The biosynthesis of plant alkaloids and nitrogenous microbial metabolites†

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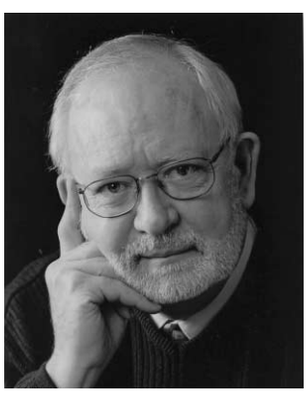
The biosynthesis of plant alkaloids and related nitrogenous microbial secondary metabolites is reviewed. This involves discussion of the outcome of studies with isotopic labels and of genetic and enzymic experiments. The review follows on from a similar, earlier account [*Nat. Prod. Rep.*, 2001, **18**, 50-65], covers the literature for the calendar years 1999 and 2000, and contains 143 references.

† This review and those that preceded it are dedicated to my three teachers who were each a marvellous scientific inspiration: F. G. Holliman, A. R. Battersby and G. Stork. They were the giants onto whose shoulders I was privileged to climb.

Richard Herbert was Reader in Bio-organic Chemistry, School of Chemistry, University of Leeds until formal retirement in 2002; BSc (Hons), Cape Town; PhD and DSc, Leeds. He is the author of The Biosynthesis of Secondary Metabolites, founder, with Tom Simpson, of The Bio-organic Subject Group of The Royal Society of Chemistry, and a member of the Editorial Board of Natural Product Reports from the journal's inception in 1984 until 2001. Research: biosynthesis of nitrogenous metabolites including alkaloids; most recently the application of isotopic labelling and NMR in solving 3D structures of membrane transport proteins and their mechanism of action, in ongoing collaboration with Peter Henderson and others in Leeds, also Oxford, Manchester, and Denmark. He is married to Margaret with two children and four boisterous grandchildren.



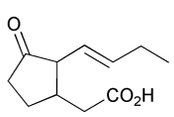
Richard Herbert

1 Introduction

The format of this report is as previously. The discussion includes background references.[1–8] CAS Online was used extensively for literature coverage. In a departure from previous practice, the review covers two calendar years.

Extensive, up-to-date information on the biosynthesis of secondary metabolites has been published in a monograph.[9] The production of alkaloids, which includes biosynthesis, has been reviewed.[10]

Jasmonic acid **1** is an important compound in the elicitation of secondary metabolite biosynthesis in higher plants.[7] A jasmonate-responsive transcriptional factor that leads to increased production of terpenoid indole alkaloids has been isolated.[11]



1

The rôle of amine oxidases in alkaloid biosynthesis has been reviewed[12] as has that of thioesterases in rifamycin bio-

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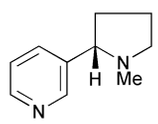
synthesis.[13] Amino-acid decarboxylases can have important rôles in the biosynthesis of alkaloids, *e.g.* terpenoid indoles and benzylisoquinolines. The decarboxylases involved have been the subject of review.[14]

2 Pyrrolidine and piperidine alkaloids

2.1 Nicotine and cocaine

Putrescine *N*-methyl transferase catalyses the first committed step in the biosynthesis of nicotine **2**. The methyl jasmonate-induction of the transferase genes has been studied in the roots of *Nicotania sylvestris*.[15] Ethylene suppresses jasmonate-induced gene expression in nicotine biosynthesis.[16] Differential induction by methyl jasmonate of genes encoding ornithine decarboxylase and other enzymes of nicotine biosynthesis has been studied in tobacco cell cultures.[17] Jasmonate-induced responses of *N. sylvestris* result in fitness costs due to impaired competitive ability for nitrogen. It was concluded that inducibility functions to minimise these costs.[18] ^{15}N NMR has been used *in vivo* to probe agropine synthesis in transformed root cultures of *N. tabacum*.[19]



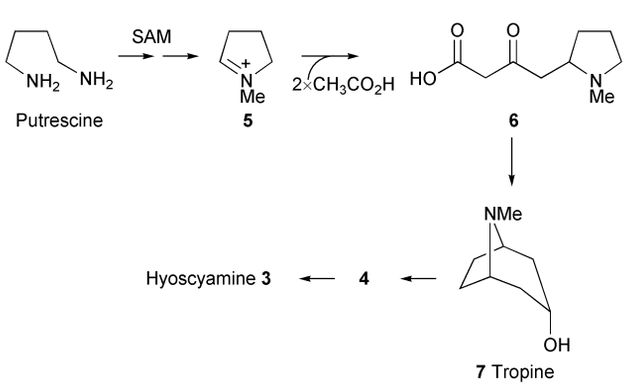
2 Nicotine

The biosynthesis of alkaloids in tobacco has been reviewed.[20] Clues to the evolutionary origin of cultivated tobacco have been provided by examining the structure and expression of the gene family encoding putrescine *N*-methyl transferase.[21] Labelled nicotine metabolites have been obtained using rabbit homogenates.[22] Stereochemistry associated with cocaine biosynthesis from *N*-methyl putrescine has been reported.[23]

2.2 Tropane alkaloids

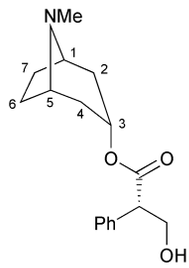
The biosynthesis of tropane alkaloids (ref. 7) has been reviewed. The focus of the first review[24] is the formation of the tropic acid moiety in, *e.g.* hyoscyamine **3**. In the second account a new proposal is made for the mechanism of assembly of the acetate-derived C_3 unit.[25]

Tropane alkaloids are biosynthesised as outlined in Scheme 1 from putrescine and acetate. The pyrrolidine **6** is firmly identified as a biosynthetic intermediate (against simple mechanistic prediction) (ref. 7, p. 50). Uncertainty surrounds the formation of **6** and the way the acetate units are used. From careful new experiments[26] in root cultures of *Datura stramonium* with ^{2}H/^{13}C/^{18}O-labelled acetates it was apparent that, whilst C-1 and C-2 of acetate labelled C-2/C-3/C-4, and more heavily than C-1/C-5/C-6/C-7, deuterium from [$^2H_3$]acetate was completely lost from littorine **4** and hyoscyamine **3**; a similar result was obtained for [$^{18}O_2$] acetate. However, up to two deuterium

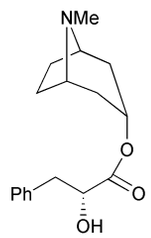


Scheme 1

atoms were incorporated, by way of acetate-to-putrescine metabolism, into each of C-6 and C-7 (the labelling of both atoms arises from the symmetry of putrescine). The deuterium results differ from earlier ones (ref. 3, p. 446).



3 Hyoscyamine



4 Littorine

Unexpectedly, the biosynthesis of the tropic acid moiety in hyoscyamine **3** evolves from littorine **4**, and this is by a direct intramolecular rearrangement on **4** (ref. 7, p. 51; ref. 6, p. 199). This has been clearly demonstrated to be an intramolecular rearrangement in comparative incorporations into **3** with [2′-^{13}C, 3-^{2}H] littorine, as **4**, and [3-^{2}H] tropine, as **7**, plus [2′-^{13}C] phenyllactate.[26]

Littorine **4** that was added to *D. stramonium* cultures has been found to be efficiently converted into hyoscyamine **3**, and in such a way as to support a direct precursor–product relationship between **4** and **3**. Evidence has also been obtained of P-450 activity[27] (ref. 7, p. 51).

N-methylation of putrescine is the first committed step in tropane alkaloid biosynthesis (Scheme 1). The cDNAs encoding the *N*-methyltransferase responsible have been isolated[28] from *Atropa belladonna* and *Hyocyamus niger*. The effect of methyl jasmonate has been examined and the regulation of tropane alkaloid biosynthesis has been discussed and compared with that of nicotine biosynthesis.

Elicitation of alkaloid production has been studied independently.[29] It appears that increased alkaloid production, which occurs as a result of methyl jasmonate treatment in hairy root cultures of *A. belladonna*, is due to the differential enhancement of the biosynthesis of tropine **7**.

In the biosynthesis of tropane alkaloids, two tropinone reductases catalyse the reduction of tropinone to two diastereoisomeric alcohols, namely tropine **7** and pseudotropine. The structures and expression patterns of the two enzymes have been studied in *H. niger*.[30] Insight into the molecular evolution of the two reductases has been obtained.[31] The substrate tolerances of *N*-methylputrescine oxidase and of other enzymes of tropane alkaloid biosynthesis have been examined.[32]

The effects of the rolC gene on induction and development of alkaloid production have been studied[33] in hairy root cultures of *D. stramonium*.

