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Mechanisms for incorporation of glycerol-derived precursors into polyketide metabolites

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Abstract Several polyketide secondary metabolites are shown by feeding experiments to incorporate glycerol-derived 3-carbon starter units, 2-carbon extender units, or 3-carbon branches into their hydrocarbon chains. In recent years, genetic studies have begun to elucidate the mechanisms by which this occurs. In this article we review the incorporation of glycerol-derived precursors into polyketides and propose new mechanisms for the incorporation processes.

Keywords Polyketide · Glycerol · Methoxymalonyl-ACP · Lactyl-ACP · FkbH

Introduction

Polyketide metabolites, by definition, result from the biosynthetic polymerisation of acetate units (via the activated acetate equivalent malonyl-CoA) [46]. Polyketide synthases can also incorporate other substrates during the process of chain elongation and often use a starter unit other than acetyl-CoA [36, 37, 46]. The chain may be processed further by different enzymatic or non-enzymatic mechanisms, leading to cyclisation or lactonisation for instance, generating a large diversity of chemical structures [46].

The classical extender units in polyketide biosynthesis are malonyl-CoA and methylmalonyl-CoA [46]. Decarboxylative condensation of these molecules with the growing polyketide chain results in the addition of successive two-carbon units (with or without a methyl branch) to the polyketide backbone. Occasionally, other extender units are utilised, such as ethylmalonyl-CoA, which incorporates two carbon atoms and a branching ethyl group into the polyketide backbone [46]. Recently, several examples of the utilisation of glycerol-derived extender units, which result in incorporation of β-methoxy or β-hydroxy 2-carbon units into a polyketide chain, have been reported (Fig. 1) [4, 5, 14, 15, 20, 25, 26, 28, 31, 40, 41, 45, 51, 61, 63].

The boron-containing polyketides tartrolons, boromycin and aplasmomycin have been shown to incorporate 3-carbon glycerol-derived starter units (Fig. 2) [7–9, 29, 52]. Bryostatins and the lankacidins are also believed to be biosynthesised from a 3-carbon glycerol-derived starter unit, although this hypothesis awaits confirmation by feeding studies (Fig. 2) [19].

Some metabolites consist of a polyketide chain condensed with a non-polyketide moiety. In several cases, the non-polyketide moiety is a three-carbon unit, derived from glycerol, which branches the carbon chain. For example, acaterin and the agglomerins contain butenolide and tetronic acid groups, respectively, which are assembled via the condensation of a glycerol-derived 3-carbon precursor with a pentaketide and a hexaketide, respectively [34, 54–56]. In a similar manner, the tetronic acid moieties present in chlorothricin and versipelostatin arise from annealing a 3-carbon glycerol-derived precursor with a polyketide chain to append three additional branches (Fig. 3) [10, 23, 30, 33].

Butyrolactones constitute another class of compounds which result from the condensation of a branching glycerol-derived 3-carbon unit with a polyketide chain [50]. In this case, however, the branch-point involves attachment of the polyketide chain to a different carbon atom of the glycerol-derived precursor and therefore the glycerol-derived precursor is probably different (Fig. 3).

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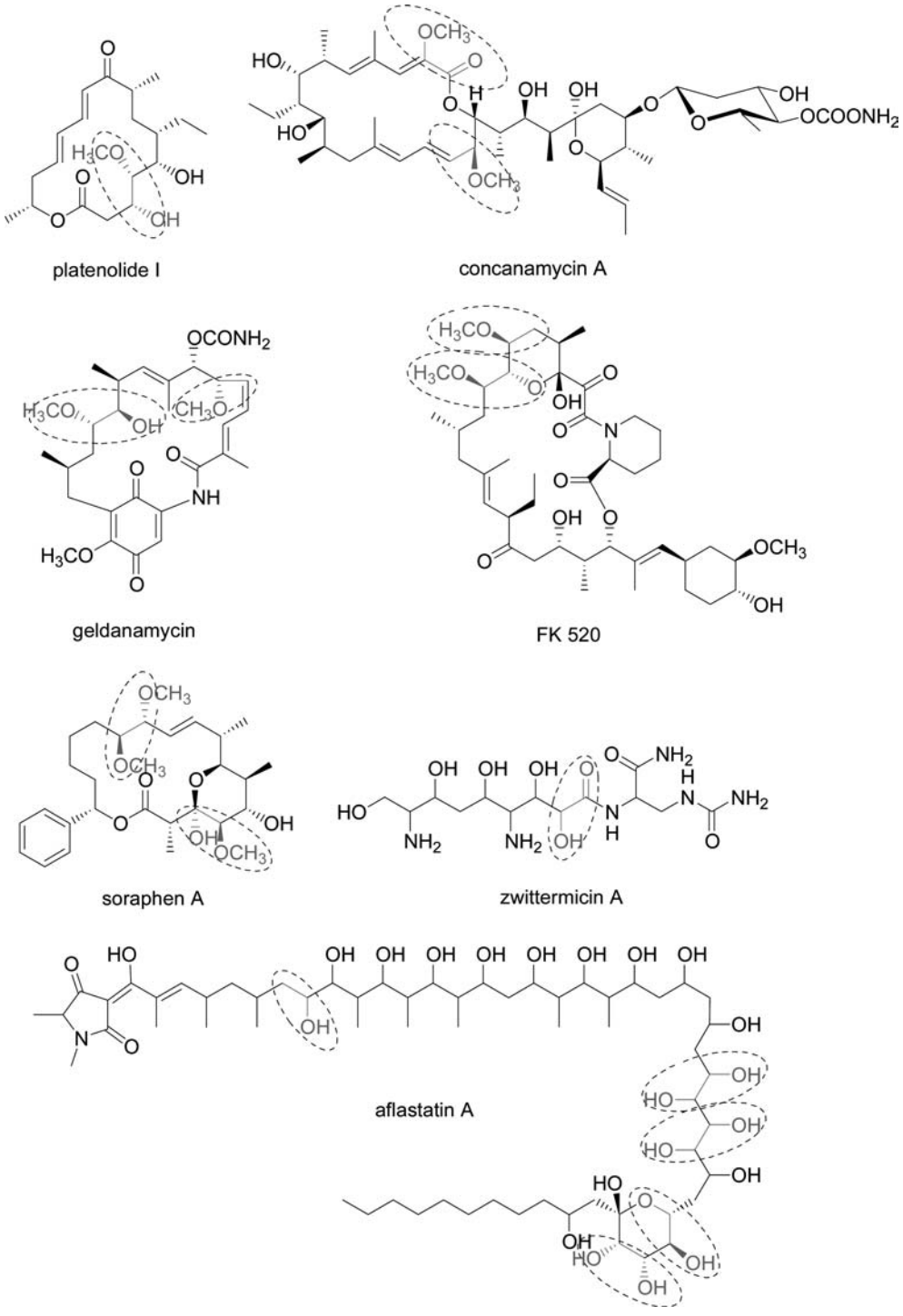


Fig. 1 Metabolites incorporating 2-carbon glycerol-derived extender units into their polyketide chains. The site(s) of incorporation into each metabolite are indicated

The biosynthesis of other secondary metabolites appears to involve the condensation of a glycerol-derived 3-carbon precursor with a polyketide chain (Fig. 3) [6]. However, in some cases, the glycerol-derived 3-carbon unit is incorporated into a larger primary metabolite, which is subsequently condensed with a polyketide chain. Indeed glycerol is a precursor in several primary metabolic pathways including the pentose phosphate pathway and the shikimate pathway [11, 37].

Through several incorporation studies over the last three decades utilising labelled precursors, and more recent genetic investigations, mechanistic insights into the incorporation of glycerol-derived 2- and 3-carbon precursors into polyketide metabolites have begun to emerge. In this article we critically review these studies,

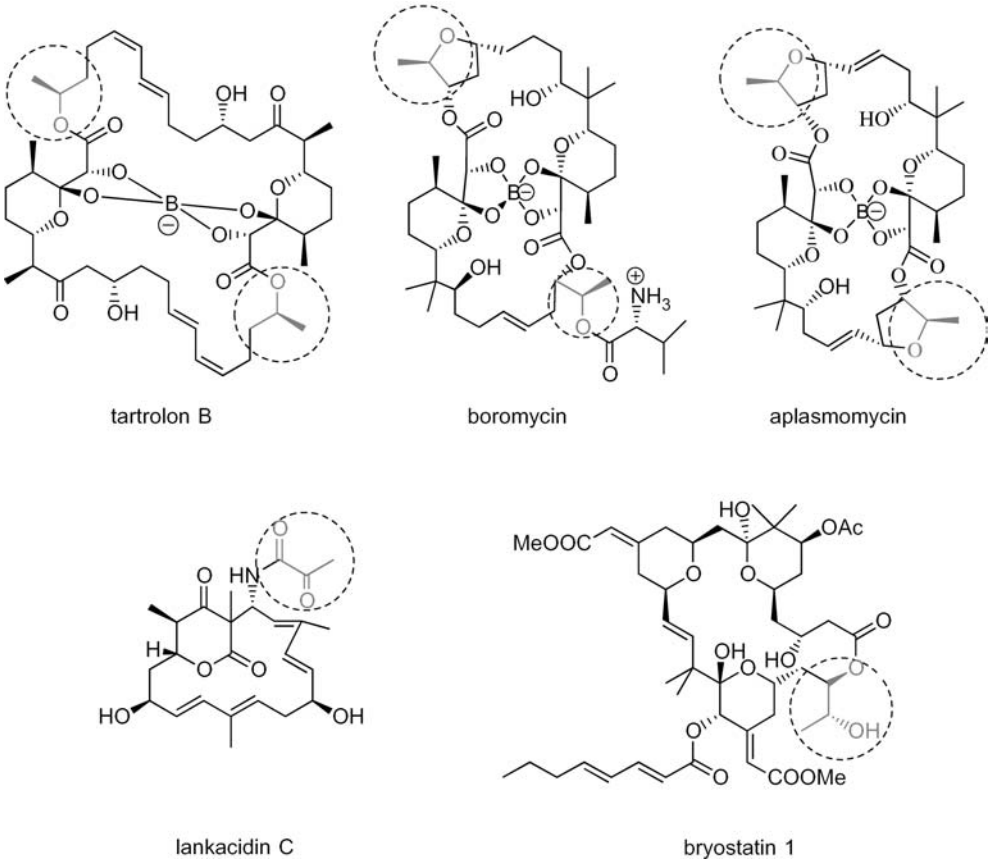


Fig. 2 Metabolites proposed to incorporate a glycerol-derived 3-carbon starter unit. The position of the starter unit is highlighted in each structure

put forward further mechanistic hypotheses and suggest directions for future research.

