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Review Article

Isotope tracer investigations of natural products biosynthesis: the discovery of novel metabolic pathways†

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Abstract: Feeding experiments using isotopically labeled precursors remain crucial elements in the study of natural product biosynthesis. Through careful isotope tracer studies, a number of previously unrecognized biosynthetic pathways have now become obvious. One of the remarkable examples is the discovery of the non-mevalonate pathway in plants, bacteria, algae, and plasmodium, whereas others include the discovery of a new shunt pathway that connects the mevalonate pathway and branched-chain fatty acids in myxobacteria and the involvement of an unusual HMG-CoA synthase-like mechanism in polyketide biosynthesis. Significant progress has also been made in the biosynthetic studies of aminocyclitol-derived natural products and other bioactive secondary metabolites. This review highlights some of the more recent discoveries of novel mechanisms and metabolic pathways involved in natural product biosynthesis based on isotope tracer experiments.

Keywords: natural products biosynthesis; isoprenoids; branched-chain fatty acids; polyketides; aminocyclitols

Introduction

Biosynthetic studies of natural products have established themselves to be one of the more exciting areas of research and have become an important part of modern drug discovery and development efforts. The advances of molecular genetics, protein science, biotechnology, and analytical instrumentation have revolutionized the way studies on natural product biosynthesis are conducted. Using combinations of contemporary molecular genetic approaches, enzymology, and chemistry, coupled with powerful and sophisticated mass spectroscopy and NMR, it is now possible to dissect mechanisms and processes involved in natural product biosynthesis at the molecular level. Nevertheless, classical isotope tracer experiments continue to play an important role in understanding how and from what precursors a particular natural product is constructed. This in turn provides meaningful information that serves as the basis for further investigation using genetic and enzymatic approaches. Isotope tracer experiments have been commonly employed to confirm the biosynthetic origin and identity of natural products, in which simple isotopically labeled precursors, e.g. acetic acid, glycerol, glucose, and amino acids, are used. In some cases, however, more elaborate studies are needed, involving more complex biosynthetic intermediates that often require long and complicated syntheses.

The wealth of knowledge obtained from various studies carried out during the past half-century has provided basic tools for biosynthetic chemists to more easily identify the origin of natural products, in most cases based on the labeling patterns in the products obtained from isotope tracer experiments. There are, however, exceptions where coupling and labeling patterns of the isotopes were inconsistent with those expected to arise from commonly known pathways. Such observations have led to the discovery of novel enzyme mechanisms and biosynthetic pathways. Over the past 20 years, these findings have provided underpinning knowledge about the diversity and complexity of natural product formation and have played a pivotal role in the identification of new drug targets. This

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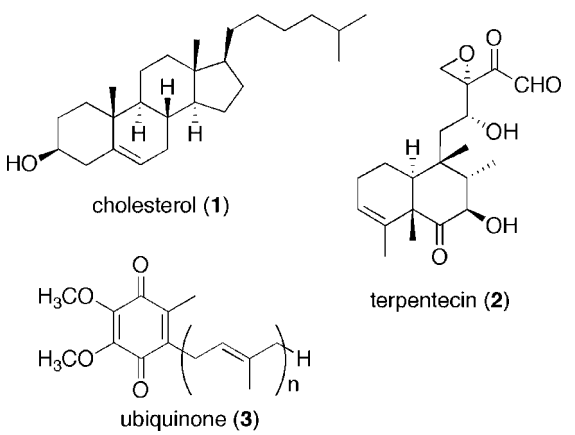
review highlights the importance of isotope tracer investigations in the discovery of several novel biosynthetic pathways and enzyme mechanisms involved in primary and secondary metabolism.

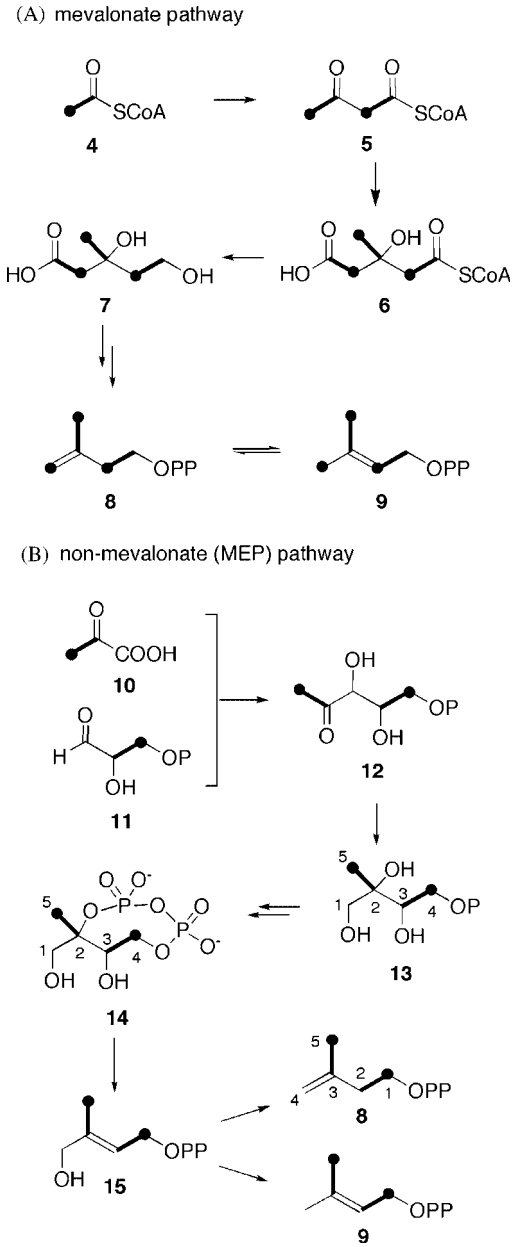
The discovery of the non-mevalonate or 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway

The mevalonate pathway has long been recognized to be a prominent metabolic pathway in microorganisms, plants, and animals. It is involved in the biosynthesis of isoprene units, isopentenyl diphosphate (**8**, IPP) and dimethylallyl diphosphate (**9**, DMAPP), precursors of many important compounds, e.g. sterols (**1**) and terpenoids (**2**) (Scheme 1).[1,2] The pathway is initiated by tandem coupling reactions of three units of acetyl-CoA (**4**) to form 3-hydroxy-3-methylglutaryl-CoA (**6**, HMG-CoA), which is then reduced to mevalonic acid (**7**) (Scheme 2A). The latter compound is subsequently converted, through a decarboxylation–dehydration process involving three molecules of ATP, to **8**, which can then be isomerized to **9** by the action of isopentenyl diphosphate isomerase.

The mevalonate pathway was first established by detailed studies in yeast and mammalian liver tissue.[3–5] It was then universally accepted as the sole biosynthetic pathway to all isoprenoids in all living organisms and routinely probed by isotope tracer experiments using labeled acetate, which gives specific labeling and coupling patterns of the isotopes in the isoprene units, indicating whether or not a natural product is derived from **8**. However, through the course of more than a half-century of biosynthetic studies of the isoprenoids, a number of contradictory results, initially in plants and later in eubacteria, were obtained.[6,7] These data suggested that an alternative pathway may lead to **8** formation. It was observed that isotopically labeled acetate and mevalonate were incorporated well into the triterpenes and the sterols, but not or very poorly into mono- and diterpenes, in plant systems. Also, incorporation patterns of [1-^{13}C]- and [2-^{13}C]-acetate into the triterpenoids of the hopane series in several Gram-negative bacteria, including *Rhodopseudomonas palustris*, *R. acidophila*, and *Methylobacterium organophilum*, were evident in con-



Scheme 1 Chemical structures of isoprene-derived natural products.