2.3 Pyrrolizidine alkaloids

The first pathway-specific intermediate in the biosynthesis of pyrrolizidine alkaloids, *e.g.* senecionine *N*-oxide **10**, is homospermidine **9**, which is synthesised from two molecules of putrescine in a pivotal metabolic reaction catalysed by the enzyme homospermidine synthase (HSS) (ref. 7, p. 52; ref. 4, p. 46). In really important work, the HSS from *Senecio vernalis*, a typical pyrrolizidine alkaloid-producing plant, has been purified to apparent homogeneity, has been subject to microsequencing and the cDNA has been cloned.[34] Sequence comparison provided direct evidence for the evolutionary recruitment of an essential gene of primary metabolism, namely deoxyhypusine synthase (DHS), for this first committed step with HSS in the biosynthesis of pyrrolizidine alkaloids.[34] DHS and HSS have strikingly similar biochemistry: *inter alia* they catalyse similar reactions (Fig. 1A and B).[34,35] The cDNA of HSS from *S. vulgaris* has been cloned and expressed.[36] The

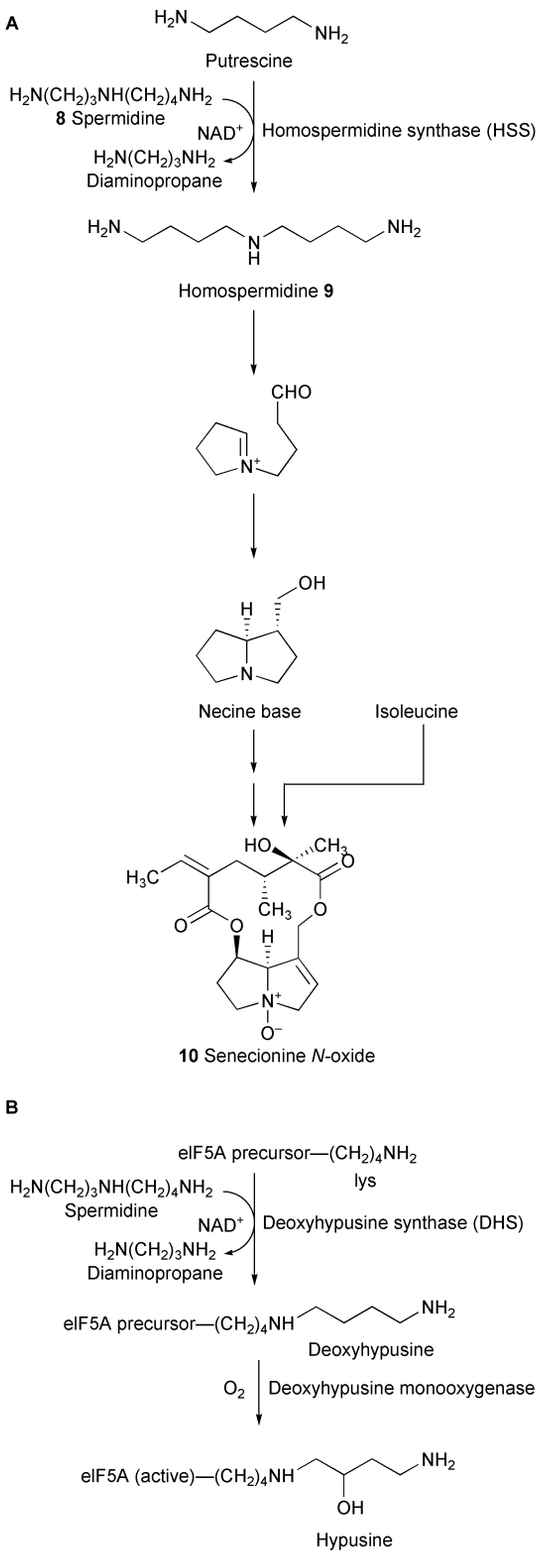


Fig. 1 Reactions catalysed by HSS **(A)** and DHS **(B)**. DHS catalyses the first step in the activation of the eukaryotic translation initiation factor 5A (eIF5a), which is essential for eukaryotic call proliferation and which acts as a cofactor for the HIV-1 Rev regulatory protein.

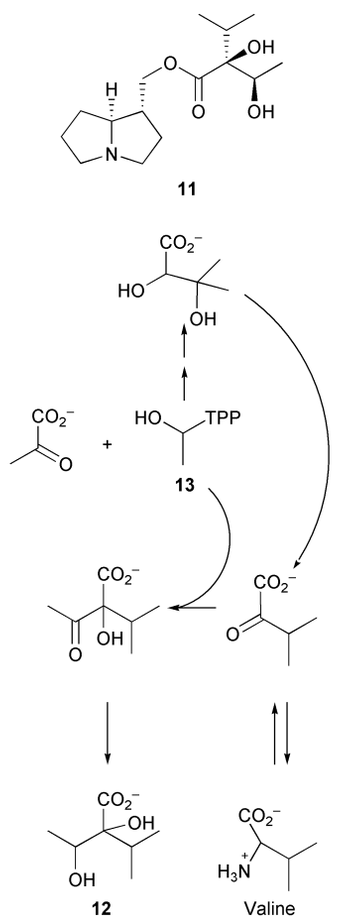
significance of the foregoing work in relation to the evolution of secondary metabolism from primary metabolism appears considerable.

The biosynthesis and metabolism of pyrrolizidine alkaloids in plants and specialised insect herbivores has been authoritatively reviewed[37] as has their chemical ecology.[38] The cDNA of DHS from tobacco has been cloned and expressed in active form in *Escherichia coli*[39] and it has been found that the chlorella virus PBCV-1 encodes a functional HSS.[40]

S-Adenosylmethionine decarboxylase and spermidine synthase are enzymes involved in the biosynthesis of spermidine **8**, an essential precursor in the biosynthesis of pyrrolizidine alkaloids. These enzymes from *S. vulgaris* root cultures have been partially purified and characterised.[41]

Tracer feeding experiments have been used to identify the biochemical mechanisms of pyrrolizidine alkaloid sequestration in chrysomelid leaf beetles.[42]

Plant pyrrolizidine alkaloids are esters formed from a necine base joined to a necic acid. Whilst the biosynthesis of the former component is now clear, with some brilliant detail (above), the origins of the necic acid moieties is much less clear.[8] New experiments in a root culture of *Eupatorium clematideum* with [U-$^{13}C_6$]glucose as a (powerful) probe of biosynthetic events has led to deductions about the trachelanthic acid part **12** of, *e.g.* trachelanthamine **11**.[43] It was concluded that **12** arises in the same manner as valine (identical labelling pattern) by addition of a C_2 unit from hydroxyethyl-TPP **13** to 2-oxoisovaleric acid followed by a reduction step (Scheme 2).

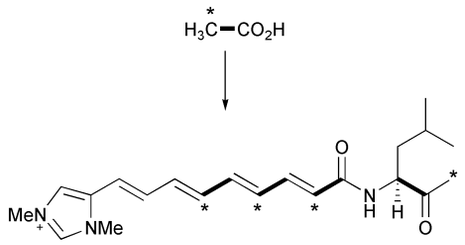


Scheme 2

2.4 Chrysophysarin A, stevensine, streptopyrrole and marcfortine

Chrysophysarin A **14** is a yellow pigment produced by the slime mould *Physarum polycephalum*. Results of experiments with [2-^{13}C]- and [$^{13}C_2$,$^2H_3$]-acetate have led to the conclusion that **14** is formed from three intact acetates, one acetate methyl, histidine and leucine.[44]

Results of experiments in cell cultures of the marine sponge, *Teichaxinella morchella* with ^{14}C-labelled histidine and arginine show that the former, but not the latter, is a precursor for stevensine **15**.[45] Both ornithine and proline were also incorporated, presumably *via* an intermediate such as pyrrole-2-carboxylic acid **16**. The specificity of the incorporations was not established.



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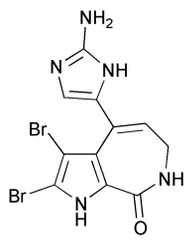

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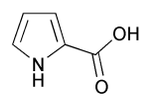
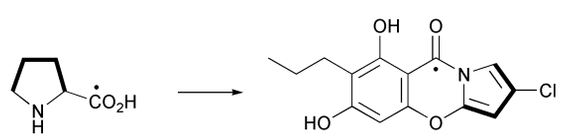


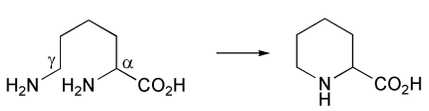
16

Streptopyrrole **18** (metabolite XR587) from *Streptomyces rimosus* is formed from proline **17** and two polyketide chains.[46] The results are in accord with the formation of an amide bond by way of an unprecedented rearrangement of a proline-derived starter unit.



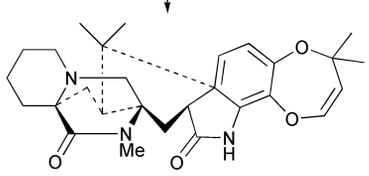
17 18

The pipecolic acid **19** moiety in marcfortine **20** is biosynthesised in a *Penicillum* species by loss of the α-amino, rather than γ-amino, group of lysine.[47] NMR spectroscopy was an essential aid in analysis of labelling.



Lysine

19



20

3 Isoquinoline alkaloids

Evidence relating to the intriguing question of whether mammals make their own morphine has been discussed.[48] The biochemical effects of allelopathic alkaloids, including isoquinoline bases, has been reviewed.[49] Morphine synthesis and biosynthesis has been the subject of review.[50]

The most notable recent results in connection with the biosynthesis of *Erythrina* alkaloids, *e.g.* erythraline **23**, have been discussed in this journal (ref. 7, p. 53). Full details that were obtained with *Erythrina crista-galli* have now been published with experimental detail.[51] In summary, (*S*)-norprotosinomine is not an intermediate in *Erythrina* alkaloid biosynthesis and there is no symmetrical intermediate, in contrast to previous supposition and "supporting experimental results". ‡ Instead the provenance of these bases is by the common isoquinoline pathway and involves (*S*)-coclaurine **21** and (*S*)-norreticuline **22**. Importantly, (*S*)-[1-^{13}C]norreticuline **22** labels C-10 of erythraline **23** exclusively (labelling at C-8 as well was required by the "symmetrical-intermediate" hypothesis). A mechanism for the biosynthesis of erythraline **23**, which is based on earlier ideas, is illustrated in Scheme 3.

That the biosynthesis of a number of plant alkaloids now rests securely is the result of rigorous experimental work carried out by Professor Zenk and his co-workers. A not small part of this is getting high levels of incorporation of precursors by choice of the best plant tissue for the experiments. In the case of *E. crista-galli* it is the use of fruit-wall tissue, which is the major site of alkaloid biosynthesis.

The reader may like to follow up the biosynthesis of *Erythrina* alkaloids by reading about the definition of the biosynthesis of Ipecac alkaloids and of colchicine (ref. 7, p. 54).