Incorporation of glycerol-derived 2- and 3-carbon precursors into linear polyketide chains

Glycerol-derived extender units

The classical extender units in polyketide biosynthesis result in the addition of successive two- or three-carbon units to the polyketide backbone [46]. They generally originate from malonyl-CoA or methylmalonyl-CoA. Some less common extender units, such as ethylmalonyl-CoA, can also be found, for instance in the 16-membered macrolide metabolites such as the platenolides [28]. This unusual extender originates from two molecules of acetate as shown by incorporation studies [32].

On the other hand, some polyketides incorporate another unusual extender unit, which does not originate from acetate. Leucomycins, spiramycin and niddamycin, which all share a similar 16-membered macrolide skeleton, are examples of these metabolites [25, 28, 40]. By analogy with the classical extender units, methoxymalonyl-CoA has been proposed as the unusual extender unit incorporated into such metabolites (Scheme 1).

Leucomycin biosynthesis has been examined by feeding the producing organism *Streptoverticillium kitasatoensis* with labelled precursors. These experiments revealed the origin of two ''non-acetate'' carbon atoms, which derive instead from glycerol [32, 40]. However, the true nature of the glycerol-derived glycolytic intermediate incorporated is still uncertain. It is conceivable that spiramycin and niddamycin also incorporate the same or a similar extender unit (methoxymalonyl-CoA) [25, 28]. The niddamycin gene cluster has been sequenced but the acyltransferase (AT) involved in the incorporation of methoxymalonyl extender unit was reported to fall in the methylmalonyl CoA-selective family of AT [25].

The plecomacrolides concanamycin A and bafilomycins, both V-Type ATPase inhibitors produced by different *Streptomyces* spp., are other secondary metabolites that incorporate glycerol-derived extender units within their polyketide chains. One of these unusual extender units is incorporated in the middle of the polyketide chain and another one is incorporated as the last extender unit. The derivation of these units from glycerol has been established by feeding studies [51]. Labelled 2-hydroxy- and 2-methoxymalonyl-*N*-acetylcysteamine (SNAC) thioesters have also been fed but were found not to be incorporated [51]. This result suggests that the glycerol-derived extender unit resides on an acyl carrier protein.

With the development and understanding of polyketide synthase (PKS) genetics, the gene clusters that direct biosynthesis of some ansamycin antibiotics, which incorporate two glycerol-derived extender units [15, 18], have been investigated [45, 63]. A set of five contiguous

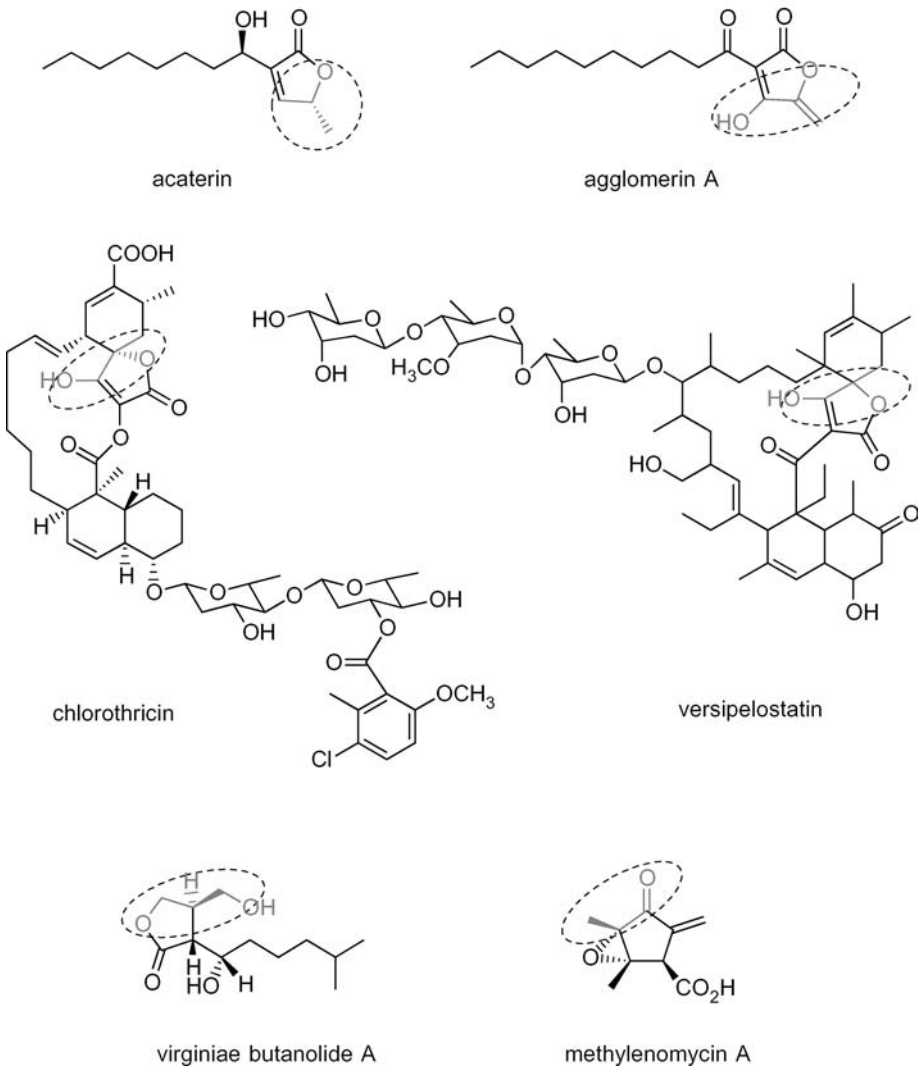


Fig. 3 Metabolites that consist of branching glycerol-derived 3-carbon precursors fused to a polyketide chain. The site of incorporation of the 3-carbon precursor is highlighted in each metabolite

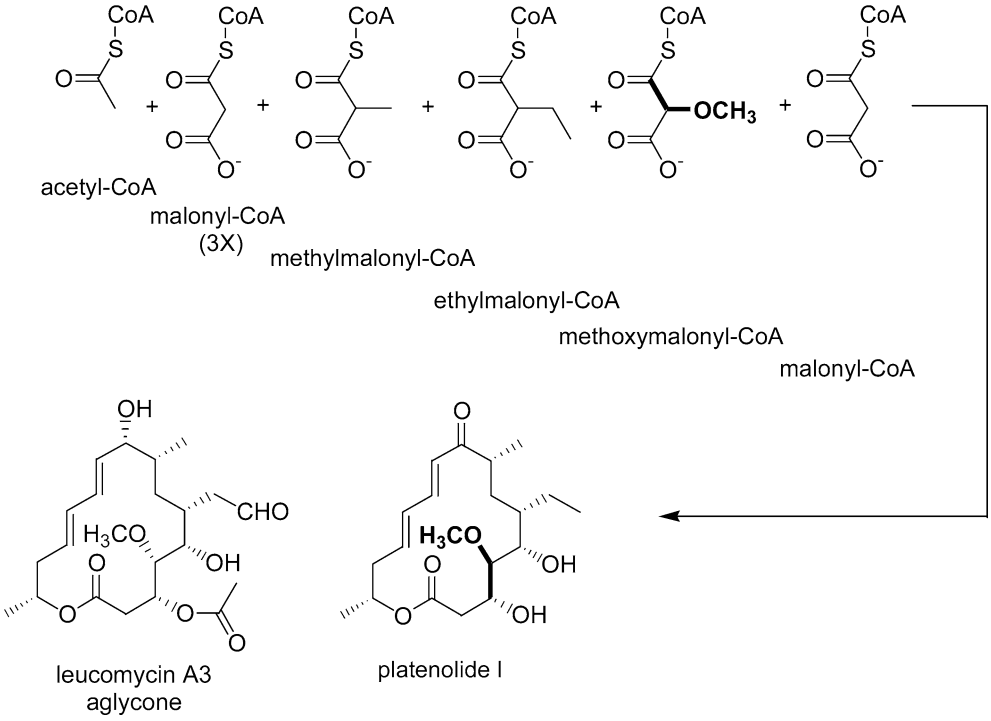
genes *asm13-17* were identified in the ansamitocin biosynthetic gene cluster [5]. Using mutagenesis and feeding experiments, this set was found necessary and sufficient for methoxymalonyl extender unit biosynthesis proposed to culminate in methoxymalonyl-ACP [5, 26]. The putative methoxymalonyl-ACP-utilising PKS modules involved in the biosynthesis of geldanamycin and ansamitocin have been identified, sequenced, analysed and utilised in biosynthetic engineering experiments [5, 26, 42, 45, 63].