Scheme 2 Biosynthetic pathways to isopentenyl diphosphate and dimethylallyl diphosphate via the mevalonate pathway (A) and the methylerythritol phosphate (MEP) Pathway (B).

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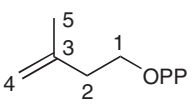
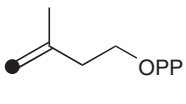
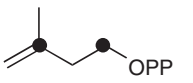
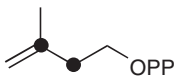
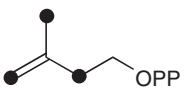
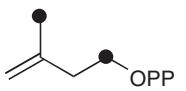
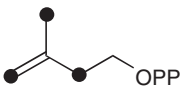
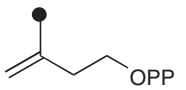
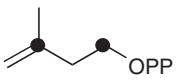
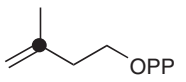
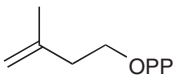
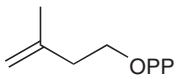
tradiction with the conventional mevalonate pathway.[8] A similar phenomenon was also observed in feeding studies using [1,2-$^{13}C_2$]acetate into the isoprenic side chain of ubiquinone (**3**) in *Escherichia coli*.[9] It was not until the early 1990s, however, that full evidence for the existence of a novel pathway to IPP (**8**) and DMAPP (**9**) in bacteria and plants was reported.[10] Using ^{13}C-labeled glucose, acetate, pyruvate, and erythrose to study the origin of carbon atoms of triterpenes and ubiquinones in a number of bacteria, Rohmer *et al.* proposed a new biosynthetic route for the early steps of isoprenoid biosynthesis (Scheme 2).[10] This has led to more elaborate efforts in studying isoprenoid biosynthesis in many other organisms. Useful information regarding the biosynthetic origin of the isoprenic units derived from the non-mevalonate pathway in bacteria was originally obtained from isotope tracer experiments using ^{13}C-labeled glucose isotopomers with *Zymomonas mobilis*, a facultative anaerobic bacterium that produces the hopanoids.[10] Carbon atoms corresponding to C-3 and C-5 of IPP (**8**) were derived, equally and, respectively, from C-2/C-5 and C-3/C-6 of glucose, whereas those corresponding to C-1, C-2, and C-4 of **8** originated from C-6, C-5, and C-4 of glucose, respectively.

To date, it is known that a variety of eubacteria[10] including Actinomycetes (soil bacteria)[2,11] and cyanobacteria proficiently utilize this pathway.[12,13] In fact, a number of bacteria solely utilize the MEP pathway for the biosynthesis of isoprenoids and do not carry the genetic information for the mevalonate pathway.[14] Many eukaryotic organisms, including a number of protists (i.e. plasmodium,[15] red and green algae[16]), as well as plants, including non-vascular varieties such as liverwort[17] and higher-order plants,[18] can use this alternative pathway. More detailed information on the MEP pathway can be found in review articles by Eisenreich *et al.*,[19] Rohmer,[20] Kuzuyama and Seto,[2] and others.

In contrast to the mevalonate pathway, the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway (also known as the deoxyxylulose phosphate (DOXP) pathway)[21] involves a direct condensation of pyruvate (**10**) with glyceraldehyde 3-phosphate (**11**) to form deoxyxylulose 5-phosphate (**12**), which is converted to IPP (**8**) and DMAPP (**9**) by a number of biosynthetic steps (Scheme 2B). First, **12** undergoes rearrangement coupled to a reduction step to give 2-*C*-methyl-D-erythritol 4-phosphate (**13**),[22] which is subsequently converted into its cyclic diphosphate (**14**), mediated by unique activation and cyclization reactions.[19] The resulting 2-*C*-methyl-D-erythritol 2,4-diphosphate (**14**) is converted into **8** and **9** through 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate (**15**).

The isotope incorporation and labeling patterns in IPP derived via the mevalonate and the non-mevalonate pathways are characteristically different (Table 1), making it possible to distinguish these two pathways by feeding experiments. Incorporation of [2-^{13}C]pyruvate, [1-^{13}C]acetate, and [5-^{13}C]glucose would normally give IPP labeled at C-1 and C-3 in the mevalonate pathway, and C-2 and C-3 in the MEP pathway. Feeding experiments with [3-^{13}C]pyruvate, [2-^{13}C]acetate, and [6-^{13}C]glucose would give IPP labeled at C-2, C-4, and C-5 in the mevalonate pathway and C-1 and C-5 in the MEP pathway. An isotopic label at C-1 of pyruvate or C-4 of glucose is normally not incorporated into the IPP product of the mevalonate pathway, as this carbon is cleaved off during pyruvate decarboxylation to give acetate (Scheme 2). This is also true for C-3 of glucose, when the latter is metabolized via the Embden–Meyerhof pathway, e.g. in *E. coli*.[19] In *Z. mobilis*, however, glucose catabolism occurs via the Entner–Doudoroff pathway, in which C-3 of glucose would eventually becomes C-2 of acetyl-CoA and C-1 of glucose is lost during pyruvate decarboxylation.[20] In the MEP

Table 1 Expected ^{13}C labeling patterns in isopentenyl diphosphate from either the mevalonate pathway or the MEP pathway after feeding experiments with various precursors

	Predicted [^{13}C]IPP	
	Mevalonate pathway	MEP pathway
[1-^{13}C]pyruvate or [4-^{13}C]glucose [3-^{13}C]glucose (via E–M pathway)[a]		
[2-^{13}C]pyruvate or [1-^{13}C]acetate or [5-^{13}C]glucose or [2-^{13}C]glucose (via E–M pathway)[a]		
[3-^{13}C]pyruvate or [2-^{13}C]acetate or [6-^{13}C]glucose or [1-^{13}C]glucose (via E–M pathway)[a]		
[3-^{13}C]glucose (via E–D pathway)[b]		
[2-^{13}C]glucose (via E–D pathway)[b]		
[1-^{13}C]glucose (via E–D pathway)[b]		

[a] E-M = Embden–Meyerhof.
[b] E-D = Entner–Doudoroff.

