous phase was analyzed by opa-hplc. The average concentration of HON was 1.5 mmol/L and the standard deviation was 0.49. The very large standard deviation meant that all results between 0.5 and 2.5 mmol/L must be considered equivalent (95% confidence limit). Since individual injections of large numbers of replicate samples was impractical, metabolite solutions were applied to five replicate plugs which were combined for the aqueous extraction step. Each opa-hplc measurement represents an average of five individual plug assays, and lower standard deviations were calculated for ten groups of five nitrate-starch plugs (Table 10). The relative standard deviations for two concentrations of L138 metabolite (20- and 4-fold) were similar and slightly higher than that of the control assays. Larger

Table 10. Determination of agar plug assay variability.

Sample	[HON] (mmol/L)		
	Mean	Standard deviation	Relative standard deviation (%)
20-Fold dialysate	11.4	1.1	10
4-Fold dialysate	4.3	0.5	12
Glucose/H_2O control	0.15	0.01	7

Sample (120 µL) was added to plugs (12 mm O.D., 6 mm I.D. and 4 mm thick) cut from 96-h nitrate-starch cultures and after an additional 46-h incubation, plugs were extracted for 6 h.

variations between agar plug assays were obtained on different days, and a series of dialysate standards of defined concentration were included in each experiment.

L127 agar plug assay - Summary

The assay based on the stimulation of HON production by L127 cultures grown on semi-solid medium provides a means for estimating the level of L138 metabolite in samples. The optimum conditions are summarized in Table 11.

Table 11. Optimum conditions for the L127 agar plug assay.

Variable description	Optimum
Sample introduction	Well method
Medium	
Carbon source	Soluble starch
Nitrogen source	Nitrate
Agar	2.0%
pH	6.0
Culture age	96 h
Plug dimensions	
Outer diameter	12 mm
Inner diameter	6 mm
Plug thickness	4 mm
Incubation period	48 n
Diffusion time	3.5 h
Glucose supplement	500 mmol/L

Agar plug assay interferences

Initial attempts to purify the L138 metabolite by ion exchange chromatography were unsuccessful because the agar plug assay provided no indication as to the location of the metabolite One possible cause of the negative assay results was interference by salts. Sodium acetate above a concentration of 10 mmol/L completely inhibited the production of HON by mutant L127. At 100 mmol/L and higher concentrations, sodium and ammonium formate completely inhibited HON production, and partial inhibition was obtained at lower concentrations (Figure 19). Neutralization of acidic or basic samples produced sodium chloride, but the concentration of NaCl which is inhibitory is significantly higher (*e.g* , 0.5 mol/L NaCl causes a 25% reduction in HON production).

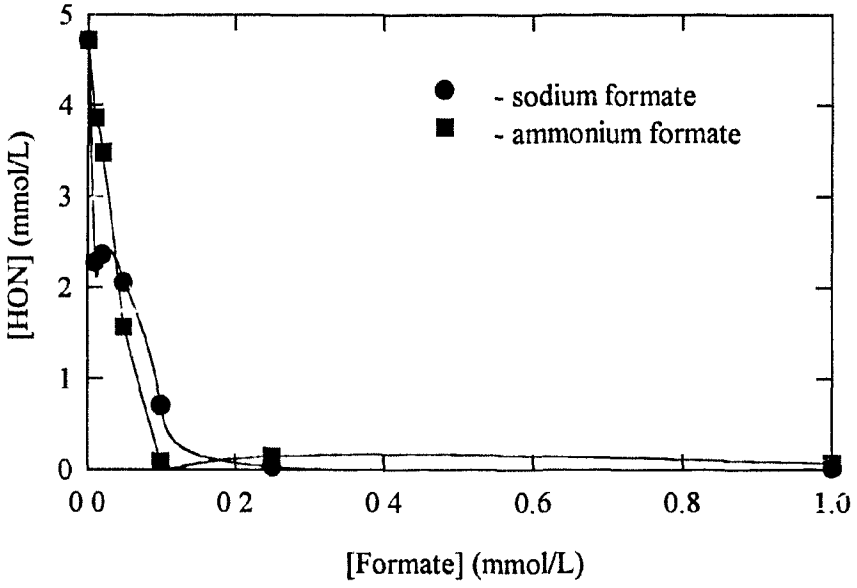


Figure 19. Effect of formate on agar plug assay

HON production by mutant L127 is inversely proportional to the concentration of NaCl (Figure 20), allowing HON production to be corrected for known sodium chloride concentrations in test solutions.

The reagents sodium borohydride and periodate, which react with the hydroxyketone group of HON, were used to probe the chemical nature of the L138 metabolite. However, no HON was produced when mutant L127 was supplemented either with treated samples or treated samples mixed with fresh L138 metabolite (*e.g.* positive control). Although the effective inhibitory concentration of these reagents has not been determined, it is clear that they create significant problems in the agar plug assay.

An inhibitory effect by D-aspartate was first suspected after estimates of L138 metabolite in a culture known to contain D-aspartate were about 25% of that in similar cultures not containing aspartate. Samples of dialysate (20-fold concentration) supplemented with D-aspartic acid (1.8 - 35 mmol/L) resulted in decreasing levels of HON production up to 7 mmol/L D-aspartate, but no additional inhibition was observed at higher aspartate concentrations (Figure 21). It is not clear why there is no increase in D-aspartate inhibition beyond 50%, even when the concentration of D-aspartate in the test sample is increased from 7 to 35 mmol/L.

Initial characterization and purification of the L138 metabolite

Metabolite production in L138 cultures

To determine the optimum harvest time of L138 cultures, the titres of L138

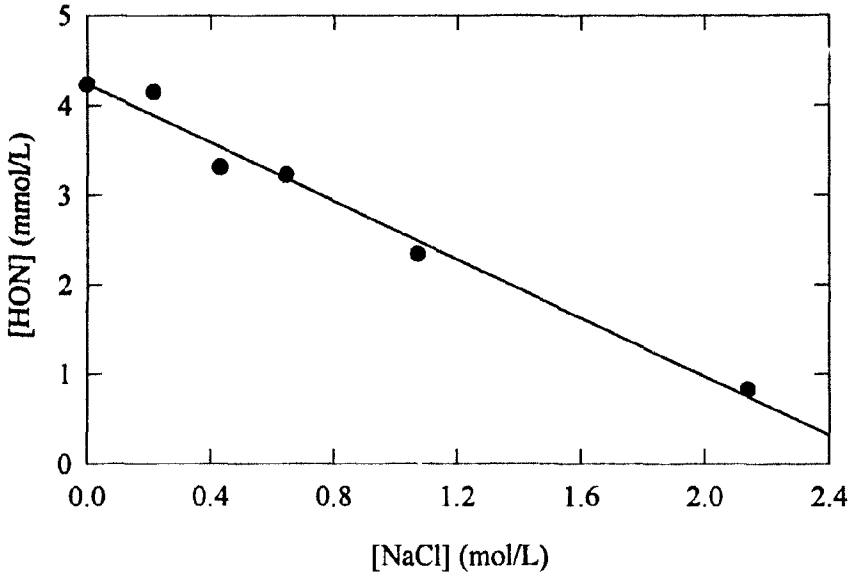


Figure 20. Effect of NaCl on agar plug assay

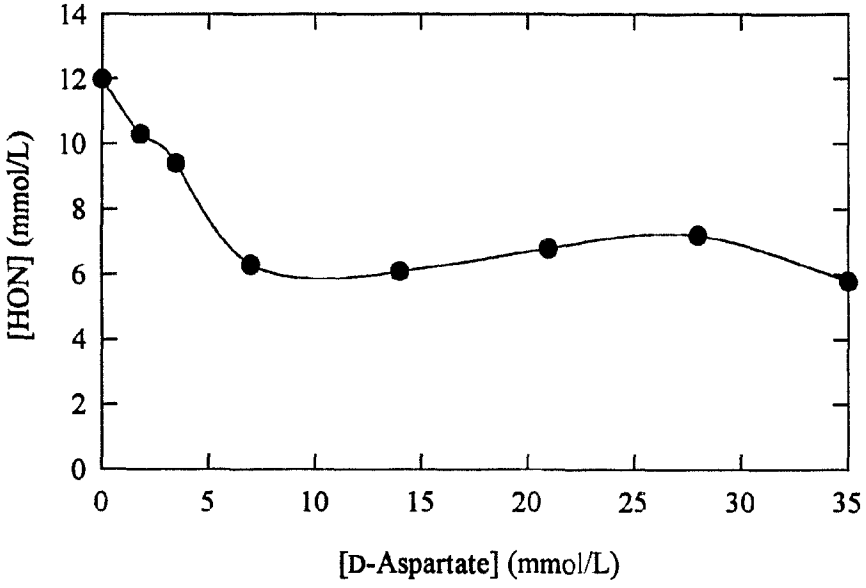


Figure 21. Effect of D-aspartate on agar plug assay

metabolite were determined over a 96-h period of growth. Samples of culture broth were removed and centrifuged; the supernatant was lyophilized and redissolved at a 5-fold concentration. Metabolite formation correlated with the initial rapid decrease of culture components, indicated by the decreasing mass of dissolved solids. The maximum amount of HON was detected in the 48-h sample by the agar plug assay (Figure 22); only 5% of the maximum level was measured at 96 h. Therefore, the most appropriate time to harvest the L138 culture was after 2 days growth.

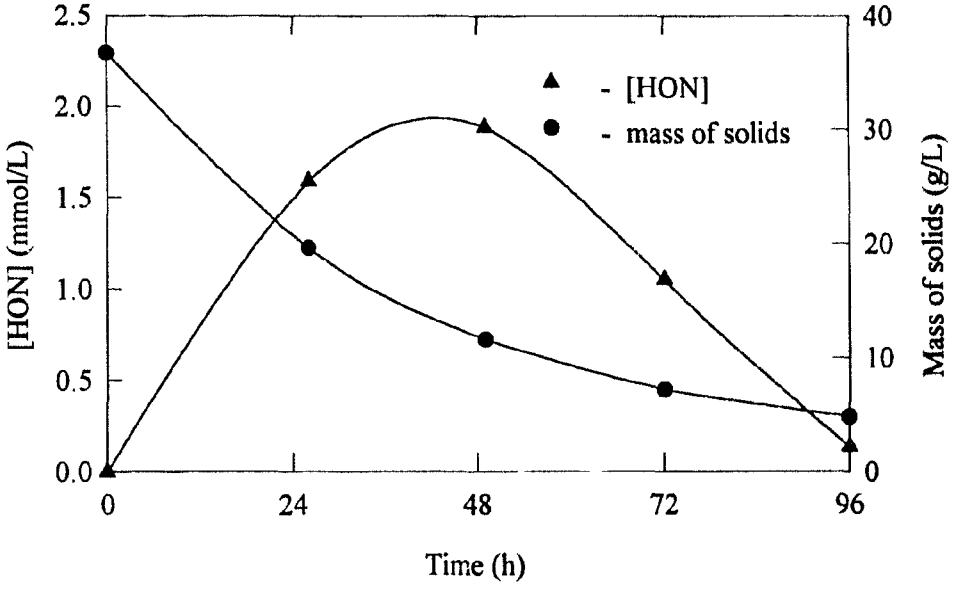


Figure 22. L138 Metabolite production time course. The amount of HON produced in the agar plug assay was assumed to be proportional to the concentration of metabolite in L138 cultures.

Stability

To effectively design a protocol for the isolation of an unknown compound from a complex mixture such as a culture broth, an understanding of the compound's stability was required. The L138 metabolite showed excellent thermal stability in solutions of pH 6.4 and pH 1 (Table 12). The loss in activity (25%) shown in treatments at pH 1 can be attributed to a reduced response in the L127 assay due to NaCl (Figure 20) as discussed above. Heating L138 dialysate (20-fold concentration) at pH 12 and 100°C for as little as ten minutes destroyed the component which stimulates HON production by mutant L127. During heating, the basic solution turned a very dark brown colour which did not lighten on neutralization. The loss of assay activity was not due to assay interferences; a second basified and heat treated sample supplemented with untreated dialysate retained activity in the L127 assay, providing support for base-induced decomposition of L138 metabolite.

Ionizable functional groups

The ionic properties of the L138 metabolite were probed using ion-exchange resins. L138 dialysate (20-fold concentration) was not retained by the strong cation-exchange resin Amberlite IR-120 (H^+ form) suggesting the absence of a cationic substituent (*e.g.*, a protonated amino group).

When L138 dialysate (20-fold concentration × 10 mL) was applied to Dowex 1-X8 (OH^- form), the activity was partitioned between sample effluent (71%) and 0.5 mol/L HCl eluate (29%). The partitioning may be caused by overloading the ionic

Table 12. Stability of L138 metabolite to heating under acidic, neutral and basic conditions.

Treatment	[HON] (mmol/L)
Untreated L138 dialysate	12.0
pH 6.4	
100°C for 10 min	12.8
100°C for 30 min	12.3
100°C for 1 h	12.2
pH 1	
20°C for 1 h	8.9
100°C for 10 min	9.2
100°C for 1 h	8.8
pH 12	
20°C for 1 h	9.2
100°C for 10 min	0.32
100°C for 1 h	-0.06

Sample (120 µL) was added to plugs (12 mm O.D., 6 mm I.D. and 4 mm thick) cut from 96-h nitrate-starch cultures and after an additional 46-h incubation, plugs were extracted for 6 h.

capacity of the column A smaller sample (3 mL) was chromatographed on a column of similar size, and about 25% of the activity originally applied to the column was recovered in the 0.5 mol/L HCl eluate. Since the neutralized and concentrated eluant contains NaCl (*ca.* 3 mol/L), the L127 agar plug assay will underestimate the level of metabolite recovered by about 80% (Figure 20) This strongly suggests that the L138 metabolite has an acidic functional group

When the L138 metabolite was passed through Dowex 1-X8 (AcO^- form) followed by elution with 0.2 mol/L NaCl and 0.5 mol/L HCl, active metabolite was not detected in the sample effluent or eluate. Presumably the negative result was due to interference in the L127 assay by sodium acetate. When Dowex 1-X8 (Cl^- form) was used, metabolite was detected in the sample effluent but not in fractions eluted with 0.2 mol/L NaCl and 0.5 mol/L HCl

Polarity

Precipitation of L138 metabolite with methanol and ethanol

The slow addition of methanol to L138 dialysate (20-fold concentration) to a final concentration of 75% (v/v) methanol precipitated 10% of the dissolved solids, but no L138 metabolite was detected in the precipitate. Addition of ethanol precipitated larger fractions of the dissolved solids (Table 13). After removal of the ethanol by rotary evaporation and dilution to the original volume, analysis of the resulting supernatants showed a significant loss of L138 metabolite at 75% ethanol precipitation. It is not clear if this reduced HON production in the L127 assay was due to inactivation

Table 13. Effect of ethanol precipitation on fresh L138 culture supernatant.

Sample	Mass (g/20 mL)	[HON] (mmol/L)
Original broth	0.2985	3.6
25% Ethanol supernatant	0.2865	3.5
50% Ethanol supernatant	0.2636	2.8
75% Ethanol supernatant	0.2160	1.7

Sample (120 μL) was added to plugs (12 mm O.D., 6 mm I.D. and 4 mm thick) cut from 74-h nitrate-starch cultures and after an additional 65-h incubation, plugs were extracted for 6 h.

of the metabolite, precipitation of the metabolite or absorption of the metabolite to larger molecules (*e.g.*, starch) which precipitate on addition of ethanol.

Chloroform, ethyl acetate and butanol extraction

The L138 metabolite was not extracted from acidified dialysate with chloroform However, when acidified dialysate (20-fold concentration) was triply extracted with ethyl acetate or butanol, significant L138 metabolite was detected in the organic phase (Table 14). Butanol was a more effective than ethyl acetate for the extraction of the L138 metabolite from the aqueous dialysate. These results indicate that the L138 metabolite is a relatively polar molecule.

Table 14. Ethyl acetate and 1-butanol extraction of L138 dialysate (20-fold)[a].

Extraction	[HON] (mmol/L)	
	Aqueous phase	Organic extract
Ethyl acetate	11.2	1.4
1-Butanol	2.8	6.5

Sample (120 µL) was added to plugs (12 mm O.D., 6 mm I.D. and 4 mm thick) cut from 96-h nitrate-starch cultures and after an additional 46-h incubation, plugs were extracted for 6 h.

[a]Untreated dialysate (20-fold) produced 12.0 mmol/L HON.

Binding of L138 metabolite to reverse phase (C_{18} and CN) resins and charcoal

In an attempt to discover a method to separate the L138 metabolite from the large mass of salts and carbohydrates in the dialysate, samples were examined for their ability to bind to reverse phase materials. Application of an acidified dialysate (20-fold concentration) to C_{18} reverse phase silica gel indicated that only a small portion (7%) of the total L138 metabolite applied was retained and eluted from the column with methanol. When a cyanopropyl (CN) reverse phase resin was used, activity was detected in the sample effluent but not in the methanol eluate.

The L138 metabolite and 65% of the total mass of the applied culture supernatant bound to a charcoal column (Darco G-60). Most of the active metabolite (80%) and 20% of the applied mass were eluted with 50% aqueous acetone (Table 15). The ^1H

nmr spectra recorded for the 20, 40 and 60% aqueous acetone eluates from a second charcoal column contained resonances attributed to carbohydrate only, suggesting that the L138 metabolite associates with starch fragments. The nmr signals due to the L138 metabolite may be hidden by the stronger carbohydrate resonances or the quantity of the active L138 metabolite may be below the nmr detection limits for a sample containing relatively large amounts of other substances due to the limiting dynamic range of the instrument.

Table 15. Darco G-60 charcoal treatment of fresh L138 culture supernatant.

Sample	Mass (g)	[HON] (mmol/L)
Applied sample	0.6433	1.6
Sample effluent	0.2215	0.16
50% Acetone eluate	0.1960	1.3
100% Acetone eluate	0.0042	0.019

Sample (120 µL) was added to plugs (12 mm O.D., 6 mm I.D. and 4 mm thick) cut from 72-h nitrate-starch cultures and after an additional 48-h incubation, plugs were extracted for 6 h.

Preparative hplc

Reverse phase hplc was performed on a 5-cm C_{18} column with H_2O-methanol gradient elution (2 mL/min). Figure 23 shows a chromatogram from a single injection

showing the separation of absorbing (260 nm) compounds. The effluents from twelve separate chromatographic injections were combined. The 0 - 2 min fraction contained most of the recovered solids (94%) and all the L138 metabolite.

More uv-absorbing material was retained on the reversed-phase column when tetra-*n*-butylammonium hydrogen sulfate was included in the mobile phase. Hydrophobic binding of the tetra-*n*-butylammonium ion to the reverse phase resin generates a charged matrix where separation depends on both the ionic nature of the species and hydrophobic interactions. Analysis of fractions by the agar plug assay did not locate the L138 metabolite. Addition of tetra-*n*-butylammonium hydrogen sulfate to dialysate inhibited HON production in the agar plug assay, indicating that the ion-pairing salts were not compatible with the assay.

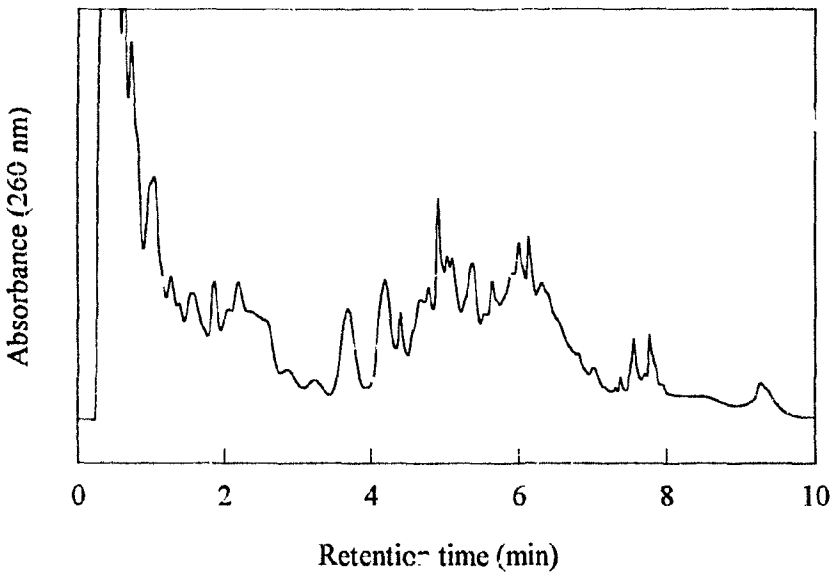


Figure 23. Preparative hplc chromatogram of L138 dialysate

Dialysis

When the metabolite concentration is at its maximum in the L138 cultures, about 30% of the initial mass of dissolved solids remains in the culture supernatant (Figure 22). Reconstitution of the freeze-dried culture supernatant yields a viscous solution, indicating that large molecules (*e.g*, starch and casein) comprise a large fraction of the unmetabolized solids. A solution of lyophilized L138 supernatant (5-fold concentration) was dialysed against water for 9.5 h and the resulting solutions from inside and outside the sack were lyophilized. The majority of the mass (78%) remained inside the sack while only 22% was found in the dialysate. The lyophilized residues were dissolved in water (10 mL) and assayed using both 24- and 48-h incubation periods. Metabolite was located in both dialysate and retentate (Table 16), indicating that the active component was small enough to pass through the dialysis membrane.

The 24- and 48-h assays indicated that the metabolite was predominantly in the dialysate and retentate, respectively. The most obvious explanation for this discrepancy was the presence of two components in the L138 culture broth, one that is limiting during short incubation periods and a second that controls the production of HON by mutant L127 during the longer incubation times. One culture component that may play a role in the production of HON is carbohydrate; wild-type mycelium resuspended in phosphate buffer continue to produce HON only when a carbon source is included (26). The carbohydrate content of the retentate was higher than that of the dialysate, a large amount of viscous material remained in the sacks after dialysis, most likely remnants of starch with molecular weights greater than the cellulose sack cutoff (12600)

Table 16. Partial purification of L138 metabolite by dialysis through cellulose membrane.

Sample	Lyophilized mass (g)	[HON] (mmol/L) Incubation period 24-h	48-h
Retentate	1.56	0 83	2.9
Dialysate	0.45	1.7	2.4
Ratio: Retentate/dialysate	3.5	0.49	1.2

Sample (120 µL) was added to plugs (12 mm O.D., 6 mm I.D. and 4 mm thick) cut from 75-h nitrate-starch cultures. Plugs were extracted for 6 h.

Therefore, the high molecular weight saccharides in the retentate would support HON production by mutant L127 over prolonged periods, and the smaller sugars in the dialysate would be quickly assimilated to support a rapid initial production of HON. To overcome this discrepancy, samples require supplementation with a carbohydrate source such as glucose prior to their addition to the assay plugs.

Time course of dialysis

In a second experiment, lyophilized L138 supernatant was dissolved in water (8-fold x 40 mL) and dialysed against water (300 mL) for 20 h. At various times, portions (25 mL) of the dialysate were removed, freeze dried, and reconstituted in one-fifth of

their original volume. The level of L138 metabolite in the dialysate increased with time to a maximum at about 6 h, whereas the mass of dissolved solids in the dialysate increased over 20 h (Figure 24). A standard curve constructed from a series of dilutions of L138 culture supernatant during this experiment indicated that the concentration of L138 metabolite in the dialysate was below the agar plug assay's upper threshold Therefore, the observed levelling effect was a valid measure of L138 metabolite indicating that 6 h was an adequate period to reach equilibrium during dialysis

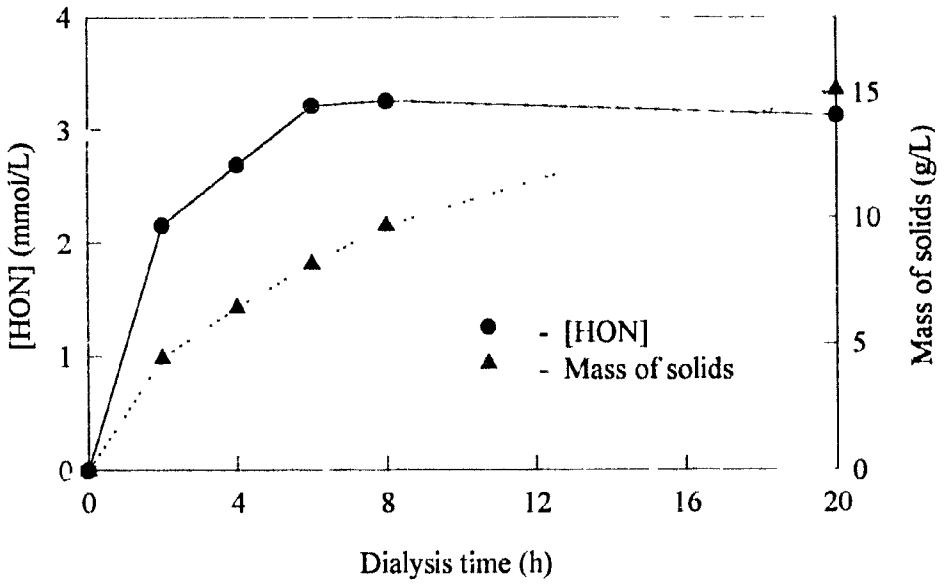


Figure 24. Time course of dialysis

Gel filtration

Dialysis and size exclusion filtration (14) suggest that the molecular weight of the L138 metabolite is less than 10000 g/mol. A semi-preparative method which has

the potential for separating the L138 metabolite of interest from unrelated material, such as partially digested casein and starch, is gel filtration, a technique compatible with the stability (Table 12) and solubility (Tables 13 and 14) properties of the metabolite. When individual fractions collected from an initial Sephadex G-10 column (2.5 × 61.5 cm) were assayed, a relatively small amount of HON was produced, corresponding to a low recovery of metabolite from the sample applied. However, when fractions were combined before the assay, the level of HON production was very similar to that of the sample applied. This result eliminated both decomposition and irreversible binding of the metabolite to the gel. The different levels of HON production obtained when individual and combined fractions were assayed supported the dialysis results (Table 16), which indicated that two or more components of the culture supernatant were required to stimulate HON production by mutant L127. Separation of the components by Sephadex G-10 chromatography would account for the above result. This possibility was further examined by assaying combinations of individual Sephadex G-10 fractions. Combinations of Sephadex G-10 fractions usually had significantly higher HON production than the sum of the HON production in individual fractions (Tables 17 and 18). The synergistic effect was most apparent with fractions II and IV, (3.9 *versus* 1.8 mmol/L). Calibration of the Sephadex G-10 column with a mixture of blue dextran, maltose and glucose showed that fraction II contains oligosaccharides where as fraction III contains di- and monosaccharides. The metabolite present in fraction IV elutes after most of the carbohydrate. The combination of fraction II and IV is effectively adding carbohydrate to metabolite. To examine the effect of adding carbohydrate to metabolite

Table 17. Agar plug assay of individual Sephadex G-10 fractions

Fraction	Effluent volume (mL)	Mass (g/group)	[HON] (mmol/L)
I	0 - 105	0 0048	0 006
II	105 - 139	0 2265	0 15
III	139 - 173	0.8290	3.2
IV	173 - 207	0.2090	1 6
V	207 - 412	< 0 01	0 000

Sample (120 µL) was added to plugs (12 mm O.D., 6 mm I.D. and 4 mm thick) cut from 74-h nitrate-starch cultures and after an additional 57-h incubation, plugs were extracted for 6 h

Table 18. Agar plug assay of combined Sephadex G-10 fractions

Combined fractions	[HON] (mmol/L)		Ratio (observed/ expected)
	Expected	Observed	
II & III	3 4	2 7	0 79
II & IV	1 8	3.9	2 2
III & IV	4 8	5 8	1 2
II - IV	5.0	7.8	1.6
I - V	5 0	9.7	1.9

Sample (120 µL) was added to plugs (12 mm O.D., 6 mm I.D. and 4 mm thick) cut from 74-h nitrate-starch cultures and after an additional 57-h incubation, plugs were extracted for 6 h.

during the agar plug assay, fractions from a second Sephadex G-10 column were supplemented with 50 mmol/L glucose, a concentration significantly less than the concentration of carbohydrate in e dialysate The later Sephadex G-10 fractions, similar to fraction IV above, showed a doubling of HON production on addition of glucose to the agar plug assay These results confirm that two components, each of which plays an integral function in the agar plug assay, are present in the L138 dialysate and can be separated by gel filtration

A separation of the components in L138 dialysate (75-fold concentration) was obtained on a Sephadex G-10 column twice the length (2.5 × 118 cm) of the column used above Solid was recovered in lyophilized fractions collected between 200 - 440 mL, most of the mass applied eluted between 270 - 350 mL (Figure 25) A majority

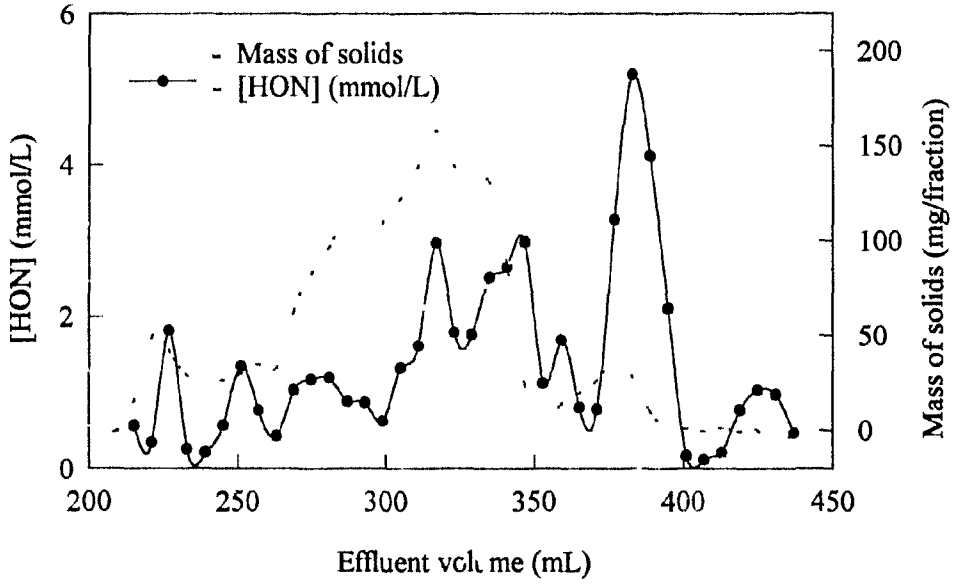


Figure 25. Sephadex G-10 chromatography of L138 dialysate

of the fractions collected between 200 and 440 mL supported HON production in the agar plug assay when supplemented with 0.5 mol/L glucose. The most active fractions (numbers 30 - 33 or 374 - 398 mL) contained only a small portion (75.8 mg) of the total mass of sample applied (2.05 g), and ^{1}H nmr spectra were recorded for fractions 30 - 32 (Figure 26). The major peaks in the ^{1}H nmr spectrum of fraction 30 were attributed to the large water resonance at 4.8 ppm and to contaminants in the solvent. In addition to the impurity peaks, the ^{1}H nmr spectra of fractions 31 and 32 showed a singlet at 1.85 ppm and resonances at 4.28 (dd, J = 4.9, 8.6 Hz), 2.94 (dd, J = 4.8, 14 Hz) and 2.68 ppm (dd, J = 8.7, 14.1 Hz), possibly due to the protons on the α- and β-carbons of an amino acid, and multiple peaks due to an unsymmetrically substituted aromatic ring.