The first completely acetogenic nature of an isoquinoline alkaloid, namely dioncophylline A **24**, has been uncovered by a labelling study with [$^{13}C_2$]acetate (Scheme 4) in *Triphyophyllum peltatum*.[52]

Codeinone reductase is the penultimate enzyme that is implicated in morphine biosynthesis (see, *e.g.* refs. 8 and 48). Its molecular cloning, functional expression and characterisation has been achieved.[53] It was found to be related to 6′-deoxychalcone synthase and a family of functionally related NADH-dependent oxidoreductases. Cloning, expression and characterisation of *O*-methyl-transferases involved in isoquinoline biosynthesis have been reported.[54] Analysis of promoters from tyrosine/dihydroxyphenylalanine and berberine-bridge-enzyme genes involved in benzylisoquinoline alkaloid biosynthesis have been examined in *Papaver somniferum*.[55] The distribution of morphinan and benzo[*c*]phenanthridine (sanguinarine) gene transcript accumulation in this plant has been tabled and discussed.[56]

4 Metabolites derived from tryptophan

The biosynthesis of microbial prenylated bases derived from tryptophan has been reviewed.[57] Attention is drawn to the biosynthetic route to the pipecolic acid moiety of marcfortine **20** that was discussed above.[47]

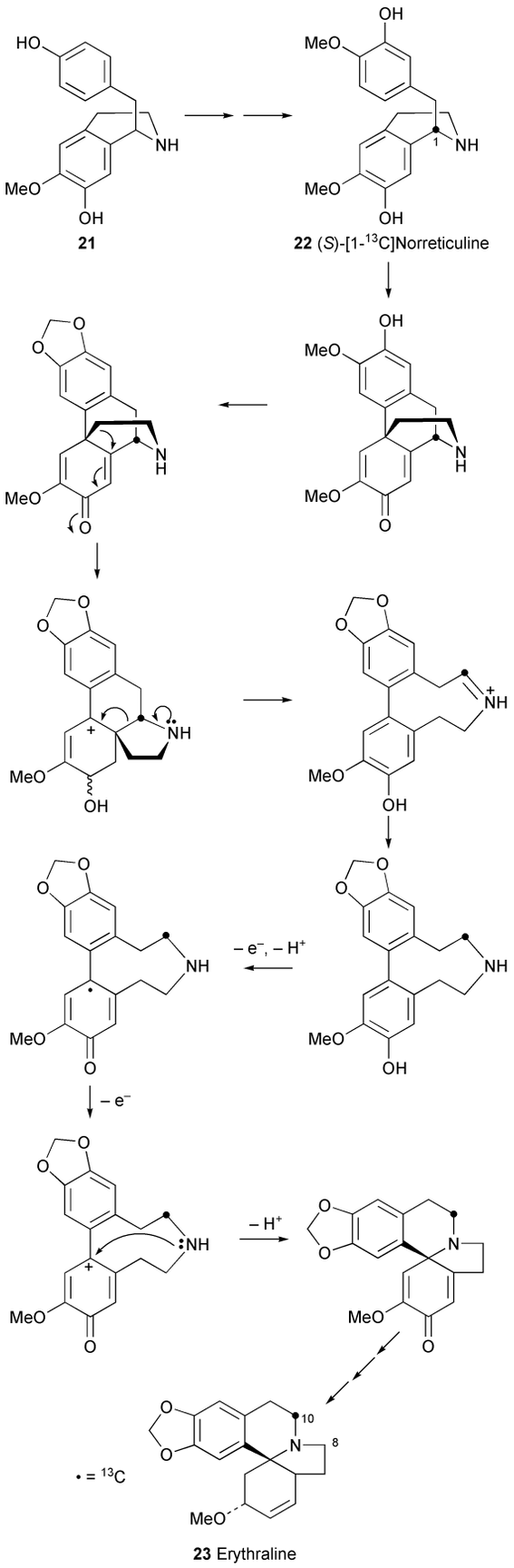
4.1 The mevalonate-independent (deoxyxylulose) pathway

Some terpenes are formed by a biosynthetic pathway that does not involve mevalonate, but pyruvate and glyceraldehyde 3-phosphate leading to isopentenyl diphosphate (ref. 7, p. 57). It has now been shown[58] that the terpenoid moiety in teleocidin B-4 **25** in *Streptomyces blastmyceticum* apparently arises exclusively by this route.

On the other hand, carquinostatin B, another *Streptomyces* metabolite originates through the deoxyxylulose pathway during the early stages of fermentation changing to the mevalonate route later on (ref. 7, p. 57).

The terpene loganin **28** is a key terpene involved in the biosynthesis of (plant) terpenoid indole alkaloids.[8] Careful analysis has been carried out on incorporation patterns in **28** arising from feeding experiments with ^{13}C-labelled glucose, ribose/ribulose, pyruvate and glycerol in *Rauwolfia serpentina* cells.[59] The results are in excellent agreement with the deoxyxylulose pathway. Earlier results with low incorporations of mevalonate were attributed to metabolite exchange between the two pathways (and finally explains why in the 1960′s in

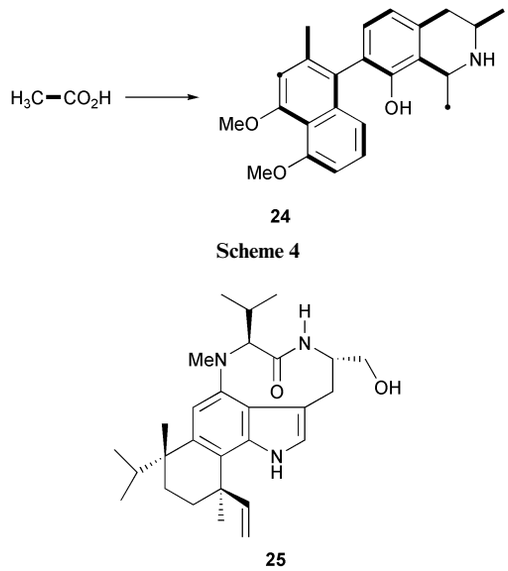
‡ Edward Leete referred to another case in alkaloid biosynthesis where experimental evidence apparently supported an incorrect hypothesis as ESP: Eager to Satisfy the Preconceptions of one's supervisor. A famous parallel example concerns the black peppered moth (J. Hooper, *Of Moths and Men: An Evolutionary Tale*, Fourth Estate, London, 2002).



Scheme 3

Liverpool my colleagues were having such difficulty in getting "decent" mevalonate results with terpenoid indole alkaloids while I was happily mining the gold seam of colchicine biosynthesis).

The cDNA of 1-deoxy-D-xylulose 5-phosphate synthase has been identified and expressed in *Catharanthus roseus* cultures.[60]

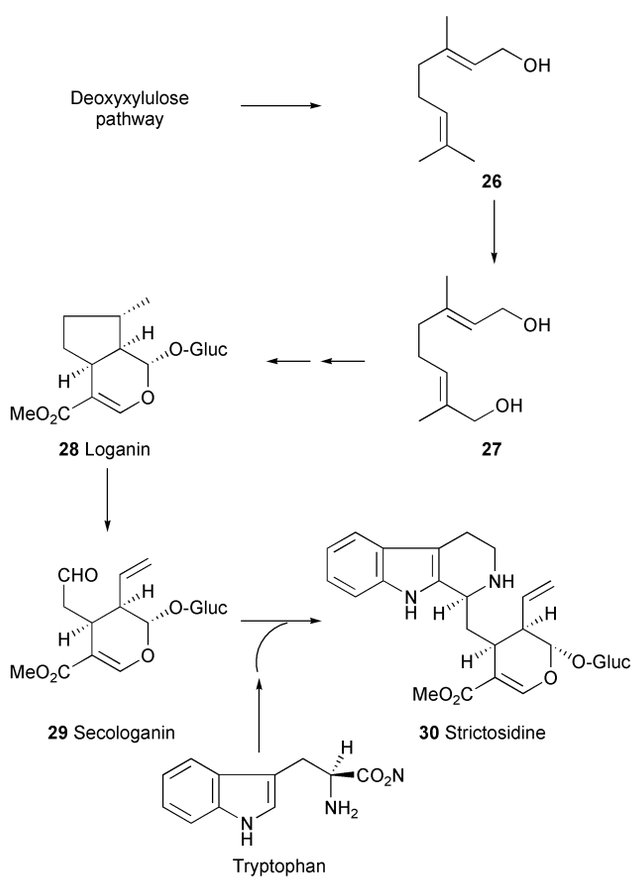


Scheme 4

The results associated with alkaloid accumulation are in accord with the labelling results above.

4.2 Terpenoid indole alkaloids

Part of the biosynthetic pathway to these alkaloids is illustrated as Scheme 5 (ref. 7, p. 56; refs. cited; ref. 8).