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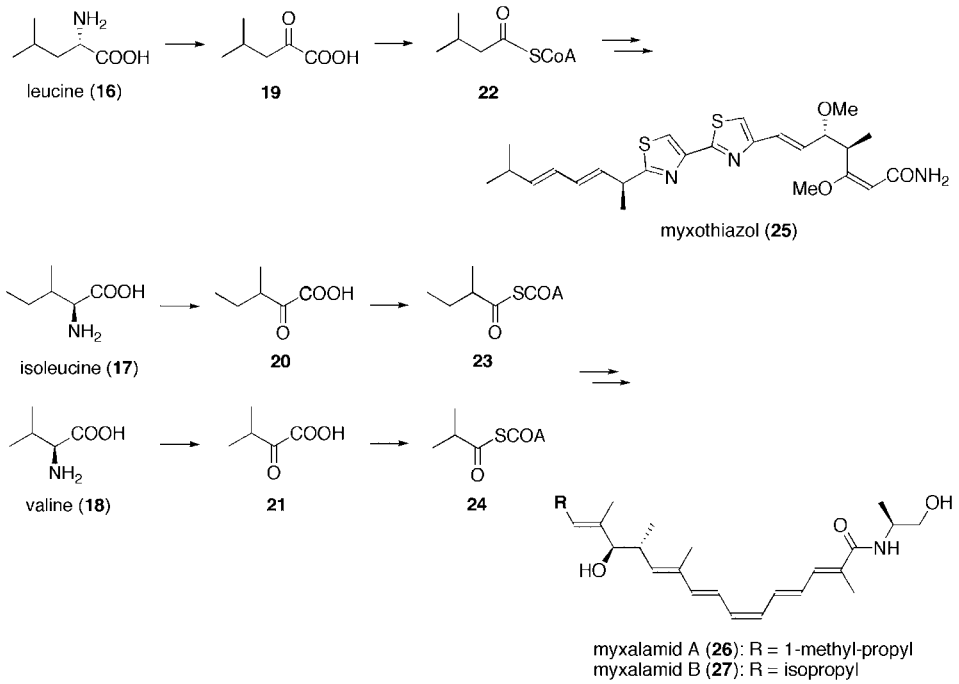
pathway, however, C-3 of glucose is incorporated via the Embden–Meyerhof and Entner–Doudoroff pathways into C-4 and C-5 of IPP, respectively (Table 1).

The convergence of the mevalonate pathway and branched-chain fatty acid biosynthesis

In addition to the discovery of the MEP pathway, isotopic feeding experiments have been instrumental in the elucidation of a shunt pathway between the mevalonate pathway and branched-chain fatty acid biosynthesis. While the mevalonate pathway has been recognized as one of the pathways to IPP (**8**) and DMAPP (**9**), recent studies in a number of microorganisms from the class of myxobacteria indicated the presence of a novel shunt pathway that also connects the mevalonate pathway and branched-chain fatty acid biosynthesis.[23–26] Branched-chain fatty acids are important components of cellular membranes in many bacteria; they constitute more than half of the cellular fatty acids in myxobacteria and a number of Gram-positive bacteria. In general, they are important for the control of cell membrane fluidity, but in myxobacteria they are also involved in cell–cell communication and signaling during development. Isovaleryl-CoA (**22**), 2-methylbutyryl-CoA (**23**), and isobutyryl-CoA (**24**) serve as primers in the biosynthesis of branched-chain fatty acids and some secondary metabolites (e.g. myxothiazol (**25**) and the myxalamids (**26** and **27**)). They are normally derived from the branched-chain amino acids leucine (**16**), isoleucine (**17**), and valine (**18**), respectively (Scheme 3). An aminotransferase is involved in the conversion of the branched-chain amino acids to α-keto acids and an evolutionarily conserved multi-enzyme assembly, the branched-chain α-keto acid dehydrogenase complex (Bkd), is responsible for the oxidative decarboxylation of the keto acids.

Interestingly, when this normal branched-chain amino acid degradation pathway was inactivated in the myxobacterium *Stigmatella aurantiaca*, an alternative biosynthetic pathway to isovaleryl-CoA appeared to be turned on.[23] Feeding experiments with DL-[$^2H_{10}$]leucine to the wild-type strain confirmed that the wild type utilizes leucine to make isovaleryl-CoA (**22**). However, an analogous experiment with the *bkd* mutant, in which the branched-chain α-keto acid dehydrogenase complex gene had been inactivated, revealed that the mutant retains the production of all its branched-chain fatty acids and of the secondary metabolite **25**, albeit at a reduced level. Feeding experiments with [1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]-acetate showed that leucine (**16**) was no longer the immediate precursor of the starter unit, which is instead derived from acetate through a novel branch of the mevalonate pathway.[23] These findings indicate the existence of a *bkd*-independent pathway for the production of these fatty acid species. Further feeding experiments with [4,4,4,5,5,5-$^2H_6$]dimethylacrylate,



Scheme 3 Formation of branched-chain fatty acid precursors and their involvement in myxothiazol and the myxalamids biosynthesis by myxobacteria.

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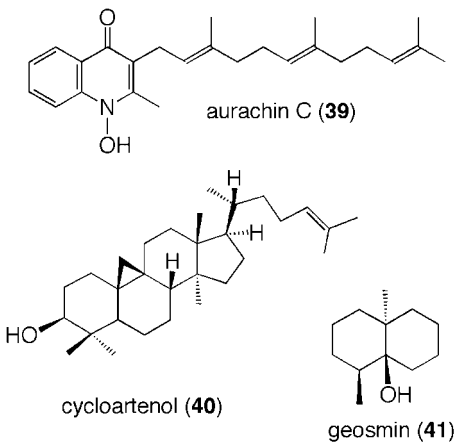
the deuterated version of **32**, and 3-[4,4,5,5,5-$^2H_5$]methylbut-3-enoate, the deuterated version of **30**, to the *bkd* mutant resulted in the incorporation of both compounds into myxothiazol (**25**) (Scheme 4). These results shed light on a novel metabolic route that links the mevalonate pathway with isovaleryl-CoA biosynthesis in myxobacteria.[24]

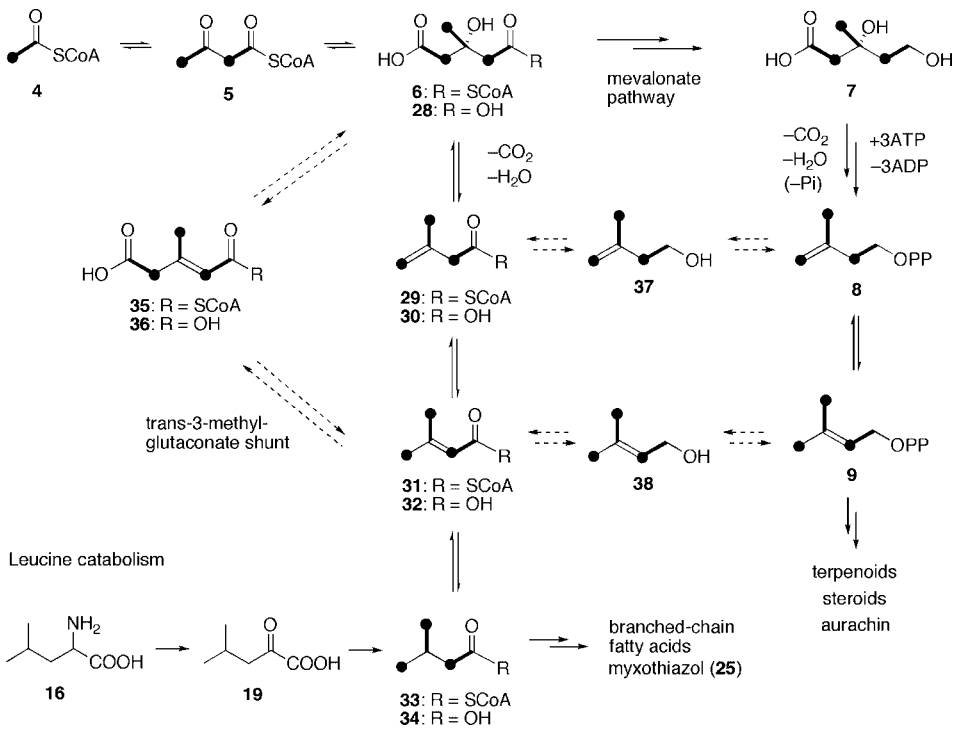
The novel shunt pathway connecting the mevalonate pathway and branched-chain fatty acids was found to be reversible. In many organisms, leucine is catabolized to acetyl-CoA before entering the mevalonate pathway. However, in some species of *Leishmania*, leucine was found to be efficiently incorporated as intact isovaleric acid backbone into isoprenoids.[27] Similar results were found regarding the production of aurachin C (**39**) by the myxobacterium *S. aurantiaca* Sg a15.[24] Aurachin C (**39**) is a quinoline alkaloid containing a sesquiterpene side chain that is normally derived from the mevalonate pathway (Scheme 5). Feeding experiments with [4,4,4,5,5,5-$^2H_6$]dimethylacrylate, 3-[4,4,4,5,5-$^2H_5$]methylbut-2-enoate, and [U-$^2H_9$]isovalerate revealed that these compounds were incorporated intact into aurachin C (**39**), while 3-methylglutaconic acid (**36**), isopentenyl alcohol (**37**), and dimethylallyl alcohol (**38**) were not (Scheme 4). The results provide evidence for the participation of the former compounds in the biosynthesis of isoprenoids, indicating a direct link between isovaleryl-CoA and HMG-CoA, without the involvement of acetyl-CoA. Very similar results were also obtained for the incorporation of isotopes into cycloartenol (**40**)[25] and geosmin (**41**) in *Myxococcus xanthus*,[26] suggesting that this novel shunt pathway may be widely distributed among myxobacteria.