Analysis by ion-pairing hplc with uv detection at 214 nm (Figure 27) showed two clearly resolved peaks in fractions 30 - 33 at retention times (t_R) of 3.1 and 3.8 min (gradient 3a, Appendix A). When eluted with gradient 3b (Appendix A) the retention times shifted to 2.7 and 3.5 min. The size of the hplc peaks did not correlate with the response in the agar plug assay (Table 19); the peaks were barely detectable in fraction 30 but a high level of HON was produced in the assay. This suggests that the metabolites producing the hplc peaks are not responsible for stimulating HON production by mutant L127, although the carbohydrate effect discussed above clearly demonstrates that the L138 metabolite level is not the sole factor controlling HON production. Analysis of the culture supernatants from mutant L167, L127 and wild-type *S. akiyoshiensis* showed that the hplc peak (t_R = 3.5 min, gradient 3b) was produced by

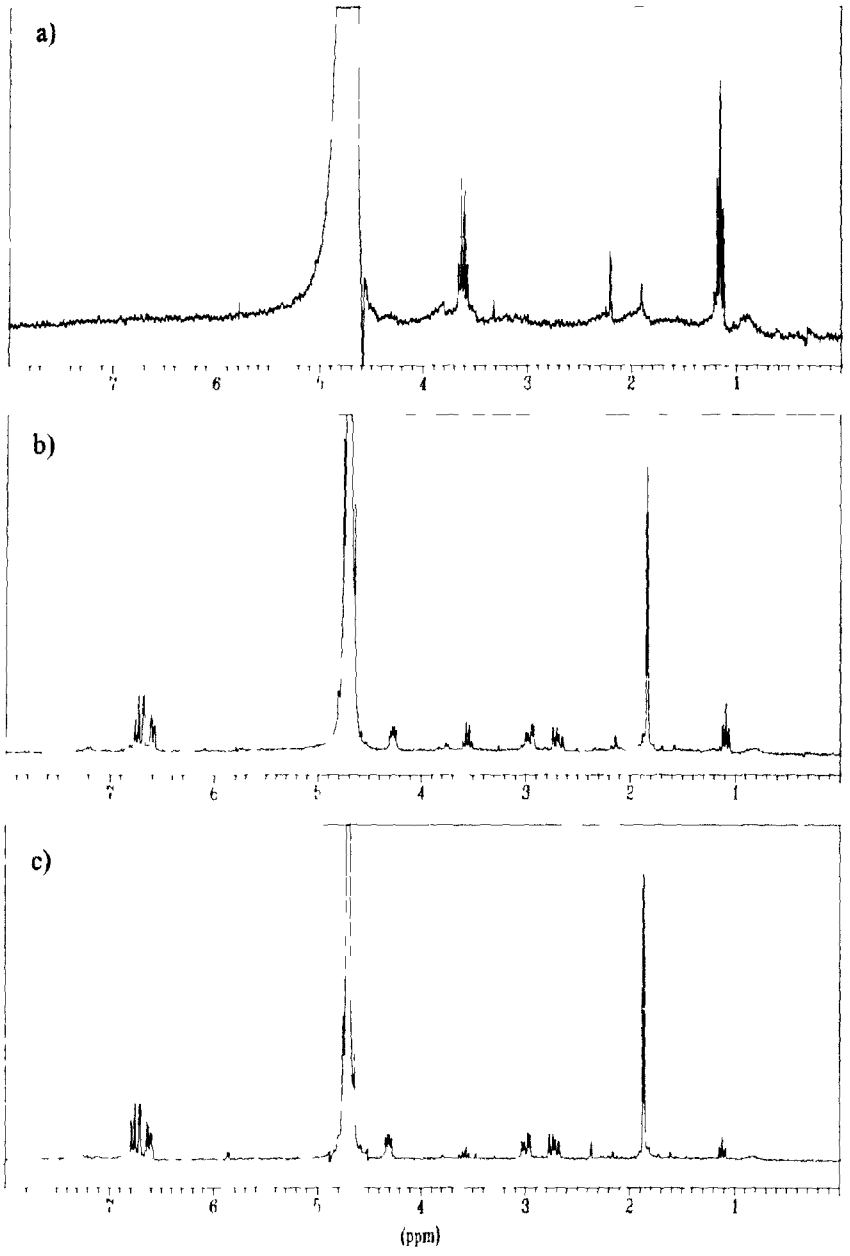


Figure 26. ^{1}H Nmr spectra of Sephadex G-10 fractions a) 30, b) 31 and c) 32.

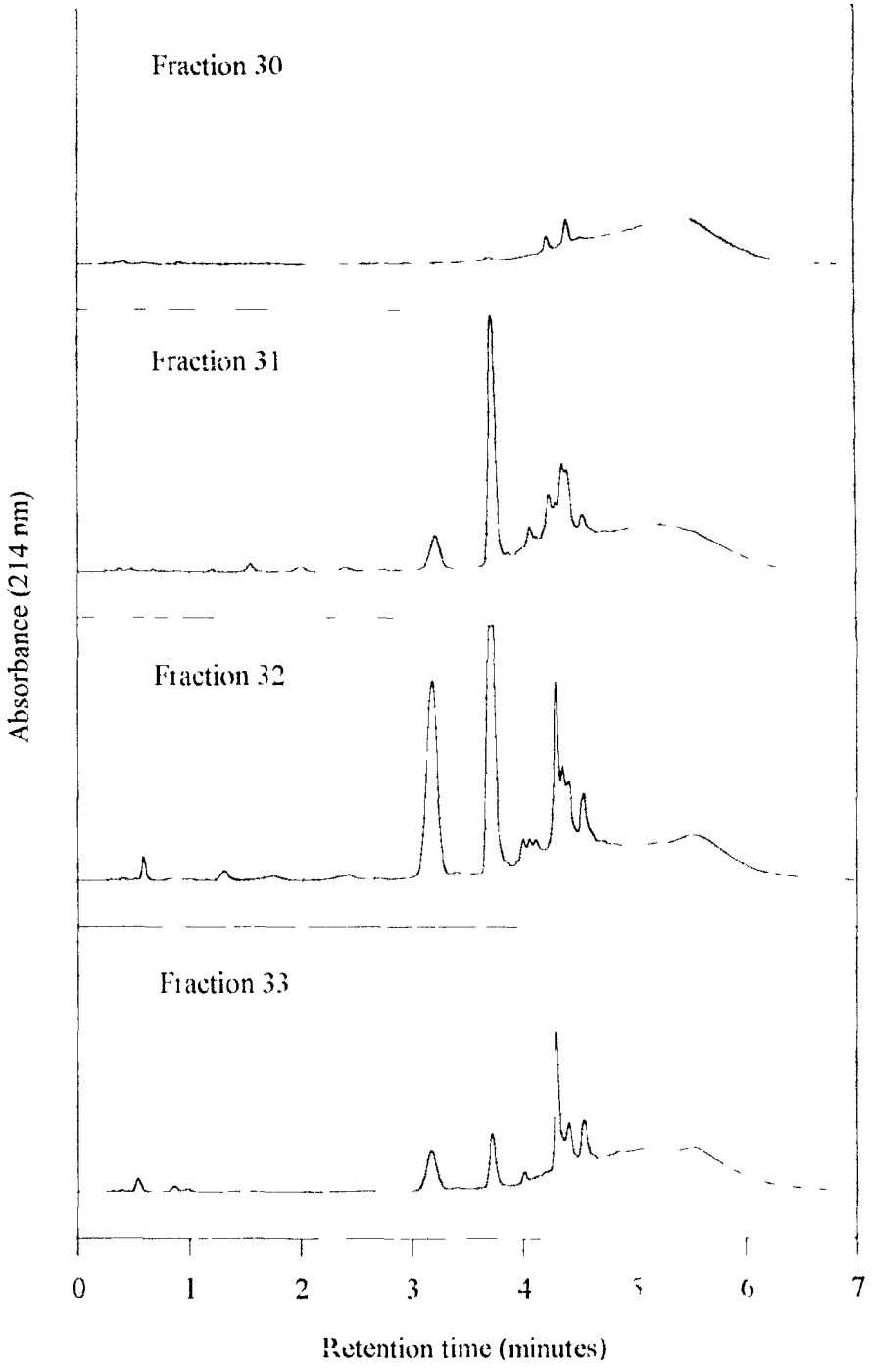


Figure 27. Ion-pairing hplc chromatograms of Sephadex G-10 fractions

Table 19. Summary of mass and HON produced for Sephadex G-10 fractions

Fraction	Mass (mg)	HON[a] (mmol/L)	Estimated L138 metabolite	
			Mass (mg)	% of Total mass
30	36 4	3 3	0 97	2 7
31	28 6	5.3	1 6	5 4
32	10.2	4 1	1 2	12
33	1 6	2 1	0 62	40

[a]Sample (120 µL) in 0.5 mol/L glucose was added to plugs (12 mm O D , 6 mm I D and 4 mm thick) cut from 72-h nitrate-starch cultures and after an additional 48-h incubation, plugs were extracted for 4 h

all the bacterial strains. If this substance is present in all mutants and in wild-type cultures, it cannot be responsible for the stimulation of HON production by mutant L127

Isolation of the substance from 96-h culture supernatant was accomplished by extraction with butanol and chromatography on Amberlite XAD-2 and Sephadex G-10 The spectroscopic and chromatographic properties for the isolated compound were identical to those of synthetic *N*-acetyl-L-dihydroxyphenylalanine (*N*-acetyl-L-dopa), and the addition of synthetic *N*-acetyl-L-dopa at various concentrations to L127 liquid cultures and agar plug assays did not stimulate HON production (Table 20) To ensure that this negative result was not due to a nutrient requirement, *N*-acetyl-L-dopa was added to agar plug assays supplemented with three concentrations of L138 dialysate No increase in HON production was observed above levels produced without the

Table 20. Agar plug assay effect of supplementing with synthetic *N*-acetyl-L-dopa

N-Acetyl-L-dopa (mmol/L)	[HON] (mmol/L)			
	Dialysate concentration			
	None	1.6-fold	4-fold	12-fold
9.62	0.04	1.1	5.8	9.5
2.40	0.07	1.9	5.5	8.7
0.481	0.04	2.5	5.1	10.0
None	Blank	2.2	5.6	10.8

Sample (120 µL) in 0.5mol/L glucose was added to plugs (12 mm O.D., 6 mm I.D. and 4 mm thick) cut from 96-h nitrate-starch cultures and after an additional 46-h incubation, plugs were extracted for 6 h

addition of the *N*-acetyl-L-dopa

N-Acetyl-L-dopa is the predominant proton containing substance (excluding HOD) detected in the ^{1}H nmr spectrum of fraction 32 (Figure 26), and it accounts for 25% of the mass. Fraction 32 stimulates mutant L127 to produce HON at 4.1 mmol/L in the agar plug assay. If the L138 metabolite is a HON intermediate and has molecular mass of 150 g/mol, then this metabolite must comprise 12% of the total mass of fraction 32 (Table 19). A small molecule present at one-half the concentration of *N*-acetyl-L-dopa would be clearly visible in the ^{1}H spectrum. Since no other significant nmr signals are present, the amount of L138 metabolite is less than that expected for an intermediate of HGN biosynthesis.

The partial purification of the metabolite in fractions 31-33 with t_R = 3.1 min provided only a small mass of lyophilized residue (0.2 mg). The ^{1}H spectrum of this

residue showed, in addition to the HOD signal, singl onances at 5.99 and 6.73 ppm, but no structural information was deduced from the spectrum. Since the residue did not stimulate the production of HON in the L127 agar plug assay, the identification of this metabolite was not pursued.

The next several sections of the thesis describe work conducted to determine whether the L138 metabolite which stimulates the production of HON in mutant L127 is an intermediate of HON biosynthesis.

Examination of L138 cultures for biosynthetic intermediates

Cross-feeding experiments in which the culture supernatant of mutant L138 and the mycelium of mutant L127 are incubated for 48 h typically produce HON at levels of 1-2 mmol/L. If the accumulation of a HON intermediate in cultures of mutant L138 is to account for this cross-feeding phenomenon, the intermediate must be present at a corresponding concentration of 1-2 mmol/L, a concentration large enough to be detected by ^{13}C nmr if the substance is enriched in ^{13}C. The mass of dissolved solids in the L138 culture supernatant is 19.6, 11.6, 7.2 and 4.8 g/L at 24, 48, 72 and 96 h, respectively, and an intermediate at 1 mmol/L would account for only a very small portion of the total mass. The ^{1}H nmr spectra of 51- and 91-h L138 culture supernatants (Figure 28) are similar to those of soluble starch and maltose (Figure 29), indicating that oligosaccharides constitute a major portion of the supernatant solids. A corresponding group of oligosaccharide signals was located between 63 and 102 ppm in the ^{13}C nmr spectra of the culture supernatant (Figure 30).

Figure 28. ^{1}H Nmr spectra of L138 culture supernatant after a) 51-h incubation and b) 91-h incubation

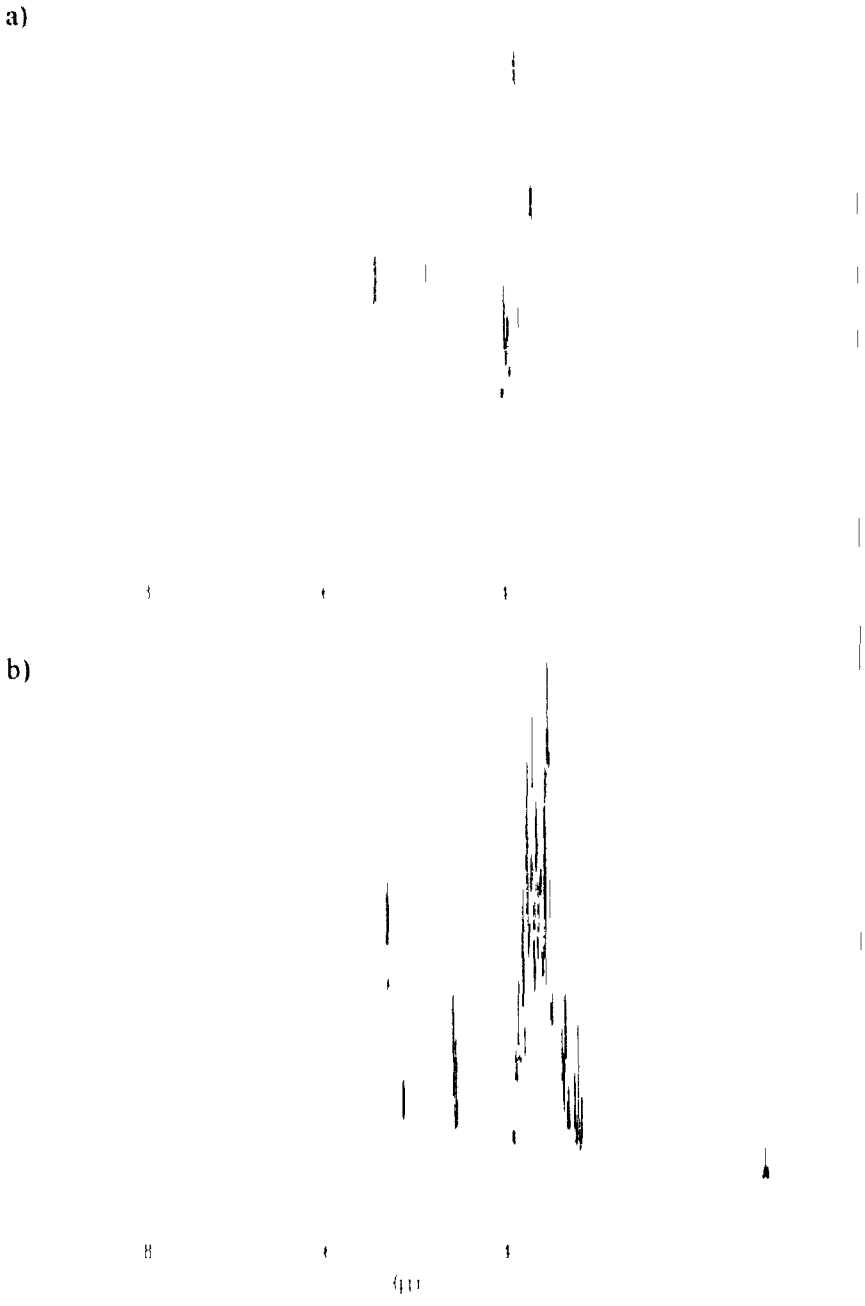


Figure 29. ^{1}H Nmr spectra of a) soluble starch and b) maltose

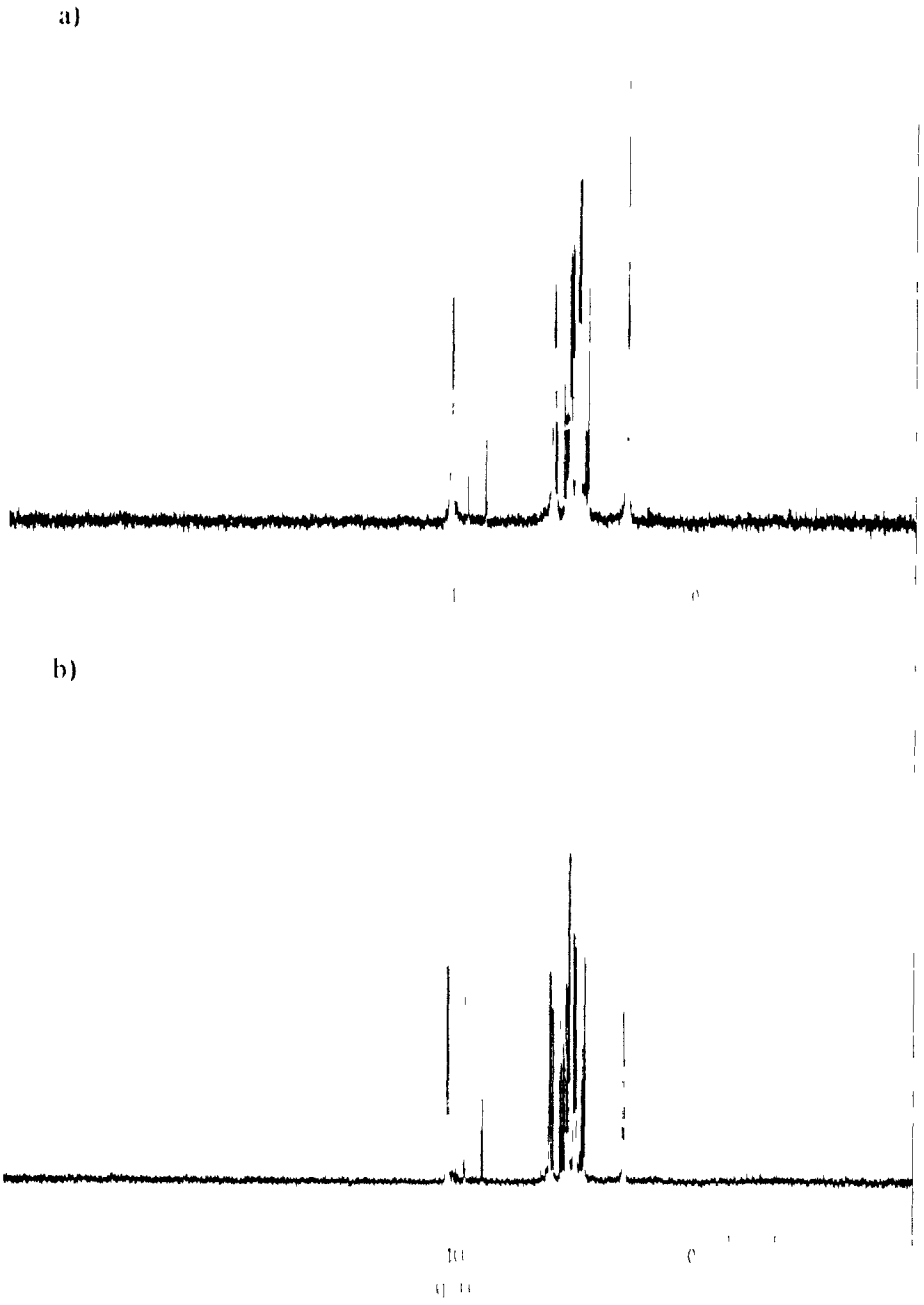


Figure 30. ^{13}C Nmr spectra of L138 culture supernatant after a) 51-h incubation and b) 91-h incubation

Since an intermediate that is structurally similar to HON, or the postulated intermediates (Figure 4), would provide ^{13}C signals outside of the carbohydrate region, experiments were undertaken using ^{13}C-labelled aspartate and acetate, the known biosynthetic precursors of HON (21, 22) Cultures of mutant L138, growing on casein-starch medium (50 mL) were supplemented with either DL-[4-^{13}C]aspartic acid, sodium [2-^{13}C]acetate or sodium [1,2-$^{13}C_2$]acetate at 25, 39, 51 5 and 65 5 h of incubation The concentration of labelled substrate after each addition was 2 0 mmol/L A portion of the culture (10 mL) was removed at 51 h, centrifuged and the supernatant was lyophilized (Table 21). After 91 h, the remaining culture was treated in the same fashion Each lyophilized solid was redissolved in D_2O and analyzed by ^1H and ^{13}C nmr spectroscopy

Table 21. Mass of lyophilized powders from sampling supplemented cultures

Experiment	Mass of solids from lyophilization	
	51-h Sampling (g/50 mL)	91-h Sampling (g/50 mL)
Control	1.10	0 61
[2-^{13}C]acetate	1 20	0 70
[1,2-$^{13}C_2$]acetate	1 25	0 71
[4-^{13}C]aspartate	1.10	0 72

Aspartate as a precursor

The ^{13}C nmr spectra of the 51-h and 91-h samples from DL-[4-^{13}C]aspartic acid supplemented cultures (Figure 31) showed an aliphatic signal with a chemical shift at 54 8 ppm as well as a more intense signal at 180 3 ppm, most likely due to the ^{13}C enriched β-carboxyl of [4-^{13}C]aspartic acid remaining in the L138 culture supernatant A doublet at 39 1 ppm (J 50 9 Hz) was also present in the 91-h sample A DEPT experiment showed that the resonances at 54 8 and 39 1 ppm corresponded to tertiary and secondary carbons, respectively These results are very much in agreement with those expected for the α- and β-carbons of aspartic acid, the β-carbon of [4-^{13}C]aspartic acid would appear as a doublet due to coupling with the adjacent, 99% enriched carboxyl carbon *S akiyoshiensis* cultures take D-aspartic acid into the mycelia at a significantly slower rate than the natural L-enantiomer (22), and residual D-aspartate would account for the nmr signals

The addition of a known amount of natural abundance aspartic acid (5 6 mg) to this sample resulted in the appearance of an uncoupled signal positioned 0 013 ppm down-field from the centre of the β-carbon doublet and an increase in intensity of the α-carbon and β-carboxyl signals (Figure 31) A previously unobserved resonance due to the α-carboxyl was now apparent at 176 5 ppm By using the known mass of unlabelled material added and ratios of the observed signal intensities, the residual amount of aspartate remaining in the culture was calculated to be 11 6 mg or 22% of the initial amount of DL-[4-^{13}C]aspartate added

The 51-h supernatant from the L138 culture supplemented with

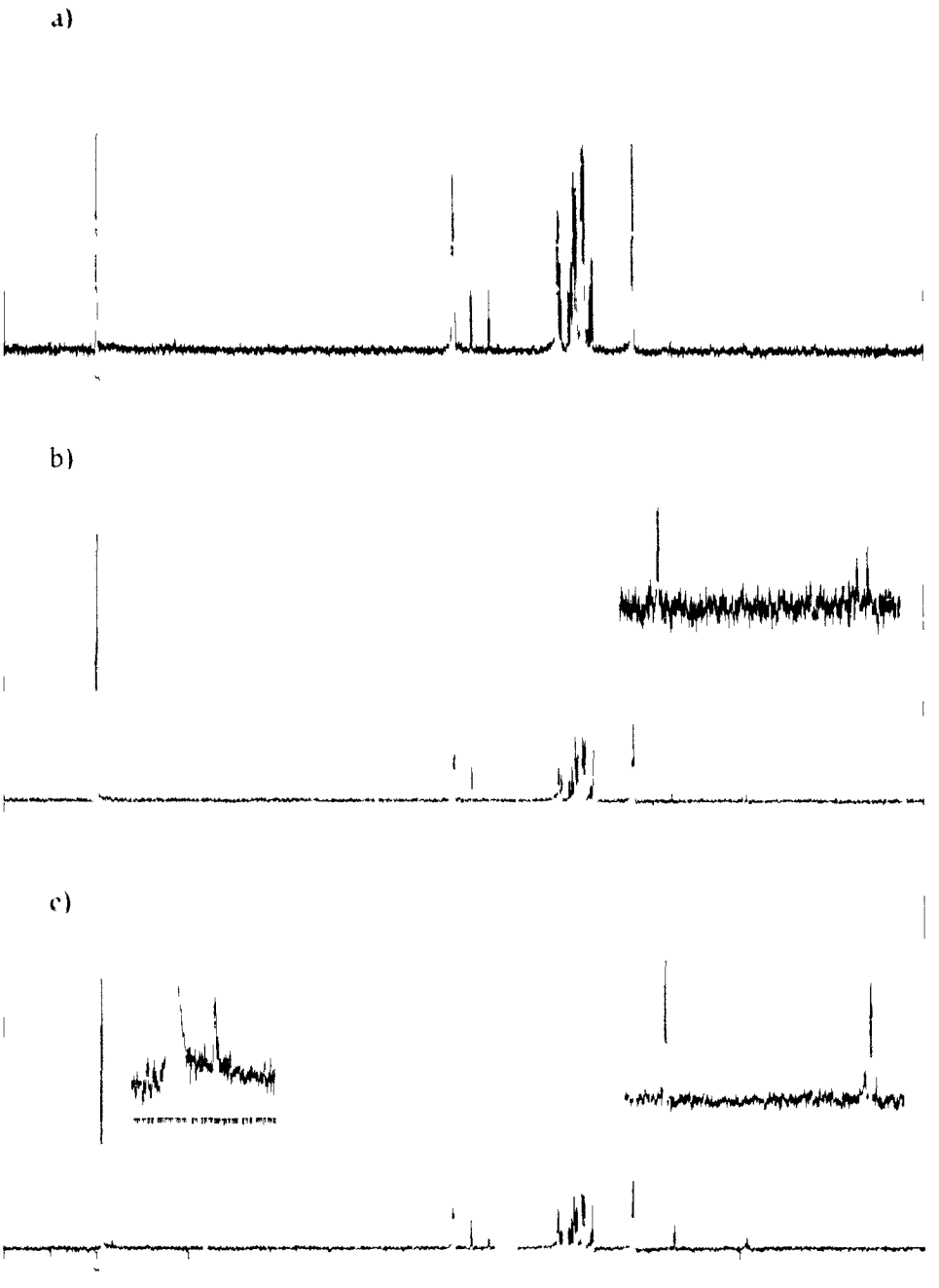


Figure 31. ^{13}C Nmr spectra of culture supernatant from a [4-^{13}C]aspartate supplemented culture after a) 51-h incubation, b) 91-h incubation and c) 91-h incubation with natural abundance aspartic acid added

DL-[4-^{13}C]aspartic acid produced HON at 0.36 mmol/L in the L127 agar plug assay, about 25% of the HON produced from control culture supernatant and supernatants from cultures supplemented with sodium [2-^{13}C]acetate or sodium [1,2-$^{13}C_2$]acetate (see below). The D-aspartate present in this sample would cause a 50% reduction in HON production (Figure 21). Therefore, a HON intermediate should be present in this culture supernatant at 0.72 mmol/L or approximately 36 μmol in 50 mL of culture.

From the intensity of the α-carboxyl resonance in the ^{13}C nmr spectrum of the [4-^{13}C]aspartic acid supplemented culture supernatant to which natural abundance aspartic acid has been added (Figure 31), the minimum ^{13}C enrichment necessary for a carboxyl signal to be detectable was calculated. The intensity of the doublet at 39.1 ppm (Figure 31), which corresponds to seven times the background noise level, was taken as minimum intensity to ensure signal detection. An intermediate with a 2-3% enrichment above natural abundance would be readily detected (Table 22). Previous feeding experiments (22) in which DL-[4-^{13}C]aspartic acid was added to wild-type *S. akiyoshiensis* cultures produced enrichments of 2.3% at C-4, indicating that a 2-3% incorporation of labeled aspartic acid into a HON intermediate is feasible.

From these results, it is unlikely that a HON intermediate derived from aspartic acid accumulates in L138 cultures. However, it is possible that an L138-produced intermediate may be derived from acetate, the second primary biosynthetic precursor of HON

Table 22. Estimated detection limit for a carbonyl resonance of an intermediate *via* nmr spectroscopy

^{13}C Enrichment (%)	Total ^{13}C (%)	Intermediate detectable	
		(μmol/nmr sample)	(μmol/50-mL culture)
0	1 1	27	95
1	2 1	14	49
2	3.1	9 4	33
3	4 1	7 1	25
5	6 1	4 8	17
10	11 1	2 6	9 3

Acetate as a precursor

The ^{13}C nmr spectrum of 51-h [2-^{13}C]acetate supplemented cultures (Figure 32a) contains three signals (24.8, 26 1 and 33 3 ppm) not detected in the spectrum of the control culture (Figure 30a) Only the signal at 24 8 ppm was present in the spectrum of the 91-h supernatant (Figure 32b), indicating that these signals were associated with carbons from different compounds. An additional signal (38 4 ppm) just above the noise level in the 51-h sample was clearly visible in the nmr spectrum of the 91-h sample. Dialysis of the 91-h culture supernatant provided a solid which was soluble in one-fifth the volume of the pre-dialysis sample (0 5 mL *versus* 2 5 mL) The spectrum recorded on the dialysate (Figure 32c) showed an increased signal-to-noise ratio and three additional signals at 33 3, 34 4 and 207 6 ppm

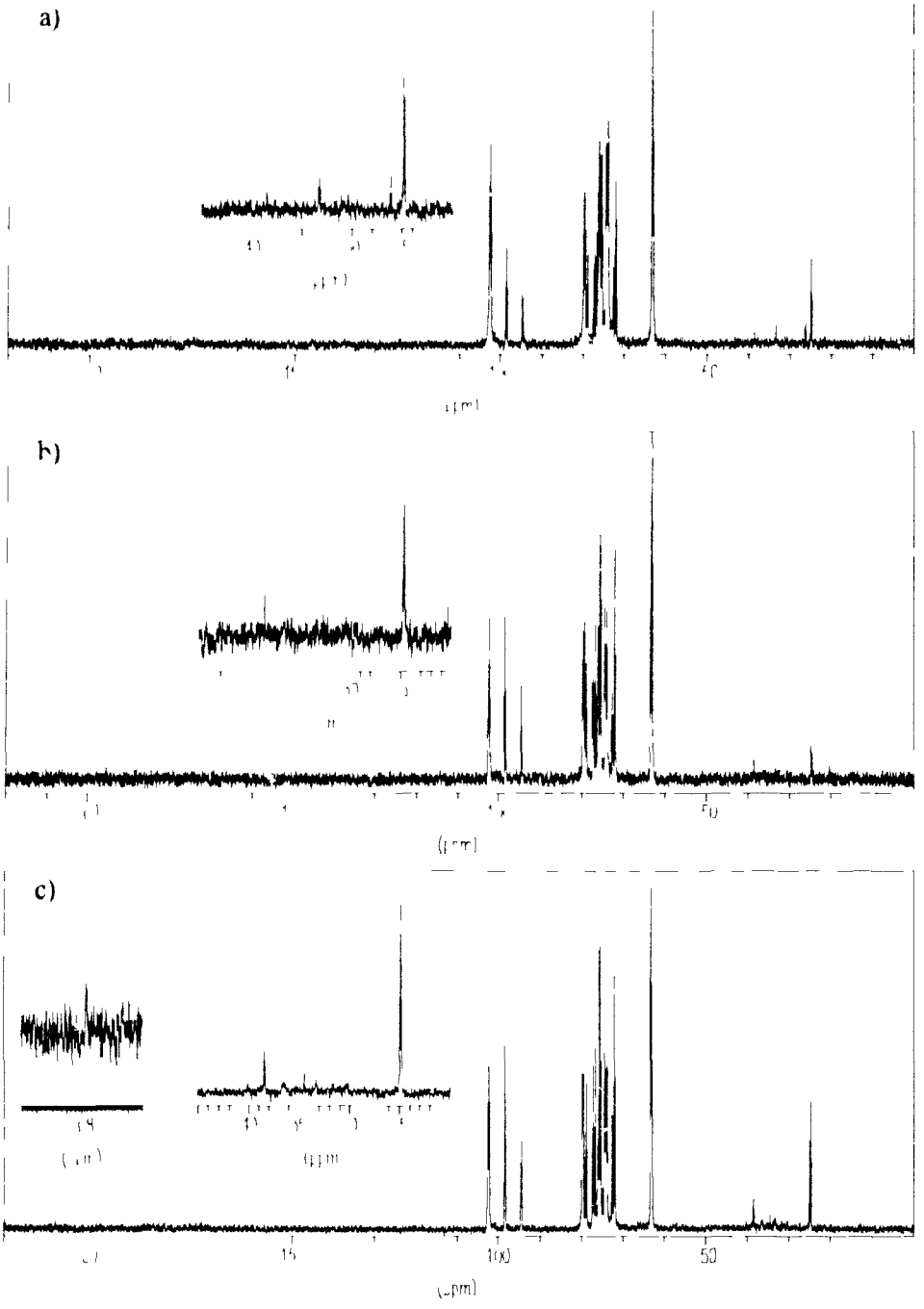


Figure 32. ^{13}C Nmr spectra of culture supernatant from a [2-^{13}C]acetate supplemented culture after a) 51-h incubation, b) 91-h incubation and c) dialysate of 91-h incubation.