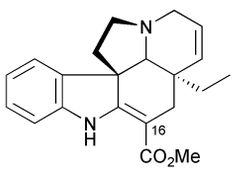


Scheme 5

The induction of a P450 hydroxylase which converts geraniol **26** into 10-hydroxygeraniol **27** [61] has been studied in *C. roseus* cell cultures.[62] Work has been reported on genes associated with tryptophan decarboxylase,[62,63] strictosidine **30** synthase [63,64] and tryptophan synthase.[65] A key P450 enzyme, which catalyses hydroxylation of the alkaloid tabersonine **31** at C-16[8] in *C. roseus*, has been shown to be light-induced.[66] Raucaffricine *O*-β-D-glucosidase which is involved in the biosynthesis of

ajmaline **33** from raucaffricine **32** in *R. serpentina* has been cloned and functionally expressed in *Escherichia coli* (ref. 5, p. 365).[67] The molecular cloning and characterisation of stricto-sidine **30** β-glucosidase from *C. roseus* has been reported.[68]

Polyneuridine aldehyde esterase catalyses a central reaction in ajmaline **33** biosynthesis by transforming polyneuridine aldehyde **34** into 16-epi-vellosimine **35** the immediate precursor for the ajmaline skeleton.[8,69] The enzyme has been purified from *R. serpentina* and characterised.[70]



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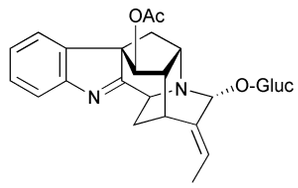

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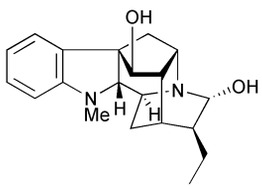
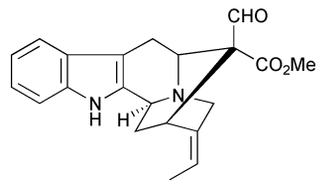


33 Ajmaline



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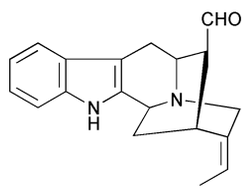

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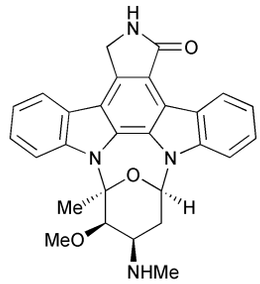
4.3 Staurosporine, camalexin, violacein and pyrrolnitrin

Incorporation of methionine, labelled on the methyl group with ^{13}C and ^{2}H, has shown[71] that both the *O*- and *N*- methyl groups in staurosporine **36** derive conventionally from methionine without hydrogen loss; *N*- and *O*-methylation may, respectively, be the last two steps of biosynthesis.[71,72] The provenance of the rest of the staurosporine **36** skeleton has been reviewed (ref. 6, p. 202; ref. 7, p. 57).

The biosynthetic origins of the phytoalexin camalexin **37** have been defined in *Arabidopsis* cultures (ref. 7, p. 57). A P450 mono-oxygenase associated with the biosynthesis of **37** has been identified.[73]

The results[74] of experiments with ^{15}N- and ^{13}C-labelled precursors in *Chromobacterium violaceum* demonstrate, in the biosynthesis of violacein **38** from two molecules of tryptophan (ref. 5, p. 364), that the 5-hydroxyindole moiety is the product of an intramolecular rearrangement (1, 2-shift of the indole fragment in tryptophan) and that, in the oxindole moiety, the ring carbon remains attached to the β-carbon of the original tryptophan side chain; appropriate incorporation of [2-^{13}C, α-^{15}N]-tryptophan showed that the "central" nitrogen atom is associated with the tryptophan that provides the oxindole moiety. Overall then, the right-hand part of **38** derives simply from tryptophan, with decarboxylation. Metabolites related to **38** have been isolated from a cell-free preparation of *C. violaceum*.[75] Incorporation of [3-^{13}C] serine was reported.

The first step in the biosynthesis of pyrrolnitrin **39** involves chlorination to give 7-chlorotryptophan **40**. The tryptophan 7-halogenase which catalyses this step has been purified to homogeneity from *Pseudomonas fluorescens*.[76] Its activity depends on the presence of a flavin reductase and it has an absolute requirement for oxygen, FAD and NADH. A bio-synthetic scheme for the chlorination has been proposed (Scheme 6).



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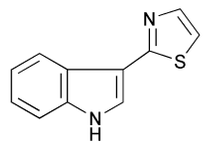

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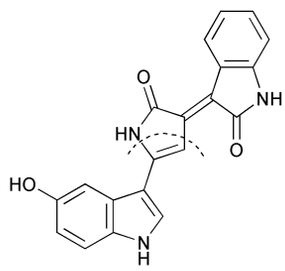

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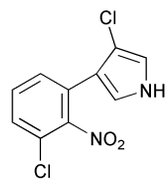

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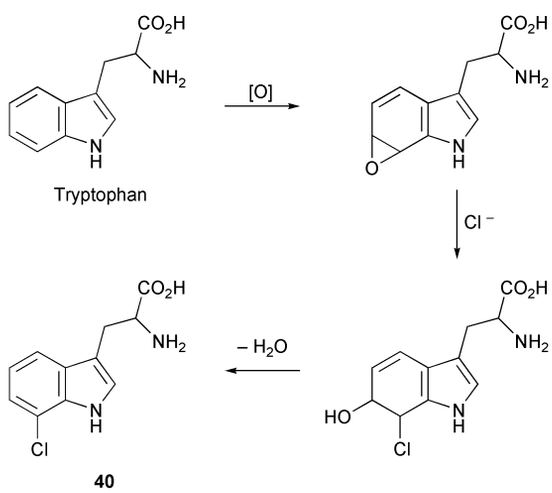


40

Scheme 6

The pyrrolnitrin gene cluster was first characterised in *P. fluorescens*. This gene assembly has been used to probe those of five pyrrolnitrin-producing organisms. The results show that the pyrrolnitrin biosynthetic pathway is highly conserved in the six micro-organisms.[77]

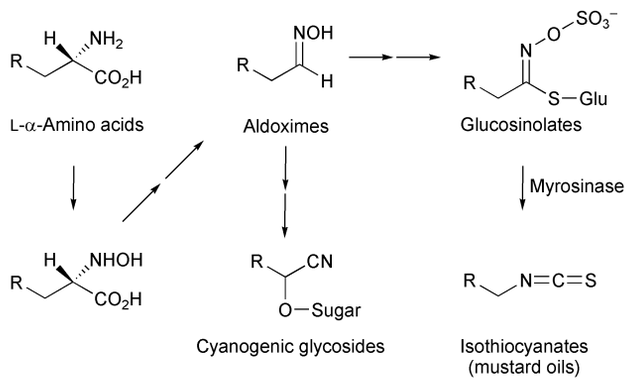
The biosynthesis of pyrrolnitrin and other phenylpyrroles has been authoritatively reviewed.[78] The biosynthesis of the metabolites in this section have been differently surveyed for 1999 and 2000 in another Report.[79]

5 Glucosinolates and cyanogenic glycosides

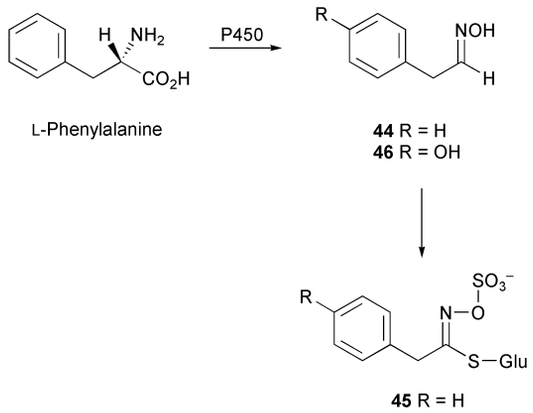
Glucosinolates[80] and cyanogenic glycosides[81] have been reviewed in accounts that incorporate biosynthesis. The last survey in this series of Reports is in ref. 7, p. 57. The general map of biosynthesis is presented as Scheme 7.

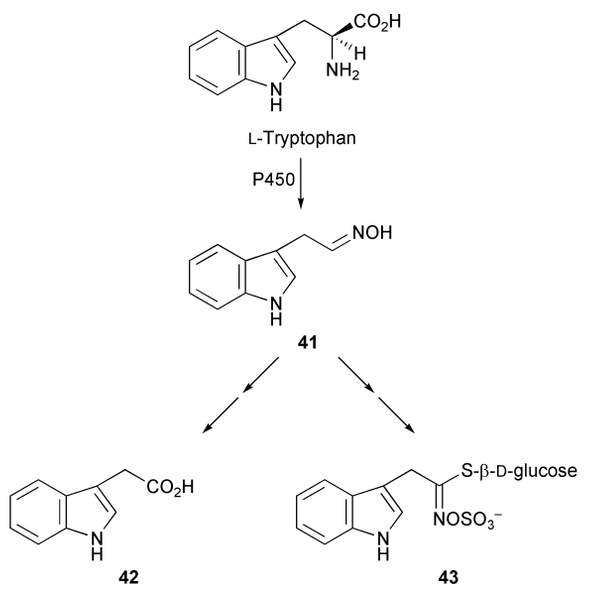
There is a link between the biosynthesis of indole-3-acetic acid **42** and indolyl-3-methylglucosinolate **43** from tryptophan by way of a common intermediate, the aldoxime **41** (Scheme 8) (*cf*. ref. 7, p. 58). Two genes encoding P450 enzymes have been isolated from *Arabidopsis* and characterised. The enzymes can catalyse the conversion of L-tryptophan into **41**.[82] Other work has been reported linked to the production of **42** and indole glucosinolates in *A. thaliana*.[83]

Following Scheme 7, phenylacetaldehyde oxime **44** would be implicated in the biosynthesis of benzylglucosinolate **45** (*cf.* ref.



Scheme 7





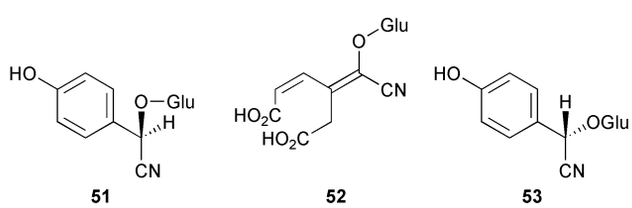
Scheme 8

7, p. 58; see especially Scheme 15). In strong support is the cloning, functional expression in *E. coli* and characterisation of a P450 oxidase that catalyses the conversion of L-phenylalanine into **44** in *A. thaliana*, which produce **45**.[84] The specificity of the oxidase is indicated by the observation that none of L-tryptophan, L-methionine and DL-homophenylalanine is a substrate for the enzyme.