FK520 (ascomycin), a secondary metabolite of *Streptomyces hygroscopicus*, is another polyketide that incorporates a glycerol-derived extender unit [4]. The gene cluster that directs its biosynthesis has been analysed and the crystal structure of an acyl-ACP dehydrogenase proposed to be involved in the biosynthesis of methoxymalonyl-ACP has been reported [60, 61]. This crystal structure suggests that the enzyme utilises an acyl-ACP substrate rather than acyl-CoA substrate providing further evidence that the unusual extender unit is methoxymalonyl-ACP [60]. A set of five contiguous genes, *fkbGHIJK*, homologous to *asm13-17* was identified in the FK520 biosynthetic gene cluster [61]. FkbH was proposed to load a glycerol-derived glycolytic intermediate onto an ACP (FkbJ) to form glyceryl-FkbJ, which is then successively oxidised by FkbK and by FkbI to yield hydroxymalonyl-FkbJ. Finally, FkbG is proposed to *O*-methylate hydroxymalonyl-FkbJ to give methoxymalonyl-ACP (Scheme 2) [60, 61].

FK506 (tacrolimus), a metabolite also produced by *S. hygroscopicus*, shares a very similar backbone with FK520. It is also very likely to incorporate two adjacent methoxymalonyl-ACP extender units. A set of five genes, similar to *fkbGHIJK* and responsible for methoxymalonyl-ACP biosynthesis, is expected to reside within the FK506 biosynthetic gene cluster [38], although this hypothesis cannot be confirmed until the sequence of the cluster has been completed.

The biosynthesis of the potent antifungal polyketide soraphen A has been investigated by incorporation experiments [20]. Two glycerol-derived extender units were found to be incorporated by feeding experiments and hydroxymalonate was excluded as an immediate precursor [20]. The gene cluster directing soraphen A

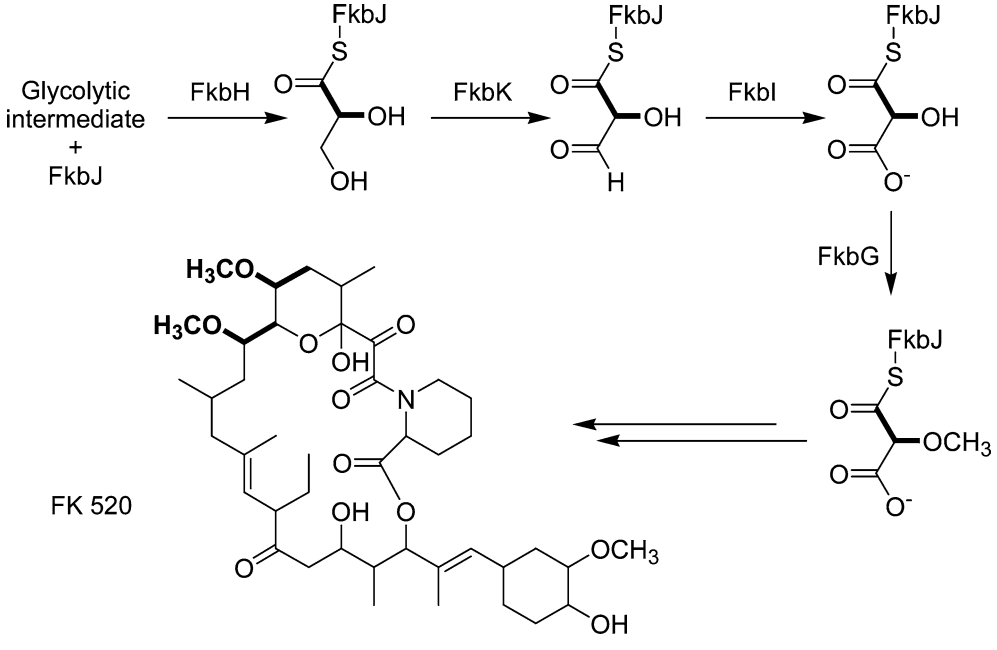
Scheme 1 Proposed biosynthetic origin of platenolide I—the aglycone of spiramycin and niddamycin—and the structure of leucomycin A3 aglycone



biosynthesis has been sequenced and analysed, but the enzymes thought to be involved in the incorporation of methoxymalonyl-ACP into soraphen are quite different from the above examples [31, 53]. No FkbH (unknown function) and no FkbG (methyltransferase) homologue appear to be encoded within the gene cluster. Instead, *sorC* encoding a three-domain protein (AT, ACP and methyltransferase) has been proposed to be involved [31]. This suggests that SorC may be involved in loading and methylating a precursor of the hydroxy/methoxymalonyl moiety, which is then converted into methoxymalonyl-ACP. The different nature of the enzymes involved in methoxymalonyl-ACP biosynthesis in the soraphen A pathway could be explained by the origin of this metabolite from the non-actinomycete *Sorangium cellulosum*.

Other metabolites incorporate glycerol-derived extender units that are hydroxylated at the β-carbon, but not methylated, such as the aflastatins [41], inhibitors of aflatoxin biosynthesis produced by *Streptomyces* sp.

Scheme 2 Proposed pathway for biosynthesis of methoxymalonyl-ACP and utilisation as an extender unit in FK-520 biosynthesis



MRI142, and blasticidin [49], which has a similar polyketide skeleton to aflastatin A. Incorporation experiments revealed that five 2-carbon units of aflastatin A originated from γ[1-^{13}C]glycolic acid [41]. However no genetic studies of aflastatin biosynthesis have yet been reported.

The biosynthesis of the linear aminopolyol antibiotic zwittermicin A, produced by *Bacillus cereus*, has also been investigated [14, 57]. The genes involved in the formation of hydroxymalonyl- and aminomalonyl-ACP intermediates incorporated into the mixed nonribosomal peptide/polyketide backbone of the molecule are of particular interest. However only three out of the four genes proposed to be involved in the formation of hydroxymalonyl-ACP in the FK-520 cluster have yet been identified: *orfs1, 3* and *4*, which encode homologues of FkbI, FkbJ and FbK, respectively [14]. A malonyl CoA ACP transacylase, encoded by *orf2*, is proposed to catalyse acylation of the ACP encoded by *orf3* with glyceryl CoA [14], although the biosynthetic origin of glyceryl CoA remains unclear (Scheme 3).

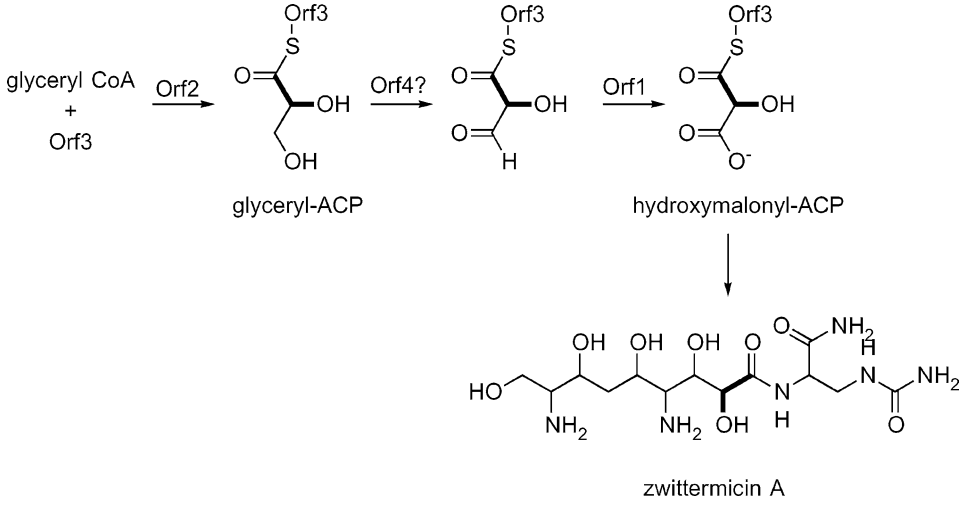
Despite the structural diversity of the above metabolites and their different origins (*Streptomyces* spp., *Sorangium* spp., *Bacillus* spp.), they all appear to utilise a similar methoxymalonyl- or hydroxymalonyl-ACP extender unit. A cassette of five genes for methoxymalonyl-ACP, and four genes for hydroxymalonyl-ACP (consistent with the absence of a gene encoding a methyltransferase), seems to be required in most cases for the biosynthesis of these unusual glycerol-derived extender units. At the moment, the function of FkbH remains unclear, but it has been proposed to be involved in the loading of an intermediate in the glycolytic pathway onto the ACP [60, 61].

Glycerol derived starter units

Several macrolides incorporate glycerol-derived starter units. Feeding experiments have shown that a group of boron-containing antibiotics (aplasmomycin, boromycin and tartrolons) are all acetate-derived polyketides whose branching methyl groups originate from methionine [7–9, 52]. In each case, glycerol has been shown to be an intact precursor of the three carbon starter unit, which is proposed to derive from phosphoglycerate or phosphoenolpyruvate [7–9, 29, 52]. Other natural products such as lankacidin and bryostatin also appear to be derived from these unusual 3-carbon starter units [19, 27].