The involvement of HMG-CoA synthase-like enzymes in polyketide biosynthesis

Polyketide natural products represent a major fraction of bioactive secondary metabolites found in plants, bacteria, blue-green algae, and fungi.[28] They are synthesized via a chain elongation process resembling



Scheme 5 Chemical structures of isoprene-derived secondary metabolites from myxobacteria.



Scheme 4 Proposed shunt pathway that connects the mevalonate pathway and branched-chain fatty acid biosynthesis.

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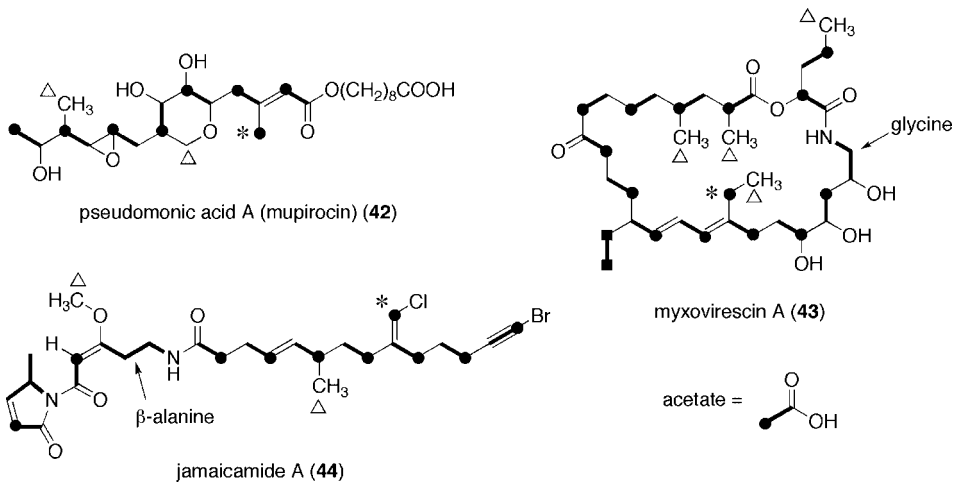
that of fatty acid biosynthesis by a set of complex enzymes called polyketide synthases. The backbone structures are normally derived from acetate (malonyl-CoA) and propionate (methylmalonyl-CoA), and in some cases, also ethylmalonyl- and methoxymalonyl-CoA.[28] Recent studies by feeding experiments with isotope-labeled precursors have shown the involvement of 1,3-bisphosphoglycerate as the source of the methoxymalonyl moiety in the biosynthesis of ansamitocin and soraphen A.[29]

Unique tailoring modifications of the polyketide backbone occur either during or after ketide assembly and add a repertoire of diversity and biological activity to polyketide natural products. Many polyketide natural products contain pendant methyl groups in their structures. Most of these methyl groups are derived from C-3 of propionate or as a result of the activity of *S*-adenosylmethionine-dependent C-methyltransferases. However, through isotope tracer investigations, a new mechanism has been increasingly appreciated as an alternative means of pendant methyl group formation on polyketide backbones. Biosynthetic studies by feeding experiments with a number of polyketide-derived natural products revealed that certain pendant, sometimes functionalized, methyl groups are not derived from C-3 of propionate or *S*-adenosylmethionine or other common C-methyl donors. Instead, they are donated by C-2 of acetate. This has been observed in the biosynthesis of the pseudomonic acids (mupirocin) (**42**) in *Pseudomonas fluorescens*,[30] the myxoveriscins (antibiotic TA) (**43**) in *M. xanthus*,[31] and of the jamaicamides (**44**) in the marine cyanobacterium *Lyngbya majuscula* (Scheme 6).[32] The results have prompted bioorganic chemists to scrutinize the formation of these pendant functional groups at the molecular level, taking advantage of advanced molecular genetic methodologies. A number of homologous genes believed to be involved in this process have been identified in several biosynthetic gene clusters of antibiotics, e.g. leinamycin,[33] the jamaicamides,[32] the curacins,[34] myxoveriscin,[35,36] mupirocin,[37] pederin,[38] and bacillaene.[39] Among other unique genetic features, those clusters commonly contain freestanding homologues of HMG-CoA synthase, which catalyzes the aldol condensation of acetoacetyl-CoA (**5**) and acetyl-CoA (**4**) to form HMG-CoA (**6**) in the mevalonate pathway (Scheme 2). *In vitro* studies by Walsh and Sherman and their co-workers in the pksX (**45**, bacillaene) and the curacin A pathways, respectively, have demonstrated the functional roles of proteins involved in the addition of C-2 of acetate onto the polyketide chain to generate a pendant functional group.[40,41] Walsh and co-workers successfully reconstituted a set of proteins from the pksX cluster and demonstrated the catalytic sequence of those proteins by using differentially radiolabeled malonyl-CoA analogs (Scheme 7).[41] The critical role of HMG-CoA synthase homologues in pendant functional group formation in polyketide biosynthesis has also been confirmed recently by Simunovic and Müller through an *in vivo* study of myxovirescin biosynthesis by *M. xanthus*.[42]

Biosynthesis of BE-40644, a hybrid isoprenoid–cyclitol-derived natural product

BE-40644 (**49**) (Scheme 8) is an isoprenoid-derived inhibitor of the human thioredoxin system produced by *Actinoplanes* sp. A40644.[43] Isotope tracer experiments



Scheme 6 Labeling patterns observed in mupirocin, myxovirescin A, and jamaicamide A after feeding experiments with [1,2-$^{13}C_2$]acetate and other potential precursors. Triangles indicate functional moieties that are derived from *S*-adenosylmethionine; asterisks indicate pendant functional moieties that are derived from C2 of acetate; connected squares indicate an ethyl moiety that is derived from C2 and C3 of succinate.