In the 51-h ^{13}C nmr spectrum of [1,2-$^{13}C_2$]acetate supplemented cultures (Figure 33a), the singlet at 24.8 ppm (Figure 32c-e) appeared as a doublet ($J_{C,C}$ = 50.4 Hz) coupled to a carbonyl signal (176.4 ppm, $J_{C,C}$ = 50.1 Hz). The chemical shifts of the coupled signals (24.8 and 176.4 ppm) are close to those expected for unmetabolized [1,2-$^{13}C_2$]acetate. However, the addition of unlabelled acetic acid to the nmr sample provided natural abundance signals that did not coincide with the doublets (Figure 33b). The methyl signal (24.4 ppm) appeared between the doublet peaks, about 0.4 ppm upfield from the centre of the doublet. Although the chemical shift of this doublet would be affected by a ^{13}C isotope effect, the shift would be upfield and much smaller (*ca.* 0.012 ppm (90)) than the shift observed here. The ^{13}C spectrum recorded for a mixture of sodium [1,2-$^{13}C_2$]acetate and natural abundance sodium acetate showed upfield isotopic shifts of 0.0095 and 0.001 ppm for the methyl and carboxyl signals, respectively. The chemical shift of the acetic acid carboxyl signal (181.2 ppm) did not coincide with the doublet signals (176.3 ppm), clearly showing that the compound responsible for the coupled signals in the [1,2-$^{13}C_2$]acetate supplemented cultures is not unmetabolized [1,2-$^{13}C_2$]acetate.

Another potential candidate for producing the coupled resonances is *N*-acetyl-L-dopa. The carbons of the *N*-acetyl group of *N*-acetyl-L-dopa have chemical shifts of 24.5 and 176.1 ppm, and ion-pairing hplc analysis showed that *N*-acetyl-L-dopa was present in the 51-h culture supernatant, although at a concentration of only 0.047 mmol/L. Addition of *N*-acetyl-L-dopa to the 91-h sample of the [1,2-$^{13}C_2$]acetate supplemented cultures resulted in a signal 0.0655 ppm upfield from centre of the

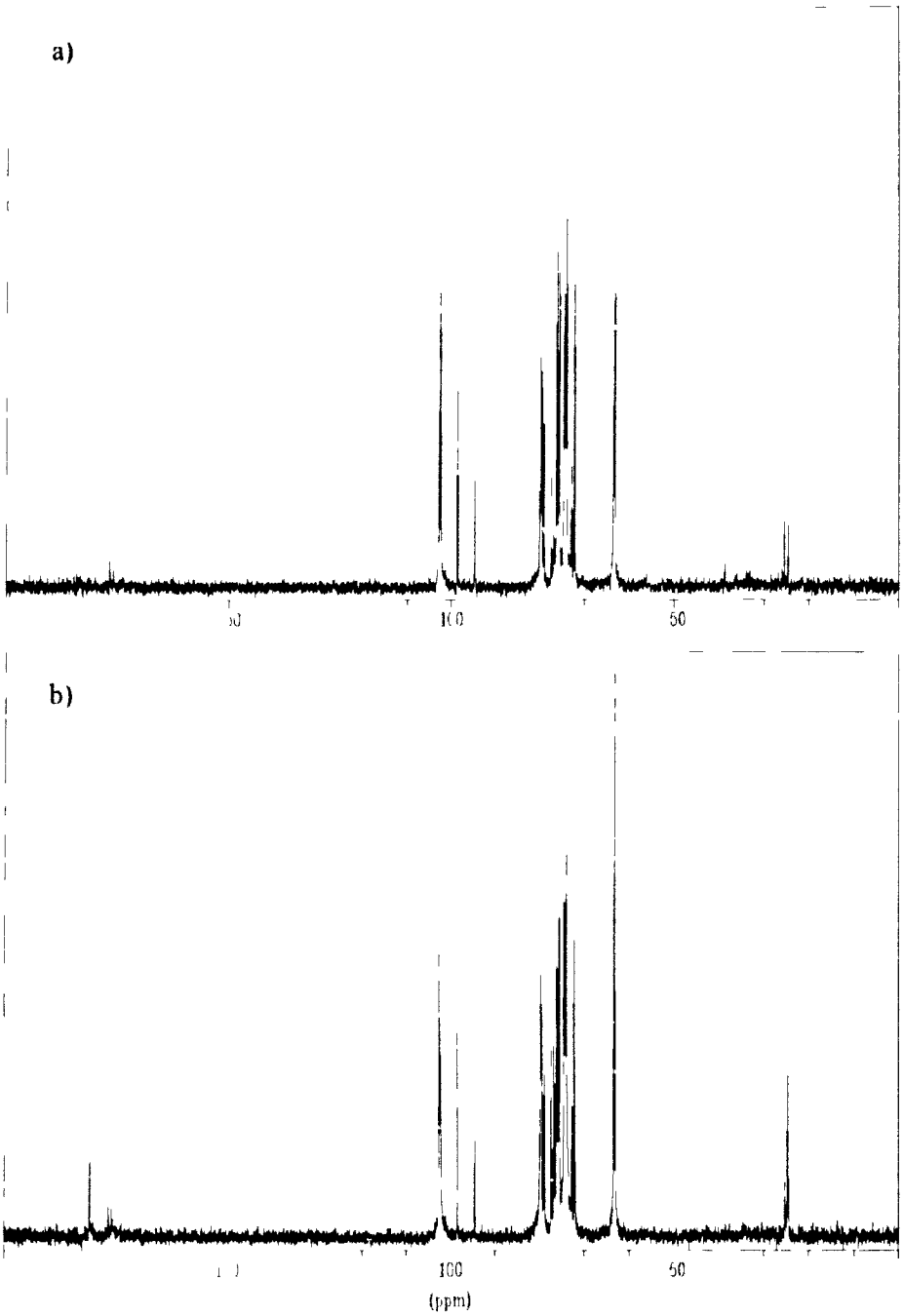


Figure 33. ^{13}C Nmr spectra of a) 51-h culture supernatant from [1,2-$^{13}C_2$]acetate supplemented L138 culture and b) same sample after the addition of natural abundance acetic acid.

aliphatic doublet and a carbonyl resonance 0.345 ppm upfield from the centre of the downfield doublet in the 91-h sample (Figure 34b). To confirm that the coupled signals were not due to *N*-acetyl-L-dopa, the sample was extracted with butanol. In the ^{13}C nmr spectrum of the butanol extract (Figure 34c), the *N*-acetyl-L-dopa signals were enhanced relative to the doublet, indicating that the metabolite responsible for the coupled signals has a different partition coefficient. The similar chemical shifts of the acetyl carbons of *N*-acetyl-L-dopa and the signals present in the acetate supplemented culture suggest that another *N*-acetyl amino acid may be present in the culture. The detection of an acetyltransferase enzyme in cell free extracts of *S. akiyoshiensis* is described later in the thesis.

The concentration of an accumulated intermediate in the 51-h culture supernatant was estimated by the agar plug assay to be 1.2 mmol/L or 60 µmol/50 mL. At this concentration, an intermediate enriched with ≥1% ^{13}C would generate readily detectable nmr signals (Table 23). Thus, it is possible that one or more of the nmr signals (Figures 32 and 34) correspond to a biosynthetic intermediate derived from acetate. If a labelled intermediate accumulates in L138 cultures, then isotopically labelled HON would be formed by the following sequence.

$$\text{Labelled Acetate} \xrightarrow{\text{Mutant L138}} \text{Labelled Intermediate} \xrightarrow{\text{Mutant L127}} \text{Labelled HON}$$

To definitively ascertain whether a biosynthetic intermediate accumulates in cultures of HON mutant L138 and is converted to HON by mutant L127, two feeding

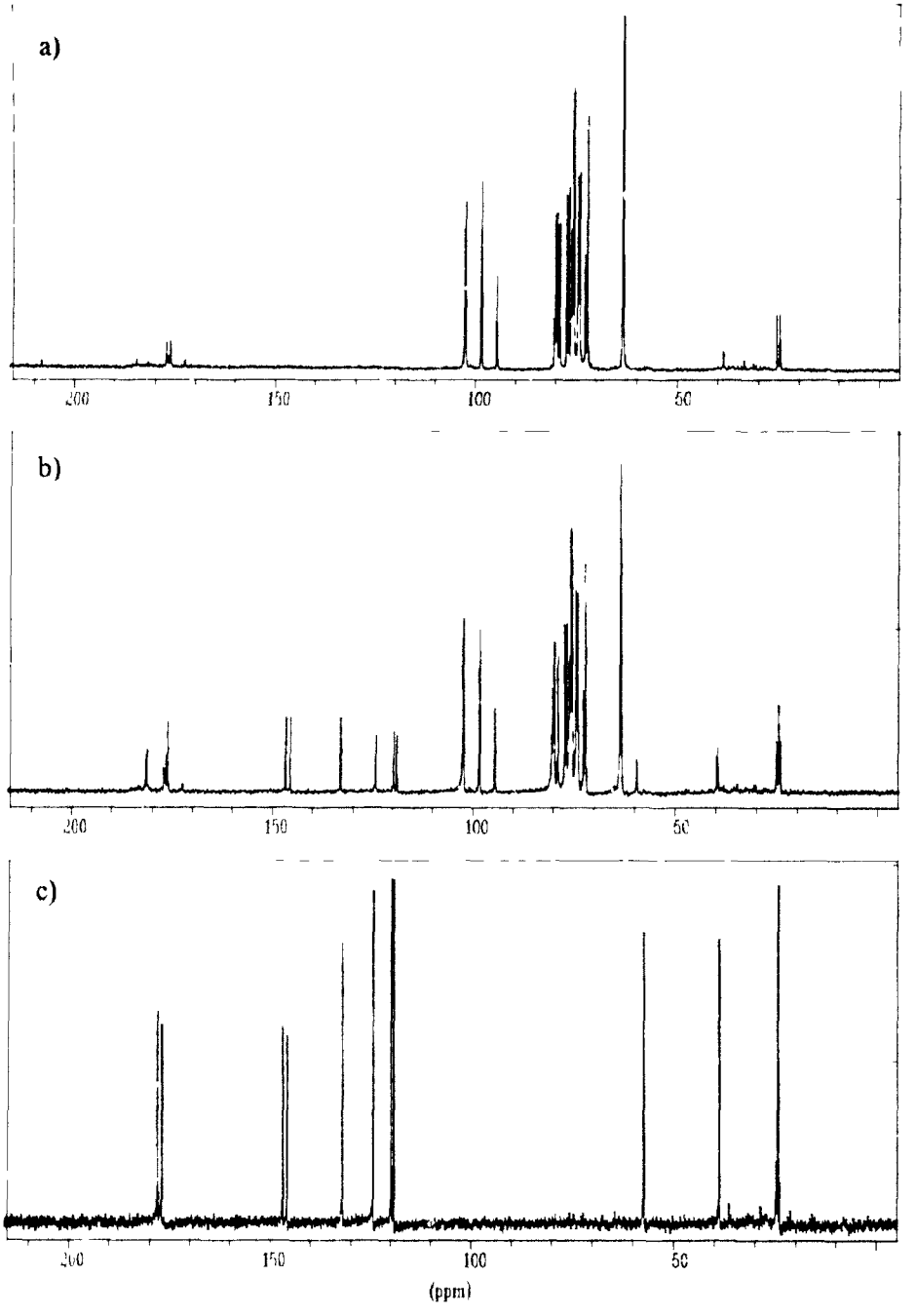


Figure 34. ^{13}C Nmr spectra of a) 91-h dialysate from [1,2-$^{13}C_2$]acetate supplemented L138 culture supernatant, b) after addition of *N*-acetyl-L-dopa and c) butanol extract of 91-h dialysate containing *N*-acetyl-L-dopa.

Table 23. Estimated detection limit for the methyl resonance of an intermediate *via* nmr spectroscopy

^{13}C Enrichment (%)	Total ^{13}C (%)	Intermediate detectable	
		(μmol/nmr sample)	(μmol/50-mL culture)
0	1.1	14	72
1	2.1	7.5	37
2	3.1	5.1	25
3	4.1	3.9	19

experiments employing [2-^{14}C]acetate and the L138 and L127 mutants were conducted In each experiment, the supernatant from a 48-h L138 culture was used to resuspend the mycelium from a 48-h L127 culture, but four [2-^{14}C]acetate additions were made to either to the L138 culture to generate a labelled intermediate (Expt 1) or to the suspension of L127 mycelium in L138 supernatant to probe for *de novo* HON biosynthesis (Expt. 2). Several different distributions of radioactive label in HON can result from these experiments (Figure 35 and Table 24). The carbon atoms of HON derived from an intermediate would indicate whether the intermediate is derived from acetate, aspartate, or both of these precursors. If *de novo* HON biosynthesis is initiated by another factor, then radioactive HON would be formed in Expt. 2, but not in Expt 1

A control experiment (Expt. 3) conducted by supplementing wild-type *S akiyoshiensis* with sodium [2-^{14}C]acetate was done to provide a level of specific incorporation and isotope distribution for comparison with those observed in Expts 1 and 2. The production of radiolabelled HON in the cultures was followed by both opa-

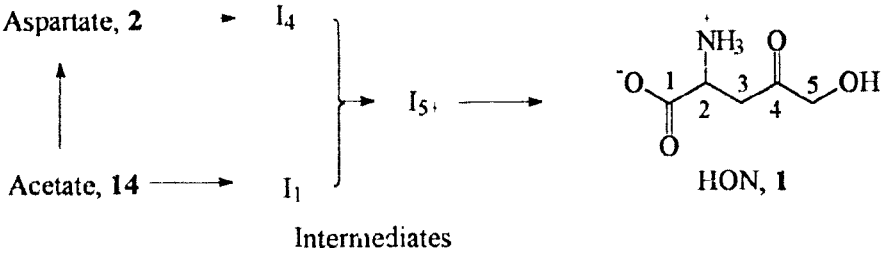


Figure 35. Biosynthesis of HON: a convergent pathway

Table 24. Predicted radiolabelling of HON from various precursor derived intermediates

Intermediate (derived)	HON isolated	
	Expt. 1[a]	Expt. 2[b]
I_1 (Acetate)	Radioactive in C-5	Radioactive in C-1 to C-4
I_4 (Aspartic acid)	Radioactive in C-1 to C-4	Radioactive in C-5
$I_{5,}$ (Acetate + aspartic acid)	Radioactive in C-1 to C-5	No radioactivity incorporated
Initiator of *de novo* biosynthesis	No radioactivity incorporated	Radioactive in C-1 to C-5

[a] Addition of [2-^{14}C]acetate to L138 culture 48 h before the addition of L127 mycelium to L138 supernatant.

[b] Addition of [2-^{14}C]acetate to L127 mycelium resuspended in L138 supernatant.

hplc and tlc HON was produced by wild-type cultures (Expt 3) after 36 h (0 085 mmol/L) and increased to 0.38 mmol/L at 48-h incubation The radioactivity associated with two components in the culture supernatant (R_f = 0 43 and 0 61) increased over the incubation period The band with the lower R_f was not observed until after 36-h incubation and the radioactivity associated with this band increased about 5-fold after an additional 12 h incubation On ninhydrin treatment of the tlc plate from the 48-h sampling, the band at R_f = 0 43 turned yellow, a property characteristic of HON (5) The second radioactive band (R_f = 0 61), not detected until 24-h incubation, increased in size during the growth of the cultures to result in a total radioactivity about equal to that of HON. Since this radioactive metabolite did not bind to a cation exchange resin, it was readily separated from HON during workup. Cultures of mutant L138 also accumulated two radioactive metabolites, one having an R_f similar to that of HON (0 41) and a second band associated with a red pigment (R_f = 0 82) produced in large quantities by these cultures Although a band was present in the L138 culture having an R_f similar that of HON, opa-hplc analysis of the culture supernatant throughout the incubation period verified that no HON was produced. The size of this band increased slightly (20%) during the incubation of the L138 supernatant with L127 mycelia (Expt 1). Two radioactive bands with R_f values (0.45 and 0 84) similar to those observed in the L138 culture were observed in tlc's performed on samples taken from the incubation mixture of Expt. 2, although the band associated with HON was significantly larger (3-fold) in this experiment.

HON produced in each experiment was isolated by ion-exchange

chromatography, diluted with unlabelled HON, and recrystallized. Additional carrier was added and each sample was cleaved with periodate to yield aspartic acid (C-1 to C-4) and formaldehyde (C-5) (22). The formaldehyde was trapped as its dimethone derivative, formaldomedone (Figure 36). Each sample of HON isolated was radioactive; the specific incorporation in Expt. 1 (7.0%) was lower than that observed in Expt. 2 (26.9%, Table 25) The distribution of radioactivity between the two degradation products in Expt 1 and 2 was similar to the distribution obtained in the parallel experiment using wild-type organism (Table 26). That the sum of the specific radioactivities of the degradation products is greater than the specific radioactivities of HON, can be attributed to the purity of the HON used for carrier dilution Although a crystalline sample was used, it had been isolated from *S. akiyoshiensis* cultures and stored at -20°C for several months It is possible that this sample was not 100% pure, a purity of 84% would account for the discrepency in the specific activities. The RSA for the degradation products, aspartic acid and formaldomedone, were corrected by assuming that the sum of their specific radioactivities provides the correct specific radioactivity for HON and are shown in brackets under the uncorrected RSA values (Table 26). The corrected RSA values of aspartate (56.6%) and formaldomedone (43 4%) derived from HON produced by wild type *S. akiyoshiensis* (Expt 3) are very similar to the relative isotopic enrichments observed in aspartate (59%) and formaldomedone (41%) of HON derived from sodium [2-^{13}C]acetate (22).

The observed difference in the distribution of label between C-1 to C-4 and C-5 of HON in Expt 1 and 2 was most likely due to differences in the [2-^{14}C]acetate levels

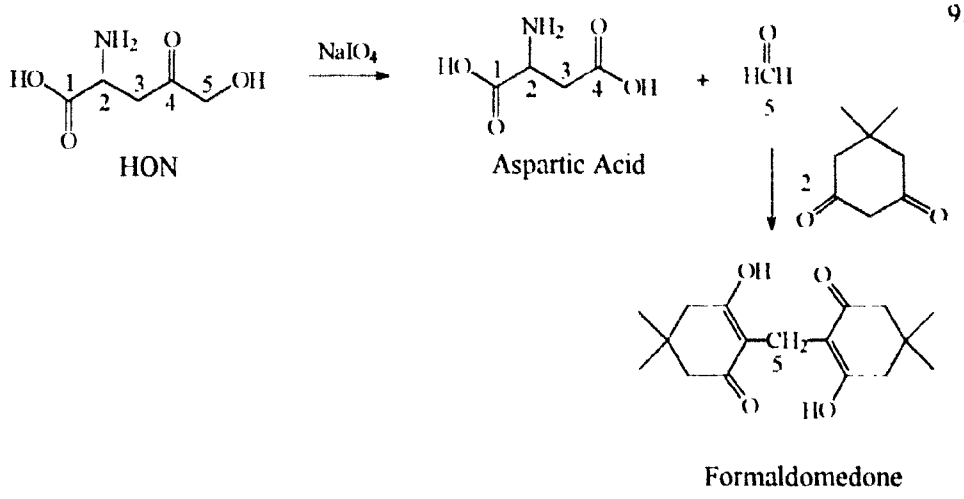


Figure 36. $NaIO_4$ degradation of HON and formation of formaldomedone.

Table 25. Specific incorporation of sodium [2-^{14}C]acetate into HON

Expt.	Specific incorporation %[a]
1 L138 +[2-^{14}C]acetate s/n transferred to L127 mycelium	7.0
2 L127 mycelia + L138 s/n + [2-^{14}C]acetate	26.9
3 Wild Type + [2-^{14}C]acetate	97.6

[a] $\frac{\text{Specific radioactivity of HON produced} \times 100}{\text{Specific radioactivity of Acetate fed}}$

Table 26. Specific radioactivity of HON and HON degradation products

Expt	Specific radioactivity (10^6 dpm/mmol)				
	HON	Asp.	RSA[a] %	Formal. dimedone	RSA[a] %
1	0.332	0.273	82.3	0.108	32.5
L138 +[2-^{14}C]acetate; s/n transferred to L127 mycelium			(71.7)		(28.3)
2	1.64	1.305	79.7	0.733	44.7
L127 mycelia + L138 s/n + [2-^{14}C]acetate			(64.0)		(36.0)
3	2.86	1.94	67.8	1.485	51.9
Wild-type + [2-^{14}C]acetate			(56.6)		(43.4)
[2-^{13}C]acetate Expt. (lit (21,22))	--	--	59	--	41

[a]Specific radioactivity relative to HON. Normalized RSA are given in parentheses

at various stages of HON biosynthesis The incorporation of [2-^{14}C]acetate into aspartate provides a radiolabelled cellular pool of aspartate that is longer lived than the cellular pool of [2-^{14}C]acetate During the maximum rate of HON production in Expt 1 (72 - 96 h), the level of radioactivity in the culture fluids had dropped to a constant level indicating that [2-^{14}C]acetate had been virtually depleted from the culture supernatant (Figure 37a). The level of [2-^{14}C]acetate in the culture supernatant in Expt 2 (Figure 37b) also becomes depleted but somewhat later in HON production (84 - 96 h), providing radiolabelled precursor for a longer time Similarly, the greater availability of [2-^{14}C]acetate during HON production by wild-type *S akiyoshiensis* in Expt. 3 (Figure 37c) could account for the higher relative specific activity measured at C-5 HON.

The similar distribution of radioactivity over C-1 to C-4 and C-5 of HON in Expts. 1 and 2 is inconsistent with the accumulation of an intermediate derived from only one of the two precursors, and the higher incorporation of radioactivity measured in Expt. 2 suggests that an intermediate derived from both precursors is not formed (Table 24). The incorporation results are most consistent with the accumulation of a metabolite in the L138 cultures that initiates HON biosynthesis by mutant L127, but is not directly incorporated. The smaller incorporation observed in Expt 1 could be due to sodium [2-^{14}C]acetate carried over in the supernatant of L138 and incorporated when *de novo* HON biosynthesis begins in mutant L127. The level of radioactivity in the culture supernatant was measured throughout the growth of mutant L138 and after the resuspension of L127 mycelium in L138 supernatant (Figure 37a) The level of

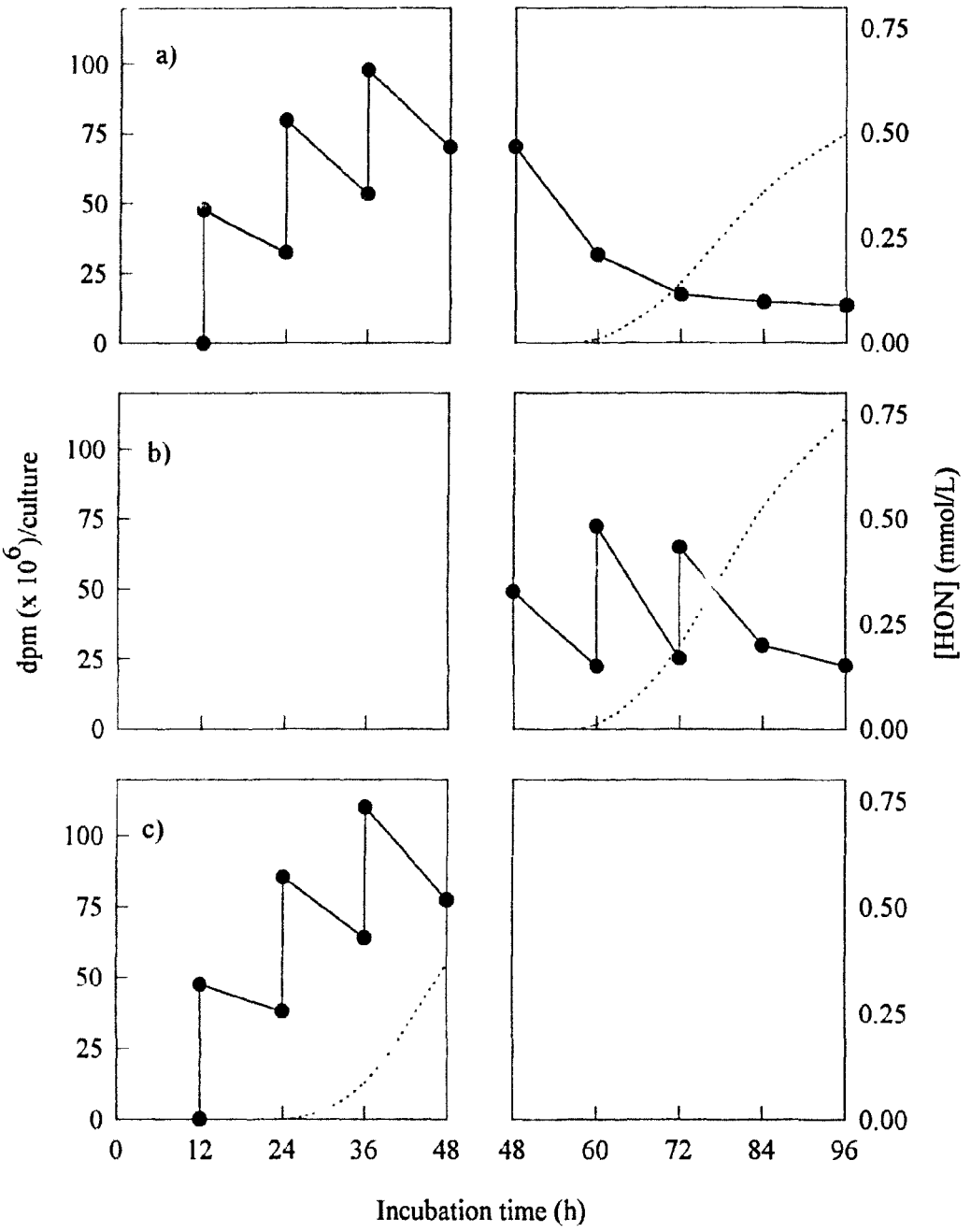


Figure 37. Radioactivity uptake from culture supernatants in sodium [2-^{14}C]acetate feeding studies. a) Expt. 1, b) Expt. 2 and c) Expt. 3

—●— radioactivity and ·· [HON]

radioactivity in the L138 culture supernatant at the time of crossing was 50 4% of the total amount added Of this, approximately 80% was assimilated by mutant L127 mycelia during the next 48 h

To determine the portion of radioactivity in the supernatant due to [2-^{14}C]acetate, a known amount of carrier sodium acetate was added to samples of supernatant collected at 48 and 72 h The acetate was derivatized with 4-bromophenacyl bromide in the presence of dicyclohexano-18-crown-6 and recrystallized from aqueous ethanol. The quantity of [2-^{14}C]acetate remaining in each sample was calculated from the specific radioactivities of the bromophenacyl acetate derivatives and the [2-^{14}C]acetate initially added to the culture Most of the radioactivity in the supernatant at 48 h was due to [2-^{14}C]acetate (15.2 mg, 3 69 mmol/L, 36 7% of the total amount added), but at 72 h only a small portion of the initial [2-^{14}C]acetate remained (0 86%, 0 131 mg, Table 27) Therefore, a significant amount of [2-^{14}C]acetate was available for incorporation into HON by mutant L127 in Expt 1

Table 27. Isolated [2-^{14}C]acetate from Expt 1

Sampling time[a]	Specific radioactivity of 4-bromophenacyl acetate (dpm/mmol)	[2-^{14}C]Acetate remaining (mg/50 mL)
48 h	313000	15 2
72 h	2670	0 131

[a] Sample removed at 48 h was initial sample removed after the crossing of the L138 supernatant and L127 mycelia. The 72 h sample was removed from the culture following an additional 24 h incubation

These results suggest that the cross feeding or co-synthesis effect observed between mutants L127 and L138 is due to a substance that initiates *de novo* HON biosynthesis by mutant L127 and not to the accumulation of an intermediate in the culture supernatant of mutant of L138. The exact nature of the substance in L138 culture supernatant is not clear, but the information provided by the initial purification and stability studies indicates that it is a small molecule.

Initiation of HON biosynthesis by *S. akiyoshiensis* metabolite

Stimulation of HON production by mutant L127 on supplementation with culture supernatant from wild-type *S. akiyoshiensis*

During the isolation of HON$^-$ mutants, it was noted that combining the culture supernatant from wild-type *S. akiyoshiensis* with mycelium from mutants L167, L127 or L138 did not result in HON levels above that already present in the wild-type supernatant (14). However, when HON was removed by ion-exchange chromatography from the 68-h culture supernatant of wild-type *S. akiyoshiensis* grown on casein-starch medium, the resulting effluent stimulated HON production in the L127 agar plug assay in a fashion identical to L138 dialysate of similar concentration. This suggests that the metabolite which stimulates the production of HON in mutant L127 is not solely present in mutant L138 but is also present in the wild-type strain.