Aplasmomycin is a novel ionophoric macrolide antibiotic isolated from *Streptomyces griseus*. Incorporation experiments have established the origin of the carbon skeleton of aplasmomycin (Fig. 4) [7, 9, 29]. Each half of the molecule contains a polyketide chain made up of a starter unit, and seven acetate-derived extension units. Some of the latter are modified by *C*-methylation with methionine-derived methyl groups [7, 9]. Unusually, the starter unit is not derived from propionate, lactate or pyruvate [7]. Instead it was found to derive from an intact glycerol molecule (Fig. 4) [7, 9, 29]. Several experiments suggested either phosphoglyceric acid or phosphoenolpyruvate as the starter unit [29]. Phosphoenolpyruvate was deemed an attractive possibility because an addition-elimination ring closure mechanism can be postulated (Scheme 4) [29]. This process has analogies with the reaction catalysed by 5-enoylpyruvylshikimate-3-phosphate synthase. A second hypothetical mechanism for ring closure involves a S_N2-type displacement of phosphate from C-16 by the C-13 hydroxyl group (Scheme 4) [29]. An analogous mechanism can be postulated using phosphoglycerate as the starter unit [29].

Boromycin, isolated from *Streptomyces antibioticus*, was the first natural product found to contain the trace element boron [8]. Apart from the D-valine moiety, the carbon skeleton of boromycin is identical to that of aplasmomycin [8]. The backbone of each carbon chain is derived from seven acetate units extending from C-1 (the C-terminus) to C-14 [8]. Three branching methyl groups in each monomer are derived from methionine [8]. This



Scheme 3 Proposed pathway for incorporation of hydroxymalonyl-ACP into zwittermicin A

zwittermicin A

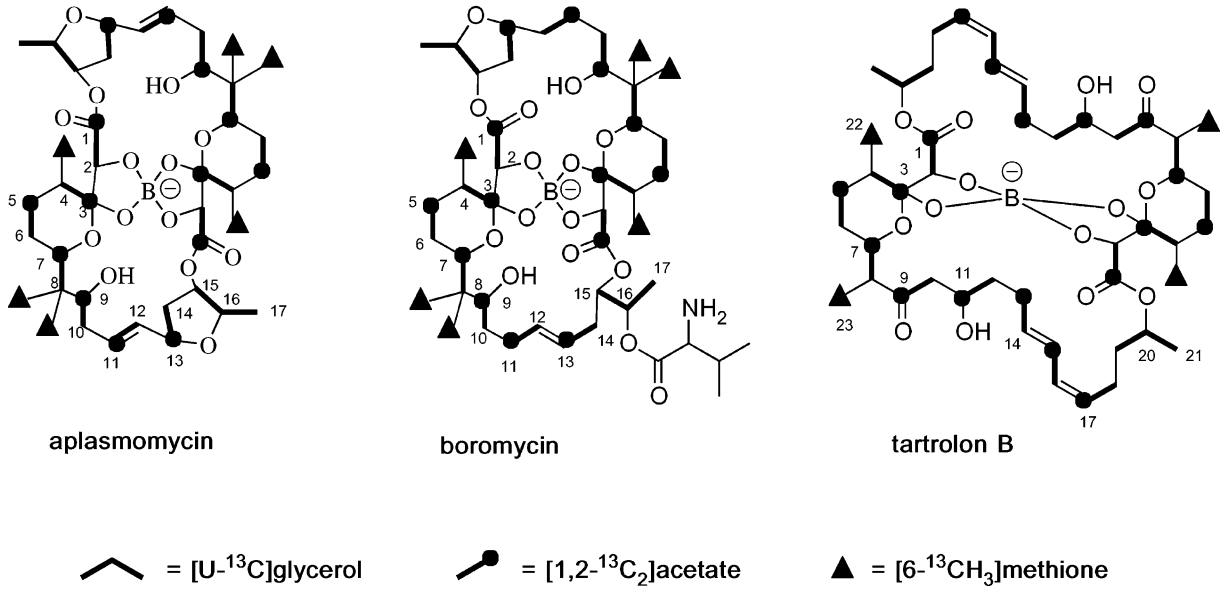
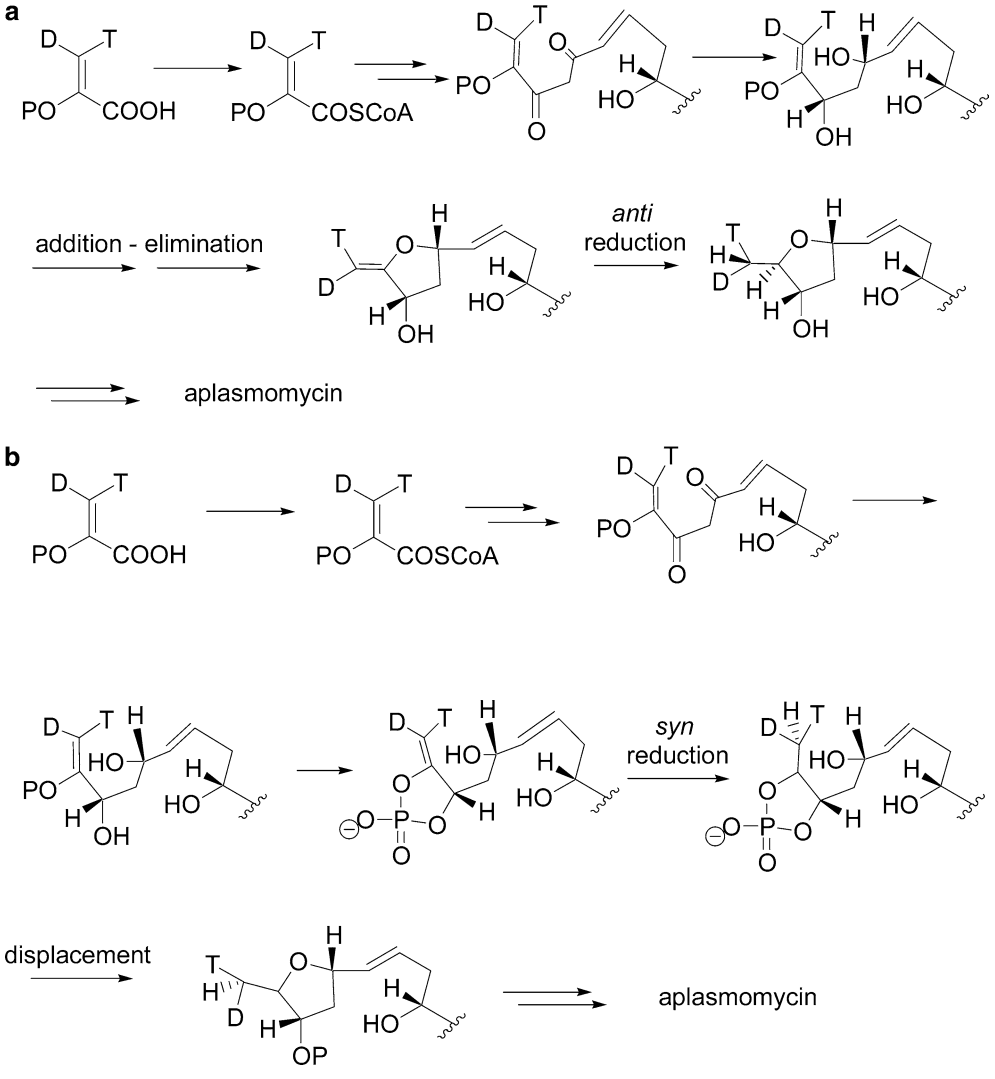


Fig. 4 The biosynthetic origins of aplasmomycin, boromycin and tartrolon B

Scheme 4 Mechanisms proposed by Floss and co-workers for the incorporation of phosphoenolpyruvate into aplasmomycin



incorporation pattern is very similar to that determined for aplasmomycin [7, 9, 29]. A further parallel is that C-15, C-16 and C-17 are not derived from acetate, but instead are derived from an intact glycerol molecule (Fig. 4) [8].

The tartrolons are also boron-containing macrolides isolated from the myxobacterium *Sorangium cellulosum* [52]. Feeding experiments determined that the carbon skeleton was derived from nine intact acetate units, with branching methyl groups C-22 and C-23 derived from the methyl group of methionine [52]. However, the three carbon starter unit C-19, C-20 and C-21 was not derived from acetate, propionate or methionine [52]. GC and GCMS studies of a degradation product containing C19-C21 of [1,3−$^{13}C_2$]glycerol-derived tartrolon B revealed an isotope pattern consistent with the incorporation of a glycerol-derived starter unit into tartrolon B (Fig. 4) [52].

The lankacidin group of antibiotics are another group of natural products which appear to incorporate an unusual three-carbon starter unit [59]. Uramoto et al. [59] used ^{13}C NMR experiments to probe the biosynthetic pathway of these macrolides (Fig. 5). They clearly showed that sodium [1−^{13}C]acetate enriches eight carbons of the macrolide [59]. This was corroborated by the incorporation of [1,2−^{13}C]acetate [59]. Glycine was determined to be the source of the C-3 amino group [59]. The use of [$^{13}CH_3$]methionine confirmed the origin of the branching methyl groups C-19, C-20, C-21 and C-22 [59]. Only the origin of the three-carbon unit attached to the nitrogen remains unclear.