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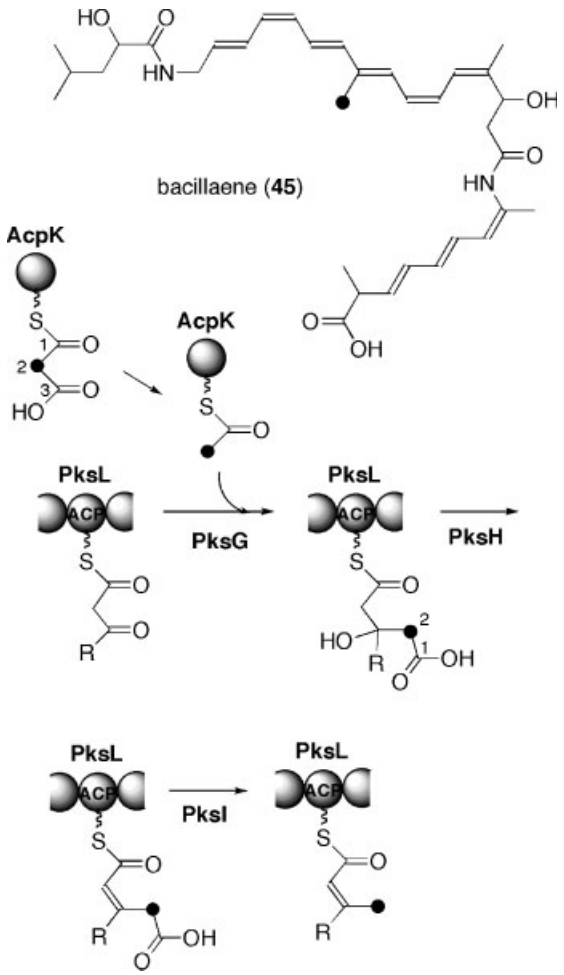


Scheme 7 Chemical structure of bacillaene and the proposed pendant methyl group formation via a HMG-CoA synthase-like mechanism during bacillaene biosynthesis. Acpk, thiolation domain; PksL, the 2nd PKS module of the bacillaene cluster; PksG, HMG-CoA synthase homologue; PksH and PksI, enoyl-CoA hydratases.

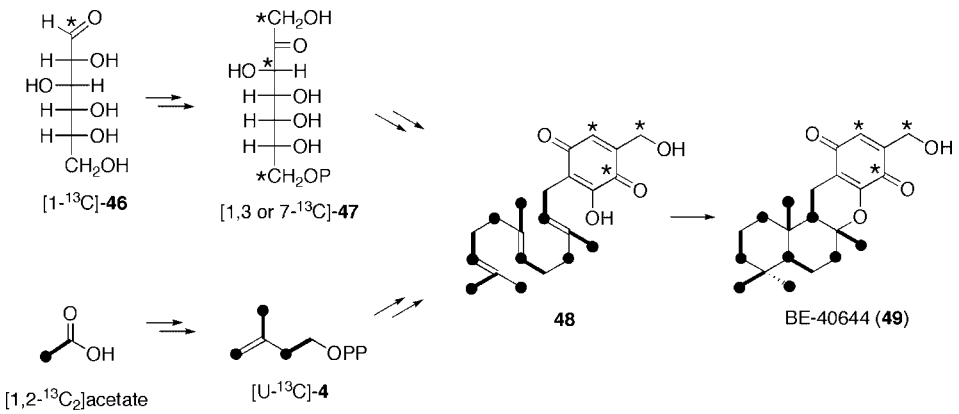
with [1-^{13}C]acetate and [1-^{13}C]glucose revealed that the isoprenoid portion of the compound is derived from the classical mevalonate pathway, whereas the cyclitol is derived from a pathway similar to those reported for some C_7N-aminocyclitol-containing natural products.[44]

The C_7N-aminocyclitol-containing natural products,[45] e.g. acarbose (**50**), validamycin A (**51**), cetoniacytone (**52**), and pyralomicin (**53**), were initially considered to be the aliphatic version of the aromatic C_7N-units found in many antibiotic natural products, e.g. rifamycin (**54**), mitomycin (**55**), and geldanamycin (**56**) (Scheme 9), which are derived from the aminoshikimate pathway – a metabolic pathway parallel to the shikimate pathway.

In 1987, Floss[46] and Rinehart[47] independently reported the results of their feeding experiments with [U-$^{13}C_3$]glycerol (**57**) and [U-$^{13}C_6$]glucose ([U-$^{13}C_6$]-**46**) to the acarbose and validamycin A producers, respectively, in which they concluded that the C_7N-aminocyclitol in both compounds are not derived from the aminoshikimate pathway or any branch of the shikimate pathway, but rather from a branch of the pentose–phosphate pathway. The new pathway gave a unique C_2–C_2–C_3 labeling pattern in the C_7N moiety as opposed to the characteristic C_3–C_4 correlation of the shikimate pathway (Scheme 10). Although the initial results shed some light on the biosynthetic origin of acarbose (**50**) and validamycin A (**51**), it was not until more recent experimental evidence emerged to support the existence of alternate biosynthetic pathways involving 2-*epi*-5-*epi*-valiolone (**59**) as a common intermediate.

The discovery of the 2-*epi*-5-*epi*-valiolone pathway

Based on the evidence from their feeding studies, Rinehart and co-workers proposed valiolone (**60**) as a



Scheme 8 Proposed biosynthetic pathway to BE-40644 based on feeding experiments with [1,2-$^{13}C_2$]acetate and D-[1-^{13}C] glucose.

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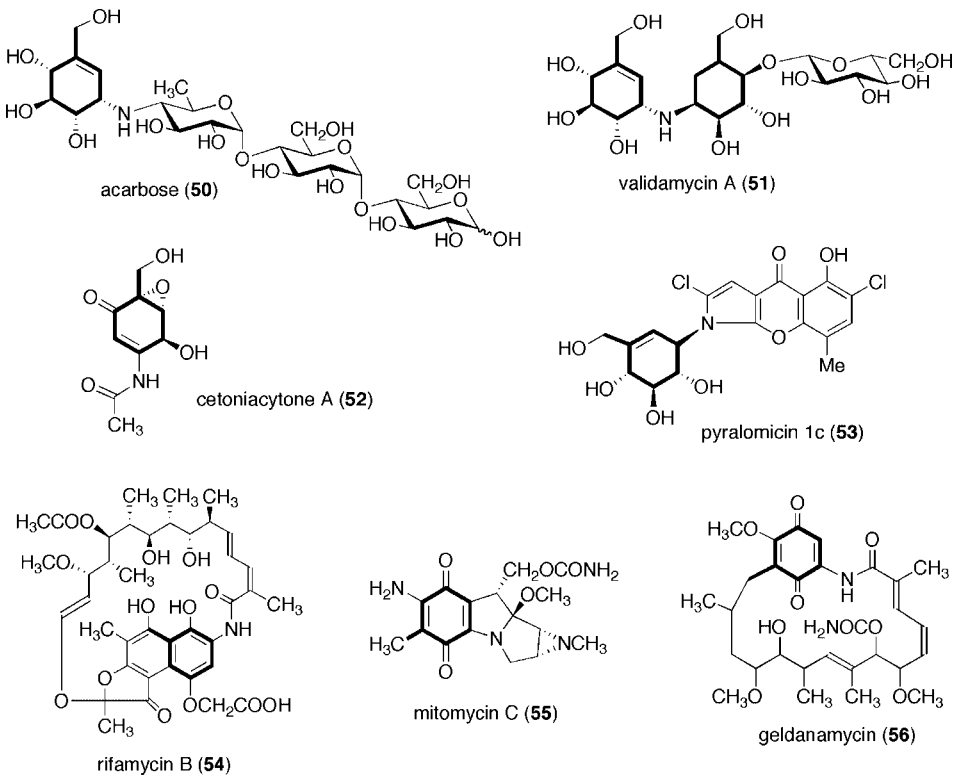