Production of HON by wild-type *S. akiyoshiensis* on nitrate starch agar

Understanding the role of the L138 metabolite in stimulating the production of

HON in cultures of L127 may be enhanced by studying the effect of the metabolite on HON production in wild-type *S akiyoshiensis* This was examined by using L138 dialysate to supplement donut-shaped plugs containing mycelial lawns of wild-type *S akiyoshiensis* grown on nitrate-starch agar medium Assay plugs containing wild-type *S. akiyoshiensis* mycelium did not produce HON when supplemented with glucose solution (500 mmol/L). However, HON was produced when these plugs were supplemented with L138 dialysate The concentrations measured were comparable to those produced by L127 plugs supplemented with L138 supernatant This indicates that a component of L138 cultures grown in casein-starch medium is necessary for HON production on semi-solid nitrate medium

Effect of casein-starch medium on HON production in L127 agar plug assays

The production of HON in liquid culture is limited to a small number of media, of which casein-starch provides the highest titres (88) To ensure that the substance present in L138 culutures which stimulates HON production in mutant L127 agar plug assays is not present in the medium, uninoculated casein-starch medium containing 500 mmol/L glucose was added to agar plug assays However, no HON was produced, confirming that the substance is a L138 metabolite rather than a component present in the medium prior to inoculation

HON production by mutant L127 grown on malt extract-yeast extract-maltose medium

Liquid cultures of wild-type *S akiyoshiensis* grown on malt extract-yeast extract-

maltose medium do not support HON production (26) However, when agar plug assays were performed with mutant L127 grown on malt extract-yeast extract-maltose agar, plugs to which only water was added produced a significant amount of HON (13.0 mmol/L) Supplementing plugs with L138 supernatant (1-fold concentration) resulted in only a slight increase in the level of HON produced (15.2 mmol/L). However, when L127 agar plug assays on nitrate-starch medium were supplemented with yeast or malt extracts, no stimulation of HON production was observed

Effect of known cofactors

One explanation for a lack of HON production in mutant L127 is an inability to produce the necessary level of a required cofactor. Since mutant L127 is not auxotrophic, a missing cofactor must not be required for bacterial growth but only for HON production Non-auxotrophic mutants of *Streptomyces cattleya* unable to produce the β-lactam antibiotic thienamycin have been shown to be deficient in vitamin B_{12} (91). To determine if the L127 mutant is stimulated to produce HON by known cofactors, a commercial multi-vitamin was partly dissolved and added to agar plug assays containing 500 mmol/L glucose solution The multi-vitamin supplemented assay produced a small amount of HON (0 30 mmol/L) but the result was not reproducible. Also, when assays containing dialysate were supplemented with the multi-vitamin, no increase in HON production over unsupplemented assays was observed Supplementing agar plug assays with either vitamin B_1, B_2, B_6, B_{12} or folic acid failed to stimulate HON production in mutant L127

The isolation of *N*-acetyl-L-dopa from cultures of *S. akiyoshiensis*

The metabolite detected in Sephadex G-10 fractions by ion-pairing hplc (retention time of 3 5 min, gradient 3b, Appendix A) is produced by the HON mutants as well as wild-type *S akiyoshiensis* It was isolated from a culture grown on casein-starch medium to provide sufficient material to fully elucidate its structure Supernatant from a 3.6-L culture was acidified and extracted with butanol The presence of the acidic group indicated by ion-pairing hplc was confirmed by the extraction of the metabolite from butanol into aqueous base Acidification of the basic solution and subsequent lyophilization afforded a brown residue which was chromatographed on two successive Amberlite XAD-2 columns Combination of fractions containing the metabolite of retention time 3 5 min and lyophilization yielded a wh te solid which gave colored solutions in methanol or water Colored material was removed on a Sephadex G-10 column The purified material (12 mg) was hygroscopic, and it could not be left in contact with the atmosphere for more than a minute without absorbing a large amount of water.

The purified metabolite showed an ABX splitting pattern in its ^{1}H nmr spectrum (Figure 38a) consistent with the CH-CH_2 unit of an amino acid (92) The only other resonance present in the aliphatic region was a three-proton singlet at 1 85 ppm, most likely due to a methyl group attached to a carbonyl Corresponding signals of appropriate chemical shift (59 1, 39 6 and 24 5 ppm) and DEPT multiplicity (d, t, and q, respectively) were present in the ^{13}C nmr spectrum (Figure 38b), along with two signals in the carbonyl region A carboxylic acid (3500-2500 and 1728 cm^{-1}) and an

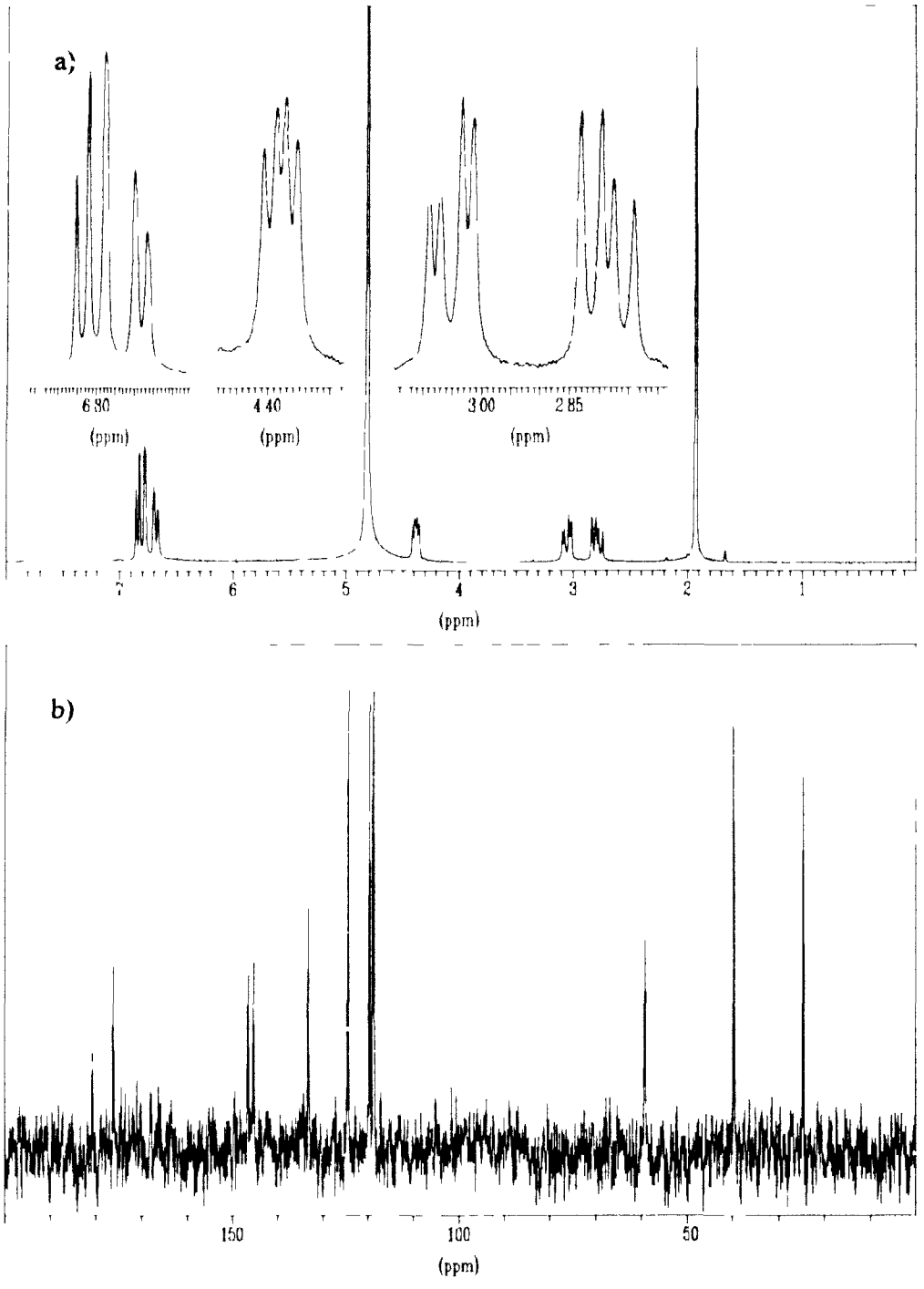


Figure 38. a) ^{1}H and b) ^{13}C nmr spectra of *N*-acetyl-L-dopa isolated from *S. akiyoshiensis*.

amide (1654 cm^{-1}) were indicated by the ir spectrum, and the lack of peaks observed by hplc analysis of *o*-phthalaldehyde-treated samples is consistent with a derivatized nitrogen. The odd value observed for the molecular ion (*m z* 239) in the mass spectrum supports the presence of a nitrogen atom, and the loss of acetamide from the molecular ion $(M-59)^+$ is characteristic of *N*-acetyl aromatic amino acids (93).

An unsymmetrical, trisubstituted aromatic ring was indicated in the ^1H nmr spectrum by the pattern of resonances (6.6-6.9 ppm) which integrated for 3 protons and by six signals in the aromatic region of the ^{13}C nmr spectrum Three resonances between 118 and 124 ppm corresponded to carbons bonded to hydrogen and the other three signals between 133 and 147 ppm corresponded to substituted carbon atoms. The downfield shift of two signals in the latter group indicated that they were attached to electronegative atoms, probably hydroxyl groups. An *N*-acetylated dihydroxyaromatic amino acid is consistent with the observed molecular ion (*m z* 239) in the mass spectrum and a major fragment ion (*m z* 123) corresponding to a dihydroxytropylium ion

Of the six possible arrangements (**52** - **57**) for two identical and one other substituent on an aromatic ring (Figure 39), two are symmetrical (**55** - **57**) and a third (**52**) contains three adjacent substituents. The latter is not consistent with the one ortho (J = 8.2 Hz) and one meta coupling (J = *ca.* 1.5 Hz) indicated in the most upfield aromatic resonance of the ^1H nmr spectrum. Of the three remaining possible structures (**53**, **54** and **56**), the substitution pattern of **56** is most consistent with the ortho and meta couplings and corresponds to that of the well-known amino acid 3,4-dihydroxyphenylalanine or dopa A sample of *N*-acetyl-L-dopa (**56**) was prepared by

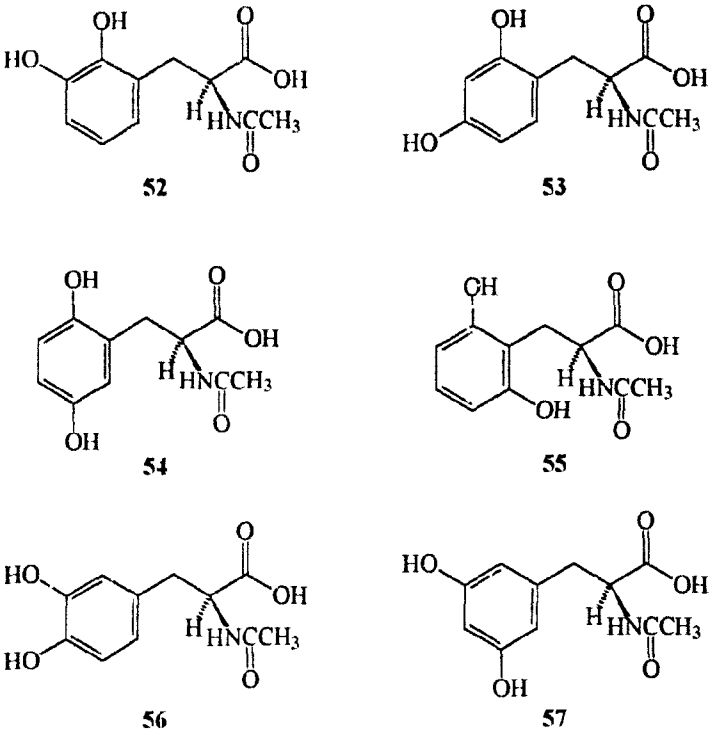


Figure 39. Possible ring substitution patterns of *N*-acetyl-L-dihydroxyphenylalanine

acetylation of L-dopa in aqueous solution (94, 95) and provided nmr, ir and mass spectra which were identical to those of the isolated compound The isolated and synthetic samples had identical retention times by hplc and a mixture coeluted as a single, symmetrical peak The optical rotation of synthetic *N*-acetyl-L-dopa prior to Sephadex G-10 chromatography (+ 45 8°) was lower than that of the isolated material but was similar to the literature value (+ 37.7° (94)). After further purification by Sephadex G-10 chromatography, the rotation (+ 68 2°) agreed with that of the isolated sample (+ 67 2°) Therefore, it can be concluded that *N*-acetyl-L-dopa (56) is produced by

S akiyoshiensis

Production of *N*-acetyl-L-dopa

In preparation for biosynthetic studies, the effect of culture conditions on the production of *N*-acetyl-L-dopa was investigated to increase yields Levels of *N*-acetyl-l-dopa in culture fluids were determined by ion-pairing hplc with uv detection at 280 nm A calibration curve was prepared using synthetic *N*-acetyl-L-dopa

The *N*-acetyl-l-dopa concentration in cultures of wild-type *S akiyoshiensis* and HON mutants L167, L127 and L138 grown on casein-starch medium and initiated from vegetative inoculum were monitored over 8.5 days. The concentrations of *N*-acetyl-l-dopa increased in all cultures over this period but did not reach a maximum (Figure 40) However, the greatest increases were observed over the initial 5 days Mutants L138 and L127 produced the highest concentrations (0.099 and 0.085 mmol/L, respectively), mutant L167 and wild-type *S akiyoshiensis* produced smaller amounts (0 066 and 0 060 mmol/L, respectively) Subsequent experiments were performed with mutant L138

Inoculum

Generally, levels of *N*-acetyl-L-dopa produced in cultures initiated from spore inoculum were less than 60% of those produced in cultures initiated from vegetative inoculum (Table 28). The production of *N*-acetyl-L-dopa was most rapid in the first 5 days, and the cultures showed a high level of pigmentation In cultures initiated with 1 - 5% spore inoculum, larger volumes of inoculum resulted in higher levels of *N*-acetyl-

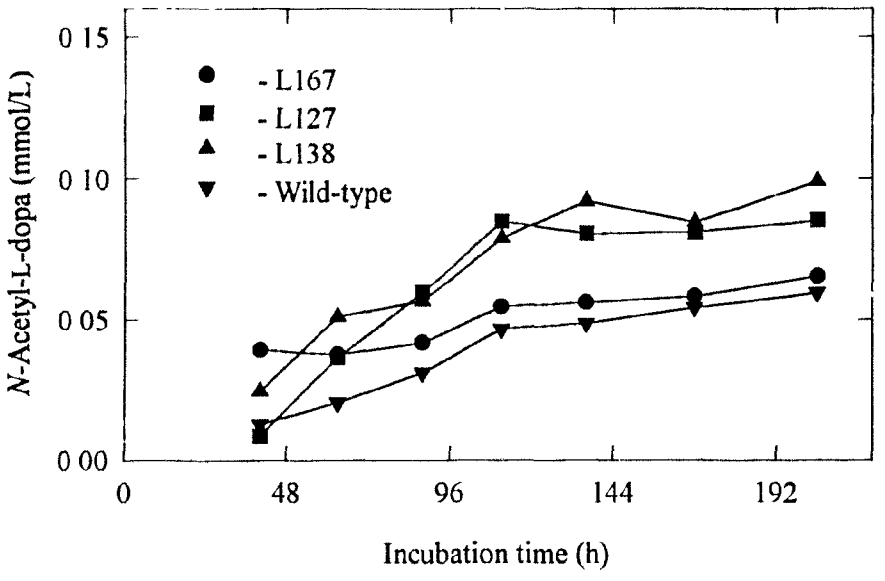


Figure 40. Production of *N*-acetyl-L-dopa in mutants and wild-type

L-dopa For spore inocula below 1%, this trend was reversed; lower levels of *N*-acetyl-L-dopa were obtained as the inoculum increased from 0 1 to 1.0%. For vegetative inocula, larger volumes produced higher initial levels of *N*-acetyl-L-dopa (Figure 41), but cultures inoculated with 3% vegetative inoculum showed a marked decrease at long incubation times. A 1% vegetative inoculum provided a similar high level of *N*-acetyl-L-dopa at 6 days without a decrease at longer incubation times.

Initial pH

The initial pH of the culture medium showed a substantial effect on the production of *N*-acetyl-L-dopa (Figure 42). At pH 5.5, *N*-acetyl-L-dopa levels were

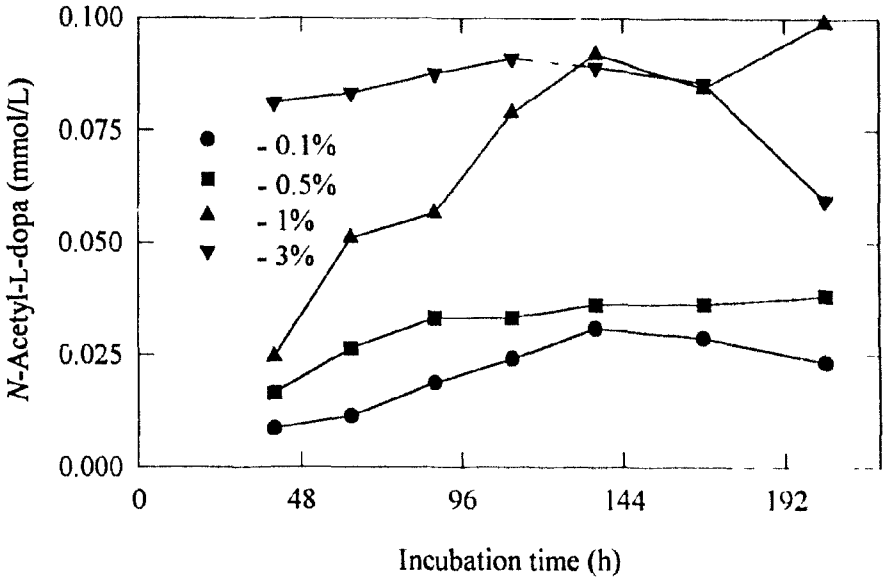


Figure 41. Effect of volume of vegetative inoculum on production of *N*-acetyl-L-dopa

Table 28. Effect of inoculum on the concentration of *N*-acetyl-L-dopa at 111 h.

	[*N*-Acetyl-L-dopa] (mmol/L)	
Volume (% (v/v))	Spore inoculum	Veg. inoculum
0.1	0.045	0 024
0.5	0.029	0 033
1	0.023	0 079
3	0.037	0 091
5	0.058	--

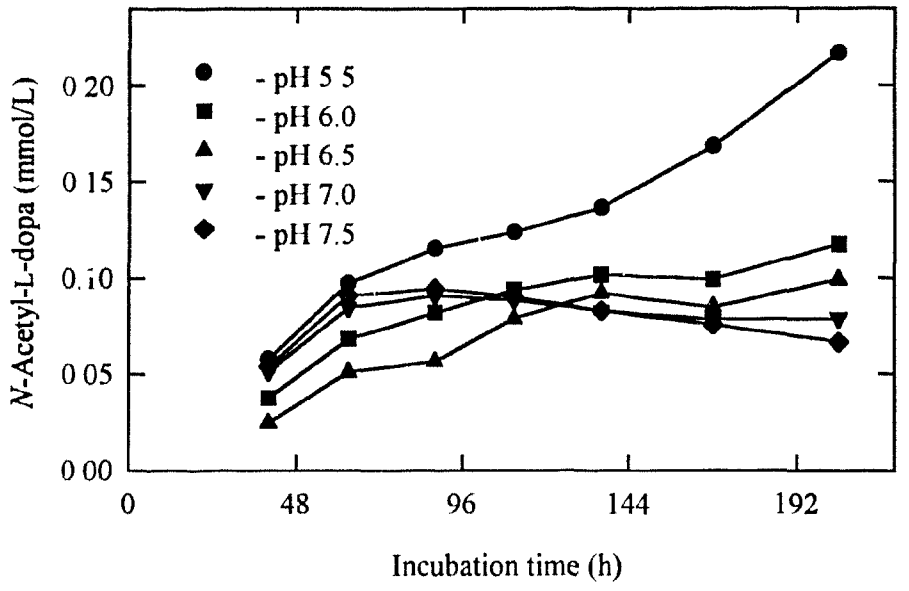


Figure 42. Effect of pH on production of *N*-acetyl-L-dopa by mutant L138

higher than in all other cultures throughout the culture period, and the rate of production was most rapid in the first 54 h and at longer incubation times (136 - 204 h). *S. akiyoshiensis* shows low growth rates at pH 5.5 (89), a factor that has been known to cause increased production of secondary metabolites (96). With increasing pH, lower levels of *N*-acetyl-L-dopa were produced at long incubation times.

Carbon source

Mutant L138 was grown on glucose, maltose, sucrose or starch (3%). The disaccharides, maltose and sucrose, proved to be poor carbon sources for sustaining *N*-acetyl-L-dopa production (<0.030 mmol/L). The monosaccharide glucose provided a

higher level of *N*-acetyl-D-dopa (0 0644 mmol/L at 204 h), but the best carbon source for supporting the production of *N*-acetyl-D-dopa was starch (0 0991 mmol/L at 204 h)

Cultures containing 1% starch produced *N*-acetyl-L-dopa at a substantially lower level than cultures containing 3% starch (0 031 *versus* 0 079 mmol/L at 111 h) The level of *N*-acetyl-D-dopa in the 1% starch cultures decreased after 111 h whereas the cultures containing 3% starch produced *N*-acetyl-L-dopa throughout the entire incubation period (0 0991 mmol/L at 204 h) In cultures containing 5% starch, production was initially more rapid than in cultures containing 3% starch (0 0806 mmol/L and 0 0567 mmol/L at 88 h, respectively), but a lower level of *N*-acetyl-D-dopa (0 060mmol/L) was obtained at 204 h

Aeration

The production of *N*-acetyl-L-dopa was monitored in L138 cultures of various volumes while maintaining a constant flask size (125 mL Erlenmeyer) Cultures of 17 5 and 25 mL produced the highest levels of *N*-acetyl-L-dopa (0 088 and 0 099 mmol/L, respectively), lower *N*-acetyl-L-dopa concentrations were produced by smaller and larger culture volumes (Figure 43) In addition to decreased production of *N*-acetyl-L-dopa, the cultures with a volume of 10 mL per flask were intensely red in colour Under high oxygenation, a biosynthetic intermediate may be diverted to pigmented products, whereas in cultures of larger volumes, oxygen uptake becomes the rate limiting factor, lowering the production of *N*-acetyl-L-dopa

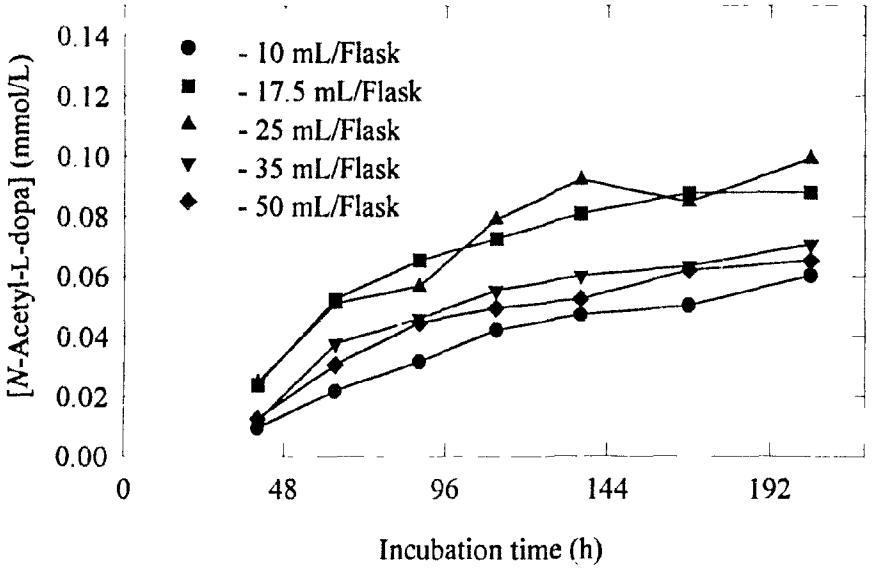


Figure 43. Effect of aeration on production of *N*-acetyl-L-dopa in mutant L138

Biosynthesis of *N*-acetyl-L-dopa: precursor identification

On structural grounds, the most likely precursor of *N*-acetyl-L-dopa is either L-phenylalanine (**58**) or L-tyrosine (**59**). These protein amino acids are derived from the shikimate pathway (97), and only hydroxylation and acetylation steps are required to convert them to *N*-acetyl-L-dopa (Figure 44). The role of these amino acids and related compounds was examined in a series of experiments using whole cultures, mycelium resuspended in buffer, and cell-free extracts.

In the initial experiment, cultures of *S. akiyoshiensis* mutant L138 supplemented with an amino acid or an *N*-acetylamino acid (5 mmol/L) prior to inoculation, and the concentrations of *N*-acetyl-L-dopa and the added substrate were determined by ion-pairing hplc at various times up to 204 h. In both the unsupplemented and the L-

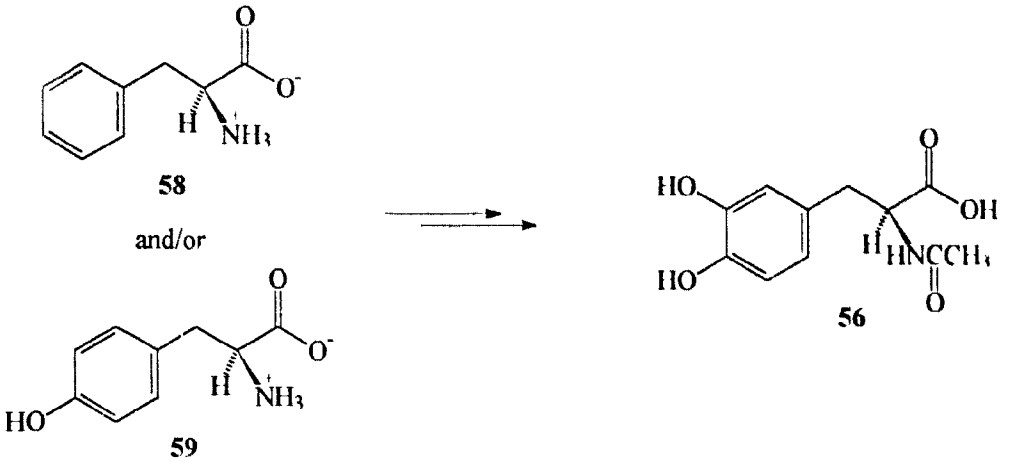


Figure 44. Possible biosynthetic precursors of *N*-acetyl-L-dopa

phenylalanine (**58**) supplemented cultures, a low-level production of *N*-acetyl-L-dopa was observed over a 204 h incubation period (Table 29), and the slight initial stimulation of *N*-acetyl-L-dopa production observed in the culture supplemented with *N*-acetyl-L-phenylalanine dropped to control levels by 168 h On the other hand, greatly elevated levels of *N*-acetyl-L-dopa were observed in each of the cultures supplemented with L-tyrosine (**59**), L-dopa (**60**) and *N*-acetyl-L-tyrosine (**61**) For each culture, *N*-acetyl-L-dopa production was complete by 72 h, and the concentration remained at the maximum during the remainder of the incubation period (Figure 45)

When maximum levels of *N*-acetyl-L-dopa were reached in cultures supplemented with either L-dopa, *N*-acetyl-L-tyrosine, or L-tyrosine, the concentration of L-dopa in the culture supernatant had fallen below detectable levels (Figure 45a), but significant levels

Table 29. Stimulation of *N*-acetyl-L-dopa production by unlabelled substrate

Addition	Concentration at 88 h (mmol/L)	
(initially 5 mmol/L)	[Substrate]	[*N*-acetyl-L-dopa]
None	--	0 057
L-Phenylalanine	n/a	0 042
N-Acetyl-L-phenylalanine	n/a	0 11
L-Tyrosine	1 6	0 40
N-Acetyl-L-tyrosine	3 6	0 91
L-Dopa	0	1 15

of *N*-acetyl-L-tyrosine (Figure 45b) and L-tyrosine (Figure 45c) remained The amount of *N*-acetyl-L-tyrosine consumed corresponded closely to the amount of *N*-acetyl-L-dopa formed in culture (0 91 mmol/L at 88 h), suggesting that *N*-acetyl-L-tyrosine was converted to *N*-acetyl-L-dopa and underwent no other metabolism On the other hand, the amount of *N*-acetyl-L-dopa formed in the culture supplemented with L-dopa or L-tyrosine at 88 h was only a small fraction (23% and 8%, respectively) of the amount added An intense black-brown coloration formed in both the L-dopa and L-tyrosine supplemented cultures during the incubation but not in the control or other supplemented cultures The color derived from L-dopa was associated with suspended particulate which sedimented on centrifugation (15850×g, 5 min) leaving a colorless supernatant, while the color produced in L-tyrosine supplemented cultures did not sediment on centrifugation It is not unexpected that L-dopa and L-tyrosine are consumed in other metabolic processes These amino acids are biosynthetic precursors of melanin

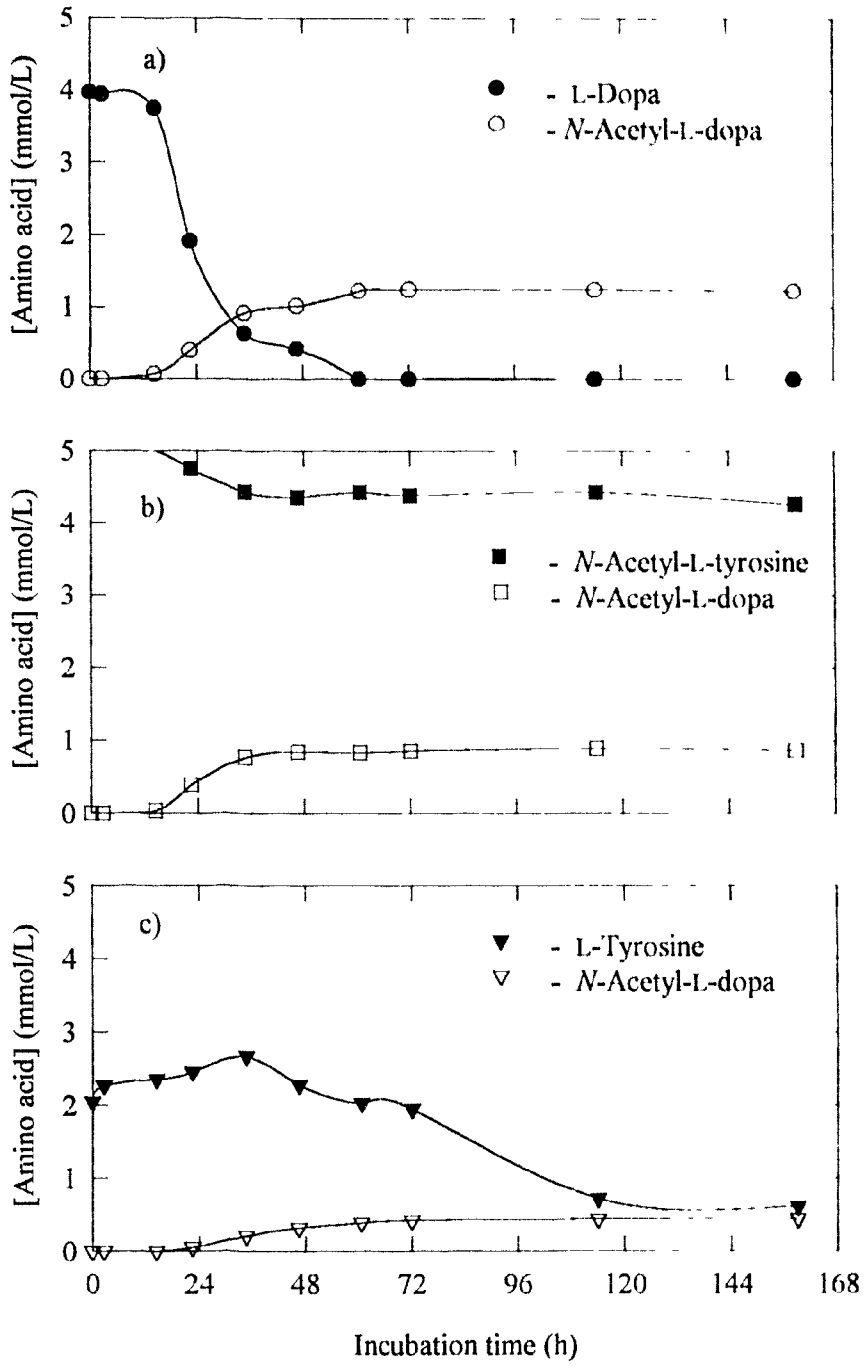


Figure 45. Production of *N*-acetyl-L-dopa in L138 cultures supplemented with a) L-dopa, b) *N*-acetyl-L-tyrosine and c) L-tyrosine

pigments, and about 50% of the streptomycetes isolated from nature produce melanoid type pigments (98)

The high concentration of *N*-acetyl-L-dopa formed in cultures supplemented with L-tyrosine, L-dopa, or *N*-acetyl-L-tyrosine suggests that these substances overcome the rate-limiting step in *N*-acetyl-L-dopa biosynthesis and that they are more likely precursors than L-phenylalanine and *N*-acetyl-L-phenylalanine However, the similar stimulations of *N*-acetyl-L-dopa production by L-dopa and *N*-acetyl-L-tyrosine (Table 29) does not permit the two possible routes to *N*-acetyl-L-dopa from L-tyrosine (Figure 46) to be distinguished Instead, the initial results support the presence of both pathways. To further examine this possibility and to provide a cleaner system from which *N*-acetyl-L-dopa could be isolated, the production of *N*-acetyl-L-dopa by mycelium resuspended in buffer was examined

Washed mycelia from cultures of mutant L138 (46 h) grown on casein-starch medium were resuspended in 20 mmol/L potassium phosphate buffer (pH 6.5) containing glucose (100 mmol/L), and one of the substrates, L-tyrosine, L-dopa or *N*-acetyl-L-tyrosine, was included at a concentration of 2 mmol/L.