Bryostatins are a family of cytotoxic macrolides found in the marine bryozoan *Bugula neritina* [43]. Hildebrand et al. [19] have recently reported that the first module of the bryostatin PKS BryA consists of four domains including a dehydratase-like domain (DH*), a ketoreductase-like domain (KR*), a FkbH-like domain and an ACP domain. FkbH homologs have recently been found in other PKS gene clusters and are thought to participate in the formation of methoxymalonyl-ACP extender units from a glycolytic pathway intermediate. Hildebrand et al. [19] proposed that a sequence of three reactions catalysed by the FkbH domain, the DH* domain and the KR* domain would yield a D-lactyl thioester: the expected starter unit for bryostatin biosynthesis. In the first reaction, a glycolytic intermediate (proposed to be phosphoglycerate) is transferred to the ACP by the FkbH domain [19]. This is followed by DH*-catalysed dehydration to enoylpyruvyl-ACP, which could spontaneously rearrange to form enzyme-bound pyruvate [19]. Finally an α-ketoreduction catalysed by KR* would yield lactyl-ACP (Scheme 5) [19]. This is consistent with earlier work carried out by Kerr et al. [27], which showed radiolabelled glycerol was incorporated into bryostatin.

A BLAST search using BryA as the query revealed that the amino acid sequence of the DH*-KR* domains shows high similarity to a putative didomain protein encoded by *orf19* within the lankacidin/lankamycin biosynthetic gene cluster of *Streptomyces rochei* [19]. In the vicinity of *orf19*, two additional open reading frames, *orf21* and *orf22* encoding an ACP and FkbH homolog, respectively, were identified (Fig. 6) [19]. The lankacidin group of antibiotics contains a 3-carbon starter unit which has different oxidation states in different components; lankacidin contains a pyruvyl moeity, whereas lankacidinol contains a D-lactyl moiety (Fig. 2). Hildebrand et al. [19] suggested that their proposed scheme for incorporation of the 3-carbon starter unit into bryostatin could also be utilised for incorporation of the 3-carbon starter unit into lankacidin and lankacidinol. In the case of lankacidinol, use of all the domains would produce the D-lactyl moiety, whereas for lankacidin, skipping of the final domain would yield the pyruvyl moiety [19].

It seems likely that the same mechanism is utilised for incorporation of glycerol-derived starter units into the boron-containing antibiotics aplasmomycin, boromycin and tartrolons. This hypothesis could be tested by probing for the presence of *fkbH* homologues in the genomes of the bacteria producing these secondary metabolites. Such homologues would be expected to be constituents of larger ORFs coding for type I modular PKSs or standalone ORFs clustered with such PKS genes. The possibility that lactyl-ACP (derived from a glycolytic intermediate rather than lactic acid) is the starter unit for aplasmomycin and boromycin biosynthesis leads us to propose a plausible pathway for formation of the tetrahydrofuran ring in these metabolites via conjugate addition of the hydroxyl group derived from C-2 of lactyl ACP onto the β-carbon of an enoyl-ACP intermediate during PKS-mediated assembly of the

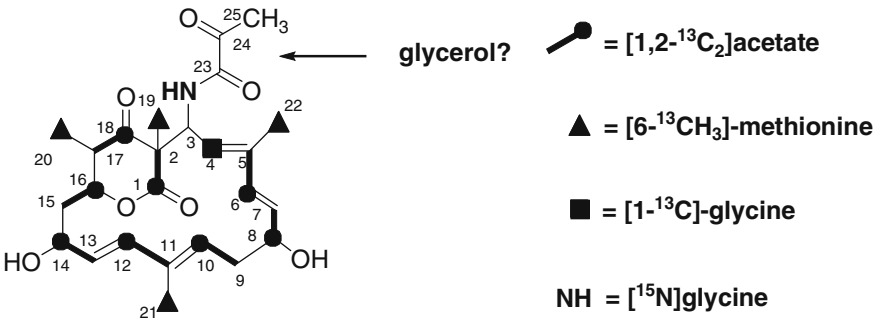
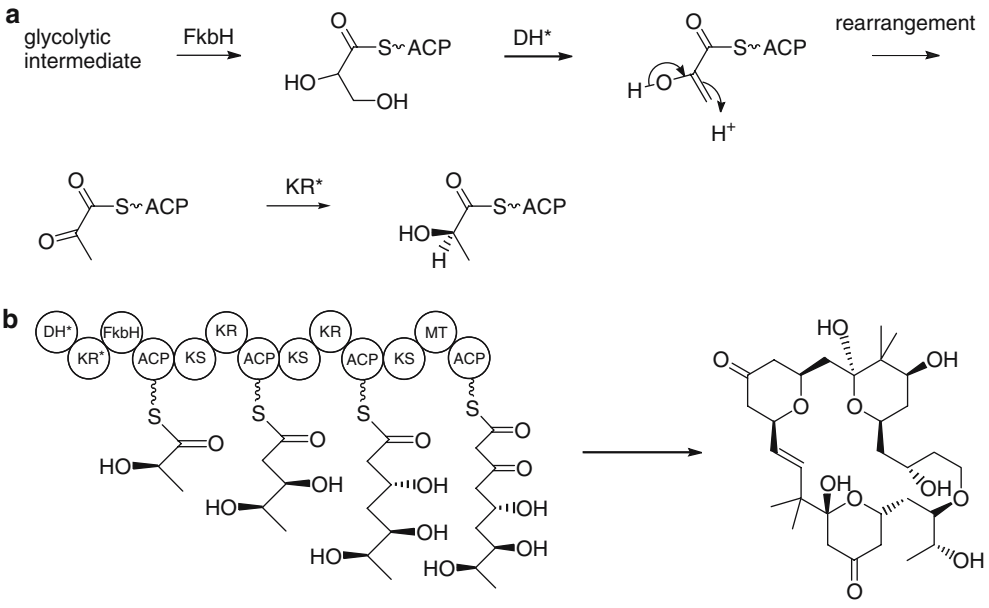


Fig. 5 The biosynthetic origin of lankacidin C



Scheme 5 Proposed mechanism for formation of D-lactyl ACP in bryostatin biosynthesis (**a**) and the proposed intermediates attached to each ACP domain of BryA on the biosynthetic pathway to bryostatin (**b**)

polyketide chain. This could be catalysed by a second DH-like domain within module 2 of the PKS (Scheme 6). A similar mechanism for the formation of a tetrahydropyran ring in pederin has recently been proposed [44]. Tartrolon B could be derived from the corresponding acyl-ACP intermediate, which cannot undergo the conjugate addition reaction because the α,β-double bond is reduced by an enoylreductase (ER) domain within module 2 of the PKS (Scheme 6).

Sequence analysis and proposed substrate of FkbH

FkbH and its orthologues have been proposed to catalyse transfer of an unidentified glycolytic intermediate onto an acyl carrier protein [5, 61, 63]. A multiple sequence alignment of the five FkbH-like proteins associated with the production of known metabolites indicates that they probably contain two domains, suggests possible catalytic roles for each domain and points to a specific intermediate in glycolysis as the substrate. Thus, the conserved motifs DXDXT and FXDD along with the universally conserved residues Ser50 and Lys80 (FkbH numbering) in the N-terminal domain strongly suggests that it has phosphatase activity [12, 39, 47]. The absence of any conserved sequence motifs characteristic of nucleotide triphosphate (NTP) utilising enzymes in the C-terminal domain suggests that FkbH-like proteins are not capable of activating the carboxyl group of a glycolytic intermediate by reaction with an NTP. Such activation would be required prior to the formation of a thioester linkage with an ACP to make the reaction kinetically and thermodynamically favourable. On the other hand, the conserved motif SCR is found in the C-terminal domain, suggesting that this domain may have AT activity, capable of catalysing the transfer of an already activated intermediate in glycolysis to the phosphopantotheine thiol of an ACP via an acyl enzyme intermediate. 1,3-Biphosphoglycerate (1,3-BPG) is the only intermediate in glycolysis that contains an activated carboxyl group and we therefore propose that this is the substrate utilised by FkbH-like enzymes. It seems likely that the C-terminal domain of these enzymes catalyses acylation of an ACP with C-1 of 1,3-BPG, while the N-