Scheme 9 Chemical structures of C_7N unit-containing natural products.

potential cyclization product of the pentose–phosphate pathway-derived heptulose 7-phophate. At the time, this prediction was entirely logical given that all stereocenters in valiolone are consistent with those in the valienamine (**61**) moiety of acarbose and validamycin A. Furthermore, additional metabolites isolated from the validamycin producer, *S. hygroscopicus* var. *limoneus*, including valiolamine (**62**), the amino analog of **60**, together with valienamine (**61**) and validamine (**63**) also retained this stereochemistry (Scheme 11). To investigate this prediction, Floss and co-workers prepared radio and stable isotopically labeled valiolone (**60**), valienamine (**61**), valiolamine (**62**) and validamine (**63**) and fed them to the acarbose- and validamycin-producing bacteria.[48,49] Surprisingly, none of these compounds were incorporated into acarbose (**50**) or validamycin A (**51**). After extensive synthetic studies of and feeding experiments with other analogs of valiolone, it was finally found that one of the epimers of valiolone, 2-*epi*-5-*epi*-valiolone (**59**), was incorporated into **50** and **51**.[48–51]

2-*epi*-5-*epi*-Valiolone (**59**) appeared to be a common intermediate for all C_7N-aminocyclitol-derived natural products.[50,51] However, it appears that downstream of this initial step the pathways to various aminocyclitols involve different intermediates. For example, 5-*epi*-[6,6-$^2H_2$]valiolone ([6,6-$^2H_2$]-**64**), [6-^{2}H]valienone ([6-^{2}H]-**65**), and [6-^{2}H]validone ([6-^{2}H]-**66**) were found to be incorporated into validamycin A (**51**), but not into acarbose (**50**).[48,49] More recent biochemical analysis of enzymes involved in the biosynthesis of acarbose (**50**) and validamycin (**51**) has revealed the presence of two distinct kinases operating in the two systems that may explain the observed discrepancies in the results of the feeding experiment. The kinase in acarbose biosynthesis (AcbM) has been shown to phosphorylate 2-*epi*-5-*epi*-valiolone (**59**), suggesting that all other downstream intermediates in the pathway are phosphorylated compounds.[52] However, the kinase in validamycin biosynthesis (ValC) only recognized valienone (**65**) and validone (**66**) as substrates, not 2-*epi*-5-*epi*-valiolone (**59**) or 5-*epi*-valiolone (**64**),[53] suggesting that phosphorylation plays a role further downstream in the biosynthesis of validamycin when compared with acarbose.

Probing the mechanism of 2-*epi*-5-*epi*-valiolone synthase using isotopically labeled substrates

To establish the C7 sugar phosphate precursor of 2-*epi*-5-*epi*-valiolone (**59**), Lee *et al.* prepared D-*sedo*-[1-^{13}C]heptulose 7-phosphate and *ido*-[1-^{13}C]heptulose 7-phosphate from ribose 5-phosphate and xylose 5-phosphate,[54] respectively. The products were incubated

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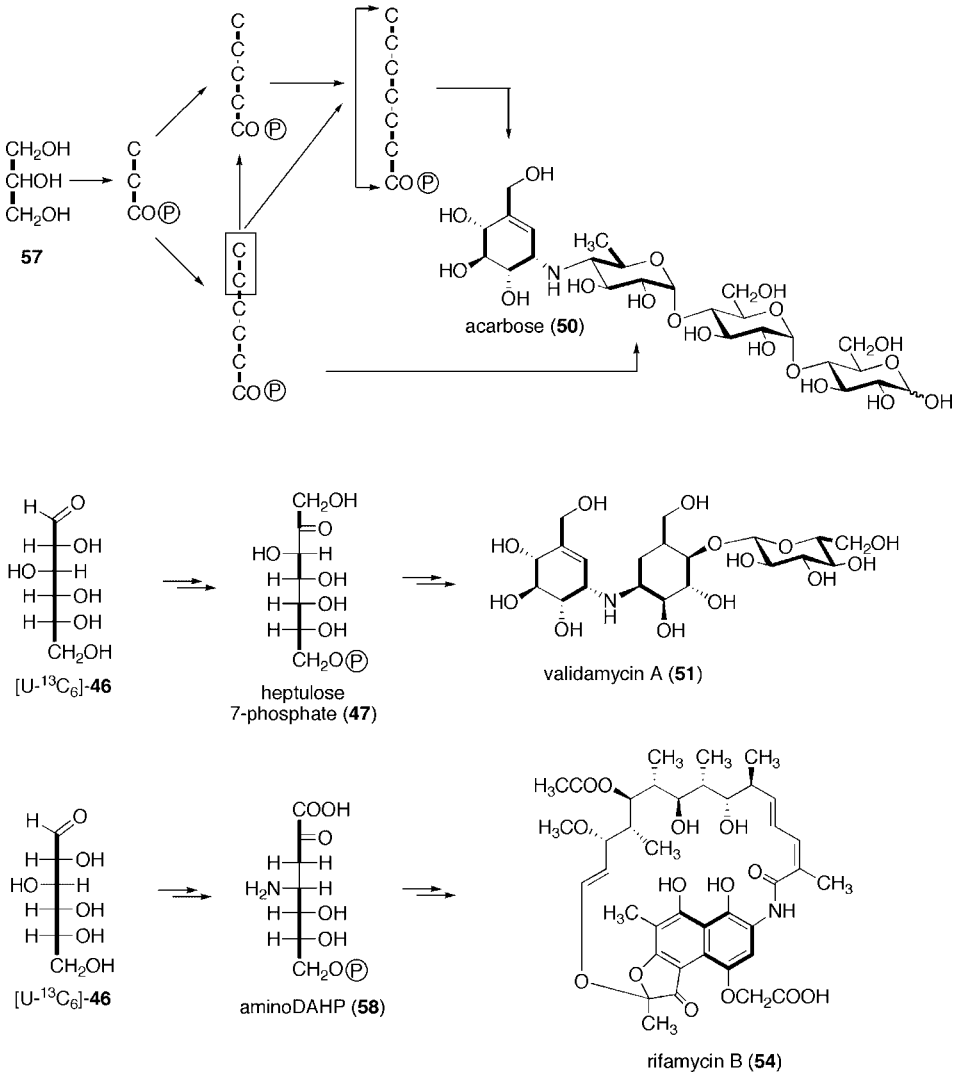

Scheme 10 Biosynthetic origin of the C_7N units of acarbose, validamycin A, and rifamycin B.

with recombinant 2-*epi*-5-*epi*-valiolone synthase (AcbC). The enzyme takes D-*sedo*-heptulose 7-phosphate, but not *ido*-heptulose 7-phosphate, as substrate, which directly confirmed the involvement of the pentose phosphate pathway.[55] There are two principal mechanisms that have been described for the cyclization of open-chain sugar phosphates to cyclitols. One is exemplified by the DHQ synthase reaction of the shikimate pathway, and the other by the cyclization of glucose 6-phosphate catalyzed by *myo*-inositol 1-phosphate synthase. The latter mechanism, when applied to the synthesis of the acarbose precursor, predicts the loss of both hydrogens from C-7 of the substrate, whereas in a DHQ synthase-like cyclization, these two hydrogen atoms would be retained in the product (Scheme 12).