Resuspended L138 mycelium supplemented with L-tyrosine produced smaller amounts of *N*-acetyl-L-dopa (0 6% of the initial L-tyrosine) than whole cultures containing similar concentrations of L-tyrosine. An increased level of *N*-acetyl-L-dopa (1 5% of the initial L-tyrosine added after 42-h incubation) was produced a larger amount of mycelium (4-fold) resuspended in the same volume of buffer (Figure 47). Excluding glucose or doubling its concentration did not significantly affect the yield of

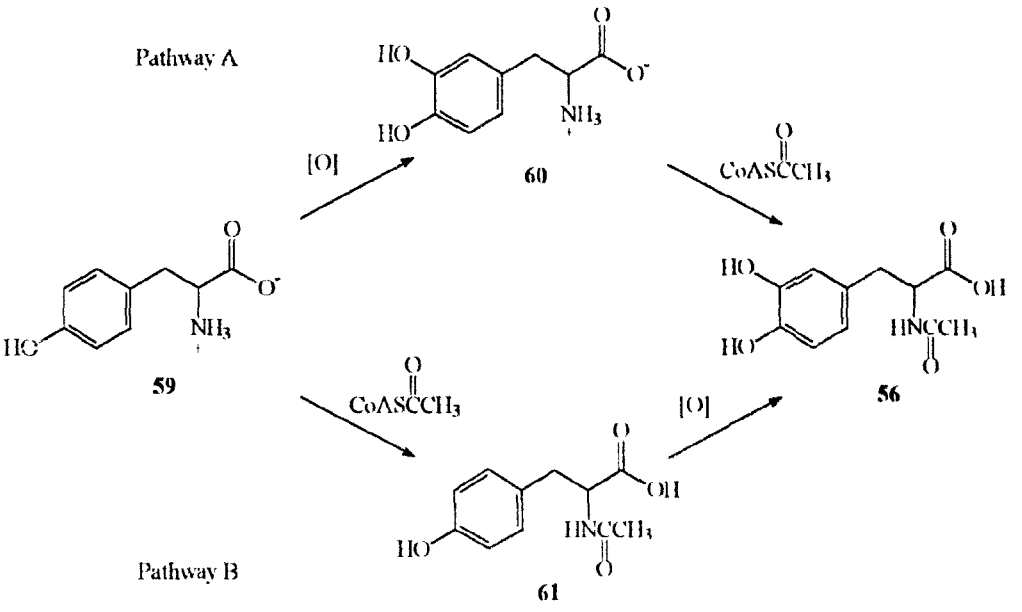


Figure 46. Possible biosynthetic pathways for *N*-acetyl-L-dopa formation from L-tyrosine

N-acetyl-L-dopa.

The resuspension experiments with L-dopa as the substrate produced *N*-acetyl-L-dopa in a similar fashion to that observed with supplemented whole cultures with *N*-acetyl-L-dopa, *i.e.*, *N*-acetyl-L-dopa was produced only when L-dopa was available (Figure 48). The maximum concentration of *N*-acetyl-L-dopa produced in mycelial resuspensions was obtained at 6 h, corresponding to 11% of the initial L-dopa, and the concentration of *N*-acetyl-L-dopa stayed constant over the remainder of the incubation time. As in the whole cultures, the low conversion of L-dopa to N-acetyl-L-dopa can be attributed to the diversion of substrate into pigment formation as the resuspension

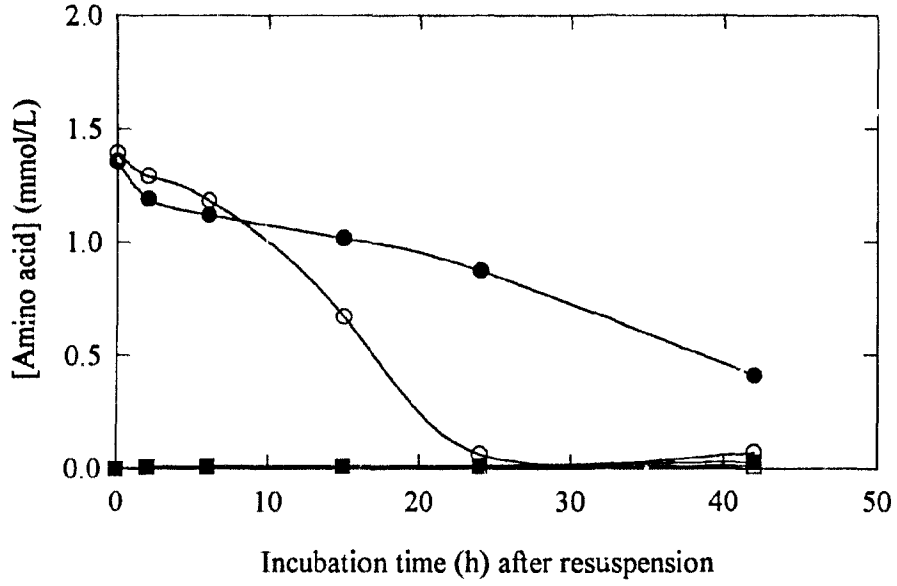


Figure 47. *N*-Acetyl-L-dopa (■ and □) and L-tyrosine (● and ○) levels in mycelial resuspensions containing normal mycelia (filled symbols) and a 4-fold increase in mycelium (open symbols).

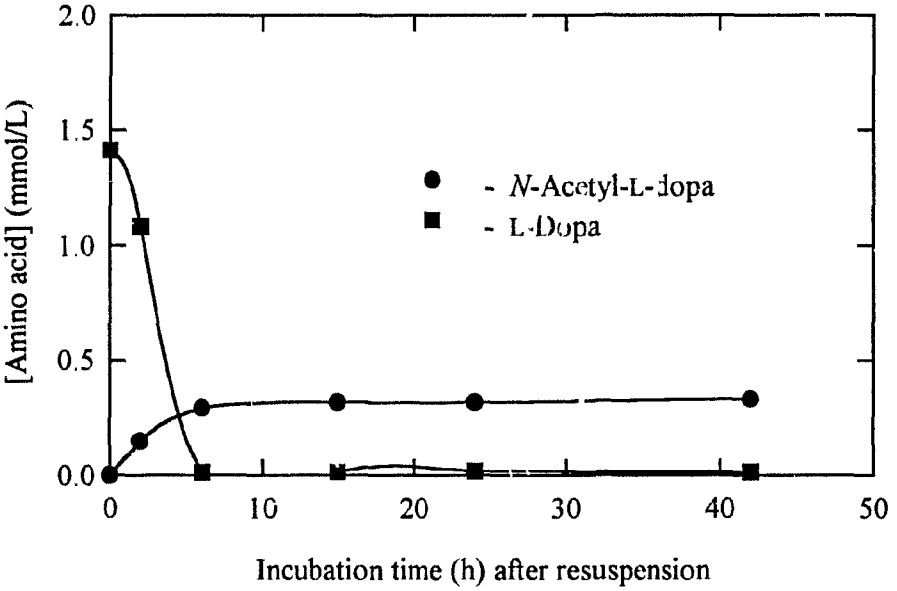


Figure 48. Production of *N*-acetyl-L-dopa by L138 mycelium resuspensions supplemented with L-dopa and glucose (100 mmol/L).

mixture became very dark during incubation Omission of glucose from the resuspension mixture resulted in a 40% decrease in the level of *N*-acetyl-L-dopa produced although higher glucose levels (200 mmol/L) did not effect the yield

When the amount of L138 mycelium in the resuspension mixture was increased 4-fold, the overall yield of *N*-acetyl-L-dopa was higher and a very rapid production was obtained over the initial 2 h (Figure 49) *N*-Acetyl-L-dopa production continued at a slower rate over the next 18 h reaching a concentration corresponding to 74% of the initial L-dopa after 42 h. The higher level of *N*-acetyl-L-dopa production can be attributed to a smaller loss of L-dopa to pigment formation, since the resuspension mixtures containing the 4-fold concentration of mycelium did not show the dark pigmentation observed with the lower concentration of mycelium As a result, significant levels of L-dopa were available for longer periods, and production of *N*-acetyl-L-dopa ceased when L-dopa was depleted

The amount of *N*-acetyl-L-dopa produced by resuspended L138 mycelia supplemented with *N*-acetyl-L-tyrosine was significantly smaller than that in whole cultures (Figure 50 and Figure 45b) After 42 h, the level of *N*-acetyl-L-dopa was only 0 030 mmol/L (1 5% of initial *N*-acetyl-L-tyrosine added) Similar production was obtained in resuspension cultures supplemented with 200 mmol/L glucose and when mycelium was resuspended in a 4-fold higher concentration However, the yield of *N*-acetyl-L-dopa was boosted significantly by omitting glucose from the resuspension buffer (0.18 mmol/L at 42 h, 9% of initial *N*-acetyl-L-tyrosine added) The production of *N*-acetyl-L-dopa w. not limited by substrate availability since approximately 90% of the

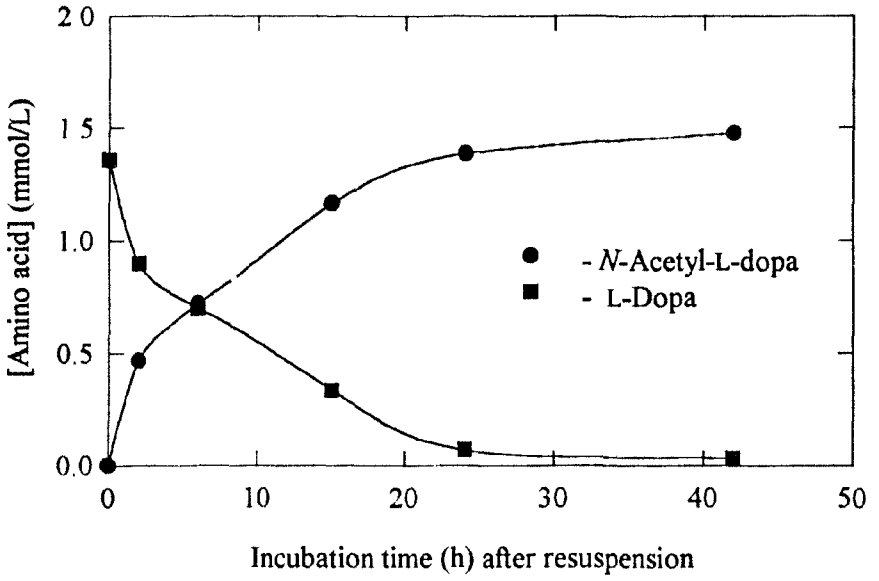


Figure 49. Effect of increased mycelium on the production of *N*-acetyl-L-dopa by mycelium resupensions containing L-dopa

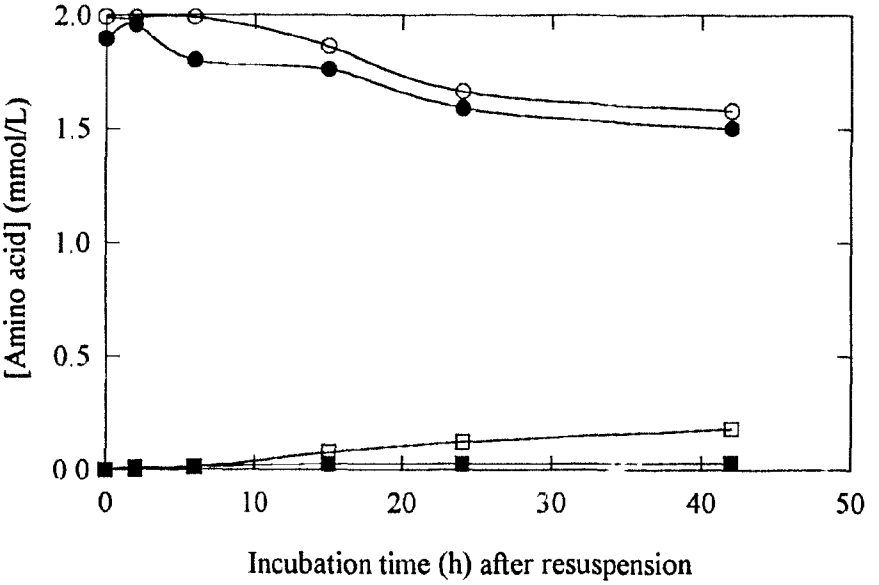


Figure 50. *N*-Acetyl-L-dopa (■ and □) and *N*-acetyl-L-tyrosine (● and ○) levels in mycelial resuspensions containing 100 mmol/L glucose (filled symbols) and without glucose (open symbols).

initial *N*-acetyl-L-tyrosine remained after 42-h incubation

It is possible that the mycelium collected at 46 h and resuspended in buffer was too old to convert *N*-acetyl-L-tyrosine to *N*-acetyl-L-dopa; the production of *N*-acetyl-L-dopa in cultures supplemented with *N*-acetyl-L-tyrosine was complete by 36 h (Figure 45b). When the experiment was repeated with mycelium collected 20 h after inoculation, no *N*-acetyl-L-dopa was detected.

Alternatively, the conversion of *N*-acetyl-L-tyrosine to *N*-acetyl-L-dopa may occur extracellularly. Tyrosinase, the enzyme which catalyzes the conversion of L-tyrosine to L-dopa as the initial step in melanin biosynthesis has been shown to be excreted by several streptomycetes (99-103) and *N*-acetyl-L-tyrosine has been documented as a substrate of the purified enzyme (99). In whole cultures supplemented with *N*-acetyl-L-tyrosine (2 mmol/L) prior to inoculation, the level of tyrosinase, determined using the dopachrome assay (99), increases to a maximum at 20 h and then decreases to below detectable levels by 36 h (Figure 51) The production of *N*-acetyl-L-dopa over this initial 36 h period was closely correlated with the tyrosinase activity In a parallel culture not supplemented with *N*-acetyl-L-tyrosine, higher tyrosinase activity was detected over a similar period (Figure 51) However, addition of *N*-acetyl-L-tyrosine at 20 h led to only a small production of *N*-acetyl-L-dopa (<0 1 mmol/L) indicating that the conversion of *N*-acetyl-L-tyrosine to *N*-acetyl-L-dopa may not be solely mediated by tyrosinase in the culture supernatant.

When culture supplemented with *N*-acetyl-L-tyrosine (2 mmol/L) prior to inoculation was centrifuged after 20 h of incubation and the supernatant was

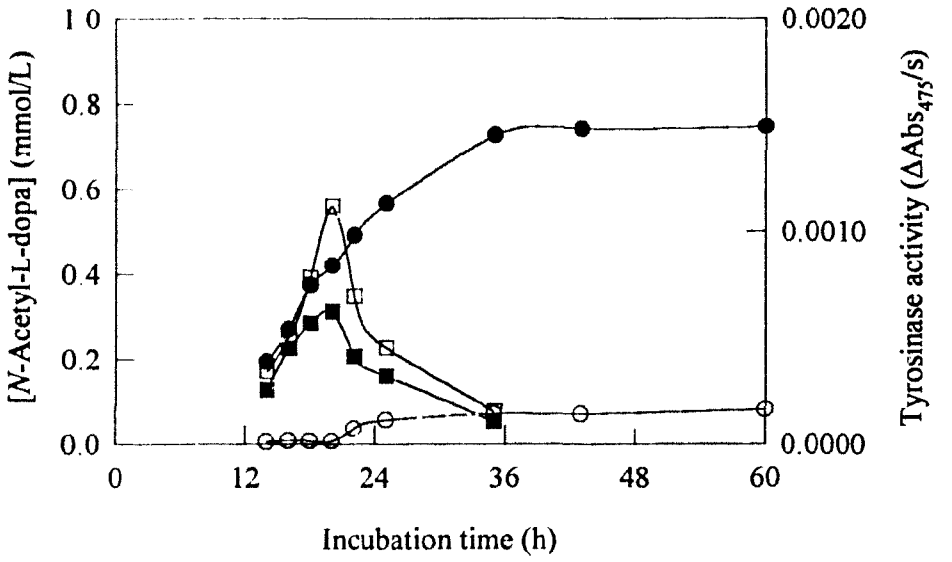


Figure 51. *N*-Acetyl-L-dopa (● and ○) and tyrosinase (■ and □) levels in cultures supplemented with *N*-acetyl-L-tyrosine prior to inoculation (filled symbols) or 20 h after inoculation (open symbols).

reincubated, the levels of *N*-acetyl-L-tyrosine and *N*-acetyl-L-dopa remained unchanged over 40-h of further incubation (Figure 52). The corresponding mycelium was washed and resuspended in 20 mmol/L potassium phosphate buffer (pH 6.5) containing glucose (100 mmol/L) and *N*-acetyl-L-tyrosine (5 mmol/L), but no *N*-acetyl-L-dopa was formed. Production of *N*-acetyl-L-dopa continued up to 36 h in a duplicate culture in which mycelium had not been separated from culture fluids (Figure 52). Therefore components provided by both mycelium and culture fluids (*e.g.*, tyrosinase) must be needed to facilitate the conversion of *N*-acetyl-L-tyrosine to *N*-acetyl-L-dopa.

When $FeSO_4$ and ascorbic acid were added to L138 resuspended mycelium containing 100 mmol/L glucose, 165% increase in the level of *N*-acetyl-L-dopa was observed. However, when a similar experiment was conducted without adding mycelium, *N*-acetyl-L-dopa was produced at a similar level, indicating that the

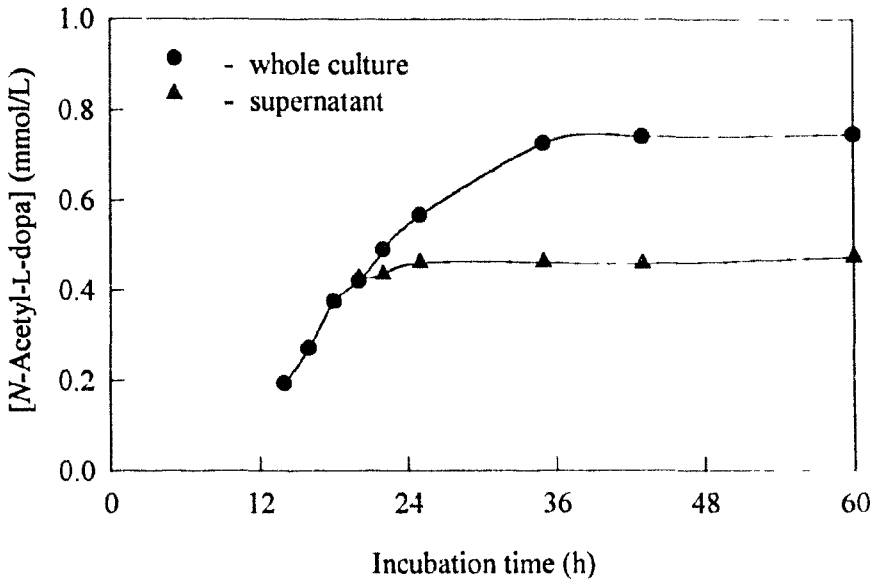


Figure 52. Production of *N*-acetyl-L-dopa in whole cultures and culture supernatants

conversion of *N*-acetyl-L-tyrosine to *N*-acetyl-L-dopa occurs as a non-enzymatic process The observation of non-enzymatic conversion of *N*-acetyl-L-tyrosine to *N*-acetyl-L-dopa suggests that *N*-acetyl-L-tyrosine is not an intermediate, but is rather an artifact unrelated to the biosynthesis of *N*-acetyl-L-dopa. The commercial importance of the non-enzymatic conversion of *N*-acetyl-L-tyrosine to *N*-acetyl-L-dopa has been recognized by the filing of a patent (104). Although this conversion was observed non-enzymatically, the amount of *N*-acetyl-L-dopa produced in this experiment was low (4% of initial *N*-acetyl-L-tyrosine added). Therefore, the non-enzymatic reaction cannot be fully account for the significantly larger conversion (18.3%) in whole cultures not supplemented with $FeSO_4$ and ascorbic acid. Several commercial patents are held for the conversion of *N*-formyl- and *N*-acetyl-L-tyrosine to *N*-acetyl-L-dopa by microbial cultures (105-107). In almost all cases, iron salts and ascorbic acid are included to increase the yield of the *N*-

acyl-L-dopa.

Acetyltransferase activity in cell-free extracts

The two possible biosynthetic pathways from L-tyrosine to *N*-acetyl-L-dopa (Figure 46) require the acetylation of either L-tyrosine or L-dopa. A cell-free extract of mutant L138 was prepared by sonicating mycelium suspended in MOPS buffer (100 mmol/L, pH 7.3) containing toluenesulfonyl fluoride (PMSF, 3 mmol/L) and β-mercapto-ethanol (6 mmol/L). Supernatant from a 47807×g centrifugation was incubated with an amino acid and acetyl CoA; the formation of an acetylated product was monitored by ion-pairing hplc. The level of *N*-acetyl-L-dopa produced in assays containing L-dopa (0.6 mmol/L) and acetyl CoA (0.6 mmol/L) over 15 and 60 min incubation at 30°C was 0.18 and 0.30 mmol/L, corresponding to 30 and 50% conversions of the amino acid substrate. When L-tyrosine was added to the cell-free extract, no more than 0.0082 mmol/L of *N*-acetyl-L-tyrosine (<1% conversion) could be detected by hplc. A cell-free extract desalted by gel filtration on Sephadex G-25 acetylated L-dopa with high efficiency (53.2% conversion after 120 min). However, a chromatographic peak corresponding to *N*-acetyl-L-tyrosine could not be detected above the smooth baseline provided by the desalted preparation. *N*-Acetyldopa was not produced in assays containing desalted cell-free extract, D-dopa (0.6 mmol/L) and acetyl CoA (0.6 mmol/L), even after 4 h of incubation at 30°C

Although samples taken from assay mixtures were heated in a boiling water bath (2 min) to denature the enzyme, a significant amount of *N*-acetyl-L-dopa was produced

from L-dopa and acetyl CoA in the 0 time sample (0.074 mmol/L, 12 3%) Similarly, assay blanks conducted with enzyme boiled for 5 min showed that the acetylation of L-dopa still proceeded, but at a significantly reduced rate (about 7% of unheated preparation). Since no *N*-acetyl-L-dopa is formed in a mixture of L-dopa and acetyl CoA containing no cell-extract, these results suggest that the L-dopa acetyltransferase has significant thermal stability. A heat stable puromycin acetyltransferase (PAT) isolated from *Streptomyces alboniger* (57) acetylates the amino group of the *O*-methyltyrosine moiety of puromycin This enzyme, which has been proposed to play a role in puromycin biosynthesis (54), also provides antibiotic resistance to the producer organism (56, 57, 108). A chloramphenicol acetyltransferase isolated from *Staphylococcus aureus* (61) was also reported to be heat stable although the corresponding *Escherichia coli* enzyme was rapidly inactivated at 75°C (61)

DISCUSSION

Initial isotopic feeding experiments in *S. akiyoshiensis* demonstrated that HON is formed from aspartate and acetate, and led White and coworkers to propose a plausible biosynthetic route (Figure 4) involving ligation of the β-carboxyl group of aspartate and the methyl group of acetate (21, 22). Subsequent experiments using cell-free systems yielded no further details of HON biosynthesis as only very small amounts of HON were formed in extracts supplemented with aspartic acid and acetyl CoA (14, 26). On the other hand, analysis of *S. akiyoshiensis* mutants blocked in the biosynthesis of amino acids in the aspartate family confirmed that the HON pathway branches before the enzymatic steps catalyzed by homoserine dehydrogenase and lysine decarboxylase (Figure 1) (14, 24). A group of nonauxotrophic mutants unable to produce HON was also isolated, and they appeared to be blocked in the HON pathway. Certain combinations of these mutants in mixed cultures (*e.g.*, L127 and L138) were capable of HON production, suggesting that an intermediate excreted by one mutant was converted to HON by the second organism (14, 24) The amount of HON produced in these experiments was similar to that produced in wild-type cultures (6-8 mmol/L after 96-h incubation), indicating that a relatively large amount of an intermediate was available for isolation

Attempted bioassay-directed purification of biosynthetic intermediates

Through cross-feeding experiments (14, 24, 25), it was determined that a metabolite excreted by mutant L138 stimulated HON production by mutant L127. A

direct means of detecting and quantifying the active metabolite of unknown structure was not available; since the L138 culture supernatant is a complex matrix containing highly colored pigments, salts and partially digested starch and casein To overcome this deficiency, an indirect assay based on the transformation of mutant L127 to a HON producer was developed The stimulatory effect was observed when L127 was grown on liquid or semi-solid medium; the latter required smaller amounts of sample and was routinely used. It was assumed that the stoichiometric relationship between the amount of HON produced in the assay and a biosynthetic intermediate excreted by mutant L138 is 1·1. The HON produced was determined by hplc (20) Since the complete conversion of an intermediate may not occur, the assay provided an estimation of the minimum level of metabolite present

The reliability and reproducibility of the L127 assay were significantly improved by optimizing the pH and the composition of the medium, the agar plug dimensions, the age of the L127 mycelium, and the incubation time Nitrate-starch medium provided more rapid and higher overall production of HON with older mycelium than casein-starch medium, although younger mycelium (48 h) grown on casein-starch medium produced HON rapidly during short periods of incubation Substitution of other carbon sources for soluble starch or mixtures of soluble starch and glucose did not significantly affect the production of HON A pH of 6 0 was slightly better than the more acidic or the basic conditions investigated. The highest levels of HON were generally produced on assay plugs of the smallest volume. This was attributed to dilution of the metabolite on addition to the assay plug, thus providing a

lower effective concentration of L138 metabolite to the organism. Replicate assays performed under the optimized conditions (Table 11) had relative standard deviations of 7 - 12% (Table 10), a large improvement over the initial values of approximately 32%

However, several limitations of the assay became apparent when it was employed to analyze components separated from crude L138 culture supernatant. The presence of various substances in the solutions assayed severely reduced the level of HON produced by the L127 mycelium Several of these substances, HCOONa and $HCOONH_4$, CH_3COONa and NaCl, are routinely used as eluents in ion exchange chromatography or are produced on the neutralization of treated samples. With the exception of NaCl, these substances reduced the assay response to such a degree that it could not be used to analyze chromatographic fractions For NaCl, assay response could be corrected using a standard curve (Figure 20) Due to these limitations, purification of the L138 metabolite was restricted to separation methodologies compatible with the L127 assay.

The major portion of the mass in crude supernatant, as well as the active metabolite, did not bind to cation-exchange resin. However, about 80% of the active metabolite was retained by an anion-exchange resin containing hydroxide ion as the counter ion and eluted with HCl. The ion-exchange behavior was consistent with the presence of an acidic functional group and the lack of a cationic substituent such as a protonated amino group.

When acidified culture supernatant was applied to a charcoal column, 80% of

the active metabolite was retained and it eluted with aqueous acetone However, a significant portion of carbohydrate was also bound to the charcoal and eluted with acetone. Attempts to improve the separation and recovery by extending the hydrophobic binding to C_{18} and cyanopropyl reversed phase resins proved unsuccessful A lack of extractability into chloroform or ethyl acetate and recovery of active metabolite from butanol extracts clearly indicated that this metabolite has substantial polar character.

When the L138 supernatant was dialyzed in cellulose sacks against H_2O, the active L138 metabolite was detected in the dialysate. This result supported previous ultrafiltration evidence (14) which showed that the L138 metabolite had a molecular mass less than 10000. The use of dialysis facilitated the large scale separation of L138 metabolite from polysaccharides with a molecular weight exceeding 12600, and provided a solution which was significantly less viscous than the crude supernatant

Gel filtration provided another method for separating the active L138 metabolite from unwanted culture components without adding salts or other substances which have a deleterious effect on the agar plug assay. Since the active component was retained by Sephadex G-10, which has a molecular weight cutoff of 700, it probably had a fairly low molecular mass. During analysis of Sephadex G-10 fractions, it became apparent that if a stoichiometric relationship held between the active metabolite and HON, sufficient metabolite was present to be detected by ^1H nmr spectroscopy However, the only ^1H nmr signals present were those due to a solvent impurity and *N*-acetyl-L-dopa, which was shown not to stimulate HON production in mutant L127

Although *N*-acetyl-L-dopa did not account for the entire mass of the sample, no other nmr signals were detected, indicating that only a small amount of the active component was present. A stoichiometric relationship with HON was not supported by this, suggesting that the active metabolite was not a biosynthetic intermediate.

Other more circumstantial evidence that is not consistent with the conversion of an intermediate to HON by mutant L127 was also available. A considerable lag period (6 - 24 h, Figures 10 - 12) between the addition of L138 metabolite and the detection of HON production was observed. The lag could be required to establish the biosynthetic enzymes or cofactors which, due to the non-functioning biosynthetic pathway, may not have been available. On the other hand, lag times in the production of the antibiotic streptomycin have been noted in the literature for the stimulation of mutants of *Streptomyces griseus* IBKh 1439 with A-factor, an autoregulator (77, 109). The lag has been attributed to the time required for cytomorphological alterations characteristic of high streptomycin production.

Another observation inconsistent with the conversion of an accumulated intermediate to HON is the very long production period observed after the addition of L138 metabolite to L127 liquid assay cultures. Figure 12 shows that HON production by 36-h L127 mycelium only begins to level off after 4-5 days of additional incubation. This pattern of production is very similar to that observed in wild-type *S. akiyoshiensis* (88, 89). If the rate limiting step in HON biosynthesis precedes the biosynthetic lesion of mutant L138, then it would be expected that the transformation of intermediate to HON would be significantly faster than the production of HON by

the wild-type organism However, a longer production time, similar to that seen in wild-type cultures, may be expected if biosynthesis is initiated by an autoregulator or if the rate limiting step is located in the later steps of the pathway.

For both liquid and semi-solid surface cultures, the L138 metabolite must be present at a minimum or threshold concentration before HON is produced by mutant L127. When L138 culture supernatant was added to the assay below this threshold concentration, HON was not observed (Figure 16), even though HON can be detected by opa-hplc analysis at concentrations significantly lower (0 1 nmol/L) than the threshold concentration (*ca* 0.5 mmol/L). The threshold effect was lowered by supplementing the assay solution with glucose Although glucose is a simple carbon source capable of overcoming conditions of nutritional limitation, thus providing the energy to drive metabolic processes including HON production, glucose is known to suppress rather than stimulate the production of many secondary metabolites (110) However, a carbon source requirement has been observed for resuspended mycelium of wild-type *S. akiyoshiensis*, HON was only produced if the resuspension buffer contained glucose or a similar carbon source (26).