Evidence has been obtained for the methionine elongation pathway in the biosynthesis of 4-methylthiobutyl glucosinolate **50** in *Eruca sativa.*[85] The patterns of [U-^{13}C]methionine and ^{14}C- and ^{13}C-labelled acetate incorporations into **50** were consistent with a three-step chain elongation cycle that is initiated by the condensation of acetyl-CoA with the 2-oxo-acid **47** derived from L-methionine (incorporation of the complete skeleton, minus the carboxyl). It ends with an oxidative decarboxylation forming a new 2-oxo-acid **48** containing an additional methylene group (Scheme 9). In the formation of 4-methylthiobutylglucosinolate **50** the cycle turns twice with the incorporation of two C_1 units from the methyl group of acetate. The second and final oxo-acid is **49** that derives from **48**. Incorporation of [^{15}N]methionine indicates that the amino acid can furnish the nitrogen source and implies also that biosynthesis is confined to a single compartment.

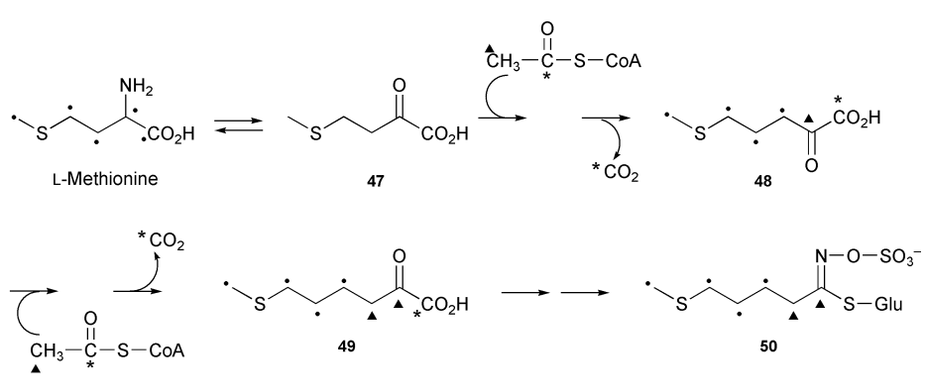
As already mentioned, there is a commonality in the biosynthesis of glucosinolates and cyanogenic glycosides (Scheme 7). Thus there are (two) P450 enzymes that are involved in the biosynthesis of taxiphyllin **51** and triglochinin **52** in *Triglactin maritima*.[86] The encoding genes have been cloned and over-expressed in *E. coli.* The enzymes convert L-tyrosine into 4-hydroxyphenylacetaldehyde oxime **46**; significantly for the formation of **52**, L-3,4-dihydroxyphenylalanine is not a substrate.

Results have been published on the expression of [87] and specificity[88] of two P450 enzymes (ref. 7, p. 58) involved in the biosynthesis of dhurrin **53** (the epimer of **51**). The last step in dhurrin biosynthesis is catalysed by a UDP-glucose:*p*-hydroxymandelonitrile *O*-glucosyltransferase. This enzyme, from *Sorghum bicolor*, has been isolated, cloned, heterologously expressed, and tested for substrate specificity.[89]



6 Other metabolites of the shikimate pathway; related compounds

The reader's attention is drawn to different coverage in a sister Report.[79]

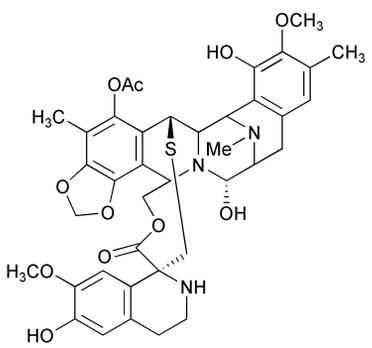


Scheme 9

6.1 Ecteinascidin and barbamide

Ecteinascidin **54** is a baroque metabolite from the tunicate *Ecteinascidia turbinata*, in which three isoquinoline residues may be discerned. The diketopiperazines **55** and **56** have been efficiently incorporated in cell-free extracts, where L-tyrosine was a poor precursor and **57** was incorporated not at all.[90] A sequence involving tyrosine to **56** to **55** to **54** is reasonable. This was supported by the observed conversion of **56** into **55** in a crude enzyme preparation.

Barbamide **58** is a metabolite from the marine cyanobacterium *Lyngbya majuscula.* The origins are as shown, with the proposal that cysteine is the precursor of this thiazole ring being deduced from results with glycine.[91] It appears that direct, unactivated chlorination of the *pro*-R methyl group of leucine affords the precursor trichloroleucine.



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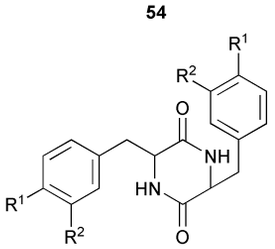
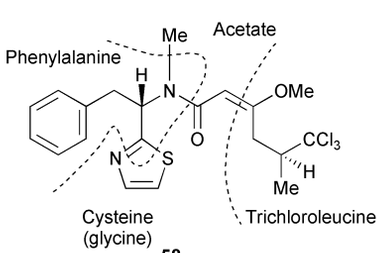


55 R^1 = OH, R^2 = OH
56 R^1 = OH, R^2 = H
57 R^1 = H, R^2 = H



58

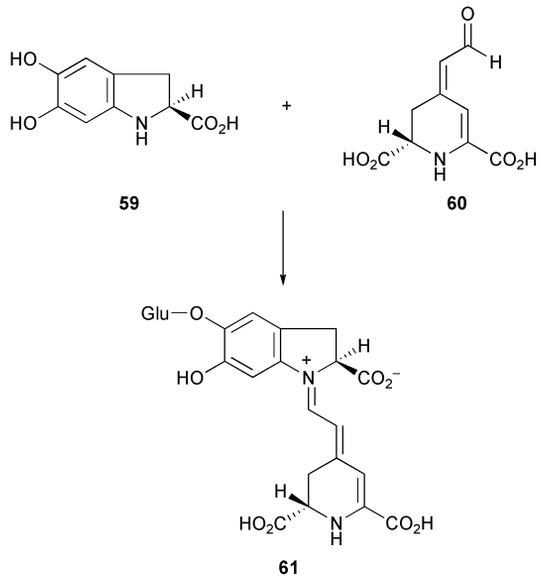
6.2 Betalains

The betalains, *e.g.* betanin **61**, are common plant pigments and the providers of beautiful colours. Elucidation of the biosynthesis of these compounds has been a long, and surely harmonious, endeavour.[8]

A cDNA encoding betanidin 5-*O*-glucosyl-transferase has been cloned, heterologously expressed and characterised.[92] A tyrosinase (tyrosine-hydroxylating enzyme) has been partially purified from *Portulaca grandiflora* and characterised.[93] It appears that the decisive step in betalain biosynthesis involves a *spontaneous* condensation between the aldehyde function of betalamic acid **60** and amino acids, possibly including cyclo-Dopa **59**, and amines (Scheme 10).[94]

6.3 Rifamycins, 3-amino-5-hydroxybenzoic acid, ansatrienins, naphthomycin, ascomycin and mitomycin

Work on rifamycin type I polyketide synthase has been



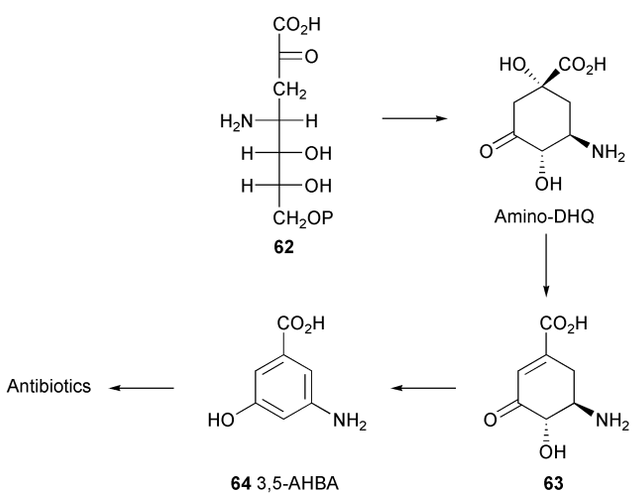
59 **60**

61

Scheme 10

reviewed authoritatively.[95] Advances[96] in the biosynthesis of the rifamycin polypeptide moieties have been published; they lie beyond the compass of this review.

At the heart of antibiotics such as rifamycin B **65** there is a C_7–N aromatic moiety (thickened bonds) that is encircled by a polyketide handle (ansa). Until recently the provenance of this unit was obscure. Then some inspired rooting around in the literature followed by appropriate experiments led to the uncovering of the biosynthetic origin of this fragment as 3-amino-5-hydroxybenzoic acid (3,5-AHBA) **64** (Scheme 11) (ref. 7, p. 58).[8]



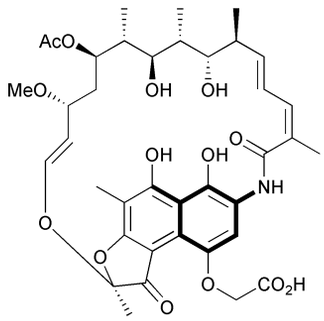
62 **63**

64 3,5-AHBA

Scheme 11

Now,[97] to cap it all, the X-ray crystallographic structure (to 2 Å resolution) of 3,5-AHBA synthase from *Amycolatopsis mediterranei* has been determined.