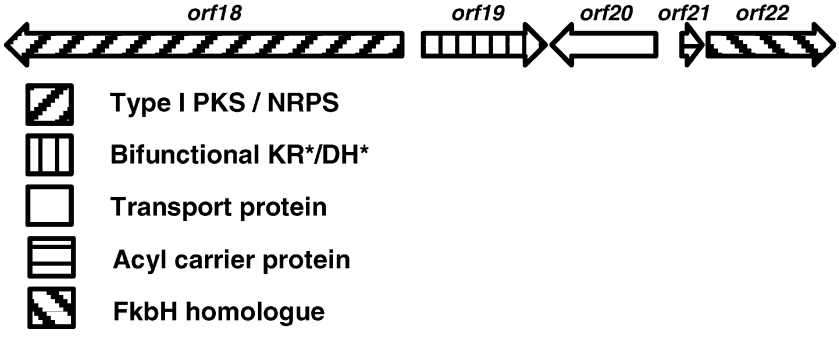
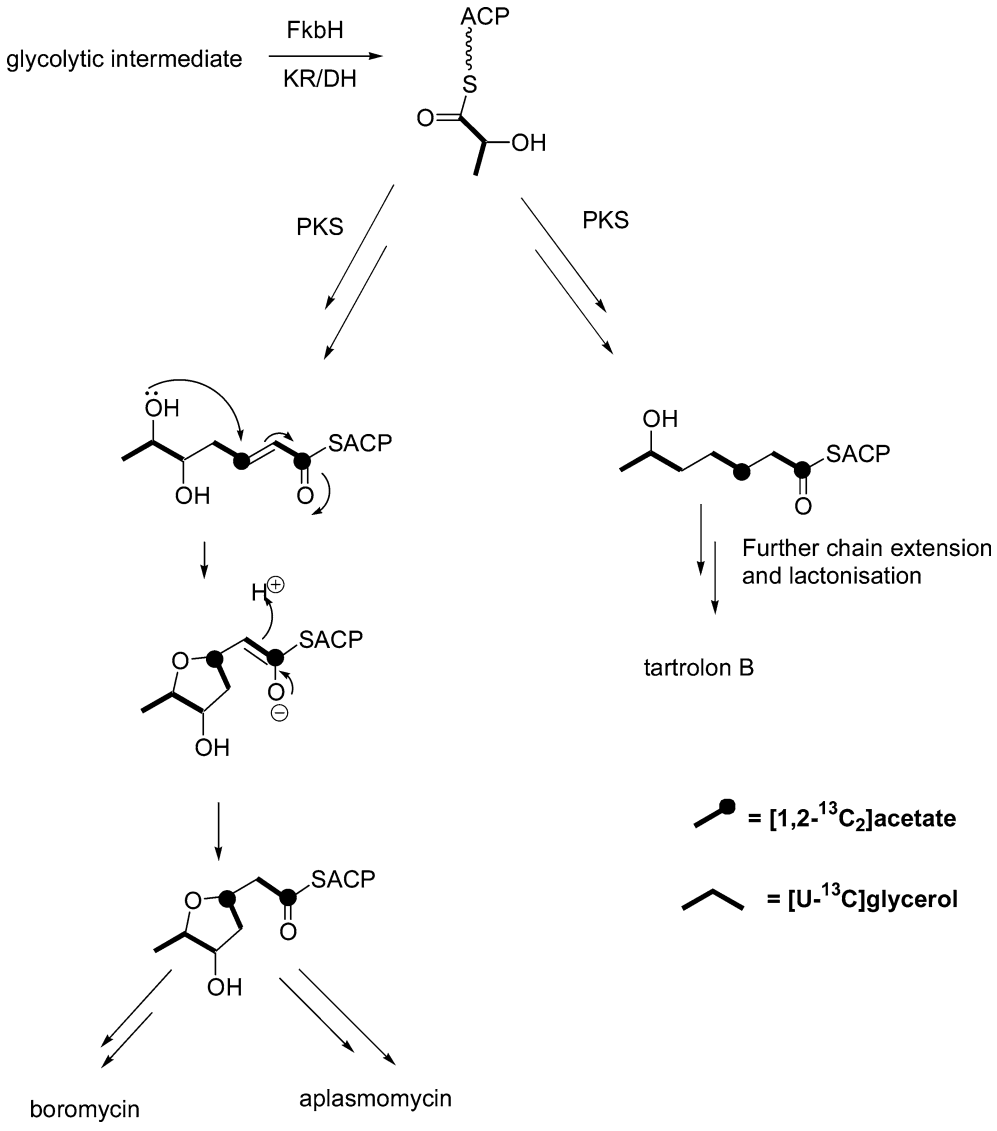


Fig. 6 A region of the lankacidin gene cluster containing ORFs proposed to be involved in the formation of the pyruvyl and D-lactyl starter units incorporated into lankacidin and lankacidinol, respectively



Scheme 6 Proposed mechanism for the formation of the tetrahydrofuranyl ring in aplasmomycin and boromycin as part of the PKS-mediated assembly process

terminal domain catalyses hydrolysis of the phosphate group attached to C-3 of 1,3-BPG to yield glyceryl-ACP. In vitro experiments utilising a recombinant FkbH-like protein, an appropriate ACP and 1,3-BPG will be required to test this hypothesis.

Addition of glycerol-derived 3-carbon branches to polyketide chains

Tetronic acids and butenolides

Incorporation of glycerol-derived precursors as a branch off the main linear chain of several polyketide metabolites has also been reported. Tetronic acid-containing natural products are a prominent example and as a result there have been several independent biosynthetic investigations of such metabolites over the last 20 years. Although the biosynthesis of the α-acyltetronic acid moieties in caloric acid and protoanemonin has been reported to involve C_4 compounds from the Krebs cycle (succinate and α-ketoglutarate, respectively) [3, 58], most α-acyltetronic acid moieties appear to be biosynthesised from 3-carbon glycerol-derived units.

Acaterin was isolated from *Pseudomonas* sp. A92 as an inhibitor of acyl-CoA:cholesterol AT [54]. 4-Dehydroacaterin, also isolated from *Pseudomonas* sp. A 92, has been shown to be a precursor of acaterin [54]. Feeding experiments determined that the linear C_{10} β-hydroxy ester moiety is derived from five acetate units, whereas the branched three-carbon unit is derived from another source [54]. Further feeding experiments with ^{13}C- and ^{2}H-labelled glycerols revealed that the branched C_3 moiety (C-3, C-4, C-5) of acaterin and 4-dehydroxyacaterin originates from glycerol (Fig. 7) [54, 55]. The direct precursor of C-3, C-4 and C-5 has been proposed to be 1,3-BPG [55]. Further studies were carried out to elucidate whether acaterin is assembled by the attachment of the C_3 precursor to the α-position of a decanoate derivative or via the coupling of an octanoate derivative with a five-carbon lactone [56]. The results led Sekiyama et al. [56] to propose that acaterin is biosyn-

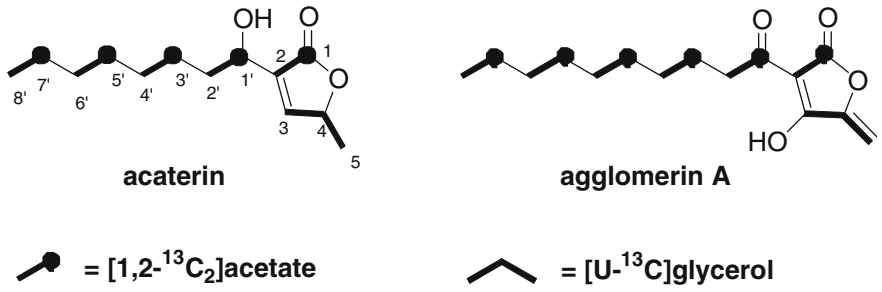


Fig. 7 Biosynthetic origins of acaterin and agglomerins

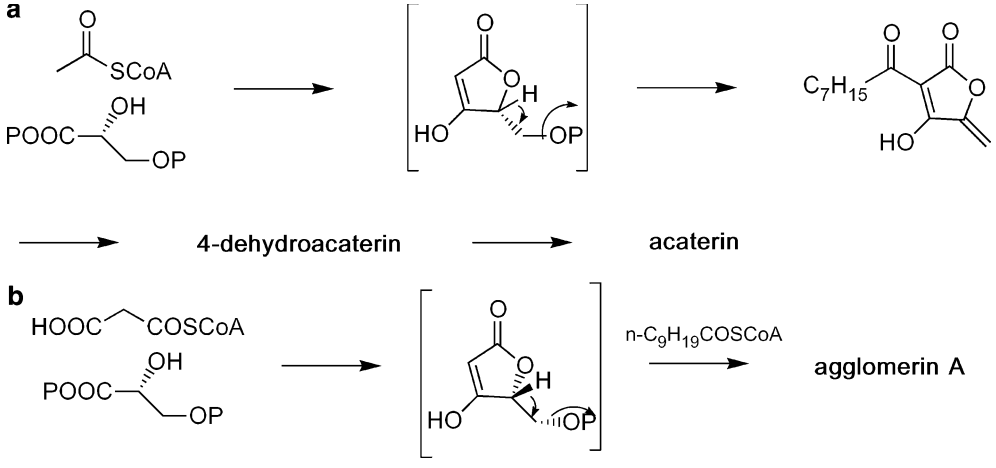
thesised via the later mode i.e. coupling of a hypothetical five-carbon lactone and octanoate. Elimination of phosphoric acid followed by reduction at C-3 and C-1' would produce 4-dehydroacaterin which could finally be reduced to furnish acaterin (Scheme 7) [21, 56].

Agglomerins A, B, C and D are structurally similar to acaterin. The biosynthesis of agglomerin A was studied to determine the origin of the branched three-carbon moiety forming part of the tetronic acid [34]. Feeding experiments showed that glycerol was incorporated in the same direction as incorporated into acaterin (Fig. 7) [34]. Stereospecific incorporation of chirally-labelled glycerols ruled out pyruvate as a possible intermediate therefore leaving 1,3-BPG as the most likely precursor of C-3, C-4 and C-5 [34]. The biosynthetic pathway to agglomerin A proposed by Fujimoto and coworkers is outlined in Scheme 7 [34].

Chlorothricin is an unusual macrocyclic antibiotic which belongs to the tetronic acid family. It is isolated from *S. antibioticus* strain Tü 99. Its structure consists of three components, a modified 6-methylsalicylic acid moiety, two identical 2,6-dideoxyhexose moieties and the aglycone chlorothricolide. Early biosynthetic studies established the origin of most of the carbon framework [23, 33]. The modified 6-methylsalisylic acid is derived from four acetate units via a polyketide pathway; the additional *O*-methyl group is derived from methionine; the two 2,6-dideoxyhexose moieties are derived directly from glucose; and the aglycone is biosynthesised primarily via a polyketide pathway from ten acetate and two propionate units which account for all but three of the carbon atoms of chlorothricolide (C-22, C-23 and C-24), which are not labelled by acetate or propionate (Fig. 8) [23, 33]. Large numbers of compounds were tested in order to identify the missing precursor of these three carbon atoms [30]. While [U–^{14}C]pyruvic acid and [1–^{14}C]lactic acid were not found to be specifically or efficiently incorporated, it was determined that [2–^{14}C]glycerol was an efficient precursor of chlorothricin [30]. To test if glycerol was specifically incorporated into the missing carbons as an intact 3-carbon unit, [U–$^{13}C_3$]glycerol fed to *S. antibioticus* Tü 99 [30]. NMR analysis showed that glycerol was incorporated intact into C-22, C-23 and C-24 (Fig. 8) [30]. In order to determine the orientation of the 3-carbon moiety, glycerol was stereospecifically labelled at C-1 (the *pro-R* hydroxymethyl group which undergoes phosphorylation during metabolism) giving rise to C-3 of triose phosphates, 3-phosphoglyceric acid and phosphoenolpyruvate [30]. Incorporation experiments with this labelled precursor determined that the C-1 of glycerol gives rise to C-22 of chlorothricolide [30].