In addition to the threshold effect observed at low metabolite concentrations, a levelling effect is apparent at high concentrations of L138 metabolite (Figure 16) A similar effect has been observed on addition of A-factor to mutants of *S. griseus* IBKh 1439. The addition of 0 1 to 2 µg/mL of A-factor to submerged cultures at inoculation was effective in inducing streptomycin production (77, 109), but no further increase in production was observed when levels greater than 2 µg/mL were added to

the non-producing mutants However, the time required for induction of cytomorphological changes was shortened when more A-factor was present A similar effect has been noted with supplementation of mutants *Nocardia* sp KB-993 which are blocked in the biosynthesis of rifamycin (83) The concentration of rifamycin produced varied directly with the level of B-factor added within the range of 2 to 30 ng/mL, but moderately higher concentrations of B-factor did not provide higher rifamycin levels

Although a significant amount of circumstantial evidence suggests that the HON mutants isolated by Le (14) do not contain lesions in biosynthetic or structural genes of HON biosynthesis, isotopic feeding studies were initiated to provide direct and conclusive evidence

Attempted isotopic labelling of an accumulated intermediate

The accumulation of a ^{13}C-labelled intermediate was probed by adding labelled substrates to cultures of mutant L138 and examining culture supernatant by ^{13}C nmr spectroscopy The spectra collected for L138 culture supernatant supplemented with DL-[4-^{13}C]aspartate showed only the presence of unmetabolized aspartate, although an intermediate at the concentration indicated by the L127 assay would have been clearly visible above the background noise if enriched with 2-3% ^{13}C at a carboxyl, or a ketone carbon This result indicated that if an intermediate accumulates in the L138 culture supernatant, it is probably not derived from aspartic acid However, since HON is derived from two primary precursors, aspartate and acetate (21, 22), it is

possible that a HON intermediate could be derived solely from acetate, if the genetic lesion in these mutants occurs prior to the ligation step

Several ^{13}C enriched substances were shown by nmr to accumulate in the culture supernatants of mutant L138 supplemented with either [2-^{13}C]- or [1,2-$^{13}C_2$]acetate. Two doublet signals, resulting from the intact incorporation of an acetate unit, were located at chemical shifts expected for an *N*-acetyl group Addition of authentic *N*-acetyl-L-dopa to the nmr sample showed that these signals were not due to this *S. akiyoshiensis* metabolite (111), although the similarity of the chemical shifts are consistent with another *N*-acetylated amino acid. *N*-Acetylated metabolites function as intermediates in biosynthetic pathways For example, *N*-acetylglutamate, *N*-acetylglutamyl phosphate, *N*-acetylglutamate semialdehyde and *N*-acetylornithine are intermediates of ornithine and arginine biosynthesis (112). *N*-Acetyldiaminopimelate is an intermediate on the biosynthetic pathway to diaminopimelate (DAP) and lysine in Bacilli (64), and *N*-acetylpuromycin is a biosynthetic precursor to the antibiotic puromycin (54). The acetylation of antibiotics is also a known resistance mechanism used by both antibiotic producers (55-59) and non-producing organisms (60-63)

The detection of labelled L138 metabolites derived from [2-^{13}C]acetate and the known incorporation of [2-^{13}C]acetate into HON (21, 22) provides some support for the accumulation of a biosynthetic intermediate by mutant L138 To test this hypothesis, the incorporation of label from an L138 metabolite into HON was investigated in mutant L127 Radiolabelled [2-^{14}C]acetate was chosen for these experiments since lower levels of acetate incorporation could be detected, and

radioactive HON was isolated in each experiment (Table 25) The incorporation of radioactivity was lower when [2-^{14}C]acetate was added during the growth of mutant L138 than when [2-^{14}C]acetate was added after L138 supernatant and L127 mycelium were mixed in a parallel experiment. These results are consistent with the incorporation of an acetate derived intermediate produced by mutant L138 and the incorporation of acetate into the remaining portion of HON by mutant L127. To account for the different levels of incorporation, the latter incorporation would have to be more efficient and produce HON with a quite different distribution of radioactivity than HON isolated in the first experiment. This is inconsistent with the similar distributions of radioactivity observed in the HON isolated from these two cultures (Table 26). Alternatively, the high incorporation of radioactivity by mutant L127 can be explained by the initiation of *de novo* HON biosynthesis by the L138 metabolite The low incorporation of radioactivity into HON when [2-^{14}C]acetate was added to growing mutant L138 could result from unmetabolized acetate in the L138 supernatant when it was combined with L127 mycelium. Isolation of radioactive acetate as its bromophenacyl ester showed that 36.7% of the acetate added to the L138 culture was still present in the L138 supernatant when it was mixed with the L127 mycelium.

Therefore, the isotopic experiments provided no definitive evidence for accumulation of an intermediate in L138 cultures fluids. Instead, it appears that the metabolite produced by mutant L138 initiates *de novo* HON production in mutant L127 and is not structurally related to HON. L127 assay results have shown that the

initiator is not present in sterile casein-starch medium However, concentrated supernatant from a wild-type *S akiyoshiensis* culture stimulates the production of HON by mutant L127, suggesting that the wild-type organism also produces this initiator The presence of autoregulators in a significant number of wild-type strains of various *Streptomyces* species has been observed (76)

The results of the above experiments suggests that the series of HON mutants previously characterized by Le are not blocked in HON biosynthesis Since mutants L167 and L127 are capable of producing HON in cosynthesis with each other and with mutant L138, it is probable that mutants L167 and L127 are blocked in the biosynthesis of the initiator. However, there is no evidence indicating that mutants of the latter group (Figure 5), which includes mutant L138, are blocked in initiator biosynthesis If these mutants are blocked in the initiator biosynthetic pathway, they would be stimulated to produce HON by addition of wild-type culture supernatant This still remains an outstanding question for the classification of this group of mutants.

Structural information of the L138 metabolite

Although the metabolite was not purified to homogeneity, some structural information was obtained by examining its stability and the nature of its binding to various chromatographic materials. The metabolite was stable in acid but its activity was lost rapidly when heated in a basic solution. The binding of the metabolite to a anion exchange resin containing an OH^- counter anion, but not to the same resin

containing a chloride counter anion, suggests that the metabolite contains a weakly acidic group, possibly a phenolic substituent

The polar nature of the L138 metabolite is indicated by the good recovery of activity obtained by extraction with butanol and the inability to extract the active metabolite from aqueous solution with chloroform and ethyl acetate The metabolite does not significantly bind to C_{18} and CN hydrophobic resins but binds almost quantitatively to Darco G-60 charcoal, from which it can be eluted with aqueous acetone This may suggest that the metabolite contains an unsaturated or aromatic substituent (*e g* phenol) which facilitates its binding to charcoal

Mutants lacking the ability to produce autoregulatory factors, including A-factor and virginiae butanolides, are not usually capable of sporulating or forming pigments in addition to lacking the ability to produce their usual antibiotic (109) However, mutants L167, L127 and L138 do not differ in spore and pigment formation from the wild-type organism (14) Unlike the butyrolactone autoregulatory factors, the L138 metabolite is not extractable with chloroform This evidence does not support an A-factor-like compound as the initiator of HON biosynthesis in mutant L127.

Since only HON biosynthesis appears to be affected in mutant L127, the initiator of HON production may be a biosynthetic cofactor that is required for HON production These mutants are not auxotrophic and thus, a missing cofactor must be specific to HON biosynthesis A nonauxotrophic *Streptomyces cattleya* mutant blocked in thienamycin biosynthesis has been characterized and the inability of this mutant to produce thienamycin has been attributed to insufficient levels of vitamin B_{12} (91) The

activity of vitamin B_{12} is catalytic with a minimum required concentration of 0 1 µg/mL However, supplementing mutant L127 mycelium in agar plug assays with individual vitamins, including B_1, B_2, B_6, B_{12} and folic acid or mixtures of vitamins and minerals, in the form of commercial multivitamins, did not restore HON production

A compound which has similar solubility and chromatographic behavior to the L138 metabolite is cosynthetic factor 1 (CF-1, 7,8-didemethyl-8-hydroxy-5-deazariboflavin, Figure 9, **51**) CF-1 was isolated initially from *Streptomyces aureofaciens* W-5 and shown to catalyze a biological reduction step in the biosynthesis of chlorotetracycline (85) Addition of CF-1 to cultures of *Streptomyces lincolnensis* mutant NTG-3 blocked in the production of the nucleoside antibiotic lincomycin restores antibiotic production (113) CF-1 is thought to be involved in the steps leading to propylproline *via* the 2,3-extradiol cleavage of the biosynthetic precursor, L-dopa (114, 115) CF-1 has been found in almost all of 45 species from 16 genera of actinomycetes screened (113) The phenolic functional group of CF-1, which has been previously utilized for its isolation (113), would account for the anion exchange behaviour of the L138 metabolite The highly polar ribityl (2*R*,3*R*,4*R*,5-tetrahydroxypentanyl) side chain attached to an aromatic deazariboflavin backbone would also account for the highly polar nature of CF-1 but also facilitates its binding to charcoal.

Identification *N*-acetyl-L-dopa

The metabolite detected in Sephadex G-10 fractions and subsequently isolated from *S. akiyoshiensis* cultures was identified as *N*-acetyl-L-dopa by spectroscopic methods. The structure was confirmed and the stereochemistry assigned by comparison to a sample prepared by acetylation of L-dopa.

Although L-dopa is widely distributed in nature, previous reports on the natural occurence of its acetylated derivative have been limited to microbial (116, 117) and plant cell (118) cultures supplemented with *N*-acetyl-L-tyrosine. These investigations were initiated to explore new methods for producing L-dopa, a precursor of the neurotransmitter dopamine that is used in the treatment of neurological disorders such as Parkinson's disease (119). *N*-Acetyl-L-dopa has also been of some interest as it exhibits a dose-dependent cytotoxicity to melanoma cell lines and prolongs the life span of melanoma-bearing mice (95).

Since *N*-acetyl-L-dopa was the only substance detected by nmr in the Sephadex G-10 fractions shown to stimulate HON production by mutant L127, this acetylated amino acid was investigated as the active culture component. However, synthetic *N*-acetyl-L-dopa did not transform mutants L127 and L167 into HON producers, and *N*-acetyl-L-dopa was subsequently detected in culture fluids of the mutants L127, L167 and L138. Thus, it is unlikely that this acetylated aromatic amino acid is involved in HON biosynthesis.

Production of *N*-acetyl-L-dopa by *S. akiyoshiensis*

In an attempt to increase yields of *N*-acetyl-L-dopa in preparation for biosynthetic experiments, a systematic variation of culture parameters was carried out using mutant L138 which yielded the highest titres under the initial conditions Higher levels of *N*-acetyl-L-dopa were achieved using a vegetative inoculum and an initial pH of 5 5 - 6 0 At this pH, Glazebrook *et al* (89) showed that *S akiyoshiensis* grew at a slow rate, and growth rate is an important factor affecting the production of secondary metabolites (96) At high pH (*e g*, 7 0 and 7 5), the level of *N*-acetyl-L-dopa declined during the incubation, suggesting that *N*-acetyl-L-dopa is degraded During these investigations, it was observed that *N*-acetyl-L-dopa, as well as free L-dopa, darkened rapidly in basic solution in the presence of air although this was slowed by passing a stream of nitrogen over the solution

Cultures grown on media containing the disaccharides, maltose and sucrose, or 1% starch as a carbon source produced less than one-third the level of *N*-acetyl-L-dopa as cultures grown on media containing 3% starch A higher level of starch (5%) resulted in a rapid initial production of *N*-acetyl-L-dopa, but at longer incubation times, the level of *N*-acetyl-L-dopa decreased

The production of *N*-acetyl-L-dopa, was also influenced by the volume of the culture placed in the flask The turbulence, and therefore the aeration resulting from the orbital shaking motion increases with decreasing culture volume The highest level of *N*-acetyl-L-dopa was produced at intermediate culture volumes (17 5 and 25 mL per 125 mL Erlenmeyer flask) Cultures having a smaller volume (10 mL) produced an

intense red pigment These results suggest that at larger culture volume, the production of *N*-acetyl-L-dopa is limited by the level of oxygen transfer into the culture and that at very small culture volumes (*i.e.*, at high oxygenation), another secondary process may divert biosynthetic intermediates to colored metabolites.

From these investigations, the only change in culture parameters which showed improved *N*-acetyl-L-dopa production over that of cultures grown at initial conditions was a decrease in the initial pH to 5.5. This resulted in a doubling of the *N*-acetyl-L-dopa produced after a 204-h incubation.

Biosynthesis of *N*-acetyl-L-dopa

The addition of L-tyrosine, L-dopa or *N*-acetyl-L-tyrosine to cultures of *S. akiyoshiensis* mutant L138 greatly increased the production of *N*-acetyl-L-dopa, suggesting that these compounds were precursors that overcame the rate-limiting step in the pathway. Similar additions of L-phenylalanine or *N*-acetyl-L-phenylalanine had no significant effect, and these compounds were not investigated further.

The conversion of L-tyrosine to *N*-acetyl-L-dopa requires two steps: hydroxylation and acetylation. Depending on the sequence of these two steps, either L-dopa (Pathway A, Figure 46) or *N*-acetyl-L-tyrosine (Pathway B) would be an intermediate in the pathway. The results of the supplementation experiments suggest that each of these possible intermediates is converted to *N*-acetyl-L-dopa. For each, the production of *N*-acetyl-L-dopa was larger and more rapid than that in the culture supplemented with L-tyrosine. This is consistent with the extra step required to

convert L-tyrosine to *N*-acetyl-L-dopa

There is literature precedent for the enzymatic conversion of L-tyrosine to L-dopa in *Streptomyces* bacteria Tyrosinase catalyzes the ortho-hydroxylation of monophenols to catechols and the dehydrogenation of catechols to orthoquinones using molecular oxygen as the electron acceptor (98). Tyrosinase has been purified and characterized from both intra- and extracelluar extracts of several *Streptomyces* species, including *S glaucescens* (99, 100), *S michiganensis* DMS 40015 (101), *S* Sp KY-453 (102) and *S. antibioticus* (103) In melanin biosynthesis, tyrosinase catalyzes the conversion of L-tyrosine to L-dopa which is then converted to dopaquinone in a second tyrosinase catalyzed step (120) The ability to produce melanin pigments has long been regarded as a characteristic for the identification and classification of *Streptomyces* and pigment formation was noted during the initial classification of *S akiyoshiensis* (3) About 50% of *Streptomyces* species isolated from nature produce melanin pigments (98).

Cultures of *S. akiyoshiensis* supplemented with L-dopa or L-tyrosine turned dark brown-black during the incubation period, most likely due to the increased production of melanin pigments by tyrosinase-catalyzed oxidation Therefore, the conversion of L-tyrosine to L-dopa, the initial step of pathway A (Figure 46), is expected to operate in this organism.

The production of *N*-acetyl-L-dopa by mycelium resuspended in phosphate buffer containing glucose and L-dopa (Figure 48) was similar to that in whole cultures supplemented with L-dopa (Figure 45), and the formation of *N*-acetyl-L-dopa ceased

when the substrate L-dopa was depleted. Approximately 11% of the initial L-dopa was acetylated, the remainder being used for pigment formation. When L138 mycelium was resuspended at a 4-fold higher concentration, pigments did not form and a higher production of *N*-acetyl-L-dopa was due to increased availability of substrate (Figure 49). Presumably the increased concentration of mycelium leads to reduced oxygen levels that are unable to support pigment formation. Under these conditions, an efficient conversion (74 %) of substrate to product was attained.

In the cultures supplemented with L-tyrosine or *N*-acetyl-L-tyrosine, substantial quantities of the aromatic substrate remained when *N*-acetyl-L-dopa levels reached their maximum values, and similar results were observed in the resuspension experiments. It appears that the termination of *N*-acetyl-L-dopa production, at least when L-tyrosine or *N*-acetyl-L-tyrosine are added to cultures, is not due to the depletion of substrate but rather to other factors (*e.g.*, depletion of required cofactors or a loss of biosynthetic enzyme activity). Omission of glucose from the resuspension mycelium containing *N*-acetyl-L-tyrosine resulted in a 6-fold increase in the level of *N*-acetyl-L-dopa produced (9% of the initial *N*-acetyl-L-tyrosine). If cellular respiration was increased by the glucose, then less oxygen would have been available for the hydroxylation step.

The conversions of L-tyrosine and *N*-acetyl-L-tyrosine to *N*-acetyl-L-dopa were significantly lower in resuspended 46-h L138 mycelium than those observed in whole cultures. Since most of the *N*-acetyl-L-dopa formed within 48 h after inoculation of cultures supplemented with N-acetyl-L-tyrosine, younger mycelium (20 h) were used in a resuspension experiment. However, no *N*-acetyl-L-dopa was detected. The lower

conversions of L-tyrosine and *N*-acetyl-L-tyrosine to *N*-acetyl-L-dopa in resuspension experiments suggest that the culture supernatant may play a significant role in the hydroxylation step. Tyrosinase, an enzyme capable of catalyzing this conversion, is produced by a number of *Streptomyces* species (99-103), and both L-tyrosine and *N*-acetyl-L-tyrosine have been documented as a substrates for the purified tyrosinase from *S. glaucescens* (99). The k_{cat} for the hydroxylation of *N*-acetyl-L-tyrosine by tyrosinase was 14-fold larger than that of L-tyrosine (99). Therefore, there is justification for the second step of pathway B (Figure 46).

Tyrosinase activity was excreted by *S. akiyoshiensis* into the culture medium and reached a maximum level (20 h) when the rate of *N*-acetyl-L-dopa production was maximum (Figure 51). Tyrosinase activity declined quite rapidly after it had reached its maximum level. However, incubation of *N*-acetyl-L-tyrosine with 20-h culture supernatant resulted in only a very small production of *N*-acetyl-L-dopa (<0.1 mmol/L), and the level of *N*-acetyl-L-dopa was unchanged when the supernatant from a 20-h L138 culture supplemented with *N*-acetyl-L-tyrosine at the time of inoculation was incubated (Figure 52).

From these results, it is evident that both the mycelium and the culture fluids are required for the conversion of *N*-acetyl-L-tyrosine to *N*-acetyl-L-dopa. The mycelium may generate a cofactor that is needed for tyrosinase-catalyzed oxidations. A mixture of $FeSO_4$ and ascorbic acid, when added to an L138 culture supplemented with *N*-acetyl-L-tyrosine, increased the production of *N*-acetyl-L-dopa about 6-fold. However, further investigation revealed that this same effect was observed in incubated

mixtures which lacked mycelium. A non-enzymatic Fe-ascorbate catalysed reaction has been demonstrated previously for the conversion of *N*-acetyl-L-tyrosine to *N*-acetyl-L-dopa (104)

To provide more direct evidence for the acetylation step in one or both of the proposed pathways, a search for enzymatic activity capable of acetylating L-tyrosine or L-dopa was undertaken. A cell-free extract that efficiently acetylated L-dopa using acetylCoA as a cosubstrate was prepared. Desalting of the extract on Sephadex G-10 gave a flat baseline in the hplc chromatograms, thus allowing for the detection of small levels of *N*-acetyl-L-tyrosine. However, there was no evidence of *N*-acetyl-L-tyrosine being produced when L-tyrosine was used as a substrate, although L-dopa was acetylated efficiently. The desalted extract did not acetylate D-dopa to any extent, indicating that the acetyltransferase was quite specific for L-dopa. An acetyltransferase isolated from baker's yeast acetylated the D-isomer of all the common protein amino acids but could not acetylate the L-isomers (121) and an acetyltransferase with high specificity for the D-isomer of tryptophan has been isolated from *Penicillium viridicatum*. An acetyltransferase highly specific for L-aspartic acid has been isolated from mammalian brain tissue (122). However, an acetyltransferase isolated from *Streptomyces roseus* MA 839-A1, which acetylates L-leucine in the initial step to leupeptin biosynthesis, acetylates a number of other L-amino acids but also can acetylate D-leucine and D-phenylalanine, although at rate only about 10% of their respective L-isomers (53, 123).

Since evidence has been presented for both the hydroxylation of L-tyrosine to

L-dopa and the acetylation of L-dopa to *N*-acetyl-L-dopa, it must be concluded that pathway A (Figure 46, p. 117) is operational in *S. akiyoshiensis*. The alternative route (Payhway B) is less likely as no evidence was found for the acetylation of L-tyrosine. Although it appears that *N*-acetyl-L-tyrosine is converted to *N*-acetyl-L-dopa by tyrosinase, this is possibly not the normal course of *N*-acetyl-L-dopa biosynthesis

The current perspective of HON biosynthesis

It is surprising that, of the 4230 single colonies tested by Le (14), a series of nonauxotrophic HON mutants blocked in HON biosynthesis could not be isolated This may suggest that some steps involved in HON biosynthesis are catalyzed by enzymes associated with primary metabolism rather than enzymes specific to a secondary metabolic pathway. Researchers unable to isolate mutants blocked in several steps in the biosynthesis of the phosphinothricin moiety of bialaphos found that these steps were catalyzed by primary enzymes normally involved in the tricarboxylic acid cycle (48). Lesions in the genes which encode enzymes catalyzing primary processes would result in auxotrophism. Therefore, mutants blocked at primary metabolic pathways related to HON biosynthesis could have been discarded during the search for nonauxotrophic mutants.

Although no primary enzyme is known to catalyze the ligation of aspartate with acetate, the initial step in diaminopimelic acid and lysine biosynthesis which is catalyzed by dihydrodipicolinate synthase involves a similar nucleophilic attack of the methyl group of pyruvate with the aldehyde function of aspartic β-semialdehyde. The

product rapidly cyclizes and loses water to form 2,3-dihydropicolinate. An analogous reaction using acetate as the nucleophile rather than pyruvate was proposed as a possible initial step of HON biosynthesis by White, Smith and DeMarco (21, 22). If the nitrogen of aspartate β-semialdehyde was derivatized, *e.g.*,acetylated, cyclization of the nitrogen with the carboxyl group provided by the acetate would not occur. An *N*-acetyl group prevents the cyclization of glutamic acid during the biosynthesis of ornithine and arginine (65) and *N*-acetyltransferase activity has been demonstrated in cell-free extracts from *S. akiyoshiensis*, although the specificity of the enzyme for aspartate has not been investigated.

Alternatively, the biosynthesis of HON may proceed by a pathway slightly more complicated than originally proposed by White, Smith and DeMarco (21, 22). HON, which is an inhibitor of homoserine dehydrogenase would be expected to be toxic to its producing cells. Therefore, there must be a mechanism by which *S. akiyoshiensis* can protect itself from HON. Since opa-hplc analysis showed that HON is not present in cellular extracts, the organism avoids a buildup of HON. This may be accomplished by an efficient efflux and exclusion system which transports newly synthesized antibiotic out of the cell and then prevents it from returning to the cytoplasm. A more effective way would be to avoid free HON in the cell during its biosynthesis. The inhibitory activity of HON results from its structural similarity to homoserine and this activity would be significantly reduced if the amino or carboxyl groups were derivatized. The detrimental effects of the glutamine synthase inhibitor, phosphinothricin, is prevented during its biosynthesis by acetylation of the amino group

of the precursor DMPT, and the resulting *N*-acetyl DMPT is then incorporated into the tripeptide, bialaphos (48)

The formation of bialaphos and many peptide antibiotics, including pepstatin, is believed to be catalyzed by a multifunctional enzyme complex, similar to the fatty acid and polyketide synthase complexes (124, 125) These multifunctional enzymes form thiotemplates with multiple cofactors which allow for peptide formation from non-protein amino acids All intermediates leading to the final peptide remain enzyme bound, as do intermediates of fatty acid and polyketide antibiotic biosynthesis on fatty acid and polyketide synthases, respectively Structural modification of individual amino acids can occur while the peptide is still bound to the complex or after its release from the enzyme (126). Therefore, if HON is synthesized as part of a peptide formed on a multifunctional enzyme, it may not be possible to obtain viable HON mutants containing genetic lesions for these steps. During the investigation of bialaphos biosynthesis, mutants blocked in the intermediate steps of peptide formation were not isolated after chemical mutagenesis (48).

As with phosphinothricin from bialaphos (48), HON could be liberated from the peptide by the action of non-specific proteases. Investigations have indicated that the culture supernatant and the cellular extracts contain a large amount of protease activity (26) It is interesting to note that most of the best nitrogen sources for the production of HON in culture are complex proteins such as casein and Pharmamedia (88) which require high levels of protease activity to generate amino acids

One other possible reason for a lack of mutants blocked in a step of the HON

pathway may be associated with the final biosynthetic step and the mechanism by which the antibiotic is exported If HON was inactivated during its biosynthesis, *e g*, acetylation of the free amino group, the final step of the biosynthesis would be the removal of the acetyl substituent Coupling of this step with an active export process would prevent HON mutants from releasing their intermediates into the culture supernatant This would prevent the accumulation of HON biosynthetic intermediates and therefore, no cosynthetic pairs could be characterized

At present, it is known that C-1 to C-4 and the nitrogen of HON is derived from the intact incorporation of aspartic acid and C-5 is obtained from the incorporation of an acetate methyl group The point at which the HON biosynthetic pathway branches from the pathway of the aspartic acid amino acids must be located prior to the steps catalyzed by homoserine dehydrogenase and lysine decarboxylase Viable mutants blocked at aspartate semialdehyde dehydrogenase are needed to more clearly define the branch point Evidence presented here indicates that HON biosynthesis is mediated by an initiator substance, although it is still not clear if this substance stimulates a general mechanism which controls a number of secondary metabolic processes or acts to mediate HON biosynthesis only To understand this, the initiator substance produced by mutant L138 must be isolated and characterized

Mutants L167 and L127 are blocked in the production of an initiator substance Mutant L138 may also be blocked in the production of initiator, and therefore may accumulate an initiator intermediate which results in its cosynthesis and cross-feeding properties Alternatively, this later mutant may produce the initiator substance in a

fashion similar to the wild-type organism but may be blocked in HON production by a different mechanism from L167 and L127 For this to be known, it must be determined if mutant L138 is stimulated to produce HON by the culture supernatant of wild-type *S akiyoshiensis*

EXPERIMENTAL

General

All chemicals (ACS grade or better) and chromatographic materials were obtained from commercial suppliers and were used without prior purification unless stated. All water was deionized (resistivity of 18.3 MΩ-cm) and filtered (0.20 μm) using a Barnstead/Thermolyne NANOpure water system Model D4741. Methanol was distilled in glass. A Beckman Model Φ31 pH meter fitted with a Beckman combination gel-filled electrode was used for pH measurements.

Culture media were sterilized at 121°C for 20 min in a Market Forge Sterilmatic Model STM-EL autoclave. Manipulations requiring sterile conditions were performed in a Canadian Cabinets Model HE5-97-T laminar flow hood. Cultures grown on semi-solid media were incubated in a National Model 3221-14 incubator at 30°C. Liquid cultures were incubated at 27°C in either a Queue Environmental shaker Model 4750 or a New Brunswick Scientific shaker Model G-53 at an orbital speed of 220 rpm. Micro-centrifugations were performed using a Beckman Microfuge "E" equipped with a fixed angle rotor. An International Equipment clinical centrifuge Model CL fitted with a IEC #221 rotor was used for semi-preparative scale centrifugations. Preparative-scale centrifugations were performed on a DuPont Instruments Sorvall Model RC-5B centrifuge equipped with either a Sorvall Model GSA or SS-34 fixed angle rotor or on a Beckman Model J2-21 centrifuge equipped with a JA-17 rotor. Centrifugations were carried out at room temperature unless

otherwise stated, and maximum centrifugal forces are reported Freeze drying was performed using a Edwards Modulyo freeze dryer connected to an Edwards Model E2M8 rotary high vacuum pump Optical densities (640 nm) of spore suspensions and uv spectra were obtained in 1-cm cuvettes using a Hewlett Packard 8452A diode array spectrophotometer.

The hplc system consisted of two Beckman Model 110B solvent delivery modules, a Beckman Model 340 gradient mixer equipped with a Model 210A injection valve and a 20-µL injection loop. Detection was accomplished by one of two modes 1) fluorescence using a Beckman Model 157 fluorescence detector equipped with a 9-µL flow cell and excitation and emission filters of 305-395 nm and 420-650 nm, respectively, or 2) ultraviolet absorbance using a Hewlett Packard 1050 multiple wavelength detector Gradient control, data collection and analysis were facilitated by a CompuPartner 486 computer interfaced to the hplc system.

The nmr spectra were recorded on a Bruker AC 250F spectrometer using standard Bruker programs. Chemical shifts are reported in ppm referenced to HOD (4.80 ppm) for proton spectra and external dioxane (66.66 ppm) for carbon spectra DEPT spectra were acquired using standard Bruker programs The ir spectra were recorded on a Nicolet 510P FT-IR spectrometer in a N_2 atmosphere, and mass spectra were obtained by electron impact (70 eV) on a CEC 21-104 mass spectrometer Optical rotations were measured on a Perkin Elmer 141 polarimeter at 589 nm Melting points were taken using a Gallenkamp melting point apparatus and are uncorrected.

Chemicals enriched in ^{13}C and ^{14}C were obtained from MSD Isotopes and International Chemical and Nuclear, respectively Scintillation counting was performed on an LKB Model 1215 Rackbeta scintillation counter. Samples were dissolved in a small amount (0 20 mL) of an approriate solvent and dispersed in scintillation cocktail (10 mL, Scintiverse II, Fisher Scientific) Thin layer plates were scanned for radioactivity using a Packard Radiochromatogram scanner Model 7201 at a scan speed of 5 cm/h and a slit width of 5 mm

Microorganisms

A lyophilized culture of *Streptomyces akiyoshiensis* (ATCC 13480) was obtained from the American Type Culture Collection. HON mutant strains of *S. akiyoshiensis* (L127 and L138) prepared by chemical mutagenesis using *N*-methyl-*N*'-nitro-*N* nitrosoguanidine (NTG) were provided by Y. Le and L C. Vining (14, 25)

Media

MYM agar

MYM agar medium (per L) contained malt extract (Bacto, Difco Laboratories, 10 0 g), yeast extract (Bacto, Difco Laboratories, 4.0 g), maltose (Bacto, Difco Laboratories, 4 0 g) and agar (Bacto, Difco Laboratories, 20.0 g) in water.

TO agar

TO agar medium (per L) contained tomato paste (Primo Foods, 20.0 g), oatmeal pablum (H. J. Heinz Company, 20.0 g) and agar (20.0 g) in water. The pH was adjusted to 6.8 with NaOH (3 mmol/L) prior to autoclaving.

Casein-starch agar

Casein-starch agar medium (per L) contained casein (light white soluble, BDH, 4.0 g), soluble starch (Anal'R B10271, BDH, 20.0 g) and agar (15.0 g) in basal medium (pH 6.0).

Nitrate-starch agar

Nitrate-starch agar medium (per L) contained KNO_3 (3.04 g, 30 mmol/L), soluble starch (20.0 g) and agar (15.0 or 20.0 g) in basal medium (pH 6.0).

MYG

MYG medium (per L) contained malt extract (10.0 g), yeast extract (4.0 g) and glucose (Fisher Scientific, 4.0 g) in water. The pH was adjusted to 6.0 with HCl (3 mol/L) prior to autoclaving.

Casein-starch

Casein-starch medium (per L) contained casein (4.0 g) and soluble starch (30.0 g) in basal medium (pH 6.5).