3,5-AHBA is biosynthesised by a parallel pathway to the shikimate pathway with the making of amino-DHAP **62** (DHAP is not itself a precursor). The synthase acts on amino-DHS **63** in a pyridoxal (PLP)-assisted reaction to give 3,5-AHBA (ref. 7, p. 59). The enzyme is dimeric with pronounced sequence homology to a number of PLP enzymes involved in the biosynthesis of antibiotic sugar moieties. X-ray data were obtained, satisfyingly, with bound PLP and then with PLP plus gabaculine. With the latter, compared to the former, an internal linkage to Lys 188 is broken and a covalent bond is formed between cofactor and inhibitor.[97]



65 Rifamycin B

The cloning, sequencing and expression of the 3,5-AHBA synthase gene from *A. mediterranei* has been reported.[98]

Despite commonality in biosynthesis the ansatrienin and naphthomycin gene clusters show clear organisational differences and carry separate sets of genes for 3,5-AHBA biosynthesis in *Streptomyces collinus*.[99]

The cyclohexanecarboxylic acid and dihydroxycyclohexanecarboxylic acid derivatives that are constituents of ansatrienins and ascomycin, respectively, are elaborated as deviations of the shikimate pathway (ref. 7, p. 58). An enoyl-CoA reductase has been identified in *S. collinus* and characterised: it is implicated in the biosynthesis of a cyclohexanecarboxylic acid residue of ansatrienin A. Curiously, the gene is not associated with the ansatrienin biosynthetic gene cluster.[100] A dehydroquinase from *S. hygroscopicus* has been implicated in the biosynthesis of the shikimate-derived moiety of ascomycin.[101]

The gene cluster for the biosynthesis of the antitumour antibiotic mitomycin in *S. lavendulae* has been characterised and analysed[102] and molecular characterisation of the enzymes involved, has been reported.[103]

6.4 3-Amino-4-hydroxybenzoic acid, vancomycin and acarbose

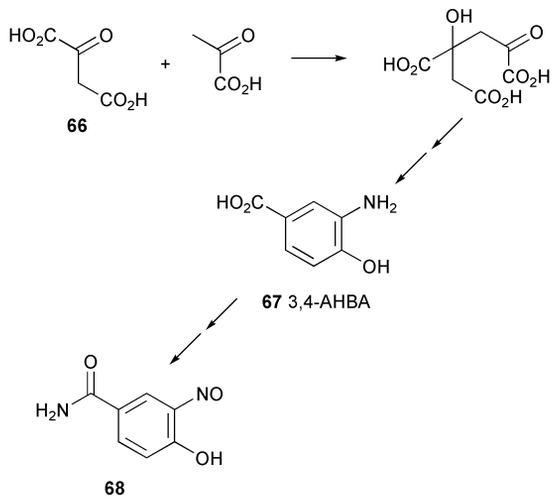
3-Amino-4-hydroxybenzoic acid (3,4-AHBA) **67**, a C_7N unit, is a defining precursor for several *Streptomyces* metabolites, *e.g.* 4-hydroxy-3-nitrosobenzamide **68**, mannumycin and asukamycin (ref. 7, p. 58; refs. cited). Curiously, unlike 3,5-AHBA **64** (Section 6.3) 3,4-AHBA **67** is not derived from the shikimate pathway. The provenance has been deduced to be a C_4 dicarboxylic acid and a C_3 triose phosphate.

Further experiments have been carried out in *S. murayamaensis* on what may be considered a simple model, namely the naturally-occurring 4-hydroxy-3-nitrosobenzamide **68** (and its ferrous chelate).[104] The conclusion from careful experiments with labelled potential precursors is that oxaloacetate **66** is the key C_4 building block (rather than its transamination product aspartic acid); methionine and homoserine and the corresponding keto acids were deduced not to be directly involved. It remains uncertain what the last C_3 precursor is. The proposed biosynthesis is illustrated (Scheme 12).

(*S*)-3,5-dihydroxyphenylglycine **69** is an unusual amino acid found in the glycopeptide antibiotic vancomycin. As the upshot of elegant and welcome new work in this area, the biosynthesis of **69** has been deduced to be by a polyketide route catalysed by a type III polyketide synthase.[105]

The carbocyclic ring (valienamine fragment) in the glucosidase inhibitor acarbose **73** has been shown, in *Actinoplanes* sp., to derive from the pentose-phosphate pathway (ref. 7, p. 62) and evidence[106] has been obtained that sedoheptulose phosphate **70** is an intermediate and that it is the substrate for ring formation. The role of the enzyme is similar to dehydroquinate synthase at the beginning of the shikimate pathway.

It has now been shown[107] in feeding experiments with a set of cyclitols that only 2-*epi*-5-*epi*-valiolone **71** is a precursor for the valienamine residue in acarbose **73**. The stereochemistry at C-5 and, particularly, C-2 in the precursor defines whether or not it will be incorporated: it must be that shown in **71**. The conversion of **71** into **72** requires four steps: epimerisation, dehydration, condensation and reduction (Scheme 13). The evidence points to this happening on a single enzyme without free intermediates.[107] The reader is referred to related work on the cyclitols gabosines A, B and C from *S. cellulosae*.[108]

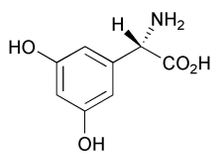


66

67 3,4-AHBA

68

Scheme 12



69

6.5 Phenazines, DIMBOA and DIBOA

The biosynthesis of microbial phenazines is from two molecules of shikimic acid **74**. The first phenazine to be formed is phenazine-1,6-dicarboxylic acid **75** and all others appear to be its derivatives (for recent work on phenazines see: ref. 7, p. 60).[8]

Study, in *S. antibioticus*, of the biosynthesis of the saphenyl esters **78** and esmeraldins **79** shows that **75** is again involved as an intermediate and biosynthesis is as illustrated in Scheme 14.[109] The C_1 unit, which forms part of the acetyl group in **76**, has its provenance in C-2 of acetate, presumably being introduced *via* an intermediate β-keto acid. Experiments with chirally labelled acetic acid reveal that decarboxylation of the putative β-keto acid occurs with inversion of configuration.

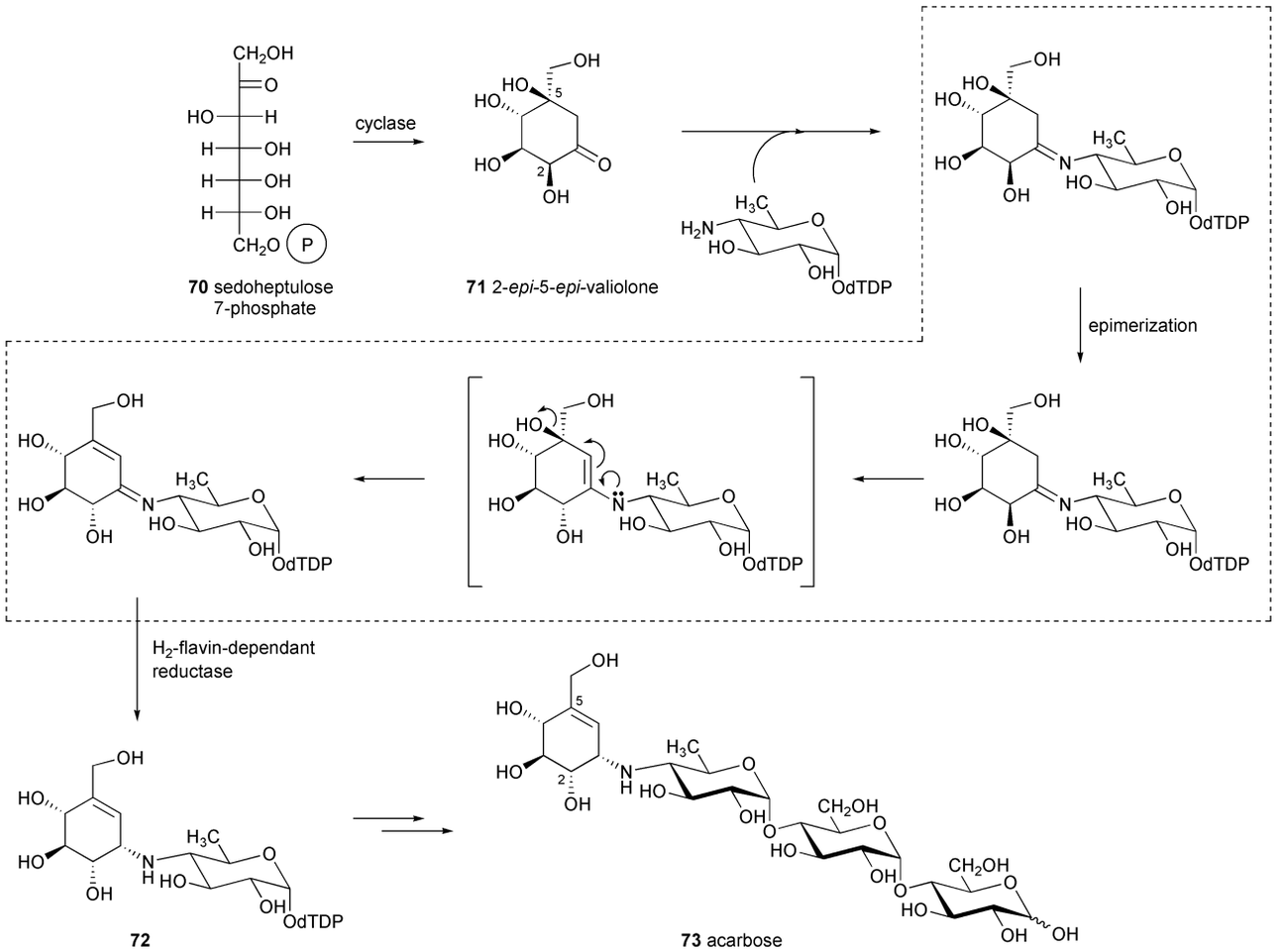
Saphenic acid **77** was incorporated differentially into the two halves of the esmeraldins **79**. This suggests that the two compounds involved in dimerisation have different biosynthetic histories.