Versipelostatin has been shown to be biosynthesised via condensation of a linear polyketide chain and a C_3 branching unit [10]. Feeding experiments established that C-28, C-29 and C-30 are derived from an intact glycerol molecule rather than pyruvic acid or succinic acid (Fig. 8) [10]. It seems likely that chlorothricin and versipelostatin share a common biosynthetic pathway.

Several tetronic acid- or butenolide-containing polyketides have been shown to be assembled via condensation of a linear polyketide chain with a 3-carbon



Scheme 7 Biosynthetic pathways to acaterin (**a**) and agglomerin A(**b**) proposed by Fujimoto et al. [34, 56]

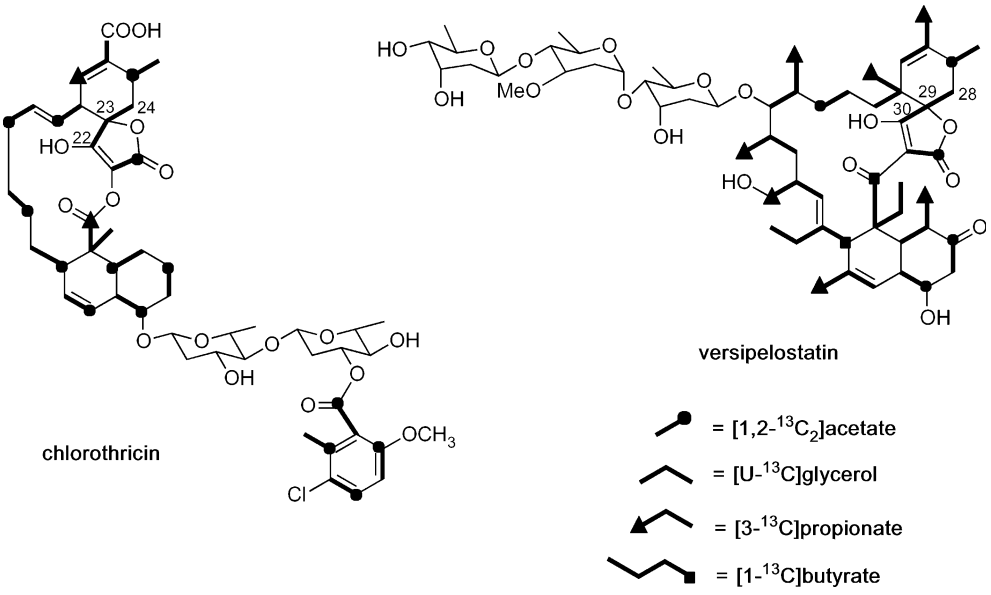


Fig. 8 Biosynthetic origins of chlorothricin and versipelostatin

precursor derived from glycerol. It is tempting to speculate that this three-carbon precursor is a glyceryl ACP, which is derived from 1,3-BPG by FkbH-mediated transfer and dephosphorylation. Condensation of this precursor with the requisite β-ketothioester polyketide chains would yield the corresponding 4-hydroxymethyl tetronic acids. Elimination of water from these would yield the exomethylene tetronic acids, which would undergo reduction to yield 4-methyl butenolides as found in acaterin or formal intramolecular Diels-Alder cycloaddition with a diene at the opposite end of the polyketide chain to yield the spirotetronic acid moieties found in chlorothricin, versipelostatin and many other similar metabolites.

Butyrolactones and furanylcarbonyl-containing metabolites

In *Streptomyces* spp., γ-butyrolactone signalling molecules regulate secondary metabolite production and/or cytodifferentiation. They possess a unique 2,3-disubstituted-butanolide skeleton [62]. The biosynthesis of these molecules is difficult to study because they are usually present at subnanomolar concentrations in culture broths (a few micrograms per litre). However biosynthetic studies of one of them, the virginiae butanolide A (VB-A), which induces the production of virginiamycin in *S. virginiae*, have been possible in *S. antibioticus*, a high producer of VB-A (several milligrams per litre) [50]. Feeding experiments with [1,3$-^{13}C_2$]glycerol revealed the intact incorporation of a 3-carbon unit into C-4, C-3 and C-5 suggesting that a β-keto acid derivative and a 3-carbon unit derived from glycerol, such as dihydroxyacetone or a derivative, are condensed in the biosynthesis of VB-A [50]. A plausible biosynthetic pathway to VB-A has been proposed, involving a butenolide intermediate (Scheme 8) [50].

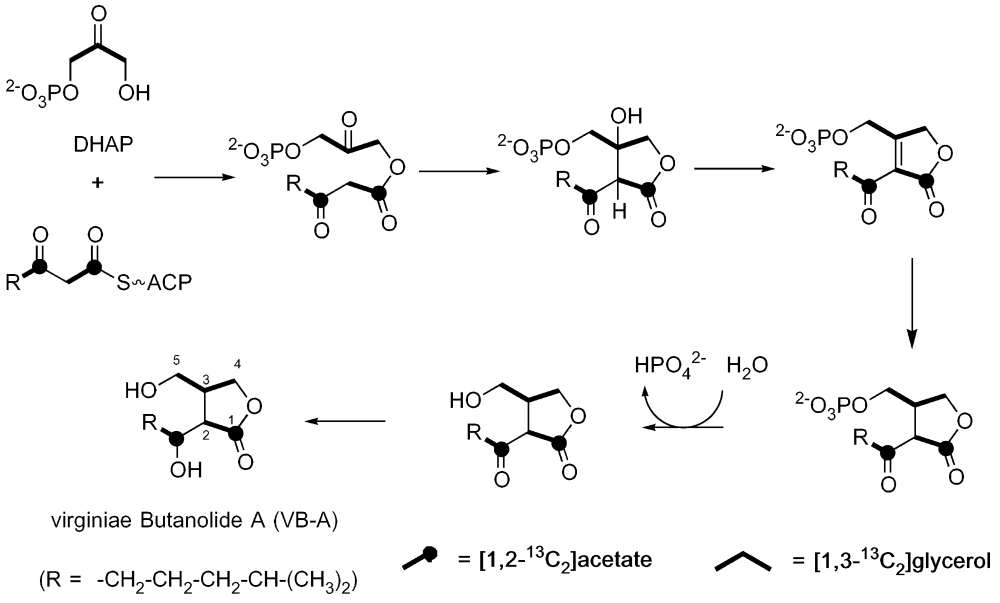
Recently, novel furanylcarbonyl- and butyrolactone-containing metabolites were purified from rhamnoside-producing *Streptomyces* strain GT 61150 and strain Tü 3634 [22]. It was postulated that their carbon skeleton could be biosynthesised from both the acetate and the carbohydrate pools [22]. Feeding experiments with labelled acetate and glycerol indicated that the formation of both the furan- and lactone-containing metabolites could arise from the same mixed acetate-glycerol biosynthetic route (Scheme 9) [22]. This study concluded that a β-ketoacyl-CoA thioester, presumably arising from a dedicated biosynthetic pathway, is coupled with a dihydroxyacetone-derived 3-carbon unit by an aldol-like condensation to form an intermediate (different to the one proposed for VB-A biosynthesis) that condenses to give either the butyrolactone or the furanylcarbonyl structure [22]. However, the β-ketoacyl-CoA thioester could also be coupled to different glycerol-derived precursors via two independent biosynthetic mechanisms. 1,3-BPG and DHAP are plausible precursors for the furan-containing polyketides and the butyrolactones, respectively.

Methylenomycins and syringolides

The methylenomycins are antibiotics produced by diverse *Streptomyces* spp. [16, 17, 24]. [U–^{13}C]glycerol is incorporated intact into C-3, C-4, and C-8 of methylenomycin A, as well as indirectly (via metabolism to acetyl-CoA) into C-5/C-9 and C-1/C-6 (Scheme 10) [6]. These experiments were carried out with *S. coelicolor* U9 in complete medium, which contains multiple carbon sources, resulting in low incorporation levels (~0.1%). The metabolic origin of C-2 and C-7 was not identified.