To investigate which of the above possibilities is more likely, AcbC, the acarbose 2-*epi*-5-*epi*-valiolone synthase, was incubated with double radiolabeled D-*sedo*-[7-^{14}C, 7-^{3}H]heptulose 7-phosphate ([7-$^{3}H_2$, 7-^{14}C]-**47**) and the $^{3}H/^{14}C$ ratio of the substrate and the product were measured (Scheme 12). D-*sedo*-[7-^{14}C, 7-^{3}H]Heptulose 7-phosphate was enzymatically prepared with transketolase from xylulose 5-phosphate and D-[5-^{14}C, 5-^{3}H]ribose 5-phosphate.[54] The change in the isotope ratio from 5.5 (in the substrate) to 3.7 (in the product) indicates 67% retention of tritium in the cyclization of the substrate to 2-*epi*-5-*epi*-valiolone (**59**), a result that supports the DHQ synthase-like mechanism for this cyclase.[55]

Exploring the steric course of 5-*epi*-valiolone dehydratase using stereospecifically labeled compounds

In parallel to the shikimate pathway, the 2-*epi*-5-*epi*-valiolone pathway in validamycin biosynthesis requires

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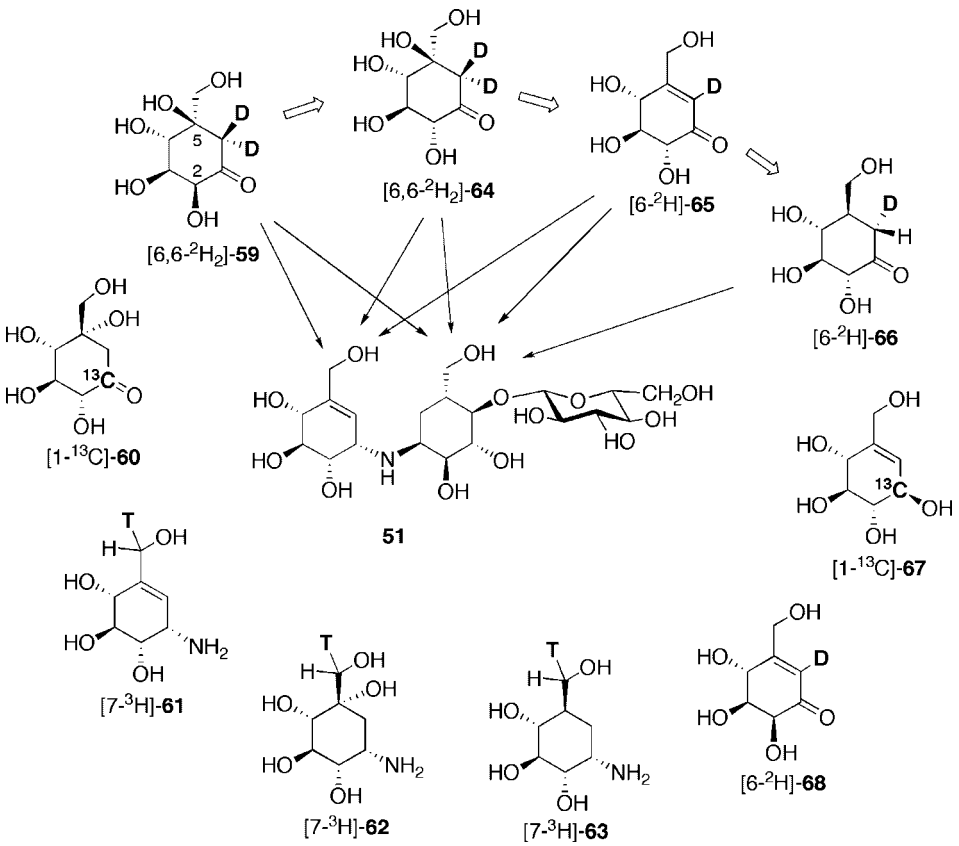

Scheme 11 Isotopically labelled precursors synthesized and fed to the acarbose and validamycin A producing bacteria. Compounds 59, 64, 65, and 66 were incorporated into validamycin A (51). Broad arrows show proposed pathway from 59 to 66 during early steps of validamycin biosynthesis.

a dehydration reaction that converts 5-*epi*-valiolone to valienone. In the shikimate pathway, dehydration of dehydroquinate (DHQ) to dehydroshikimate may be catalyzed by either one of two evolutionarily and mechanistically unrelated types of DHQ dehydratases. Type I enzymes catalyze a *syn* elimination of the elements of water, whereas the type II enzymes catalyze an *anti* elimination.[56] To probe the steric course of 5-*epi*-valiolone dehydratase, 5-*epi*-[6α-^{2}H]valiolone ([6α-^{2}H]-**64**), and 5-*epi*-[6β-^{2}H]valiolone ([6β-^{2}H]-**64**) were stereospecifically synthesized from D-glucose and fed to the cultures of *S. hygroscopicus* var. *limoneus*.[57] The key step in the synthesis involves desulfurization of the intermediate tetrabenzyl-6,6-bis(methylthio)-5-*epi*-valiolone (**78**) and stereospecific introduction of the deuterium using active Zn, $NiCl_2$, ND_4Cl/D_2O, and THF to give tetrabenzyl-5-*epi*-[6α-^{2}H]valiolone ([6α-^{2}H]-**80**) and tetrabenzyl-5-*epi*-[6β-^{2}H]valiolone ([6β-^{2}H]-**80**) (Scheme 13). The synthesis of [6β-^{2}H]-**80** began with the reduction of the first methylthio group of **78** with $Zn/NH_4Cl/H_2O$/THF to give **79** and its 6-epimer. Treatment of **79** with $Zn/ND_4Cl/D_2O$/THF in the presence of $NiCl_2$ gave [6β-^{2}H]-**80** with 93 atom% D and 81% d.e.[57] On the other hand, treatment of **78** with $Zn/ND_4Cl/D_2O$/THF gave [6-^{2}H]-**79** and its 6-epimer. Subsequent treatment of [6-^{2}H]-**79** with $Zn/NH_4Cl/H_2O$/THF and $NiCl_2$ yielded [6α-^{2}H]-**80** with 93 atom% D and 78% d.e. Deprotection of ([6α-^{2}H]-**80**) and ([6β-^{2}H]-**80**) furnished 5-*epi*-[6α-^{2}H]valiolone ([6α-^{2}H]-**64**) and 5-*epi*-[6β-^{2}H]valiolone ([6β-^{2}H]-**64**), respectively. When [6α-^{2}H]-**64** was fed to the culture of the validamycin A producer, it was found that the deuterium was incorporated (24%) into validamycin A, whereas feeding with [6β-^{2}H]-**64** only gave about 4% incorporation of deuterium, suggesting that the dehydration of 5-*epi*-valiolone occurs via a *syn*-elimination of the elements of water.