DIBOA **80** and DIMBOA **81** (ref. 7, p. 60) are natural pesticides, the biosynthesis of which involves oxidation. The specificity and conservation of P450-dependent monooxygenases in grasses has been studied as has the general occurrence of hydroxylases in juvenile wheat.[110]

7 β-Lactams

Recent advances in the structure of isopenicillin N synthase (Section 7.1) have been reviewed[111], as have the biosynthesis and molecular genetics of cephamycins,[112] of carbapenems,[113] and of clavulanic acid (Section 7.2).[114]

Deacetoxycephalosporin C synthase (DAOCS) is a non-heme iron-binding and α-ketoglutarate-dependent enzyme implicated in the biosynthesis of cephalosporins and cephamycins. The three-dimensional X-ray structure has been determined of DAOCS, alone and with Fe^{2+} and α-ketoglutarate. The structures of DAOCS and other enzymes in the biosynthetic pathway to the cephalosporins may be used as guides for



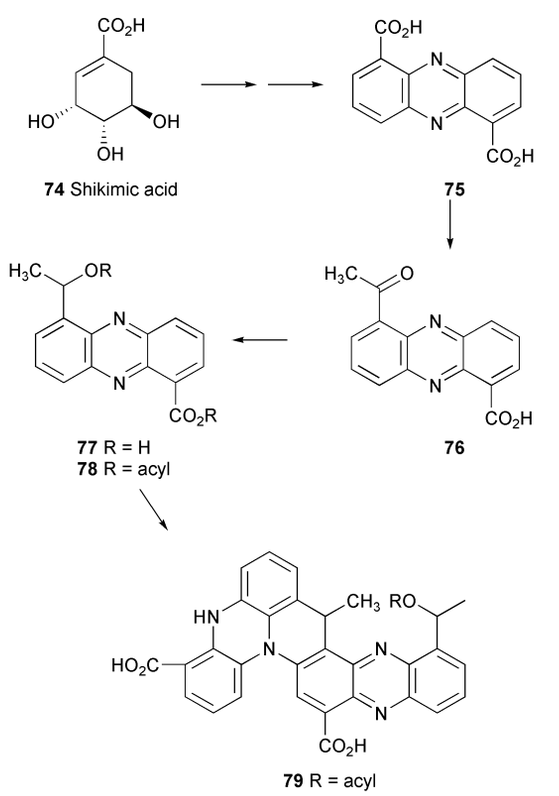
70 sedoheptulose 7-phosphate

71 2-*epi*-5-*epi*-valiolone

72

73 acarbose

Scheme 13



74 Shikimic acid

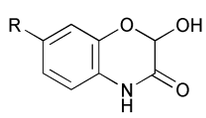
75

76

77 R = H
78 R = acyl

79 R = acyl

Scheme 14



80 R = H
81 R = OMe

the preparation of enzymes modified to take unnatural substrates.[115] Mutational evidence has been obtained[116] that histidine-183, aspartate-185, and histidine-243, deduced from the crystal structure of DAOCS to be the iron-binding residues, are essential for enzyme (ring-expansion) activity.

The carboxy-terminal thioesterase domain of 5-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ref. 7, p. 60) catalyses the release of the tripeptide LLD-ACV that is involved in penicillin biosynthesis. A synthetase mutated in a serine residue (S3599A) within a highly conserved GXSXG motif resulted in loss of only 50% of activity and LLL-ACV as the dominating product.[117] It was suggested that LLL-ACV is an intermediate in penicillin biosynthesis and this motif is involved in the control of tripeptide epimerisation (LLL to LLD) by selection of the epimer to be released.

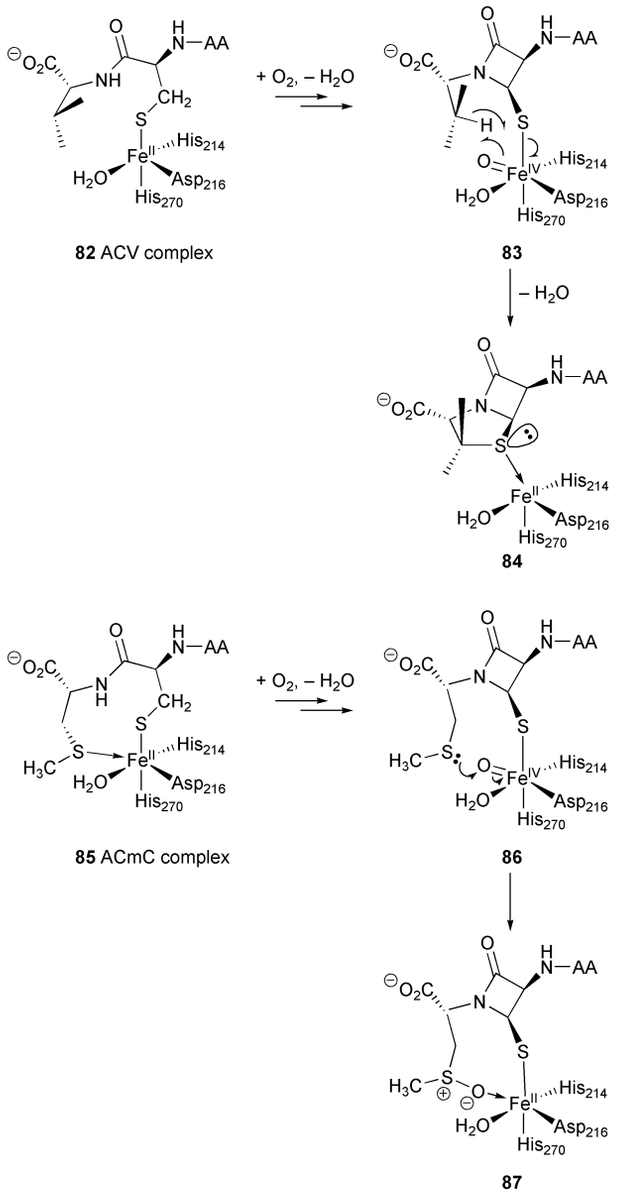
The cloning and characterisation of a gene from *Aspergillus nidulans* that is involved in the regulation of penicillin biosynthesis has been reported.[118] The molecular control of expression of penicillin biosynthesis genes in fungi has been reviewed.[119]

The screening of potential affinity ligands and the developments of affinity purification of an *S*-adenosylmethionine-dependent tranferase involved in nocardicin A biosynthesis has been reported.[120]

7.1 Isopenicillin N synthase

The X-ray crystal structure of isopenicillin N synthase (IPNS) complexed to Fe^{2+} and the substrate LLD-ACV (see above) has previously been reported (ref. 7, p. 61). More impressively,

now remarkable structural snapshots of the pair of ring closures that convert LLD-ACV through to isopenicillin N have been definitively revealed (Scheme 15). The key to the revelations come from growing IPNS·Fe^{2+}·substrate crystals anaerobically then exposing them to high pressures of oxygen to promote what is an oxygen-dependent sequence of reactions and then frozen; product structures were elucidated by X-ray crystallography.[121] With the natural ACV substrate the IPNS·Fe^{2+}·IPN product complex **84** was obtained (some unchanged starting substrate lingered).



Scheme 15

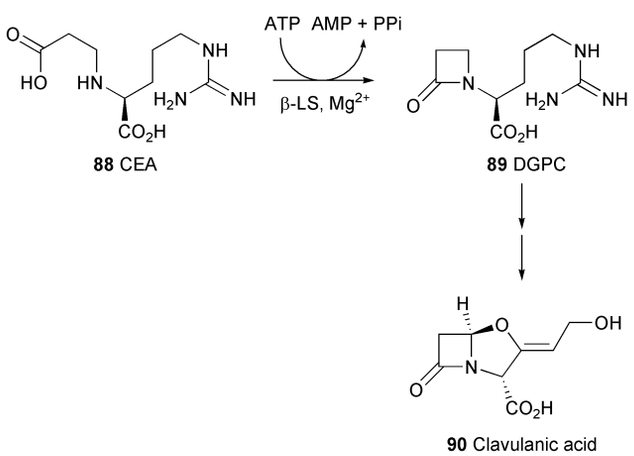
The most significant differences that were observed between the IPNS·Fe^{2+}·IPN structure **84** and the IPNS·Fe^{2+}·ACV complex **82** (Scheme 15, **82** through **84**) are in the positions of the β-lactam carbonyl and the cysteinyl sulfur. During reaction the carbonyl moves 60° around the cysteinyl C–1–C–2 bond; this was deduced to correlate with a lack of well-ordered hydrogen bonds in the substrate complex driving the reaction. In the reaction course the sulfur migrates around the iron forming the pentacoordinate product complex **84** (Scheme 15). The new structure also provides some evidence concerning product release from the active site.

A modified ACV substrate was designed (see Scheme 15, **85** through **87**) to interrupt the reaction sequence, namely δ-[L-α-aminoadipoyl]-L-cysteinyl-D-*S*-methylcysteine (ACmC). This interruption did indeed happen in the subsequent experiment with the formation of **87**. This provides direct evidence that β-lactam formation is the first of the two cyclisation reactions. This time the carbonyl moves, but not the sulfur. Notably the β-lactam carbonyl in both product structures (**84** and **87**) is hydrogen bonded to two well-ordered water molecules and stabilised by them in contrast to the substrate complex where this is lacking (above). It was suggested that this is crucial to stabilisation of a C–S bond during cyclisation, but in being absent in **82** reduction in electron density on nitrogen, which would be deleterious for the first cyclisation, does not occur.