Basal

Basal medium contained $MgSO_4$ (0.20 g), KH_2PO_4 (0.75 g), K_2HPO_4 (1.75 g), $FeSO_4 \cdot 7H_2O$ solution (0.2% (w/v), 4.5 mL), salt solution (1% (w/v) of each NaCl and $CaCl_2$, 1.0 mL) stock mineral solution (4.5 mL) and water to total volume of 1000 mL. The pH was adjusted to 6.0 or 6.5 using HCl (3 mol/L) prior to autoclaving. Stock mineral solution contained H_3BO_3 (1.6 mg), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (10 mg), $CuSO_4 \cdot 5H_2O$ (10 mg), $MnCl_2 \cdot 4H_2O$ (10 mg), $ZnCl_2$ (40 mg), $FeCl_3 \cdot 6H_2O$ (120 mg) and water to total volume of 1000 mL.

Preparation of stock spore suspension

Stock spore suspensions of *S. akiyoshiensis* were prepared on petri dishes (9.0 cm diameter) containing sterile MYM agar or TO agar medium. Plates were inoculated by streaking with a sterile inoculating loop containing stored spore suspension (10 µL, optical density at 640 nm of 0.2 measured after 1:50 dilution). After seven days of growth, sterile water (5 mL) was added, and the spores were dislodged from the surface with a sterile wire loop. The suspension was filtered through a plug of sterile cotton to remove mycelia, and the filtrate was centrifuged (1750×g, 10 min). The supernatant was discarded, and the pellet was resuspended in sterile water (5 mL) by vortexing (30 s). The suspension was recentrifuged (1750×g, 10 min), and the resulting pellet was resuspended in 20% (w/v) glycerol to give a final optical density of 0.2 (measured at 640 nm after 1:50 dilution). Stock spore suspensions were stored at -20°C and were used to inoculate seed, production and

assay cultures, as well as fresh MYM and TO agar plates for preparation of new stock spore suspensions

Seed cultures

Vegetative inoculum was prepared by inoculating MYG medium (pH 6.0 30 mL per 250-mL baffled flask) with spore suspension (50 µL) and incubating with rotary shaking for 48 - 72 h. Vegetative inoculum was used without prior washing

Production of L138 metabolite

Optimization

Casein-starch medium (300 mL per 2-L Erlenmeyer flasks, 4 flasks) was inoculated with mutant L138 seed culture (3.0 mL per 300 mL medium) and incubated at 27°C and 220 rpm. At 24-h intervals, samples (10 mL) were removed aseptically from individual flasks and combined. Supernatants (25 mL) from centrifugations (11951×g, 20 min) were lyophilized, and the L138 metabolite was estimated by the agar plug assay.

Large scale production

L138 cultures (48 h) grown on casein-starch medium (300 mL per 2-L Erlenmeyer flasks, 5 flasks) were centrifuged (16274×g, 20 min) to remove mycelia This supernatant is referred to as "fresh L138 supernatant" The mycelial pellet was washed with H_2O (200 mL) and recentrifuged The combined supernatant and H_2O

wash was filtered (Whatman #1) to remove floating debris and the filtrate (1435 mL) was lyophilized The resulting light brown hygroscopic solid (16.97 g) was stored at -20°C Solutions of lyophilized solid in H_2O are referred to as "L138 supernatant", and the fold concentration is referenced to the original culture volume.

Dialysis

The lyophilized solid (16 97 g) obtained from a large scale production of L138 metabolite (5 × 300 mL per 2-L Erlenmeyer flask) in H_2O (150 mL) was placed in preswollen cellulose dialysis sacks (6, Sigma 250-7U) and dialyzed against H_2O (350 mL) for 5 h The dialysate was replaced with fresh H_2O (350 mL) and dialysis was continued for an additiona 5 h The combined dia ysates were concentrated *in vacuo* (65 mL), partitioned into small portions (*e g.*, 10 mL), and stored at -20°C. No loss of activity was observed on storage This solution is referred to as "L138 dialysate", and the fold concentration is referenced to the original culture volume.

Assay of L138 metabolite

Liquid culture assay

Liquid cultures of *S akiyoshiensis* mutant L127 were prepared by addition of spore suspension (0 4% (v/v)) to casein-starch medium (10 mL per 125 mL Erlenmeyer flask) and, after a period of growth, test solutions (1 mL) were added. Samples (0 30 mL) were removed and centrifuged (15850×g, 5 min) The concentration of HON in the supernatant was determined by opa-hplc.

Agar plug assay

Assay plates were prepared by sterile pipetting a desired volume (2, 3, 4 and 6 mm thickness required 11.3, 17 0 22 7 and 34 1 mL, respectively) of hot sterile casein-starch agar or nitrate-starch agar medium into petri dishes The plates were dried in the laminar hood (15 min) prior to use. Surface cultures of *S akiyoshiensis* mutant L127 were prepared by addition of spore suspension (50 µL per 9 0-cm plate), and the spores were spread with a sterile bent glass rod. After a period of incubation at 30°C (typically 72-96 h), donut-shaped agar plugs were cut from the mycelial lawn using sterile cork borers (#2, 4, 6, 7 and 9 cork borers provided 6, 9, 12, 14 and 16 mm diameters, respectively) and placed in petri dishes (5 plugs per 6 0-cm dish) Test solutions (120 µL) were placed in the plug's centre well, and the plugs were incubated at 30°C. After a period of incubation (typically 48 h), water (3 mL) was added, and the agar plugs were allowed to stand for at 4°C (typically for 3 5 h) The concentration of HON in the aqueous phase was determined by opa-hplc Controls consisted of plug assays in which the test solution was replaced with H_2O When samples to be tested were supplemented with glucose, it was also added to control assays. Concentrations of HON from agar plug assays are corrected for the HON produced in control assays and is expressed amount of HON produced per volume of sample applied (600 µL) to the agar plug assay.

Opa-hplc analysis

Hplc analysis of HON was carried out according to a literature procedure (20)

Pre-column derivatization was performed by adding o-phthalaldehyde (opa) reagent (Pierce, 40 µL) to a mixture of test solution (20 µL) and an internal standard solution (cysteic acid, 20 µL, typically 0 05 mmol/L) After standing for 1 min at room temperature with occasional mixing, sodium acetate solution (120 µL, 100 mmol/L, pH 6 2) was added, and a 20-µL portion was chromatographed

Separations were obtained on a Beckman Ultrasphere ODS (5 µm, 4 6 × 45 mm) column fitted at the inlet with an Upchurch Scientific Model C-130B guard column (2 · 20 mm) containing Perisorb RP-18 (30-40 µm) using a binary gradient (Appendix A) formed between sodium acetate (100 mmol/L, adjusted to pH 6 2 with 3 mol/L HCl)-methanol-tetrahydrofuran (900 95 5)) and methanol The sodium acetate solution was filtered (0 45 µm) and degassed under reduced pressure (water aspirator) prior to use The column effluent was monitored by fluorescence detection.

The HON response was corrected for variability in the derivatization reaction using a correction factor calculated from the ratio of the measured peak area to the expected peak area for the concentration of cysteic acid injected The average response (peak area/mmol/mL) for cysteic acid was calculated for 20 injections at three concentrations (0 048, 0 024 and 0 010 µmol/L) The average relative standard deviation for the peak area of cysteic acid was 3% HON concentrations were estimated using a standard curve constructed from triplicate injections of a purified HON sample (127) at 10 concentrations over the range 0 002 to 1 mmol/L.

Initial characterization and purification of the L138 metabolite

Ion-exchange chromatography

Amberlite IR-120

Amberlite IR-120 resin (H^+ form, 16-50 mesh, 100 mL) was stirred in a solution of HCl (5 mol/L, 500 mL) for 3 h. The mixture was decanted, a second portion of HCl was added, and stirring was continued for an additional 3 h. The resin was washed with water (4 × 500 mL) until the pH of the wash was neutral. The resin was packed in a column (1.5 × 28.3 cm) and washed with water (500 mL). L138 dialysate (20-fold, 10 mL) was applied to the column at a flow rate of 1 mL/min, and fractions (10 mL) were collected. The column was washed with water (100 mL) followed by an ammonia solution (0.3 mol/L, 200 mL). Fractions were neutralized (HCl) and concentrated *in vacuo*. The resulting residues were assayed using the agar plug assay.

Dowex 1-X8 (OH)

Dowex 1-X8 resin (Cl^-, 20-50 mesh, 100 mL) was stirred in a solution of sodium hydroxide (3 mol/L, 500 mL) for 3 h. The mixture was decanted, a second portion of sodium hydroxide was added and stirring was continued for an additional 3 h. The resin was washed with water (4 × 500 mL) until the pH of the wash was neutral. The resin was packed in a column (1.5 × 12 cm) and washed with water (500 mL). L138 dialysate (20-fold, 3 or 10 mL) was applied to the column at a flow rate of 1 mL/min, and fractions (10 mL) were collected. The column was washed with

water (50 mL) followed by an HCl solution (0 5 mol/L) Fractions were neutralized (HCl or NaOH, 0 5 mol/L) and concentrated *in vacuo* The resulting residues were assayed using the agar plug assay.

Dowex 1-X8 (Cl)

Dowex 1-X8 resin (Cl, 20-50 mesh, 100 mL) was stirred in a solution of HCl (3 mol/L, 500 mL) for 3 h The mixture was decanted; a second portion of HCl was added and stirring was continued for an additional 3 h. The resin was washed with water (4 · 500 mL) until the pH of the wash was neutral. The resin was packed in a column (1.5 × 27.3 cm) and washed with water (500 mL). L138 dialysate (20-fold, 10 mL) was applied to the column at a flow rate of 1 mL/min, and fractions (20 mL) were collected The column was washed with water (100 mL) followed by solutions of NaCl (0 2 mol/L, 200 mL) and HCl (0.5 mol/L, 100 mL). Fractions were neutralized (NaOH) and concentrated *in vacuo*. The resulting residues were assayed using the agar plug assay.

Adsorption chromatography

C_{18} Reversed-phase resin

A C_{18} reversed-phase Sep-Pak (Fisher Scientific) containing 600 mg of chromatographic material was conditioned by washing with methanol (3 × 10 mL) and water (3 × 10 mL). L138 dialysate (20-fold, 10 mL) was acidified with HCl (0 5 mol/L) to pH 1 and applied to the resin at a flow rate of 0.25 mL/min. The resin

was washed with water (10 mL) and methanol (10 mL). The effluent and methanol eluant were neutralized with NaOH (0.5 mol/L) and concentrated *in vacuo*. The resulting residues were assayed using the agar plug assay.

Cyanopropyl reversed-phase resin

A CN reversed-phase Sep-Pak (Fisher Scientific) containing 300 mg of chromatographic material was conditioned by washing with methanol (3 × 10 mL) and water (3 × 10 mL). L138 dialysate (75-fold, 1.5 mL) was acidified with HCl (0.5 mol/L) to pH 1 and applied to the resin at a flow rate of 0.25 mL/min. The resin was washed with water (5 mL), 30% (v/v) aqueous methanol (2 mL) and methanol (3 mL). The effluent and the 30% (v/v) aqueous methanol and methanol eluants were neutralized with NaOH (0.5 mol/L) and concentrated *in vacuo*. The resulting residues were assayed using the agar plug assay.

Charcoal

Acidified supernatant (50 mL, pH 1.5) from a mutant L138 culture (48 h) was applied to a column (1.4 × 4.5 cm) of charcoal (Darco G-60, Aldrich) at 0.25 mL/min. The column effluent and water wash (15 mL) were combined, concentrated to 10 mL, and lyophilized (221.5 mg). The column was eluted with 50% (v/v) aqueous acetone (25 mL) and acetone (25 mL). The neutralized eluants were concentrated and lyophilized to give 196.0 mg and 4.2 mg of residue, respectively. The mass of lyophilized residue from an untreated 50 mL portion of supernatant was 643.3 mg.

The residues were taken up in water (15 mL) and assayed using the agar plug assay

Amberlite XAD-2

Amberlite XAD-2 resin (20-60 mesh, 25 mL) was washed with 50% methanol (3 × 100 mL) and water (3 × 200 mL) During the water washing, the resin beads which floated were decanted off and discarded The washed resin was packed in a column (1 4 × 4 5 cm) and further washed with water (50 mL). Acidified supernatant (50 mL, pH 1 5) from a mutant L138 culture (48 h) was applied to the column at 0 25 mL/min The column effluent and water wash (15 mL) were combined, neutralized with NaOH (0 5 mol/L), concentrated to 10 mL, and lyophilized (591 3 mg) The mass of lyophilized residue from an untreated 50 mL portion of supernatant was 643.3 mg The residue was taken up in water (15 mL) and assayed using the agar plug assay

Gel filtration chromatography

Sephadex G-10 chromatography resin (100 g) was swelled in water overnight. After decanting many times to remove the fine particles, a column was prepared (2 5 × 61 6 cm), fitted with a flow adapter, and washed with 1 L of water. L138 dialysate (75-fold concentration, 4 mL) was applied at a flow rate of 1 2 mL/min and eluted with water Fractions of varying size (Table 17) were collected and lyophilized. The residue from individual and combinations of fractions were assayed using the agar plug assay

A second Sephadex G-10 column (2.5 × 118 cm) was prepared to provide inproved resolutions L138 dialysate (75-fold, 5 mL) was applied (1 2 mL/min) and eluted with water. Large fractions (4 × 50 mL) and subsequent smaller fractions (6 mL) were collected and lyophilized. Residues were analyzed by ion-p 3 hplc and assayed using the agar plug assay.

Sephadex columns were calibrated by chromatographing mixtures of blue dextran (10.5 mg), maltose (62.5 mg) and glucose (52 0 mg). Blue dextran was detected by absorbance at 600 nm. Glucose and maltose were detected using the BCA assay for reducing compounds.

BCA assay for reducing compounds (128)

BCA reagent consists of equal parts solution A, prepared by dissolving bicinchonic acid (860 mg), $Na_2CO_3 \cdot H_2O$ (31.76 g) and $NaHCO_3$ (12 10 g) in water (500 mL), and solution B, prepared by dissolving $CuSO_4 \cdot 5H_2O$ (624 mg) and L-serine (631 mg) in water (500 mL). Equal volumes (2 mL) of sample solution and fresh BCA reagent were mixed and heated at 100°C for 15 min The assay solutions were allowed to cool to room temperature, and the absorbance (560 nm) was determined A reagent blank consisting of water (2 mL) was treated in a similar fashion as described above.

Solvent extraction with butanol, chloroform and ethyl acetate

Acidified L138 dialysate (20-fold concentration, 28 mL, pH 1 5) was extracted

with butanol (3 × 10 mL) The resulting phases were separated by centrifugation (1750 × g, 10 min) The combined butanol extracts and the aqueous phase were each concentrated *in vacuo* to 5 mL Butanol remaining in the sample was removed by twice diluting with water (10 mL) and reconcentrating The resulting aqueous solution was lyophilized The solid residues were taken up in water (28 mL) and assayed using the agar plug assay. Extractions of L138 dialysate with chloroform and ethyl acetate were performed in a similar fashion although chloroform and ethyl acetate extracts were concentrated to dryness *in vacuo* and the residue was dissolved in H_2O (28 mL) prior to assaying

Preparative hplc chromatography

Water-methanol gradient elution:

L138 dialysate (40-fold concentration) was acidified to pH 1.5, and a portion (85 µL) was chromatographed on a Beckman Ultrasphere ODS (5 µm, 4 6 × 45 mm) column fitted at the inlet with an Upchurch Scientific Model C-130B guard column (2 × 20 mm) containing Perisorb RP-18 (30-40 µm) using a binary gradient (Appendix A) formed between water and methanol (2 mL/min). Water was filtered (0.45 µm) and degassed under reduced pressure (water aspirator) prior to use. The column effluent was monitored simultaneously by uv detection at 214 and 260 nm and fractions (4 mL/fraction) were collected from twelve separate chromatographic runs were combined The combined breakthrough fraction (0 - 2 min) contained 208.8 mg of residue where the combined mass of fractions 2 - 5 (2 - 10 min) contained only

12 7 mg of residue The residues were taken up in 2 mL of water and assayed using the agar plug assay

Ion-pairing conditions

L138 dialysate (40-fold concentration) was acidified to pH 1 5, and a portion (85 µL) was chromatographed on a Beckman Ultrasphere ODS (5 µm, 4 6 × 45 mm) column fitted at the inlet with an Upchurch Scientific Model C-130B guard column (2 × 20 mm) containing Perisorb RP-18 (30-40 µm) using isocratic elution with a solution of 20 mmol/L KH_2PO_4 containing 10 mmol L tetra-*n*-butyl ammonium hydrogen sulfate, pH 5 0 at a flow rate of 1 0 mL/min over 8 min Solvent was filtered (0 45 µm) and degassed under reduced pressure (water aspirator) prior to use The column effluent was monitored simultaneously by uv detection at 214 and 280 nm, and fractions (2 mL) collected from eight separate chromatographic runs were combined The corresponding fractions from each injection were combined and concentrated *in vacuo* Combined fractions 1, 2, 3 and 4 contained 359 5, 114 3, 112 2 and 111 3 mg of residue, respectively The mass of salts from an equivalent volume of hplc solvent had a mass of 97 7 mg The residues from combined fractions were taken up in 2 mL of water and assayed using the agar plug assay

Treatment of L138 culture supernatant with methanol

L138 dialysate (5 mL, 20-fold concentration) was treated dropwise with methanol (15 mL) and the resulting solution were centrifuged (26891 × g, 20 min) The

supernatant was concentrated to dryness *in vacuo* (0 98 g), and the pellet was dried *in vacuo* (0 10 g) Residues were dissolved in water (5 mL) and assayed using the agar plug assay

Treatment of L138 culture supernatant with ethanol

L138 culture supernatant (150 mL, 48 h) was treated dropwise at a rate of 3 mL/min with absolute ethanol to give 25, 50 and 75% (v/v) aqueous ethanol. After each successive addition, the mixture was allowed to stand for an additional 10 min and portions (26 7, 40 and 80 mL, respectively) of the resulting solution were centrifuged (26891 × g, 15 min), and the supernatants were concentrated *in vacuo* to remove ethanol. Solutions were diluted to their original volume and assayed using the agar plug assay

Examination of L138 cultures for biosynthetic intermediates

^{13}C Isotopic labelling of L138 produced intermediate

Cultures of *S. akiyoshiensis* mutant L138 were initiated by inoculating casein-starch medium (50 mL per 250-mL Erlenmeyer flask) with seed inoculum (1.5% (v/v)) Cultures were incubated with orbital shaking at 27°C. Sterile solutions of DL-[4-^{13}C]aspartate, [2-^{13}C]acetate and [1,2-$^{13}C_2$]acetate (10 mL, 40 mmol/L) were added in four 2.5-mL portions to separate L138 cultures at 25, 39, 51 and 65 h growth. A culture to which additions of sterile H_2O (2.5 mL) were made was used as a control culture Prior to the third addition (51 h), a portion (10 mL) of each supplemented

culture was removed aseptically, centrifuged (17210×g, 20 min), and the resulting supernatants were lyophilized. The lyophilized solids were dissolved in D_2O (0.5 mL) and ^1H and ^{13}C nmr spectra (128 and 2000 scans, respectively) were recorded. After 91-h growth, the remainder of the culture was centrifuged (17210×g, 20 min), and the resulting supernatant was lyophilized. Portions of each lyophilized residues (ca. 25%) were dissolved in D_2O (0.75 mL) and nmr spectra were recorded. Following the recording of nmr spectra, the nmr sample and the remaining lyophilized residue were dissolved in H_2O (5 mL) and dialyzed against fresh H_2O (250 mL) for 4 h. The dialysate was concentrated *in vacuo* to 10 mL, lyophilized, and the resulting solid was dissolved in D_2O (0.5 mL) and the ^{13}C nmr spectrum (2000 scans) was recorded. Synthetic *N*-acetyl-L-dopa (41.7 mg) was added to the dialyzed supernatant from the L138 culture supplemented with [1,2-$^{13}C_2$]acetate and the ^{13}C nmr spectrum was recorded again. The nmr sample was acidified to pH 1 and transferred to an 1.5 mL microcentrifuge tube. The solution was extracted with butanol (2 × 0.5 mL) and the combined butanol extracts were concentrated to dryness. The residue was dissolved in D_2O and again, the ^{13}C nmr spectra was recorded.

Cross feeding experiments using mutants L127 and L138 and [2-^{14}C]acetate

Expt. 1: Addition of [2-^{14}C]acetate to mutant L138

Separate cultures of *S. akiyoshiensis* mutants L127 and L138 were initiated by inoculating casein-starch medium (50 mL per 500-mL Erlenmeyer flask) with stock spore suspension (1.5% (v/v)). The cultures were incubated with orbital shaking at

27°C, and a sterile solution of sodium [2-^{14}C]acetate (1 00 mL, 168 mmol/L, 0 125 mCi/mmol) was added to the L138 culture at 12, 24 and 36 h Samples (0 50 mL) were removed aseptically before and after each addition After 48 h, the cultures were centrifuged (39800×g, 25 min, 4°C), and the mycelium of mutant L127 was resuspended in the supernatant from the mutant L138 culture Incubation was continued for an additional 48 h The culture was centrifuged (39800×g, 25 min, 4°C), and the supernatant was stored at -20°C During the second incubation period, samples (0 50 mL) were aseptically removed at 12 h intervals and centrifuged (15850×g, 5 min) The supernatants were stored at -20°C and subsequently analyzed by opa-hplc, liquid scintillation counting, and in some cases, thin layer chromatography.

Expt 2 Addition of [2-^{14}C]acetate to L127 mycelium suspended in L138 supernatant

Cultures of *S akiyoshiensis* mutant L127 and L138 were grown as described above (Expt 1) The mutant cultures were centrifuged (39800×g, 25 min, 4°C) after 48 h of growth The mycelium collected from the mutant L127 culture was resuspended in the supernatant obtained from the mutant L138 culture and incubated with shaking at 27°C Sterile sodium [2-^{14}C]acetate (1 00 mL, 168 mmol/L, 0 125 mCi/mmol) was added to the mixture immediately, and also at 12 and 24 h Samples (0 50 mL) removed aseptically before and after each addition were centrifuged (15850×g, 5 min) The supernatants were stored at -20°C and subsequently analyzed by opa-hplc, liquid scintillation counting, and in some cases, thin layer chromatography After 48 h, the culture was centrifuged (39800×g, 25 min, 4°C), and

the supernatant was stored at -20°C

Expt. 3: Addition of [2-^{14}C]acetate to wild-type S. akiyoshiensis

Casein-starch medium (50 mL per 500-mL Erlenmeyer flask) was inoculated with wild-type *S. akiyoshiensis* spore inoculum (1.5% (v/v)) and incubated with orbital shaking at 27°C. Sterile sodium [2-^{14}C]acetate (1.00 mL, 168 mmol/L, 0.125 mC/mmol) was added at 12, 24 and 36 h. Samples (0.50 mL) removed aseptically before and after each addition were centrifuged (15850×g, 5 min). The supernatant was frozen (-20°C) and subsequently analyzed by opa-hplc, liquid scintillation counting, and in some cases, thin layer chromatography. The culture was centrifuged (39800×g, 25 min, 4°C), after 48 h of growth, and the supernatant was stored at -20°C

Analysis by thin layer chromatography

Culture supernatant (50 µL) was spotted in a 3 mm band 1 cm from the bottom of the thin layer chromatography plate (5 × 20 cm, silica gel HLF, 250 µm thickness). After drying for 30 min, the plates were developed in a solvent consisting of butanol-acetic acid-1.5 mol/L $NaHSO_4$ (20:20:20). After air drying in a fumehood overnight, the top and bottom of each plate was marked with radioactive ink and scanned for radioactivity using a Packard Radiochromatogram scanner Model 7201 at a scan rate of 5 cm/hr and a slit width of 5 mm. Bands due to HON gave a characteristic yellow color when sprayed with ninhydrin solution (0.25%(w/v) in acetone) and heated (10 min, 95°C)

Isolation of radiolabelled HON

Thawed culture supernatant was centrifuged (39800×g, 20 min, 4°C) and the resulting supernatant (45 mL) was applied to an Amberlite IR-120 (H^+) ion exchange column (1.5 × 12.2 cm) at a flow rate of 1.0 mL/min. The column was washed with H_2O (250 mL) and eluted with NH_3 (0.3 mol/L) at a flow rate of 2.0 mL/min. Fractions (20 mL) were collected, and those containing HON (numbers 5 - 12) were combined, concentrated *in vacuo* to 24 mL, and applied to a Dowex 1-X8 (^-OAc) column (1.5 × 13.5 cm) at a flow rate of 1.0 mL/min. The sample effluent and water wash fractions containing HON were combined, and authentic unlabelled HON (5.080 mg, 0.03452 mmol) was added as carrier. After concentration *in vacuo* (1 mL), the sample was applied to a Dowex 1-X8 (HSO_3^-) column (1.5 × 26.5 cm) at a flow rate of 0.5 mL/min. The column was washed with H_2O (30 mL) and eluted with 10% aqueous acetone. Fractions (10 mL) containing HON (numbers 2 - 7) were combined, concentrated to about one-half the initial volume, and lyophilized to provide a solid residue (7.2 mg). In Expt. 2, authentic HON (5.288 mg, 0.03594 mmol) was added as carrier and 7.4 mg of HON was obtained and in Expt. 3, authentic HON (4.229 mg, 0.02874 mmol) was added as carrier and 6.9 mg of HON was obtained.

Sodium periodate degradation of HON

The isolated HON (*ca.* 7 mg) was dissolved in H_2O (5.00 mL). A portion (1.50 mL) was removed and diluted to 5.0 mL with H_2O. After the removal of triplicate samples (0.050 mL each), authentic HON (38.579 mg, 0.2622 mmol) was

added as carrier Solid $NaHCO_3$ (205 mg) was added to a concentration of 0 5 mmol/L, and $NaIO_4$ (75 mg) was added with swirling over 1 min After an additional 1 min with occasional swirling, a solution of 5,5-dimethyl-1,3-cyclohexandione (130 mg) in ethanol (1 mL) was added The mixture was warmed to dissolve the resulting solid, cooled to room temperature, and stored at 4°C overnight The resulting white solid was filtered and dried *in vacuo* (26 9 mg) Neutralization of the mother liquor with AcOH (0 5 mol/L) provided a second crop (56 0 mg) which on recrystallization from 50% aqueous ethanol gave white formaldomedone as needle-like crystals (47.1 mg) mp. 188-189°C (lit (129) mp 189-190°C) Triplicate samples (*ca* 1 mg each) of formaldomedone were dissolved in freshly distilled toluene (0.20 mL) and dispersed in liquid scintillation cocktail for radioactivity measurements

The mother liquors were applied to an Amberlite IR-120 (H^+) column (1.5 × 10.0 cm) at 0 6 mL/min The column was washed with H_2O (150 mL) and eluted with NH_3 (0.3 mol/L) Fractions (20 mL) containing aspartic acid (numbers 6 - 13) were combined and concentrated to dryness *in vacuo* The residue was taken up in H_2O (10 mL) and applied to a Dowex 1-X8 (OAc) column (1 5 · 10 cm) at a flow rate 0 5 mL/min After washing with H_2O (100 mL), the column was eluted with AcOH (0.5 mol/L) Fractions (20 mL) containing aspartic acid (numbers 2 - 5) were combined, concentrated, and dried over KOH *in vacuo* to give 29 7 mg Recrystallization from 55% aqueous ethanol gave 24 9 mg of aspartic acid Triplicate samples (*ca* 1 mg each) of aspartic acid were dissolved in H_2O (0 20 mL) containing 1 drop of NaOH (5 mol/L) and dispersed in liquid scintillation cocktail (10 mL) for

radioactivity measurements

Formation of 4-bromophenacyl [2-^{14}C]acetate esters

A mixture of culture supernatant from Expt 1 (48 h, 0 10 mL) and unlabelled NaOAc (26.9 mg, 0.328 mmol) in 0.90 mL of H_2O was adjusted to pH 9 5 with KOH (0.5 mol/L) and lyophilized. The lyophilized powder was suspended in acetonitrile (15 mL) containing 4-bromophenacyl bromide (250 2 mg, 0 90 mmol) and dicyclohexano-18-crown-6 (17.0 mg, 0.045 mmol) The mixture was refluxed for 4 h, cooled to room temperature and filtered The filtrate was concentrated *in vacuo* to give a yellow solid (264 mg). The solid was extracted with CCl_4 (2 × 5 mL), and the extract was flash chromatographed on silica gel (type 60, BDH) using a gradient system consisting of varying proportions of CCl_4 and CH_2Cl_2 starting with pure CCl_4 Fractions (10 mL) were collected and those shown to contain 4-bromophenacyl acetate by tlc (R_f = 0.38, silica gel (Whatman PE Sil G/UV), CH_2Cl_2, uv detection) were combined and concentrated to dryness *in vacuo* to give 75 3 mg of pale yellow solid The solid was dissolved in hot ethanol (3 mL) and treated with charcoal (15 mg) The charcoal was removed by filtration, and the filtrate was diluted at the boiling point with H_2O (3 mL). The white plate-like crystals which formed on cooling were filtered to provide 50.1 mg of 4-bromophenacyl acetate. mp 83 5 - 84 5°C (lit (130) 85-86°C) A second sample of culture supernatant from Expt 1 (72 h, 0 10 mL) was mixed with unlabelled NaOAc (27.4 mg, 0 334 mmol) in 0.90 mL of H_2O and treated in an identical fashion to that described above The reaction mixture filtrate was

concentrated *in vacuo* to give 270 mg of yellow solid Flash chromatography provided 77 6 mg of pale yellow solid which after charcoal treatment and recrystallization gave white plates of 4-bromophenacyl acetate (49 7 mg): mp 83 5 - 84 5°C. Triplicate samples (*ca* 2 mg each) of the 4-bromophenacyl acetate esters were dissolved in freshly distilled toluene (0 20 mL) and dispersed in liquid scintillation cocktail (10 mL) for radioactivity measurements.