Conclusions and perspectives

Despite the broader use of contemporary molecular genetic approaches in the study of natural product biosynthesis, the classical isotope tracer experiments remain as an important avenue to identify the origin of natural products and, in several examples, to explore the steric course or catalytic mechanism of enzymes

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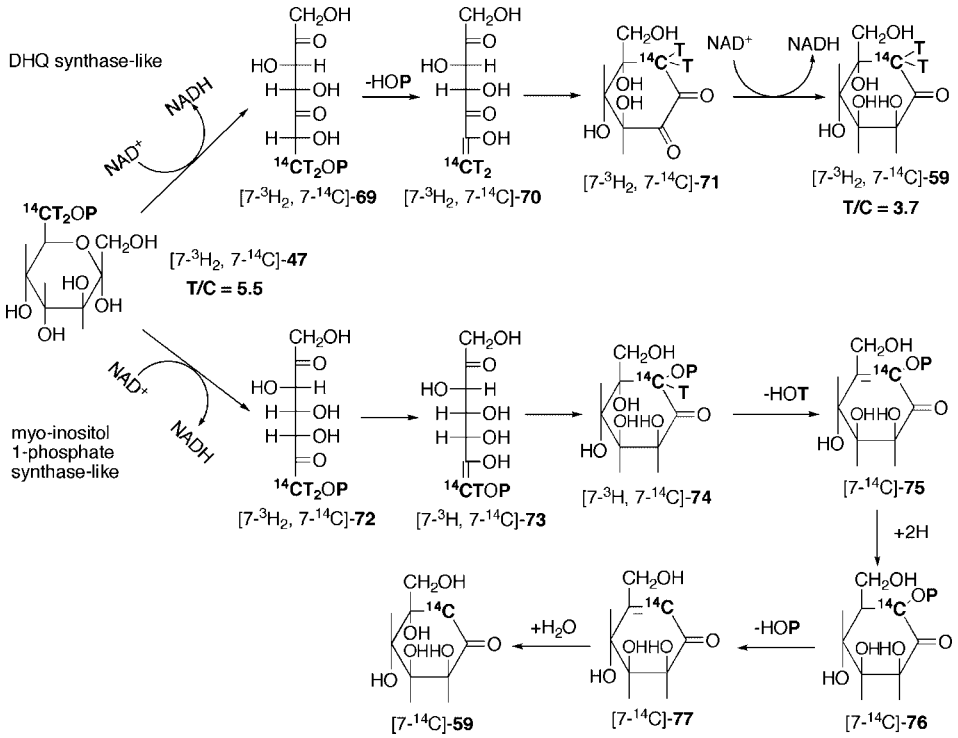
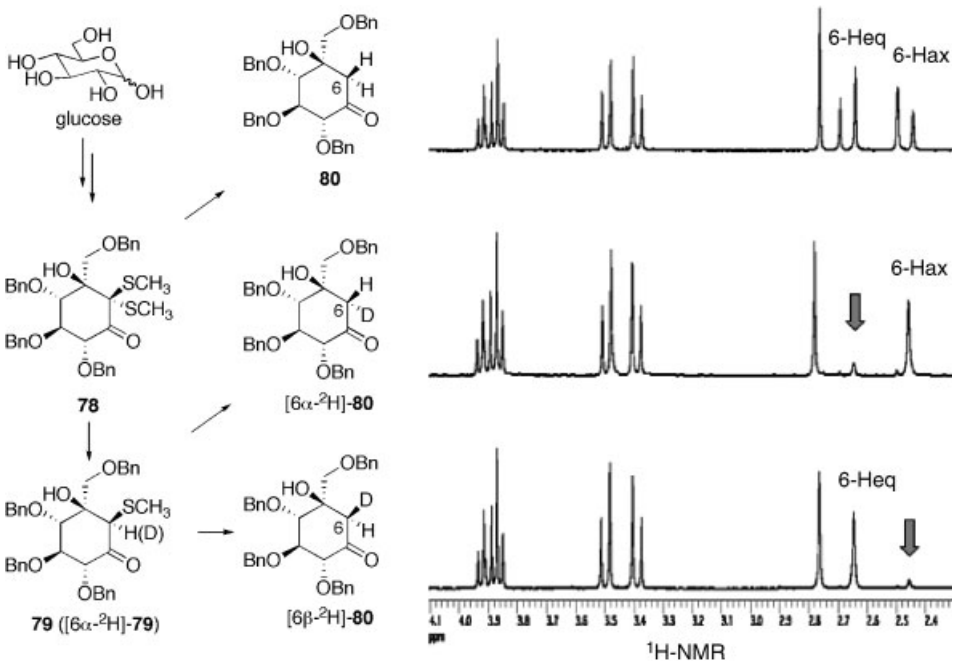


Scheme 12 Possible reaction mechanisms for 2-*epi*-5-*epi*-valiolone synthases. The change in the isotope ratio from 5.5 (in the substrate) to 3.7 (in the product) indicates 67% retention of tritium in the cyclization of the substrate to 2-*epi*-5-*epi*-valiolone (59).



Scheme 13 Synthesis and partial ^{1}H NMR spectra of stereospecifically labeled tetrabenzyl-5-*epi*-valiolones.

involved in natural product biosynthesis. Based on the results of isotope tracer experiments, a number of novel metabolic pathways have recently been identified. These include the MEP pathway, an alternative biosynthetic pathway to the building blocks of sterols and terpenoids, which is distributed across several king-

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doms including plants, bacteria, algae, and plasmodium, but not in humans or animals. This pivotal finding not only exemplifies the diversity of pathways involved in natural product biosynthesis, but also has provided new antimicrobial and antimalarial drug targets that are being actively explored using compounds such as fosmidomycin.[15,58] A number of isotope tracer studies have also confirmed close relationships between primary and secondary metabolisms, as shown in the shunt pathway that connects HMG-CoA and the branched-chain fatty acid precursor, isovaleryl-CoA. Other studies have demonstrated inter-relationships between different metabolic pathways, such as the convergence of isoprene and polyketide biosynthesis through the HMG-CoA synthase homologues.

Some natural products are produced by hybrid systems involving two or more biosynthetic pathways. Polyketide–nonribosomal peptide and isoprenoid–polyketide hybrid systems are examples that are commonly found in nature. More rare hybrid systems include the biosynthetic machinery responsible for BE-40644 formation, which consists of a combination of the mevalonate pathway and the 2-*epi*-5-*epi*-valiolone pathway. The common precursor 2-*epi*-5-*epi*-valiolone is the product of a cyclization of sedoheptulose 7-phosphate, an intermediate of the pentose phosphate pathway, catalyzed by 2-*epi*-5-*epi*-valiolone synthase. The latter enzyme is a homologue of DHQ synthase, an enzyme involved in the biosynthesis of aromatic amino acids via the shikimate pathway. Altogether isotope tracer experiments have played and continue to play a critical role in elucidating the complex interrelationships between various metabolic pathways involved in primary and secondary metabolism. Combined with the advances of science on many fronts and the availability of more powerful analytical instruments, isotope tracer experiments promise to continue to play an important role in the dissection of complex molecular systems in living organisms.

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