The PCR cloning, heterologous expression and characterisation of isopenicillin N synthase using *S. lipmanii* has been reported.[122]

7.2 Clavulanic acid

Iron/oxygen chemistry related to that of IPNS is carried out by clavaminate synthase in the course of the biosynthesis of the structurally similar β-lactam clavulanic acid **90** (an important, clinical β-lactamase inhibitor). The formation of the β-lactam rings in clavulanic acid and penicillin biosynthesis is distinctly different. In the case of **90**, N^2-(carboxyethyl)-L-arginine (CEA) **88** is converted into deoxyguanadineproclavaminic acid (DGPC) **89** catalysed by an ATP/Mg^{2+}-dependent β-lactam synthetase (β-LS) (Scheme 16) (ref. 7, p. 61). The kinetic mechanism has been deduced as consistent with an ordered bi-ter process and ordered substrate binding with ATP binding occurring first.[123]



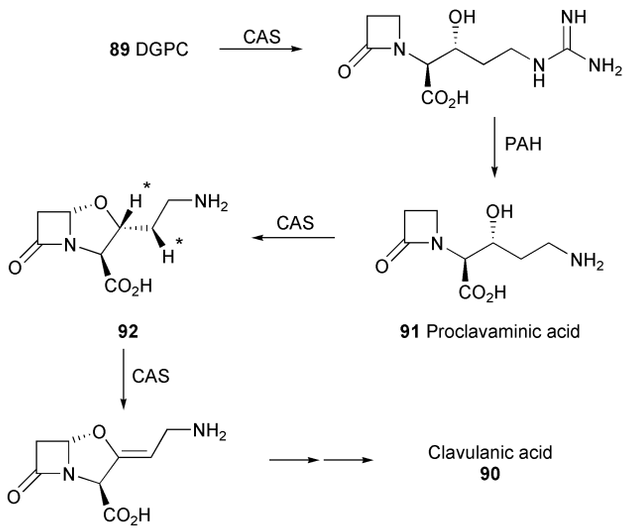
Scheme 16

Most impressive and defining X-ray crystal structures have been reported of the way a later enzyme in clavulanate biosynthesis namely clavaminic acid synthase (CAS) works its magic catalysis (the reader is encouraged to read the paper as only incomplete justice can be done here to the work).[124]

CAS is an Fe^{2+}/2-oxoglutarate oxygenase which remarkably catalyses three separate oxidation reactions (hydroxylation, oxidative cyclisation and desaturation) with the interposition of proclavaminate amidinohydrolase (PAH) (Scheme 17).

Crystal structures of CAS have been obtained with Fe^{2+}, 2-oxoglutarate and first substrate analogue α-N-acetyl-L-arginine or second substrate proclavaminic acid **91** bound. They reveal how CAS catalyses the construction of the clavam nucleus, by means of quite exceptional organic chemistry. They suggest how it discriminates between substrates and controls reaction of its highly reactive ferryl intermediates. The deduced mechanism is outlined in Scheme 18A and the relationship between the ferryl intermediate and the two substrates in the hydroxylation and cyclisation reactions is shown as Scheme 18B and 18C, respectively.[124]

β-Secondary kinetic isotope effects in the CAS-catalysed oxidative cyclisation of **91** into **92** have been carefully



Scheme 17

studied,[125] as has the consequences of site-directed mutagenesis in the ferrous active site of CAS.[126] In an expansion of the clavulanic acid biosynthesis gene cluster, three more genes required for its biosynthesis in *S. clavuligerus* have been identified.[127]

Genes specific for the biosynthesis of clavam metabolites antipodal to clavulanic acid in *S. clavuligerus* are clustered with the gene for CAS-1.[128] Enzymes catalysing the early steps of clavulanic acid biosynthesis in this organism are encoded by two sets of paralogous genes.[129] Deletion of a gene (pyc: pyruvate converting) in *S. clavuligerus* blocks clavulanic acid biosynthesis except in a glycerol medium, suggesting the two sources for C_3 units in biosynthesis.[130]

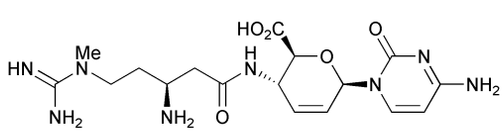
8 Miscellaneous metabolites

8.1 Taxol (paclitaxel)

Two *O*-acetyltransferases that catalyse acylation in the course of taxol biosynthesis (ref. 7, p. 62) have been studied.[131] A "pseudomature" form of taxadiene synthase involved in taxol biosynthesis has been heterologously expressed and characterised together with evaluation of potential intermediates and inhibitors for the multistep diterpene cyclisation reaction.[132] The biosynthesis of taxol has been reviewed.[133]

8.2 Blasticidin S

The final steps in the biosynthesis of blasticidin S **93** (ref. 7, p. 62) the antifungal peptidyl-nucleoside, have been revised [134] to include a novel resistance mechanism whereby the putative final precursor, demethylblasticidin S, is modified with a leucine residue; this intermediate has reduced antibiotic activity.

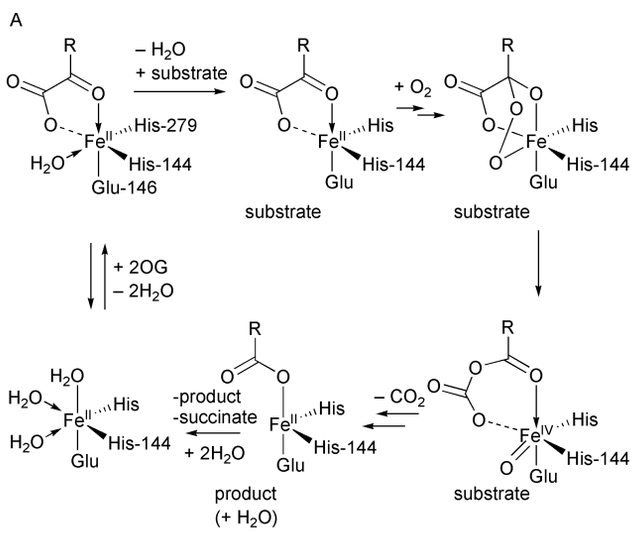


93 Blasticidin S

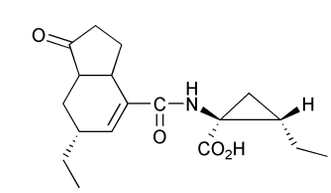
8.3 Coronatine and caffeine

The biosynthesis of the polyketide moiety, coronafacic acid (left half of coronatine **94**) has been studied in relation to the polyketide synthases involved.[135] For related work see ref. 7, p. 63.

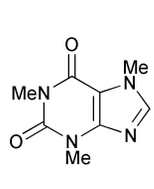
The contribution of *de novo* nucleotide synthesis to the biosynthesis of caffeine **95** in young tea leaves seems to be important.[136] Caffeine synthase has been purified and characterised.[137] The biosynthesis and metabolism of caffeine has been reviewed.[138]



Scheme 18



94 Coronatine

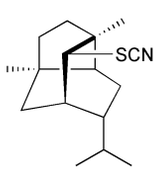


95 Caffeine

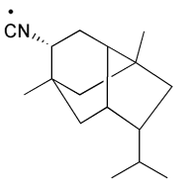
8.4 Isocyanopupukeanane and isothiocyanatopupukeanane

Natural products containing isocyano, cyano and isothiocyanato functionality are unusual and interesting.[139] Marine examples have been subject to close scrutiny. Circumstantial evidence had been obtained, from the incorporation of thio-

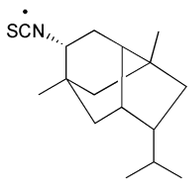
cyanate into both isocyano and isothiocyano examples in *Acanthella cavernosa*[140] and of cyanide into both an isocyano example in *A. cavernosa* and a thiocyanate, 2-thiocyanatoneopupukeanane **96**, in *Axinyssa* n.sp.,[141] that metabolites with isocyano groups might be interconvertible in the sponges with those bearing isothiocyanato functionality. Now, in experiments with ^{14}C-labelled 9-isocyanopupukeanane **97** and 9-isothiocyanatopupukeanane **98** (• = label) in *Axinyssa* this interconvertiblity is demonstrated (0.12–0.16% incorporation with apparent specificity).[142] Supporting, earlier results[143] were also obtained with diisocyanoadociane **99** (incorporation of **100** and thiocyanate).



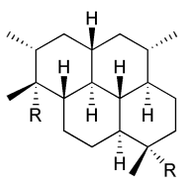
96



97



98



99 R = NC
100 R = NCS

9 Conclusion

What? how? and why? are three cardinal questions; and surely the greatest of these is why? The questions naturally apply, and historically in this order, to the biosynthesis of secondary metabolites.

When structures were first obtained for secondary metabolites questions were raised: what are the building blocks and what are the patterns of structural repeats? Biosynthetic studies with labelled compounds provided concrete answers to these questions, later augmented with results of defining enzyme experiments. Many examples are to be found earlier in this account.

How? has become more dominant recently with the developing involvement of the powerful tools and devices of molecular biology and X-ray crystallography. Now excitingly we are beginning to answer the question why? See, *e.g.*, the important results on pyrrolizidine alkaloids in Section 2.3.

The contents of this Report (as of all those that came before) attest again and again to decent experiments of high calibre and to conclusions, sometimes delightfully unexpected, that advance our knowledge and understanding of biosynthetic pathways and mechanisms. In all of this Duilio Arigoni has been a brilliant question master and solver of puzzles – a personal hero.

The recent crystal structure results from Oxford relating to β-lactam biosynthesis (Section 7) are readily singled out in this Report for their special and detailed revelations based on exquisite experiments (see also 3,5-AHBA, Section 6.3).

After reviewing the literature on the biosynthesis of nitrogenous metabolites for many years (coverage: 1969 through 2000) the time has come to quit and perhaps contemplate the ultimate question, to which the answer is surely not always 42.

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