Isolation of *N*-acetyl-3,4-dihydroxy-L-phenylalanine (*N*-acetyl-L-dopa)

S akiyoshiensis cultures (96 h) from 14 2-liter Erlenmeyer flasks were centrifuged (23435×g, 20 min), and the supernatant was filtered to remove floating debris The filtrate (3650 ml) was supplemented with NaCl (50 g/L) and acidified (6 mol/L HCl to pH 1.5) The resulting solution was divided into three equal portions, and each was extracted with butanol (2 × 500 mL). The combined butanol extracts were washed sequentially with HCl (0.5 mol/L, 150 mL), H_2O (2 × 300 ml) and K_2CO_3 (0 05 mol/L, 3 × 200 mL). The combined K_2CO_3 extracts were acidified (6 mol/L HCl to pH 1 5), concentrated *in vacuo* (*ca.* 100 mL), and freeze dried. The brown residue (8 09 g) was dissolved in H_2O (60 ml) and centrifuged (1400×g, 5 min) to remove particulate material. The supernatant was acidified (6 mol/L HCl to pH 1.5) and applied to an Amberlite XAD-2 column (2.5 × 48 cm) equilibrated in H_2O. The column was eluted with H_2O, and fractions (20 mL) were collected. Fractions containing metabolite (numbers 56 - 77) were combined and freeze dried. The residue (0.05 g) was dissolved in H_2O (3 mL). The solution was acidified (6 mol/L HCl to

pH 1 5) and applied to a second Amberlite XAD-2 column (1 5 × m) Fractions 20 - 38 (20 mL each) were combined, concentrated *in vacuo* (*ca* 50 mL) and lyophilized. The resulting white hygroscopic solid (16 2 mg) was dissolved in H_2O (1 mL) and applied to a Sephadex G-10 column (1.5 × 87 cm) The column was eluted with H_2O, and fractions 37 - 53 (2.5 mL each) were combined After concentration to 10 mL *in vacuo* and lyophilization, a white hygroscopic solid (12.0 mg), giving a single tlc spot (R_f = 0.36, silica gel, CH_2Cl_2-MeOH-AcOH (4.5:0.5:0.4), detected by I_2). was obtained: $\{\alpha\}_D^{24.5}$ + 67.2 (*c* 1 09, MeOH), uv (H_2O) λ_{max} 198 ε (34 200), 224 (sh, 5360), and 280 nm (2210); ir υ_{max} (KBr) 3342, 3039, 2936, 2624, 1728, 1654, 1609, 1530, 1451, 1381, 1287, 1124, ^{1}H nmr (D_2O, 250 MHz) δ 6.87 (d, J = 8.2 Hz, 1 H), 6.81 (d, J = *ca.* 1.5 Hz, 1H), 6 72 (dd, J 8 2 and *ca.* 1.5 Hz, 1 H), 4.42 (dd, J = 8.7, 4.7 Hz, 1 H), 3.09 (dd, J 14.0, 4.9 Hz, 1 H), 2.82 (dd, J = 13.9, 8.7 Hz, 1 H), 1.96 (s, 3 H); ^{13}C nmr (D_2O, 62.9 MHz) δ 180.88 (s), 176.06 (s), 146.47 (s), 145.29 (s), 133.20 (s), 124.30 (d), 119.60 (d), 118.84 (d), 59 12 (d), 39.61 (t), 24.54 (q); eims *m z* (initial rel. int.) 239 {M$^+$} (10), 222 (2), 221 (4), 181 (6), 180 (54), 124 (11), 123 (100) Thermal decomposition of isolated material and authentic *N*-acetyl-L-dopa occurred in the probe of the mass spectrometer resulting in a decreasing intensities of the molecular ion and the peak at *m z* 180 and an increase in the height of peaks at *m z* 221 and 222 in successive scans.

Synthesis of authentic *N*-acetyl-L-dopa (94, 95)

Acetic anhydride (2.0 mL) was added dropwise over 1 h to a stirred suspension

of l-dopa (0 20 g, 1 0 mmol) in H O (2 5 mL) The mix ure was stirred for an additional hour at room temperature and concentrated *in vacuo* to a light yellow oil The oil was dissolved in H_2O (5 mL), reconcentrated *in vacuo* to remove acetic acid, and chromatographed on an Amberlite XAD-2 column as described above to yield a slightly yellow hygroscopic solid (168 mg, 68%) $\{\alpha\}_D^{22}$ + 45 8 (c 1 10, MeOH) {lit (94) $\{\alpha\}_D$ + 37 7 (c 0 82, MeOH)} After Sephadex G-10 purification as above, a white hygroscopic solid was obtained $\{\alpha\}_D^{19.5}$ + 68 2 (c 1 10, MeOH), uv (H_2O) λ_{max} 198 ε (39 500), 224 (sh, 7390), and 280 nm (2670), ir υ_{max} (cm^{-1}, KBr) 3345, 3035, 2936, 2633, 1727, 1653, 1608, 1528, 1446, 1376, 1287, 1118, ^1H nmr (ppm, D_2O, 250 MHz) δ 6 88 (d, J = 8 2 Hz, 1 H), 6 82 (d, J = ca 1 5 Hz, 1H), 6 73 (dd, J = 8 2 and ca 1 5 Hz, 1 H), 4 41 (dd, J = 8 4, 4 7 Hz, 1 H), 3 10 (dd, J = 14 0, 4 9 Hz, 1 H), 2 82 (dd, J = 13 9, 8 7 Hz, 1 H), 1 97 (s, 3 H), ^{13}C nmr (D_2O, 62 9 MHz) δ 181 08 (s), 176 03 (s), 146 53 (s), 145 33 (s), 133 26 (s), 124 28 (d), 119 60 (d), 118 83 (d), 59 21 (d), 39 68 (t), 24 56 (q), eims *m z* (initial rel int) 239 {M } (8), 222 (2), 221 (3), 181 (6), 180 (52), 124 (11), 123 (100)

Ion-pairing hplc analysis

Separations were obtained on a Beckman Ultrasphere ODS column (5 μm, 4 × 45 mm) column fitted at the inlet with an Upchurch Scientific Model C-130B guard column (2 × 20 mm) containing Perisorb RP-18 (30-40 μm) using a binary gradient (Appendix A) between 2 mmol/L KH_2PO_4 containing 10 mmol/L tetra-*n*-butyl-ammonium hydrogen sulfate (pH adjusted to 5 0 with 5 mol/L NaOH) and a mixture

of 4 mmol/L KH_2PO_4 containing 20 mmol/L tetra-*n*-butylammonium hydrogen sulfate (pH adjusted to 5.0 with 5 mol/L NaOH) and methanol (1 1) KH_2PO_4/tetra-*n*-butyl-ammonium hydrogen sulfate solutions were filtered (0.45 µm) and degassed under reduced pressure (water aspirator) prior to use. The absorbance of the column effluent was monitored at 260 nm or simultaneously at 214 and 280 nm (4 nm bandwidth) referenced against 500 nm (80 nm bandwidth)

L-Dopa, L-tyrosine, *N*-acetyl-L-dopa and *N*-acetyl-L-tyrosine concentrations were estimated using standard curves constructed from triplicate injections of synthetic *N*-acetyl-L-dopa and commercial L-dopa, L-tyrosine and *N*-acetyl-L-tyrosine over the range of 0.022-4.3 mmol/L.

Supplementation of liquid cultures with possible *N*-acetyl-L-dopa precursors

Triplicate sets of flasks containing sterile casein-starch medium (50 mL per 250 mL Erlenmeyer flask) were prepared. Supplement (L-phenylalanine, L-tyrosine, L-dopa, *N*-acetyl-L-phenylalanine and *N*-acetyl-L-tyrosine, 0.90 mmol each) was prepared by dissolving the compound in sterile water (25 mL) in a sterile volumetric flask. L-Dopa and L-tyrosine were only partially soluble (*ca* 80% and 50%, respectively) and were used as suspensions. The supplement was added (8.0 mL per flask) to cooled flasks using a sterile syringe. After mixing, each flask was inoculated with 1% (v/v) vegetative culture (72 h). Flasks were incubated at 27°C and 220 rpm on a rotary shaker. Samples (0.50 mL) were removed aseptically, centrifuged (15850×g, 5 min), and the supernatant was stored (-20°C). Equal portions (100 µL)

of each supernatant were combined and analyzed by ion-pairing hplc

Resuspension experiments

Mutant L138 cultures (46 h) from five 2-L Erlenmeyer flasks were centrifuged (23435×g, 15 min). The mycelium from each flask was resuspended in phosphate buffer (200 mL, 20 mmol/L, pH 6.5) and centrifuged as above. After two additional washes, 46.6 g of moist mycelium was obtained. The moist mycelium (0.78 g) was transferred to 125 mL Erlenmeyer flasks containing phosphate buffer (25 mL, 20 mmol/L, pH 6.5) and possible precursors (2 mmol/L). In addition, components such as glucose (100 or 200 mmol/L), $FeSO_4$ (20 mmol/L) or ascorbic acid (5.7 mmol/L) were added to some flasks. Control flasks contained mycelium suspended in phosphate buffer (25 mL, 20 mmol/L, pH 6.5) or phosphate buffer containing glucose (100 mmol/L). Flasks were incubated at 27°C and 220 rpm on a rotary shaker. Samples (0.50 mL) were removed aseptically, centrifuged (15850×g, 5 min), and the supernatants were stored (-20°C) prior to analysis by ion-pairing hplc.

Detection of enzyme activities

Acetyltransferase

Casein-starch medium (2 × 300 mL per 2-L Erlenmeyer flasks) was inoculated with mutant L138 seed culture (3.0 mL) and incubated at 27°C and 220 rpm. After 26.5-h growth, the cultures were centrifuged (16274×g, 15 min, 4°C). The mycelium was resuspended in phosphate buffer (150 mL, 20 mmol/L, pH 6.5) and recentrifuged

(16274×g, 15 min, 4°C) The washed mycelia (8 29 g) were immediately frozen at -20°C To disrupt cells, frozen mycelia were thawed in ice cold MOPS buffer (10 ml , 100 mmol/L, pH 7 3) containing toluenesulfonyl fluoride (3 mmol/L) and β-mercaptoethanol (6 mmol/L) and sonicated with a Branson Sonifier (model 210) for 6 × 10 s continuous bursts (power 8), with cooling (1 min) in a $CaCl_2$-ice bath between bursts The homogenate was centrifuged (47807×g, 20 min, 4°C), and the resulting supernatant (12 mL) was used as a crude extract Desalted extracts were prepared by chromatographing crude extract (2 5 mL) on a Sephadex G-25 column (1 5 × 14 cm) and eluting with MOPS buffer Fractions (2 5 mL) containing protein (numbers 4 - 6) were combined and used in enzyme assays

Equal volumes (150 µL) of 2 mmol/L acetyl CoA and 2 mmol/L l -dopa were mixed and allowed to stand at room temperature for 5 min Acetylation was initiated by the addition of either crude or desalted extract (300 µL) A sample (100 µL) was removed immediately, heated in a boilinig water bath (2 min) and then cooled in an ice bath (5 min). After centrifugation (15859×g, 5 min), the supernatant was analyzed by ion-pairing hplc. The assay mixture was incubated at 30°C and samples (100 µL) were removed after 15 min and 1 h, and treated as discussed above

Tyrosinase

Tyrosinase activity was determined spectrophotometrically according to a literature procedure (99) A solution of L-dopa (1 mL, 5 mmol/L) in phosphate buffer (20 mmol/L, pH 6 5) was mixed with 100 µL of culture supernatant and the formation

of dopachrome was detected as a change in absorbance (475 nm) The rate of absorbance change was determined from the slope of the linear portion of the plot.

SUMMARY AND FUTURE WORK

These investigations strongly suggest that the cosynthetic and cross-feeding behaviour exhibited by the HON-nonproducing mutants is not due to the accumulation of HON biosynthetic intermediates but to an initiator that is capable of transforming the nonproducing mutants into HON-producing strains To provide further evidence for this "initiator" hypothesis, the metabolite should be isolated from culture broths of mutant L138 and the wild-type strain and its structure determined Charcoal chromatography and butanol extraction would be the most suitable initial purification steps. The nature of the initiation effect could also be proved by examining the effect of known autoregulatory substances, such as cosynthetic factor 1, on the HON-nonproducing mutants.

No evidence was provided in this investigation to indicate whether the block in the third group of mutants (*e.g.*, L138) was related to those in mutants L167 and L127. An investigation of the effect of wild-type supernatant on HON production by the mutants of the third group (*e.g.*, L138) would elucidate the relationship

The investigation of *N*-acetyl-L-dopa biosynthesis indicated that L-tyrosine and L-dopa were precursors, and the efficient acetylation of L-dopa, but not L-tyrosine, was detected in crude cell-free extracts supplemented with acetyl CoA Further analysis of the substrate specificity of a purified acetyltransferase should be undertaken Also, the observed production of *N*-acetyl-L-dopa in cultures supplemented with *N*-acetyl-L-tyrosine requires a closer examination to determine the involvement of the tyrosinase observed in the supernatant of mutant L138.

APPENDIX A

Table A1 Gradient 1 Used for Separations by Opa-HPLC[a]

Time (min)	Solvent A[b] (%)	Methanol (%)
0.00	100	0
0.50	90	10
1.50	90	10
2.00	55	45
3.00	55	45
3.50	0	100
4.00	0	100
4.50	100	0
5.00	100	0

[a] Total flow rate was 2.5 mL/min.

[b] Solvent A consisted NaOAc (0.1 mol/L)-Methanol-THF (900:95:5)

Table A2 Gradient 2 Used for Preparative HPLC[a]

Time (min)	Water (%)	Methanol (%)
0.00	100	0
3.00	100	0
5.00	80	20
7.00	0	100
8.00	0	100
9.00	100	0
10.00	100	0

[a] Total flow rate was 2.0 mL/min

Table A3a Gradient 3a Used for Separations by Ion-Pairing HPLC[a]

Time (min)	Solvent A[b] (%)	Solvent B[c] (%)
0 00	100	0
1 00	100	0
3 00	75	25
4 00	30	70
5 00	0	100
6 00	100	0
7 00	100	0

[a] Total flow rate was 2.0 mL/min.

[b] Solvent A consisted of K_2HPO_4 (2 mM) and tetrabutylammonium hydrogen sulfate (10 mM) adjusted to pH 5.0 with 1-M KOH.

[c] Solvent B consisted of K_2HPO_4 (2 mM) and tetrabutylammonium hydrogen sulfate (10 mM) in 50% methanol (v/v) adjusted to pH 5 0 with 1-M KOH

Table A3b. Gradient 3b Used for Separations by Ion-Pairing HPLC[a]

Time (min)	Solvent A[b] (%)	Solvent B[c] (%)
0.00	100	0
1.00	100	0
2.50	80	20
4.00	0	100
5.00	0	100
6.00	100	0
7.00	100	0

[a] Total flow rate was 2.0 mL/min.

[b] Solvent A consisted of K_2HPO_4 (2 mM) and tetrabutylammonium hydrogen sulfate (10 mM) adjusted to pH 5.0 with 1-M KOH.

[c] Solvent B consisted of K_2HPO_4 (2 mM) and tetrabutylammonium hydrogen sulfate (10 mM) in 50% methanol (v/v) adjusted to pH 5.0 with 1-M KOH.

REFERENCES

1 S Shapiro *In* Regulation of Secondary Metabolism in Actinomycetes. *Edited by* S Shapiro CRC Press, Inc., Boca Raton. 1989. Preface.

2 J A Robinson Chem. Soc. Rev **17**, 383 (1988).

3. S Tatsuoka, A. Miyake, H. Hitomi, J. Ueyanagi, H. Iwasaki, T. Yamaguchi, K Kanazawa, T. Araki, K. Tsuchiya, F. Hiraiwa, K. Nakazawa, and M. Shibata J. Antibiotics. **14**, 39 (1961).

4 S Hunt. *In* Chemistry and Biochemistry of the Amino Acids. *Edited by* G.C. Barrett. Chapman and Hall, London. 1985. pp. 55-138.

5 A Miyake Chem. Pharm. Bull. **8**, 1071 (1960).

6 A Miyake. Chem Pharm. Bull. **8**, 1074 (1960).

7 G Barry and E. Roark. J. Biol. Chem. **239**, 1541 (1964).

8 Y Tsuda and H C Friedmann. J Biol Chem. **245**, 5914 (1970).

9 D Perlman, K.L. Perlman, M. Bodanszky, A. Bodanszky, R.L. Foltz, and H.W. Matthews Bioorganic Chemistry. **6**, 263 (1977).

10 K Kanazawa, K. Tsuchiya, and T. Araki. American Review of Respiratory Diseases **81**, 924 (1960).

11 A Miyake. Chem. Pharm. Bull. **8**, 1079 (1960).

12. S. Watanabe, K Numata, S. Omura, and H. Yamaguchi. Japan. Kokai Tokkyo Koho 61 243 018 [86 243 018] (1986); Chem. Abstr. **106**, 72944*r* (1987).

13. H Yamaguchi, K Uchida T. Hiratani, T. Nagate, N. Watanabe, and S. Omura. Ann N Y Acad. Sci **544**, 188 (1988)

14 Y Le Ph D. Thesis, Dalhousie University, Halifax, 1994.

15 Y Nishiyama, Y Asagi, T Hiratani, M. Yamaguchi, H. Yamaguchi, and H. Yamaki Journal of Electron Microscopy. **39**, 281 (1990).

16 H Yamaki, M. Yamaguchi, T. Nishimura, T. Shinoda, and H. Yamaguchi. Drugs Exptl. Clin Res. **14**, 467 (1988).

17 H Yamaki, M Yamaguchi, H Imamura, H Suzuki, T Nishimura, H Saito, and H Yamaguchi Biochemical and Biophysical Research Communications **168**, 837 (1990)

18 M Yamaguchi, H Yamaki, T Shinoda, Y Tago, H Suzuki, T Nishimura, and H Yamaguchi J Antibiotics **43**, 411 (1990)

19 H Yamaki, M Yamaguchi, and H Yamaguchi Recent Prog Antifungal Chemother 403 (1992)

20 R L White, A C DeMarco, and K C Smith J Chromatogr **483**, 437 (1989)

21 R L White, A C DeMarco, and K C Smith J Am Chem Soc **110**, 8228 (1988)

22 R L White, K C Smith, and A C DeMarco Can J Chem **72**, 1645 (1994)

23 C Walsh Enzymatic Reaction Mechanisms W H Freeman and Company, San Fransico 1979 Chapters 5,7, and 8

24 Y Le, L C Vining, K C Smith, and R L White *In* Proceedings of the First International Workshop on Antibiotics, Havana, Cuba, November 1-4, 1993

25 Y Le, K C Smith, L C Vining, and R L White J Antibiotics (1995) *submitted July 24*

26 R L White and K C Smith Unpublished results, Acadia University, 1989-1990

27 J Williamson, E Inamine, K E Wilson, A W Douglas. J M Liesch, and G Albers-Schonberg J Biol Chem **260**, 4637 (1985)

28 W Leanza, K Wildonger, J Hannah, D Shih, R Ratcliffe, L Barash, E Walton, R Firestone, G Patel, F Kahan, J Kahan, and B Christensen *In* Recent Advances in the Chemistry of beta-Lactam Antibiotics *Edited by* G I Gregory The Royal Society of Chemistry, London 1981 pp 240-254

29 R J Parry and J V Mueller J Am Chem Soc **106**, 5764 (1984)

30 D D Weller and J K L Rinehart J Am Chem Soc **100**, 6757 (1978)

31 D G I Kingston, M X Kolpak, J W LeFevre, and I Borup-Grochtmann J Am Chem Soc **105**, 5106 (1983)

32 H Morishima, T Sawa, T Takita, T. Aoyagi, T. Takeuchi, and H Umezawa. J Antibiotics **27**, 267 (1974)

33 H von Dohren *In* Genetics and Biochemistry of Antibiotic Production. *Edited by* L C Vining and C Stuttard Butterworth-Heinemann, Boston. 1995. pp. 129-171

34 P M Hardy *In* Chemistry and Biochemistry of the Amino Acids. *Edited by* G C Barrett. Chapman and Hall, London. 1985. pp. 6-24.

35 E Umbarger and B.D. Davis. *In* The Bacteria. Vol. 3. *Edited by* I.C. Gunsalus and R Y Stanier Academic Press, New York. 1962. pp. 167-251.

36 E Umbarger Annu Rev Biochem **47**, 533 (1978)

37 S A Brown. *In* Biosynthesis Vol 1. *Edited by* T A. Geissman. The Chemical Society, London. 1972 pp 1-40.

38 C R Hutchinson Nat Prod Reports. **3**, 133 (1986)

39 E A Adelberg. Bacteriol Rev. **17**, 253 (1953).

40 E Bayer, K Gugel, K. Hagele, H. Hagenmaier, S. Jessipow, W. Konich, and H Zaher Helv Chim Acta **55**, 224 (1972)

41 S Omura, K Hinotozawa, N Imamura, and M Murata. J. Antibiotics. **37**, 939 (1984)

42 H Kato, K Nagayama, H Abe, R Kobayashi, and E Ishihara. Agric. Biol. Chem **55**, 1133 (1991)

43 A Raibaud, M Zalacain, T G. Holt, R Tizard, and C.J. Thompson. J. Bacteriol **173**, 4454 (1991)

44 O Hara, T Murakami, S. Imai, H. Anzai, R. Itoh, Y. Kumada, F Takano, E. Satoh, A Satoh, K Nagaoka, and C. Thompson J. Gen. Microbiol. **137**, 351 (1991)

45 M Horiguchi, J S Kittredge, and E Roberts Biochem. Biophys. Acta **165**, 164 (1968).

46 T O Rogers and J Birnbaum Antimicrob. Agents Chemother. **5**, 121 (1974).

47 A Satoh, H. Ogawa, and Y. Satomura Agric. Biol Chem. **39**, 2331 (1975).

48 C J Thompson and H Seto *In* Genetics and Biochemistry of Antibiotic Production *Edited by* L C Vining and C Stuttard Butterworth-Heinemann, Boston 1995 pp 197-222

49 H Seto, S Imai, T Sasaki, K W Shimotohno, T Tsuruoka, H Ogawa, A Satoh, S Inouye, T Niida, and N Otake J Antibiotics **37**, 1509 (1984)

50 K Shimotohno, H Seto, N Otake, S Imai, and A Satoh J Antibiotics **39**, 1356 (1986)

51 K W Shimotohno, H Seto, and N Otake J Antibiotics **41**, 1057 (1988)

52 K W Shimotohno, S Imai, T Murakami, and H Seto Agric Biol Chem **54**, 463 (1990)

53 K Suzukake, H Hayashi, M Hori, and H Umezawa J Antibiotics **33**, 857 (1980)

54 E Cundliffe Annu Rev Microbiol **43**, 207 (1989)

55 T Kobayashi, T Uozumi, and T Beppu J Antibiotics **39**, 688 (1986)

56 J A Perez-Gonzalez, J Vara, and A Jimenez Biochemical and Biophysical Research Communications **113**, 772 (1983)

57 Soon-Y Paik, M Sugiyama, and R Nomi J Antibiotics **38**, 1761 (1985)

58 C J Thompson, J M Ward, and D A Hopwood Nature **286**, 525 (1980)

59 M Sugiyama, A Takeda, Soon-Y Paik, O Nimi, and R Nomi J Antibiotics **39**, 827 (1986)

60 H Tschape, E Tietze, R Prager, W Voigt, E Wolter, and G Seltmann Plasmid **12**, 189 (1984)

61 W V Shaw and R F Brodsky J Bacteriol **95**, 28 (1968)

62 W V Shaw Bioch Soc Trans **2**, 834 (1974)

63 W V Shaw and D A Hopwood J Gen Microbiol **94**, 159 (1976)

64 G Sundharadas and C Gilvarg J Biol Chem **242**, 3983 (1967)

65 G Zubay Biochemistry Addison-Wesley Publishing, Reading 1983 p 835

66. S. Imai, H. Seto, T. Sasaki, T. Tsuruoka, H. Ogawa, A. Satoh, S. Inouye, T. Niida, and N. Otake. J. Antibiotics. **38**, 687 (1985).

67. O. Hara, H. Anzai, S. Imai, Y. Kumada, T. Murakami, R. Itoh, E. Takano, A. Satoh, and K. Nagaoka. J. Antibiotics. **41**, 538 (1988).

68. H. Anzai, T. Murakami, S. Imai, A. Satoh, K. Nagaoka, and C.J. Thompson. J. Bacteriol. **169**, 3482 (1987).

69. H. Seto, S. Imai, T. Tsuruoka, A. Satoh, M. Kojima, S. Inouye, T. Sasaki, and N. Otake. J. Antibiotics. **35**, 1719 (1982).

70. H. Seto, T. Sasaki, S. Imai, T. Tsuruoka, H. Ogawa, A. Satoh, S. Inouye, T. Niida, and N. Otake. J. Antibiotics. **36**, 96 (1983).

71. K. Kamigiri, T. Hidaka, S. Imai, T. Murakami, and H. Seto. J. Antibiotics. **45**, 781 (1992).

72. A.N. Glazer and H. Nikaido. Microbial Biotechnology. W.H. Freeman and Company, New York. 1995. pp. 491-498.

73. J.L. Doull and L.C. Vining. *In* Genetics and Biochemistry of Antibiotic Production. *Edited by* L.C. Vining and C. Stuttard. Butterworth-Heinemann, Boston. 1995. pp. 9-63.

74. S. Horinouchi and T. Beppu. *In* Genetics and Biochemistry of Antibiotic Production. *Edited by* L.C. Vining and C. Stuttard. Butterworth-Heinemann, Boston. 1995. pp. 103-119.

75. Y. Yamada, K. Sugamura, K. Kondo, M. Yanagimoto, and H. Okada. J. Antibiotics. **40**, 496 (1987).

76. O. Hara and T. Beppu. J. Antibiotics. **35**, 349 (1982).

77. U. Grafe. *In* Regulation of Secondary Metabolism in Actinomycetes. *Edited by* S. Shapiro. CRC Press, Inc., Boca Raton. 1989. pp. 75-126.

78. S. Horinouchi, Y. Kumada, and T. Beppu. J. Bacteriol. **158**, 481 (1984).

79. S. Horinouchi, O. Hara, and T. Beppu. J. Bacteriol. **155**, 1238 (1983).

80. W. Piepersberg. *In* Genetics and Biochemistry of Antibiotic Production. *Edited by* L.C. Vining and C. Stuttard. Butterworth-Leinemann, Boston. 1995. pp. 531-570.

81 K Miyake, S Horinouchi, M. Yoshida, N. Chiba, K Mori, N Morikawa and T Beppu J. Bacteriol **171**, 4298 (1989)

82. P.A. McCann and B M. Pogell J Antibiotics **32**, 673 (1979)

83. T. Kawaguchi, T. Asahi, T. Satoh, T Uozumi, and T Beppu J Antibiotics **37**, 1587 (1984)

84. S. Kondo, M Katayama, and S Marumo J Antibiotics **39**, 727 (1986)

85. J R D McCormick and G.O. Morton J Am Chem Soc **104**, 4014 (1982)

86. L D Eirich, G.D. Vogels, and R S Wolfe. Biochemistry **17**, 4583 (1978)

87. T Buchan, C Roach, C Ruby, D. Taylor, C Preisig, and C Reeves J Antibiotics **47**, 992 (1994).

88. M A Glazebrook, L C Vining, R L. White, K C. Smith, and E G Chedrawy Can J Microbiol. **39**, 536 (1993).

89 M.A. Glazebrook, L C. Vining, and R L. White Can J. Microbiol **38**, 98 (1992).

90 D A Forsyth *In* Isotopes in Organic Chemistry Isotopic Effects Vol 6 *Edited by* E. Buncel and C C Lee Elsevier Science Publishers B V, Amsterdam 1984 pp. 24-25.

91. T S. Chen, B.H. Arison, C.L Ruby, A W. Dombrowski, and E S Inamine J Ind. Microbiol. **12**, 66 (1993)

92 G C Barrett and J S Davies *In* Chemistry and Biochemistry of the Amino Acids. *Edited by* G C. Barrett. Chapman and Hall, London 1985 pp 525-544

93. R.A.W. Johnston and M E. Rose. *In* Chemistry and Biochemistry of the Amino Acids *Edited by* G C Barrett Chapman and Hall, London 1985 pp 487-488

94 G Prota, G Scherilo, and R A Nicolaus Gazz Chim Ital **98**, 495 (1968)

95. S Inoue, S. Ito, Y. Imai, T Kasuga, and K Fujita Biochem Pharmacol **36**, 3537 (1987)

96 L C Vining. *In* Genetics and Biochemistry of Antibiotic Production *Edited by* L.C. Vining and C. Stuttard Butterworth-Heinemann, Boston 1995 pp 1-8

97 S Shapiro *In* Regulation of Secondary Metabolism in Actinomycetes *Edited by* S Shapiro CRC Press, Inc , Boca Raton 1989 p 167

98 R Baumann, L Ettinger, R Hutter, and H P Kocher *In* Actinomycetes The Boundary Organisms *Edited by* T Arai Toppan Co., Tokyo 1976 pp 55-63.

99 K Lerch and L Ettlinger Eur J Biochem **31**, 427 (1972)

100 R Cramer, L Ettlinger, R Hutter, K Lerch, M A Suuter, and J A Vetterli. J Gen Microbiol **128**, 371 (1982)

101 T Held and H J Kutzner J Gen Microbiol **136**, 2413 (1990).

102 T Yoshimoto, K Yamamoto, and D Tsuru J Biochem **97**, 1747 (1985)

103 V Bernan, D Filpula, W Herber, M Bibb, and E Katz Gene **37**, 101 (1985)

104 H Nakano and T Suzuki Japan Kokai 77 08,820 (1977), Chem Abstr **88**, 62608e (1977)

105 K Nakayama, H Yoshida, and Yoshitake Japan Kokai 74 12,092 (1974); Chem Abstr **80**, 146546f (1974)

106 S Watanabe, N Kitajima, and I Takeda Japan Kokai 73 19,794 (1973); Chem Abstr **78**, 157879b (1973)

107 I Chitbata, T Kakimoto, K Nabe, and T Shibatani Japan Kokai 72 39,692 (1972), Chem Abstr **78**, 111753c (1973)

108 M Sugiyama, Soon-Y. Paik, and R Nomi J. Gen Microbiol. **131**, 1999 (1985)

109 A S Khokhlov Microbial Autoregulators Harwood Academic Publishers, Reading (Translated from Russian by E M Suscenko) 1991 pp 43-93

110 A L Demain *In* Regulation of Secondary Metabolism in Actinomycetes *Edited by* S Shapiro CRC Press, Inc , Boca Raton 1989 pp 127-134

111 K C Smith, R L White, Y Le, and L C. Vining J. Nat. Products (1995) *in press*

112 C A Smith and E J Wood Molecular and Cell Biochemistry Biosythesis Chapman and Hall, London 1992 p 134.

113. M S T Kuo, D Yurek, J H Coats, and G P Li J Antibiotics **42**, 475 (1989)

114. N.M. Brahme, J.E. Gonzalez, J P. Rolls, E J Hessler, S Mizsak, and L H Hurley. J Am Chem. Soc. **106**, 7873 (1984)

115. J.H. Coats, G P. Li, M.S T. Kuo, and D Yurek J. Antibiotics **42**, 472 (1989)

116. I. Chibata, T. Kakimoto, K Nabe, and T Shibatani. Japan Kokai 72 39,692 (1972); Chem. Abstr **78**, 111753c (1973).

117. K. Nakayama, H. Yoshida, and Y. Tanaka. Japan Kokai 74 12,092 (1974), Chem. Abstr. **80**, 146546f (1974).

118. N Mukai, H Tanaka, and H Tanaka Japan Kohai 76 19,035 (1976), Chem Abstr. **86**, 60514f (1977)

119. G. Zubay. Biochemistry. Addison-Wesley Publishing, Reading 1983 p 1161

120. T Keiser, L. Ettlinger, and R Hutter. *In* Genetics of Actinomycetales *Edited by* E Freerksen, I Tarnok, and J H. Thumin Stuttgart, New York 1979 pp 59-60

121. M.H. Zenk and J.H. Schmitt Biochem. Z. **342**, 54 (1965).

122. F.B Goldstein J Biol. Chem. **234**, 2702 (1959).

123. K. Suzukake, M. Hori, H Hiyashi, and H Umezawa *In* Peptide Antibiotics Biosynthesis and Functions. *Edited by* H. Kleinkauf and H von Dohren De Gruyter, Berlin. 1982. pp. 325-336

124. P.L. Bartel, N C Conners, and W R Strohl J Gen Microbiol **136**, 1877 (1990).

125 D A Hopwood and D H Sherman Annu Rev Genet **24**, 37 (1990)

126. L C. Vining and H. von Dohren. *In* Genetics and Biochemistry of Antibiotic Production *Edited by* L C Vining and C. Stuttard Butterworth-Heinemann, Boston. 1995. pp. 121-127

127. D.T. Davis. B Sc. Thesis, Acadia University, Wolfville, 1991

128. Q. Chen, N Klemm, and I. Jeng Anal Biochem **182**, 54 (1989)

129. R.E Reeves. J. Am. Chem Soc. **63**, 1476 (1941).

130 J.L.E. Erickson, J.M. Dechary, and M.R. Kesling. J. Am. Chem. Soc. **73**, 5301 (1951).