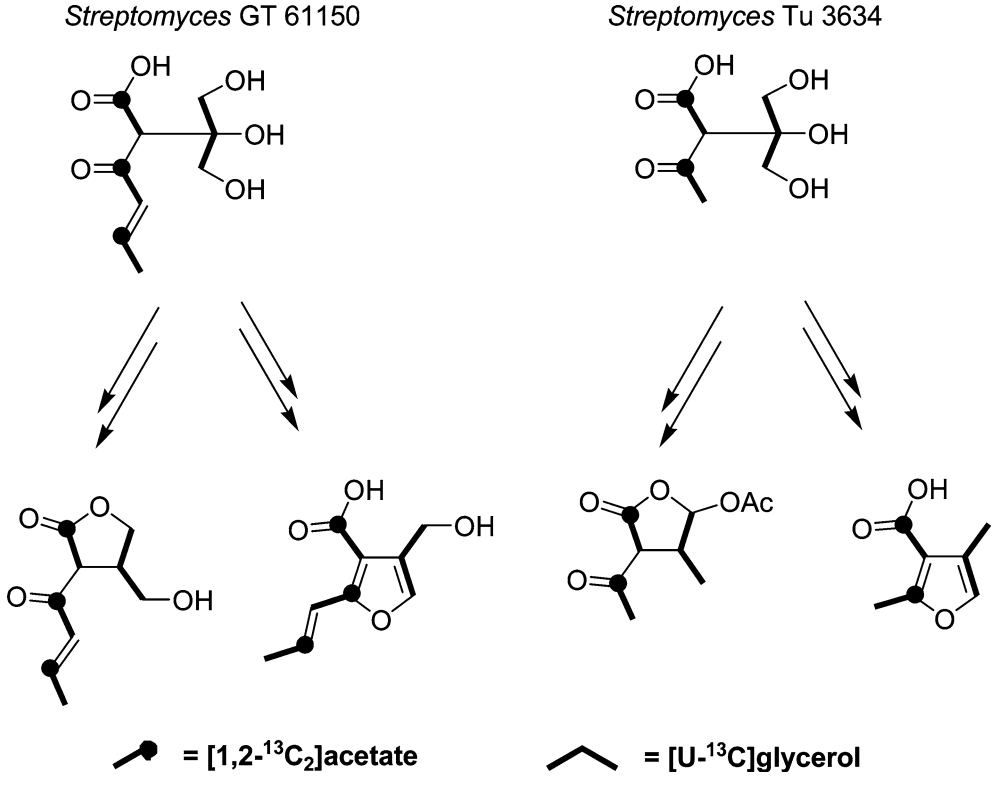
Recently, the sequence of the entire *mmy* cluster (located on the giant linear plasmid SCP1 of *S. coelicolor*),

Scheme 8 Mechanism for incorporation of a glycerol-derived 3-carbon precursor into VB-A proposed by Yamada et al. [50]

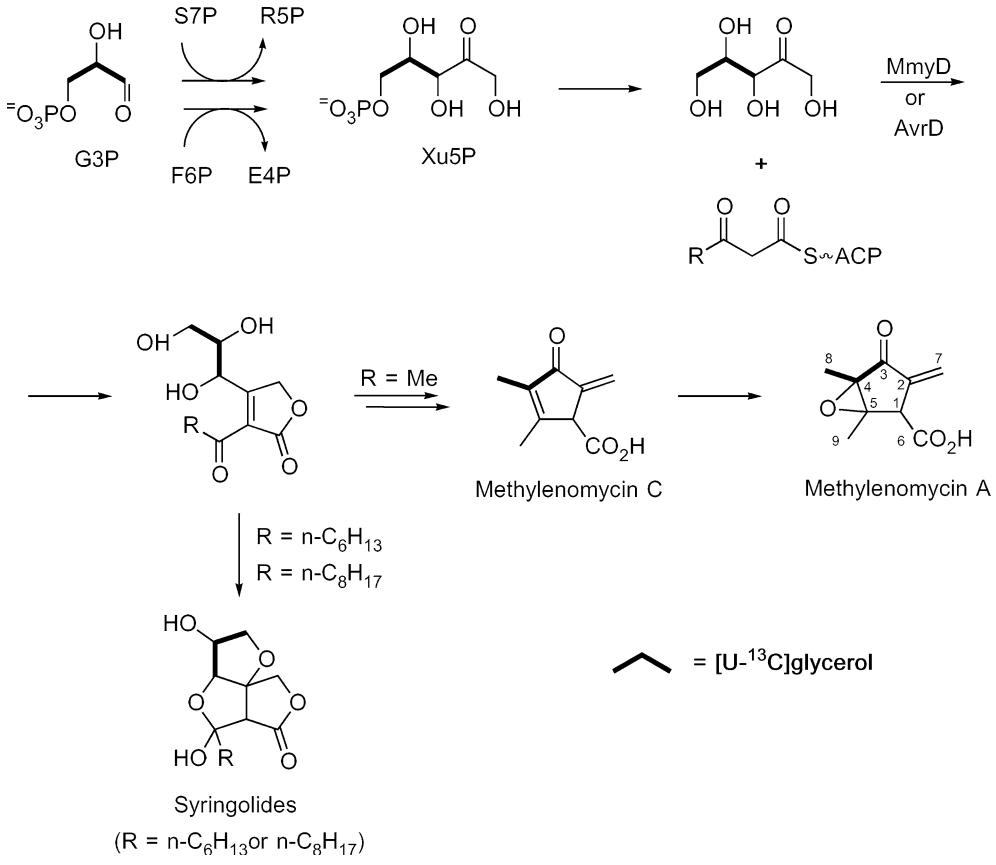


which directs the biosynthesis of the methylenomycins, has been determined [2]. A strain of *S. lividans* transformed with the integrative cosmid C73_787 (containing the *mmy* cluster) produces methylenomycins in a supplemented minimal medium [11]. The primary metabolic origins of the methylenomycins were reinvestigated using this strain in minimal medium with [U–^{13}C]glycerol as the main carbon source [11]. These experiments revealed the involvement of the pentose phosphate pathway in the biosynthesis of the methylenomycins [11]. [U–^{13}C]ribose was fed to prove the incorporation of an intact 5-carbon pentose into methylenomycin A [11]. These results were explained by the metabolism of [U–^{13}C]glycerol to [U–^{13}C]glyceraldehyde-3-phosphate (G3P), which is incorporated through the pentose phosphate pathway into a 5-carbon pentose precursor of C-2, C-3, C-4, C-7 and C-8 of the methylenomycins [11]. Transketolase-mediated condensation of [U–^{13}C]G3P with unlabelled fructose-6-phosphate (F6P) or seduheptulose-7-phosphate (S7P) would give xylulose-5-

Scheme 9 Incorporation of glycerol-derived precursors into butyrolactone and furanocarbonyl metabolites



Scheme 10 Proposed pathways for methylenomycin and syringolide biosynthesis



phosphate (Xu5P) labelled at only C-3, C-4 and C-5 with ^{13}C [11]. On the basis of the feeding experiments and the analysis of the *mmy* cluster, a plausible biosynthetic pathway involving an unusual condensation of a diketide with a pentose has been proposed (Scheme 10) [11].

The syringolides are elicitors produced by plant pathogenic *Pseudomonas* spp. that share no obvious structural relationship with the methylenomycins, but analysis of the *mmy* cluster reveals a common genetic basis for methylenomycin and syringolide biosynthesis. Thus, a homologue of the enzyme encoded by the *Pseudomons syringae* pathovar tomato gene *avrD*, which is necessary and sufficient to elicit syringolide production in *E. coli* [64], is encoded within the *mmy* cluster (MmyD shows 47% similarity over 333 amino acids to AvrD) [11]. AvrD has been proposed to catalyse the condensation of D-xylulose with a β-ketoacyl thioester in the biosynthesis of syringolides [35]. In methylenomycin biosynthesis (as has been proposed for syringolide biosynthesis) we propose that a diketide is coupled with a 5-carbon pentulose (probably xylulose) to yield a butenolide intermediate. Spontaneous cyclisation of this intermediate gives the syringolides. However in methylenomycin biosynthesis, this spontaneous rearrangement appears not to occur, perhaps as a result of modification of one or more hydroxyl group in the butenolide intermediate (e.g. by phosphorylation or oxidation to a keto group), or by reduction of the double bond in this intermediate.

To conclude, it is worth noting that the intact incorporation of glycerol or glycerol-derived precursors into polyketide metabolites does not always mean that they are direct precursors. Indeed, intact glycerol-derived 3-carbon units are incorporated into diverse primary metabolic pathways such as the pentose phosphate pathway (via G3P) and the shikimate pathway. Shikimate and pentoses have been shown to be direct precursors to secondary metabolites [11, 37]. Three-carbon units can also be involved indirectly in the biosynthesis of terpenoids via the non-mevalonate pathway that involves the decarboxylative condensation of G3P with pyruvate [48]. Such advanced precursors of non-polyketide origin are often condensed with other polyketide-derived precursors to produce secondary metabolites of mixed biosynthetic origin. Finally, it is noteworthy that the biosynthesis of all of the metabolites discussed in this section not only involves the coupling of a glycerol-derived precursor with a polyketide, but also appear to proceed via similar putative butenolide intermediates.

Conclusion

The utilisation of glycerol-derived starter and extender units in the biosynthesis of several polyketides has been established by incorporation experiments. Intriguingly, recent genetic studies suggest that the same glycolytic intermediate (probably 1,3-BPG) is channelled into

lactyl- and pyruvyl-ACP starter unit biosynthesis and methoxymalonyl-ACP extender unit biosynthesis, by FkbH-like enzymes. The biosynthesis of several other secondary metabolites that have a 3-carbon glycerol-derived branch appended to a polyketide chain has also been studied. While some of these metabolites (methylenomycin, the syringolides and butyrolactone signalling molecules) appear to use FkbH-independent pathways for incorporation of the glycerol-derived precursors, the enzymes involved in incorporating such precursors into other metabolites (tetronic acids and butenolides) are not yet known. However, it is tempting to speculate that glyceryl-ACP precursors of tetronic acids and butenolides could be formed by the action of FkbH-like enzymes on 1,3-BPG.

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